

THE INTRACELLULAR BACTERIA OF THE PROLIFERATIVE ENTEROPATHIES:
A COMPARISON OF IN VITRO AND IN VIVO INFECTION

JASNI SABRI
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DEDICATION

THIS THESIS IS DEDICATED TO MY WIFE, NORHASLIN JUMAAT, OUR CHILDREN, MUHAMMAD IRFAN SAFWAN AND NURSYAZWANI LIYANA AND BOTH OF OUR PARENTS.

DECLARATION

I DECLARE THE WORK AND COMPOSITION OF THIS THESIS ARE MY OWN

JASNI

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ABSTRACT OF THESIS (Regulation 3.5.10)

Name of Candidate JASNI SABRI

Address DEPT. VET. PATHOL., ROYAL (DICK) SCHOOL OF VET. STUDIES, SUMMERHALL.

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The intracellular bacteria from naturally occurring porcine proliferative enteropathy were cultured in an in vitro cell culture system, the morphology of the bacteria, the pathogenicity of the organism in vivo, in hamsters, and the morphological events of infection in both cultured cells and hamsters were investigated.

Bacteria purified from intestinal lesions of the disease were cultured in a rat enterocyte, IEC-18 cell line. Infection was enhanced by centrifugation of the bacteria onto the cell monolayer incubated at reduced oxygen tension in a microaerobic atmosphere. However, centrifugation was not necessary for infection to occur. Bacterial infection can be passaged and maintained several times.

Morphological observations of the bacteria grown in cell culture pelleted by centrifugation revealed that the bacterium measure from 0.1 to 0.3µm in width and 0.7 to 2.0µm in length. The bacteria were pleomorphic with a wavy trilaminar outer membrane and an often indistinct cytoplasmic membrane generally clearly separated by a periplasmic space. The pleomorphic bacteria differed in the internal structure and in the electron density of their cytoplasm; some were electron-dense and some were electron-lucent. The internal structure of the former was amorphous and consisted of numerous granules, presumably bacterial ribosomes whereas the latter demonstrated a central reticulate appearance with less dense peripheral granules. Bacterial divisions were consistently seen in the electron-lucent form and were by transverse septation.

IEC-18 cells were used as an in vitro model to study the cellular events of intestinal cell infection. Cells were artificially infected either by centrifugation or spontaneously. A method of bacterial attachment and entry into the host cell was observed in both methods of infection. In centrifuged infection, bacteria were seen attached by an electron-dense cap projection of the cell membrane. In contrast, bacteria were seen closely apposed to the cell membrane in non-centrifuged infection. However, in both cases, attachment of the bacteria was followed by entry into and escape from endocytic vacuoles free into the cytoplasm. Bacteria then multiplied to large numbers to fill the cytoplasm and were eventually released by extrusion from the host cell cytoplasm.

The aetiology and pathogenesis of the disease in hamsters infected with the pig-derived bacteria was revealed. Hamsters developed marked hyperplasia of the crypt epithelial cells associated with numerous bacteria in the apical cytoplasm when dosed with the bacteria cultivated in cell culture. Cellular events of infection, morphologically similar to that observed in the in vitro model were evident.

This study revealed that the intracellular bacteria from porcine proliferative enteritis can be cultured and passaged in rat enterocytes in vitro and suggest that the in vitro model is a relevant model of in vivo infection, in hamsters as both models showed similar stages in the pathogenesis of infection and proved that the porcine-derived intracellular bacteria grown in IEC-18 cells are pathogenic in hamsters.

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CHAPTER 1
LITERATURE REVIEW

LITERATURE REVIEW

1.1 Introduction

Proliferative enteropathy (PE) is a well known enteric disease of swine which was first reported by Biester and Schwarte (1931). Since that time, additional reports of morphologically similar diseases involving many mammalian species, including hamsters, have appeared. The disease in pigs consists of a group of clinical conditions with common underlying pathological changes, which results in clinical signs of subclinical weight loss (Roberts, Lawson, Rowland and Laing, 1979) and emaciation (Lomax, Glock and Hogan, 1982c) or in peracute or acute outbreaks, death of the affected animals (Love, Love and Edwards, 1977; Yates, Clark, Osborne, Enweani, Radostits and Theede, 1979). The economic significance of the disease is considered substantial; estimated production losses in the Australian pig industry of up to £12 per sow per annum has been reported (Cutler and Gardner, 1989; Pointon, 1989). Similar losses are postulated in the UK pig industry (MacKinnon, 1993).

The disease is present world-wide and has been reported in Australia, Belgium, Brazil, Canada, Denmark, Finland, France, Greece, Holland, India, Japan, South Africa, Sweden, Taiwan, the United Kingdom, the United States and Yugoslavia (Rowland and Lawson, 1992).

The aetiology of the disease in pigs is inextricably linked to the presence of an intracellular bacterium in the affected tissues and current evidence, although conflicting, suggests that this is an as yet uncultivated microorganism. The intracellular bacteria are curved, resemble Campylobacters in morphology and a variety of organisms belonging to this genus may be cultivated from the lesions. However, numerous studies have been unable to correlate the relationship between the lesions and cultivated Campylobacters (Lawson and McOrist, 1993).

The natural diseases in pigs and hamsters are remarkably similar clinicopathologically. Hamsters inoculated orally with intracellular bacteria purified from the lesions of pigs affected by the natural disease, appeared to develop infection (McOrist and Lawson, 1987).

Intracellular curved bacterial forms, identical in morphology to those observed in pigs were evident in the intestines of experimentally-induced and spontaneous disease in hamsters. Although Campylobacters may also be isolated from hamsters, no relevant relationship between the disease and the cultivated bacteria has been disclosed in this species.

The aetiology of the disease in pigs is still in dispute. The inability to cultivate the intracellular bacteria suggests that it is an obligate intracellular bacteria and as such first demands cultivation of the organism in an appropriate in vitro culture system before its aetiological significance can be established.

This study was conducted during the early stages of what later become successful in vitro cultivation of the bacteria, and was carried out to investigate the characteristics of infection in in vitro and in vivo experimental models using cell culture and hamsters respectively.

1.2 Clinical signs

PE has been reported to occur in all ages of pigs (Rowland and Rowntree, 1972) but is commonly observed in weaned animals between the ages of 6 and 20 weeks (Rowland and Lawson, 1974). Affected pigs are usually anorexic, thus fail to sustain the expected growth rate and may or may not have diarrhoea. The uncomplicated disease in this age group is rarely fatal and has been reported to be self-limiting; the majority of the affected animals may recover within 6 weeks of clinical recognition. The disease, however, in the presence of complications is often fatal and diarrhoea is a prominent feature. Mortality of up to 50% in young boars and sows has been documented in peracute and acute haemorrhagic outbreaks (Love, et al., 1977). Affected pigs may die suddenly without any clinical signs, or 8 to 24 hours after the onset of diarrhoea. Marked loss of body condition is not a feature of the haemorrhagic disease in young adults. Some affected animals may show inappetence. Pregnant sows at various stages of gestation have been reported to abort (Love et al., 1977).

1.3 Pathology

The disease is characterised grossly by thickening of the mucosa of the small intestine, mostly confined to the terminal ileum. The large intestines may also be affected in the caecum, proximal or the spiral colon. Characteristic histological features are replacement of the epithelium by proliferating immature epithelial cells, often with only limited inflammatory cell infiltration, and the presence of intracellular curved bacterial forms. The lesions are marked by a reduction in numbers of or absence of goblet cells, an increase in mitotic activity, and affected glands lined by undifferentiated crypt cells. Crypts may also be enlarged, elongated or branched (Rowland and Lawson, 1992).

A variety of secondary pathological changes may be superimposed on the basic lesions. The primary uncomplicated condition is often called porcine intestinal adenomatosis (PIA). If there is necrosis of the proliferating mucosa the condition may be described as, necrotic enteritis (NE) or regional ileitis (RI) and, in those cases demonstrating intestinal luminal haemorrhage,

proliferative haemorrhagic enteropathy (PHE). All these conditions consistently exhibit the same basic proliferative lesion with the presence of morphologically similar intracellular bacteria visualised by silver staining and ultrastructural observation of the affected intestines (Rowland and Lawson, 1975).

1.4 Experimental infection with diseased mucosa

A number of workers have attempted to elucidate the aetiology of the disease. Many workers have witnessed repeated success in their experiments when pigs were dosed orally with homogenates of proliferative mucosa obtained from the field disease in pigs. Such experimental pigs can develop gross lesions which manifest severe crypt hyperplasia on histology (Roberts, Rowland and Lawson, 1977; Mapother, Joens and Glock, 1987a,b; McOrist and Lawson, 1989; McOrist, Lawson, Rowland and MacIntyre, 1989). Homogenised diseased ilea from successful experimental studies were able to further transmit the disease in conventional pigs. Both fresh and previously frozen inocula accomplished serial reproduction of

proliferative lesions. The disease has been serially transferred nine times in pigs given freshly prepared and five times using previously frozen homogenised ilea. Similar success in the reproduction of the disease was also achieved when gnotobiotic pigs were dosed with 0.65µm filtered inocula (McOrist and Lawson, 1989; McOrist et al., 1989).

The type of proliferative lesions produced does not depend on the nature of the diseased homogenate employed. Pigs dosed with PHE homogenate developed NE or PHE and vice versa; this implies the involvement of a common aetiological agent in the spectrum of disease conditions (Mapother et al., 1987a).

1.5 Recovery of Campylobacter

species from the lesions of PE

Many Campylobacter spp. have been isolated from the lesions. Among these organisms, a catalase negative bacterium, Campylobacter mucosalis was the first to be isolated (Lawson and Rowland, 1974). C. mucosalis has also

been isolated from the oral cavity of healthy and affected swine but not from the faeces, normal intestines and unaffected areas of the bowels (Lawson and Rowland, 1974; Lawson, Rowland and Roberts, 1975; Roberts, 1981). However, this bacterium was isolated inconsistently and only in small numbers from cases of PHE (Lawson, Rowland, Roberts, Fraser and McCartney, 1979).

Later Gebhart, Ward, Chang and Kurtz (1983) associated proliferative lesions with a catalase positive organism, Campylobacter hyointestinalis. These authors reported that C. mucosalis and C. hyointestinalis can be usefully identified and differentiated by immunofluorescence staining of the lesions but that C. hyointestinalis was more consistently and predominantly present in the proliferating cells. Frozen sections from all affected pigs showed fluorescence when stained with hyperimmune sera raised in rabbits against C. hyointestinalis, however, C. mucosalis was less numerous and confined to the necrotic mucosa and the cellular debris (Chang, Kurtz, Ward, and Gebhart, 1984).

Campylobacter jejuni and Campylobacter coli have also been isolated but less regularly, and the latter is commonly present in the intestines of normal pigs (Doyle, 1944; Deas, 1960; Lawson and Rowland, 1974; Eriksen, Landsverk, Gondrosen and Vormeland, 1990).

1.6 Experimental infection

with cultivable Campylobacters

Although naturally occurring lesions have yielded C. hyointestinalis and C. mucosalis, numerous attempts to reproduce the disease using pure culture of either one or a combination of these organisms have not successfully fulfilled Koch's postulates. This inability to reproduce PE in specific-pathogen free or gnotobiotic pigs exposed to Campylobacter cultures suggests that these bacteria mainly colonise the altered intestines and are not the cause of PE (Lomax, Glock and Hogan, 1982b; McCartney, Lawson and Rowland, 1984; Boosinger, Thacker and Armstrong, 1985).

Conventional health status neonatal pigs dosed orally with cultures of C. mucosalis did not develop the

anticipated lesions, despite limited colonisation for up to 40 days (Roberts, Lawson and Rowland, 1980a). Anticholinergic drugs to reduce peristalsis and antibacterial agents did not increase the susceptibility of the pigs to infection with this agent. Weaned pigs were totally resistant to intestinal colonisation by C. mucosalis on its own (Roberts, Lawson and Rowland, 1980b).

C. hyointestinalis and C. mucosalis alone or in combination have been shown to be non-pathogenic in gnotobiotic pigs. Gnotobiotic pigs dosed orally with these agents did not develop gross or microscopic lesions nor were enterocytes colonised by the bacteria (McCartney et al., 1984; Boosinger et al., 1985). Gnotobiotic pigs were also dosed with other enteric pathogens or commensals along with C. mucosalis; combined infection with pathogenic Rota virus failed to cause the disease and concurrent infection with Streptococcus spp. and Escherichia coli only resulted in persistent colonisation in the gut by C. mucosalis (McCartney et al., 1984).

In a series of experiments, one group of workers

infected specific pathogen free and caesarean-derived colostrum deprived pigs with C. mucosalis and recorded evidence mainly histological, of proliferative lesions. Such experiments do not appear to have been repeated (Lomax, Glock, Harris and Hogan, 1982a; Lomax, et al., 1982b).

1.7 Identification of another

Campylobacter-like organism in the lesions of PE

The success of homogenised mucosa, in comparison with pure cultures of organisms, in initiating the disease is intriguing. Investigation by Lawson, Rowland and MacIntyre (1985) first provided evidence for the presence of another intracellular organism in the lesions of PE. The sera of rabbits immunised with bacteria directly extracted from the intestinal mucosa of a case of PHE, and sera from some apparently normal rabbits, reacted with intracellular bacteria in sections of both PIA and PHE. However, the sera did not react with any of the known Campylobacters that may be cultivated from PE in immunofluorescence assays, nor did it react with these bacteria in

agglutination tests. One possible explanation for these observations was that the intracellular bacteria are distinct from known Campylobacter spp. previously associated with the disease. Furthermore, these observations were the first indication that rabbits may also suffer from a PE-like conditions similar to the disease in pigs. These observations imply that apparent reactions in immunofluorescence tests using rabbit hyperimmune sera prepared against known cultivated Campylobacters may have on occasion been due to naturally occurring antibody to the intracellular organism.

1.8 Production of monoclonal

antibody to the intracellular bacteria of PE

The development of specific mouse monoclonal antibodies against intracellular bacteria purified from filtered (0.65µm) suspensions of affected ileal mucosa had also been successfully accomplished (McOrist, Boid, Lawson and McConnell, 1987). These reagents did not react with any of the cultivable Campylobacters; C. mucosalis, C. hyointestinalis, C. jejuni or C. coli previously associated

with PE or Campylobacter fetus in enzyme-linked immunosorbent assay or immunofluorescence tests. However, such antibody reacted strongly with the intracellular bacteria when tested on the mucosal filtrate used to prepare the antibody and also on tissue sections from pigs with either PIA, NE or PHE.

These findings suggest the presence of a unique antigen in the intracellular bacteria either not present or not exposed on the surface of known Campylobacters when cultivated in vitro but present on the intracellular organism in both the haemorrhagic and the non-haemorrhagic forms of the disease.

1.9 Molecular biology of the intracellular bacteria of PE

The intracellular bacteria purified directly from the lesions of PE were compared antigenically and genetically with the cultivated Campylobacters spp. The outer membrane protein profiles of intracellular bacteria visualised by sodium dodecyl sulphate-polyacrylamide gel electrophoretic analysis differ markedly from those of

known Campylobacter spp. and immunoblotting results with both specific polyclonal and monoclonal antibodies to the intracellular bacteria identified a 25k to 27k antigenic component present in the outer membrane of the intracellular bacterium (McOrist, Boid and Lawson, 1989a). This antigenic component was not detected in normal pig intestines nor in sonicates of Campylobacter spp., thus confirming the unique character of a major antigen of the intracellular bacterium.

The DNA fragment pattern of the intracellular bacterium on endonuclease digestion also revealed the separate identity of the intracellular bacterium and the known Campylobacters (McOrist, Lawson, Douglas and Boid, 1990). Specific DNA probes to the intracellular bacterium have also been successfully developed (Gebhart, Lin, McOrist, Lawson and Murtaugh, 1991). These probes were able to hybridize only with the porcine mucosa obtained from pigs with PE but not with normal mucosa nor any of the Campylobacter spp. Correspondingly, DNA probes prepared against the cultivable Campylobacters did not react with the intracellular bacterium (Gebhart, Murtaugh, Lin and

Ward, 1990). These studies provided evidence that the intracellular bacterium of PE are antigenically and genetically unique and that they may be a novel as yet uncultured bacterium.

1.10 Reports of PE in other animal species

Proliferative enteropathy is known to affect many other mammalian species. Natural disease has been reported in the hamster (Frisk and Wagner, 1977a), guinea pig (Elwell, Chapman and Frankel, 1981), fox (Landsverek, 1981), foal (Duhamel and Wheeldon, 1982), rat (Vandenberghe and Marsboom, 1982), ferret (Fox, Murphy, Ackerman, Prostack and Gallagher and Rambow, 1982) and rabbit (Schoeb and Fox, 1990). In all these animal species, curved bacterial forms have been reported in the hyperplastic lesions. A similar disease has also been documented in sheep (Cross, Smith and Parker, 1973), nevertheless, no intracellular Campylobacter-like bacterial forms were seen. Although the ovine disease merits the pathological description of proliferative enteropathy, the conditions can clearly be differentiated from the infectious conditions of PE which

is associated with the presence of Campylobacter-like bacteria within the affected cells. One explanation for these lesions should take into account that other enteric infections do manifest some limited degree of hyperplasia, with no evidence of intraepithelial cell infection with Campylobacter-like forms characteristic of PE (Kent and Moon, 1973). Furthermore, the intestine is an "enterocyte replenishment system" which specifically functions in response to physiological and pathological mediated changes in enterocyte population (Leblond and Stevens, 1948; Leblond and Walker, 1956; Bertalanffy, 1960; Kent and Moon, 1973).

1.11 Immunological detection of morphologically similar intracellular bacteria of PE in other animal species

Others have attempted to ascertain the antigenicity of the intracellular bacteria in the lesions of PE in the hamster (Lawson, et al., 1985; McOrist et al., 1987), ferret (Fox and Lawson, 1989), and the rabbit (Schoeb and Fox, 1990). Tissue sections from the natural disease of PE in the hamster, ferret and rabbit stained by

immunofluorescence and employing the monoclonal antibody prepared from the pig-derived bacteria showed staining of the intracellular bacteria in the lesions. This indicates that the intracellular bacteria of PE in these animal species share a common antigen with the intracellular bacteria of PE in pigs.

1.12 Naturally occurring PE in hamster

PE is a common naturally occurring disease in the Syrian hamster in the U.S.A. (Wagner, Owens and Troutt, 1973; Renshaw, Van Hoosier and Amend, 1975; Frisk and Wagner, 1977a; Lentsch, McLaughlin, Wagner and Day, 1982). Natural disease has not been reported in the U.K.. The disease in hamsters is also known as wet tail (Scheffield and Beveridge, 1962), enzootic intestinal adenocarcinoma (Jonas, Tomita and Wyand, 1965), proliferative ileitis (Booth and Cheville, 1967) and atypical ileal hyperplasia (Jacoby, Osbaldiston and Jonas, 1975). Weanling hamsters between the age of 3 to 10 weeks of age are principally affected (Jacoby and Johnson, 1981). Affected hamsters usually showed clinical manifestations of diarrhoea, rapid

weight loss and dehydration, hunched posture, ruffled hair, lethargy and or may suddenly die without any premonitory clinical signs (Jacoby and Johnson, 1981). The disease normally occurs as explosive outbreaks. Morbidity varies from 20-60% and mortality can be as high as 90% of the affected animals (Jonas et al., 1965; Booth and Cheville, 1967; Jacoby and Johnson, 1981). The aetiology of the disease has been consistently associated with curved bacteria, morphologically identical to those observed in pigs detected by silver staining and ultrastructural observations (Frisk and Wagner, 1977a,b). C. jejuni has been frequently isolated from the lesions (Frisk and Wagner, 1977b; Lentsch et al., 1982; Regina and Lonigro, 1982). Lentsch and co-workers (1982) cultured C. jejuni from 2 separate groups of affected hamsters obtained from a commercial supplier; 60 of 200 hamsters from one group and 75 of 200 hamsters from another group yielded this agent on bacteriological examinations.

1.13 Experimental reproduction of PE in hamsters

Of the animal species apart from pigs in which PE has

been reported, the disease in hamsters has been the subject of the most extensive research study, mainly in America. The inspiration for this work may have been the significant impact of the disease on laboratory animal health and the commercial hamster breeder (Wagner et al., 1973; Lentsch et al., 1982; Battles, 1985).

Experimental transmission studies have employed both cultivable *Campylobacters* and diseased mucosa. However, the disease has only been successfully reproduced by oral inoculation of hamsters with either crude or filtered (0.65 μ m) ileal mucosa from affected hamsters (Jacoby et al., 1975; Amend, Loeffler, Ward and Van Hoosier, 1976; Frisk and Wagner 1977a; Jacoby, 1978; Johnson and Jacoby, 1978). In experimentally-induced hamster disease, curved bacterial forms and typical histological lesions of the natural disease were present. Filtrates (0.22 μ m and 0.45 μ m) of the inocula were non-infective and neither homogenates of normal or diseased ileal mucosa of hamsters pre-heated at 56°C or treated with chloroform nor ilea of untreated healthy hamsters induced intracellular bacteria or cell proliferation (Jacoby et al., 1975).

Repeated attempts to reproduce the disease by oral inoculation of pure culture of C. jejuni have not been fruitful. Hamsters dosed with C. jejuni periodically for 2 to 6 weeks did not develop lesions or intracellular parasitism, despite ileal colonisation for up to 6 weeks (Regina and Lonigro, 1982; Lentsch et al., 1982). The relationship between this bacterium and experimentally-induced crypt epithelial cell hyperplasia has also been studied using monoclonal antibody specific for C. jejuni utilised in an immunofluorescence test (Stills, Hook and Sprouse, 1987). C. jejuni was only observed in the intestinal lumen but another antibody of uncertain activity detected both luminal and intracellular bacteria in the affected hamsters (Stills et al., 1987). Other methods of infecting hamsters have also been attempted; surgical inoculation of C. jejuni into the ileum of hamsters resulted only in enterocolitis and not PE and there was no intraepithelial cell infection (Humphrey, Montag and Pittman, 1985). C. jejuni was shown however, to cause a variable degree of acute intestinal inflammation in the intestines of hamsters (McOrist and Lawson, 1987). These results led to the conclusion that C. jejuni is not the

cause of the disease in hamsters.

Thus the results of experimental transmission of the disease in the hamsters are similar to the results in pigs, where diseased mucosa frequently result in successful transmission, yet the cultivated Campylobacters associated with the disease, C. jejuni in the case of hamsters, is incapable of initiating the lesions.

1.14 Relationship of PE in pigs and hamsters

Many previous experimental studies have been confined to the inoculation of hamsters with infective material of PE of hamster origin. Some workers (McOrist and Lawson, 1987) have shown that the intracellular bacteria of pigs can induce PE in hamsters, which therefore confirms that interspecies transmission can occur. Hamsters dosed with ilea of pigs affected with PHE developed pronounced hyperplasia of the crypt enterocytes whereas none of the hamsters dosed orally with cultures of C. mucosalis or C. hyointestinalis of porcine origin or C. jejuni of hamster or porcine origin developed PE. With the exception of C.

jejuni of hamster origin, all other Campylobacter spp. tested did not significantly colonise the intestines. Hyperimmune sera and monoclonal antibody against the intracellular bacteria purified from lesions of PE in pigs detected the intracellular presence of the bacteria in tissue sections of hamster dosed with diseased pig ilea (McOrist and Lawson, 1987; McOrist et al., 1989). These observations illustrates that hamsters are susceptible to the intracellular bacteria of porcine origin and that neither C. mucosalis, C. hyointestinalis nor C. jejuni from pig with PE colonise the intestines of hamsters.

1.15 Ultrastructural studies of PE in hamsters

Ultrastructural studies of naturally and experimentally-induced PE in hamsters have also been conducted. Affected hamsters showed numerous free curved bacterial profiles within the apical cytoplasm of proliferating ileal crypt epithelial cells (Wagner, et al., 1973; Johnson and Jacoby, 1978). Bacteria lay free in the cytoplasm and were not contained within membrane bound structures. The work by Johnson and Jacoby (1978) has

provided some information on the pathogenesis of the disease in hamsters. Hamsters were dosed orally with crude ileal homogenate of the spontaneous disease in hamsters, sequentially euthanised and the development of lesions studied. These workers showed that intracellular parasitism of the bacteria in the apical cytoplasm of the crypt enterocytes precedes the onset of hyperplastic lesions. Bacteria were seen replicating by binary fission and accumulated in progressively greater numbers free in the cell cytoplasm of proliferating cells. However, bacterial entry was not observed although morphologically identical bacteria were seen in the crypt lumen.

1.16 Ultrastructural comparison

of the disease in pigs and hamsters

The first attempt at immunological identification of the intracellular bacteria in ultrastructural studies of PE in pigs and hamsters both dosed orally with filtrate of homogenised affected mucosa of pigs with PE was conducted by a group of researchers in the United Kingdom (McOrist et al., 1989). The work provided additional evidence of the

pathogenesis of the disease. Diseased intestinal tissues of gnotobiotic pigs and conventional hamsters, were compared by electron microscopy and specific immunogold labelling (McOrist et al., 1989). Three of four gnotobiotic pigs and two of four groups of conventional hamsters dosed with 0.65µm filtrate of the diseased mucosa developed marked crypt hyperplasia of the ileum and the proximal colon at 10 and 21 days post-infection. Undosed hamsters and pigs did not develop any lesions.

In pigs and hamsters, 10 days after inoculation, numerous intracellular bacteria were seen in the hyperplastic crypt enterocytes and adjacent crypt lumina. The morphology of the luminal bacteria was similar to that present in the lesions and some were closely associated with the microvillus brush border. However, bacterial entry into the cells was not observed. Some bacteria were observed dividing by transverse binary fission in the proliferative mucosa.

At 21 days post-infection, in both pigs and hamsters, bacteria were only observed in the lesions; no luminal

bacteria were evident. Immunogold labelling with the monoclonal antibody prepared from the pig organism showed staining of the bacteria only in the cytoplasm of the affected cells at both day 10 and 21 after inoculation. None of the luminal organisms were specifically stained (McOrist, 1988).

These observations suggests that the bacteria of PE may undergo antigenic changes during entry from the crypt lumen into the crypt enterocytes, expressing antigens which are reactive with the monoclonal antibody only when they are present in the intracellular location. Other possible explanations for this site-specific intracellular labelling are either that antigenic sites of extracellular bacteria are damaged during the process of fixation or that the extracellular bacteria are Campylobacter spp. and are distinct from the intracellular organism.

1.17 Attempts to cultivate the intracellular bacteria of PE in vitro

Despite the successful reproduction of the disease with

crude or filtered inocula, attempts to isolate and identify the bacteria by culture in conventional cell free media have been consistently unsuccessful (McOrist and Lawson, 1987; McOrist et al., 1989; McOrist et al., 1990). PHE inocula used to reproduce PE contained numerous Campylobacter-like forms yet failed to yield bacteria on culture recognised by the monoclonal antibody to the intracellular organism (McOrist et al., 1987). In addition, cultivation of intestines of porcine gnotobiotics infected with PHE filtrates, yielded a variety of Campylobacter spp. and none of these organisms were recognised by the monoclonal antibody to the intracellular bacteria (McOrist et al., 1987; McOrist and Lawson, 1989).

Such cultural results led to the conclusion that the intracellular bacteria, apart from being antigenically and genetically distinct may require special growth conditions not satisfied by conventional cell free culture media.

1.18 Association of obligate

intracellular bacteria with the host cell

Repeated failure to cultivate the bacteria associated with PE and the consistent observation of the bacteria within the cytoplasm of affected cells suggests that the intracellular organism is an obligate intracellular bacteria. Obligate intracellular bacteria unlike facultative intracellular bacteria (eg: Brucella, Listeria, Salmonella, and Mycobacteria spp.) are truly professional intracellular bacterial forms. Microorganisms in the Orders Chlamydiales (Genus Chlamydia) and Rickettsiales (Genus Cowdria, Coxiella, Ehrlichia and Rickettsia) and also Bacillus piliformis are examples of some known obligate intracellular bacteria that strictly require eukaryotic cells for their growth. Inability to multiply extracellularly implies that the intracellular environment and metabolism is crucial to their growth and multiplication. The cytoplasm of a living host cell contains a complex of nutrient pathways some of which may be available to intracellular microorganisms. However, life in the intracellular environment is not always hospitable (Moulder, 1974). The host cell is defensively equipped with killing mechanisms that can quickly destroy invading bacteria. A series of events in the host cell,

that usually results in death of the invading bacteria are triggered following bacterial ingestion (Elsback and Weiss, 1983; Horwitz, 1982), prevents exploitation of the host cell. These microbicidal events embody oxidative killing with an increase in oxygen consumption (respiratory burst) that inevitably leads to the production of destructive products (hydrogen peroxide, superoxide radical and oxygen-derived radicals); or non-oxidative killing which occurs when lysosomes fuses with a bacteria laden phagosome with subsequent release of potent acid hydrolases (glycosidases, proteases, and lipases). These potential microbicidal events are triggered by microorganisms that lack the capability for intracellular survival (Elsback and Weiss, 1983; Horwitz, 1982). An obligate intracellular bacteria must therefore be equipped to evade the lethal consequences of their obligate intracellular restriction.

The ability of obligate intracellular bacteria to survive in this environment implies that they have evolved special survival traits (Moulder, 1974). As a result of their evolutionary transition to an intracellular existence, all obligate intracellular bacteria have adapted

to deal with the same set of basic problems experienced during the life-cycle of infection; namely how to i) enter the host cell ii) avoid host cell microbicidal mechanisms once inside the host cell iii) multiply intracellularly iv) maintain host functions essential for parasite multiplication v) release the new generation of parasite from the host cell and vi) transfer from one cell to another (Moulder, 1974, 1985). Although these events of infection are common to all intracellular parasitism, different obligate cell dependant organisms have evolved a diverse range of life-cycles which overcome the problems of cell dependant growth in different ways (Moulder, 1985; Williams and Vodkin, 1987).

1.19 Life-cycle of obligate intracellular bacteria

The life-cycle of obligate intracellular bacteria begins at the surface of the host cell. The initial events of this host-parasite relationship involve the recognition of specific adhesion molecules and receptors on the surfaces of bacteria and host cells respectively, followed by bacterial attachment and host cell entry (Williams and

Vodkin, 1987). These workers termed these initial events of host-parasite interaction the process of bacterial uptake.

However, being cell dependant, the main aim of an obligate intracellular bacterium is to reach the preferred site of the host cell (Table 1). The choice of intracellular residence varies; either in the phagosome, phagolysosome or free in the cytoplasm. Following entry, however, the bacteria have first to evade the host defence mechanisms. Intracellular parasites have evolved several tactics to gain access to the preferred sites; some quickly escape into the cytoplasm from the confines of the phagosome or entry vacuole; others are capable of inhibiting the fusion of the phagosome with the lysosome or allow fusion of the entry vacuole with the lysosome (phagolysosome) and remain in this compartment throughout their life-cycle (Moulder, 1985; Williams and Vodkin, 1987). After residing and multiplying in their chosen intracellular location, extracellular release of bacterial progeny occurs before the bacteria initiate a fresh infection in another host cell (Doughri, Storz and Altera,

Table 1. Some characteristic features of intracellular parasitism of known obligate intracellular bacteria

Genus	Intracellular replicating site	Host target cell	Arthropod transmission	Intracellular survival		
				Release from phagosome	Inhibition of phagolysosomal fusion	Allowing phagolysosomal fusion
Chlamydia	Phagosome	Epithelial	No	No	Yes	No
Cowdria	Phagosome	Endothelial Reticuloendothelial	Yes	No	Yes	No
Ehrlichia	Phagosome	Mononuclear ^a Polymorphonuclear ^b	Yes	No	Yes	No
Coxiella	Phagolysosome	Macrophage Epithelial	Yes	No	No	Yes
Rickettsia	Cytoplasm	Endothelial	Yes	Yes	No	No

References: Moulder (1985), Williams and Vodkin (1987)

^a *Ehrlichia canis, sennetsu and risticii*

^b *Ehrlichia equi and phagocytophila*

1972; Ewing, Takeuchi, Shirai and Osterman, 1978; De La Maza and Peterson, 1982; Jongenjan, Zandbergen, Van De Wiel, Groot and Uilenberg, 1991).

The differences in the choice of intracellular residence may reflect not only survival mechanisms but metabolic requirements. It also suggests that the content of each preferred intracellular location plays an important role in the growth and development of the microorganisms.

1.20 Strategies adopted by obligate

intracellular bacteria for intracellular survival

Inhibition of fusion of phagosome with the lysosome

Chlamydia are examples of obligate intracellular bacterium that are capable of intracellular survival by preventing the fusion of phagosomes with lysosomes. The life-cycle in all Chlamydia spp. involves a unique developmental cycle (Bedson and Bland, 1932; Bland and Canti, 1935; Rake and Jones; 1942; Grayston, Kuo, Wang and Altman, 1986) which occurs within the confines of the unfused phagosome. The process of inhibition of fusion

requires viable bacteria. The work of Friis (1972) has provided evidence that heat inactivated Chlamydia psittaci enters the phagosome which later fuses with lysosomes.

Persistence in the phagolysosome

Coxiella burnetti is an obligate intracellular bacterium that multiplies in phagolysosome after fusion. This implies that these microorganisms are able to resist the microbicidal effect of this intracellular compartment without escaping free into the cytoplasm (Hackstad and Williams, 1981; Roman, Coriz and Baca, 1986). Phagolysosomal vacuoles containing these bacteria maintain an acidic pH for prolonged periods in vitro (Maurin, Benoliel, Bongrand and Roullet, 1992). The acidic environment of the phagolysosome is essential for their multiplication. In this acidic environment, metabolism and growth of the organism were enhanced (Hackstad and Williams, 1981). Neutralisation of the acidic condition of the phagolysosomes with alkalinizing agents such as chloroquine, methylamine and ammonium chloride inhibit parasite multiplication and enhanced the bactericidal

effect of doxycycline on the bacteria (Hackstad and Williams, 1981, Raoult, Drancourt and Vestris, 1990).

Release from the phagosome into the cytoplasm

Members of the genus *Rickettsia* grow free in the cytoplasm and occasionally in the nucleus of the host cell (Moulder, 1985). These organisms are known to escape rapidly from the phagosome into the cytoplasm and in doing so avoid the host microbicidal activities (Rikihisa and Ito, 1980, 1982). The ability to escape from the cytoplasm is accompanied by the production of membrane lytic product by the bacteria (Winkler and Miller, 1982).

1.21 Possible cultivation of the intracellular bacteria of PE in vitro

Facultative intracellular parasites are capable of growth in cell free media. The intracellular bacteria of PE is an obligate intracellular bacteria and at the present time cannot be grown in traditional bacteriological media. An excellent example of the potential benefits of an in

vitro culture system for growth and isolation of an obligate intracellular bacteria is the genus Chlamydia. Chicken embryos have been used to culture Chlamydial agents. The pioneering work of cultivation was conducted by Burnett and Rountree in 1935, who inoculated this bacterium into the chorioallantoic membrane of fertile embryos. Small, opaque, pox-like lesions containing Chlamydial elementary bodies were observed. Later Rake, McKee and Shaffer (1940) discovered that this agent can also multiply in the yolk sac of the developing chicken embryo. However, both of these methods of cultivation have limitations and the work by Gordon, Harper, Quan, Treharne, Dywer and Garland, (1969) attracted scientific attention to a more sensitive and convenient technique, that of cell culture. These workers showed that cell culture is four time more sensitive than egg culture in detecting Chlamydial infection. Chicken embryo inoculation is a laborious and time consuming procedure. Susceptibility to infection may not be uniform as this decreases with age of the embryo (Stewart, 1962).

Cell culture has served as an important model for the

investigation of many aspects of Chlamydial-host-parasite interaction (Moulder, 1991). This include the Chlamydial developmental cycle of infection (Bedson and Bland, 1932; Bland and Canti, 1935; Ward, 1983), mechanisms and methods of host cell entry (Gregory, Byrne, Gardner and Moulder, 1979; Murray and Ward, 1984; Hodinka and Wyrick, 1986), and release (De La Maza and Peterson, 1982; Todd and Caldwell, 1985), bacterial morphology (Gordon, Quan and Trimmer, 1960; Matsumoto and Manire, 1970; Chi, Kuo and Grayston, 1987), growth and nutritional requirements (Weiss and Wilson, 1969; Kuo, 1978), and antibiotic sensitivity (Gordon and Quan, 1962; Hobson, Stefanidis, Rees and Tait, 1982).

1.22 The cultivation of the intracellular bacteria of PE in vitro

The cultivation of obligate intracellular bacteria in vitro has not proved straightforward. Many parasite, host and micro-environmental factors can affect successful and satisfactory infection (Evans and Woodland, 1983). The capacity of the lymphogranuloma venereum Chlamydial agent

to infect cultured cells, for instance, depends on the presence or absence of mechanical assistance (Pearce and Ainsworth, 1981). Infection is poor unless it is assisted by centrifugation onto the cell monolayer. Bacterial adhesion and infectivity depends on the surface charge of the bacteria and host cells. Host cell pre-treated with positively charged chemical agents, such as diethyl aminoethyl dextran, can enhance attachment and infectivity of the trachoma agent (Kuo, Wang and Grayston, 1972, 1973). Cell lines differ in their susceptibility to C. trachomatis (Croy, Kuo and Wang, 1975), although many cell lines have been tested, Hela-229 cell appeared to be the most susceptible.

Micro-environmental factors such as pH of the culture medium and temperature of incubation have been shown to influence infectivity. An optimum pH is essential and variation in pH of the culture medium can suppress the number of Chlamydial inclusions detected (Harrison, 1970; Johnson and Hobson, 1976). Centrifugation of the trachoma agent onto the cell monolayers at 33°C increases the number of inclusions detected in comparison with centrifugation at

20°C (Rota and Nicholls, 1973).

Cultivation of the obligate intracellular bacteria of PE may embrace similar problems. Additionally, preparation of inocula for cell culture infection should take into account the existing commensal flora of the gut. This demands the purification of the inocula by filtration or other means prior to infection. Also, the type of cell line to be utilised for infection should consider the cell tropism of the bacteria, in the case of the intracellular bacteria of PE, the intestinal epithelial cell.

1.23 Taxonomic identification of the intracellular bacteria of PE

Concurrent with this Ph.D study, the application of further molecular analysis of the taxonomic relationships of the intracellular organism have been successfully accomplished by a group of workers (Gebhart, Barns, McOrist, Lin and Lawson, 1993). Sequencing of the 16S rDNA of the bacterial genome following amplification by polymerase chain reaction established that the bacteria

showed an unexpected genetic relationship. Comparison of base sequence with those held in current databases showed closest similarity to the delta subdivision of Proteobacteria with 91% homology with a sulphate reducing bacteria, known as Desulfovibrio desulfuricans. Comparison of the amplified 16S rDNA sequence of the intracellular bacteria and those of other known Campylobacters and some other bacteria including Helicobacter pylori showed no specific relationship. A hypervariable region unique to the intracellular bacteria was also identified in the sequence. An oligonucleotide probe to this sequence which was synthesised and labelled with digoxigenin revealed specific hybridization only to affected porcine intestines containing intracellular bacteria and not other intestines. Despite these genetic relationships the properties of the Desulfovibrio and the intracellular organism are quite disparate, and there is doubt as to the identity and taxonomic location of an appropriate genus.

This has led to the proposal of the vernacular name Ileal symbiont intracellularis for the bacteria. This solution is not satisfactory and it seems likely that a new

genus will be created in the future; the tentative name "Ileobacter" intracellularis will therefore be used in this thesis.

1.24 Objectives of the present study

I. intracellularis is apparently dependant on eukaryotic cells and produces immature cell proliferation in the natural disease. This is unusual for obligate intracellular bacteria. Evaluation of the mechanisms of cell infection would be aided by the development of a defined cell culture infection system. The initial cultivation of this agent was pioneered by DR. G.H.K. Lawson (personal communication) who achieved bacterial multiplication in a cell culture system. Important aspects of cell culture infection have already been explored at the commencement of this thesis, namely: growth and maintenance in cell culture, assessment of the susceptibility of cell lines, suppression of cell culture contamination, early quantitation of cell culture infection and establishment of immunological detection of bacteria in infected cultured cells. However, cultivation of the intracellular organism

was at this stage in its infancy, yields of bacteria from infected cell culture were low and passage often erratic.

The aim of this research is therefore to:

- i) evaluate the dynamics of cell culture infection in order to maximise infection,
- ii) ascertain the ultrastructural morphology of the intracellular bacteria grown in cell culture,
- iii) examine the ultrastructural events of intestinal cell infection in vitro,
- iv) test the pathogenicity of the intracellular bacteria grown in cell culture in hamsters,
- v) evaluate the pathology of and the ultrastructure of the intracellular bacteria in infected hamsters and
- vii) compare the ultrastructural events of cell culture and hamster infections in an effort to assess the validity of the model system.

It is therefore anticipated, that this study will provide a better understanding of the pathogenesis of the disease. This will additionally provide a sound foundation for further research into the Proliferative Enteropathies.

CHAPTER 2
GENERAL MATERIALS AND METHODS

GENERAL MATERIALS AND METHODS

This chapter presents the general materials and methods that were routinely employed throughout this study. Further details and specific additional techniques are described in the relevant Chapters.

2.1 Growth of IEC-18 cell line

Rat small intestinal epithelial cells (IEC-18, American Type Culture Collection CRL 1589) were used throughout the study and were grown in Dulbecco's Modification of Eagles medium (DMEM, Gibco no. 074.021.00) supplemented with L-glutamine (2mM), amphotericin B (2.0ug/ml) and 10% vol/vol foetal calf serum (FCS). 25cm² tissue culture flasks were seeded with 5ml of cell suspension containing 0.5×10^5 cells ml⁻¹, and incubated at 37°C in an atmosphere of 5% CO₂ and 21% O₂. Cells were observed daily for confluence with a phase contrast microscope.

2.2 IEC-18 cell line passage

The cells were passaged by trypsinisation (Paul, 1975) at weekly intervals. The growth media was discarded and the monolayers washed briefly with 1.0ml of 0.02% ethylenediamine tetra-acetic acid (EDTA) (Flow, U.K.) in physiological saline. The EDTA solution was decanted and the monolayers were covered with 5ml of 0.05% trypsin/0.02% EDTA (Flow, U.K.) for 90 seconds. After decanting the trypsin/EDTA solution, the flask was placed in an incubator at 37°C for 10 minutes. Five ml of freshly prepared growth media with supplements as described was pipetted into the flasks and the cells detached from the flasks by further gentle pipetting. Cell concentration was estimated with a Neubauer counting chamber and adjusted to a concentration of 0.5×10^5 cells ml⁻¹. Five ml of this cell suspension was then added to 25cm² tissue culture flasks and the cells incubated at 37°C for further passage for experimental infection.

2.3 Preparation of IEC-18 cell line for infection

Cells were prepared for infection by trypsinisation, and seeded at the required cell concentration onto 16mm

glass or 13mm diameter plastic (Thermanox) coverslips (Miles Scientific, U.K.) in universal containers and in 25 cm² tissue culture flasks. I. intracellularis purified from the lesions of PE was suspended in media and used to infect cells at variety of times after establishment of the monolayer.

2.4 Inocula for IEC-18 cell line infection

The inocula were prepared from pigs which died naturally of PE in 1ml vials and stored at -70°C until required for IEC-18 cell line infection (Table 2). For preparation of pig-derived bacterial inocula, mucosas that had histologically confirmed PE were processed fresh or thawed from storage (-70°C). The intestines were opened, the affected mucosa removed by scraping and homogenised with a blender in 5ml saline. An equal volume of 1% trypsin (Difco, U.K.), at pH 7.4 was added and the suspension was incubated in a water bath for 35 minutes at 37°C. DMEM (40ml) or sucrose potassium glutamate (SPG) with 10% FCS was added to 10ml of trypsinised mucosa and the diluted tissue ground in a tissue grinder, filtered through

Table 2. Some features of *I. intracellularis* inocula used for IEC-18 cells infection

Strain	Farm of origin	Age of animal (mth)	Storage (-70°C) history ^a	Intestinal smear	Dilution
871/86	Mountmarle	6	Prepared after 3 years of storage and stored frozen ^b	++	1/12
1482/89 (NCTC No. 12656)	Mountmarle	4	Prepared fresh and stored frozen ^c	+++	1/12
1378/90	Easter Howgate	4	Prepared fresh and stored frozen ^d	+++	1/12
916/91 (NCTC No. 12657)	Easter Howgate	3	Prepared fresh and stored frozen ^e	+++	1/10

^a Terminal ileum of pigs affected with PHE

^{b,c,d} DMEM with 10% FCS as diluent plus 10% DMSO to final volume

^e SPG with 10% FCS as diluent

++,+++ Quantitative assessment of numbers of bacteria observed on smears after Modified Ziehl Neelsen staining
NCTC National Collection of Type Cultures

a 200-mesh stainless steel filter, through a glass fibre filter (Whatman GF/D) and finally through 1.2, 0.8, and 0.65µm pore-size membrane filters (Millipore, U.K.). For storage at -70°C, dimethyl sulphoxide (10%) was added to filtered preparations diluted in DMEM.

2.5 IEC-18 cell line infection

Primary infection

Filtered pig-derived bacterial suspensions were rapidly thawed and mixed with growth media and supplements including antibiotics and used to infect IEC-18 cell monolayers on coverslips and/or tissue culture flasks. The inoculated cell monolayers were centrifuged at 2,020 x g for 30 minutes and then incubated microaerobically in steel jars which were partly evacuated with a vacuum pump and replaced with hydrogen and carbon dioxide to give a final gas mixture of 7.0% O₂ and 10.0% CO₂.

Passage of infection

Infection was passaged by lysing infected IEC-18 cell

monolayers grown in tissue culture flasks by treating the cells with potassium chloride (KCl). The growth medium in the flasks was decanted and replaced with 5ml of 0.2% KCl in distilled water for 5 minutes, the supernatant again decanted, and the monolayer then treated with 0.1% KCl for 30 minutes at 37°C in an incubator. The KCl was decanted and replaced with freshly supplemented medium. The infected cell monolayers were then detached from the plastic with a cell scraper and the suspension drawn into and expressed from a 1 1/2 inch 19 G needle connected to a 10ml syringe six times. The cell lysate and intact bacteria were then diluted in growth media and supplements and used to infect IEC-18 cell monolayers with centrifugation (2,020 x g) for 30 minutes, and incubated as previously described.

2.6 Monoclonal antibody for immunological identification of the intracellular bacteria

Specific mouse monoclonal antibody IG4 was used throughout to identify the intracellular bacteria in immunological tests. This monoclonal antibody had been

prepared previously as described by McOrist et. al., (1987). Briefly, filtered and washed bacteria from histologically confirmed PHE intestinal mucosa had been used to immunise BALB/c mice. Spleen cells from each mouse were fused with mouse myeloma cells. Hybridoma cell supernatant fluids showing positive reactions in an immunofluorescence assay with intracellular bacteria antigen were cloned and grown in bulk in vitro. Mouse ascites fluid was prepared by standard methods using cloned hybridoma IG4 cells.

2.7 Necropsy procedure

Pigs

The mucosas of pigs used for preparation of bacterial inocula for IEC-18 cell infection (Section 2.4) were examined for PE by necropsy removal of portions of the terminal ileum which were fixed in 10% buffered formalin. Sections of these tissues were prepared by routine methods and stained with haematoxylin and eosin and silver staining (Section 2.14) to confirm the presence of proliferative lesions and intracellular bacteria.

Hamsters

All hamsters were euthanised by intracardiac injection of a solution of sodium pentobarbitone. After death, a mid line incision was made to expose the abdominal viscera. The entire small intestines and portions of the caecum and proximal colon were immersed in 10% buffered formalin to be processed for light microscopy. The small intestine was also flushed with buffered formalin using a 19 G needle connected to a 10ml syringe to remove intestinal contents, and opened longitudinally to expose the mucosal surface. Gross observations were recorded and the entire small intestine was then rolled onto a wooden applicator stick and fixed in place with a pin. This technique enables the whole length of the small intestine to be examined on one slide. For electron microscopic examination, 1mm³ intestinal samples were taken from the terminal ileum, caecum and proximal colon and fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) (Appendix AI) at 4°C overnight. The samples were then washed at 10 minutes intervals with two changes of cacodylate buffer alone and processed for transmission electron microscopy (TEM)



(Section 2.11).

2.8 Immunofluorescence staining

Infected IEC-18 monolayers

Infected cell monolayers on 16mm glass coverslips were harvested at appropriate times, washed in Locke's balanced salt solution (LBSS) (Appendix AI) at 37°C for 5 minutes and fixed in two changes of acetone for 60 seconds. The coverslips were then mounted with glue (Loctite, U.K.) on glass slides and allowed to dry before staining. The cell monolayers were washed briefly with phosphate buffered saline (PBS) solution (Appendix AI), then incubated with ascites monoclonal antibody IG4, diluted 1/200 in PBS, for 30 minutes in a moist chamber at 37°C. After washing in three changes of PBS for 30 minutes, the cell monolayers were incubated with sheep anti-mouse IgG fluorescein isothiocyanate (FITC) conjugate (Sigma, U.K.), diluted 1/20 in PBS, for 30 minutes in a moist chamber at 37°C. The cell monolayers were washed three times for 30 minutes in PBS and counterstained with Erichrome Black (1/20 dilution in PBS) for 30 seconds, washed for 5 minutes in PBS and

mounted with glass coverslips in 9:1 glycerol/PBS and observed with a fluorescence microscope.

Smear preparation

Cell culture derived inocula for in vitro and in vivo infections were lightly smeared on glass slides, air dried, fixed in two changes of acetone for 60 seconds and stained by immunofluorescence as described in Section 2.8 but the counterstaining with Erichrome Black stain was omitted.

Paraffin sections

Immunofluorescence staining of formalin-fixed paraffin sections of intestinal tissue was performed as described by Lawson et. al., (1985). Sections (5 μ m) were cut and placed in an incubator at 37 $^{\circ}$ C overnight and dewaxed on the day of staining with 50/50 alcohol/water. The sections were rinsed briefly in distilled water, treated twice with 0.1% wt/vol trypsin (Difco, U.K.) containing 0.1% calcium chloride in distilled water (pH 7.8) for 5 minutes on each occasion at 37 $^{\circ}$ C in a moist chamber. The trypsin solution

was drained and the sections were washed with three changes of PBS for 30 minutes at room temperature. Ascitic monoclonal antibody IG4, diluted 1/200 in PBS, was applied for 30 minutes at 37°C in a moist chamber. The sections were washed with three changes of PBS for 30 minutes and then incubated at 37°C in a moist chamber with sheep anti-mouse IgG FITC conjugate, diluted 1/40 in PBS, for 30 minutes. The sections were then washed in three changes of PBS for 30 minutes and mounted with 9:1 glycerol/ PBS and observed with a fluorescence microscope.

2.9 Immunoperoxidase staining

Immunoperoxidase staining was conducted on IEC-18 cells infected with intracellular bacteria and processed for light and electron microscopic observation.

For light microscopy, IEC-18 cell monolayers on 16mm glass coverslips were washed with LBSS for 5 minutes and fixed in two changes of acetone for 60 seconds. After fixation, the monolayers were briefly rinsed in PBS and washed in two changes of PBS/azide solution (Appendix AI)

for 30 minutes to block endogenous peroxidase. The cell monolayers were then rinsed with two changes of PBS for 20 minutes, followed by immunoperoxidase buffer (IPX buffer) (Appendix AI) for 10 minutes before incubation for 30 minutes with monoclonal antibody with IG4, diluted 1/200 in PBS, at 37°C in a moist chamber. This was followed by a brief rinse in PBS and two changes of IPX buffer at 10 minutes intervals. Anti-mouse IgG horseradish peroxidase conjugate (Scottish Antibody Production Unit), diluted 1/25 in PBS, was flooded onto the monolayers for 60 minutes at room temperature in a moist chamber. Subsequent rinsing in IPX buffer and PBS was carried out for 10 minutes each. The monolayers were then treated with two changes of 3,3 - Diaminobenzidine tetrahydrochloride (Sigma, U.K.) (Appendix AI) for 5 minutes, rinsed briefly in distilled and tap water and counterstained with haematoxylin using the standard method for paraffin sections except that the initial treatment with alcohol and acetone and eosin staining were omitted.

For electron microscopic examination, infected monolayers were grown on 13mm Thermanox plastic coverslips,

then washed in LBSS for 5 minutes, fixed in 1% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) for two hours at 4°C, and permeabilised in 0.5% Triton-X-100 (Sigma, U.K.) in 0.1M sodium cacodylate buffer for 30 minutes. The cell monolayers were washed twice in two changes of cacodylate buffer for 10 minutes, and stained by the immunoperoxidase staining technique (Section 2.9), then further processed for TEM (Section 2.11).

2.10 Immunogold staining

Pre-embedded immunogold staining

The pre-embedded immunogold staining conducted was developed with the help of the manufacturer, BioCell, U.K.. IEC-18 cell monolayers on Thermanox coverslips were fixed in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4°C for two hours and permeabilised in 0.5% Triton-x-100 in cacodylate buffer for 30 minutes at room temperature. The cell monolayers were then washed in two changes of Tris-casein buffer (pH 8.2) (Appendix AI) for 20 minutes. Primary monoclonal antibody IG4 was then applied to the infected cell monolayers and incubated for two hours

at room temperature in a moist chamber using 1/200 dilution in Tris-casein buffer. The cell monolayers were subsequently washed in 5 changes of Tris-casein buffer for 50 minutes and incubated with goat anti-mouse IgG 1nm gold conjugate (BioCell, U.K.), diluted 1/100 in Tris-casein buffer, for two hours in a moist chamber at room temperature and washed again in the same buffer 5 times for 50 minutes. Post-fixation in 1% glutaraldehyde in distilled water was carried out for 10 minutes with consequent washings in two changes of 0.1M sodium cacodylate buffer for 20 minutes. Samples were then processed for TEM (Section 2.11).

Post-embedding immunogold staining

Post-embedding staining was conducted by floating the sections on gold grids on droplets of appropriately diluted reagents or PBS formed on paraffinised strips of paper in a moist chamber.

Sections on gold grids were first etched with 0.5% (w/v) periodic acid for 15 minutes at room temperature,

then washed in tap water for 30 minutes and briefly rinsed in distilled water (McOrist, 1988). After washing briefly in Tris-casein buffer, the sections were then incubated with monoclonal antibody IG4, diluted 1/50 in Tris-casein (pH 8.2) buffer for 90 minutes at 37°C. Excess antibody was removed by washing with four changes of Tris-casein buffer for 40 minutes and the sections incubated in goat anti-mouse IgG 15nm gold conjugate, which was diluted 1/10 in Tris-casein, buffer for 90 minutes at 37°C. Washing with four changes of Tris-casein buffer for 40 minutes was repeated before the sections were stained with lead citrate and uranyl acetate for TEM observation (Section 2.11).

2.11 Electron microscopy

Transmission electron microscopy

Glutaraldehyde fixed tissues or cell monolayers (on 13mm Thermanox plastic coverslips) were processed for TEM using a standard method. Briefly, post-fixation in 1% osmium tetroxide in 0.1M sodium cacodylate buffer (pH 7.4) was conducted for 45 minutes, specimens were then dehydrated through a graded series of an ascending

concentration of acetones before embedding in Araldite. Thin sections (60nm) were cut with a diamond knife on a Reichert OMU 4 ultramicrotome and mounted on 200 mesh copper or gold grids. The sections were stained with saturated uranyl acetate for 30 minutes and lead citrate (Reynold, 1963) for 5 minutes and viewed with a PHILIPS EM400 transmission electron microscope.

Scanning electron microscopy

Infected monolayers on 16mm glass coverslips were fixed in 1% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) for two hours at 4°C. The samples were washed in three changes of 0.1M sodium cacodylate buffer for 90 minutes then osmicated with 1% osmium tetroxide in 0.1M sodium cacodylate buffer for 30 minutes. The samples were then washed with distilled water and dehydrated through a graded series of an ascending concentration of acetones. Critical point drying with carbon dioxide in a POLERON E3000 CPD was conducted followed by sputter coating with 20nm gold:palladium (60:40) layer in an EMSCOPE SC500. Samples were observed with a PHILIPS EM505 scanning electron

microscope.

2.12 Bacterial culture

Infected IEC-18 cell monolayer

Infected cells cultured in universal containers (without coverslips) were washed with Brucella semi solid broth (Difco, U.K.), scraped with a sterile swab and 60ul of the scraped cell suspension was inoculated on Skirrow's medium (Skirrow, 1977) (Appendix AII), Brucella semi-solid broth and Columbia blood agar (Oxoid, U.K.) (Appendix AII) and incubated microaerobically in steel jars at 37°C for up to 7 days. Jars were evacuated with a vacuum pump and the atmosphere replaced with hydrogen and carbon dioxide to give a gas concentration of 6.0% O₂ and 10.0% CO₂ for incubation.

Intestinal swabs

Intestinal swabs were taken from the mucosa of the lower ileum of all hamsters at necropsy after washing the mucosal surface with sterile PBS. These swabs were

inoculated on Skirrow's medium, Columbia blood agar and Brucella semi-solid broth and incubated microaerobically for up to 7 days as described above.

2.13 Bacterial staining

Gram staining

Thin smears of bacteria grown in culture were made on glass slides, air dried and heat fixed. The samples were flooded with methyl violet for two minutes, washed with water and for one minute with Gram's iodine. The slides were washed with water and the samples were decolourised briefly in acetone, washed with water and counterstained with dilute carbol fuschin for 30 seconds. Finally, after washing in water, they were allowed to air dry before observation.

Modified Ziehl-Neelsen staining

Smears of pigs' and hamsters' intestines, cell culture lysates or supernatant fluids were air dried and heat fixed, then treated with dilute carbol fuschin for 15

minutes, washed in tap water, decolourised with 0.5% acetic acid for 10 seconds before being washed again in tap water. The samples were then counterstained with methylene blue for 8 seconds, washed in tap water and allowed to air dry for observation.

2.14 Histological staining

Haematoxylin and eosin

Formalin fixed tissue samples were trimmed into cassettes and processed overnight through a vacuum infiltration automatic tissue processor, embedded in wax, sectioned at 5µm and stained with haematoxylin and eosin and mounted in DPX mounting medium.

Giemsa staining

Acetone fixed cytopsin preparations of cells on glass slides were treated with 1:10 Giemsa in distilled water for 10 minutes, differentiated in distilled water for 5 minutes, briefly dehydrated in isopropyl alcohol, washed in xylene and mounted in with Gurr's neutral mounting

medium.

Young's modification of Warthin Starry silver staining

Silver staining of sections was conducted as described by Young (1969). Paraffin sections were incubated at 37°C overnight, immersed in xylene to dewax and dehydrated through alcohol. The sections were then washed thoroughly in water and rinsed in Young's buffer pH 3.8 (Appendix A1).

Solutions A to E were prepared:

- A. 1% silver nitrate in Young's buffer in a Coplin jar.
- B. 0.2g silver nitrate in 9ml buffer in a universal bottle.
- C. 0.3g quinol in 8ml Young's buffer in a universal bottle.
- D. 3.0g gelatin in 45ml Young's buffer in a conical flask.
- E. 50ml Young's buffer in a Coplin jar.

The sections were incubated in solution A for one hour at 60°C. At the same time, solutions B,C,D and E were incubated at 60°C to allow the quinol and gelatin to dissolve. Sections were transferred to solution E to reduce background deposits and prevent premature reduction

of the silver before the sections were completely developed. Sections were then agitated in a Coplin jar containing a mixture of the stated volumes of solutions B,C, and D.

When the sections became golden brown, the developing solutions were poured off, the sections were washed briefly in warm tap water and rinsed in cold water for 20 minutes. Finally, the sections were dehydrated through a series of alcohol solutions and mounted in Gurr's neutral mounting medium.

CHAPTER 3

ESTABLISHMENT OF A METHOD OF IEC-18 CELL LINE INFECTION BY THE INTRACELLULAR BACTERIA

ESTABLISHMENT OF A METHOD OF IEC-18 CELL LINE INFECTION BY

THE INTRACELLULAR BACTERIA

3.1 General Introduction

PE is an enteric disease of swine in which the identity of the aetiological agent is unclear. Until this time, the cause of the disease has not been resolved (Lawson and McOrist, 1993). Previously, several Campylobacter spp. have been considered as candidates responsible for the disease but failure of experimental transmission with the isolated microorganisms, has inevitably suggested that these agents are not involved in the initiation of the condition. Recently, it has been proposed that an as yet to be cultivated obligate intracellular bacterium, Ileobacter intracellularis might be the causative organism of the disease (Lawson et al., 1985; Lawson, 1991; Gebhart et al., 1993). This implies that experimental confirmation that this agent is the cause of the disease would not be possible unless this bacteria is first cultivated in a cell associated culture system. Cultivation of the organism has been undertaken but the bacterial yield so far achieved is still unsatisfactory (Lawson, personal communication).

The work described in this chapter was designed to examine the dynamics of interaction of this microorganism with the host cell in vitro, using the IEC-18 cell line, the eventual aim being to maximise infection.

3.2 Effect of centrifugation on infection of IEC-18 cells incubated at atmospheric oxygen tension.

3.3 Introduction

Centrifugation is a popular method of enhancing host cell infection by microorganism in vitro. The extent of cell infection of many intracellular microorganisms including Chlamydia, Rickettsia and viruses has been shown to increase when mechanically assisted by this method (Hughes, 1993).

The ability of this technique to enhance IEC-18 cell infection by the intracellular bacteria of PE was investigated.

3.4 Materials and Methods

IEC-18 cell line infection

IEC-18 cells were trypsinised (Section 2.3) and seeded at a cell concentration of 0.25×10^5 cells ml^{-1} on 16mm coverslips in universal containers. After 24 hours, the cells were infected with PE inoculum (Section 2.4) designated, 871/86. The inoculum was processed for IEC-18 cell infection as follows; the inoculum (in 1ml vial) was rapidly thawed at 37°C and diluted in 30ml of DMEM supplemented with L-glutamine, Amphotericin B and 8% vol/vol FCS containing vancomycin ($100\mu\text{g}/\text{ml}$) and neomycin ($50\mu\text{g}/\text{l}$). Aliquots of diluted inocula were used to infect IEC-18 cell monolayers, either with centrifugation at $2,020 \times g$ or without centrifugation for 30 minutes at room temperature. The infected cells were then incubated at atmospheric oxygen tension with 5% CO_2 in an incubator at 37°C and were harvested at 0 hour (immediately after centrifugation), and at days 1, 2, 3, 4 and 5 post-infection, and processed for fluorescence microscopy (Section 2.8). Infected cells were fed with similar growth media and supplements with 5% vol/vol FCS at day 2 and 4 post-infection.

3.5 Results

Immunofluorescence staining showed bright particulate fluorescence when IEC-18 cells, both infected with and without the assistance of centrifugation were specifically stained with monoclonal antibody IG4. In both methods of infection, fluorescence stained curved organisms were observed associated with the cell cytoplasm. However, the number of infected cells and total bacterial counts were higher at all stages of infection in centrifuge assisted infection in comparison to those not infected by centrifugation (see Table 3, Figures 1 and 2). Infection was highest at day 0, immediately after centrifugation and thereafter steadily declined. The number of organisms per cell did not exceed 20 bacteria. Uninfected controls did not show any fluorescence.

3.6 Comment

The first study showed that cells exposed to infection demonstrated bacteria in close association with the cells but all quantitative assessment indicated that bacterial declined thereafter. Centrifugation enhanced IEC-18 infection by the intracellular bacteria. This effect was

Table 3. IEC-18 cells exposed to *I. intracellularis* strain 871/86 incubated at atmospheric oxygen tension

Days PI	Infected cells ^a		Number of bacteria ^b		Number of cells ^c	
	Centrifuge	Non centrifuge	Centrifuge	Non centrifuge	Centrifuge	Non centrifuge
0	0.88 (0-20)	0.20 (0-2)	2.54	1.60	2.27	2.28
1	0.86 (0-13)	0.16 (0-1)	2.53	1.50	2.79	2.76
2	0.78 (0-12)	0.14 (0-1)	1.96	1.41	3.05	3.04
3	0.48 (0-3)	0.14 (0-1)	1.86	1.17	3.14	3.15
4	0.36 (0-2)	0.12 (0-1)	1.84	1.08	3.26	3.28
5	0.28 (0-1)	0.10 (0-1)	1.73	1.00	3.24	3.24

a Percentage of infected cells counted for 1 coverslip, with 50 cells counted on each

b Number of bacteria counted for 1 coverslip, from 10 different areas (0.014 mm²)

c Number of cells counted for 1 coverslip, from 5 different areas (1.5 mm²)

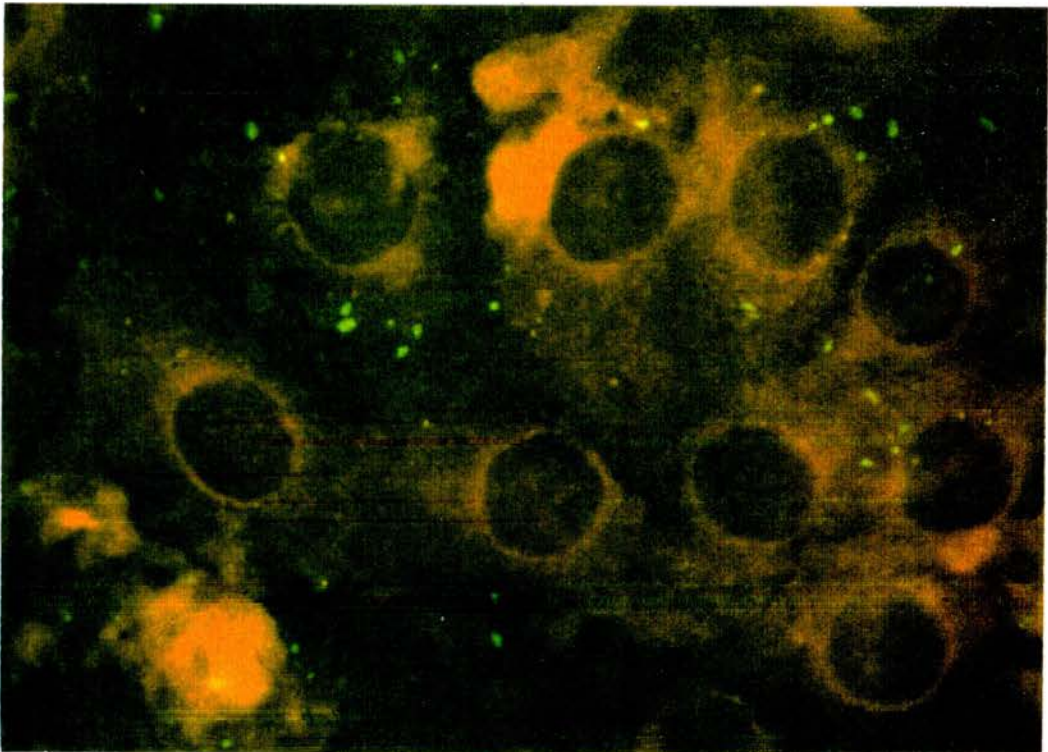
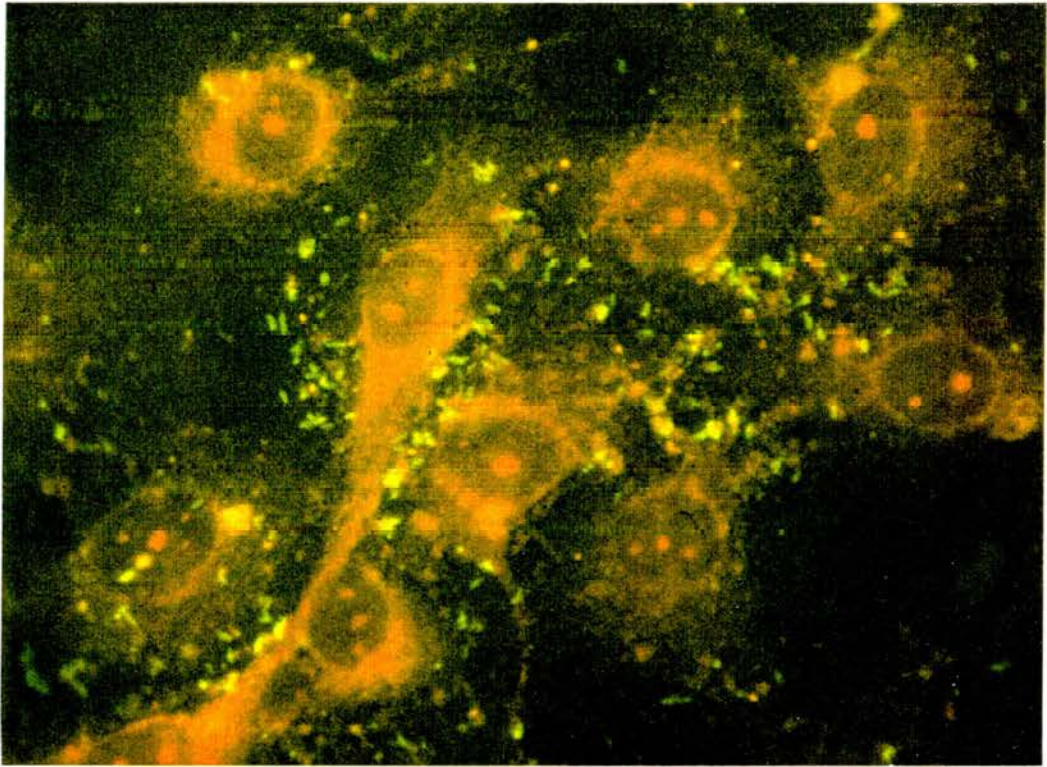
Number in parentheses represents range of number of bacteria per cell

Figure 1

IEC-18 cells infected with the intracellular bacteria by centrifugation incubated at atmospheric oxygen tension (0 hour after centrifugation). Note many infected cells showing numerous brightly fluorescing bacteria. Immunofluorescence staining with monoclonal antibody IG4 and sheep anti-mouse FITC conjugate. Erichrome Black counterstain x 712.

Figure 2

IEC-18 cells infected with the intracellular bacteria at atmospheric oxygen tension at 0 hour post-infection in the absence of centrifugation. Note fewer infected cells showing fewer brightly fluorescing bacteria. Immunofluorescence staining with monoclonal antibody IG4 and sheep anti-mouse FITC conjugate. Erichrome black counterstain x 712.



observed immediately after centrifugation and to an extent thereafter, however there was no evidence of bacterial multiplication as a consequence of centrifugation. This study provides no information on the location of the bacteria (either extracellularly or intracellularly) and a variety of factors could explain failure of the bacteria to multiply.

3.7 Bacterial culture of intracellular bacteria grown in IEC-18 cells infected by centrifugation and incubated at atmospheric oxygen tension.

3.8 Introduction

Before further experiments were undertaken to develop a successful cell based infection system, it is important to ascertain whether purified intracellular bacteria in IEC-18 cells can be cultivated in conventional cell free media. Successful cultivation of the intracellular organism in cell free media would radically alter the strategic approach to the investigation of the disease; despite the assumption of the obligate character of the organism this feature requires to be continually monitored

during early investigation into cell culture. It is also necessary to determine the presence or absence of Campylobacter spp. in the purified inocula and their ability to persist in the cell culture model.

This study was conducted in attempt to culture the intracellular bacteria grown in IEC-18 cells at different times in standard conventional cell free media.

3.9 Materials and Methods

IEC-18 cell line infection

IEC-18 cells were set up on coverslips and infected with intracellular bacteria strain 871/86 as described previously (Section 3.4). However, the initial inocula was diluted 1 in 20ml of growth media and supplements containing 8% vol/vol FCS and antibiotics. Cells were only infected by centrifugation. Bacterial culture of infected IEC-18 cell was also conducted.

Bacterial culture

IEC-18 cells were exposed to infection, sampled at day 2 and 4 post-infection and cultivated in Columbia blood agar, Skirrow's medium and Brucella semi-solid broth as described previously (Section 2.12). The inoculum used to infect the IEC-18 cells was also similarly cultivated on these media. Preparations of the infected cells and inoculum prepared on glass slides were also stained by Gram and Modified Ziehl Neelsen and examined (Section 2.13).

3.10 Results

Bacterial culture of the intracellular bacteria grown in IEC-18 cell line and inoculum used for cell infection failed to yield bacteria although parallel infection of IEC-18 cells showed numerous Campylobacter-like bacteria reactive to specific monoclonal antibody IG4 in immunofluorescence and in Gram and Modified Ziehl Neelsen stained preparations. The bacteria stained Gram-negative and were modified acid fast. The bacteria were rod-shaped, S-shaped or curved. The pattern and level of infection appeared similar to that observed in the previous experiment (Section 3.2) (see Table 4).

3.11 Comment

The intracellular bacteria was incapable of growing in conventional cell free media in a microaerobic atmosphere. This suggests that the bacteria strictly requires eukaryotic cells. These examinations were not exhaustive and require amplification when larger numbers of intracellular bacteria can be obtained. Campylobacters were not recovered from the inocula providing further evidence that these organisms were not related to the intracellular bacterium. The absence of bacterial multiplication in cells was again revealed (see Table 4). Further studies should be conducted to overcome this problem.

3.12 Effect of centrifugation on infection of IEC-18 cells incubated at reduced oxygen tension

3.13 Introduction

Previous attempts to achieve in vitro bacterial multiplication have been unsuccessful. The effect of centrifugation on IEC-18 cell infection appeared to

Table 4. Bacterial culture of *I. intracellularis* strain 871/86 grown in IEC-18 cells (centrifuge only) in cell free media and reassessment of centrifuge infection

Days PI	Bacterial culture	Infected cells ^a	Number of bacteria ^b	Number of cells ^c
0	NR	0.92 (0-19)	2.64	2.29
1	NE	0.90 (0-18)	2.40	2.75
2	NR	0.68 (0-16)	2.25	3.05
3	NE	0.48 (0-6)	1.86	3.15
4	NR	0.30 (0-2)	1.77	3.28
5	NE	0.28 (0-1)	1.76	3.25

^a Percentage number of infected cells counted for 1 coverslip, with 50 cells counted on each

^b Number of bacteria counted for one coverslip, from 10 different areas (0.014 mm²)

^c Number of cells counted for one coverslip, from 5 different areas (1.5 mm²)

NR Not Recovered on Culture

NE Not Examined

increase the numbers of bacteria associated with the cells, but not to allow bacterial multiplication. A host of reasons may account for this failure but the atmospheric requirement of the intracellular bacteria may be a critical contributory factor, it is unlikely that the oxygen tension of the enterocyte approaches that of the atmosphere and bacteria growing in such cells may have adapted to an oxygen concentration lower than atmosphere. The bacteria may require a microaerobic environment to grow. The antibiotics used, in particular neomycin, to suppress bacterial contamination may have an adverse effect on the bacteria. In separate experiments, delaying the use of the antibiotic during infection has been shown to allow an increase in bacterial numbers associated with the cells (Lawson, personal communication). This study was conducted to investigate the effect of centrifugation on IEC-18 cells infection subsequently incubated at reduced oxygen tension.

3.14 Materials and Methods

IEC-18 cell line infection

IEC-18 cells were grown on coverslips as described

previously (Section 3.4) but the cells were infected with PE inoculum strain 1378/90 (Section 2.4). The inoculum was prepared for IEC-18 infection as follows; the inoculum (in 1ml vial) was rapidly thawed at 37°C and diluted in 10ml of growth media and supplements with 8% vol/vol FCS (without antibiotics), refiltered (the stored inoculum is contaminated with small numbers of coliform bacteria and has to be refiltered at 0.65 µm for further purification; (Lawson, personal communication) and made up to a final volume of 40ml. The cells were then infected by centrifugation and incubated microaerobically in anaerobic jars (Section 2.5) for 3 hours, fed with similar growth media and supplements with 8% vol/vol FCS and vancomycin and neomycin. Coverslips were further incubated in jars and harvested at 1, 3, 6 and 8 days, fixed and stained by the immunoperoxidase method (Section 2.9). The infected cells were refed with similar growth media and supplements, containing 5% vol/vol FCS and antibiotics on days 2 and 4 post-infection.

Bacterial culture

Parallel culture of IEC-18 cells were sampled at days 1 and 8 post-infection and cultured on Columbia blood agar, Skirrow's medium and Brucella semi-solid broth as described in Section 2.12. The inoculum for infecting the cells was also similarly cultured on these media. Preparations of both infected cells and inoculum were also stained by Gram and the Modified Ziehl Neelsen method (Section 2.13).

3.15 Results

IEC-18 cells infected by centrifugation and incubated in reduced oxygen tension showed evidence of bacterial multiplication. Unlike previous experiments some cells contained more than 30 specifically stained bacteria. Individual cells might contain around 30 bacteria, in some bacteria were too numerous to count. Such cells were first observed at day 3, and increased in number until day 8 post-infection (see Table 5). Cells containing more than 30 organisms appeared as groups of infected cells, with adjacent cells which were also infected but containing fewer than 30 bacteria (Fig. 3). Many cells on each coverslip remained free of bacteria. Bacteria were

Table 5. IEC-18 cells exposed to *I. intracellularis* strain 1378/90 incubated at reduced oxygen tension

Days PI	Infected cells ^a	Highly infected cells/infected foci ^b	Number of bacteria ^c	Number of cells ^d
1	0.89 (0-21)	0/0	2.93	2.76
3	0.48 (0-8)	8/7	2.13	3.14
6	0.12 (0-1)	51/10	1.20 (+)	2.11
8	0.09 (0-1)	164/51	0.87 (2+)	3.27

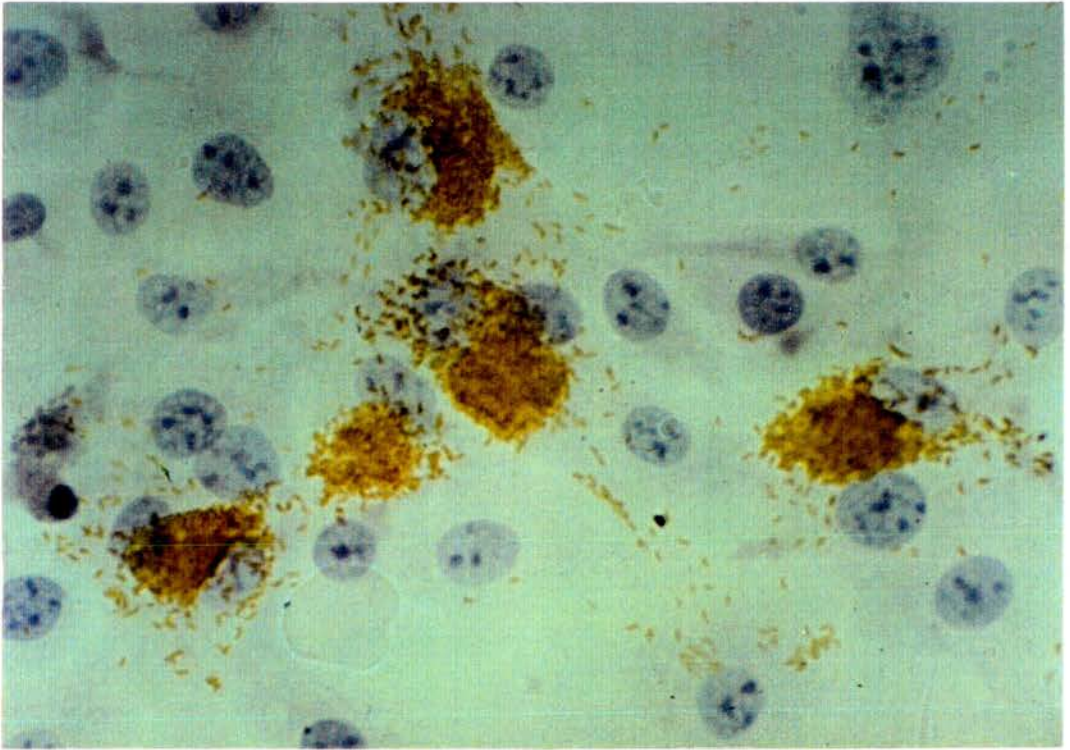
- a Percentage number of infected cells counted for 2 coverslips, with 50 cells counted on each
b Total number of highly infected cells (>30 organism per cell) and infected foci counted for 2 coverslips (200 mm²)
c Number of bacteria counted for 2 coverslips, from 10 different areas (0.0672 mm²)
d Number of cells counted for 2 coverslips, from 5 different areas (1.5 mm²)

Numbers in parentheses represents range in numbers of bacteria per cell

- + Represents bacteria too numerous to count in a single cell

Figure 3

IEC-18 cells infected at day 8 post infection. Note groups of HIC and adjacent cells which are lightly infected. Immunoperoxidase staining with monoclonal antibody IG4 and sheep anti-mouse peroxidase conjugate. Haematoxylin counterstain x 640.



morphologically similar to those observed previously (Section 3.10). Since cells at day 1 post-infection in this experiment, and at day 0 after exposure by centrifugation in previous study (Sections 3.2 and 3.7) did not reveal more than 20 or 21 bacteria, cells with more than 30 bacteria per cell were termed highly infected cells (HIC) and the number of HIC was taken as a measure of bacterial multiplication. HIC were frequently arranged in groups, (infected foci) and this was taken as a measure of infectivity. Each infected focus might contain as many as 28 HIC. Cells unexposed to infection did not show any immunoperoxidase stained bacteria. Conventional culture failed to yield bacteria.

3.16 Comment

This study suggests that microaerobic atmosphere is conducive to *Ileobacter* multiplication. IEC-18 cells were observed highly infected and such cells appear to increase in numbers. However, the percentage of infected cells do not appear to increase which implies that many cells remained uninfected. The techniques of cultivation

employed at this stage of the investigation have proved rewarding but techniques that can enhance yield of bacteria and cell infection should be further developed.

3.17 Effect of passaging infection in IEC-18 cells

3.18 Introduction

Passing microbial infection artificially to new susceptible host cells, in vitro, is a known method of maintaining and increasing infectivity. The bacterial yield from one passage can be used to generate a new cycle of infection in new cells, thus the level of infection can be economically maintained and increased. Pollard, Starr, Tanami and Moore (1960) have reported increase numbers of the Trachoma agent in infected McCoy cells when serially passaged.

This study was conducted to investigate the effect of passage of *Ileobacter* in IEC-18 cells to increase bacterial yield.

3.19 Materials and Methods

IEC-18 cell line infection

IEC-18 cells were set up at a cell concentration of 0.25×10^5 cells ml^{-1} on 16mm coverslips and 25 cm^2 tissue culture flasks as described in Section 2.3. After 24 hours, the cell monolayers were infected with PE inoculum strain 916/91 (Section 2.4). A 1ml volume of the inoculum was rapidly thawed at 37°C and added to 14ml of DMEM and supplements with 7% vol/vol FCS. Diluted inocula were then used to infect IEC-18 cell monolayers by centrifugation (2,020 x g) for 30 minutes. The infected cells were incubated microaerobically in steel jars for 3 hours, fed with similar growth media and supplements containing 7% vol/vol FCS, and vancomycin and neomycin, further incubated in an incubator (8.0% CO₂, 8.8% O₂) at 37°C and passaged at either 5, 6 or 7 days post-infection by treatment with KCl (Section 2.5). 10ml of DMEM and supplements with 7% vol/vol FCS were added to each flask, the cells were mechanically detached and further diluted before infecting fresh monolayers (Section 2.5). Infected cells were refed at days 2 and 4 post-infection with similar growth media

and supplements containing 5% vol/vol FCS, vancomycin and neomycin.

To monitor infection, at each passage, IEC-18 cells on coverslips were also simultaneously infected and harvested (days 5, 6 and 7 post-infection).

To study the development of passage infection, IEC-18 cells on coverslips were infected with bacteria passaged once and harvested at different time intervals (days 1, 2, 4 and 6 post-infection). Harvested coverslips were all stained by immunoperoxidase method as described in Section 2.9.

Bacterial culture

Prepared inocula used to infect IEC-18 cells at every passage were cultured on Columbia blood agar, Skirrow's medium and Brucella semi-solid broth as described in Section 2.12. Preparations of inocula were stained by Gram and Modified Ziehl Neelsen as described in Section 2.13.

3.20 Results

Passage of *Ileobacter* infection in IEC-18 cells results in numerous HIC and the maintenance of the level of infection (see Table 6). The number of infected cells may reach 100%. Discrete infected foci were not observed but infected cells covered the coverslips (Fig. 4).

IEC-18 cells sequentially harvested and observed for infection showed enhanced yields of HIC and infected cells (see Table 7). HIC were observed early, at 24 hours post-infection and increased thereafter. IEC-18 cell density was generally low.

Infection of IEC-18 cells in flasks allowed observation of infected cells in situ. No cytopathic effect, syncytia, vacuoles, or rounding of cells were observed in association with infection. A minority of infected and uninfected cells detached from the monolayer and floating in the culture media, these cells appeared more numerous in infected monolayers. Neither extracellular nor intracellular bacteria were visible in the infected flasks

Table 6. Passage of infection of IEC-18 cells infected with *I. intracellularis* strain 916/91

No of passage	Dilution at passage	Days PI	Highly infected cells ^a	Infected cells cells ^b
1	1/2.8	6	0.40	1.00
2	1/3.2	6	0.46	1.00
2	1/4.0	7	0.16	1.00
3	1/4.0	5	0.15	1.00
4	1/2.0	6	0.21	0.97
4	1/2.0	7	0.18	0.96
5	1/4.0	6	0.17	0.93
5	1/4.0	7	0.16	0.91

^a Percentage of highly infected cells counted for 2 coverslips, with 100 cells counted on each

^b Percentage of cells, counted for 2 coverslips, with 100 cells counted on each

Figure 4

Passage infection of the intracellular bacteria in IEC-18 cells showing numerous infected cells. Immunoperoxidase staining with monoclonal antibody IG4 and sheep anti-mouse peroxidase conjugate. Haematoxylin counterstain x 640.

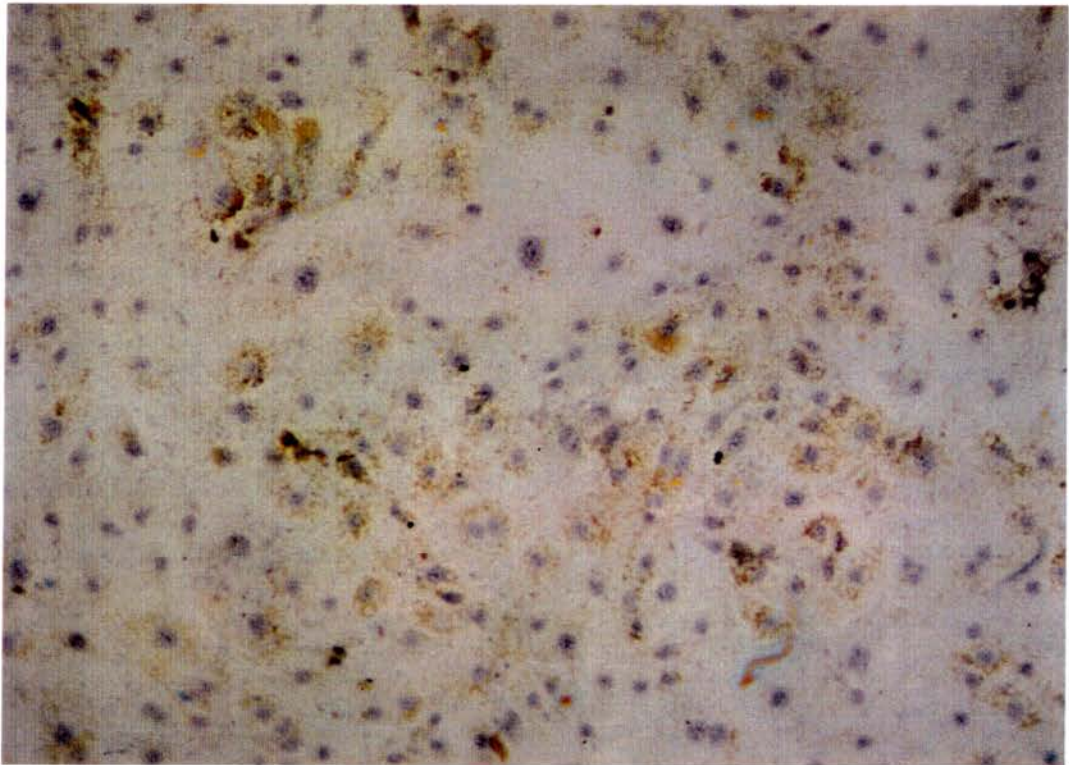


Table 7. IEC-18 cells exposed to *I. intracellularis* strain 916/91: Quantitation of infection with passaged bacteria

Days PI	Highly infected cells ^a	Infected cells ^b	IEC-18 cell density
1	0.07	0.95	1.67
2	0.23	0.88	2.04
4	0.31	1.00	2.02
6	0.46	1.00	1.99

^a Percentage of highly infected cells counted for 2 coverslips, with 100 cells counted on each

^b Percentage of cells infected counted for 2 coverslips, with 100 cells counted on each

^c IEC-18 cells counted from 5 different areas (1.5 mm²)

and coverslips without immunoperoxidase staining. Bacteria stained with haematoxylin were not observed. Bacteria were not cultured from the inocula used to infect the IEC-18 cells when cultured onto conventional cell free media.

3.21 Comment

The bacteria can be passaged in IEC-18 cells and the infection level maintained. HIC cells were numerous, and large numbers of cells were infected. Similar results were obtained in a separate experiment which suggest an inoculum of higher level of infectivity (Lawson, personal communication). HIC were observed earlier at day 1 post-infection when IEC-18 cells were infected with passaged bacteria in comparison to cells primarily infected with the bacteria; HIC were first observed at 3 days post-infection in primary infection (Section 3.15). Passage of infection was harvested at either days 5, 6 or 7; the time of passage infection harvest was based on findings in separate experiments which shows that peak levels of infection generally occurs at 5-7 days post-infection. The number of HIC begin to decline at day 8 post-infection and therefore

harvest of passage infection was not conducted at this time after infection (Lawson, McOrist, Jasni and Mackie, 1993). The success of passaging the organism in IEC-18 cells enables the production and maintenance of infectious material for further experiments in vitro and in vivo.

3.22 Discussion

IEC-18 cells were infected with the intracellular bacteria of PE and the effect of centrifugation, both at atmospheric and reduced oxygen tension were studied. Centrifugation was capable of increasing the level of infection of IEC-18 cells. However, this enhancing effect only resulted in substantial bacterial proliferation when infection was carried out at reduced oxygen tension. The ability of *Ileobacter* to multiply under this atmospheric conditions suggests that the bacteria is a microaerobic microorganism and requires such an environment to grow and multiply. The inability to grow the organism in conventional cell free media suggests that the bacteria are strictly cell dependant.

The benefit of centrifugation in enhancing infection is indisputable. This method of assisting infection has been shown to be a sensitive method of isolation and identifying microorganism. Infection of cell cultures of C. burnetti, Trachoma agent (Weiss and Dressler, 1960), E. coli (Vesikari, Bromerska and Maki, 1982), Shigella flexneri (Sansone, Ryter, Clerc, Maurelli and Mounier, 1986), Rickettsia prowazekii (Weiss and Dressler, 1960), Rickettsia conorii (Peter, Raoult and Gilot, 1990), Yersinia enterocolitica (Vesikari et al., 1982) and many viruses (Huges, 1993) are also increased by centrifugation. The mechanism involved in the enhancement of infection by centrifugation is poorly understood. It is believed that centrifugation have increased the chances of contact between *Ileobacter* and host cells which leads to the internalisation of the bacteria into the host cell. Infection in the absence of centrifugation depends on bacterial random contact or affinity towards specific receptors on the cell surface that mediate entry (Finlay and Falkow, 1989). Thus the number of bacteria that are able to adhere and enter the cells in a unit of time would be less in comparison with centrifuged infection where

large numbers of bacteria are propelled to the cell surface at the same time. In the intracellular location the bacteria undergo replication and the number of progeny produced would depend on the number of bacteria that have entered the cells and would be less in the case of non-centrifuged infection. The possible mechanisms that lead to the enhancement of infection of IEC-18 cell by *Ileobacter* by centrifugation is discussed in Chapters 5 and 6.

Ileobacter infection can be passaged in IEC-18 cells. and the infection level maintained. The important implication of passaging infection is that it serves as a economical method of obtaining and maintaining inocula.

The work in this study suggests that the intracellular bacterium of PE is an obligate intracellular bacterium that can be cultured and passaged in IEC-18 cells.

CHAPTER 4
MORPHOLOGICAL OBSERVATIONS OF THE CELL CULTURE DERIVED
INTRACELLULAR BACTERIA

MORPHOLOGICAL OBSERVATIONS OF THE CELL CULTURE DERIVED

INTRACELLULAR BACTERIA

4.1 Introduction

Since the novel identity of the intracellular organism was first suggested (Lawson et al., 1985), morphological observation of the intracellular bacteria was mostly limited to tissue sections prepared for both light and electron microscopy. These observations were mostly concerned with confirming that the intracellular bacteria in the lesions of the disease were morphologically similar to the Campylobacters that could be isolated from the tissues and identifying antigens which supported this relationship. Although Campylobacters that may be isolated from the lesions have been well described (Rajasekhar, 1981), other than brief descriptions (McOrist et al., 1989) little attention has been paid to the intracellular organism.

Cell culture supports the growth of many microorganisms and until this stage of the investigation, this work has concentrated on the isolation of the intracellular bacteria

and the demonstration that conventional bacteria could not be grown from passaged material. The original cell culture inocula were prepared from field material, there is always the opportunity for other microorganisms to multiply, particularly viruses, in the cultures even though cytopathic effects have not been observed (Chapter 3).

One group of workers (Mapother et al., 1987a) suggested the possible involvement of a viral agent in the lesions of PE. In their experiments, five of eight conventional pigs dosed orally with an 0.22 μ m filtrate prepared from diseased mucosal homogenate of pigs with PE developed gross lesions of haemorrhagic enteritis. Histologically the lesions manifested mild crypt hyperplasia with some inflammatory cell infiltration. Virus particles suggestive of enterovirus were present in the filtered inocula (Mapother, Finn and Joens, 1988), but curved intracellular bacteria were not seen in the apical cytoplasm of crypt epithelial cells of affected pigs.

Others have demonstrated a Chlamydial agent in PE lesions in hamsters (Fox, Stills, Paster, Dewhirst, Taylor

and Yan, 1991). Curved bacteria within enterocytes accompanied Chlamydial elementary bodies in immunofluorescence tests which employed monoclonal antibodies against these agents (Fox et al., 1991). Unusually these Chlamydia appeared to react with C. trachomatis antisera and not C. psittaci. A Chlamydial agent had also been isolated in INT-407 cells infected with extracts of ilea of hamsters with PE (Stills, Fox, Paster and Dewhirst, 1991). Immunological and iodine staining of McCoy cells subsequently infected with this agent (Fox, Stills, Paster, Dewhirst, Yan, Palley and Prostack, 1993), confirmed that it is Chlamydia trachomatis. Infected McCoy cells showed intracytoplasmic inclusions reactive with monoclonal antibody against C. trachomatis and positive with iodine staining.

This study was undertaken for two main purposes. Firstly, to identify the major ultrastructural features of Ileobacter cultured in cells, secondly to assess the possible presence of other cell dependant agents in those cultures.

4.2 Materials and Methods

Preparation of bacterial pellet

IEC-18 cell monolayers in two 75cm² Costar flasks that had been infected with the intracellular bacteria strain 1482/89 (passage 19) and harvested after 7 days of incubation were used to prepare a bacterial pellet. Growth media was removed from the flasks and the monolayers were treated with 15ml of KCl (Section 2.5). The KCl was decanted and replaced with 10ml of DMEM supplemented with L-glutamine, fungizone and 5% vol/vol FCS. Cells were removed from the flasks after being scraped with a cell scraper, then lysed by passing 3 times through a Luer lock 20 G needle connected to a 50ml syringe. The contents of the two flasks were bulked and centrifuged at 100 x g for 5 minutes to deposit the cell nuclei. Five mls of the supernatant fluids (total of 20ml) was then removed and centrifuged at 2700 x g for 25 minutes in Eppendorf tubes to form a bacterial pellet. The pelleted organisms were fixed in 1% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) for 2 hours at 4°C, then washed twice for 10 minutes in cacodylate buffer before being processed for

electron microscopy (Section 2.11). The remaining 15ml of the cell lysate was similarly centrifuged at 2700 x g, the pellet resuspended in 15ml DMEM with supplements and 7% vol/vol FCS (without antibiotics) and used to infect IEC-18 cells in a separate experiment.

Bacteriology

The infected cell lysate was cultured microaerobically on Columbia blood agar, Skirrow's medium and Brucella semi-solid broth for 7 days as described in Section 2.12.

Immunological detection of the intracellular bacteria

Smears of the infected cell lysate prepared on glass slides were fixed in acetone and immunofluorescence staining conducted with monoclonal antibody IG4 and sheep anti-mouse IgG FITC conjugate (Section 2.8). Infected IEC-18 cell monolayers on coverslips, set up in parallel to the infected flasks used to prepare the bacterial pellet, were also fixed in acetone and stained with similar monoclonal antibody and sheep anti-mouse IgG peroxidase conjugate in

an immunoperoxidase test (Section 2.9).

Immunological detection of other microorganisms

Some infected IEC-18 cell monolayers on coverslips set up in parallel to the infected flasks were fixed in methanol for 5 minutes and stained with a mixture of FITC conjugated monoclonal antibodies prepared against the outer membrane proteins present in 12 known serovars of C. trachomatis (Syva, U.S.A.) according to the manufacturer's instructions. Appropriate positive and negative controls were also included in the staining schedule. Coverslips were treated with 30ul of the monoclonal antibody and incubated for 15 minutes in a moist chamber at room temperature. After rinsing in distilled water for 10 seconds, the coverslips were allowed to air dry, mounted, and observed with a fluorescence microscope.

4.3 Results

Light microscopy of IEC-18 cells infected in parallel to the flasks and smear preparations of the lysate used to

prepare the pellet showed bacteria reactive with monoclonal antibody IG4. The bacteria were morphologically similar to those described previously in Chapter 3. Occasional sea-gull and filamentous-shaped bacteria were also observed (Fig. 5). All bacteria detected stained immunologically and no bacteria were observed stained with the haematoxylin dye.

Ultrastructural examination of the pellet derived from lysed IEC-18 cell cultures contained numerous bacteria (Fig. 6). The bacteria measured from 0.1 to 0.3 μ m in width and 0.7 to 2.0 μ m in length and appeared pleomorphic, curved or straight rods. Ovoid, comma and sigmoid-shaped cells were all present. The majority of bacterial cells were curved and 0.3 μ m in cross-section. Organisms had a wavy trilaminar outer membrane and, often an indistinct cytoplasmic membrane which was generally clearly separated from the cell wall by a periplasmic space, characteristic of gram negative bacterium. Some bacteria had their cytoplasmic content separated from their outer membrane (Fig. 6). The bacteria differed in internal structure and the electron density of their cytoplasm; some were

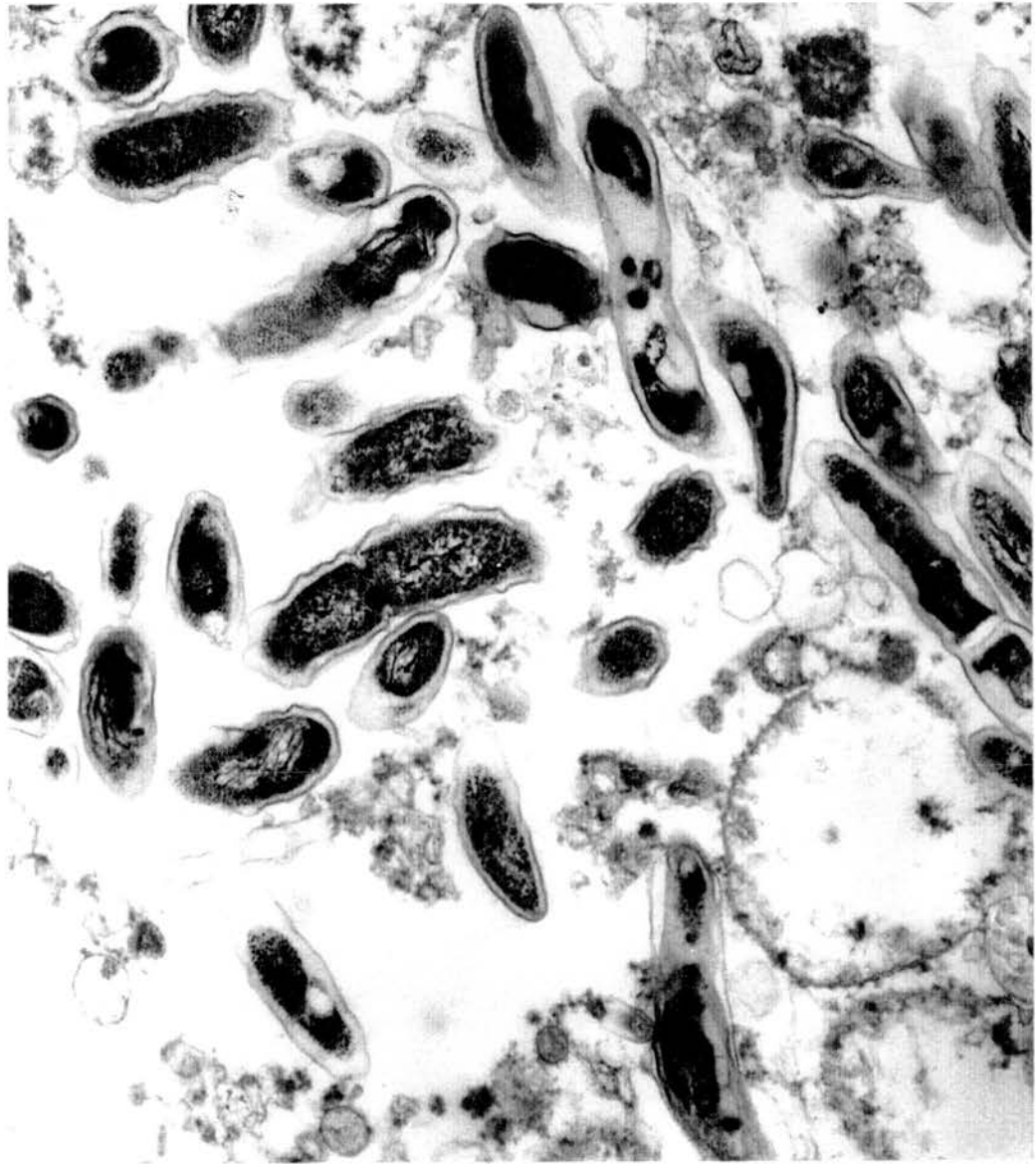
Figure 5

IEC-18 cells infected with the intracellular bacteria. Note immunoperoxidase stained filamentous-shaped bacteria (arrows). Immunoperoxidase staining with monoclonal antibody IG4 and sheep anti-mouse peroxidase conjugate. Haematoxylin counterstain x 1343.



Figure 6

Transmission electron micrograph of the intracellular bacteria pelleted by centrifugation from IEC-18 cells. Numerous bacteria are evident among cellular debris. Note pleomorphic curved or straight rods, ovoid, comma and sigmoid-shaped cells are all present. Organisms have a wavy trilaminar outer membrane and, often an indistinct cytoplasmic membrane which is generally clearly separated from the cell wall by a periplasmic space. Some bacteria have distinct separation of their cytoplasmic content from the outer membrane. Lead citrate and uranyl acetate stain x 33000.



electron-dense (ED) and some were more electron-lucent (EL) (Figs. 7 and 8). The internal structure of the former was amorphous and consisted of numerous granules presumably bacterial ribosomes whereas the latter demonstrated a central reticulate appearance with less dense peripheral granules. Some ED forms had pale circular non-membrane bound granule within the cytoplasm (Fig. 9). Stages of bacterial division were more frequently seen in EL forms and consisted of transverse septation (Figs. 10 to 13). Many EL forms, in particular those that were dividing appeared to have distinct electron-dense nucleoids. The nucleoid contained a small central mass, sometimes elongated or cylindrical often with fine radiating fibrils which lay in a poorly delineated electron transparent space in the centre of the bacterial cytoplasm. Bacteria were not observed to have spores, capsules or flagella.

Ultrastructurally distinct agents such as Chlamydia or viruses were not detected. The bacterial pellet contained considerable amounts of amorphous sometimes membrane bound debris representing the residual protoplasmic material from the lysed IEC-18 cells. Staining of infected IEC-18 cells

Figure 7

Transmission electron micrograph of the intracellular bacteria pelleted by centrifugation from IEC-18 cells. Note electron-dense and electron-lucent forms. Lead citrate and uranyl acetate stain x 60350.

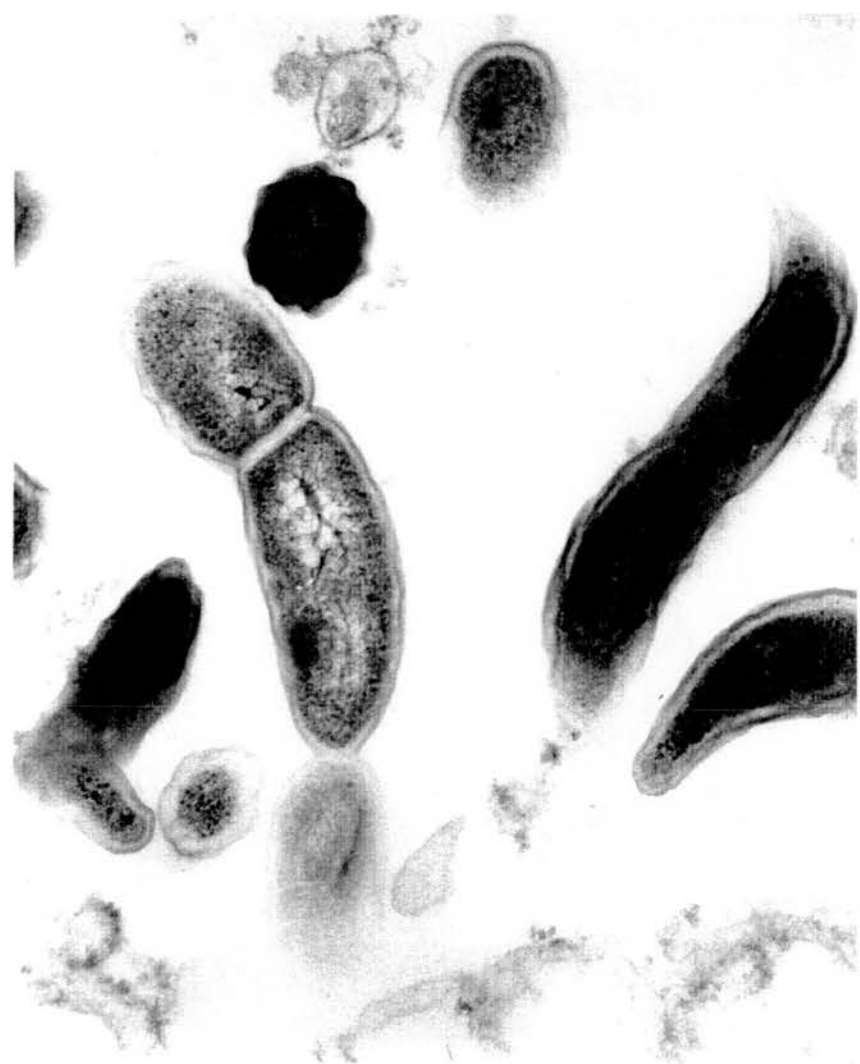


Figure 8

Transmission electron micrograph of the intracellular bacteria pelleted by centrifugation from IEC-18 cells. Electron-dense and electron-lucent forms at higher magnification. Note electron-dense form has numerous granules presumably ribosomes whereas electron-lucent form has fewer granules and with localised electron-lucent cytoplasm (arrows). Lead citrate and uranyl acetate stain x 225000.

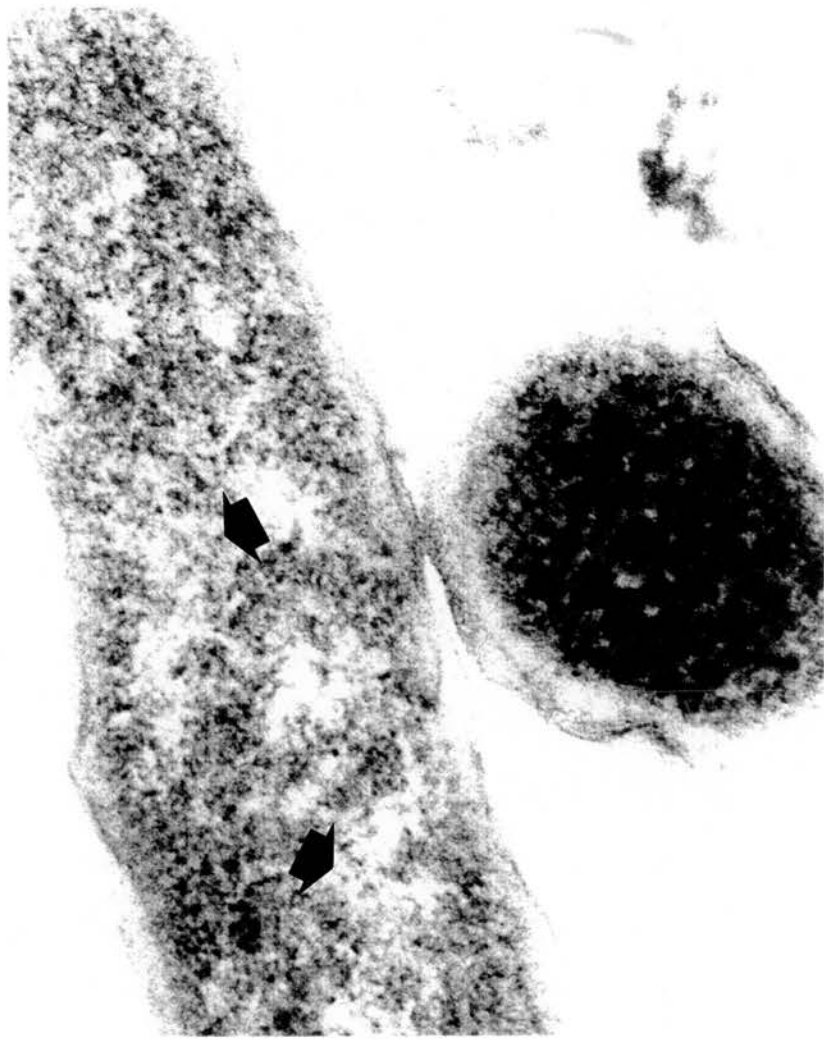


Figure 9

Transmission electron micrograph of the intracellular bacteria pelleted by centrifugation from IEC-18 cells. Note an electron-dense bacterium with pale area within the cytoplasm. Lead citrate and uranyl acetate stain x 42600.

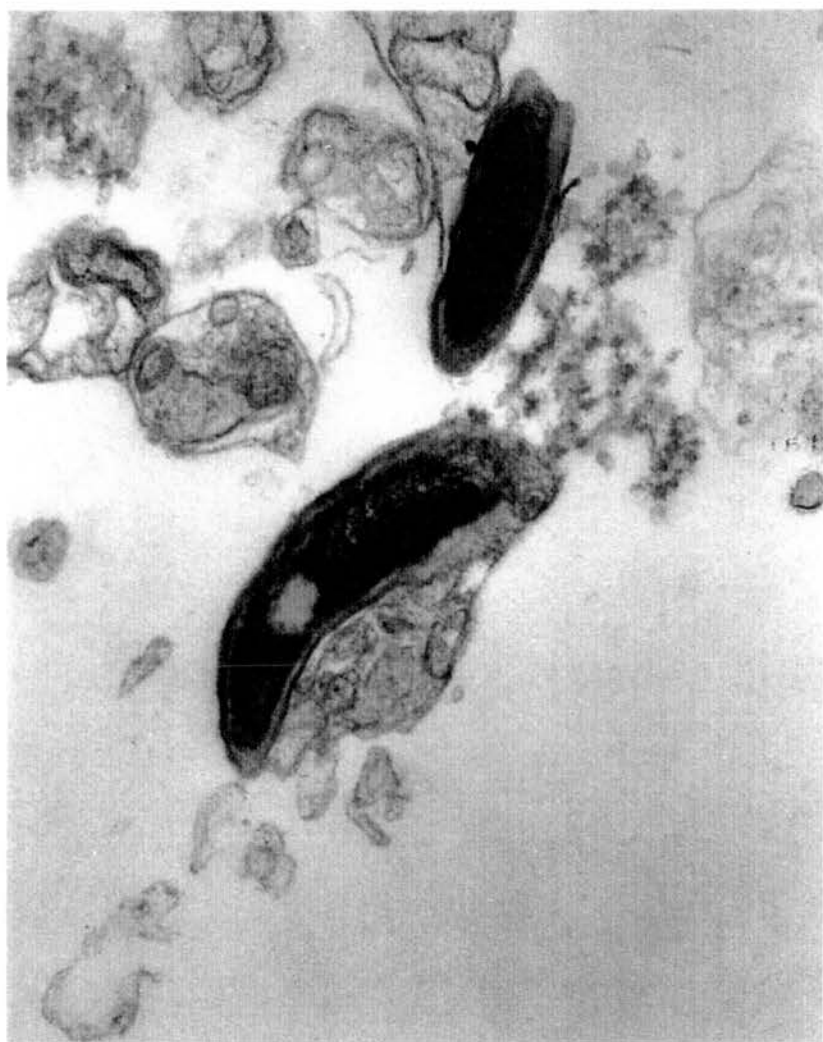


Figure 10 to 13

Transmission electron micrograph of the intracellular bacteria pelleted by centrifugation from IEC-18 cells. Sequence of events of bacterial division.

Figure 10

Electron-lucent form at an early stage of division. Nucleoids are evident at both poles of the bacteria. Fine fibrils radiate from the nucleoids. Lead citrate and uranyl acetate stain x 92000.

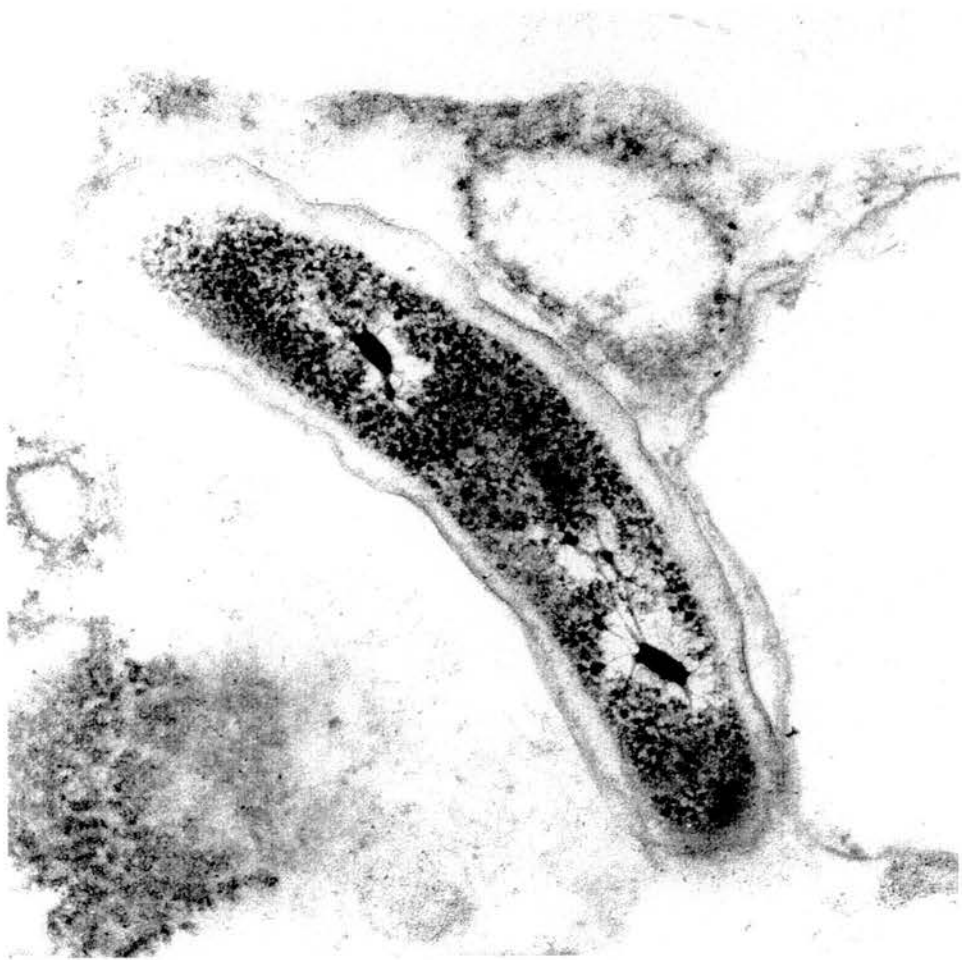


Figure 11

Transmission electron micrograph of the intracellular bacteria pelleted by centrifugation from IEC-18 cells. A later stage of transverse binary fission of electron-lucent form showing early septation. Lead citrate and uranyl acetate stain x 55200.

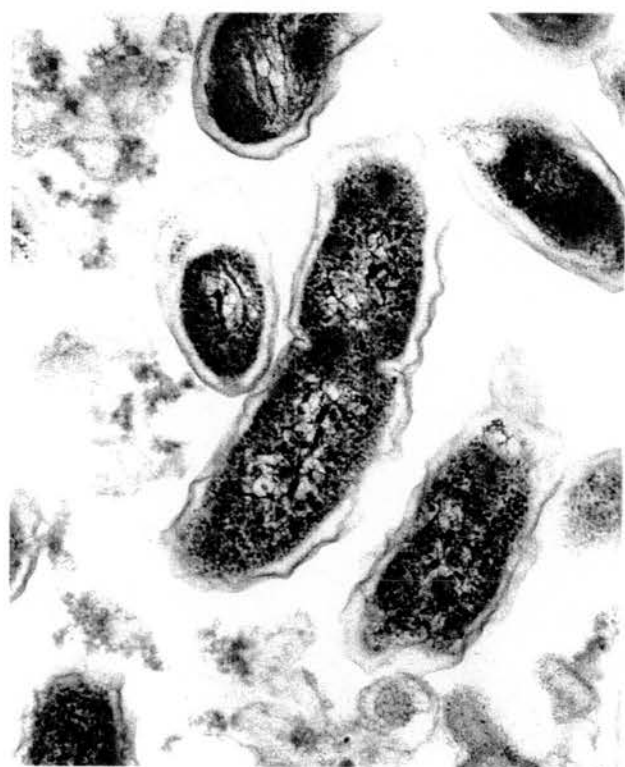


Figure 12

Transmission electron micrograph of the intracellular bacteria pelleted by centrifugation from IEC-18 cells. A further stage of transverse binary fission of the electron-lucent form showing complete septation. Lead citrate and uranyl acetate stain x 63900.

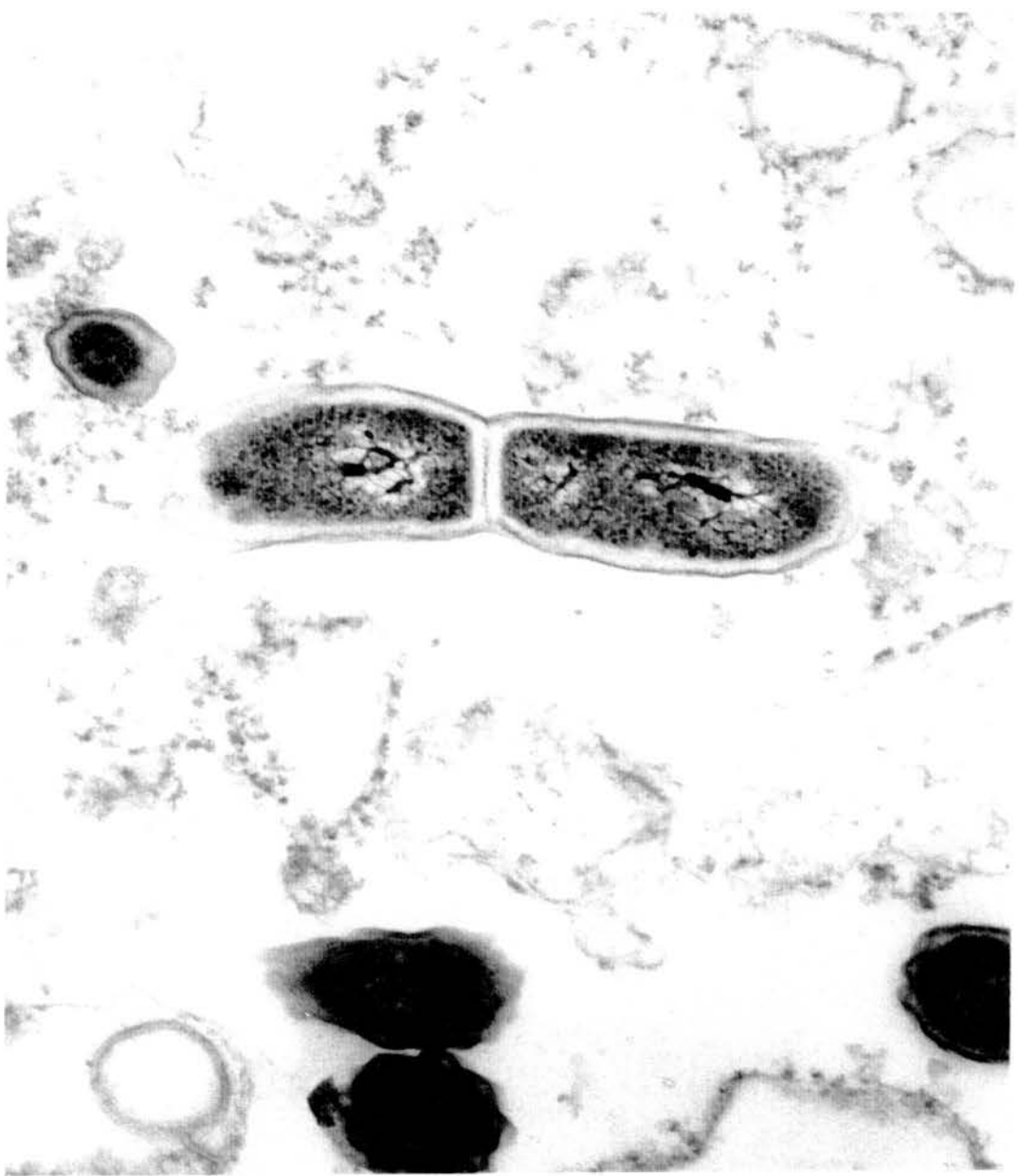
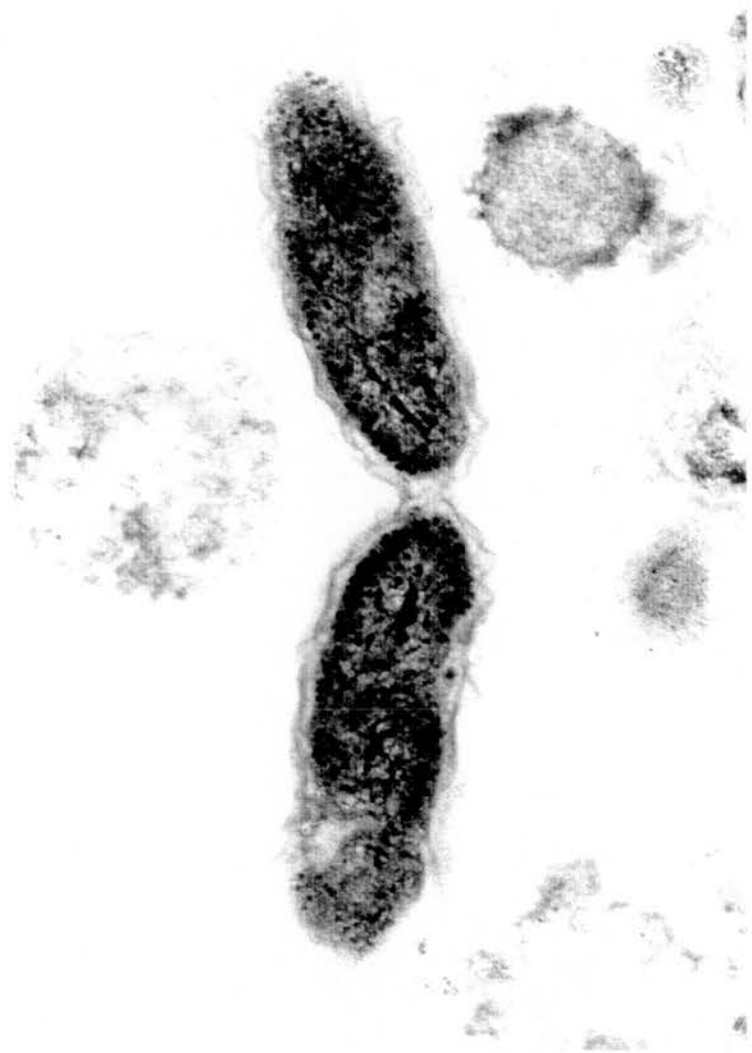


Figure 13

Transmission electron micrograph of the intracellular bacteria pelleted by centrifugation from IEC-18 cells. An end stage of transverse binary fission of the electron-lucent form showing separation of septated bacteria. Lead citrate and uranyl acetate stain x 92000.



infected on coverslips and smears of the infected cell lysate used to prepare the bacteria pellet revealed numerous bacteria reactive with monoclonal antibody IG4 in immunoperoxidase and immunofluorescence tests respectively. Immunofluorescence assay of infected IEC-18 cells on coverslips with monoclonal antibody to Chlamydia did not identify any Chlamydial forms nor was there evidence of cell inclusions evident in haematoxylin counterstaining of infected monolayers. Standard bacterial culture of the infected cell lysate was "sterile", ie. failed to demonstrate Campylobacter spp. or any other bacteria.

4.4 Comment

Ultrastructural observation has clearly revealed the morphology of I. intracellularis that cannot be fully resolved by light microscopic observation of the bacteria (Chapter 3). Growth of Ileobacter in pure culture suggests the presence of a single cultured agent. This conclusion was confirmed by bacteriological study and additionally supported by the immunological and ultrastructural examination of the bacteria. Many bacteria were analysed

following passage and pelleting by centrifugation. A morphological feature commonly seen in other obligate intracellular bacteria, showing with more than one morphological form was also evident in *Ileobacter*. Stages of transverse septation of the bacteria have for the first time been fully revealed and described.

4.5 Discussion

This study describes the ultrastructural morphology of cell culture derived *I. intracellularis* and confirms the absence of other intracellular microorganisms such as Chlamydia or viruses, Campylobacter spp. or other bacteria. This confirms the isolation and examination of a single cultured agent in the IEC-18 cell line. The intracellular bacteria grown in cell culture, appeared pleomorphic and exhibited two morphological forms, the ED and EL forms, differing slightly in internal structure and electron density of the cytoplasm. The presence of more than one morphological form of other obligate intracellular bacteria is not uncommon. It has been reported in Chlamydia spp. (Ward, 1983), Coxiella burnetti (McCauls and William,

1981), Cowdria ruminantium (Kocan, Crawford, Dilbeck, Evermann and McGuire, 1990), Ehrlichia spp. (Rikihisa, Penny and Cordes, 1985) and Rickettsia rickettsii (Todd, Burgdorfer, Marvos and Wray, 1981). Different morphological forms of C. ruminantium have been observed in bacteria growing in cultured endothelial cells (Prozesky, Bezuidenhout and Paterson, 1986), in in vivo studies in cattle (Pienaar, 1970) and also in the midgut epithelial cells and salivary gland acini of the tick vector, Ambloyma hebraeum (Kocan, Bezuidenhout and Hart, 1987). It is possible that the differing forms of the intracellular bacteria may play different roles in infection. The EL form were more associated with stages of bacterial division implying that this form is mainly present during intracellular multiplication. Chlamydial organisms have two forms with different roles during infection; the elementary body which is the infective form and the reticulate body which is the dividing form (Ward, 1983) but Ileobacter differed from Chlamydia in that the latter are observed in phagocytic vacuoles whereas the former are free in the cytoplasm of the host cell (Ward, 1983; Rowland and Lawson, 1992). Similar variations in the electron density

of intracellular bacteria in naturally occurring PE are observed in photographs presented by Lawson, Rowland and Roberts (1976). The majority of bacteria cells were 0.3um in cross-section, smaller forms were probably bacteria sectioned obliquely.

EL forms of *Ileobacter* were observed at different stages of division and septation. Much of the information on the process of bacterial division had been obtained from studies conducted on *Escherichia coli*. It was reported that the process of septation involves a coordinated movement of three bacterial cell envelope layers, namely, the inner cytoplasmic membrane, the murein layer and the outer membrane (Cook, De Boer and Rothfield, 1989). It is believed that the driving force of septation arises from the circumferential inward growth of the murein layer (Cook et al., 1989). This layer determines the shape of the bacterial cell and is responsible for the rigidity as well as the resistance of the cell to mechanical and osmotic stresses. The murein layer which is closely apposed to the outer membrane and completely surrounds the cell is composed of an extensive thin cross-linked peptidoglycan

layer. The inward growth of the murein layer pulls the outer membrane from behind and results in the forward movement of the inner cytoplasmic membrane. The inward growth of this layer during septation involves the synthesis and insertion of new peptidoglycan units into the existing murein layer (Cook et al., 1989). However, bacterial division is preceded by chromosomal replication and partitioning of bacterial nucleoids (Begg and Donachie, 1991). A nucleoid of a prokaryotic cell is an intracellular mass of folded chromosomal DNA molecule. According to Begg and Donachie (1991), the chromosomes of nucleoids are fixed in position within a growing bacterial cell before and during chromosomal replication but are separated (partitioning of nucleoids) immediately after replication which subsequently results in the initiation of division and septation. Replicated nucleoids are spatially separated from each other and are positioned at $1/4$ and $3/4$ cell length prior to septation (Burdett, Kirkwood and Whalley, 1986; Hiraga, 1990). The observation of *Ileobacter* with nucleoids at the quarters of the bacterial cell suggests the occurrence of nucleoid partitioning and represents the stage of division before septation.

Some ED bacteria were observed with cytoplasmic granules which may represent inclusion bodies as described by Shively (1974). Bacteria with similar granules were observed in the hyperplastic crypt cells and especially in crypt lumina of pigs and hamsters with PE (McOrist, 1988; McOrist et al., 1989). Morphologically similar structures have also been reported in many bacteria including Helicobacter pylori (Shively, 1974; Caselli, Aleotti, Baldrini, Ruina and Alvisi, 1993). Inclusion bodies observed in bacterial cells contain reserve nutrients (eg. glycogen, lipid, phosphorus) whose presence and amount vary with the type of bacteria and their level of metabolic activity (Shively, 1974; Scanlan, 1988). Only a few Ileobacter contained protoplasmic granules suggesting that such inclusions developed only during certain phases of growth or metabolism of the bacteria. A type of bacterial inclusion, polyphosphate, can easily disintegrated upon exposure to an electron beam and is chipped out during ultrathin sectioning which results in the electron-dense inclusion appearing pale or vacuolated. (Bode, Mauch, Ditschuneit and Malfertheiner, 1993). Pale protoplasmic granules in Ileobacter may represent polyphosphate granules

that were damaged.

Bacteria accumulate nutrients as reserves during optimum nutritional condition and utilise these nutrients when nutritional condition in the micro-environment are depleted (Dawes and Senior, 1973). The ability to accumulate these reserves and their utilization during adverse nutritional condition is an evolutionary characteristic to aid survival. However, their presence is not a prerequisite for bacterial growth (Dawes and Senior, 1973). The presence of cytoplasmic granules in *Ileobacter* suggest that this bacteria is also capable of accumulating and utilising energy reserves possibly under conditions of limited exogenous nutrient sources and depletion of reserves during survival and multiplication of the bacteria. The utilization of these reserves may inhibit the consumption of important cellular constituents such as structural proteins from the bacterial cell (Thomas and Batt, 1969 ; Dawes and Senior, 1973). The separation of cytoplasmic contents from the parietal structures observed in *Ileobacter* was also reported in H. pylori in ultrastructural observations of gastric biopsies of human

patients, and was considered to be a morphological consequence of bacteria utilising their structural components after depletion of their energy reserves (Caselli et al., 1993). It could also result from the accumulation of bacterial metabolic end products due to metabolism of structural components. Another possible explanation is that the reserves are used in the extracellular survival of *Ileobacter* during transit between cells or hosts. Being cell dependant, the extracellular environment is hostile for an obligate intracellular bacterium. Different obligate intracellular bacteria overcome this problem in different ways. Rickettsia spp. depend on arthropod vectors for host to host transmission and are enclosed in host derived membranes after release from host cells and before infecting other cells (Ewing et al., 1978; Williams and Vodkin, 1987).

Attempts to detect C. trachomatis in the infected cell culture by immunological, ultrastructural and haematoxylin staining has failed to detect the presence of this organism. Immunological detection of C. psittaci or C. trachomatis in IEC-18 cell line infected with similar and

other inocula derived from affected pigs with PE has also been unsuccessful (Lawson et al., 1993). The work by Zahn, Kunz and Pospischil (1987) confirms the lack of association between Chlamydial infection and the presence of *Ileobacter* in the lesions of PE. These workers examined the small intestines of pigs aged 1 to 12 weeks. From 185 pigs studied, 15% were shown by immunological means to have Chlamydial organisms (*C. psittaci*) in the intestines. These bacteria were present in both healthy and in diseased pigs, with and without diarrhoea. However, none of the pigs in which Chlamydia was detected in the intestines had PE lesions nor intracellular colonisation by bacteria characteristic of PE. Chlamydia were concurrently present with other enteric pathogens such as *E. coli*, Rota virus and Coccidia in diarrhoeic pigs with enteritis (Zahn et al., 1987). It was therefore believed that the presence of Chlamydia in these conditions was coincidental and merely accentuated the pathogenicity of other enteric pathogens. Pospischil and Wood (1987) reported severe pseudomembranous enteritis in pigs experimentally infected with *Salmonella typhimurium* which also had co-incidental infection with *Chlamydia spp.* Less severe lesions of the intestines were

observed in pigs infected with S. typhimurium alone. Similarly, none of the pigs infected with either or both organisms had proliferative lesions with typical intracellular bacteria of PE in the cytoplasm. The only documentation of Chlamydia in PE tissues also containing intracellular bacteria (Fox et al., 1991) may require further investigation but the former are possibly present co-incidentally (Fox et al., 1993).

The detection of Chlamydia in healthy pigs and those with diarrhoea by Zahn and co-workers (1987) in the absence of PE lesions suggests that they are not the cause of PE in pigs. This is supported by ultrastructural studies of field or experimentally-induced PE in pigs which has never disclosed the presence of Chlamydia in hyperplastic crypt cells of the intestines (Lomax et al., 1982a,b; McOrist and Lawson, 1987; McOrist and Lawson, 1989b; McOrist et al., 1989). Pospischil and Wood (1987) reported that Chlamydia spp. are common in the intestines of neonatal pigs. Chlamydia are less common in adult pigs which may explain their absence in PHE tissues. PE tissues, inoculated into embryonated eggs, a culture technique capable of growing

Chlamydial agents, also failed to isolate this agent (Ward, Harp and Jones, 1991).

The morphological characteristics of a single cultured agent, I. intracellularis, has been described providing for the first time a detailed description of the intracellular bacteria of PE grown in IEC-18 cells.

CHAPTER 5

CELLULAR EVENTS IN CENTRIFUGE-ASSISTED INFECTION OF
IEC-18 CELLS BY THE INTRACELLULAR BACTERIA

CELLULAR EVENTS IN CENTRIFUGE-ASSISTED INFECTION OF IEC-18

CELLS BY THE INTRACELLULAR BACTERIA

5.1 Introduction

Infection of host cells by intracellular bacteria involves an infection cycle which constitutes the pathogenic events of infection, these events can lead to successful infection and production of disease (Moulder, 1985; Williams and Vodkin, 1987; Falkow, 1991). Both professional (neutrophils, macrophages) and non-professional phagocytic cells (fibroblasts, epithelial cells) are possible hosts of an intracellular bacteria.

Attachment to the surface of an eukaryotic host cell is a prerequisite of infection by intracellular bacteria that eventually leads to entry of the microorganism into a host cell. An intracellular bacterium can enter host cells either by diacytosis or endocytosis (Moulder, 1985). In diacytosis, a microorganism can pass directly into the host cell cytoplasm by punching a hole through the cell membrane which closes behind the parasite after entry. In endocytosis, the microorganism does not destroy the

structural continuity of the host cell envelope and enters surrounded by a host-derived membrane. Endocytosis can be divided into host-specified or parasite-specified endocytosis. Professional phagocytes are adapted to ingest organisms and other particles with ease; this is an example of host-specified endocytosis. In parasite-specified endocytosis, it is the organism that has adapted to promote their entry.

Intracellular bacteria employ different strategies to evade the defence mechanisms of the host cell following entry. Different intracellular bacteria not only evade the cell defence mechanism in different ways but also multiply within the host cell at different intracellular locations, multiply and are eventually released from the host cell (Moulder, 1985). In field cases of proliferative enteropathy, intracellular bacteria are not membrane bound, lie free and apparently multiplying in the cytoplasm of the enterocyte. Bacteria free in the cytoplasm can either indicate entry by diacytosis or endocytosis; either penetration of bacteria into the cell cytoplasm or escape from an endocytic vacuole with subsequent release into the

cytoplasm respectively. The cellular events of infection of cells by I. intracellularis from entry, until release from the host cell are still unexplored.

This study was conducted to investigate the ultrastructural events of intestinal cell infection by I. intracellularis using the IEC-18 cell line as an in vitro model. In conjunction with this ultrastructural study, detailed morphological observation of simultaneously infected IEC-18 cells was also conducted by light microscopy.

5.2 Early and late events of infection

Materials and Methods

Early IEC-18 infection

I. intracellularis strains 1482/89 and 916/91 were passaged (19 and 2 times respectively) in IEC-18 cells and used as inocula to infect freshly prepared 24-hour IEC-18 cell monolayers, seeded at 1×10^5 cells ml^{-1} on 13mm plastic coverslips.

Strain 1482/89 was processed from two 75cm² tissue culture flasks; that is, the remainder of the purified bacteria used in the morphological study described in Chapter 4. Strain 916/91 was processed from three 25cm² tissue culture flasks of IEC-18 cells infected with this strain for 6 days. The growth medium in the flasks was decanted, and the 916/91 infected cells treated with KCl, 5ml of DMEM and supplements with 7% vol/vol FCS was added to each flask, the cells, removed mechanically with a scraper and ruptured (Section 2.5). The cell lysate was centrifuged at 100 x g for 5 minutes to deposit the cell nuclei, the supernatant made up to a total volume of 19ml with DMEM and supplements with 7% vol/vol FCS and used to infect IEC-18 cells.

IEC-18 cells were infected with both bacterial strains by centrifugation, incubated microaerobically in steel jars for 3 hours, fed with DMEM with 7% vol/vol FCS and vancomycin and neomycin added and further incubated in an incubator (8.8% CO₂, 8.0% O₂) at 37°C. The infected cells were harvested at 0 (immediately after centrifugation), 3 and 24 hours post-infection and processed for TEM (Section

2.11). Some infected cells were stained with the monoclonal antibody IG4 and goat anti-mouse gold conjugate in a pre-embedded immunogold method (Section 2.10). The coverslips were divided into 4 equal quadrants, stained and were randomly sectioned at different sites for viewing with the electron microscope.

Late IEC-18 infection

Two 25cm² tissue culture flasks of IEC-18 cells that had been infected with the intracellular bacteria strain 916/91 for 6 days were used as inoculum to infect 24 hour IEC-18 cell monolayers seeded at 0.25×10^5 cells ml⁻¹ on 13mm plastic coverslips. The infected flasks were treated with KCl, and 5ml of DMEM and supplements with 7% vol/vol FCS was added to each flask before the cells were mechanically removed and lysed (Section 2.5). The cell lysate from the two flasks was made up to a total volume of 46ml and used to infect the IEC-18 cells. Coverslips were harvested at day 6 post-infection, immunoperoxidase staining (Section 2.9) with monoclonal antibody IG4 and sheep anti-mouse peroxidase conjugate. Foci of infection were selected,

marked with a marker objective and processed for TEM (Section 2.11). Ultrathin sections were cut randomly at different sites from the marked areas for viewing with the electron microscope.

Monitoring of infection

To monitor the infection, parallel infections of IEC-18 cells with I. intracellularis set up on 16mm glass coverslips for light microscopic observation and were harvested at 3 hours (centrifuged and non-centrifuge infected), 24 hours and 6 days post-infection (centrifuge infected only). Coverslips were specifically stained with the monoclonal antibody IG4 and sheep anti-mouse peroxidase conjugate in an immunoperoxidase test (Section 2.9).

Control

Uninfected IEC-18 cell monolayers of similar ages after seeding were simultaneously harvested with infected cells. Representative infected coverslips were harvested; some after centrifugation, some after aerobic incubation and some

after microaerobic incubation procedures in steel jars.

Bacteriology

The tissue culture inocula prepared for both early and late IEC-18 cells infection studies were cultured in Skirrow's medium, Brucella semi-solid broth and Columbia blood agar (Section 2.12).

5.3 Results

IEC-18 cells infected with I. intracellularis and exposed to centrifugation revealed a clearly defined sequence of events in host cell penetration. Culture of the inocula used to infect IEC-18 cells in conventional cell free media failed to yield any bacteria. Parallel infection set up for light microscopical observation showed bacteria reactive with the specific monoclonal antibody IG4 in immunoperoxidase staining and other non-staining bacteria were not observed.

Early events of infection

0 hour post-infection

Immediately after centrifugation, bacteria were observed in close association with host cell membranes. In some instances, bacteria were closely applied to the host cell surface with a cap of electron dense material surrounding the junction of bacteria and host cell. This material lay outside the host cell membrane which was often still clearly separated from the outer bacterial membrane and appeared at its most electron dense in the area immediately between cell and bacteria (Fig. 14). The extracellular bacteria were either ED or EL and some had a pale or electron dense granules within their cytoplasm (Figs. 14 and 15). Intracellular bacteria either singly or in pairs were only observed in endocytic vacuoles just beneath the cell membrane. Some vacuoles contained ground substance morphologically similar to the cell cytoplasm outside the vacuole. In most instances, endocytic vacuoles appeared to be closely associated with host cell mitochondria (Fig. 16). In some cases, the membrane of this organelle appeared to be focally in contact or fused

with the endocytic vacuole (Fig. 17). Bacteria free in the cytoplasm were not seen during this stage of infection.

3 hours post-infection

At 3 hours post-infection, extracellular bacteria were observed near the cell membrane. However, attachment of bacteria by an adherent cap was not observed. Intracellular bacteria in endocytic vacuoles were also seen. The membrane of endocytic vacuoles appeared to be disintegrating and in some instances, bacteria were being freed into the cytoplasmic matrix (Fig. 18). Some single non-membrane bound bacteria were evident at various sites in the cytoplasm (Fig. 19).

Some extracellular and intracellular bacteria had a fuzzy electron dense layer extending from the outer membrane in immunogold stained samples. Numerous gold particles were seen on this bacterial layer when stained with the specific monoclonal antibody IG4 (Fig. 20).

Light microscopic observation of both centrifuged and

Figure 14

Transmission electron micrograph of IEC-18 cell line infected with the intracellular bacteria at 0 hour post-infection (after centrifugation). An electron-lucent bacterium closely applied to host cell membrane with a cap of electron-dense material surrounding the junction of bacteria and host cell. This material lay outside the host cell membrane which is distinctly separated from the outer bacterial membrane and appeared at its most electron-dense in the area immediately between cell and bacteria. Note pale and electron-dense granules in the bacteria protoplasm. N, Nucleus. Lead citrate and uranyl acetate stain x 115000.

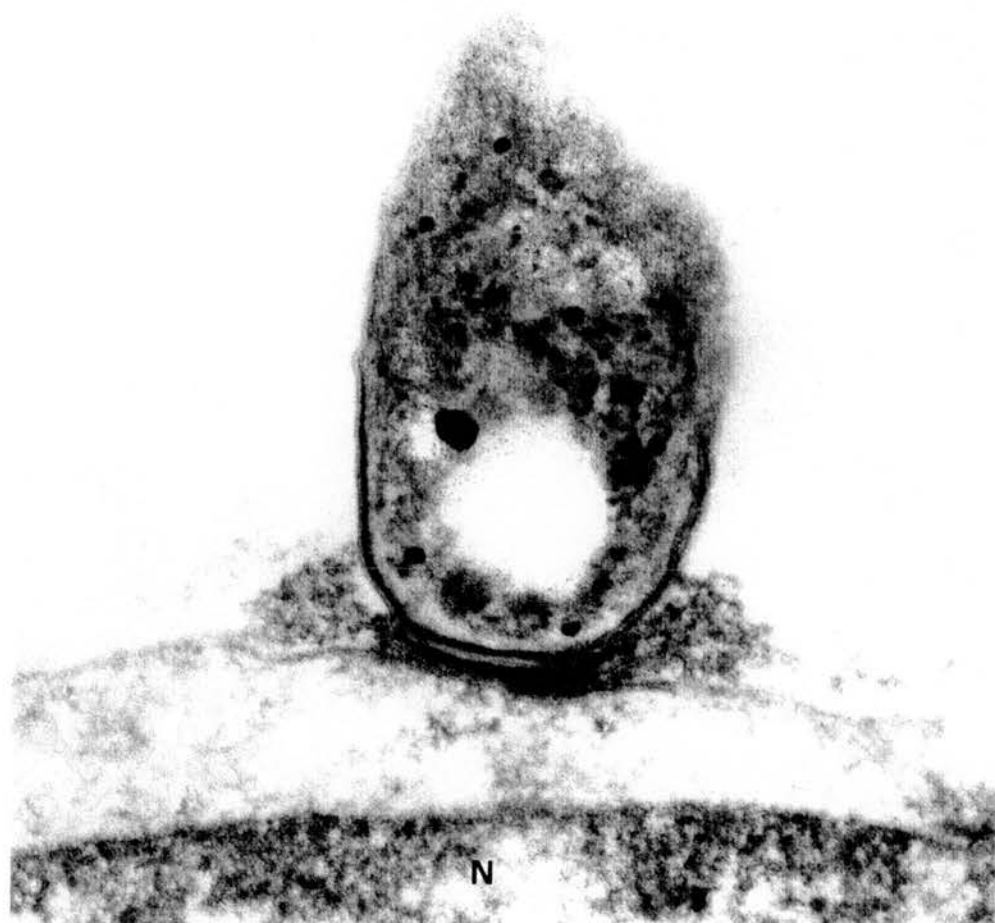


Figure 15

Transmission electron micrograph of IEC-18 cell line infected with the intracellular bacteria at 0 hour post-infection (after centrifugation). Note an electron-dense bacteria with pale granule attached to an electron-dense cap of the cell membrane. Lead citrate and uranyl acetate stain x 46000.

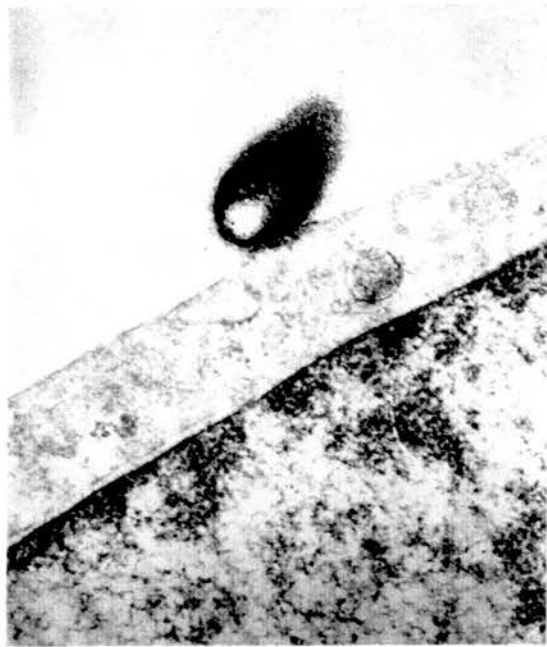


Figure 16

Transmission electron micrograph of IEC-18 cell line infected with the intracellular bacteria at 0 hour post-infection (after centrifugation). An endocytic vacuole containing bacteria just beneath the cell membrane. The endocytic vacuole appeared adjacent to a host cell mitochondria and smooth vesicles. M, mitochondria. Lead citrate and uranyl acetate stain x 66000.

Figure 17

Transmission electron micrograph of IEC-18 cell line infected with the intracellular bacteria at 0 hour post-infection (after centrifugation). An endocytic vacuole containing two bacteria is surrounded by two mitochondria. Note that the mitochondrial membrane appeared fused with the membrane of the endocytic vacuole (arrows). Ground substance morphologically similar to the cell cytoplasmic matrix is also present in the vacuole. The endocytic vacuole membrane appeared to have disintegrated focally. Lead citrate and uranyl acetate stain x 120000.

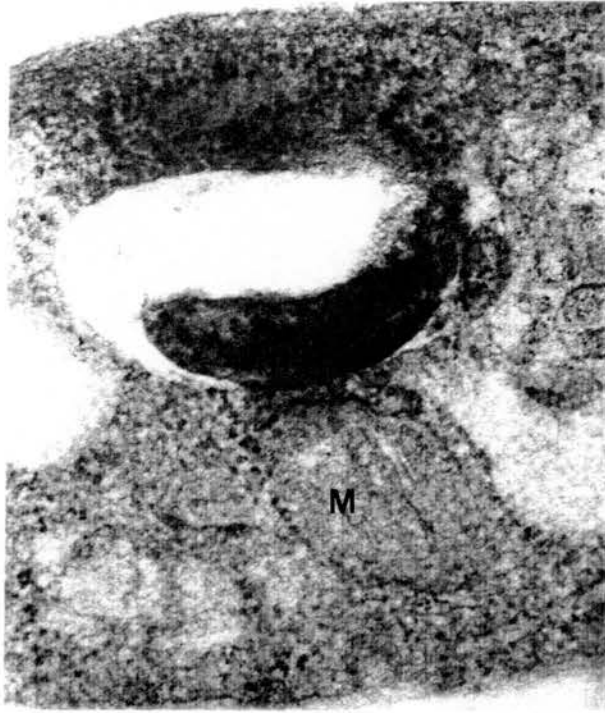


Figure 18

Transmission electron micrograph of IEC-18 cells infected with the intracellular bacteria at 3 hours post-infection. Note distinct breakdown of an endocytic vacuole and bacteria in contact with the cytoplasmic matrix (arrow). Two mitochondria (M) are also associated with the entry vacuole. Lead citrate and uranyl acetate stain x 92400.

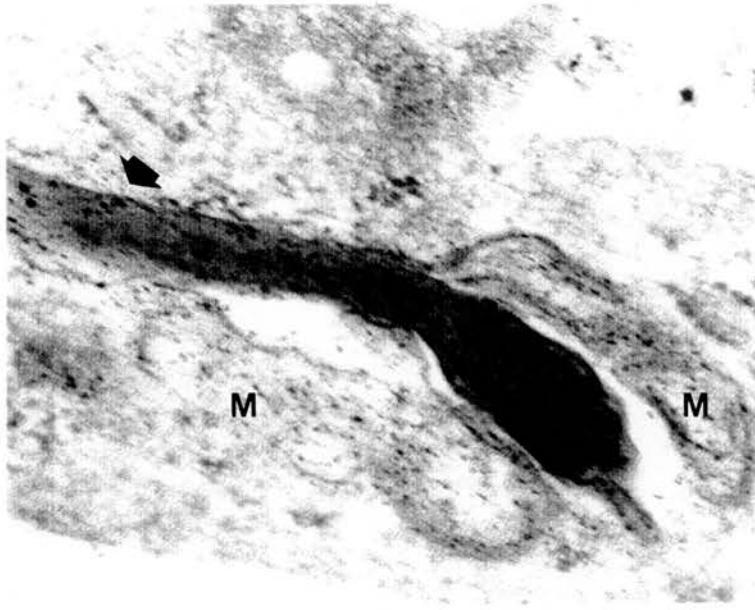


Figure 19

Transmission electron micrograph of IEC-18 cells infected with the intracellular bacteria at 3 hours post-infection. A single bacterium lying free in the cytoplasm. Note an empty entry vacuole with membrane breakdown lying just adjacent to the bacteria (arrow). N, nucleus. Lead citrate and uranyl acetate stain x 55000.

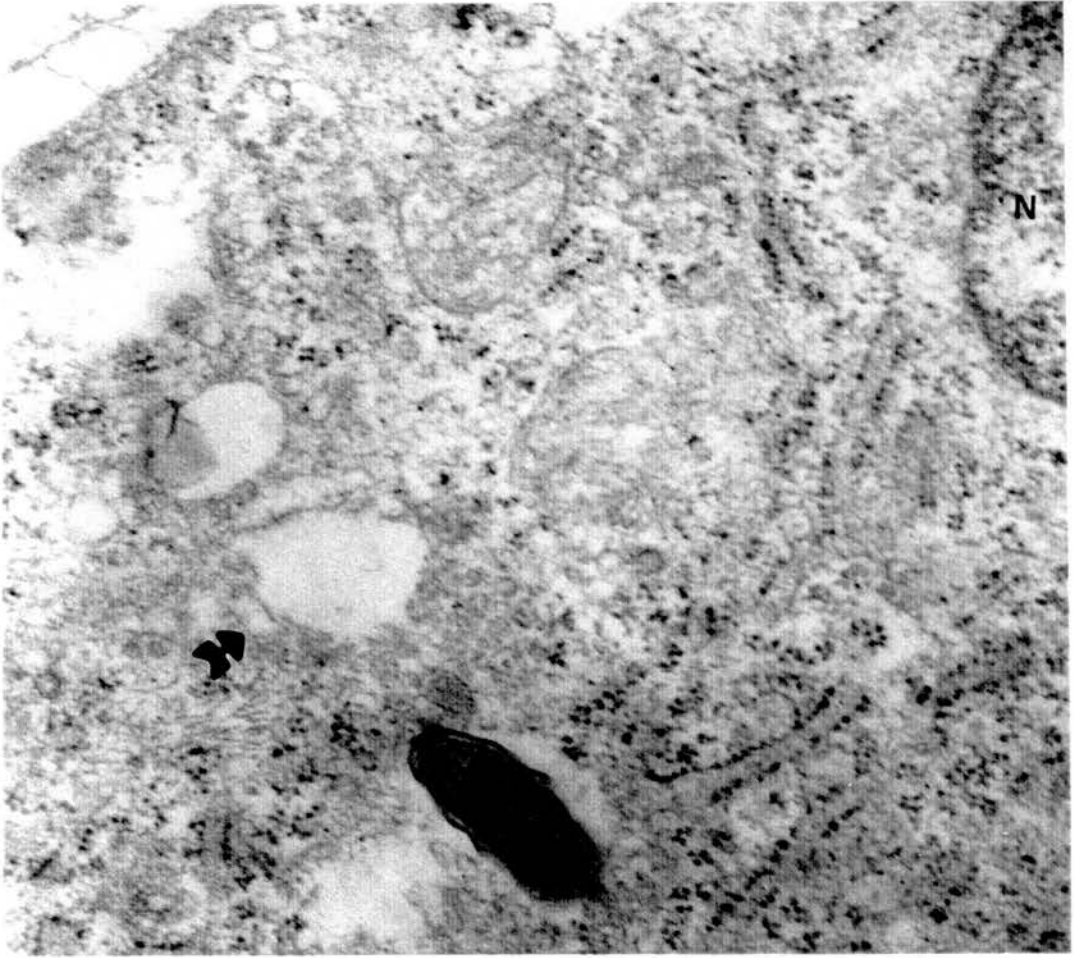
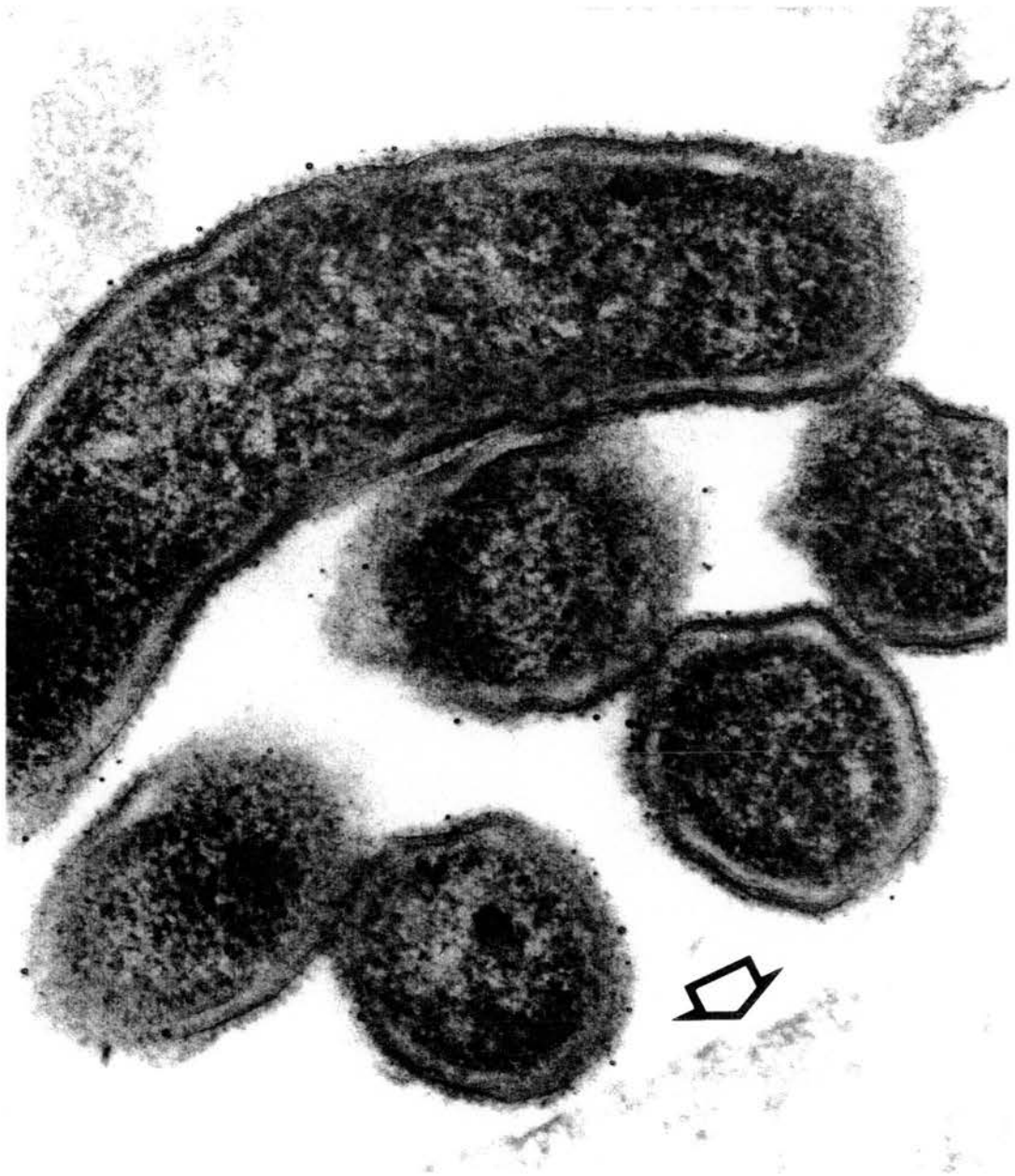


Figure 20

Transmission electron micrograph of IEC-18 cell (arrow points to the surface of a cell) and bacteria which is outside the cell at 3 hours post-infection. A fuzzy and regular electron-dense layer extends from the outer membrane of the bacteria. Note numerous 1nm gold particles on this bacterial layer. Immunogold staining with monoclonal antibody IG4 and goat anti-mouse gold conjugate. Lead citrate and uranyl acetate stain x 115000.



non-centrifuged assisted infection showed scattered bacterial infection generally involving single labelled bacilli. Infection was higher in centrifuged infection in comparison to non-centrifuged infection (see Section 5.4; Table 9).

24 hours post-infection

Extracellular bacteria were rarely seen during this stage of infection and were only present near the cell membrane. Attachment of the bacteria by cap adherence was not observed. Only free intracellular bacteria were observed in the cytoplasm. Bacteria were not seen in endocytic vacuoles. Some free bacteria were frequently seen very closely associated with host cell mitochondria (Fig. 21). Small groups of bacteria packed closely together were first evident at this site (Fig. 22). Both extracellular and intracellular bacteria were reactive with specific monoclonal antibody IG4 in immunogold staining.

Light microscopic observation showed singly scattered and small groups of bacteria. Many single bacteria were

Figure 21

Transmission electron micrograph of IEC-18 cells infected with the intracellular bacteria at 24 hours post-infection. Note a bacterium free in the cytoplasm in close contact with a mitochondria (arrow). Lead citrate and uranyl acetate stain x 782000.

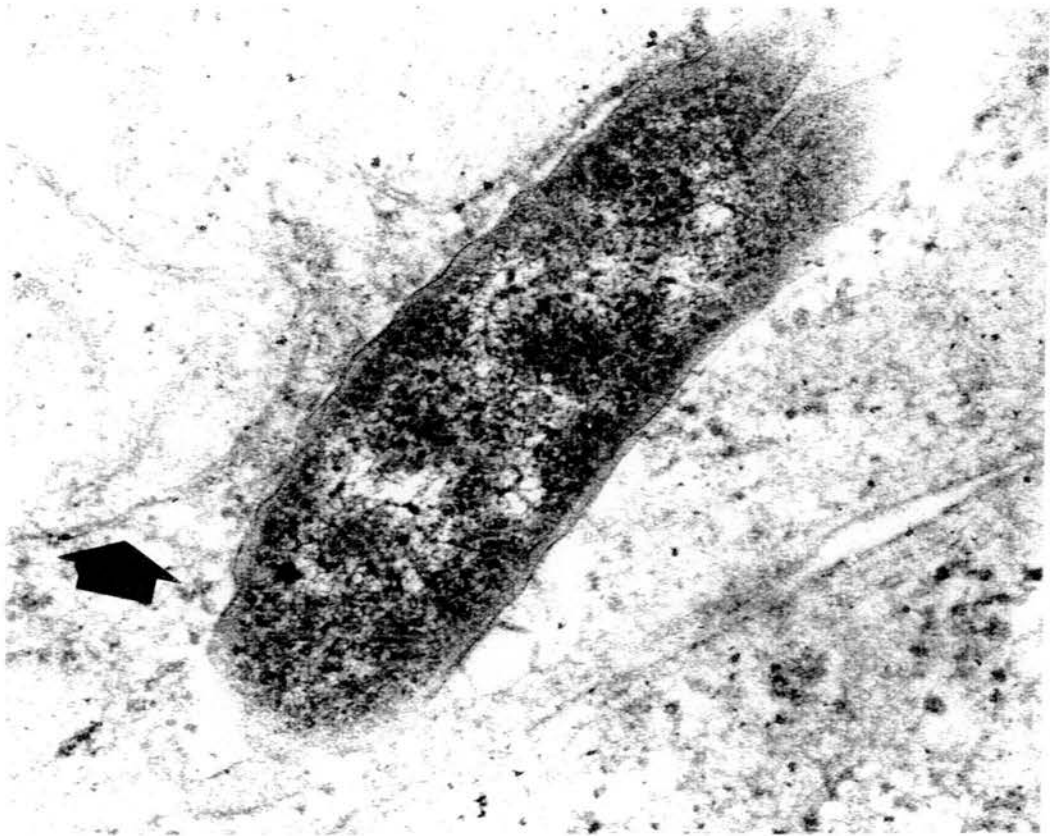


Figure 22

Transmission electron micrograph of IEC-18 cells infected with the intracellular bacteria at 24 hours post-infection. Note small group of bacteria free in the cytoplasm. Lead citrate and uranyl acetate stain. x 78200.



seen undergoing division by transverse septation (Fig. 23).

Late events of infection

Only occasional extracellular bacteria were observed. These bacteria were only seen near the cell membrane with no apparent attachment by cap protrusion. IEC-18 cells heavily infected with numerous organisms were evident. Groups of bacteria were seen either loosely scattered or closely packed together (Figs. 24 and 25) free in the cytoplasm. Some bacteria were seen closely associated with host cell mitochondria. Bacteria were also present in closely packed circular aggregations, sometimes near the nucleus and apparently close to the cytoplasmic surface of the cell membrane (Fig. 26). In a few instances, these bacterial aggregations appeared to be closely associated to granular ribosome-like structures and appeared to be surrounded by host cell mitochondria and rough endoplasmic reticulum. Morphologically, bacteria in these circular aggregations lack well defined inner cytoplasmic constituents. Apart from the highly infected cells, lightly infected cells containing single free bacteria were also evident. On very rare occasions, some single bacteria

Figure 23

IEC-18 cells infected with the intracellular bacteria at 24 hours post-infection. Bacteria are often not numerous within cells (small arrow), a few cells contain more numerous organisms (large arrow). Note a bacterium is dividing by transverse binary fission (arrowhead). Immunoperoxidase staining with monoclonal antibody IG4 and sheep anti-mouse peroxidase conjugate. Haematoxylin counterstain x 1281.

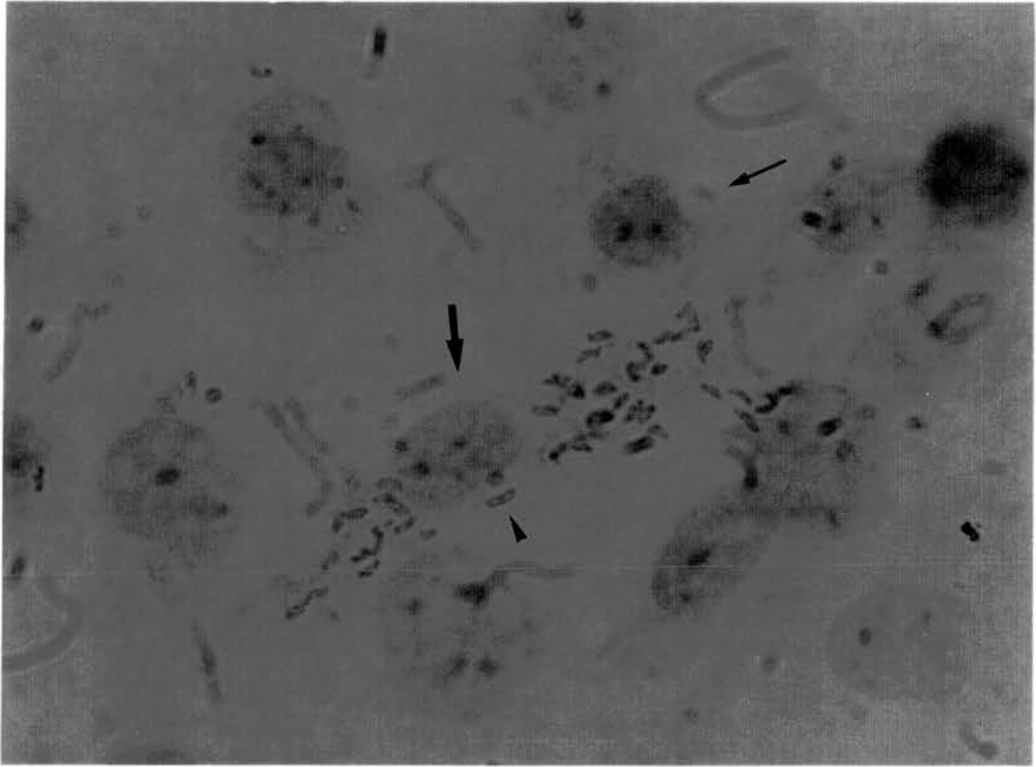


Figure 24

Transmission electron micrograph of IEC-18 cells infected with the intracellular bacteria at day 6 post-infection. Large number of bacteria are closely packed together free in the cytoplasm. Note the cytoplasmic matrix of the infected cell is disrupted. Lead citrate and uranyl acetate stain x 43000.



Figure 25

Transmission electron micrograph of IEC-18 cells infected with the intracellular bacteria at day 6 post-infection. Large number of bacteria are loosely scattered free throughout the cytoplasm. Some bacteria have a sectioning artefact in their protoplasm. Note a bacterium closely associated with a host cell mitochondrion (arrow) The cytoplasmic matrix of the infected cells appeared disrupted. Lead citrate and uranyl acetate stain x 28750.

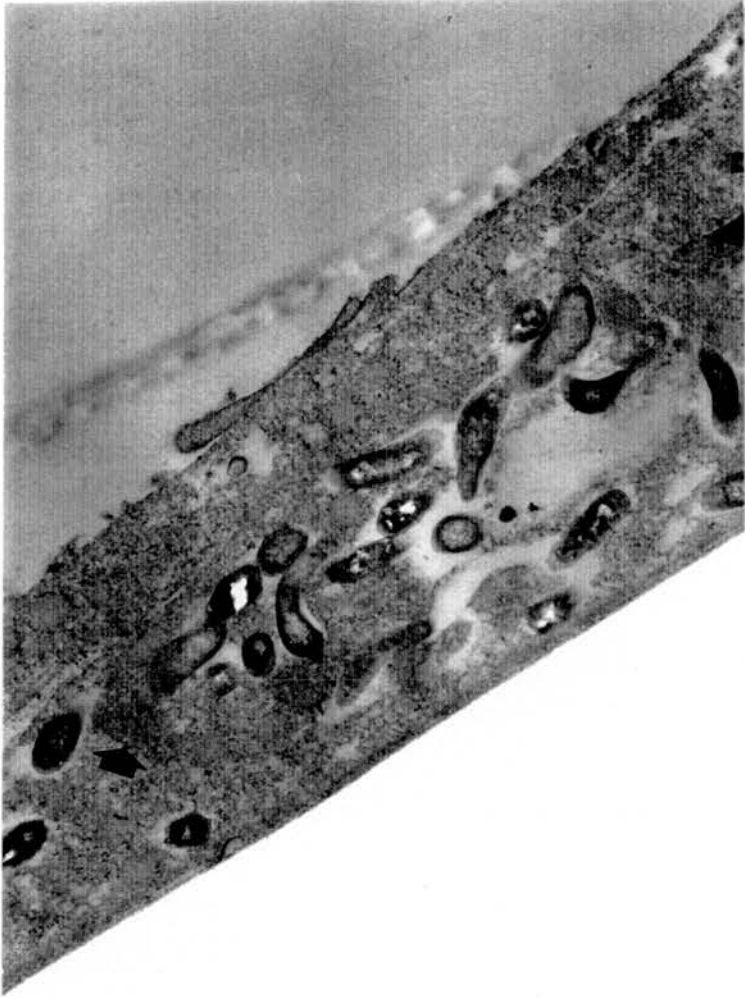
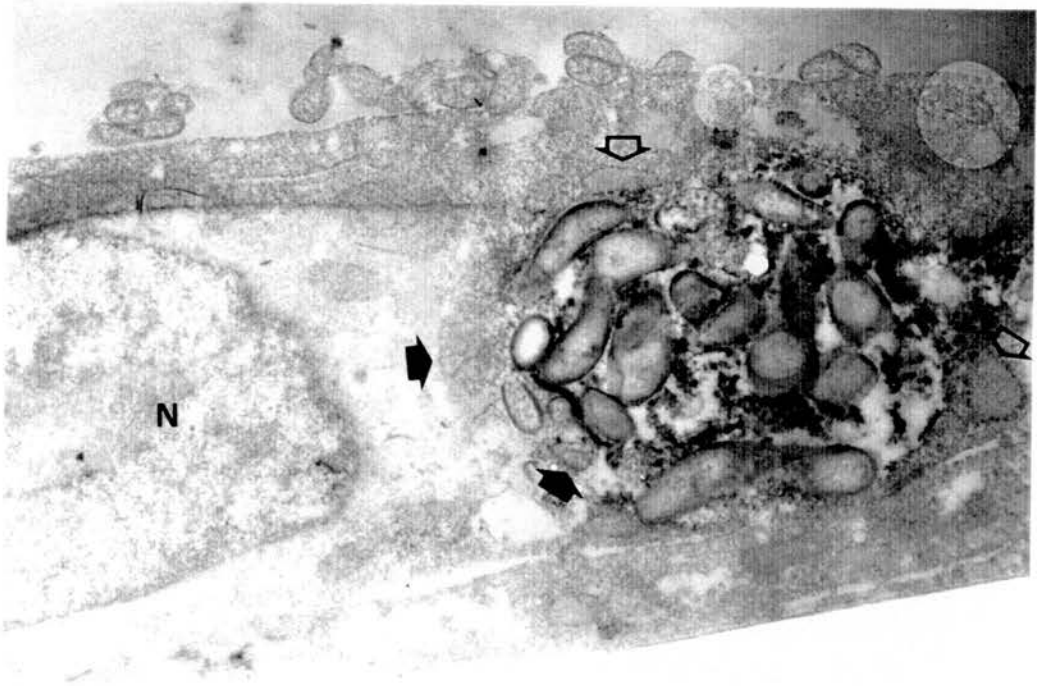


Figure 26

Transmission electron micrograph of IEC-18 cells infected with the intracellular bacteria at day 6 post-infection. Note bacteria in circular groups adjacent to the cell nucleus. Mitochondria (close arrow) and rough endoplasmic reticulum (open arrows) appeared to surround these bacteria. A granular ribosomal-like material is evident interspersed between the bacteria. Note lack of well defined bacterial inner cytoplasmic constituents. N, nucleus. Lead citrate and uranyl acetate stain x 25000.



were also seen surrounded by ribosome-like structure. Highly infected cells showed variable degrees of cytoplasmic disruption and mild to moderate enlargement of the mitochondria and the rough endoplasmic reticulum.

Light microscopy revealed foci of infected cells as observed in earlier experiment and described in Chapter 3. Each focus consisted of groups of highly infected cells with adjacent cells which were lightly infected. In an infected focus, different morphological patterns of infection were observed; lightly infected cells consisting of single scattered or small loose groups of organisms, whereas other foci contained heavily infected cells with numerous bacteria which were usually tightly packed together and completely covered the cell cytoplasm. Bacteria were observed surrounding the cell nucleus but seldom seen in the supranuclear region. Many cells, in division were also seen to be infected (Fig. 27).

In some infected foci, some cells showed variable numbers of circular bacterial aggregations staining specifically with the immunoperoxidase stain (Fig. 28).

These antigen aggregates were frequently seen at the perinuclear region of the host cell and some appeared to fuse with one another to form larger aggregates (Fig. 29).

Some cells, adjacent to highly infected cells appeared to be surrounded by bacteria, with the organisms adjacent to the periphery of the cell (Fig. 30). Heavily infected cells often containing large numbers of bacteria in the cytoplasm were sometime observed overlying other cells of the monolayer. It appeared as if such cells had lost the ability to adhere and were being extruded from the monolayer. Some bacterial aggregates also appeared to protrude from the cell surface and not to lie in the focal plane of the monolayer (Figs. 31 and 32).

Groups of bacteria were also seen very occasionally tightly packed in the nucleus of some infected cells (Fig. 33).

5.4 Comment

The sequence of events in IEC-18 cell line infection

Figure 27

IEC-18 cells infected with the intracellular bacteria at day 6 post-infection. Note an infected cell in metaphase stage of mitosis (arrow). Immunoperoxidase staining with monoclonal antibody IG4 and sheep anti-mouse peroxidase conjugate Haematoxylin counterstain x 640.

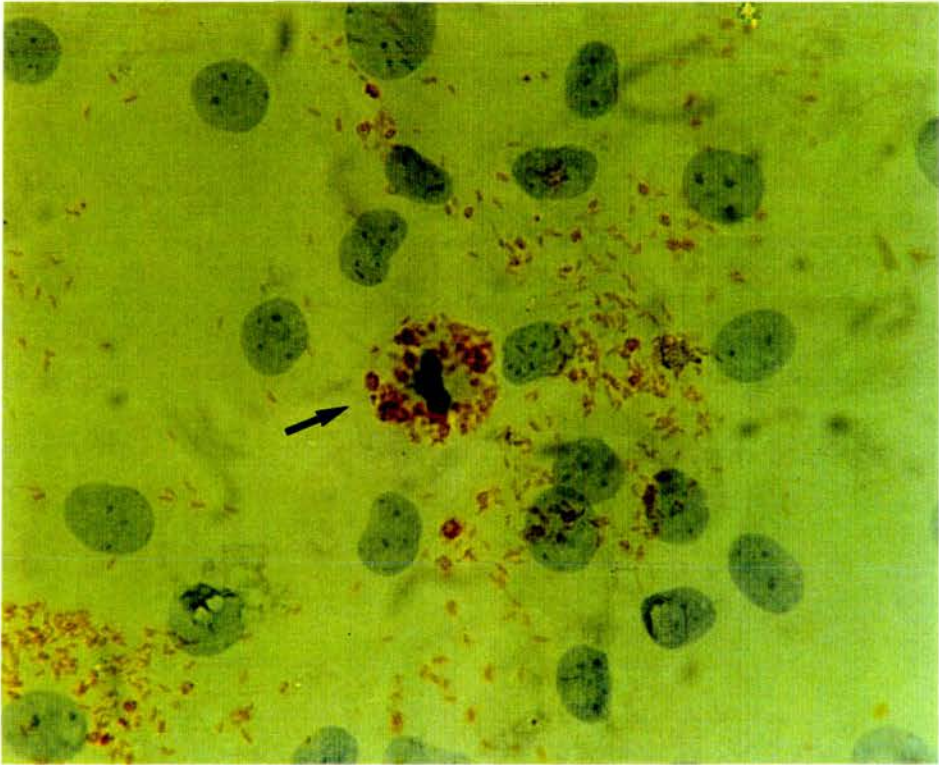


Figure 28

IEC-18 cells infected with the intracellular bacteria at day 6 post-infection. Bacteria in circular groups are often observed at the perinuclear region of cells (arrowheads). Immunoperoxidase staining with monoclonal antibody IG4 and sheep anti-mouse peroxidase conjugate. Haematoxylin counterstain x 1100.

Figure 29

IEC-18 cells infected with the intracellular bacteria at day 6 post-infection. Some circular groups of bacteria (arrowhead) coalesced with one another forming larger bacterial aggregates. Immunoperoxidase staining with monoclonal antibody IG4 and sheep anti-mouse peroxidase conjugate. Haematoxylin counterstain x 1100.

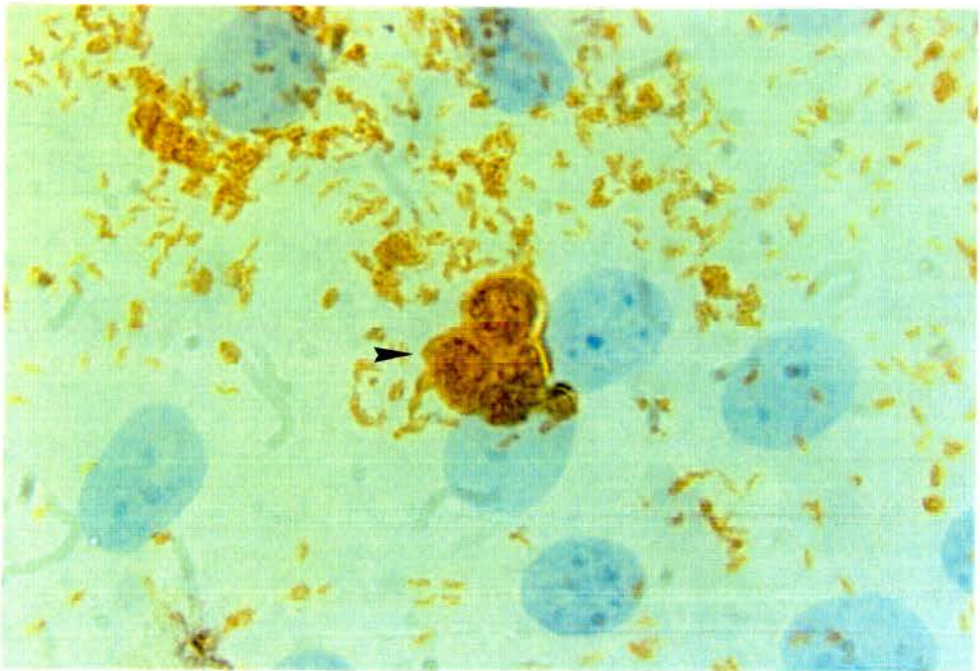
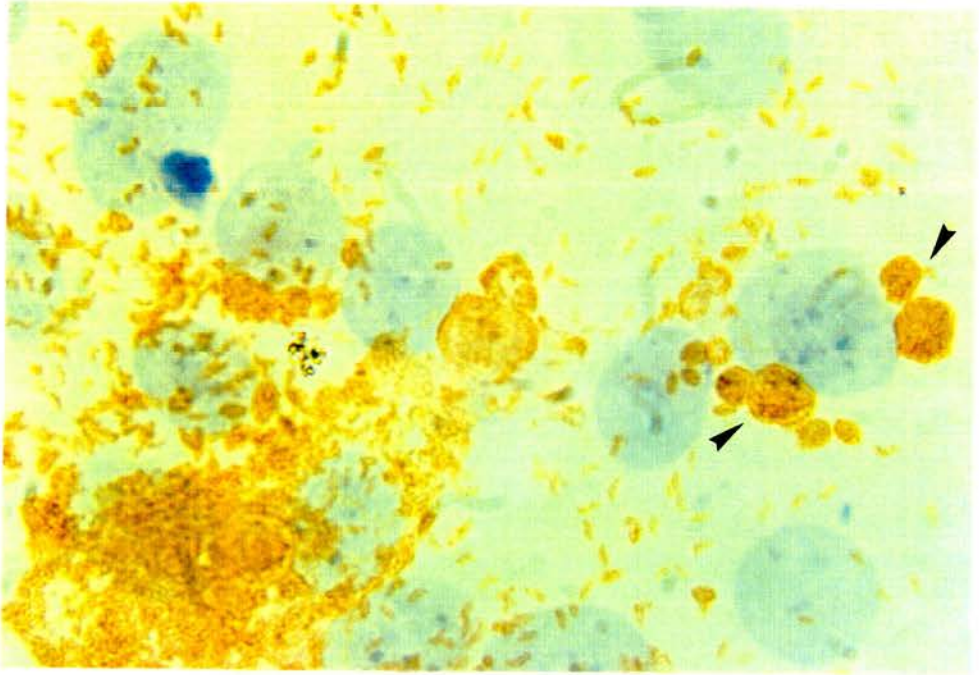


Figure 30

IEC-18 cells infected with the intracellular bacteria at day 6 post-infection. An infected cell (arrow) appeared to be surrounded only at the periphery with bacteria near a highly infected cell. Immunoperoxidase staining with monoclonal antibody IG4 and sheep anti-mouse peroxidase conjugate. Haematoxylin counterstain x 640.

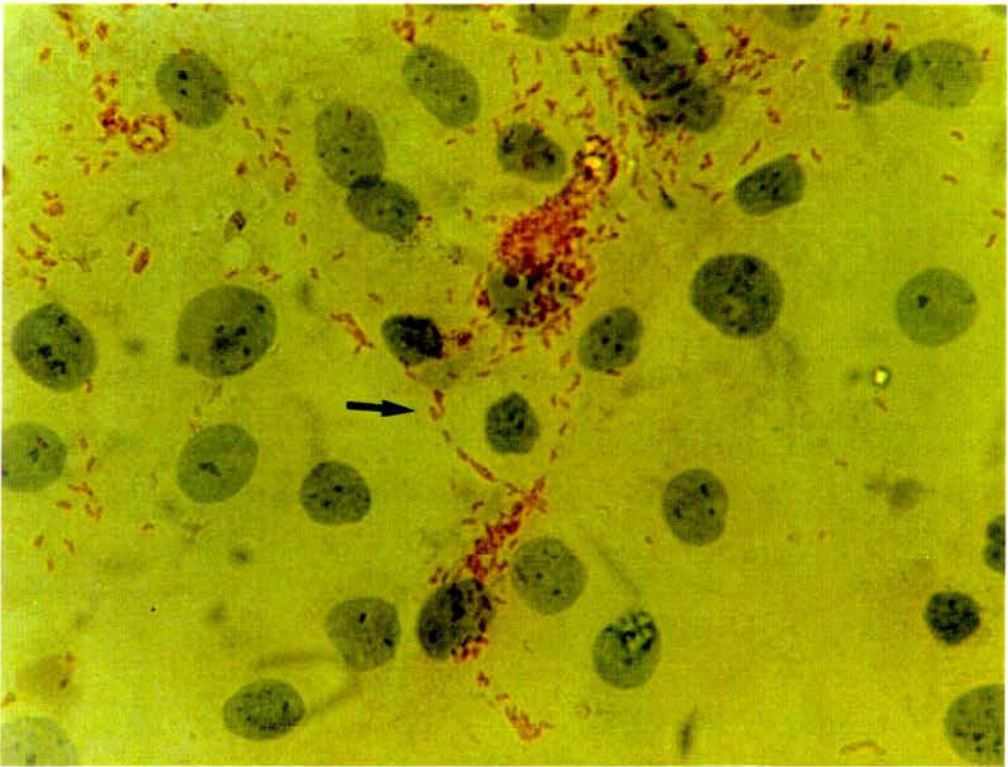


Figure 31

IEC-18 cells infected with the intracellular bacteria at day 6 post-infection. Bacteria in circular groups (arrows) in some infected cells are not in the same plane as other organisms in other infected cells and appeared to protrude from the cytoplasm. Immunoperoxidase staining with monoclonal antibody IG4 and sheep anti-mouse peroxidase conjugate Haematoxylin counterstain x 625.

Figure 32

IEC-18 cells infected with the intracellular bacteria at day 6 post-infection. Three infected IEC-18 cells (arrows) not in the same plane as other cells and appeared to protrude or on the verge of losing adherence. Immunoperoxidase staining with monoclonal antibody IG4 and sheep anti-mouse peroxidase conjugate. Haematoxylin counterstain x 656.

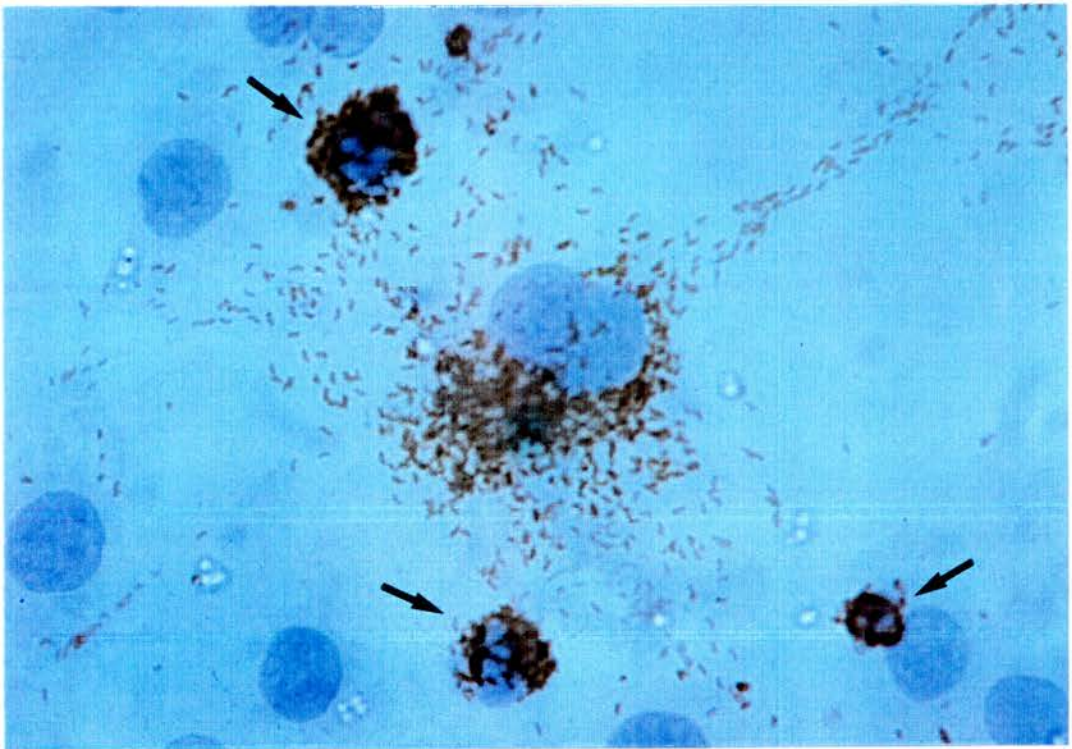
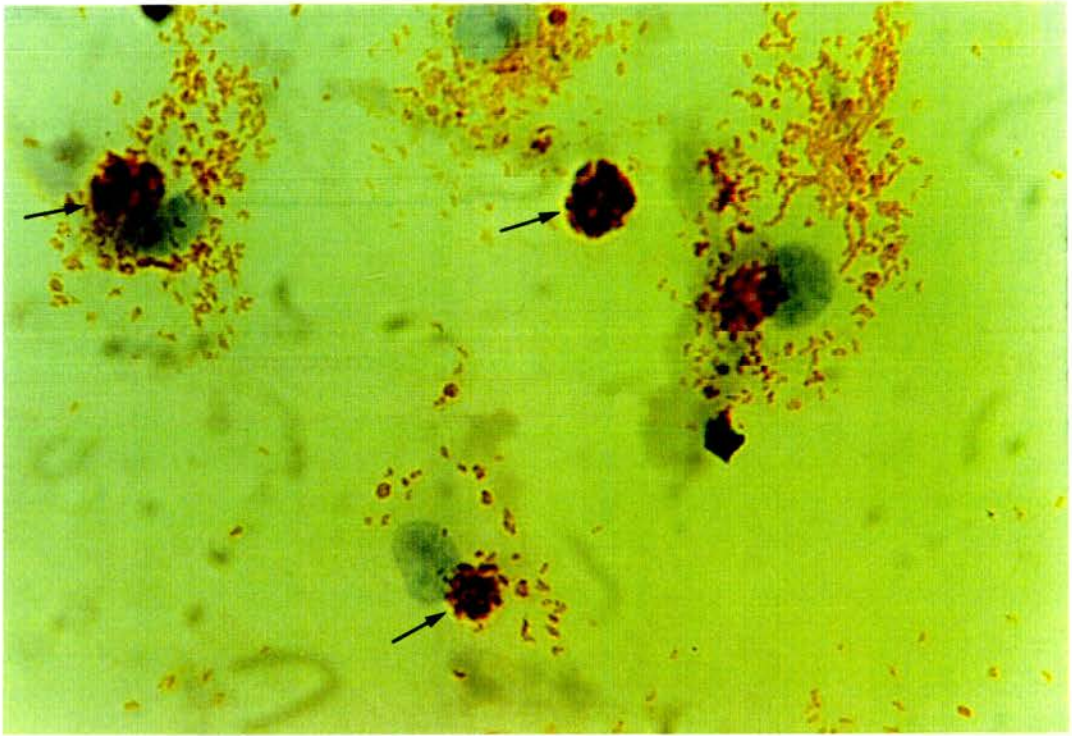
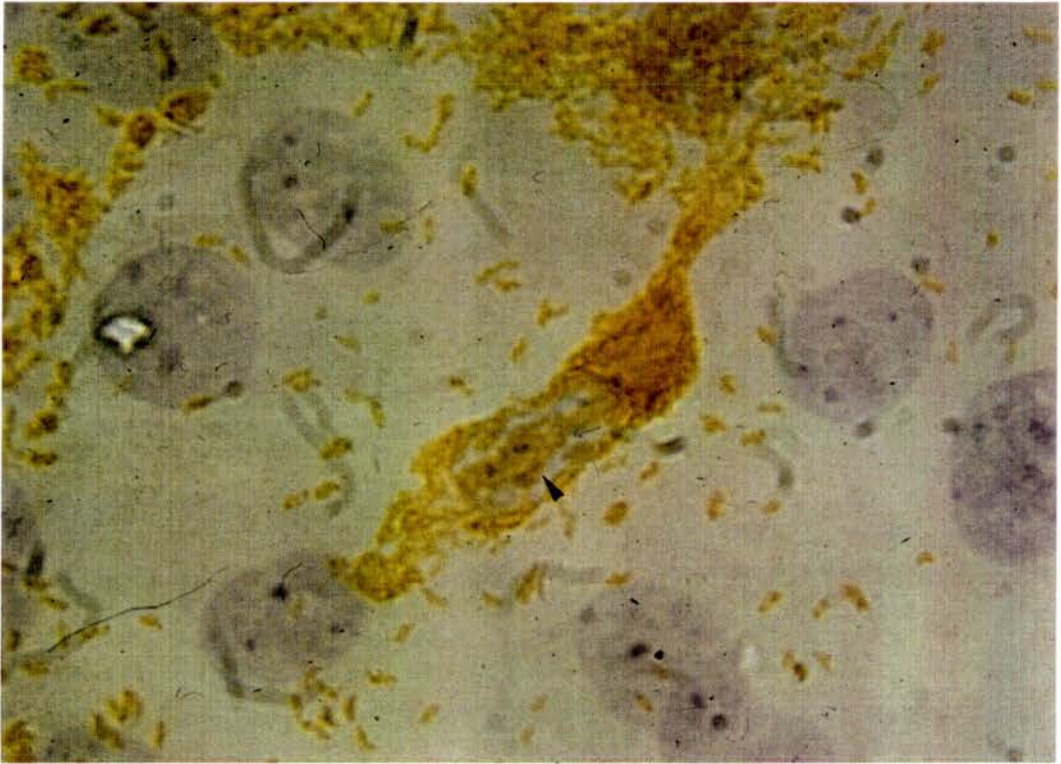


Figure 33

IEC-18 cells infected with the intracellular bacteria at day 6 post-infection. A cell with an intranuclear presence of numerous bacteria (arrow head). Note the cytoplasm of the cell is also packed with numerous bacteria. Immunoperoxidase staining with monoclonal antibody IG4 and sheep anti-mouse peroxidase conjugate. Haematoxylin counterstain x 1343.



from attachment of I. intracellularis to entry, intracellular survival and multiplication are revealed in this study. A summary of the stages of infection observed at different time of infection is depicted in Table 8. Observation of the bacteria by electron microscopy can be carried out by infecting IEC-18 cells with inocula containing high number of bacteria. The number of bacteria used to infect IEC-18 cells was estimated by the method described by McOrist, Jasni, Mackie, Neef, MacIntyre and Lawson (1993). Each highly infected cell was assumed to contain 50 organisms and the infected cells were spread evenly over the flasks (25 or 75cm²) used for inocula and the monitoring coverslips (13 or 16mm), with this assumption, the number of bacteria in the inocula was not less than the estimated value. Details of the inocula used and quantitation of infection is depicted in Table 9.

The work in this study has ultrastructurally defined the location of the bacteria not proven either by immunofluorescence or immunoperoxidase methods for light microscopical examination (Chapter 3). Entry and multiplication of the bacteria in IEC-18 were observed.

Table 8. IEC-18 cells exposed to *I. intracellularis* strains 916/91 and 1482/89: Summary of ultrastructural events in centrifuged infection

Bacterial strain	Hours PI	Extracellular event Attachment	In endocytic vacuole	Intracellular event Bacteria: Free in the cytoplasm
916/91	0	Yes	Yes	No
	3	No	Yes	Yes
	24	No	No	Yes
1482/89	0	Yes	Yes	No
	3	No	No	Yes
	24	No	No	Yes
916/91	6 days	No	No	Yes

Table 9. IEC-18 cells exposed to *L. intracellularis* strains 916/91 and 1482/89: Light and electron microscopic observations of infection

Bacterial strain	LM infection: ^a		Number of bacteria per ml inocula	Method of purification of inocula	Hour PI	LM infection: ^b		EM infection: ^c												
	Inocula parallel HIC/F	322/118				7196/NA	3.18 x 10 ⁴	100 x g	0	3	24	0	1	0	2	1	0	4	3	5
916/91																				
1482/89																				
916/91																				

^{a,b} Light microscopic (LM) observation
^c Electron microscopic (EM) observation
^a Total number of highly infected cells (HIC) counted on 1 coverslip (916/91, 200 mm²; 1482/89, 130 mm²), set up in parallel infection in flasks used to prepare inocula
^b Percentage infection counted for 1 coverslip (200 mm²), with 100 cells counted on each, set up in parallel infection on coverslips for EM observation
^c Total number of bacteria counted from 200 cells, sectioned at different sites (2 to 5 sites per quadrant) of the manox coverslips.
 NA Not Applicable. Highly infected cells too numerous to count and no discrete foci of infection. Total number of HIC estimated on 130 mm² coverslips from 5 different areas of 0.0672 mm²
 F Foci of Infection
 NCT Not Centrifuged
 NE Not Examined
 ND Not Done
 * Infection exist as foci

Electron microscopic quantitation showed that more bacteria were observed in the cytoplasm as infection progresses.

Although centrifugation is believed to facilitate bacterial entry, occasional extracellular bacteria were observed. Centrifugation may have damaged the membranes of some bacteria and host cell structures responsible for entry (bacterial adhesins and host cell receptors) which could reduce receptiveness or create a weak reversible bacterial-host cell attachment. The synthesis and receptiveness of host cell receptors may vary at different stages of cell growth which could also influence interaction of bacteria with cells. Bacteria-host cell contact may be interrupted by lysed cell protoplasmic debris present in the inocula. Inocula purified twice contained less protoplasmic debris.

Whilst this study has revealed the stages of IEC-18 cell-Ileobacter infection, another important event of intracellular bacterial infection, that is, release of bacteria from infected cells is still unknown. Extracellular bacteria seen at late stages of infection may

represent this event of infection. This merits investigation of this feature of intracellular parasitism.

5.5 Release of the intracellular bacteria from IEC-18 cells

5.6 Introduction

In order to survive as a successful intracellular pathogen, an obligate intracellular bacterium must exit from an infected host cell following replication so as to enter and initiate a fresh infection in another susceptible host (Moulder, 1985). This behaviour ensures transfer of bacterial progeny from one host to another. Evidence of release of bacteria from infected host cells both in vitro and in the natural hosts have been demonstrated in many known obligate intracellular bacteria [Chlamydia, De La Maza and Peterson, 1982, Cowdria, Prozesky and Du Plessis, 1987, Rickettsia, Ewing et al., 1978 and Coxiella spp., Khavkin and Amosenkova, 1981] and also in facultative intracellular bacteria, such as Listeria (Tilney and Portnoy, 1989) and Shigella spp. (Bernardini, Mounier, d'hauteville, Rondon and Sansonetti, 1989).

Light microscopic and ultrastructural observations of IEC-18 cells infected by I. intracellularis has suggested a method of release of the intracellular bacteria from infected IEC-18 cells. Infection of monolayers with the bacteria results in infected foci of heavily infected cells apparently amongst lightly or non-infected cells. Some heavily infected cells are still capable of division. There is no evidence of cytolytic foci in the infected monolayers. These features suggest that much of the bacterial proliferation in monolayers takes place by division of cells infected from the inoculum, but does not resolve the mechanisms of bacterial release. Two observations may be relevant, firstly, foci of infected cells demonstrated occasional cells with few bacteria located at the cell periphery and secondly some cells show aggregates of bacteria that may protrude from the cell surface. Previous work has shown that the supernatant fluids from infected monolayers to be infectious (Lawson, personal communication), the appearance of cells apparently detaching from the monolayer and possibly floating free in the supernatant (Section 5.3) suggested that these events are related. Bacteria may be released from infected cells

either as a result of cell lysis or as a result of extrusion from the cytoplasm without cell rupture.

The method of release of I. intracellularis from the host cell is still unknown and has not been explored. It is also important that the operative mechanisms are understood before proposing any in vitro infection system as a method of natural infection.

The work in this Section was designed to investigate the events of extracellular release of I. intracellularis from intestinal cell in vitro. Attempt to achieve this objective was conducted by examining culture supernatant fluids from late IEC-18 cells infection by light and transmission electron microscopy. Scanning electron microscopy of the early and late stages of infection was also conducted.

5.7 Materials and Methods

Preparation of supernatant cytospin

The supernatant from passaged IEC-18 cell infection

with I. intracellularis strains 1428/89 (passaged 19 times) and 916/91 (passaged 2 times) after 5 and 6 days post-infection respectively were used for this study. The supernatant from infected flasks was decanted and placed into a conical tube at 4°C for 30 minutes and 60ul taken from the bottom of the container and centrifuged onto a glass slide at 750 x g for 10 minutes. The centrifuged preparation was allowed to air dry, fixed in acetone for 90 seconds and stained with Modified Ziehl Neelsen (Section 2.13), Giemsa (section 2.14) or the specific monoclonal antibody IG4 and anti-peroxidase conjugate in an immunoperoxidase test (Section 2.9).

Transmission electron microscopy

Preparation of supernatant pellet

The supernatant from a passaged infection of IEC-18 cell with I. intracellularis strain 1482/89 (day 5 post-infection) passaged 19 times was used to form a pellet for ultrastructural observation.

Supernatant (30ml) from three 75cm² tissue culture

flasks was harvested and immediately fixed (1:2 vol/vol) in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4 for 2 hours at 4°C in a centrifuge tube and centrifuged at 45 x g for 10 minutes. One ml of the fluid was removed from the bottom of the centrifuge tube and transferred into an Eppendorf tube for further centrifugation at 2700 x g for 25 minutes. After the final centrifugation, the supernatant was discarded and the formed pellet was processed for TEM (Section 2.11).

Scanning electron microscopy

Scanning electron microscopy observation of the release events was conducted in parallel to that used for ultrastructural study of late infection (Section 5.2). IEC-18 cells were infected, harvested at 3, 24, 48 hours and 6 days post-infection and processed for scanning electron microscopy as described in Section 2.11.

5.8 Results

Phase microscopic examination of the infected flasks

showed many floating extruded cells in the supernatant fluids. Some adherent cells had circular protrusions from the cytoplasm. Both extruded cells and protruded cytoplasm were refractile. Bacteria were not observed free in the supernatant fluids or associated with cells or the protrusions. Examination of supernatant fluids in uninfected flasks revealed similar, but fewer cell extrusions and protrusions.

Examination of cytopsin preparations of the supernatant fluids showed many extruded cells and non-nucleated cell cytoplasm which contained aggregates of bacteria (Fig. 34). Numerous free bacteria were also observed (Fig. 35). These bacteria were modified acid fast, reactive with the monoclonal antibody IG4 and were rod-shaped, S-shaped and predominantly curved. Extruded cells and non-nucleated cell cytoplasm with no evidence of bacteria were also observed.

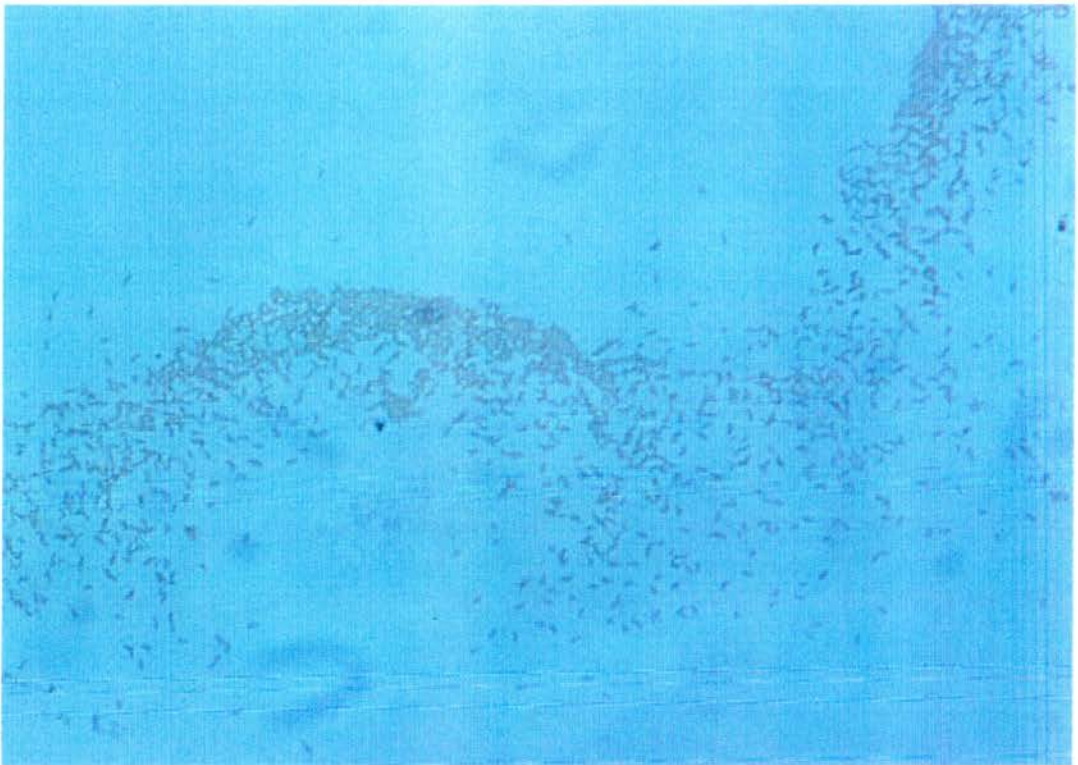
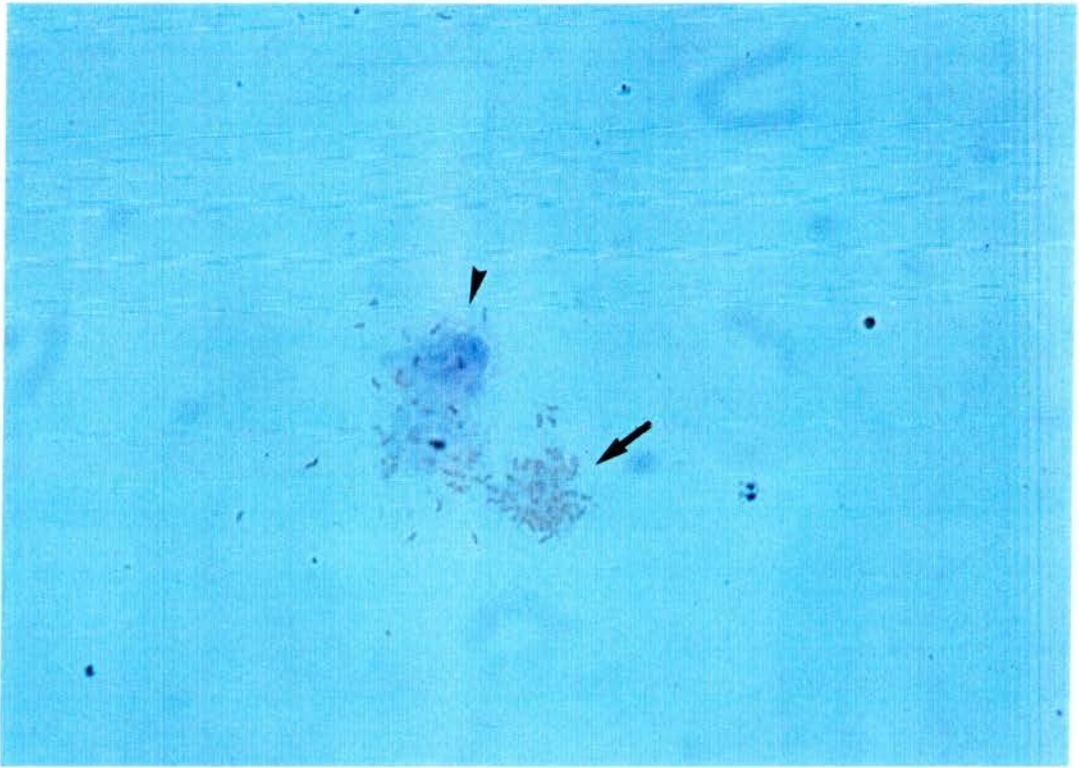
Ultrastructural observation of supernatant fluids pelleted by centrifugation correlated with the findings by light microscopy. Non-nucleated cell cytoplasm and

Figure 34

Supernatant fluid pelleted by centrifugation from infected IEC-18 cells. An extruded cell (arrowhead) and non-nucleated cell cytoplasm (arrow) containing numerous bacteria. Immunoperoxidase staining with monoclonal antibody IG4 and sheep anti-mouse peroxidase conjugate. Giemsa counterstain x 656.

Figure 35

Numerous free bacteria in the supernatant fluid of infected IEC-18 cells are stained with the monoclonal antibody IG4 and sheep-anti mouse peroxidase conjugate in an immunoperoxidase test. Giemsa counterstain x 656.



extruded cells were also evident. The cytoplasm of these extruded cells and non-nucleated cell cytoplasm contained numerous bacteria lying free in the cytoplasm in numbers ranging from 20 to 150. Both morphological forms of the bacteria, the electron-dense and electron-lucent forms were observed. Approximately 90% of the bacteria were the electron-dense and 10% the electron-lucent type. The extruded cells and non-nucleated cell cytoplasm were circular in morphology. Larger cells appeared to contain more bacteria in their cytoplasm than smaller cells. The cytoplasmic matrix of extruded cells and non-nucleated cell cytoplasm was disorganised and disrupted. Besides, cytoplasmic degeneration, apparently disrupted organelles were evident. Microvilli were sparse or totally absent (Figs. 36 and 37). Some extruded cells and non-nucleated cell cytoplasm appeared pedunculated (Fig. 38). Extruded cells and non-nucleated cell cytoplasm with no bacteria were also observed. Those that were devoid of bacteria had minimally damaged cytoplasmic matrix. Only occasional cytoplasmic disruption, and distension of the rough endoplasmic reticulum and mitochondria were observed. Microvilli and cytoplasmic processes were more abundant and

the cell membrane appeared intact (Figs. 39 and 40).

Scanning electron microscopy of infected cells revealed multifocal groups of cells with focal, circular, bulging or balloon-like cytoplasmic protrusions. The cell surface adjacent to protrusion appeared unaltered and intact. Each cell had one or a pair of cytoplasmic protrusions often arranged adjacent to one another. Some protrusions which appeared pedunculated and attached to the surface of cells were also observed in this preparation (Figs. 41 to 44).

Many bacteria were observed released extracellularly from infected cells. Bacterial release were observed both in ultrathin and scanning electron microscopic examinations. The morphology of cytoplasmic protrusions differ which suggest a possible sequence of events in release of bacteria from the infected cells (Figs. 44 to 47); some protrusions were seen with no bacteria or bacteria were observed partially embedded in the protrusions whilst some protrusions had cavity with circular opening. Many bacteria either free single or in groups were seen closely associated with the cell membrane

of neighbouring cells or lay adjacent to the opening of the cytoplasmic protrusions. Cells with bacteria present on the surface were also observed (Fig. 48). Uninfected cells showed similar protrusions, but fewer in number. Protrusions had circular morphology but no cavity or opening, and there was no evidence of bacteria.

Ultrathin sections shows extracellular free release of bacteria by extrusion from the cytoplasm through membrane breakdown of extruded cells and non-nucleated cell cytoplasm (Figs. 49 and 50). Some release bacteria had thickened outer membranes (Figs. 51 and 52).

Cytoplasmic protrusions were not seen at the early stages of infection at 3, 24 and 48 hours post-infection but there were occasional extracellular bacteria closely associated to the cell membrane (Figs. 53 to 55).

5.9 Comment

This study has revealed a method and the possible sequence of events of release of I. intracellularis from

Figure 36

Transmission electron micrograph of pelleted supernatant fluid from IEC-18 cells infected with the intracellular bacteria, at day 5 post-infection. Extruded non-nucleated cell cytoplasm containing numerous bacteria. The cytoplasm matrix is disorganised. Cytoplasmic vacuolation, disrupted and distended organelles are evident. Lead citrate and uranyl acetate stain x 8880.

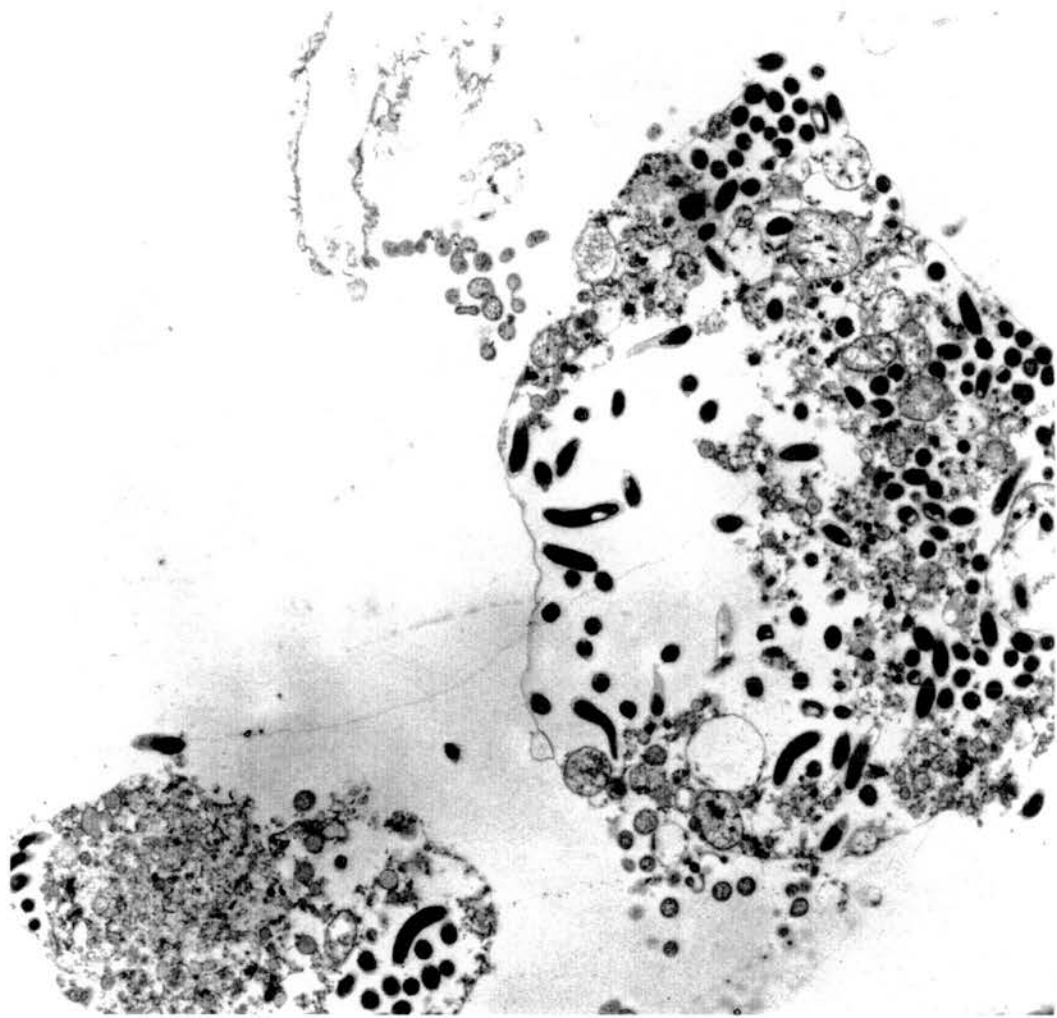


Figure 37

Transmission electron micrograph of pelleted supernatant fluid from IEC-18 cells infected with the intracellular bacteria, at day 5 post-infection. An extruded highly infected cell with distinct nucleus. Many bacteria are present amongst damaged organelles. The cytosol is also disrupted. Lead citrate and uranyl acetate stain x 8418.

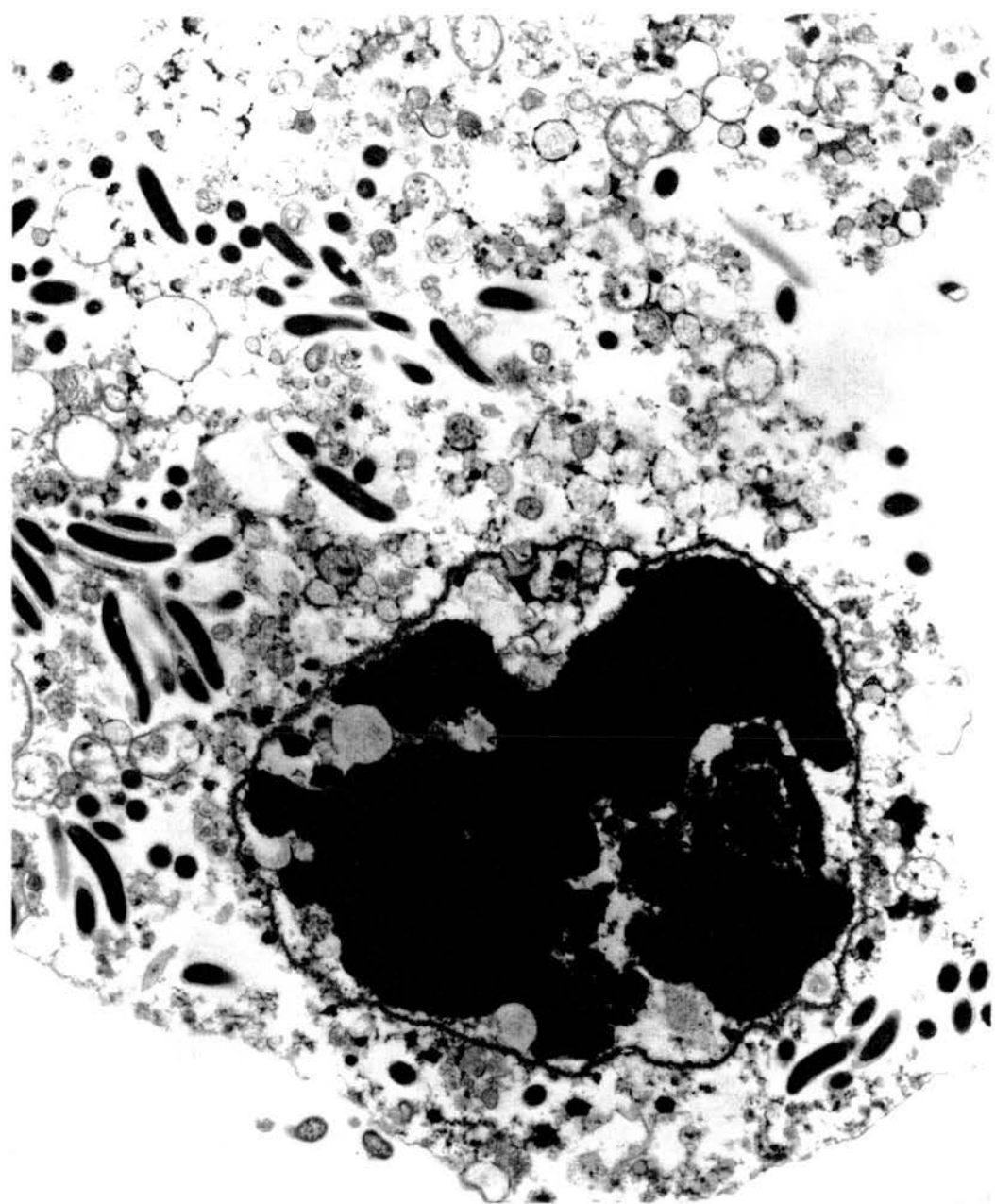


Figure 38

Transmission electron micrograph of pelleted supernatant fluid from IEC-18 cells infected with the intracellular bacteria, at day 5 post-infection. Extruded non-nucleated cell cytoplasm which appeared pedunculated (arrow shows pedunculation). Lead citrate and uranyl acetate stain x 40920.

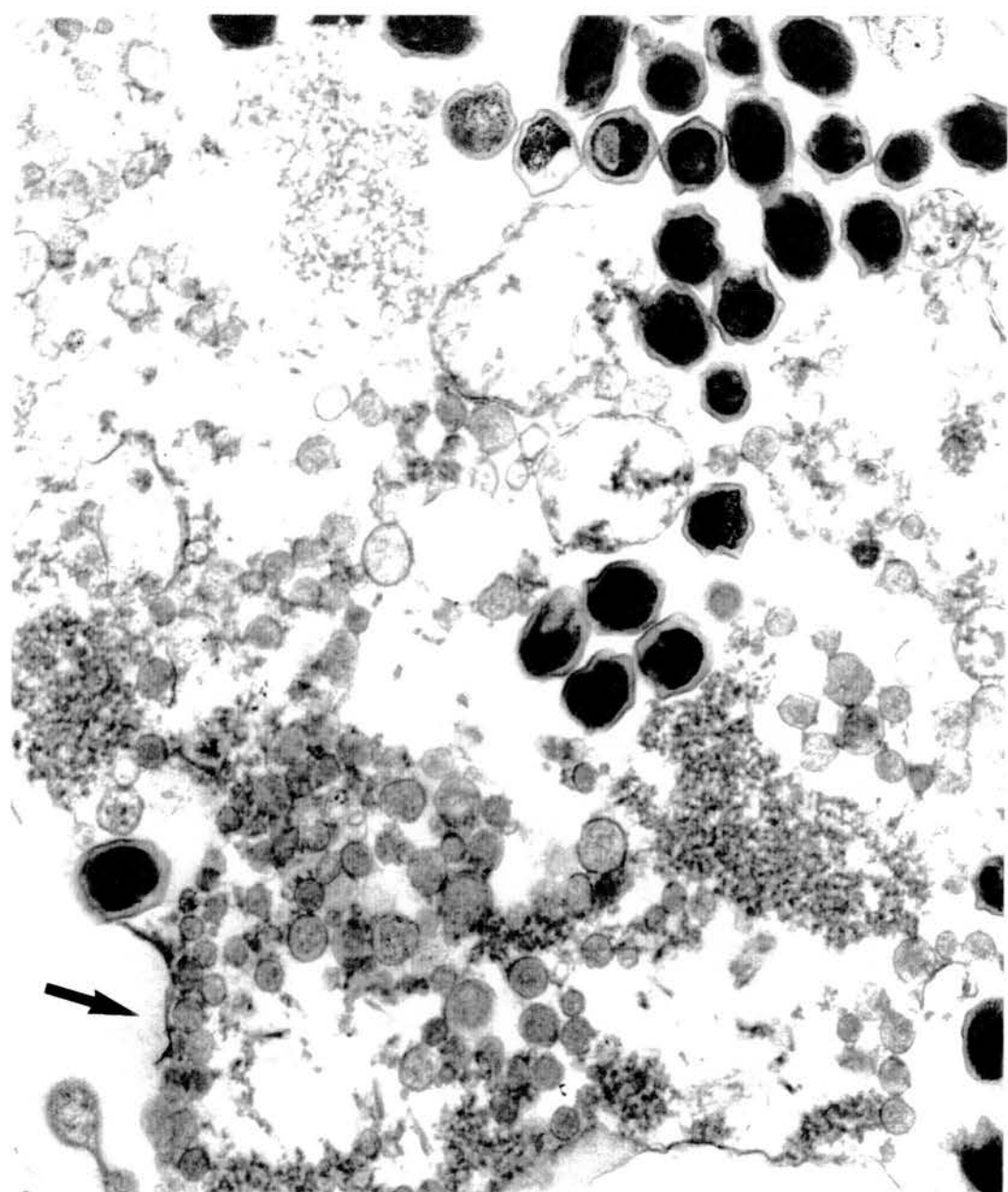


Figure 39

Transmission electron micrograph of pelleted supernatant fluid from IEC-18 cells infected with the intracellular bacteria, at day 5 post-infection. A non-adherent cell with no bacteria evident. The cytoplasmic matrix is minimally damaged. Microvilli are abundant. Cell membrane is intact with no focal rupture. Lead citrate and uranyl acetate stain x 11400.

Figure 40

Transmission electron micrograph of pelleted supernatant fluid from IEC-18 cells infected with the intracellular bacteria, at day 5 post-infection. An extruded non-nucleated cell cytoplasm with no bacteria evident. Damage to the cytoplasmic organelles is restricted to the rough endoplasmic reticulum which is distended. Microvilli and cytoplasmic process are abundant. Lead citrate and uranyl acetate stain x 14630.

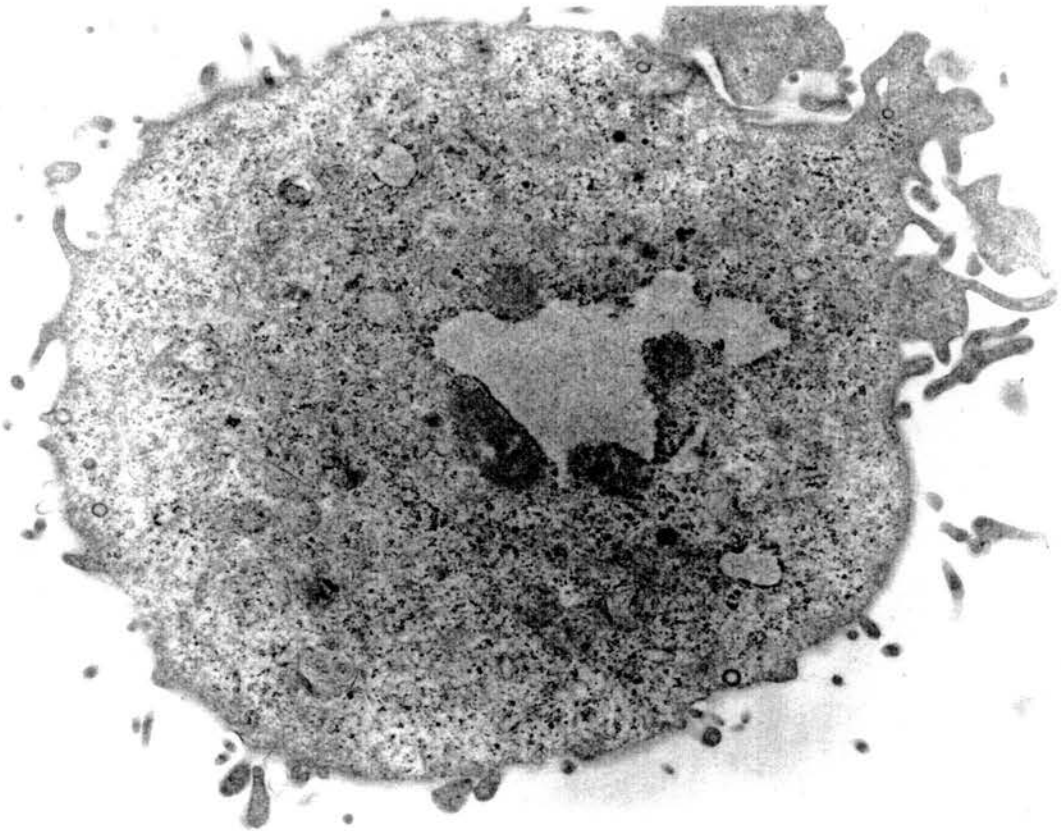
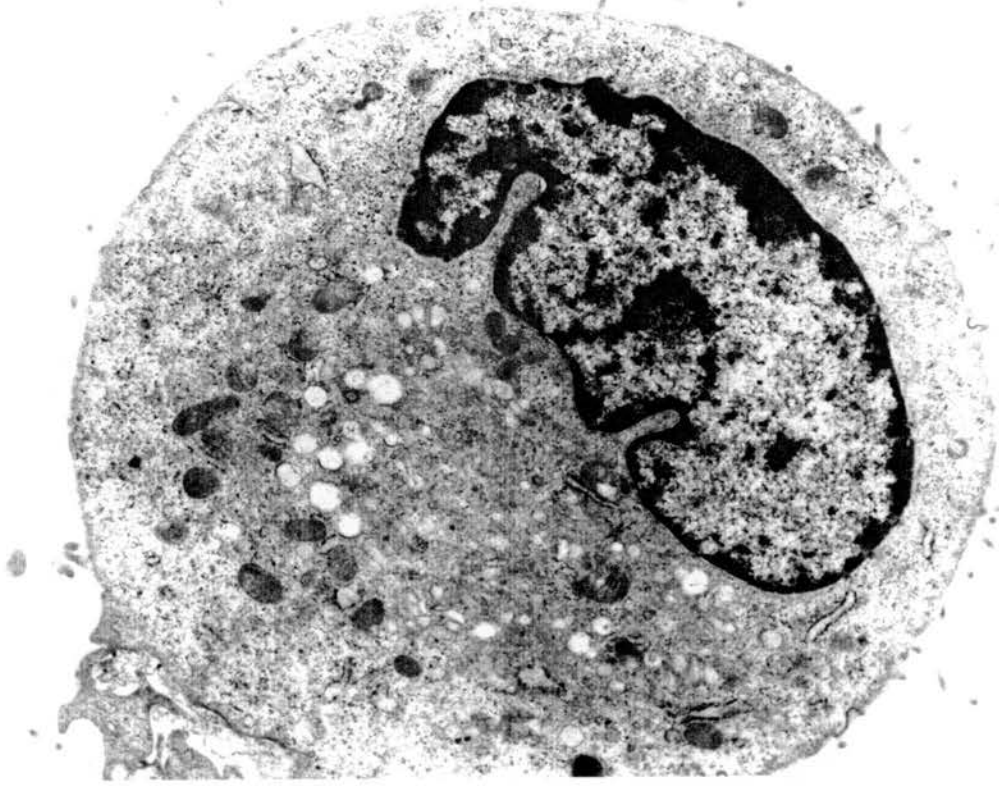


Figure 41

Scanning electron micrograph of IEC-18 cells infected with the intracellular bacteria, at day 6 post-infection. Note a single circular cytoplasmic protrusion in the cell surface (arrow). Adjacent unaffected area of the cell surface remain flat x 970.

Figure 42

Scanning electron micrograph of IEC-18 cells infected with the intracellular bacteria, at day 6 post-infection. A group of cells with cytoplasmic protrusions. Cells have either one or two protrusions of the cytoplasm, some very close to one another x 2390.

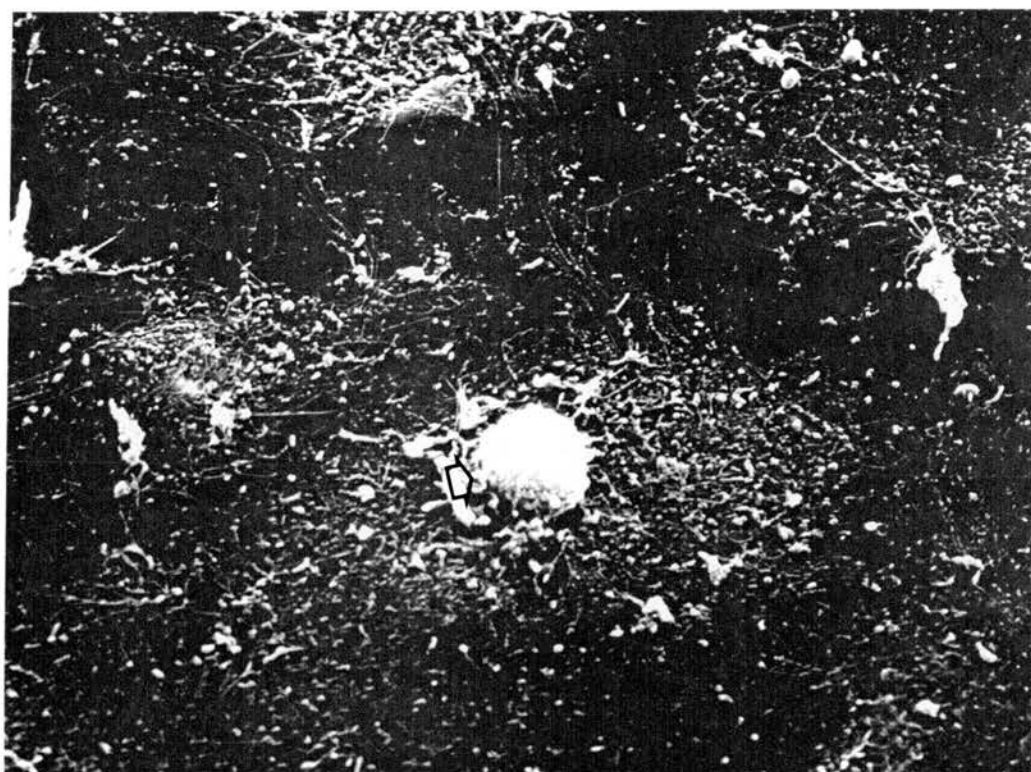


Figure 43

Scanning electron micrograph of IEC-18 cells infected with the intracellular bacteria, at day 6 post-infection. A higher magnification of a cytoplasmic protrusion showing clearly the spherical morphology. Microvilli are present on the surface of the protrusion x 7700.

Figure 44

Scanning electron micrograph of IEC-18 cells infected with the intracellular bacteria, at day 6 post-infection. A possible early event of bacterial release from the protruded cytoplasm showing numerous bacteria partially embedded in its surface (small arrows). Microvilli are not evident on the surface of the protrusions. The large arrow points to a narrow peduncle which appeared to attach the cytoplasmic protrusion on the cell surface x 9170.

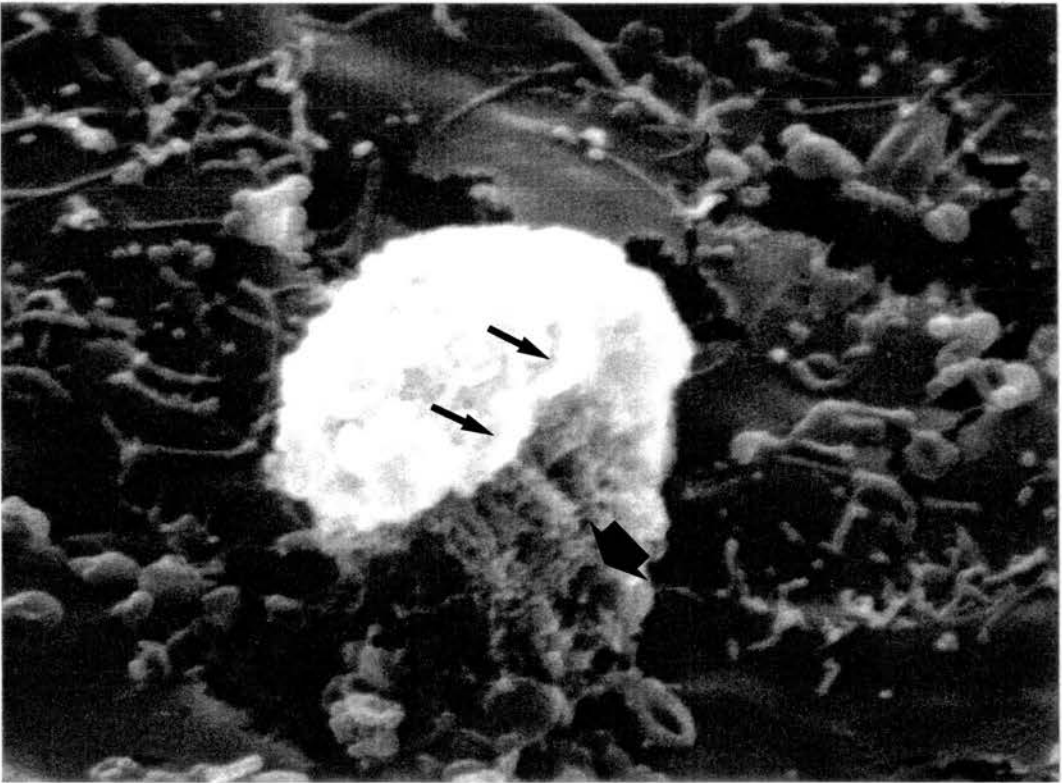
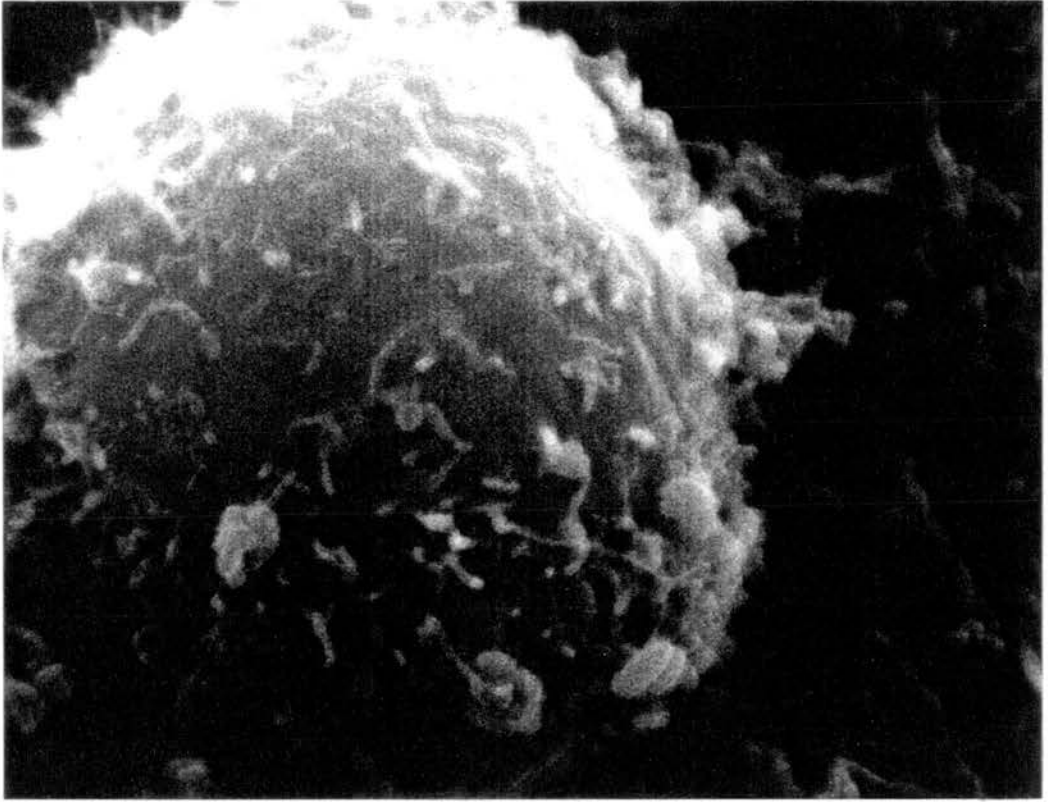


Figure 45

Scanning electron micrograph of IEC-18 cells infected with the intracellular bacteria, at day 6 post-infection. A cytoplasmic protrusion with a ragged circular opening and shallow depression. Free bacteria are seen lying very close to the opening of the depression possibly indicating a later stage of bacterial release x 1000.

Figure 46

Scanning electron micrograph of IEC-18 cells infected with the intracellular bacteria, at day 6 post-infection. A possible further stage of release of bacteria from a cytoplasmic protrusion with a larger and well demarcated circular opening and empty interior. Free bacteria are seen lying adjacent to the opening x 8780.

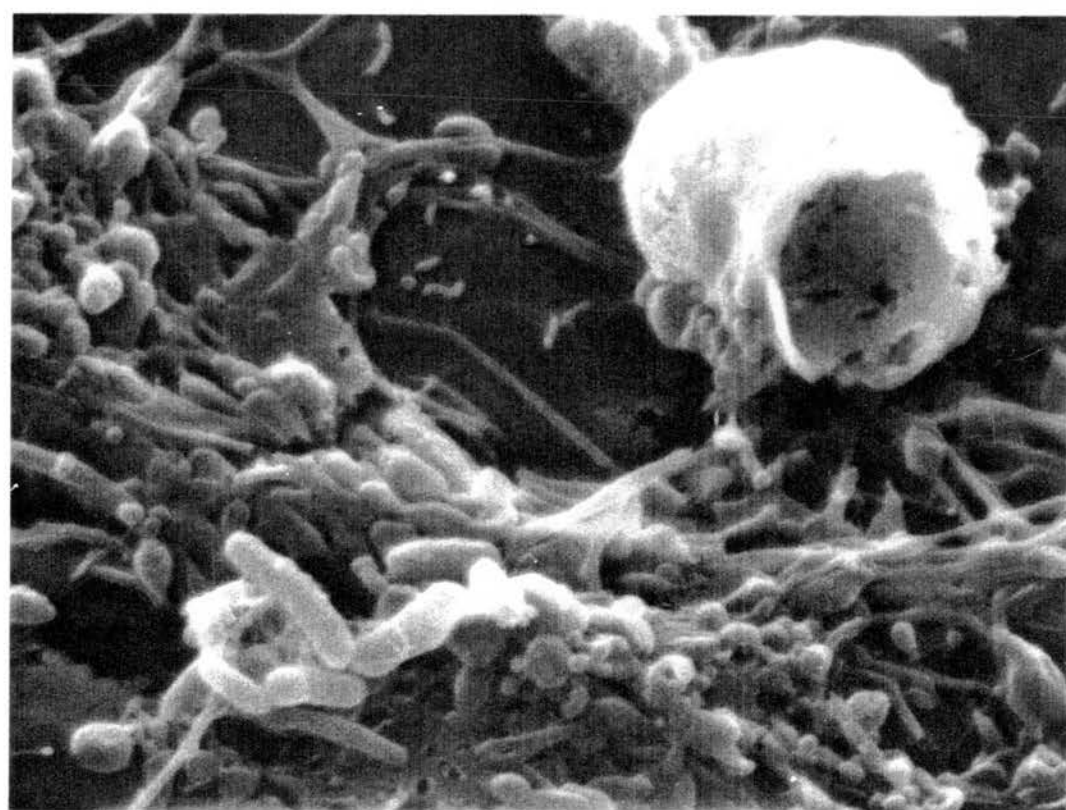
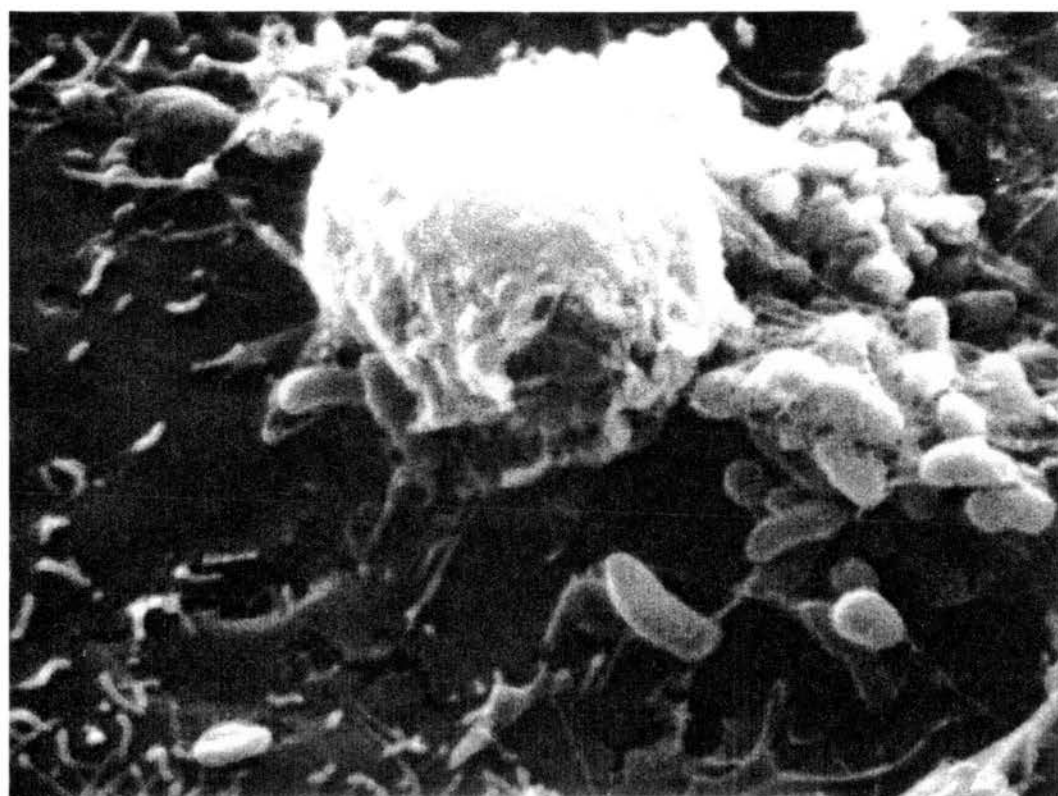


Figure 47

Scanning electron micrograph of IEC-18 cells infected with the intracellular bacteria, at day 6 post-infection. A group of free bacteria on the surface of a cell apparently entangled with cytoplasmic debris after exiting from a protruded cytoplasm x 2390.

Figure 48

Scanning electron micrograph of IEC-18 cells infected with the intracellular bacteria at day 6 post-infection. Note an apparently detaching cell with many bacteria on the surface x 5940.

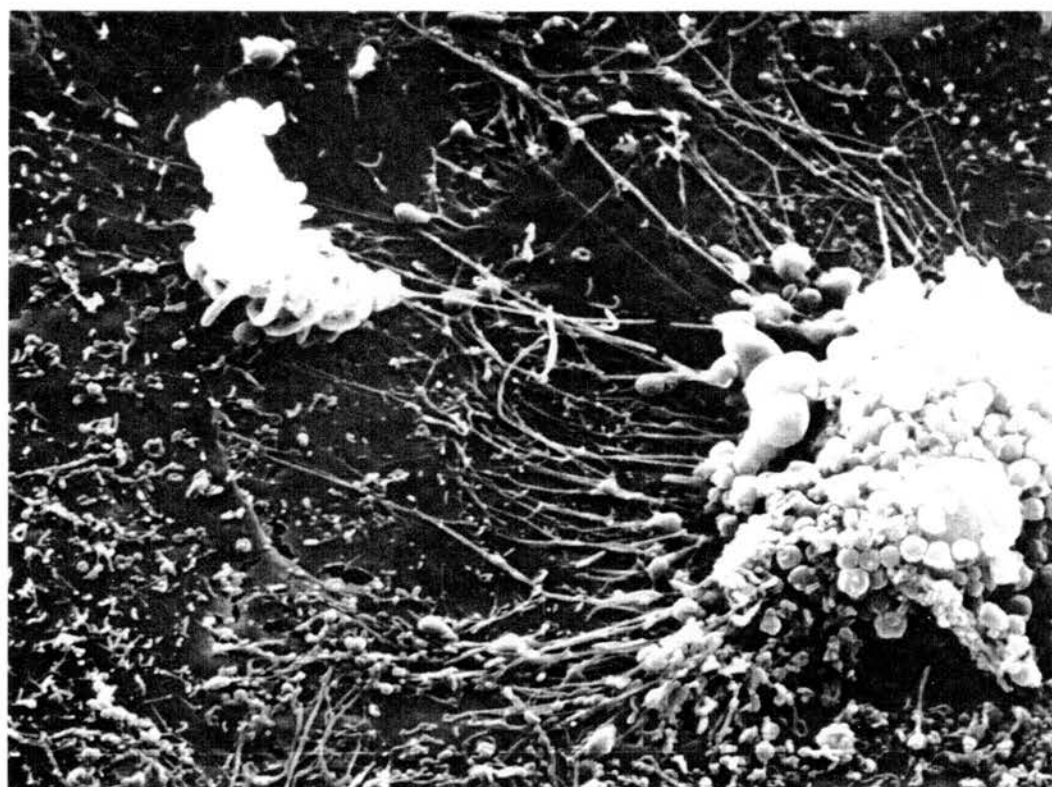


Figure 49

Transmission electron micrograph of pelleted supernatant fluid from IEC-18 cells infected with the intracellular bacteria, at day 5 post-infection. Bacteria are being released from circular cell cytoplasm together with cellular debris through focal cell membrane rupture (arrow). Lead citrate and uranyl acetate stain x 10120.

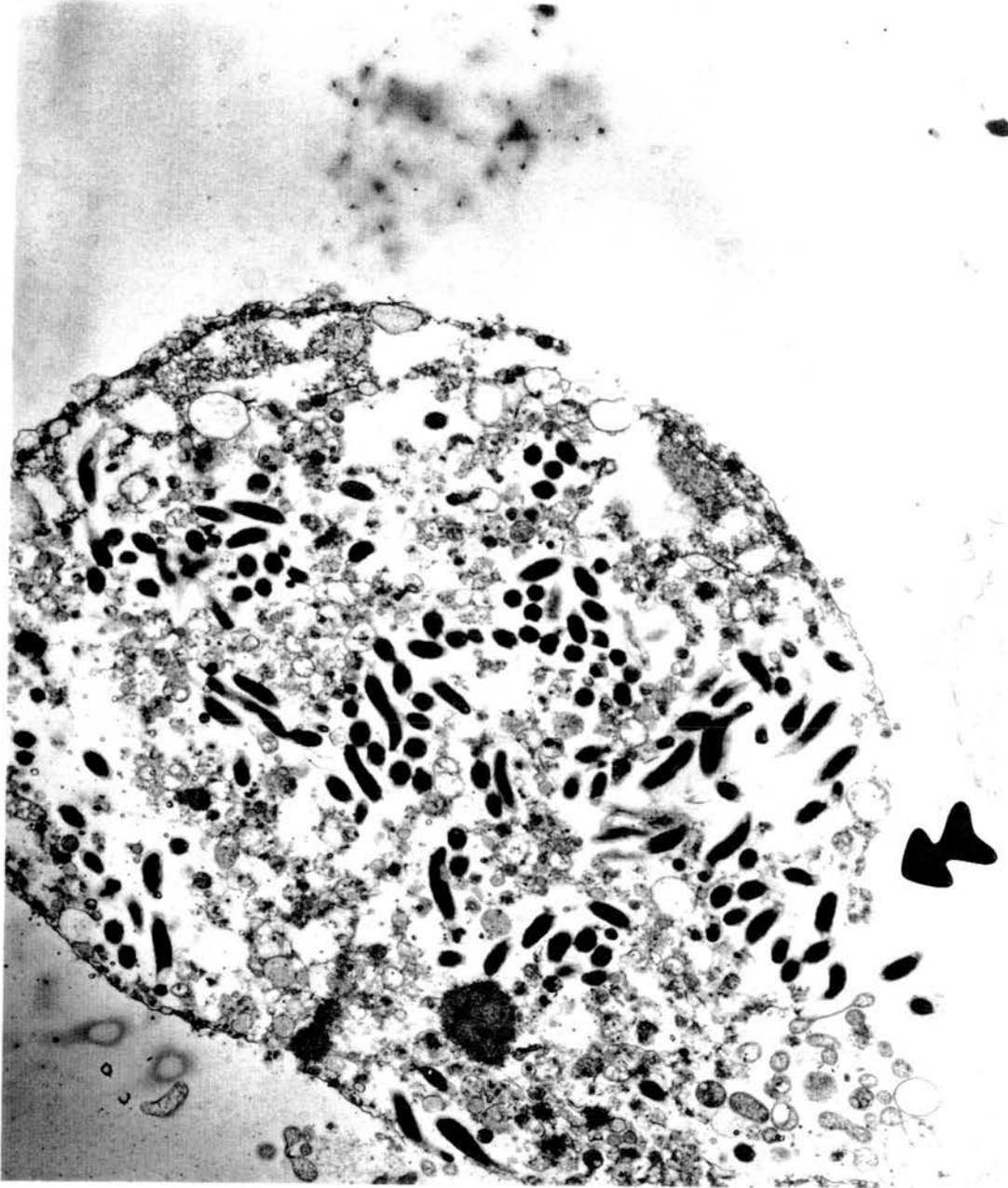


Figure 50

Transmission electron micrograph of pelleted supernatant fluid from IEC-18 cells infected with the intracellular bacteria, at day 5 post-infection. A higher magnification of bacterial release from an extruded cell cytoplasm. Bacteria both electron-dense and electron-lucent are escaping from the cell cytoplasm through cell membrane breakdown. Two bacteria (arrows) are also seen apparently entrapped in distended organelle during centrifugation to form the pellet. Lead citrate and uranyl acetate stain x 31190.

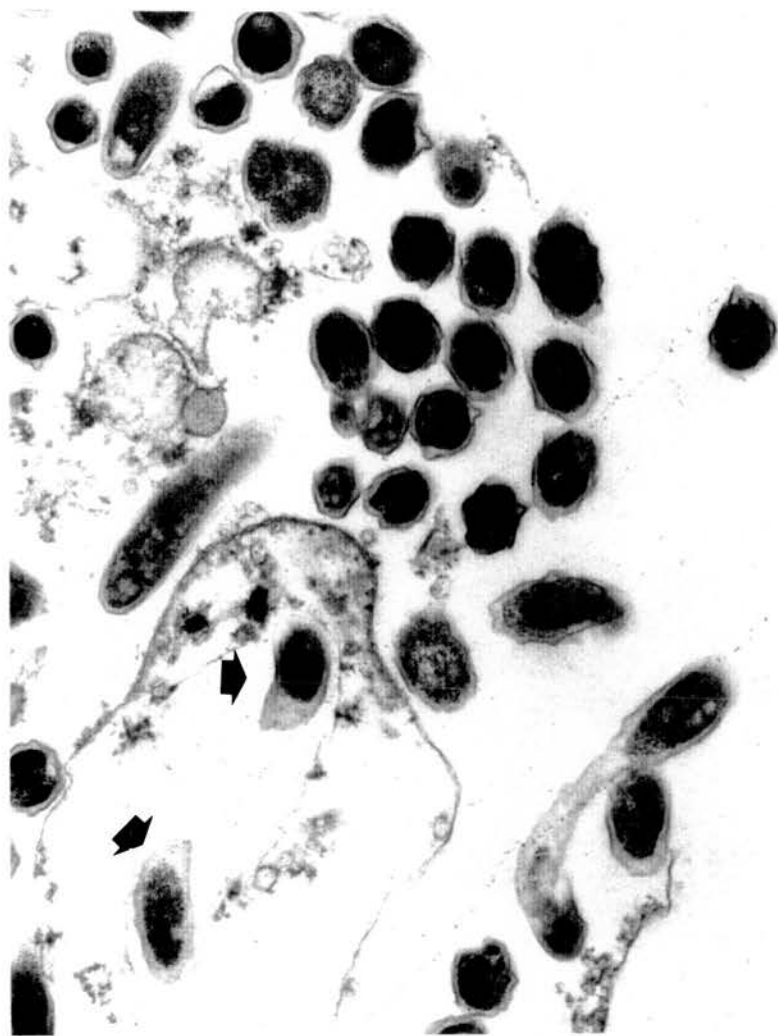


Figure 51

Transmission electron micrograph of pelleted supernatant fluid from IEC-18 cells infected with the intracellular bacteria at day 5 post-infection. Numerous free bacteria not associated with either cell cytoplasm or cells. Some bacteria (arrows) appeared to have thickened outer membrane. Lead citrate and uranyl acetate stain x 73430.



Figure 52

Transmission electron micrograph of pelleted supernatant fluid from IEC-18 cells infected with the intracellular bacteria, at day 5 post-infection. Free bacteria which are not associated with cells and cell cytoplasm at higher magnification. Note a bacterium with thickening of the outer membrane (arrow). Lead citrate and uranyl acetate stain x 204000.



Figure 53

Scanning electron micrograph of IEC-18 cells infected with the intracellular bacteria, at 24 hours post-infection. Cytoplasmic protrusions are not observed in any of the infected cells at this early stage of infection x 850.

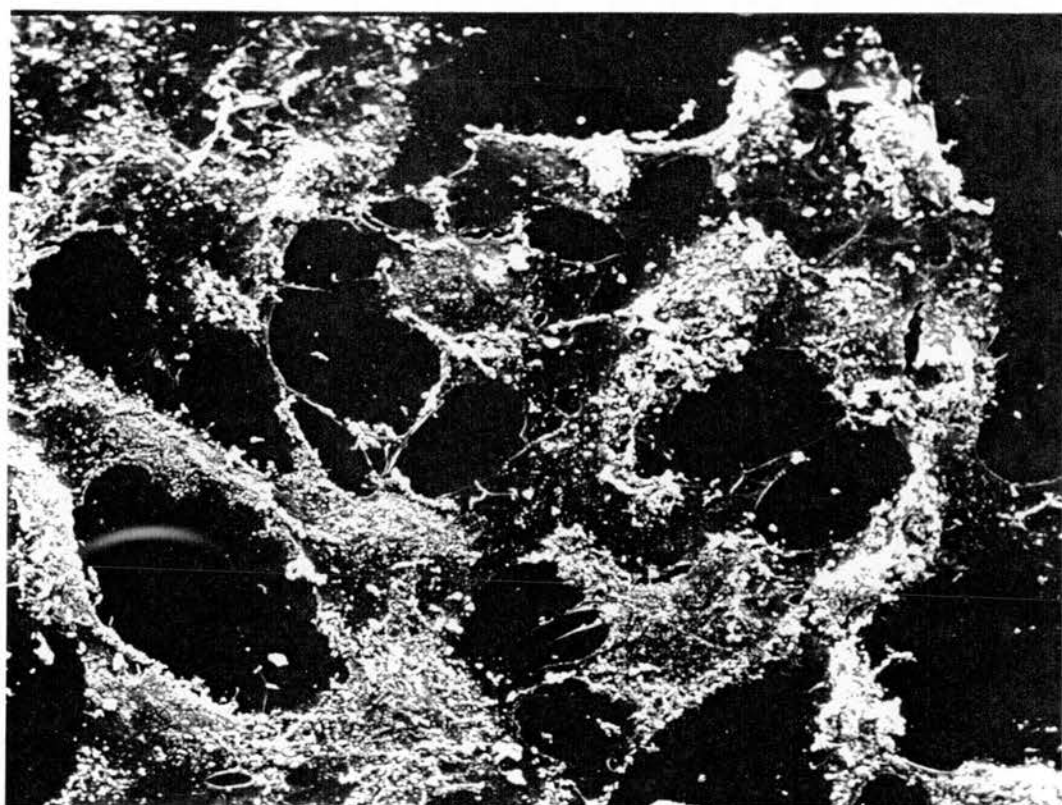
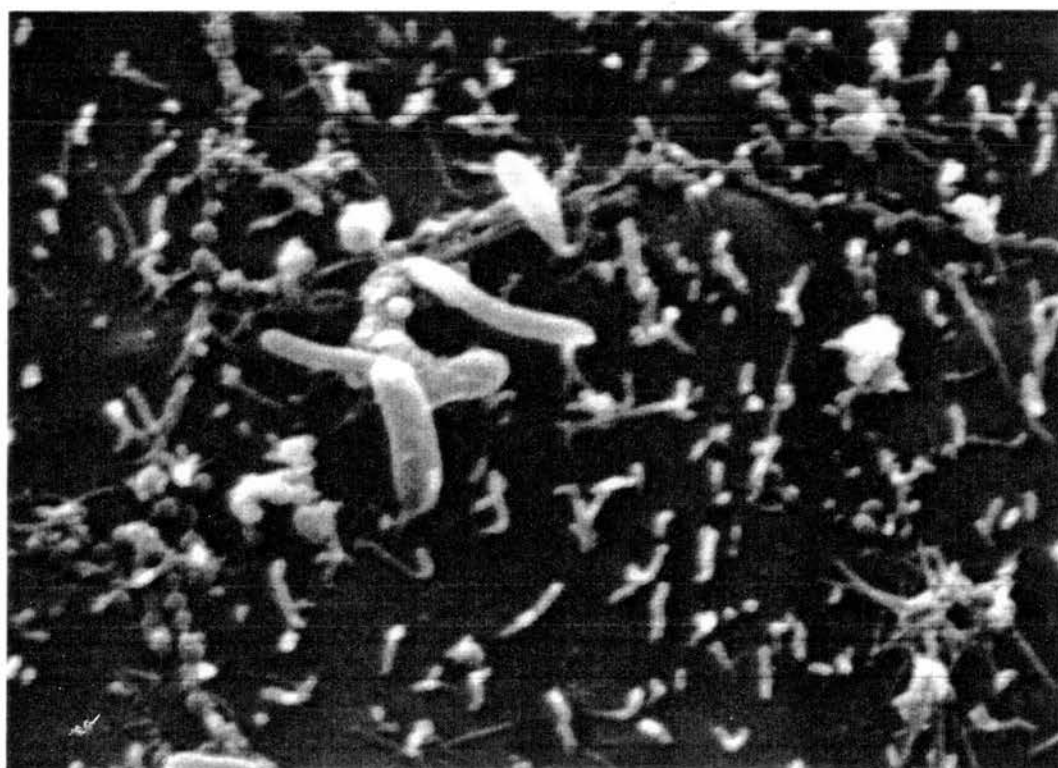
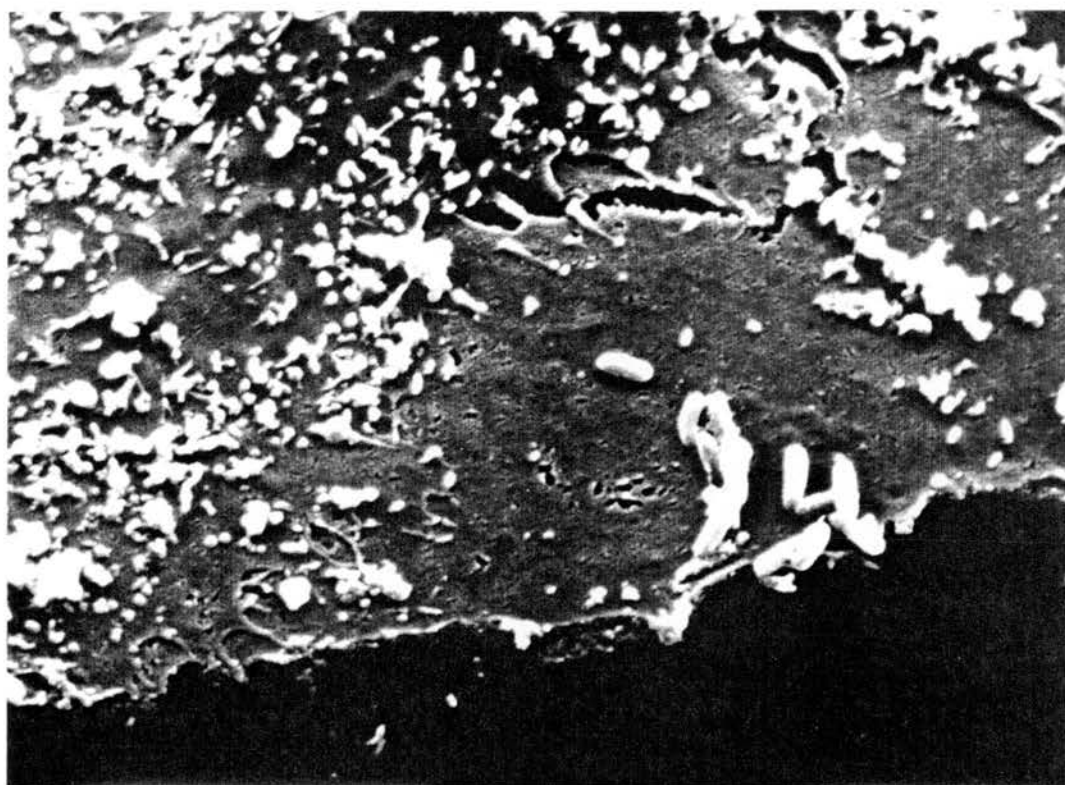


Figure 54

Scanning electron micrograph of IEC-18 cells infected with the intracellular bacteria at 24 hours post-infection. A group of bacteria are seen closely apposed to the cell membrane at the periphery of the cell x 4780.

Figure 55

Scanning electron micrograph of IEC-18 cells infected with the intracellular bacteria, at 48 hours post-infection. Bacteria are seen closely apposed to the cell membrane and microvilli x 9620.



infected IEC-18 cells. The release event is associated with numerous bacteria in the cytoplasm of IEC-18 cells. The supernatant fluids from infected cell cultures has proved an important source material to study the behaviour of the organism.

5.10 Ultrastructural observation of IEC-18 cell nuclei for the intranuclear presence of the intracellular bacteria

5.11 Introduction

Detail morphologic observation of IEC-18 cells infected by I. intracellularis has shown the presence of this organism in the nucleus of some infected IEC-18 cells (Section 5.3). This unusual phenomenon was observed in only two infected IEC-18 cells. The presence of bacteria in the nucleus is not a recognised feature of the natural disease either in pigs or in other animal species, bacteria are only seen free in the cytoplasm of infected cells (Rowland and Lawson, 1992).

This study was conducted to examine and investigate the presence of I. intracellularis in the nucleus of IEC-18

cells.

5.12 Materials and Methods

IEC-18 nuclei for this study were obtained from the procedures conducted in Section 4.2. The nuclei from KCl lysed infected IEC-18 cells grown in tissue culture flasks were collected by centrifugation as described, fixed in 1% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) for 2 hours at 4°C, washed in 2 changes of the buffer alone at 10 minutes intervals and processed for TEM (Section 2.11).

5.13 Results

Ultrastructural examination of the nuclei, pelleted by centrifugation from IEC-18 cells infected with I. intracellularis showed groups of bacteria in some cell nuclei (Fig. 56). Only 3 nuclei were observed with bacteria from a total of 200 nuclei observed. The bacteria were seen free in the nucleoplasm in numbers ranging from 3 to 10. The bacteria were rod-shaped and predominantly

Figure 56

Transmission electron micrograph of nuclei from infected IEC-cells pelleted by centrifugation. Note the presence of a group of bacteria, both electron-dense and electron-lucent in the nuclearplasm. Lead citrate and uranyl acetate stain x 32250.



curved. Both electron-dense and electron-lucent bacteria were observed. Both the nucleoplasm and the nuclear membrane appeared morphologically unaltered. Bacteria were not seen entering the nuclei.

5.14 Comment

Demonstration of the bacteria in the nucleus by light microscopy is difficult to assess and could be the result of an artefact. Intranuclear bacteria are not a characteristic feature of PE and has never been reported in the field disease. This ultrastructural observation has provided clear evidence of the presence of this bacteria in the nucleus of IEC-18 cells.

5.15 Discussion

This study has revealed a sequence of events of infection of IEC-18 intestinal cells infection by I. intracellularis. This study suggests that infection was initiated by intimate attachment of the bacteria to the cell membrane with subsequent entry in an endocytic

vacuole. Rapid breakdown of the entry vacuole occurs and bacteria then escape free into the cytoplasm. The bacteria then multiply by binary fission in the cytoplasm to produce large numbers of progeny which are eventually released extracellularly from non-adherent and adherent host cells by extrusion from the cell cytoplasm.

Attachment of bacteria to the surface of eukaryotic host cells is an initial step in the pathogenesis of infection by intracellular bacteria. This early event of infection involves the complementary interaction of adhesion molecules on the surface of bacteria and receptors on the surface of eukaryotic cells. The adhesion molecules on the surface of bacteria cells are usually proteins whereas receptors on the surface of eukaryotic cells, are a carbohydrate moiety. The complementary receptors and adhesion molecules must be accessible and arranged in such a fashion that allows the formation of multiple bonds over the area of contact between host and bacterial cells (Beachey, 1981). This leads to irreversible binding and the triggering of an endocytic pathway into the host cell (Ofek and Beachey, 1980). Attachment and entry of bacteria

into a host cell, however, are not straightforward events of infection but involve intracellular signalling systems and also the participation of host cell cytoskeletal proteins which act as the driving force in the process of endocytosis of an organism (Silverstein, Steinman and Cohn, 1977; Bliska, Galan and Falkow, 1993).

Immediately after centrifugation, *Ileobacter* cells were observed closely attached to the surface of IEC-18 cells by means of an adherent cap. The adherent cap was a cell protrusion apparently located outside the host cell membrane. The protrusion adhered to the bacterial cell but a discernable junction remained between the bacterium and the cell membrane. The method of formation of the adherent cap is unknown and it is uncertain if it originates from host cell or bacteria. Structures on the surface of bacteria such as fimbriae of some gram-negative organisms (eg. *Escherichia coli*) or hair-like structures (eg. fibrillae) of some gram-positive bacteria (eg. *Streptococci*), mediate attachment of these bacteria to epithelial cell surfaces (Peterson and Quie, 1981). These bacterial surface structures differ from the adherent cap

observed in this study; the cap also differed from the method of attachment involving pedestals which are observed in enteropathogenic and enterohaemorrhagic E. coli of infant diarrhoea (Levine and Edelman, 1984), human haemorrhagic colitis (Francis, Collins and Duimstra, 1986), H. pylori of human gastritis (Goodwin, Armstrong and Marshall, 1986), Citrobacter freundii of transmissible murine colonic hyperplasia (Johnson and Barthold, 1979) and recently, Hafnia alvei, recently claimed to be a cause of diarrhoea in humans (Albert, Alam, Islam, Montanaro, Rahman, Haider, Hossain, Kibriya and Tzipori, 1991). Such bacteria destroy the microvillus border and attach to the host cell by a pedestal, the lesions of bacterial destruction of microvilli are often therefore described as attaching and effacing lesions. Damaged or effaced microvilli could not be appreciated in association with Ileobacter attachment to IEC-18 cells. Pedestal formation with other enteropathogens involves a broadly based cell protrusion which partially encircles the bacterial cell. These morphological differences imply that the mechanism of attachment of Ileobacter differ from that involved in pedestal inducing bacteria.

Polymerization of host cell actin, which appears morphologically as electron dense material, is initiated by the attachment of pedestal producing bacteria (Knutton, Baldwin, Williams and McNeish, 1989; Smoot, Resau, Naab, Desbordes, Gilliam, Bull-Henry, Curry, Nidiry, Sewchand, Mills-Robertson, Frontin, Abebe, Dillon, Chippendale, Phelps, Scott and Mobley, 1993). This electron-dense material lies just beneath the cell membrane of effaced microvilli at the sites of attachment of the bacteria. Myosin and other cytoskeletal proteins such as x-actinin, talin and ezrin also accumulate at similar sites of attachment (Finlay, Rosenshine, Donnenberg and Kaper, 1992; Manjarrez-Hernandez, Baldwin, Aitken, Knutton and Williams, 1992). This observation suggests that the electron-dense adherent cap in *Ileobacter* attachment could be host cell cytoskeletal proteins.

The formation of the adherent cap may be an artefact of centrifugation. Centrifugation could alter the properties of the cell membrane such that the presentation or conformation of either bacterial adhesin or receptor molecules are modified resulting in morphological change in

the area of bacterial attachment. Physiochemical factors can affect the interaction between bacteria and cell surfaces (Peterson and Quie, 1981). The net charge on the surface of both bacteria and host cell is usually negative which creates a repulsive force (Beachey, 1981). Treatment of IEC-18 cells with the positively charged, diethyl aminoethyl dextran did not increase infectivity of *Ileobacter* in these cells (Lawson, personal communication). Centrifugation may modify the interaction between the intracellular bacteria and cell surface by overcoming or reducing the existing repulsive forces. The adherent cap may therefore have been initiated by the impact of the bacteria onto cells possibly combined with centrifuged induced membrane changes at the site of attachment. Uninfected centrifuged IEC-18 cells did not appear to show any such changes. Many studies, however, have suggested that centrifugation can induce changes on host cell surfaces. Consideration that the membrane lesion is an artefact of centrifugation is further discussed in the next chapter (Chapter 6).

Bacterial attachment is followed by rapid entry into an

endocytic vacuole and this is believed to occur during the 30 minute process of centrifugation, since both of these events could be observed at harvest of infection immediately after centrifugation. The membrane of the entry vacuole disintegrated with the first appearance of bacteria free in the cytoplasm 3 hours post-infection indicating that rapid escape from the entry vacuole into the cytoplasm takes place. It also suggests that the organism produces a lysin capable of disrupting the membrane. Other intracellular bacteria such as Shigella flexneri and Rickettsia spp. also enter and escape rapidly from endocytic vacuoles by producing membrane lytic agents. The mechanism of escape was investigated using an in vitro experimental model involving contact haemolysis of erythrocytes by these organisms. The haemolysis of erythrocytes results in the hydrolysis of phospholipids present in the erythrocyte membrane. Haemolysis can be prevented by inhibitors of phospholipase or excess substrate suggesting that the lytic agent is an enzyme (Sansone et al., 1986; Winkler and Miller, 1980; Winkler and Turco, 1988). The occasional observation of endocytic vacuoles containing two *Ileobacter* cells suggest that entry

vacuoles containing single organisms could have coalesced with one another. Membrane disruption of such multi-infected vacuole was also observed which indicates that a similar escape mechanism is present.

In the intracellular location, the bacteria is believed to exploit host metabolites for their growth and multiplication. The demonstration of a close association between the entry vacuole containing bacteria and host mitochondria could either be an incidental event which the bacteria exploited to acquire host cell nutrients or a mechanism adapted by the bacteria to facilitate transfer of host nutrients. The escape of bacteria from the entry vacuole may require expenditure of energy which could be obtained from this source. Perhaps the arranged association of this organelle with the entry vacuole might block contact between entry vacuoles with lysosomes or to some extent delay this fusion, thus enabling the bacteria to escape rapidly before fusion occurs. An association between host cell organelles especially mitochondria with entry vacuoles containing other intracellular microorganisms has been observed; such as C. psittaci

(Friis, 1972; Matsumoto, 1981), Ehrlichia canis (Hildebrandt, Conroy, McKee, Nyindo and Huxsoll, 1973), Legionella pneumophila (Horwitz, 1983), Rickettsia prowazekii (Silverman, Wisseman and Waddel, 1980) and Toxoplasma gondii (Jones and Hirsch, 1972) all show this feature of intracellular parasitism. These bacteria, however, unlike *Ileobacter* and *Rickettsia* multiply in endocytic vacuoles throughout their life-cycles.

For instance, Friis (1972) found that endocytic vacuoles in L-cells containing C. psittaci were surrounded by mitochondria closely apposed to the vacuolar membrane 20 hours after entry. Jones and Hirsch (1972) observed that host cell vacuoles containing T. gondii were surrounded and closely apposed to mitochondria and endoplasmic reticulum minutes after entry into mouse peritoneal macrophages, L-929 cells and HeLa cells. Horwitz (1983) described the endocytic vacuoles containing L. pneumophila in cultured human monocytes, vacuoles were initially surrounded by smooth vesicles which appeared to fuse to bud off from the vacuolar membrane, then by mitochondria and smooth vesicles at 1 hour after infection and thereafter mainly by

ribosomes.

The mechanism of endocytic vacuole-organelle interaction is not understood. It is believed that this unique behaviour is a bacterial mediated activity. *Ileobacters* could alter the vacuolar membrane in such a way that it attracts mitochondria, possibly in response to secretory or metabolic bacterial products. The failure of formalin-killed *L. pneumophila* (Horwitz, 1983) and glutaraldehyde-killed *T. gondii* (Jones and Hirsch, 1972) to exhibit this intracellular feature is a relevant indication that it involves bacterial directed activity.

Once the bacteria escaped from the entry vacuole, the organisms were in close contact with the cytoplasmic matrix and began to multiply in the cytoplasm. The presence of small groups of bacteria at 24 hours post-infection and later evidence of large numbers occupying most of the cytoplasm suggests that release of the organism from the entry vacuole into the intracellular location is a prerequisite event for bacterial multiplication. Bacteria were not observed within vacuoles at the later stages of

infection and degenerate bacteria in phagolysosomes were never observed. This confirms that the cytoplasm is the favoured location for bacterial multiplication probably through provision of host nutrients. The close contact of *Ileobacter* with the cytoplasmic matrix and in some cases with the mitochondria, rough endoplasmic reticulum and possibly ribosomes is believed to be essential for bacterial growth. Free in the cytoplasm *Ileobacter* may also trigger interaction with host organelles. *S. flexneri* has been observed surrounded by mitochondria and closely associated with the cytoplasmic matrix when free in the cytoplasm of cultured macrophages after escape from the entry vacuole (Clerc, Baudry and Sansonetti, 1988).

The detection of *Ileobacters* in the nucleus of IEC-18 cells, although very rare, is intriguing and believed to be an accidental event; it is possible that the bacteria may have been trapped in the nuclear compartment, possibly during reformation of nuclear membrane during the late stage of cell division. A similar postulation has been made for the rickettsial organism, *R. rickettsii* which are normally seen in the cytoplasm but are also detected in the

nucleus of host cells (Todd et al., 1981; Todd, Wray and Burgdorfer, 1984). The frequent observation of I. intracellularis in IEC-18 cells undergoing division supports this explanation. Moreover, this observation was extremely rare, and the majority of cells only demonstrated infection in the cytoplasm. Bacteria have not been observed in nuclei in in vivo infection, neither natural nor experimentally-induced PE (Frisk and Wagner, 1977a,b). This supports the view that bacteria are occasionally entrapped in the nucleus during cell division and this is an accidental occurrence with, as yet, no proven significance in the field disease.

This study also revealed that another important feature of intracellular parasitism, release of bacteria from host cells also occurs in I. intracellularis infection. It has been shown that following bacterial entry and escape from entry vacuoles into the cytoplasm, the bacteria multiply to large numbers and in doing so occupy most of the cell cytoplasm. IEC-18 cells infected with the bacteria and observed late during the infection showed a method of release from the host cell. The bacteria were released

extracellularly as free bacteria in the supernatant either through membrane breakdown in non-adherent host cells or in the protruded cytoplasm of adherent cells (ie: cytoplasmic protrusion). The protruded cytoplasm of adherent cells may also detach from the cell or release bacteria by extrusion from the cytoplasm.

The demonstration of numerous bacteria both in non-adherent cells or detached cytoplasmic protrusions and in adherent cells showing protrusions suggest that these are the mechanisms of bacterial release. This mechanism explains the presence of bacteria associated with non-nucleated cell cytoplasm, non-adherent cells and numerous free bacteria in cytospin preparations of supernatant fluids. These observations were supported by phase microscopy of infected supernatant fluids, some cells were seen floating free whilst other adherent cells showed protrusions. A cell can only adapt within limits to changes in demand made on it. The plasma membrane of the cell forms a barrier between the cell and its environment critical to cell homeostasis (Slauson and Cooper, 1992). The continued intracellular bacterial multiplication may

overtax the capacity of host cells to support the organism and divert nutrients essential to cell integrity, the end result is cell disruption. It is also possible that the effect is due to continued synthesis or overproduction of the membrane lytic agent used by the intracellular bacteria to escape from entry vacuoles. Such an enzyme may cause plasma membrane alteration at high bacteria to cell ratios. The infected cells lose adherence or are modified in shape to form focal spherical protrusions of the cytoplasm. The cytoplasmic protrusion maintains connection with the cell by means of a narrow neck-like cytoplasmic pedicle which later may be pinched off to form an extruded cytoplasmic sphere.

However, since both infected and non-infected cell cultures showed the presence of non-adherent cells and protruded cytoplasm, it is postulated that the method of release of *Ileobacter* from the IEC-cell may not be a specific event. Other specific methods of bacterial release may be present. Transfer of infection occurs through cell division; bacteria are clearly present in dividing cells which create an infected focus in this way.

There is good evidence that this is a major method of transfer of infection in a monolayer as protrusions are not observed until late in the infection process and the percentage of cells infected increases between 2 and 7 days (Lawson et al., 1993) at the time of host cell proliferation. The number of heavily infected cells is closely related to cell multiplication and does not increase after seven days of infection.

Cytopathic changes such as syncytia, vacuolation, inclusions or rounding of cells were not seen in association with either infection or release of the bacteria. Non-adherent cells were observed both in infected and non-infected cultures which support the view that it is a method of natural loss of effete cells from the monolayer. In infected monolayers, release of effete cells may have been aggravated by unrestricted bacterial multiplication.

Some bacteria showed thickened outer membranes. This alteration could be a preparation for extracellular survival or merely a consequence of the artificial

experimental procedure. Life outside the host cell is not an exceptionally safe environment for bacteria that strictly depend on host cells for their existence. However, exit from host cells is an important event for intracellular bacteria and necessary for the transmission of infection to another host. Obligate intracellular bacteria have also evolved extracellular survival tactics to survive transit in the extracellular environment before initiating a fresh infection. R. tsutsugamushi has been reported to retain a surrounding host cell membrane when released from cultured mouse peritoneal mesothelial cells (Ewing et al., 1978). Only those released in this way are capable of infecting other cells. The elementary body of the Chlamydia organism released from host cells and responsible for reinfection has evolved into an environmentally resistant transit form. Unlike the reticulate body which is merely involved in intracellular replication, the cell wall of the elementary body contains sulphur rich amino acids such as cysteine, linked by disulphide bonds which provides structural rigidity of this Chlamydial form. This structural pattern is associated with a reduced permeability of the bacterial outer

membrane which prevents deleterious compounds from the extracellular environment (Ward, 1983; Bavoil, Ohlin and Schachter, 1984; Schachter, 1988). *Ileobacter* cell infection in the presence of neomycin is reduced, yet bacteria released into the supernatant fluid containing neomycin at similar concentrations appear highly infective. This apparent contradiction requires further evaluation but could indicate changes in bacterial membrane permeability at different stages of infection (Lawson, personal communication).

The structural changes may be reversible; reorganisation of the Chlamydial elementary body into a reticulate body during the developmental cycle of this organism is associated with reduction in wall disulphide bonds which renders the wall more flexible and permeable. This structural reformation enables the organism to resume nutrient assimilation from the host cell after cell entry.

The occasional demonstration of an additional regularly arranged fuzzy layer which extended from and completely covered the outer membrane of *Ileobacter* is an interesting

morphological feature. Numerous gold particles were observed apparently attached to this area of the membrane when bacteria was stained with the monoclonal antibody IG4. This observation suggest that the antigen recognised by the monoclonal antibody is present in this external layer. Bacterial surface components such as capsules, slime layers and S-layers which extend from the outer membrane have been documented (Costerton, Lappin-Scott and Cheng, 1992).

Capsule and slime layers also respectively known as capsular or slime exopolysaccharide are polysaccharide containing structures of bacterial origin (Costerton and Irvin, 1981; Whitfield, 1988). S-layers, on the other hand, are regular arrays of bacterial protein or glycoprotein subunits.

There are many possible explanations to suggest that the external layer observed in *Ileobacter* is the S-layer of the bacteria. Capsule and slime layers cannot be visualised by conventional staining in ultrathin sections by electron microscopic observation (Costerton and Irvin, 1981). These bacterial surface components are easily

damaged in the dehydration process of the preparation of samples for electron microscopic observation and is a consequence of the normal hydrated nature of these surface structures (Costerton and Irvin, 1981; Costerton et al., 1992). Visualisation of these exopolysaccharide layers can be achieved by special staining procedures employing polyanion specific stains such as ruthenium red and stabilization of the structures by cross-linking agents such as lectins. Comparatively, S-layers are made up of 99.3% proteins (Sleytr, 1978) which explains the ability of this bacterial layer to resist hydration and condensation and its presence in ultrathin sections. However, better observation of S-layers is achieved by negative staining (Sleytr and Messner, 1988; Beveridge and Graham, 1991; Messner and Sleytr, 1992).

Bacterial S-layers covers the cell surface completely not only in isolated cells but during cell growth and division (Sleytr and Glaucert, 1976; Sleytr, 1978; Sleytr and Messner, 1988). S-layers have been reported in many bacteria including Chlamydia spp. such as C. psittaci and C. trachomatis and Rickettsia spp. such as R. rickettsii

and R. prowazekii (Sleytr, 1978; Sleytr and Messner, 1988; Messner and Sleytr, 1992).

S-layers help to determine and maintain cell shape and are also responsible in part for cell envelope rigidity. The protein meshwork and well-defined pore size of S-layers can act as a molecular sieve and protect the bacterial cell against damaging agents such as proteolytic enzymes. The S-layers, however, allow the exchange of nutrients from the extracellular environment and the release of bacterial metabolic waste products, it may also help in cell adhesion and surface recognition (Sleytr and Messner, 1988; Messner and Sleytr, 1992). The presence of an S-layer in *Ileobacter* may serve the same purpose and may contribute to the survival and pathogenicity of the bacteria. S-layers are known to be virulence characteristics of bacterial pathogens notably Campylobacter fetus subspecies venerealis and Aeromonas salmonicida (Messner and Sleytr, 1992).

However, the S-layer-like structure was observed in intracellular and extracellular bacteria from samples in samples treated with Triton-X-100 only. This is a

detergent that was used to permeabilise IEC-18 cells and allow penetration of gold particle labelled IgG conjugate and monoclonal antibody IG4 to antigen present in the intracellular location. The demonstration of this bacterial layer may be attributed to the effect of the detergent. The stability of S-layers differs considerably between bacteria in response to different detaching agents (Messner and Sleytr, 1992). Sleytr and Thorne (1976) reported that treatment with 0.5% Triton-X-100 did not detach the S-layers from the surface of Clostridium thermosaccharolyticum and Clostridium thermohydrosulfuricum and allowed visualisation of this layer. Similar concentrations of the detergent were used in this study.

The observation of this S-layer in *Ileobacter* in samples treated with the detergent is intriguing and may reflect a change in the hydrophilic or hydrophobic nature of the layer induced by the detergent. This detergent may have increased the affinity of the layer to electron microscopic staining which results in their visualisation.

Messner and Sleytr (1992) reported that some bacteria

have more than one S-layer superimposed on one another. S-layers of bacteria either adhere peripherally or even penetrate into the matrix of bacterial outer membrane (for reviews see Beveridge, 1981; Sleytr and Messner, 1983, 1988; Messner and Sleytr, 1992). Immunogold labelling indicates that the 25-27K antigen is located on the bacterial surface (McOrist et al., 1987; McOrist et al., 1989), it is tempting to suggest that in view of the immunodominance of this antigen that it may be located on the cell surface and equate to the S-layer.

This study has for the first time outlined the cycle of infection of the intracellular bacteria of PE in an in vitro model, using the IEC-18 cell line. Other bacteria were not observed in or cultured from the infected cells. and additional immunological results indicate the absence of Chlamydia in the cultured cells (Chapter 4). These results suggest the involvement of a single organism, I. intracellularis, in the cellular events of infection observed. The diagrammatic presentation of the purported infection cycle of the intracellular bacteria is depicted in Figure 57. The observation of an additional layer,

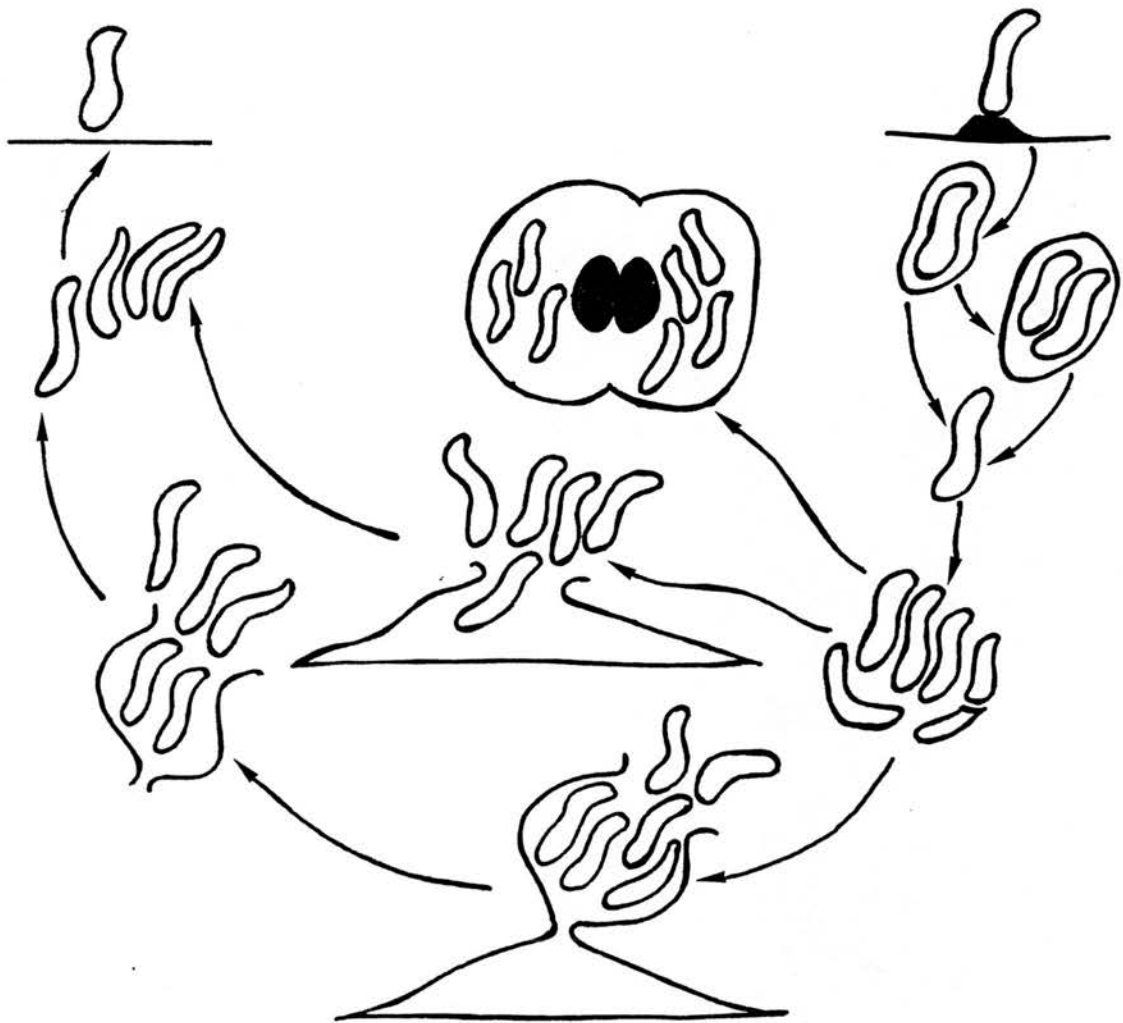


Fig. 57 Diagrammatic representation of the infection cycle of *I. intracellularis* in IEC-18 cell line. Arrows indicate the sequence of events of infection.

possibly an S-layer of the bacteria has also been reported for the first time.

CHAPTER 6

CELLULAR EVENTS IN NON-CENTRIFUGE ASSISTED INFECTION OF IEC-18 CELLS BY THE INTRACELLULAR BACTERIA

CELLULAR EVENTS IN NON-CENTRIFUGE ASSISTED INFECTION OF

IEC-18 CELLS BY THE INTRACELLULAR BACTERIA

6.1 Introduction

The cellular events of infection of IEC-18 cell line by I. intracellularis as a potential in vitro system have been described (Chapter 5). That study has provided a possible insight into the events of infection in the natural host. Since the method of infection of IEC-18 cells in that study was artificial in many ways but not least in that it was utilising centrifugation, it is also important to investigate the events of infection in the same model, but this time, in the absence of centrifugation. Such methods of infection better resemble the cellular events of infection which occur in vivo and may well involve different mechanisms. It is believed that centrifugation can bypass the spontaneous events of bacterial attachment and entry and as such there is much doubt if the events observed by this method of infection is a relevant model of the possible events of infection in the natural host.

This study was designed to investigate the

ultrastructural events of infection of IEC-18 cells by I. intracellularis in the absence of centrifugation.

6.2 Materials and Methods

Preparation of inocula

The inocula was processed from one 75cm² tissue culture flasks infected (7 days post-infection) with the intracellular bacteria strain 1482/89, passaged 19 times in IEC-18 cells. The supernatant fluid from the flask was first removed and the infected cells were treated with 0.1% KCl for 10 minutes at 37°C. The KCl from the flask was decanted and replaced with 10ml of SPG solution with 5% vol/vol FCS. The cell monolayer was removed with a scraper and the fluid passed through a 20 G needle and syringe three times to lyse the cells. The lysed cells were then centrifuged at 100 x g for 5 minutes, the supernatant removed and centrifuged at 2700 x g for 25 minutes to form a bacterial pellet. The pellet was then resuspended in 2.0ml of SPG containing 5% vol/vol FCS vol/vol. The bacterial suspension was then diluted 1/24 in DME supplemented with L-glutamine, fungizone and 7% vol/vol FCS

with vancomycin and used to infect a 24 hour IEC-18 cell monolayers grown on 13mm plastic coverslips. Cells had been seeded at a cell concentration of 0.5×10^5 cell ml^{-1} . Infected cells were then incubated microaerobically in steel jars for 3 hours before transfer to a microaerobic incubator (8.8% CO_2 and 8.0% O_2) at 37°C . Coverslips were harvested at 3, 24 and 48 hours post-infection. The infected cells were fed at 24 hours post-infection with DMEM and supplements with 7% vol/vol FCS containing vancomycin and neomycin.

Electron microscopy

Harvested coverslips were processed for transmission electron microscopy (Section 2.11), some were stained with the specific monoclonal antibody IG4 and goat anti-mouse gold conjugate in a pre-embedding immunolabelling method (Section 2.10). The coverslips were cut into 4 equal quadrants and ultrathin section were prepared at sites in each for viewing with the electron microscope.

Light microscopy

IEC-18 cells were similarly infected with the intracellular bacteria on 16mm glass coverslips, and similarly harvested and stained with the specific monoclonal antibody IG4 and sheep anti-mouse peroxidase conjugate in an immunoperoxidase test (Section 2.9) for light microscopy.

Control cells

Uninfected IEC-18 cells were set up on coverslips harvested after similar incubation periods as infected cells.

Preparation of supernatant cytospin

The supernatant fluid from the infected flask was centrifuged onto glass slides to observe for evidence of extracellular bacteria and cells as described in Section 5.7.

Bacteriology

The inoculum for IEC-18 cells was inoculated to Skirrow's medium, Columbia blood agar and brucella semi-solid broth as described in Section 2.12.

6.3 Results

IEC-18 cells that were infected with I. intracellularis and sequentially harvested at different time intervals revealed a sequence of cellular events of infection of the organism. Immunoperoxidase and immunogold staining showed bacteria reactive with monoclonal antibody IG4. Both highly and lightly infected cells and both electron-dense and electron-lucent bacteria were observed during infection. Bacteria in the nucleus and circular groups of bacteria at the perinuclear region of cells were not seen. Uninfected controls did not show any evidence of bacteria. Bacterial culture of the inoculum used for infection did not yield any bacteria. Cytospin preparations of the supernatant fluid from the infected flasks showed many free bacteria, extruded cells and non-nucleated cell cytoplasm. Bacteria were morphologically similar to those observed in infected cells.

3 hours post-infection

Many bacteria were observed near the cell membrane but they were rarely seen closely attached to the cell membrane. No cap surrounding the area of bacterial attachment was evident (Chapter 5). The outer membranes of extracellular bacteria were seen only adjacent to the host cell membrane and appeared indistinct (Fig. 58). Attaching bacteria had pale cytoplasmic granules within their cytoplasm. Intracellular bacteria were only observed singly in endocytic vacuoles and the membrane of the endocytic vacuole was indistinct and undergoing lysis (Fig. 59). Endocytic vacuoles containing bacteria were observed not restricted to the region of the cell membrane (ie: cytoplasmic surface) but at different sites of the cytoplasm, and in one occasion, at the subnuclear region. Mitochondria which lay adjacent to the endocytic vacuoles and the surrounding cytoplasmic matrix were disrupted. Bacteria were not observed free in the cytoplasm.

Light microscopical observation showed mostly lightly infected cells containing mainly singly scattered

Figure 58

Transmission electron micrograph of IEC-18 cells infected with the intracellular bacteria, in the absence of centrifugation, at 3 hours post-infection. Note a bacterium with a pale granule in its protoplasm closely associated with the cell membrane. The bacterial outer membrane adjacent to the host cell membrane is indistinct. Lead citrate and uranyl acetate stain X 120000.

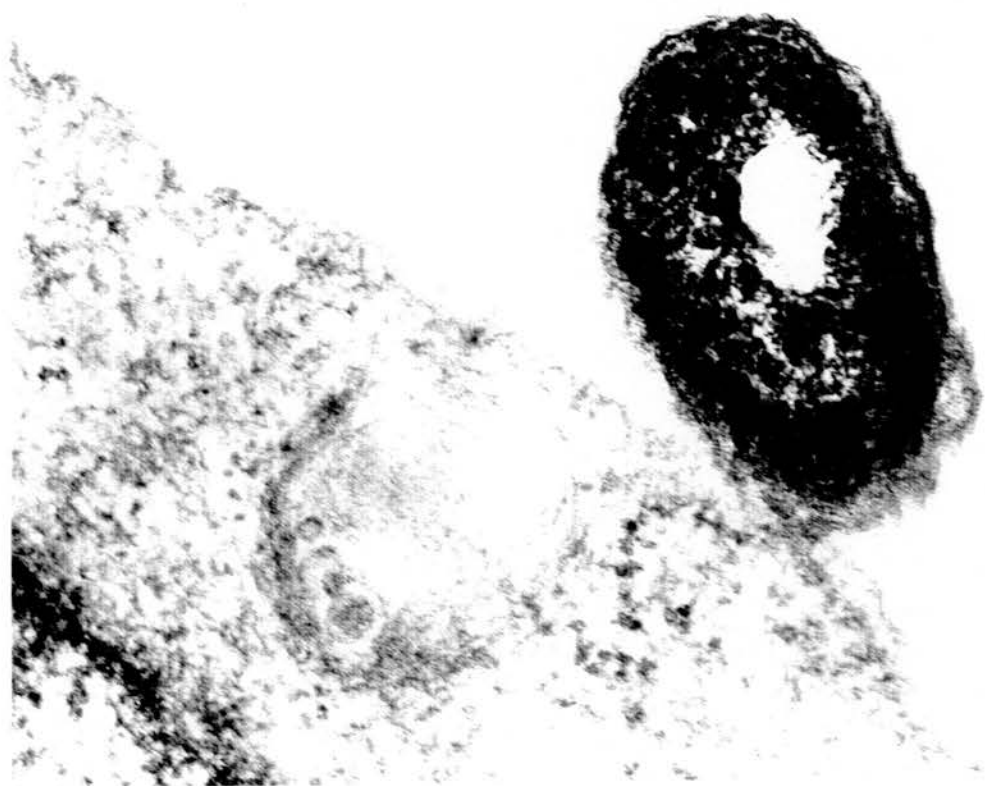
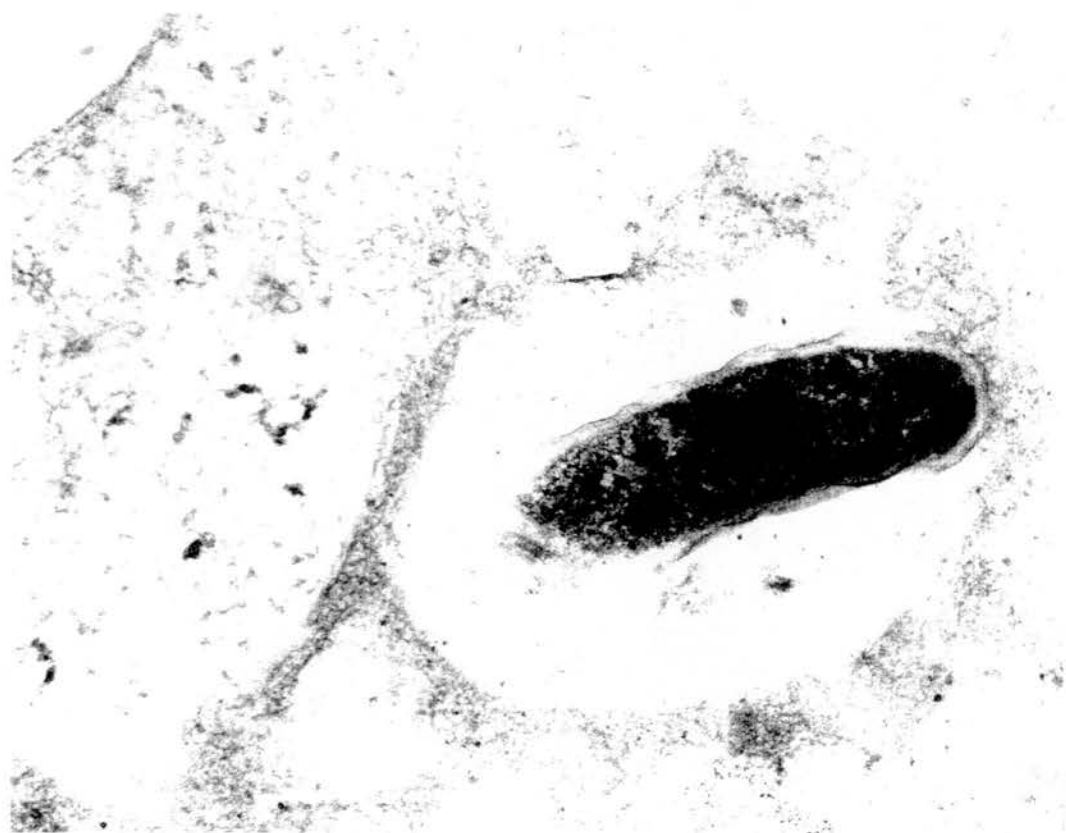


Figure 59

Transmission electron micrograph of IEC-18 cells infected with the intracellular bacteria, in the absence of centrifugation, at 3 hours post-infection. Note a single bacterium in an endocytic vacuole. The membrane of the endocytic vacuole is indistinct and undergoing lysis. The cytoplasmic matrix and organelles around the vacuole are disrupted. Lead citrate and uranyl acetate stain x 85100.



organisms.

24 hours post-infection

Both extracellular and intracellular bacteria were observed. Extracellular bacteria were only observed near but not attached to the cell membrane. Bacterial attachment by cap protrusion was not observed. Endocytic vacuoles containing single or several bacteria were evident. Endocytic vacuoles had distinct membrane breakdown and bacteria were observed escaping at the disrupted sites of the vacuolar membrane. The cytoplasmic matrix adjacent to sites of vacuole breakdown was disrupted. Mitochondria which were disrupted and had poorly defined matrix were also observed adjacent to endocytic vacuoles. On one occasion, many spherical vesicles and ground substance were interspersed between several bacteria in endocytic vacuole, the latter was similar in electron density to the cell cytoplasm outside the vacuole. The spherical vesicles were variable in size and consisted of electron-lucent amorphous material. Some vesicles were observed being extruded from the endocytic

vacuole through membrane breakdown. Some bacteria had their protoplasm distinctly separated from their outer membrane and a few bacteria especially at the periphery of the vacuole had outer membrane blebs (Fig. 60). Bacteria free in the cytoplasm singly or in groups were first evident at different sites of the cytoplasm (Fig. 61). On one occasion, a single bacterium just below the cell membrane was observed closely associated with a host cell mitochondrion and to lie very close to a coated pit and coated vesicle (Fig. 62). However, no bacteria were observed in coated pits or vesicles.

Light microscopy observation showed both lightly and highly infected cells. The percentage of infected cells was higher than that observed at 3 hours post-infection (see Section 6.4; Table 11).

48 hours post-infection

Extracellular and intracellular bacteria were both observed. Extracellular bacteria were very occasionally seen and the bacteria were seen only near the cell

Figure 60

Transmission electron micrograph of an IEC-18 cell infected with the intracellular bacteria, in the absence of centrifugation, at 24 hours post-infection. Note a group of bacteria in an endocytic vacuole. In the vacuole, ground substance morphologically similar to the cytoplasmic matrix and many spherical vesicles (arrowheads) which contains amorphous electron-lucent material are also evident. Bacteria (open arrows) especially those adjacent to the vacuolar membrane are observed with blebs of their outer membrane. The vacuole shows localised breakdown. Bacteria and some vesicles are escaping at the ruptured sites of the vacuole. The cytoplasmic matrix adjacent to membrane breakdown is disrupted (double arrows). Mitochondria (M) with disrupted matrix are observed lying adjacent to the vacuole. Lead citrate and uranyl acetate stain x 65320.

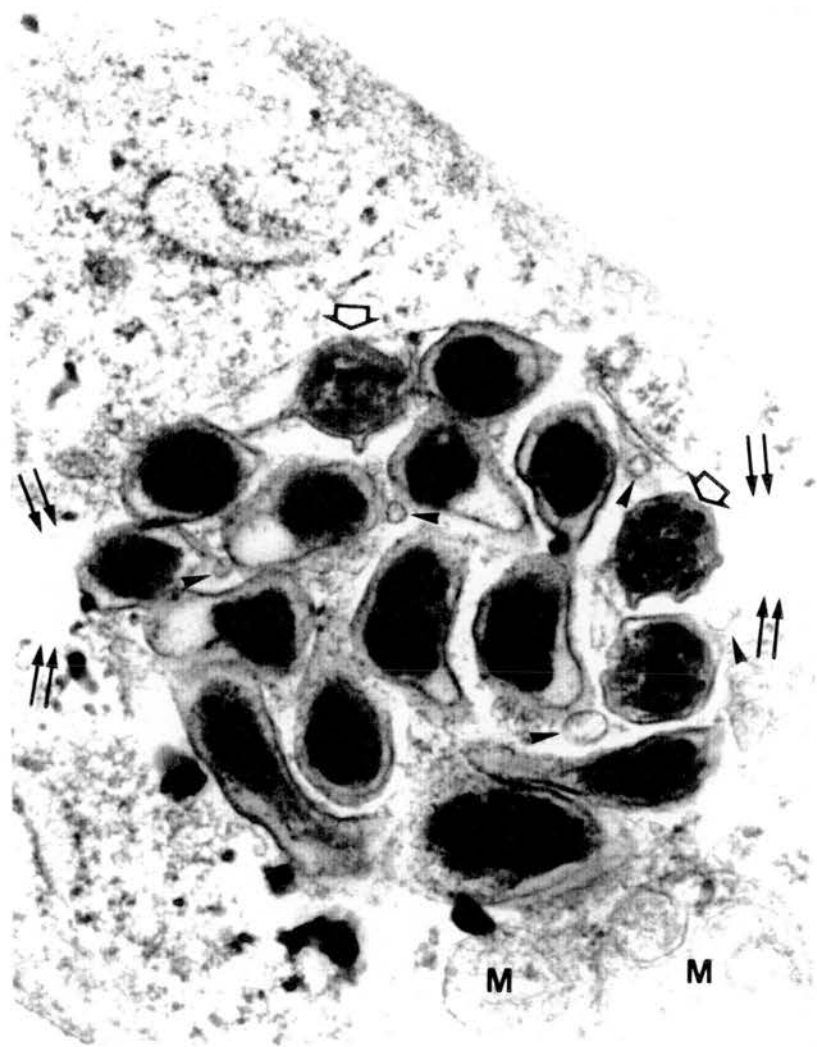


Figure 61

Transmission electron micrograph of IEC-18 cell infected with the intracellular bacteria, in the absence of centrifugation, at 24 hours post-infection. Note bacteria free in the cytoplasm. Lead citrate and uranyl acetate stain x 138600.

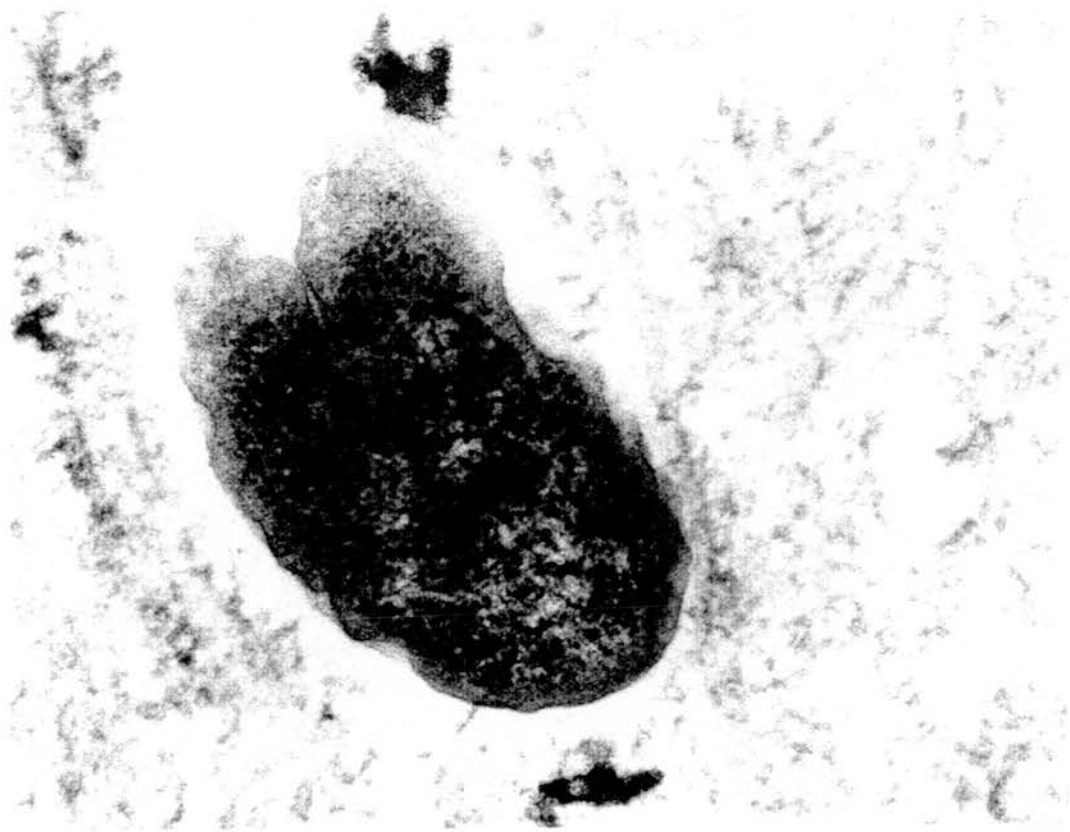
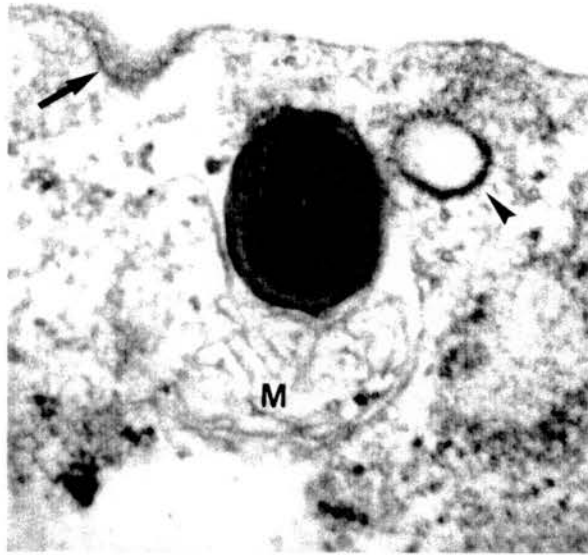


Figure 62

Transmission electron micrograph of IEC-18 cell infected with the intracellular bacteria in the absence of centrifugation at 24 hours post-infection. Note a single bacterium below the cell membrane closely adjacent to a coated pit (arrow), coated vesicle (arrowhead) and mitochondria (M). Lead citrate and uranyl acetate stain x 100000.



membrane. Attachment of bacteria to the cell surface was not evident. Immunogold staining showed specific labelling of both extracellular and intracellular bacteria with the monoclonal antibody Ig4. Extracellular bacteria had numerous gold particles in comparison to intracellular bacteria. Bacteria at both locations had a fuzzy regular additional layer which extended from the outer membrane (Figs. 63 to 65). Gold particles were observed adherent to this layer. The fuzzy coat was only observed in samples treated with Triton-x-100 for immunogold staining.

Intracellular bacteria were only observed free in the cytoplasm closely associated with the cytoplasmic matrix and on occasion with the host cell mitochondria (Fig. 66). These bacteria were often observed scattered as groups adjacent to or surrounding the cell nucleus. Single bacteria were also observed at different sites of the cytoplasm but usually near the cell membrane. On many occasions, bacteria present as groups especially those near the cell nucleus were seen lying close to coated pits and coated vesicles. Each group of bacteria was seen associated with more than one coated pit or vesicle

Figure 63

Transmission electron micrograph of extracellular bacteria with a regular fuzzy layer which extends from the outer membrane. Numerous 1nm gold particles are attached to this layer. Immunogold staining with monoclonal antibody IG4 and goat-anti mouse gold conjugate. Lead citrate and uranyl acetate stain x 100000.

Figure 64

Transmission electron micrograph of intracellular bacteria with a regular fuzzy layer which extends from the outer membrane. A few 1nm gold particles (arrowheads) are attached to this layer. Immunogold staining with monoclonal antibody IG4 and goat-anti mouse gold conjugate. Lead citrate and uranyl acetate stain x 12500.

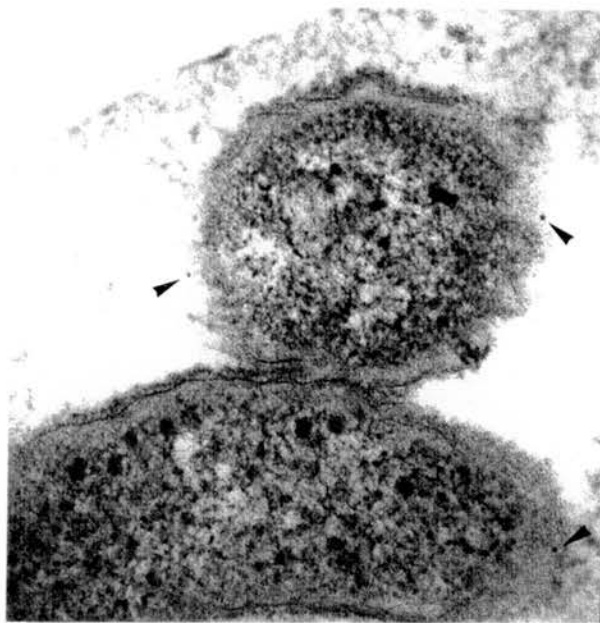
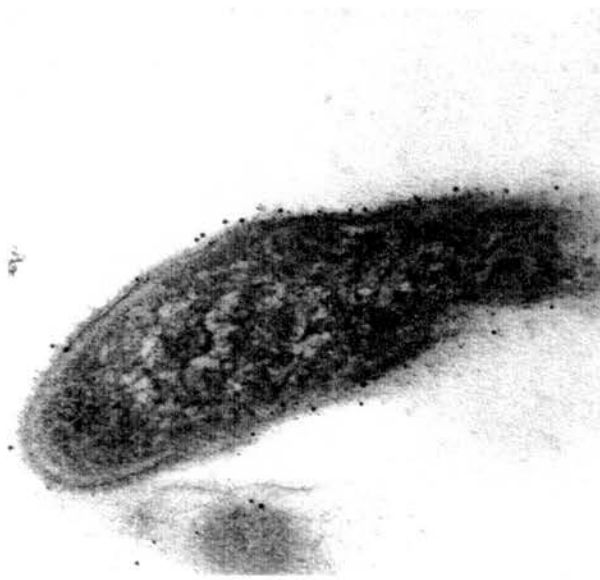


Figure 65

Transmission electron micrograph of IEC-18 cell infected with the intracellular bacteria in the absence of centrifugation at 48 hours post-infection. Intracellular bacteria with gold particles (arrows) attached to the external bacterial layer at higher magnification. Immunogold staining with monoclonal antibody IG4 and goat anti-mouse gold conjugate. Lead citrate and uranyl acetate stain x 300000.

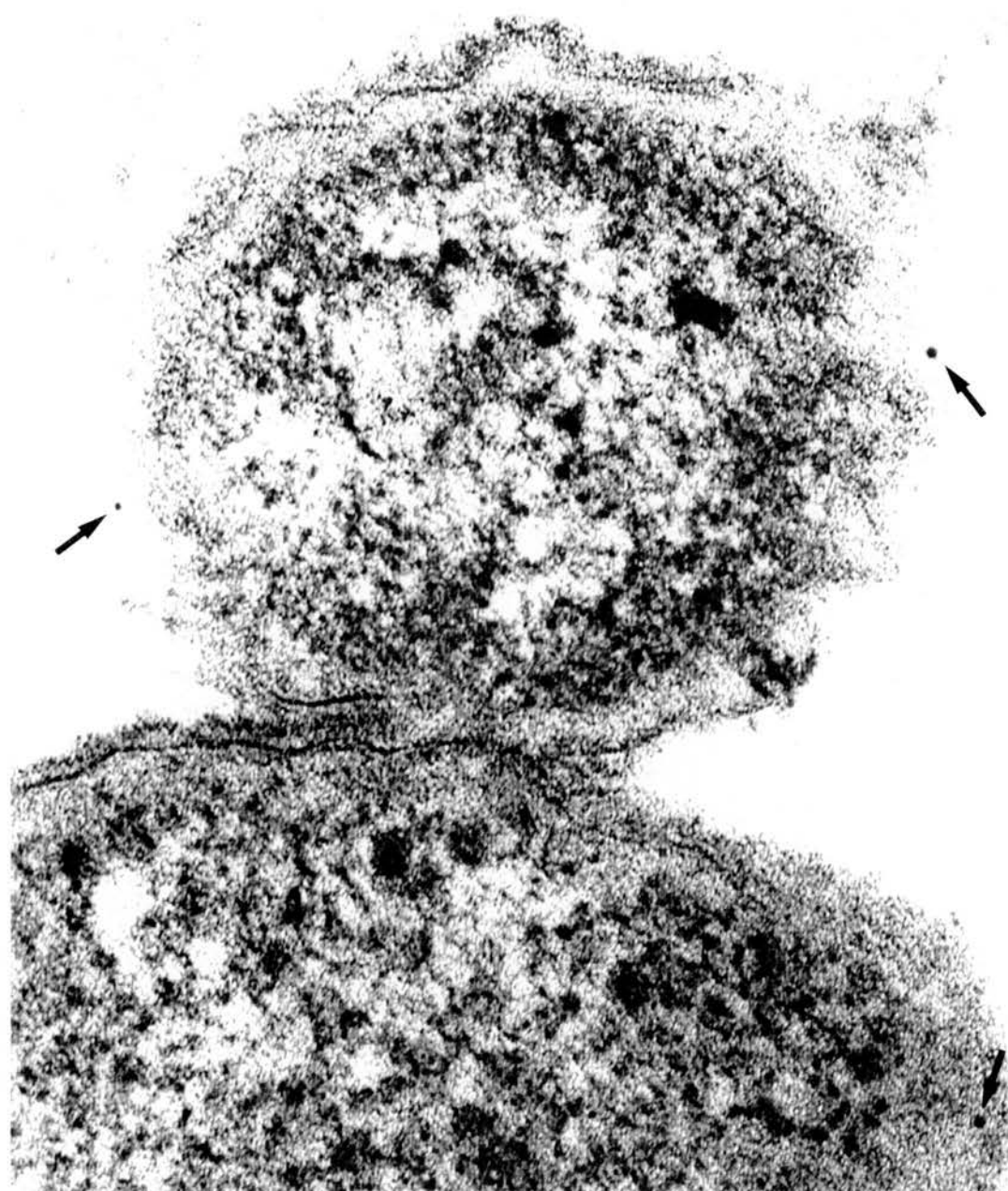


Figure 66

Transmission electron micrograph of IEC-18 cells infected with the intracellular bacteria, in the absence of centrifugation, at 48 hours post-infection. Many bacteria were evident free in the cytoplasm. Note two bacteria (double arrows) with thickened outer membrane. Lead citrate and uranyl acetate stain x 67450.



demonstrated by serial sectioning (Figs. 67 and 68). Bacteria were not observed in coated pits or coated vesicles.

Coated pits and vesicles were also evident in non-infected IEC-18 cells but on rare occasions. They were usually observed singly. Coated vesicles were more rarely observed in comparison to coated pits.

Observation of infected cells by the light microscope showed both lightly and highly infected cells. More infected cells were observed than earlier stages of infection (see Section 6.4; Table 11).

6.4 Comment

The work in this study showed that centrifugation is not necessary for *Ileobacter* to infect IEC-18 cells. The cellular events of infection revealed by infecting cells by centrifugation (Chapter 5) were also observed in non-centrifuged infection in this study. A summary of the cellular events in non-centrifuged infection observed at

Figure 67

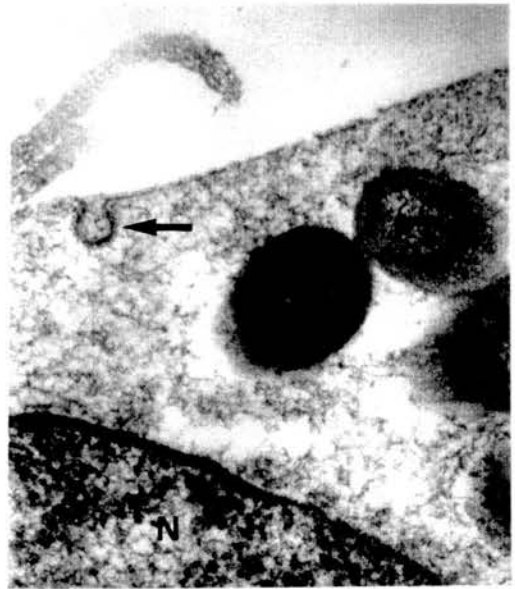
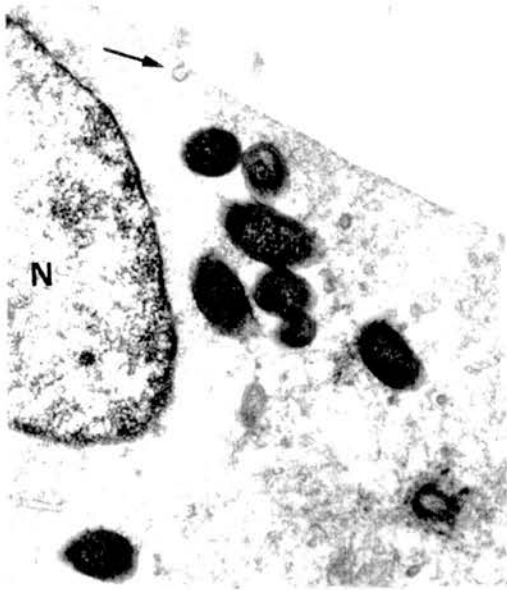
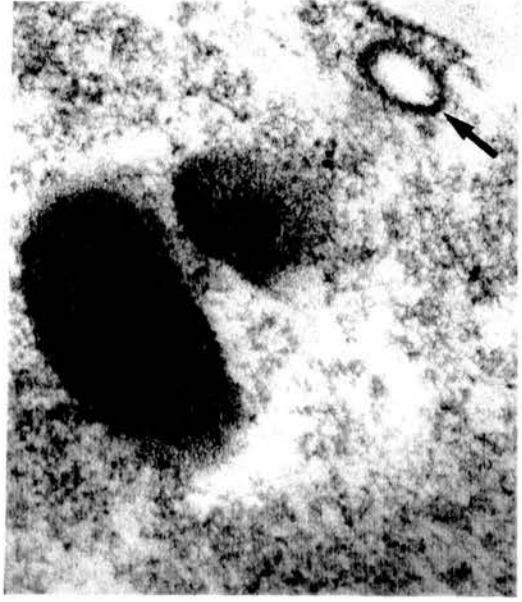
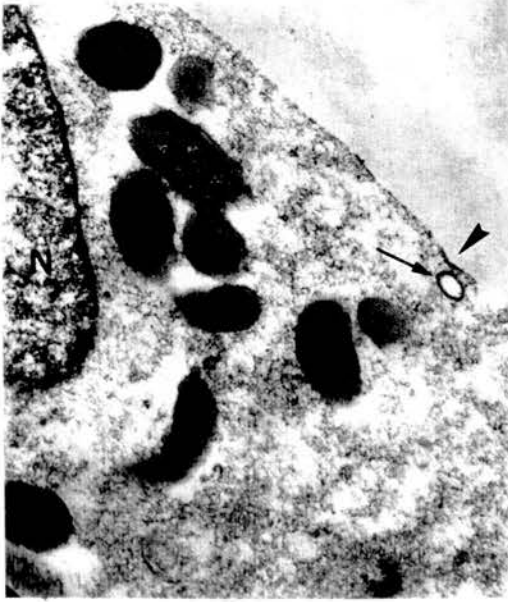
Left: Transmission electron micrograph of IEC-18 cells infected with the intracellular bacteria, in the absence of centrifugation, at 48 hours post-infection. Note a group of bacteria adjacent to the nucleus close to a developing coated pit (arrowhead) and a coated vesicle (arrow). N, nucleus. Lead citrate and uranyl acetate x 35500.

Right: Bacteria and coated vesicle (arrow) at higher magnification. Lead citrate and uranyl acetate x 100000.

Figure 68

Left: Transmission electron micrograph of IEC-18 cells infected with the intracellular bacteria, in the absence of centrifugation, at 48 hours post-infection. Note a group of bacteria and a developed coated pit (arrow). N, nucleus. Lead citrate and uranyl acetate x 21500.

Right: Bacteria and coated pit (arrow) at higher magnification. N, nucleus. Lead citrate and uranyl acetate x 60000.



different time after infection is depicted in Table 10.

From the result of this study, comparison of the cellular events of infection between centrifuged and non-centrifuged infection is now possible. This allows assessment of the validity of both models of infection as better models of in vivo infection in the natural host.

The percentage of infected cells increases as infection progresses and there is evidence of intracellular bacterial multiplication (Table 11). *Ileobacter* were exposed to the extracellular environment for 3 hours to allow infection unlike infection by centrifugation which is believed to facilitate bacterial entry and therefore reduce bacteria to extracellular exposure. The ability of *Ileobacter* to survive, infect and multiply in IEC-18 cells could have been related in part to the technique used to prepare the inoculum for IEC-18 infection. The inoculum was prepared by adding SPG (Section 6.2), the method is suitable for increasing infection of IEC-18 cells without the assistance by centrifugation (Lawson, personal communication). This reagent could have facilitate entry by reducing the repulsive electrostatic charges between bacteria and host

Table 10. IEC-18 cells exposed to *I. intracellularis* strain 1482/89: Summary of ultrastructural events in non-centrifuged infection

Hours PI	Extracellular event Attachment	Intracellular events Bacteria:	
		In endocytic vacuole	Free in the cytoplasm
3	Yes	Yes	No
24	No	Yes	Yes
48	No	No	Yes

Table 11. IEC-18 cells exposed to *I. intracellularis* strain 1482/89. Light and electron microscopic observations of infection in the absence of centrifugation

LM infection: ^a Inocula parallel HIC/F	Bacteria per ml ^b	Hours PI	LM infection: ^c EM parallel	EM infection: ^d Number of bacteria	
				Extracellular	Intracellular
190/100	2.3 x 10 ⁴	3	0.67	15	7
		24	0.75	2	19
		48	0.81	1	134

^{a,c} Light microscopic (LM) observation
^d Electron microscopic (EM) observation

^a Infection counted for 2 coverslips (130 mm²), set up in parallel to infection in flask used to prepare inocula for EM observation
^b Estimated as described in Chapter 5
^c Percentage infection counted for 100 cells on 1 coverslip (200 mm²), set up in parallel to infection on coverslips for EM observation
^d Total number of bacteria counted for 200 cells, sectioned at different sites (2 to 5 sites per quadrant) on thermanox coverslips

HIC Highly Infected Cells
 F Foci of infection

cells or create a masking effect that neutralised such forces. Bovarnick et al. (1950) reported that SPG can enhance the stability of Rickettsia thus preserving the viability of the bacteria. Additionally, host cell membrane properties (receptors) important for interaction with bacterial adhesins are believed preserved without centrifugation.

6.5 Discussion

The sequence of event when IEC-18 cells were infected with I. intracellularis in the absence of centrifugation have been described. Infection was initiated by attachment of the bacteria to the cell membrane, with subsequent single entry in the endocytic vacuole. The bacteria then escaped from this intracellular confinement free into the cytoplasm where bacterial multiplication took place. Later during the infection, the bacteria were released extracellularly from non-adherent and adherent host cells by extrusion from the cell cytoplasm.

Attachment of the bacteria to the host cell markedly

differed to that observed in centrifuge assisted infection. Bacteria were not observed attached to the cell membrane by cap projection as observed in centrifuged infection (Chapter 5). Instead, the bacterial outer membrane was observed closely associated with the cell membrane. The bacterial outer membrane at the point of contact with the host cell membrane was indistinct.

These observations suggest a possible difference in the mechanism of entry of *Ileobacter* in centrifuged and non-centrifuged infection (spontaneous infection). Centrifugation is believed to result in an artefact of attachment and entry of the organism into the cell and this has been previously discussed (Chapter 5).

A few further studies support that the mechanisms of interaction of *Ileobacter* with the host cell surface in these two different modes of infection could be different. These studies were conducted on *Chlamydia* in an effort to elucidate how centrifugation enhance infection of cultured cells by these bacteria (Pearce, Allan and Ainsworth, 1981). It is believed that centrifugation can induced

changes on the host cell surface by compression without affecting the surface of the organism. Chlamydial elementary bodies, which are responsible for initiating infection have a rigid cell wall and are therefore less likely to be affected by centrifugation in comparison with the flexible host cell surface which alters shape by means of the cytoskeletal network (Silverstein et al., 1977). Allan, Spragg and Pearce (1977) conducted a study of the effect of pressure and force of centrifugation on infectivity. These workers reported that pressure and force directed by centrifugation can deform the surface of cells. Different levels of pressure achieved by layering immiscible fluid and centrifugation of the bacteria at 69 x g onto cultured cell monolayers corresponds with the level of infectivity obtained; in raising the level of pressure increases the level of infectivity, and infection equates to that achieved by centrifugation alone at a force of 1580 x g. Cells have been found to be susceptible to infection in the first 20 minutes after centrifugation but not at later stages (Allan and Pearce, 1979).

The mechanisms of bacterial attachment at 4°C differ in

centrifuged and spontaneous infection. A temperature of at 4°C inhibit centrifuged attachment but has no effect on spontaneous attachment (Allan and Pearce, 1979). Specific antibody is active in inhibiting spontaneous Chlamydial infection whilst centrifugation can overcome this effect. These observation may suggest that centrifuged infection is non-specific (Ainsworth, Allan and Pearce, 1976; Allan et al., 1977; Horwitz, 1982) as organisms may have been able to enter cells at sites where specific receptors are absent. Centrifuged assisted bacterial entry may however involve both specific and non-specific pathways.

The interaction of *Ileobacter* with IEC-18 cells in spontaneous infection is more likely to involve a specific pathway which involves the complementary interaction between bacterial adhesin and host cell receptors. Bacterial entry in centrifuged infection may or may not be specific and it should not be assumed that the mechanisms of cell infection are similar in the two systems.

However, it should also be noted that different bacteria or host cells may respond to the effect of

centrifugation in a different manner and the artefacts produced as a result of this method of infection may also differ (Croy et al., 1975; Hughes, 1993). Some Chlamydial species are sensitive to centrifugation (enhancement effect of infection), some are not, and differences in the response to centrifugation between strains of the same species occur (Moulder, 1991). Chlamydia do not attach to host cell surface by cap projection as observed in *Ileobacter*.

This study shows that attachment of the bacteria to the cell surface is followed by their single entry into endocytic vacuoles from which the bacteria rapidly escapes to lie free in the cytoplasm. Escape from the entry vacuole may involve the production of a membrane lytic agent by the bacteria, *S. flexneri* which also employs a similar escape strategy produces a hemolysin active in this manner (Sansone, 1992). Evidence of entry vacuole membrane breakdown in *Ileobacter* infection supports such a supposition. The cytoplasmic matrix and mitochondria adjacent to the endocytic vacuole appeared disrupted, possibly also as a result of enzyme secretion during

bacterial escape from this intracellular location. Disruption of the cytoplasm was not commonly observed adjacent to the lysed endocytic membrane in centrifuged infection, mitochondria on the other hand, adjacent to entry vacuoles had a disrupted matrix. This additional evidence may suggest that escape from the entry vacuole in centrifuged infection is not typical of natural infection could also be an artefact induced by centrifugation (Chapter 5). Breakdown of the entry vacuole membrane enclosing escaping bacteria is unlikely to be an artefact in non-centrifuged infection in the absence of centrifugal forces.

The presence of several bacteria in an endocytic vacuole strongly suggest that the bacteria can multiply on occasion in this intracellular location. A less likely explanation is that endocytic vacuole containing a single organism could fuse with one another to form a larger vacuole containing several bacteria. Endocytic vacuole membrane breakdown with multiple bacteria escaping into the cytoplasm from this confinement was also observed. The cytoplasmic matrix adjacent to the endocytic vacuole was

again disrupted indicating lytic activity associated with bacterial release. Other intracellular bacteria such as S. flexneri enter and escape singly from endocytic vacuole. S. typhimurium on the other hand, also enter singly by endocytic vacuoles but the vacuoles containing single organisms are reported to coalesce with one another after entry (Finlay and Falkow, 1989). Fusion of endocytic vacuoles containing several Chlamydia has also been observed (Ridderhof and Barnes, 1989). However, S. typhimurium and Chlamydia spp. differ from Ileobacter and S. flexneri in that the former do not escape from but multiply in endocytic vacuoles.

Single entry and escape from the entry vacuole provide protection from host defence mechanisms, multiplication of Ileobacter in this intracellular location could allow time for lysosomal fusion with the entry vacuole. However, fusion of entry vacuoles with lysosomes was not observed. This is further discussed in the general discussion in Chapter 8. The escape of bacteria into the cytoplasm indicates continual enzyme synthesis which suggest that these bacteria are viable.

The presence of ground substance and spherical vesicles within the endocytic vacuole are possibly products of bacterial metabolism. Multiplication of *Ileobacter* in the endocytic vacuole suggests that such organisms are metabolically active. Bacterial multiplication is an active process which involves the synthesis of new bacterial components during division (Cook et al., 1989). The close association of mitochondria with the endocytic vacuole may indicate the energy source for this process to occur but nutrients may be less available than in the cytoplasm. The presence of some *Ileobacters* within vacuoles showing separation of protoplasm from the outer membrane may indicate a response to this situation (Chapter 4). Morphologically identical changes have been observed in *H. pylori* and it has been suggested that it is a consequence of reuse of structural components when stored energy reserves have been depleted during nutrient limitation (Caselli et al., 1993).

The evidence of outer membrane blebs and spherical vesicles were seen in *Ileobacter* endocytic vacuoles. This phenomenon has been observed in association with other

bacteria either grown in vitro (Vibro cholerae, Chatterjee and Das, 1967; E. coli, S. typhimurium, Rothfield and Pearlman-Kothencz, 1969; Neisseria meningitidis, Devoe and Gilchrist, 1973) or in an intracellular location (C. trachomatis and psittaci, Stirling and Richmond, 1980; R. tsutsugamushi (Rikihiisa and Ito, 1981) and in these in vitro grown bacteria these blebs are believed to be mainly composed of lipopolysaccharide. The blebs are formed by evagination of the bacterial outer membrane that later are pinched off as spherical-shaped vesicles (Chatterjee and Das, 1967; Devoe and Gilchrist, 1973; Rikihiisa and Ito, 1981). The vesicles in the endocytic vacuoles in this study therefore probably originated from Ileobacter as blebs of the bacterial outer membrane. R. tsutsugamushi cells and their vesicles were both labelled with antibody to the former indicating the origin of the latter (Rikihiisa and Ito, 1981).

Rothfield and Pearlman-Kothencz (1969) reported that E. coli and S. typhimurium outer membrane blebs and vesicles are formed as a result of suboptimal nutritional condition specifically a restriction of availability of some required

amino acids and consequential inhibition of protein synthesis. Some workers reported that this is a consequence of an imbalance of the synthesis of the constituents of bacterial outer membranes imposed by nutritional limitation in heavily infected cells (Knox, Vest and Work, 1966; Rikihisa and Ito, 1981). Chatterjee, Das (1967), Devoe and Gilchrist (1973) associated bleb formation and its subsequent release as vesicles, as a possible excretory mechanism for bacterial endotoxin. In this study some vesicles were observed being extruded through a disrupted vacuole membrane containing *Ileobacter*; whether such vesicles are related to the release of endotoxin or lytic enzymes of *Ileobacter* remains unclear.

Endocytic vacuole containing several bacteria were rare and the majority of bacteria were observed free in the cytoplasm, the latter therefore is the main site of multiplication of the organism. Multiplication in endocytic vacuoles may reflect the reduced ability of *Ileobacter* derived from a pig with PE to infect enterocyte of different animal species, in this case, rat enterocytes.

When bacteria were numerous in the cytoplasm they were often present scattered as groups throughout the deeper cell matrix. Cells with numerous bacteria often showed coated pits and coated vesicles. Coated pits or vesicles were only rarely observed in control uninfected cells. This may suggest an association of *Ileobacter* with the receptor mediated endocytic pathway. Receptor mediated endocytosis (RME) is a process by which host cell take up nutrients by way of an invagination of the host cells membrane called a coated pit with subsequent transfer into coated vesicles (Goldstein, Anderson and Brown, 1979). Coated pits and vesicles acts as transport organelles carrying proteins and peptides to the cytoplasm to be degraded into amino acids for host cell consumption such as cell membrane synthesis. A 180 KD protein, known as clathrin forms a major component of coated pits and vesicles (Pearse, 1976). *C. trachomatis* and *S. flexneri* aggregates in cultured cells have been observed associated with clathrin (Clerc and Sansonetti, 1989; Majeed and Kihlstrom, 1991) suggesting their association with receptor mediated endocytic pathway. However, *Chlamydia* unlike *Shigella* has been reported entering cultured cells by

endocytic pits and in vesicles (Hodinka and Wyrick, 1986; Hodinka, Davis, Choong and Wyrick, 1988). There is no morphological evidence that *Ileobacter* entry into cells is associated with endocytic pits or vesicles in this study. Coated pits and vesicles appear more prominent when bacteria have multiplied in the cytoplasm after escaping from endocytic vacuoles. Sansonetti (1992) proposed that receptor mediated endocytosis is involved in the turnover and stabilization of host cell membrane during bacterial entry. Bacterial entry involves self-interiorization of the host cell plasma membrane to form the endocytic vacuole which surrounds the organism. Cultured macrophages and fibroblast cells interiorize 186% and 54% of their surface area respectively without alteration of cell volume and surface area (Silverstein et al., 1977). The process incorporates a rapid and extensive recycling of cell membrane.

Although the study of the release of *Ileobacter* from the cells was not as extensive as in centrifuged infection (Chapter 5), it is likely that the phases of bacterial proliferation and release follows the same pattern. All

the observations made show the later stages of infection to be closely similar. The comparison of centrifuged and non-centrifuged infection is summarised in Table 12.

In conclusion, this study suggest that the cycle of infection of the intracellular bacteria in the IEC-18 cell line is morphologically similar in both centrifuged and non-centrifuged infection. Differences were present in the morphology of the entry stage and association of the bacteria with components of the receptor mediated endocytic pathway, giving rise to the suggestion that different mechanisms are involved in entry in the two systems. It seems likely that the infection without centrifugation will provide a better in vitro model of infection.

Table 12. Comparison of the ultrastructure of centrifuged and non-centrifuged infection

Parameter	Centrifuge	Non-centrifuge
ATTACHMENT		
Method of attachment	Adherent cap	Close association with cell membrane
ENTRY INTO ENDOCYTTIC VACUOLES (EV)		
Location of EV	Below cell membrane	Different cytoplasmic sites
Single entry	Yes	Yes
Fusion of EV	Possible	Possible
Multiplication in EV	No	Yes
Mitochondria associated with EV	Yes	Yes
ESCAPE FROM EV		
EV membrane breakdown	Yes	Yes
Cytoplasmic disruption associated with escape	No	Yes
FREE RELEASE INTO THE CYTOPLASM		
Multiplication	Yes	Yes
Bacterial aggregation	Present	Present
Location of aggregation	Cytoplasm perinuclear	Cytoplasm Generally perinuclear occasionally focal
Arrangement of bacterial aggregation	Tightly and loosely packed	Tightly and loosely packed
Association of bacterial aggregation with host organelles:		
Mitochondria	Yes	Yes
Rough endoplasmic reticulum	Yes	No
Coated pits	No	Yes
Coated vesicles	No	Yes
RELEASE OF BACTERIA FROM THE HOST CELL		
Free release via extrusion from cell cytoplasm:	Yes	Yes
Cytoplasmic protrusions	Yes	Yes
Non-adherent cells	Yes	Yes
BACTERIAL MORPHOLOGY		
Electron-dense and electron-lucent	Yes	Yes
Cytoplasmic globules	Yes	Yes
Thickened outer membrane	Yes	Yes

CHAPTER 7

EXPERIMENTAL REPRODUCTION OF PROLIFERATIVE ENTERITIS IN
HAMSTERS WITH PORCINE-DERIVED INTRACELLULAR BACTERIA

EXPERIMENTAL REPRODUCTION OF PROLIFERATIVE ENTERITIS IN
HAMSTERS WITH PORCINE-DERIVED INTRACELLULAR BACTERIA

7.1 Introduction

Proliferative enteropathy or enteritis presents a similar clinical and pathological syndrome in pigs and hamsters, with hyperplasia of crypt epithelial cells, primarily in the ileum and colon (Rowland and Lawson, 1974; Frisk and Wagner, 1977b). Natural disease in hamsters has not been reported in the UK, only from the USA.

The disease in both species is consistently associated with the presence of intracellular curved bacteria in the cytoplasm of the hyperplastic crypt epithelial cells. The consistent presence of these bacteria in crypt cells early in the course of natural and experimentally-induced disease in pigs and hamsters suggests that they are involved in the aetiology of the condition (Frisk and Wagner, 1977b; Jacoby and Johnson, 1981; McOrist et al., 1989). Campylobacter mucosalis, hyointestinalis and jejuni isolated from pigs with the natural disease do not colonise the intestines of experimentally infected hamsters, do not induce

proliferative lesions and cannot be identified in an intraepithelial location (McOrist and Lawson, 1987).

Previous attempts to reproduce the disease in hamsters by oral inoculation of pure cultures of C. jejuni isolated from the intestines of hamsters with the disease have been unsuccessful (Lentsch et al., 1982; Regina and Lonigro, 1982; McOrist and Lawson, 1987), whereas the disease has been reproduced with homogenised diseased ilea free of C. jejuni (Stills and Hook, 1989), and with a cell culture of Chlamydia and an unidentified intracellular bacteria from hamsters with PE (Stills, 1991). Hamsters have been shown to develop lesions of PE when dosed with mucosal homogenates and filtrates from the natural disease. It remains therefore uncertain whether the intracellular bacteria is the cause of hamster PE and whether pure cultures of the pig organism can initiate the disease in hamsters.

This study is the first to test the pathogenicity of I. intracellularis, from pigs with PE grown in cell culture in apparently pure culture using hamsters as an experimental

animal model. The study also evaluated the pathology of the disease in hamsters infected with pig-derived bacteria and described the ultrastructure of the lesions and the microorganism.

7.2 Materials and Methods

Hamsters

Hamsters (Mesocricetus auratus) were obtained from a closed colony which was naturally infected with C. jejuni. the progeny of which were used for experimental studies. Hamsters normally start to take solid food at around 10 days and are weaned at circa 21 days old of age. Weanling hamsters were fed pellets, either Mouse/Hamster diet, Special Diet Service Ltd., Witham, Essex, U.K. or Mouse Chow, Ralston Purina, St Louis, Missouri, USA. The diets are proprietary formulations containing a balanced complete ration with 5% and 3% fibre respectively. The hamsters were weaned, randomly allocated to treatment groups and dosed at 3 weeks of age. Control and inoculated hamsters were placed in separate cages after dosing.

Inocula for hamsters

The source material for cell culture infection was derived from the affected intestines of two pigs naturally affected with proliferative haemorrhagic enteropathy designated as 916/91 and 1482/89 prepared and stored frozen in 1ml vials (Section 2.4).

IEC-18 cells were seeded at a cell concentration of 0.25×10^5 cells ml^{-1} in 25cm^2 tissue culture flasks and grown for 24 hours in DMEM supplemented with L-glutamine, fungizone and 10% vol/vol FCS. A one ml vial of the prepared inoculum was added to 14ml of warm DMEM with supplements as above, but with 7% vol/vol FCS after rapid thawing at 37°C . The diluted suspension was then added to the IEC-18 monolayers. Infection of cells was assisted by centrifugation of the flasks at $2020 \times g$ for 30 min. Flasks were incubated microaerobically in steel jars for 3 hours, then fed with DMEM with similar supplements containing neomycin and vancomycin and further incubated in an incubator. At days 2 and 4 post-infection, the infected cell monolayers were further refed with the same growth

media, supplements and antibiotics but with 5% vol/vol FCS. Infected cell monolayers were passaged at day six post-inoculation by treatment with KCl followed by mechanical removal of the cells. The cells were then ruptured before they were used to inoculate fresh monolayers of IEC-18 cells (Section 2.5).

Infected cells were grown for 6 days before being prepared for passage or for the inoculation of hamsters. The inoculum for hamsters in group 1 was prepared from one flask. The media in the flask was removed and was replaced with 6ml of DMEM containing 5% vol/vol FCS, the cells removed with a scraper, homogenised for 15 seconds and each hamster dosed intragastrically with 1ml using a blunt-ended needle. The inocula for hamsters in groups 2, 3 and 4 were prepared from a number of flasks corresponding to the number of hamsters to be infected. For each flask, the media was removed and replaced with 2ml of DMEM containing 5% vol/vol FCS. Cells from the flasks were then bulked, homogenised for 15 seconds and hamsters dosed with 2ml each. Hamsters in groups 5, 6 and 7 were passaged material which had been frozen at -70°C in DMEM and 10% DMSO in 1ml

vials for approximately 3 months. Frozen inocula were rapidly thawed at 37°C before use. Inoculum for hamsters in group 5 was prepared from 4 vials without further dilution. For group 6 hamsters, 1 vial of the inoculum was diluted in 6ml of DMEM with 10% vol/vol FCS. Inoculum for hamsters in group 7 was prepared from 5 vials which was diluted in 4ml of DMEM with 10% vol/vol FCS. Inoculum for hamsters in groups 5, 6 and 7 were bulked, homogenised for 15 seconds and hamsters dosed with 1ml each. Further details including source strains, numbers of bacteria and extent of passage are given in Tables 13 and 14.

IEC-18 inocula

Control hamsters were either not dosed or dosed orally with non-infected IEC-18 cells. Confluent monolayers of these cells were detached by trypsinisation by standard methods (Paul, 1975), then homogenised for 15 seconds and each hamster dosed with 2ml.

Monitoring of inocula

Table 13. Detail of fresh inocula used to dose hamsters

Group	Strain no.	Passage no.	Infection ^a : HIC/F	No. of flask (25 cm ²) used to prepare inocula	Inocula volume	Bacteria per ml ^b
1	916/91	1	1032/94	1	6	1.07 x 10 ⁵
2	916/91	5	7351/NA	5	10	2.30 x 10 ⁶
3	916/91	6	13333/NA	5	10	4.17 x 10 ⁶
4	916/91	7	1/1	6	12	3.12 x 10 ²

^a Infection counted for 2 coverslips (200 mm²), set up in parallel to infection in flasks used to prepare inocula
^b Estimated as described in Chapter 5

HIC Highly infected cells
 F Foci of infection

NA Not Applicable. Highly infected cells too numerous to count and there is no discrete foci of infection. Total number of HIC estimated from 5 different areas (0.0672 mm²)

Table 14. Detail of frozen inocula used to dose hamsters

Group	Strain no.	Passage no.	Infection ^a : HIC/F	Preparation of source inocula in (1 ml) vials		Preparation of inocula to dose hamster		Inocula volume	Bacteria per ml ^c
				Diluent per flask ^b (ml DMEM)	No. of vial used	Diluent (ml DMEM)			
5	916/91	5	7351/NA	20	4	Not diluted	4	1.83 x 10 ⁴	
6	1482/89	2	274/91	8	1	1:6	7	4.70 x 10 ³	
7	1482/89	2	274/91	8	5	5:4	9	1.81 x 10 ⁴	

a Infection counted for 2 coverslips (916/91, 200 mm²; 1482/89, 130 mm²), set up in parallel to infection in flasks used to prepare inocula in vials

b One flask used per group of hamster to prepare inocula in vials

c Estimated as described in Chapter 5

HIC Highly infected cells

F Foci of infection

NA Not Applicable. Highly infected cells too numerous to count and there is no discrete foci of infection. Total number of HIV estimated from 5 different areas (0.0672 mm²)

DMEM Dulbecco's Modification Eagle's Medium

To monitor the number of I. intracellularis cells at each passage, IEC-18 cells on glass coverslips were infected with inocula also used to infect flasks. On day 6 post-inoculation, coverslips were harvested, washed, fixed and stained with monoclonal antibody IG4 as the primary antibody and an anti-mouse peroxidase conjugate as the secondary antibody in an indirect test (Section 2.9).

In addition, 10ul of each bulked inoculum used to dose hamsters was smeared onto a glass slide, fixed and stained by an indirect immunofluorescence assay using the same primary antibody IG4 followed by a fluorescein conjugated sheep anti-mouse antibody (Section 2.8). Some infected cells on coverslips have been tested for the presence of Chlamydia by immunofluorescence staining as described in Section 4.2.

Necropsy procedure

Light microscopy

Necropsy was conducted on all hamsters 21 days after dosing and samples of small and large intestines taken for

light microscopy as described previously (Section 2.7). Sections of formalin-fixed intestines were stained with haematoxylin and eosin or Young's modification of silver impregnation stain (Section 2.14). The ratio of crypt villus height was estimated with a measured objective for 10 undistorted crypt villus units per section and the mean values calculated. Further paraffin sections were immunofluorescence stained with the primary monoclonal antibody IG4 and fluorescein conjugated sheep anti-mouse immunoglobulin as the secondary antibody. Similar staining was conducted on all hamsters using an antiserum (diluted 1/80 in PBS) prepared in rabbits against C. jejuni strain 664/83 isolated from a normal hamster (McOrist and Lawson, 1987), and conjugated sheep anti-rabbit immunoglobulin (diluted 1/80 in PBS) as the primary and secondary antibodies respectively. Appropriate dilutions of primary and secondary antibody had been established by titrations employing antigen 664/83 prepared from bacterial cultures.

Electron microscopy

Samples of glutaraldehyde fixed small and large

intestines were processed for routine TEM (Section 2.11). Immunogold staining of further selected ultrathin sections from each group of hamsters was performed with monoclonal antibody IG4 as the primary antibody and anti-mouse IgG conjugated with 15nm gold particles as the secondary antibody (Section 2.10).

Bacteriology

Tissue culture inocula

Samples were taken from all inocula and inoculated onto Columbia blood agar, Skirrow's medium, Brucella semi-solid medium and incubated microaerobically for Campylobacter spp. and other bacteria (Section 2.12).

Intestinal culture for Campylobacter spp.

Swabs from the intestines of all hamsters were also cultured onto similar medium, smears were prepared from suspect Campylobacter colonies and stained by Gram's and Modified Ziehl Neelsen methods (Section 2.13). Subcultures were transferred to blood agar, tested for catalase and

antibiotic sensitivity (30ug Cephalothin and 30ug Nalidixic acid). Antibiotic sensitivity was read after 24 hours incubation at 37°C. Colonies with Campylobacter-like morphology that were catalase positive, sensitive to Nalidixic acid and resistant to Cephalothin were considered as C. jejuni or C. coli; representative isolates were tested for the ability to hydrolyse hippurate (see below) and identified as C. jejuni.

Hippurate hydrolysis

The tests conducted were as described by Lander and Gill (1985). The bacterial growth from an overnight blood agar culture was removed and suspended in 2ml distilled water. Sodium hippurate (0.5ml) was added and the mixture was incubated at 37°C for 2 hours. One ml of ninhydrin reagent (3.5g ninhydrin in 100ml of a 1:1 mixture of butanol and acetone) was added, a rapid development of purple blue colouration was considered evidence for the hydrolysis of hippurate.

Results

All hamsters were clinically healthy throughout and appeared normal on post mortem examination. Bacterial culture of the tissue culture derived inocula failed to result in visible growth. Immunoperoxidase staining of infected IEC-18 cells on coverslips harvested at the same time as the hamsters inocula and immunofluorescence staining of smears of the inocula derived from infected flasks showed numerous curved bacteria reactive with the primary monoclonal antibody. Examination of infected monolayers showed no discernable cytopathic effects. C. jejuni was isolated from 33/40 treated and 24/32 control hamsters.

Microscopic findings

Many hamsters including control hamsters had varying degrees of localised acute intestinal inflammation (Table 15). Infiltration of the mucosa with inflammatory cells especially neutrophils, and scanty lymphocytes and macrophages was present. Some hamsters had focal necrosis

and infiltration of the crypts with neutrophils. Crypt lumina containing numerous curved bacilli were seen consistently on silver stained sections. Numerous fluorescent curved bacilli were also evident in occasional crypt lumina in sections treated with antiserum raised against C. jejuni 664/83.

Microscopic lesions consistent with proliferative enteritis were seen only in hamsters dosed with inoculum 916/91 in groups 1, 2, and 3 (see Table 15). Infected hamsters showed marked hyperplasia of crypt epithelial cells in the terminal ileum and three of the hamsters in group 1 also had similar lesions in the jejunum. Affected crypts were enlarged, had reduced or total absence of goblet cells and many mitotic figures (Fig. 69). Adjacent villi were distorted and reduced to a leaf-like shape. There was a moderate increase in crypt to villus ratio in affected hamsters (Table 15). Silver staining showed numerous bacteria in the apical cytoplasm of affected crypt epithelial cells (Fig. 70).

Immunofluorescence staining of intestinal sections with

Table 15. Some features of hamsters dosed with tissue culture derived inocula

Group	Strain no.	Number of bacteria in inoculum	No. of hamsters inoculated	Histological changes in the intestines:			Mean crypt to villus ratio
				No. visible lesions	Acute enteritis	Proliferative enteritis	
1	916/91	1.07 x 10 ⁵	6	0	2	4	0.39
2	916/91	2.30 x 10 ⁶	5	0	2	3	0.37
3	916/91	4.17 x 10 ⁶	5	0	2	3	0.38
4	916/91	3.12 x 10 ²	6	2	4	0	0.25
5	916/91	1.83 x 10 ⁴	4	0	4	0	0.27
6 ^a	1482/89	4.70 x 10 ³	6	3	3	0	0.26
7	1482/89	1.81 x 10 ⁴	8	0	8	0	0.29
8	IEC-18 controls	None	4	1	3	0	0.28
9	Undosed controls	^b	28	12	16	0	0.27

^a Special Diet Service Ltd. used

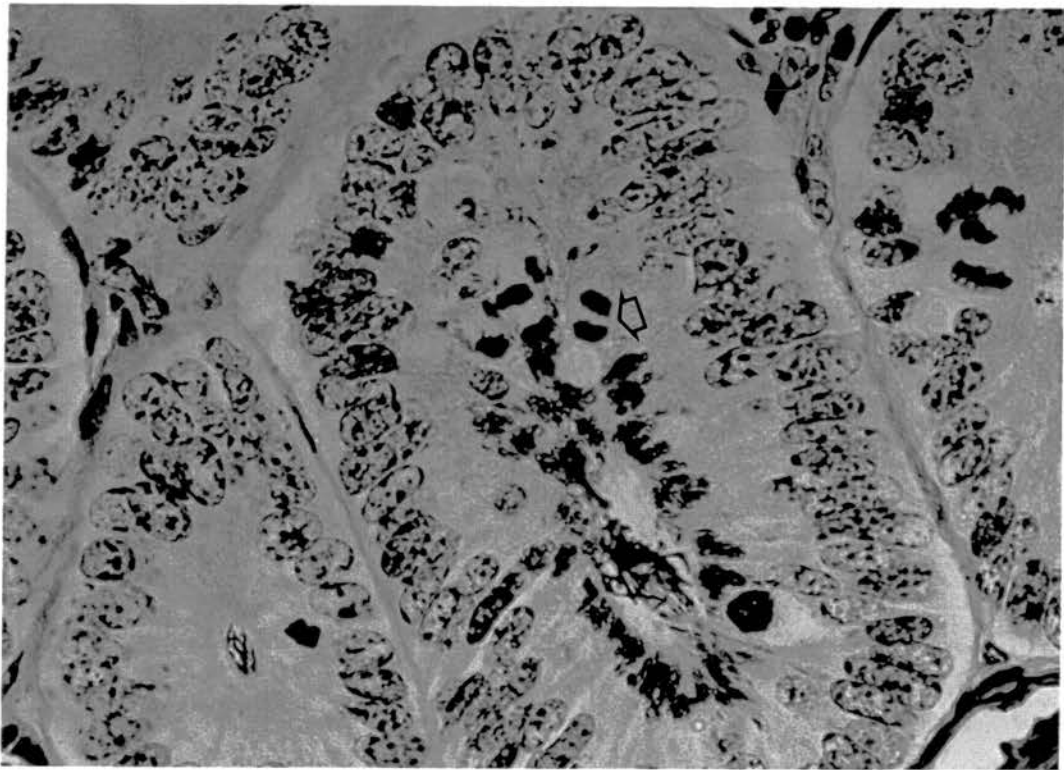
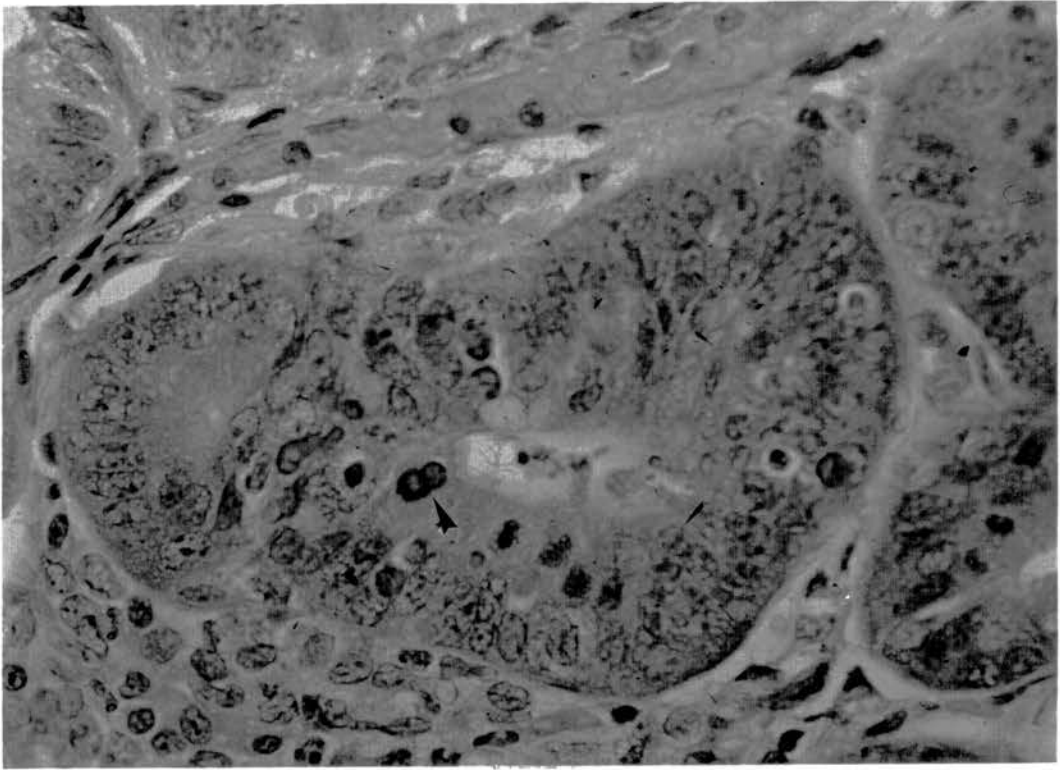
^b Not applicable

Figure 69

Terminal ileum of a hamster infected with the intracellular bacteria of pigs cultivated in IEC-18 cells. There is an increase in mitotic activity of affected crypt cells. Note that the crypt epithelial cells are hyperplastic and the affected glands are enlarged and elongated. Goblet cells are markedly reduced in numbers. Some intraepithelial lymphocytes are present in the epithelium (arrowhead). Haematoxylin and Eosin stain, x 1312.

Figure 70

Terminal ileum of a hamster infected with the intracellular bacteria of pigs cultivated in IEC-18 cells showing numerous bacteria in the apical cytoplasm of hyperplastic crypt epithelial cells. There is an increase in mitotic activity with some cells undergoing division (arrow). Note adjacent normal glands have bacteria only in the crypt lumen. Young's modification of Warthin Starry silver stain x 1341.



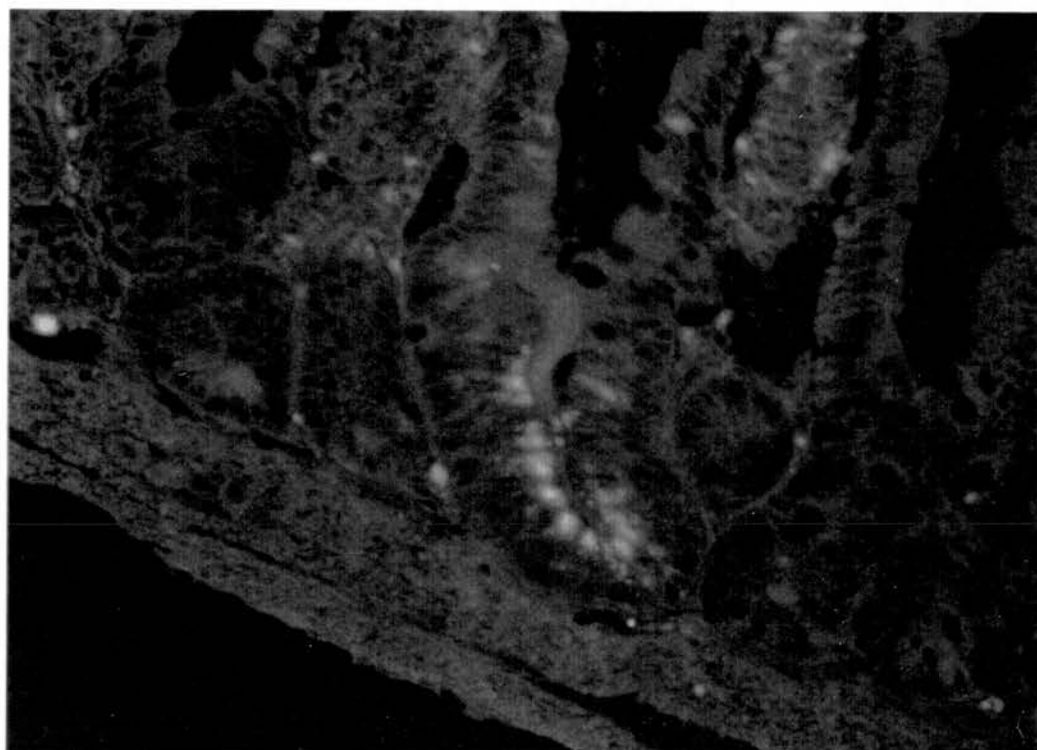
monoclonal antibody IG4 showed numerous brightly fluorescing curved bacteria especially in the apical cytoplasm of cells in the affected crypts and villi (Fig. 71). Curved bacteria were present in the lumen of some proliferative crypts in silver stains, luminal bacteria were not seen to react with the monoclonal antibody. Animals which were exposed to infected material which did not developed proliferative lesions, failed to show any evidence of luminal or intracellular infection by I. intracellularis, by monoclonal antibody staining. Proliferative lesions were not observed in the caecum or colon of any hamsters. Sections of control hamster intestines showed no mucosal proliferation and sections treated with monoclonal antibody IG4 showed no fluorescence.

Ultrastructural findings

Ultrastructural observations showed numerous intracellular bacteria in the cytoplasm of crypt epithelial cells of the terminal ileum of affected hamsters. Bacteria were not seen in the cytoplasm of control or exposed

Figure 71

Immunofluorescence staining of the lesions of PE in the terminal ileum of a hamster exposed to the intracellular bacteria of pigs cultivated in IEC-18 cells. Numerous brightly fluorescing bacteria are localised in the cytoplasm of the affected crypt. The villous wall with hyperplastic extension also showed fluorescing bacteria. Monoclonal antibody IG4 and sheep anti-mouse FITC conjugate stain x 328.



hamsters in which intestinal cells appeared normal.

Occasional crypt lumina of both exposed and control hamsters contained numerous curved bacteria (Fig. 72). Approximately 30% of the luminal bacteria had a pale or electron dense circular granules within their protoplasm.

Stages of bacterial attachment and entry into the cells were observed (Figs. 73 to 77). Bacteria at the brush border some containing pale or electron-dense globules appeared to attach and enter cells singly in endocytic vacuoles. Some preparation showed luminal bacteria not adherent to the microvillus border, these organisms could not be differentiated from those entering cells. The membrane in the luminal surface of the epithelial cells was thickened and ill-defined at the point of bacterial attachment. Bacteria enter a depression formed in the membrane underneath the bacteria which was the beginning of the formation of the endocytic vacuoles. The leading membrane of the bacteria appeared to be closely applied to the cell membrane of the endocytic vacuole at one point or another (Figs. 74 to 77). Each vacuole and associated

Figure 72

Numerous curved bacteria in the crypt lumen of the intestines. Some bacteria have a distinct pale or electron dense granules within the protoplasm. Lead citrate and uranyl acetate stain x 16632.



Figures 73 to 77

Transmission electron micrograph of crypt epithelial cell of the terminal ileum of a hamster infected with the intracellular bacteria of pig cultivated in IEC-18 cells. Stages of bacterial entry into epithelial cells.

Figure 73

A single bacterium in close contact with the microvillus brush border. Microvilli at the point of contact appeared shorter and disrupted. Note pale granule in the bacteria protoplasm. Lead citrate and uranyl acetate stain
x 116700.

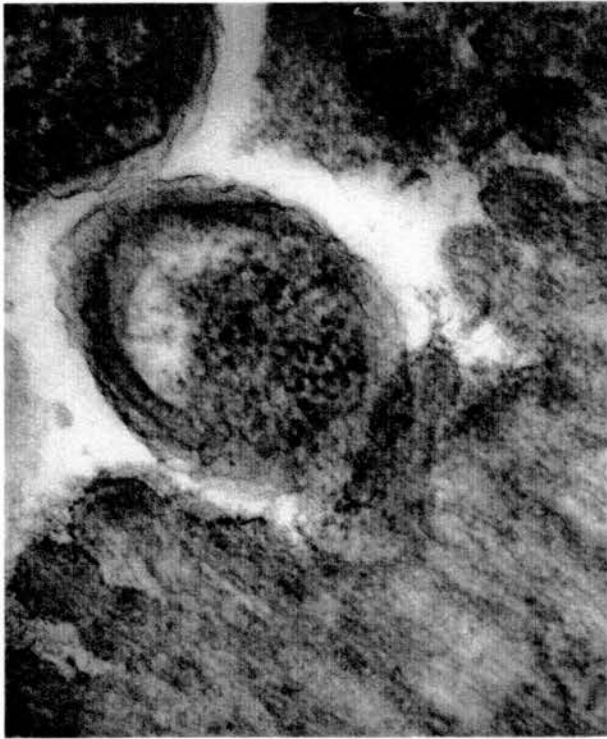


Figure 74

A transverse section of single bacterium attached to disrupted microvillous brush border with initial vacuole formation. Lead citrate and uranyl acetate stain x 65400.

Figure 75

A longitudinal section of a bacterium attaching and entering crypt epithelial cells. The bacterial membrane appeared closely applied to the thickened membrane of the forming endocytic vacuole. Microvilli are absent at site of bacterial attachment and entry. Lead citrate and uranyl acetate stain x 65400.

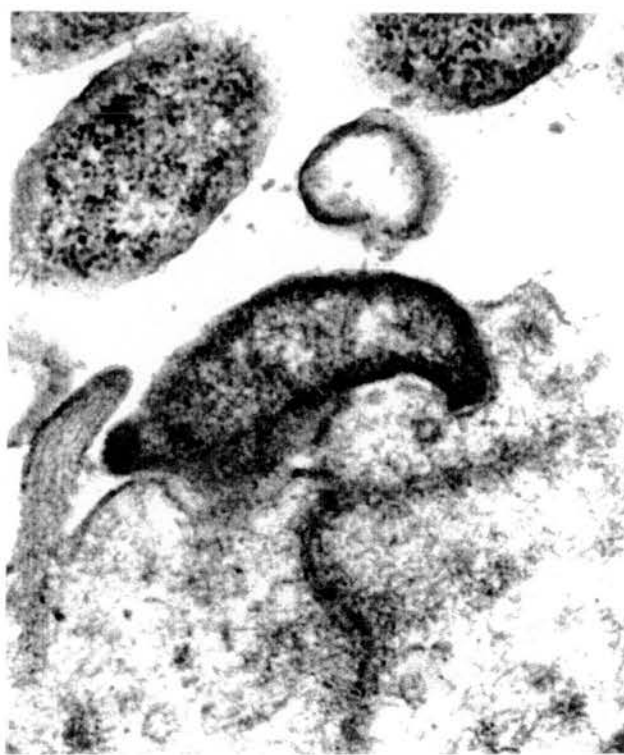
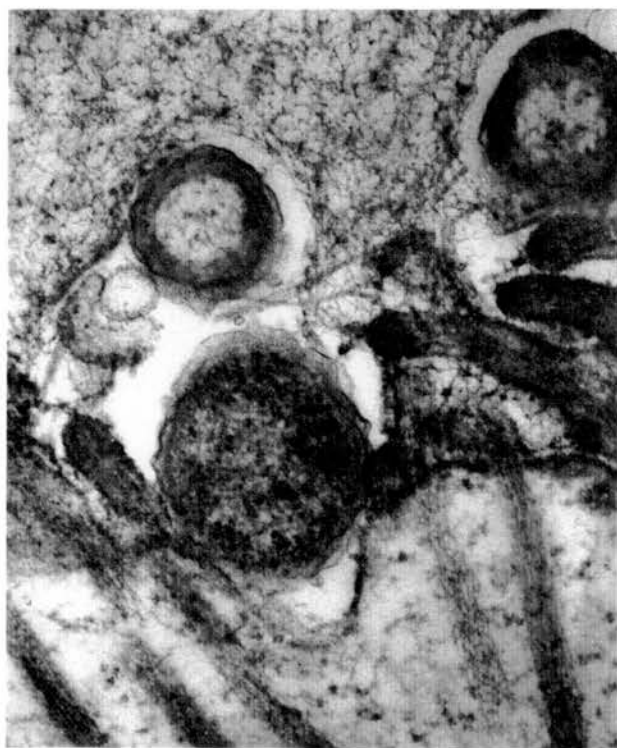


Figure 76

A single bacterium at an advanced stage of entry into a cell. Note that the microvillus brush border is disrupted. Lead citrate and uranyl acetate stain x 15400.

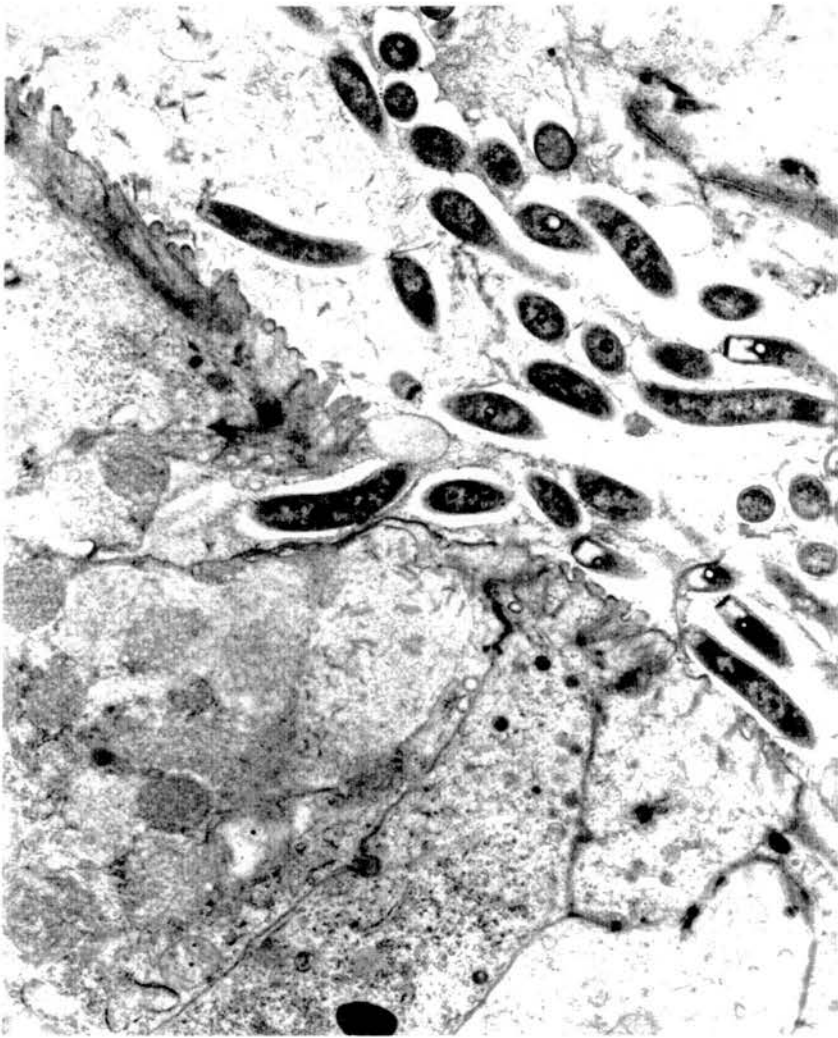


Figure 77

A bacterium with electron dense granules at an advanced stage of entry into a cell at higher magnification. Bacterial engulfment into an entry vacuole has almost completed. The bacterial membrane appears to be closely applied to the membrane of the endocytic vacuole at one point or another. The bacteria appears to have polar fibrils (arrow). Note disrupted microvilli and terminal web. Lead citrate and uranyl acetate stain x 65780.



single bacterium extended into the cell (Figs. 76 and 77). In one occasion, bacteria at an advanced stage of entry into vacuoles was observed with polar fibrils (Fig. 77). Bacterial attachment and entry resulted in loss and disruption of the microvilli and terminal web in adjacent areas of the brush border. Two further events were apparent after bacterial entry. In most instances, there was breakdown of the entry vacuoles with bacteria of both forms appearing free in the cytoplasm (Figs. 78 and 79). In a few instances, several bacteria were observed in endocytic vacuoles. Some of these vacuoles appeared to coalesce with one another to form larger endocytic vacuoles. Similarly, these vacuoles showed membrane breakdown and bacteria escaping free into the cytoplasm from the disrupted sites were in close contact with the cytoplasmic matrix. Occasionally, vacuoles containing single and several bacteria also contained amorphous and electron-dense material (Figs. 80 to 83). In a few instances, entry vacuoles were seen closely associated with host cell mitochondria (Fig. 84).

In the cytoplasm bacteria were most frequently seen in

Figure 78

An early event after bacterial entry. A single bacterium is completely engulfed in an endocytic vacuole at the terminal web. Note breakdown of the entry vacuole at one end towards the cell (arrow). Microvilli are architecturally damaged and the terminal web disrupted. Some free bacteria are lying just adjacent to the entry vacuole. Lead citrate and uranyl acetate stain x 59290.



Figure 79

A later event after bacterial entry. Entry vacuoles each containing a single bacterium are now just below the terminal web. Breakdown of entry vacuoles is more distinct and bacteria are being released into the cytoplasm (arrows). Both free intracellular electron-dense and electron-lucent forms of the bacteria are adjacent to the entry vacuoles. Microvilli is now intact. Lead citrate and uranyl acetate stain x 35640.

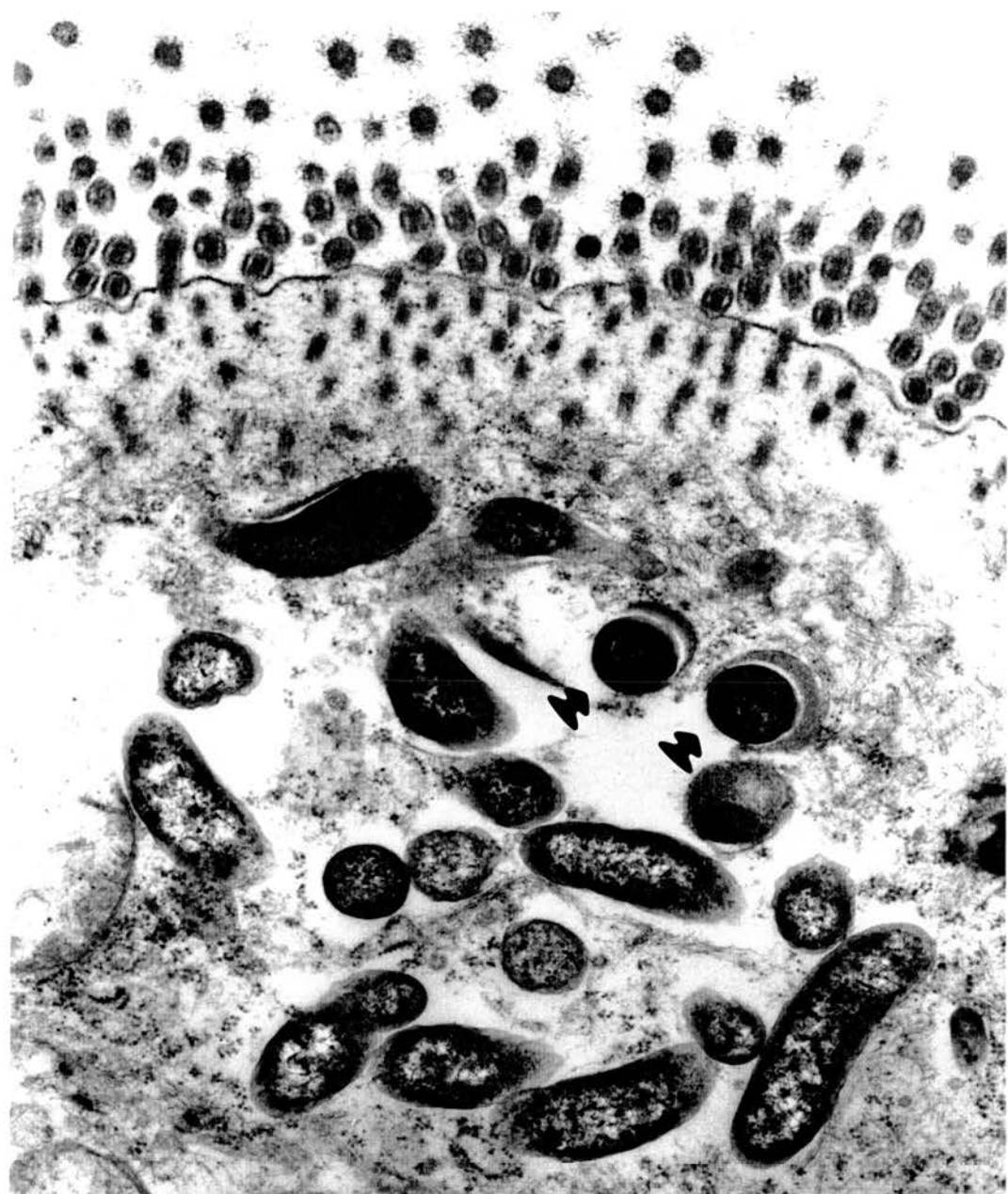


Figure 80

Entry vacuoles with electron-dense bacteria showing membrane breakdown at higher magnification. Note disruption of the cytoplasmic matrix adjacent to the site of vacuolar membrane breakdown (arrows). Crescent-shaped amorphous, homogenous electron-dense material is also evident in the vacuoles. Lead citrate and uranyl acetate stain x 154000.

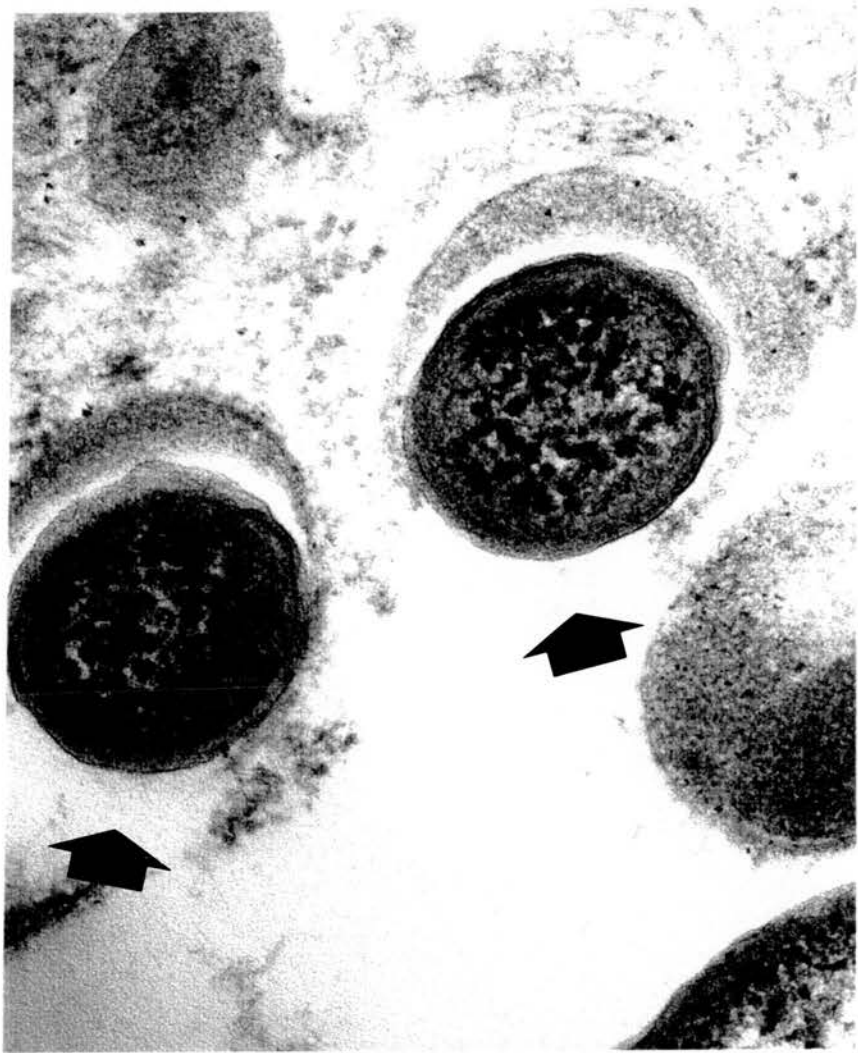


Figure 81

Three endocytic vacuoles with multiple bacteria. An amorphous and homogenous electron dense material is also present in the vacuoles. Distinct breakdown of the vacuolar membrane is evident and bacteria are seen escaping into the cytoplasm. Note disrupted organelles adjacent to one of the vacuole (arrow). Lead citrate and uranyl acetate stain x 49780.

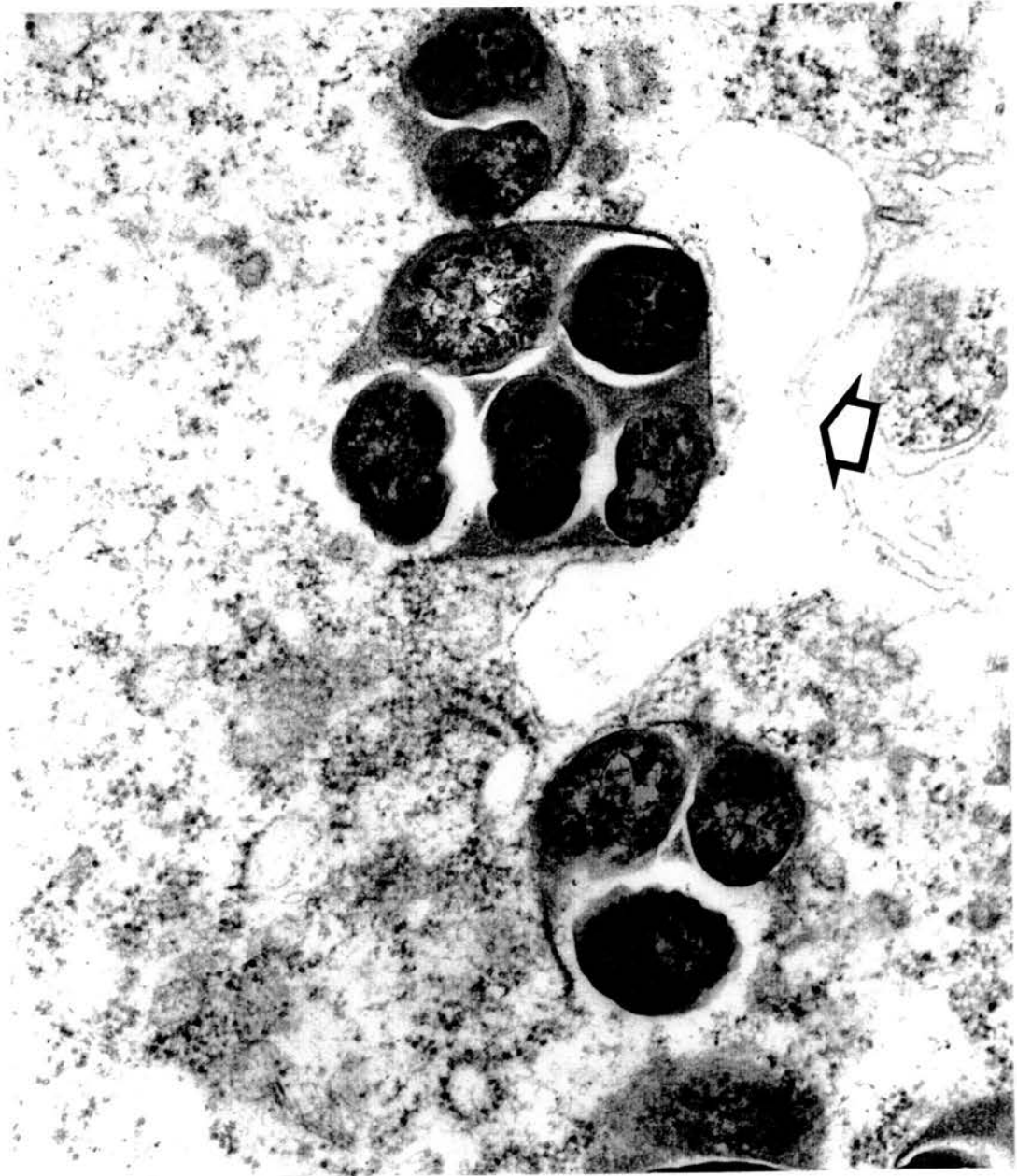


Figure 82

Three endocytic vacuoles containing several bacteria. Two of the vacuoles appeared to have coalesced with one another. The vacuolar membranes appeared to be disrupted and bacteria which are escaping into the cytoplasm appeared to be in intimate contact with host cell cytosol. Lead citrate and uranyl acetate stain x 28050.

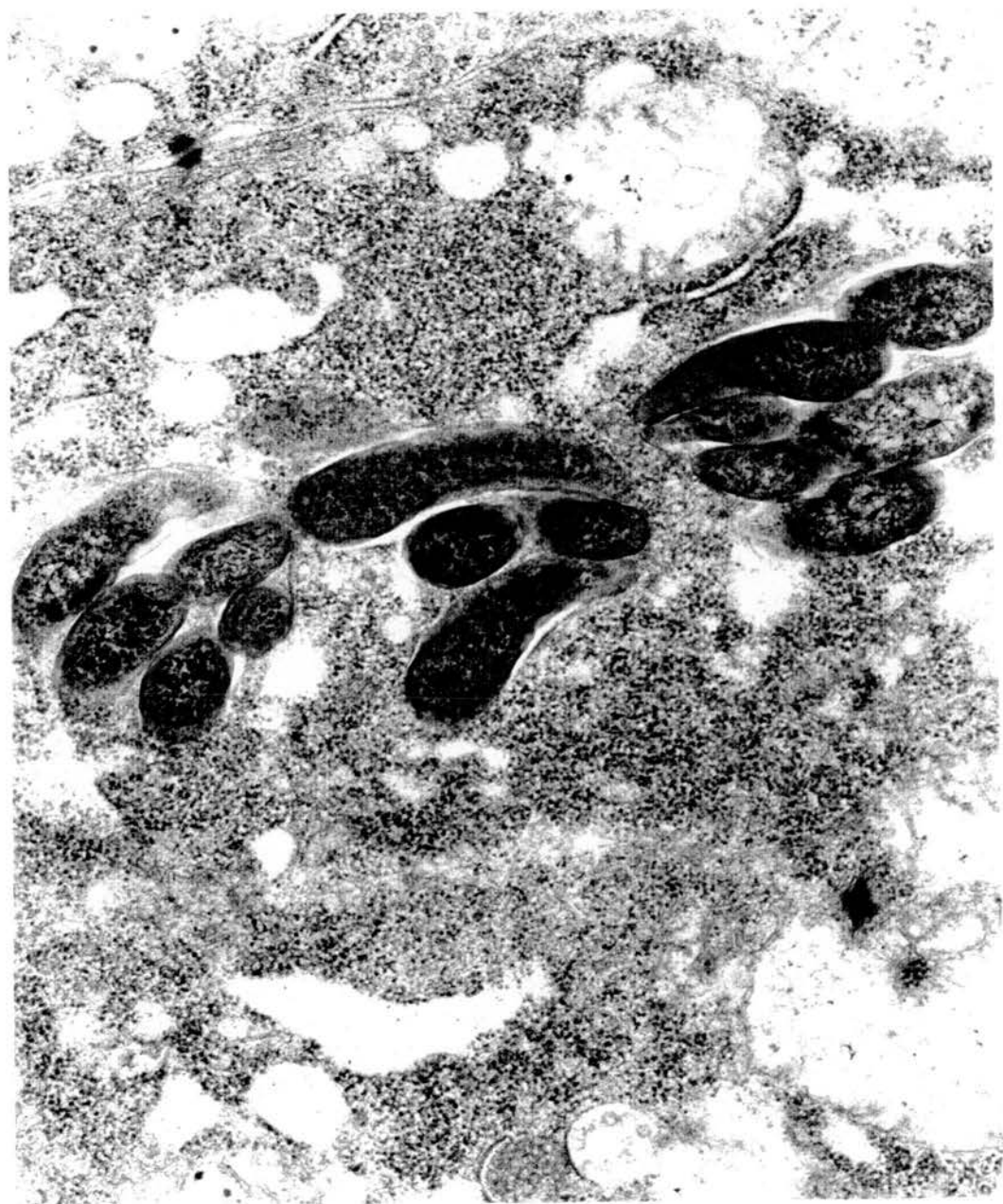


Figure 83

Bacteria in an endocytic vacuole showing the intimacy of contact of the bacteria with host cell ribosomes at the point where vacuolar membrane breakdown has occurred (arrows). Lead citrate and uranyl acetate stain x 123200.

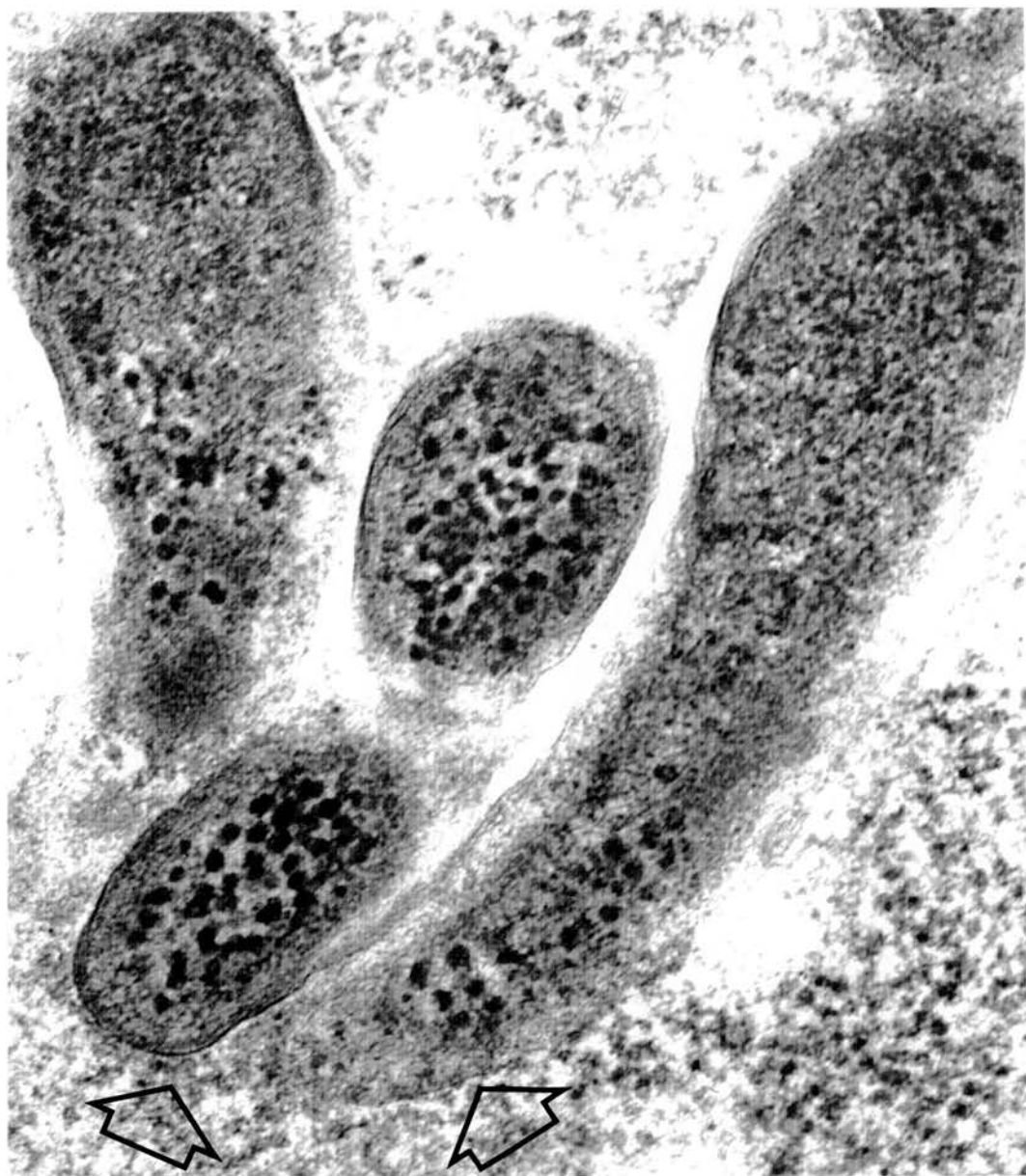
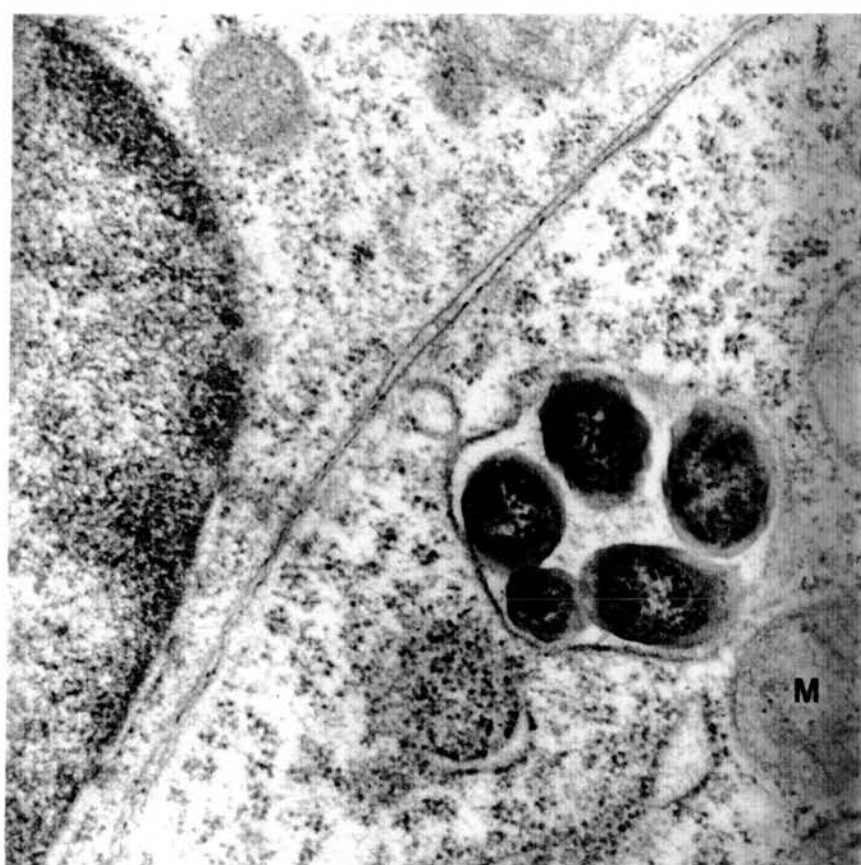


Figure 84

A single endocytic vacuole containing several bacteria close to a host cell mitochondrion, M. Lead citrate and uranyl acetate stain x 56800.



groups lying free in the apical cytoplasm (Fig. 85); they were seldom seen singly. The bacteria were apparently similar in morphology to those observed in the cell culture study (Chapters 4, 5 and 6); both pleomorphic electron-dense and electron-lucent forms were observed. Electron-lucent forms predominated in the cytoplasm. Bacterial division was only observed involving electron-lucent forms either free in the cytoplasm or within vacuoles (Fig. 86). 10% of the bacteria had pale or electron dense granules in their protoplasm. Bacteria with these protoplasmic inclusions were observed below the terminal web (Fig. 87).

In most infected cells, there was marked distension and disruption of some mitochondria and the rough endoplasmic reticulum. The terminal web adjacent to groups of bacteria was usually disrupted, disorganised and increased in electron density. In some instances, groups of bacteria in the cytoplasm were lying adjacent to normal microvillus brush border. Additionally, parasitised crypt cells had localised cytoplasmic vacuolation (Fig. 88). Many free bacteria appeared to be very closely associated with normal and distended mitochondria and rough endoplasmic reticulum

Figure 85

Bacteria are frequently seen in groups and seldom seen singly in the apical cytoplasm. Note that both electron-dense and electron-lucent forms of the bacteria are present. Lead citrate and uranyl acetate stain x 4880.

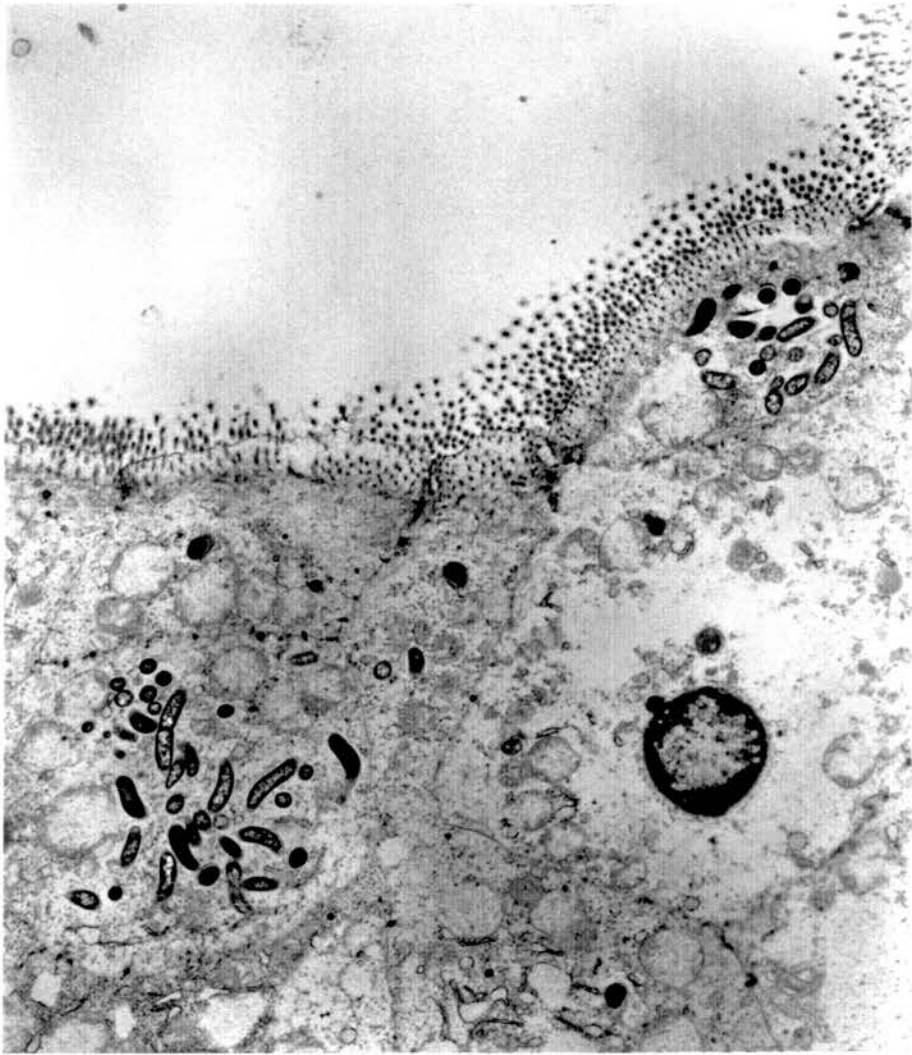


Figure 86

A group of electron-dense and electronlucent forms in the apical cytoplasm. Electron-lucent bacteria predominated in the cytoplasm. Note an electron-lucent organism is undergoing division by septation. Lead citrate and uranyl acetate stain x 17940.

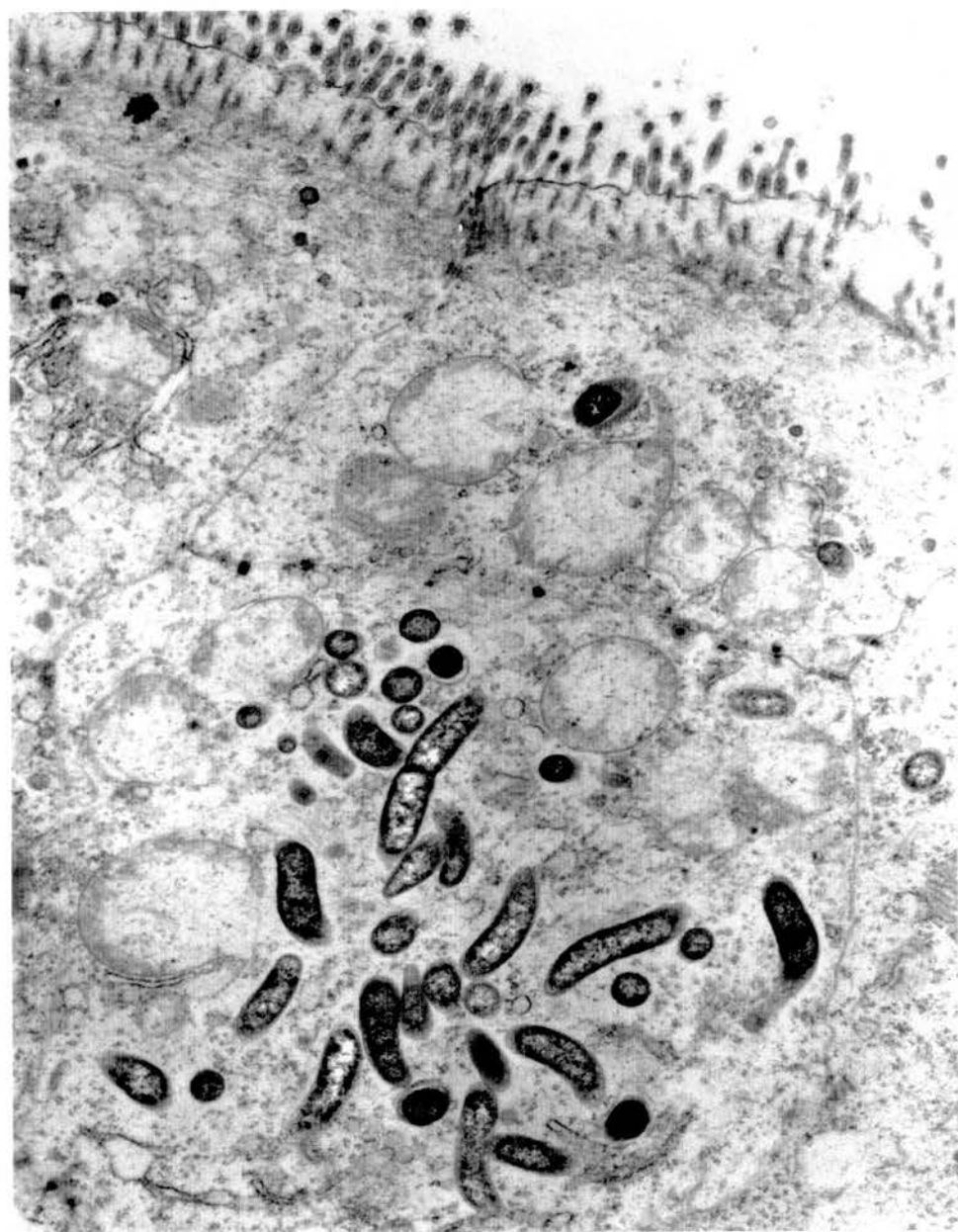


Figure 87

A bacterium free in the cytoplasm just beneath the terminal web with dense circular granule in its cytoplasm. Lead citrate and uranyl acetate stain x 34380.

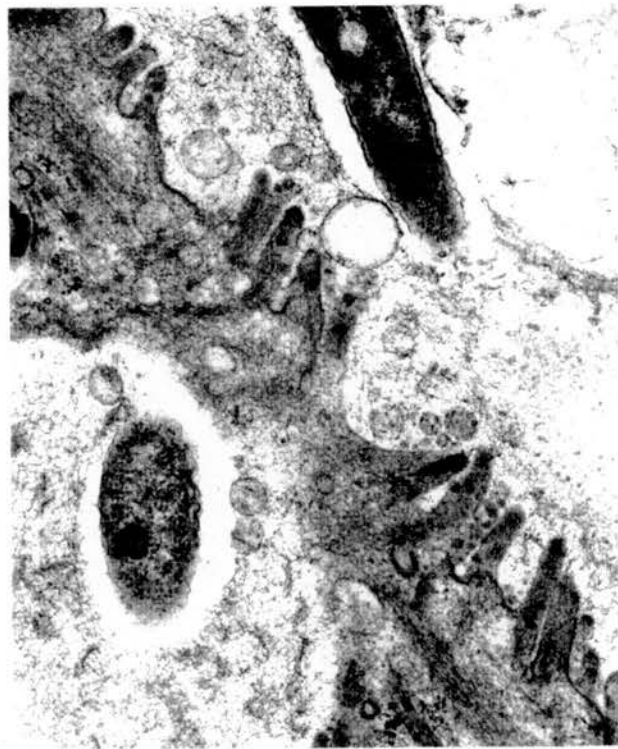
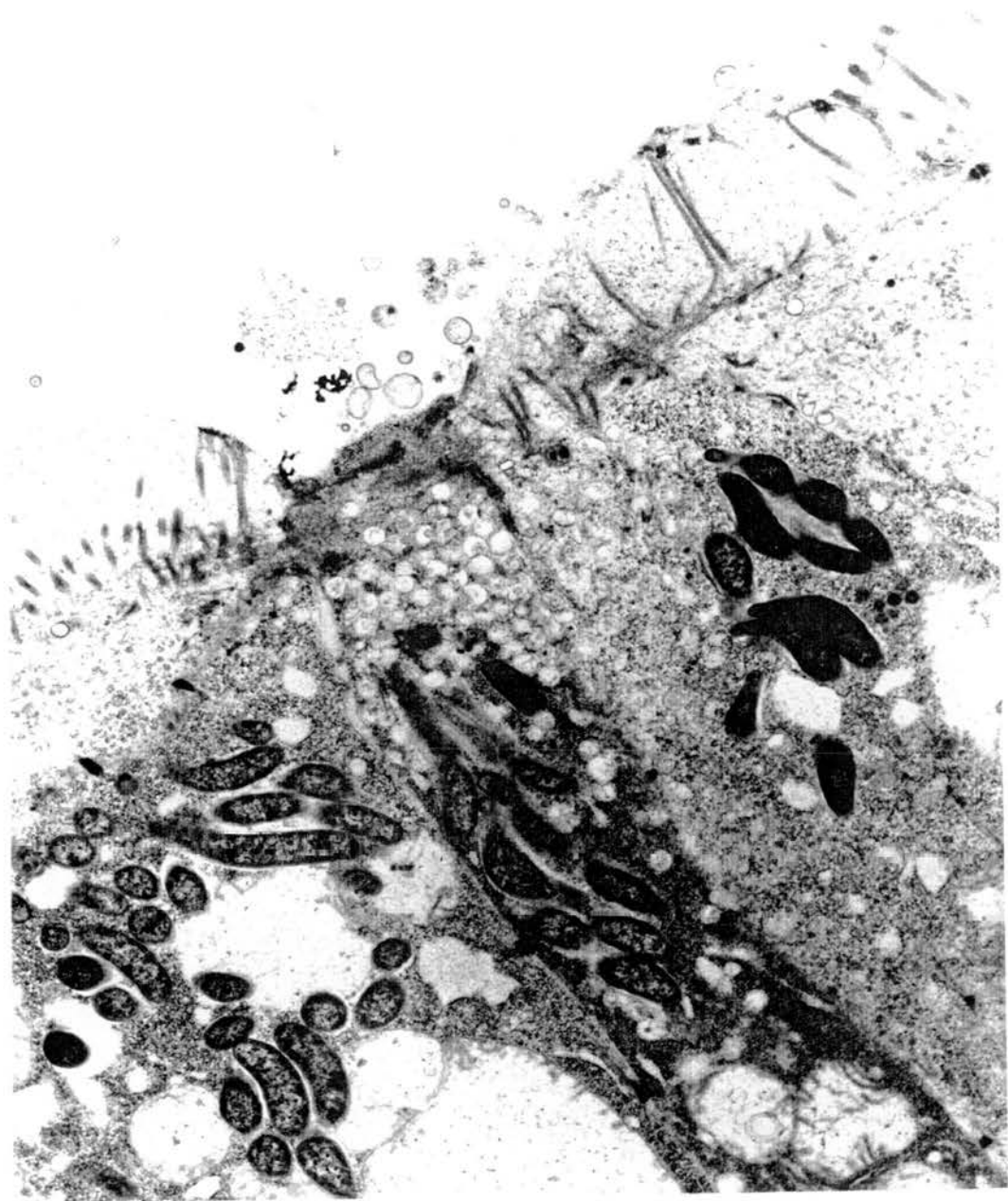


Figure 88

Parasitised crypt epithelial cells showing localised cytoplasmic vacuolation adjacent to a disrupted terminal web and microvilli. Lead citrate and uranyl acetate stain. x 15400.



(Figs. 89 to 91). Some bacteria had thickening of their outer membrane when free in the cytoplasm and close to the rough endoplasmic reticulum and mitochondria (Figs. 92 and 93).

Neutrophils and macrophages in the mucosa contained many bacteria at various stages of degeneration and lysis within phagolysosomes. Highly infected cells often ruptured, releasing bacteria both free and in endocytic vacuoles into the crypt lumina (Figs. 94 and 95). Some infected cells were apoptotic (Fig. 96).

Immunogold staining showed bacteria reactive to the monoclonal antibody IG4 only in the apical cytoplasm of the affected crypt cells. Immunogold particles were consistently seen on the outer membrane of the intracellular bacteria (Fig. 97). Morphologically similar bacteria in the crypt lumina and those just entering showed no associated gold particles. Gold particles were absent from sections of control hamsters, stained in an identical manner. Chlamydia or viruses were not observed in the lesions. Immunological staining for Chlamydia was negative

Figure 89

Intracellular bacteria free in the cytoplasm, some closely associated with normal mitochondria. Lead citrate and uranyl acetate stain x 49500.



Figure 90

Intracellular bacteria free in the apical cytoplasm, some of the organisms are closely associated with markedly distended and disrupted mitochondria. Lead citrate and uranyl acetate stain x 20000.



Figure 91

Bacteria closely associated with disrupted host cell mitochondria (M) at higher magnification. Lead citrate and uranyl acetate x 88750.

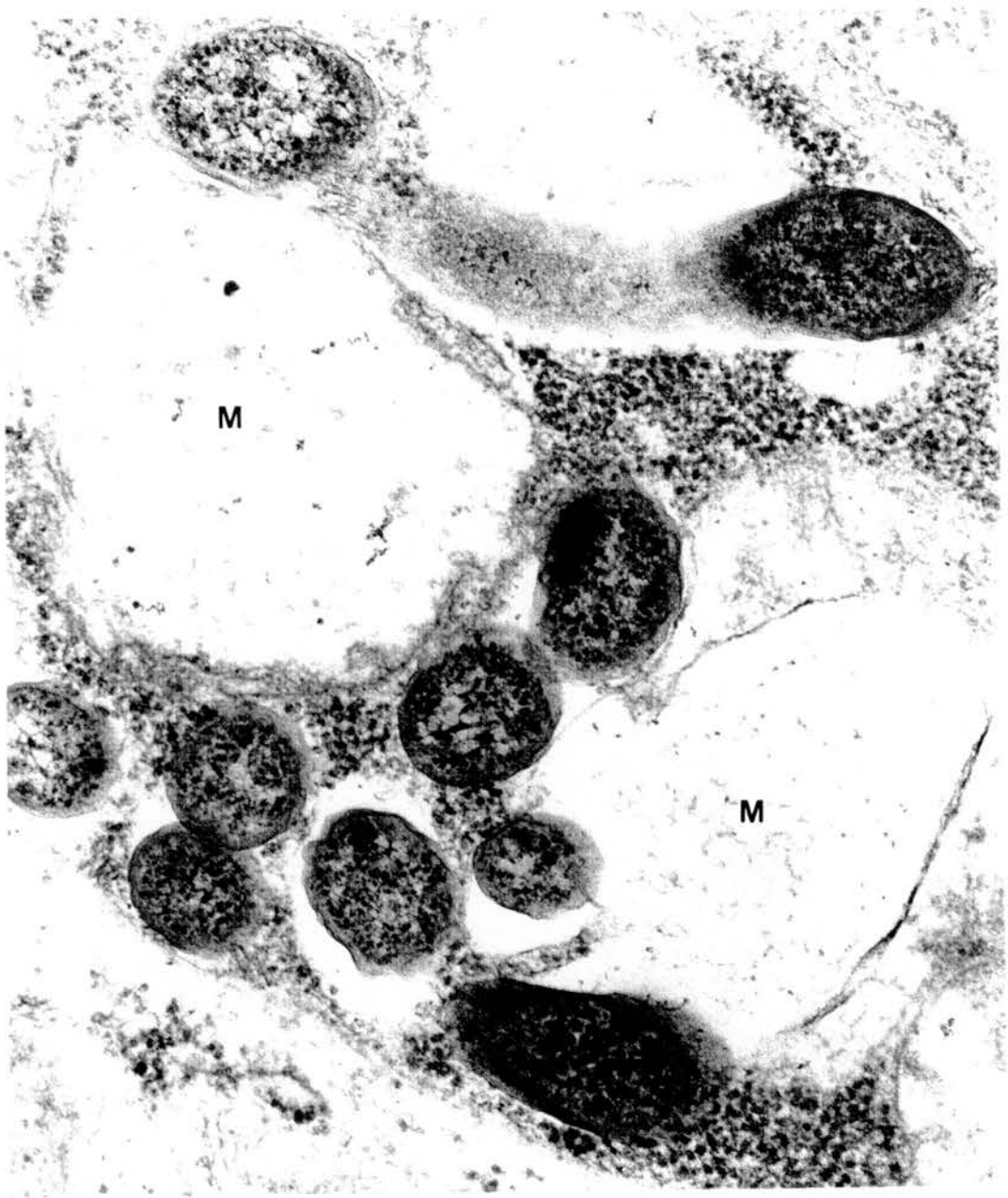


Figure 92

Many bacteria associated with distended mitochondria and rough endoplasmic reticulum. Note a bacterium with thickened outer membrane (arrow) free in the cytoplasm and close to a rough endoplasmic reticulum. Lead citrate and uranyl acetate stain x 65680.

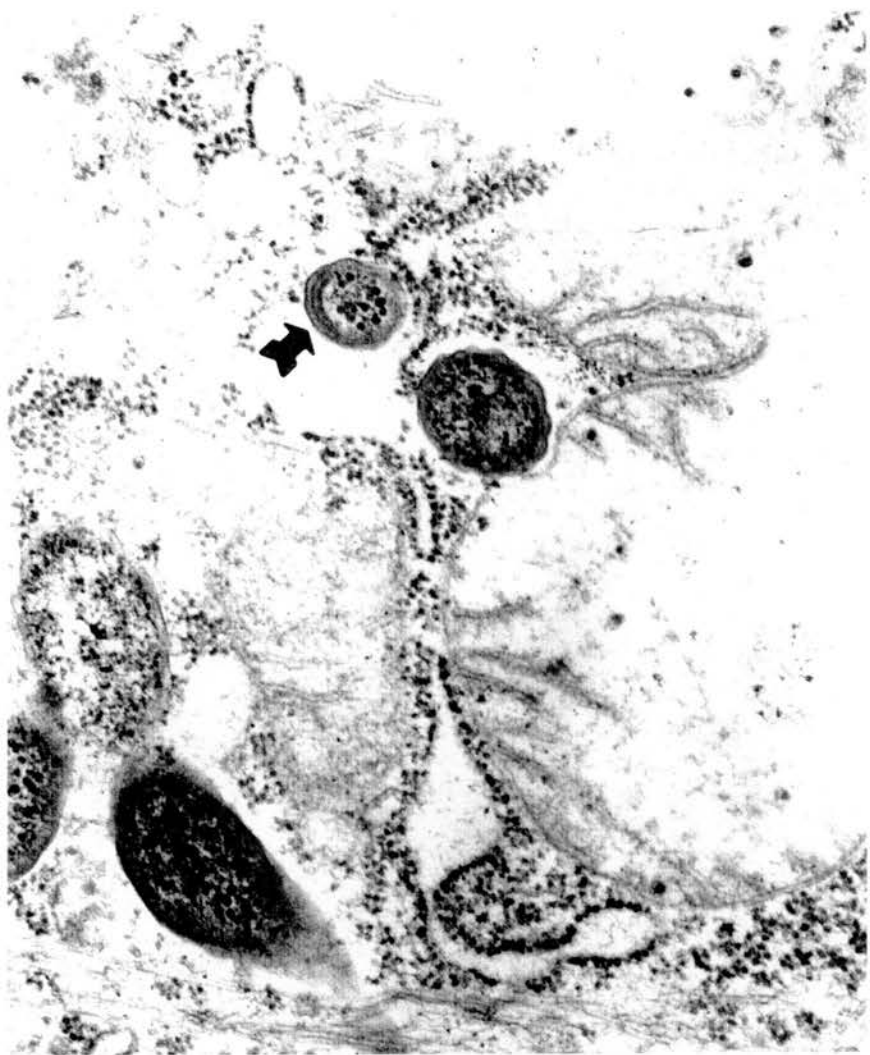


Figure 93

Bacteria with thickened outer membranes free in the cytoplasm and close to a rough endoplasmic reticulum of an epithelial cell at higher magnification. Lead citrate and uranyl acetate stain x 173750.

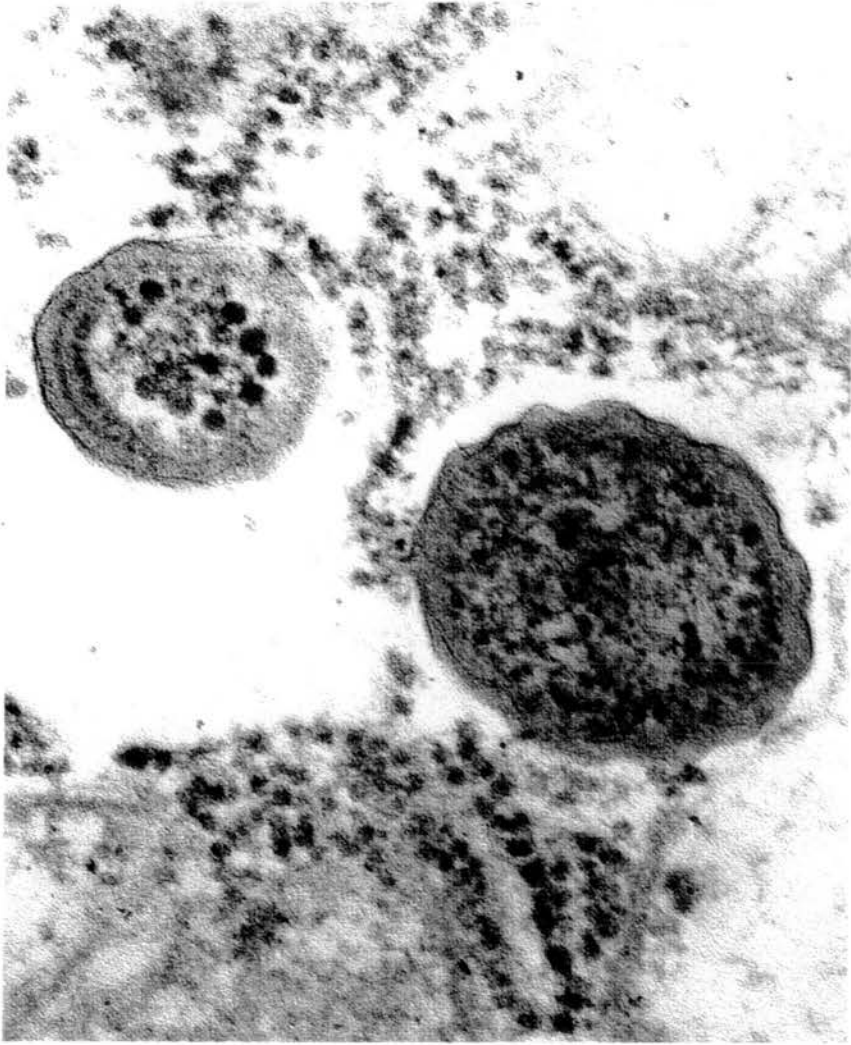


Figure 94

Rupture of an enterocyte showing release of bacteria and cellular debris into the crypt lumen. Lead citrate and uranyl acetate stain x 15000.



Figure 95

Released extracellular bacteria at higher magnification. Note disrupted cytoplasmic debris adjacent to the bacteria. Lead citrate and uranyl acetate stain. x 99600.

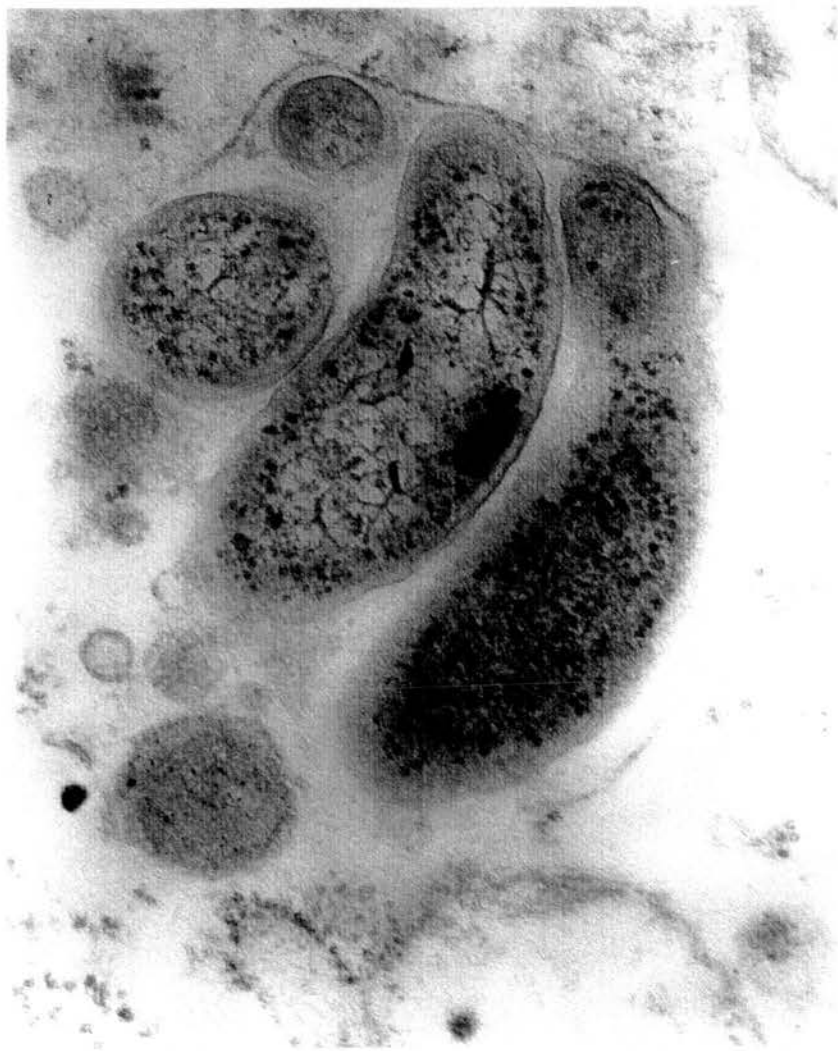


Figure 96

Apoptotic cells showing numerous intracellular bacteria. Note electron-dense cytoplasmic matrix of apoptotic cells. Lead citrate and uranyl acetate stain x 71000.

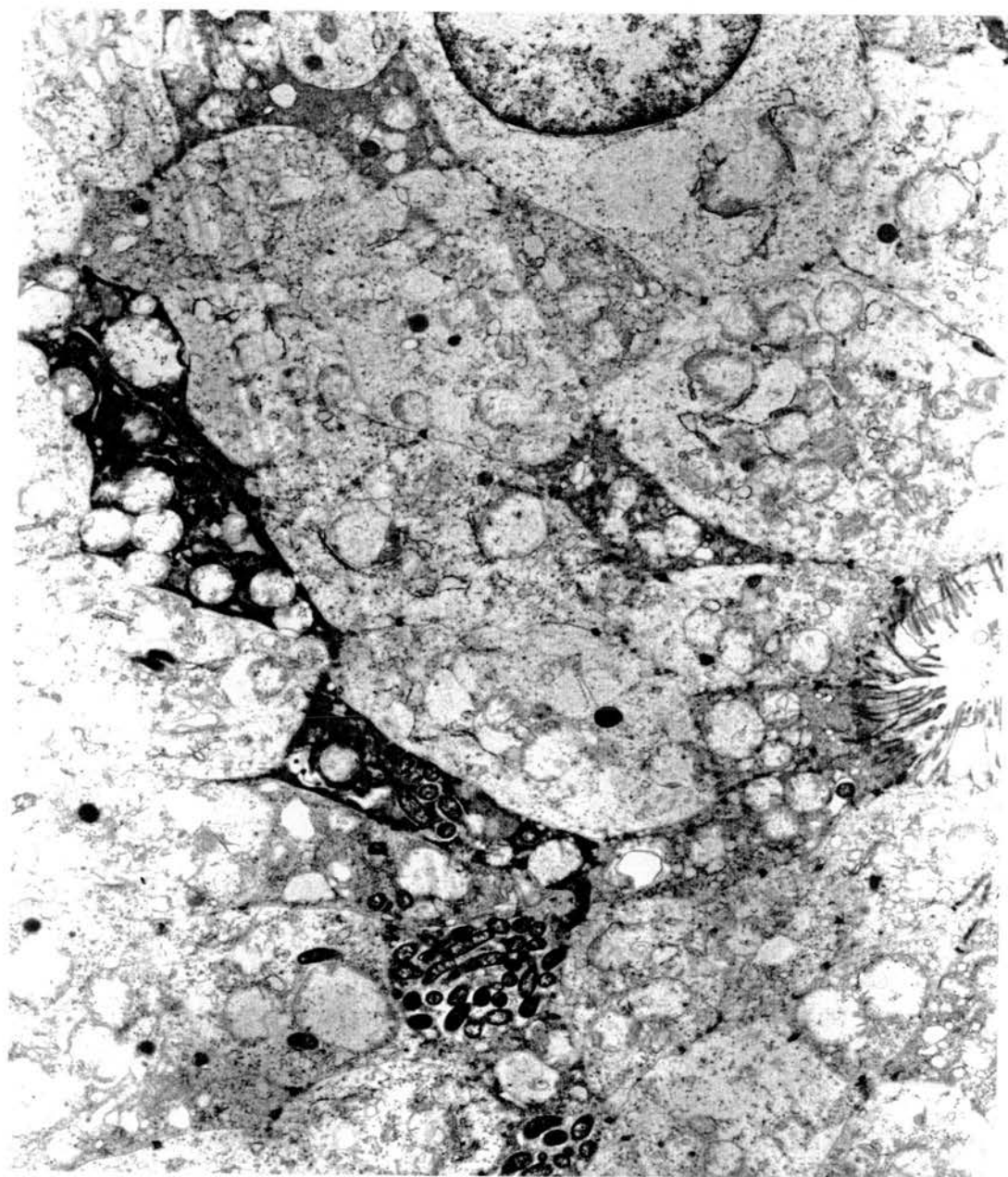
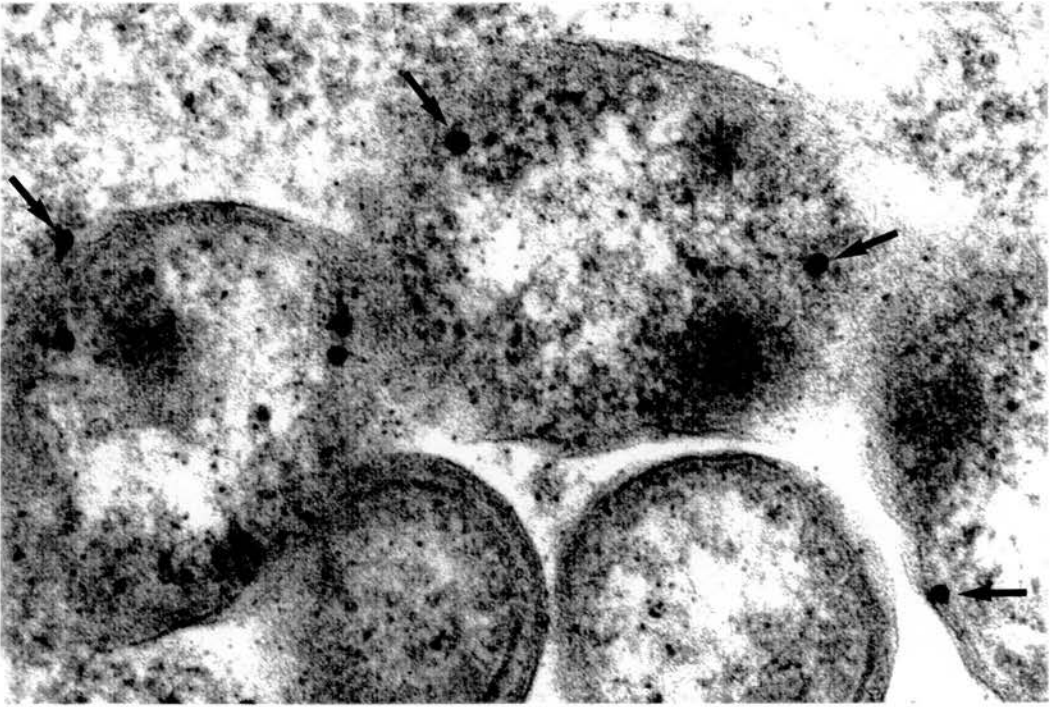


Figure 97

Intracellular bacteria showing a few 15nm gold particles on the outer membrane (arrows). Immunogold staining with monoclonal antibody to the intracellular bacteria and goat anti-mouse gold conjugate. Lead citrate and uranyl acetate stain x 197890.



(Chapter 4). Infected cell cultures did not reveal any viral-induced cytopathic effects.

7.4 Comment

The disease proliferative enteritis can be reproduced in hamsters when infected with the intracellular bacteria grown in cell culture derived from pigs with PE. Several factors may contribute to this success (see below). The pathogenesis of the disease has been revealed for the first time.

7.5 Discussion

Weanling hamsters proved to be susceptible to infection by I. intracellularis grown in cell culture derived from porcine proliferative enteropathy tissue. The severity of the disease which appeared in dosed hamsters was relatively mild but was apparently histologically identical to the naturally occurring and experimentally-induced disease in pigs and hamsters (Jacoby and Johnson, 1981; McOrist and Lawson, 1987; Rowland and Lawson, 1992).

Only hamsters given passaged 916/91 inocula in groups 1,2 and 3 developed lesions of PE. The failure of the hamsters given inocula 1482/89 to develop the disease may suggest that only particular strains are capable of infection and initiation of proliferative responses in the intestines of experimentally challenged hamsters. Ileobacter derived from different hosts of origin may differ in the ability to produce the disease. Ileobacter derived from pigs cannot be passaged in hamsters (Gebhart, 1987). However, passage of infection of pig organisms in pigs has been shown to be successful (Mapother et al., 1987b). Passage of infection may reduce virulence and hence pathogenicity of Ileobacter. The severity of the disease may also be influenced by a number of other factors, including the number of infective bacteria in the inocula. Inoculum 1482/89 contained few bacteria in comparison to inoculum 916/91. A high concentration of the intracellular organism is known to be required to elicit a successful reproduction of PE in hamsters (Frisk and Wagner, 1977b). Another possible explanation is that the organism may occur in different infective forms at different stages of development and only the infective form

is capable of crypt cell infection. The rickettsial bacterium, C. ruminantium exists in three different forms, only two of which are infective (Jongenjan et al., 1991). The inability of frozen inocula to initiate the lesions may be attributed to the reduction of infective titre by freeze thawing.

Some workers have postulated the possible role of diet in the reproduction of PE (McOrist and Lawson, 1987; Jacoby and Johnson, 1981). The severity of murine colonic hyperplasia caused by, Citrobacter freundii differs significantly under the influence of different dietary formulations (Barthold, Osbaldiston and Jonas, 1977). Certain diets may provide substrates that allow Ileobacter to accumulate and later utilise energy reserves which enable the bacteria to survive in the intestinal lumen before initiating infection (Chapter 4). The type of diet may also provide a suitable environment for infection to occur possibly by disrupting the defence mechanisms of the mucosal surface. C. freundii, however, differs from Ileobacter in that the former induces hyperplasia in the colon by attachment without intracellular colonisation of

the colonic enterocytes (Barthold et al., 1977).

Diets may influence the intestinal flora, successful infection of enterocytes by *Ileobacter* may require the synergistic presence of an intestinal microflora of the hamsters. Gnotobiotic hamsters are not commercially available to assess this phenomenon. The microflora may be involved in *Ileobacter* pathogenicity in a variety of ways, these include reduction of host resistance to infection, environmentally induced alteration of *Ileobacter* virulence or provision of a suitable environment that facilitates surface colonisation prior to infection. Successful reproduction of swine dysentery by *Serpulina hyodysenteriae* in pigs has been shown to require the synergistic presence of the intestinal microflora (Harris and Glock, 1981).

Numerous intracellular curved bacterial forms were observed in the cytoplasm of hyperplastic crypt cells and in adjacent crypt lumina. The intestinal and crypt lumina are known to be colonised by numerous bacteria which include curved or spiral microorganisms (Lee, O'Rourke, Barrington and Trust, 1986). The definite relationship of

curved bacterial forms within crypt lumina and adjacent cells in affected hamsters is still not clear and these experiments although supporting earlier evidence leave uncertainty over the identity of the luminal bacteria. However, the experimental evidence strongly support the opinion that the organism responsible for the reproduction of PE in hamsters in the present study was I. intracellularis.

Immunological staining with specific monoclonal antibody IG4 identified the organism in the lesions. Cell culture inocula used to dose the hamsters are free of any Campylobacter spp. and other bacteria at all stages. Specific immunolabelling of infected cell cultures for Chlamydia failed to identify such bacteria (Chapter 4). Ultrastructural examination of infected hamsters' enterocytes also did not show the presence of Chlamydia or viruses. Observation of infected cell cultures failed to show viral type cytopathic effects. Although C. jejuni was isolated from many control and infected hamsters and formed colonies in the crypt lumina of the intestines with associated acute intestinal inflammation, there is no

indication of any significant relationship of this organism to the proliferative lesions. If C. jejuni were involved in entry and production of proliferative lesions, such events would have similarly been observed in control hamsters since this organism were also cultured from these animals. C. jejuni was present in control and infected hamsters, similar findings have been reported in previous experimental studies (Regina and Lonigro, 1982; McOrist and Lawson, 1987; Stills and Hook, 1989), this confirms that the intracellular bacteria are distinct from C. jejuni. C. jejuni forms part of the intestinal microflora of hamsters; and is often detected in the intestines of healthy hamsters (Fox, Zanotti and Jordan, 1981). Lesions of PE were only produced in hamsters infected with cell culture inocula believed to contain a single agent, I. intracellularis.

A sequence of events of enterocyte infection by Ileobacter was observed in this study. Examination of the brush border of affected and adjacent crypt epithelial cells indicated the method of attachment and entry of these bacteria from the crypt lumen into the cells. This study has shown that pig derived I. intracellularis enters

hamsters crypt epithelial cells from the crypt lumen by attachment to the microvillus brush border, followed by ingestion, singly in endocytic vacuoles, and final release into the cytoplasm. Released bacteria then multiply in the cytoplasm. This work is consistent with previous studies of the early lesions of proliferative enteritis in pigs and hamsters which indicated a similar pathogenesis (McOrist et al., 1989), but which did not involve lesions experimentally induced with a single cultured agent. Detailed examination of the exact method of entry into the cell and the pathogenesis inside the cell have not been previously explored. Attachment and entry of the bacteria into the epithelial cells results in microvillus and terminal web disruption. In a few instances, *Ileobacter* that have entered crypt cells appeared to lie adjacent to normal microvillus brush border suggesting that rapid reformation had occurred or that such observations are a reflection of the plane of section. An in vitro study conducted by a group of workers has shown that microvilli reform rapidly 5 minutes after experimentally induced destruction (Goligorsky, Menton and Hruska, 1986).

Bacteria that were observed entering the enterocytes and those especially below the terminal web were observed with electron-dense or pale cytoplasmic granules. These bacterial structures have been observed in *Ileobacter* in cell culture lysates (Chapter 4). *Ileobacter* attaching to the cell membrane of IEC-18 cells in centrifuged and non-centrifuged infections were also observed exhibiting this morphology (Chapters 5 and 6). Attachment or entry of the bacteria is believed to require the expenditure of energy which may be reflected in this morphological change. The cytoplasmic granules (Shively, 1974; Scanlan, 1988) which are the bacterial energy reserves may play a role either in extracellular survival or may also be utilised by *Ileobacter* during entry. If the latter explanation is true, it would suggest that only viable bacteria can enter intestinal non-professional phagocytic cells, however, the presence of bacterial granules in organisms at the cell surface suggests that energy expenditure had preceded entry.

The hamsters in this study were examined 3 weeks after inoculation. Initial bacterial entry in PE is believed to

occur as early as 5 days post-infection (Johnson and Jacoby, 1978), with the onset of hyperplasia occurring shortly after entry, being clearly visible 7 and 10 days post-infection (Johnson and Jacoby, 1978; Mcorist et al., 1989; Jones, Ward, Murtaugh, Lin and Gebhart, 1993b). Bacterial release and host cell reinfection is a common feature in the life-cycles of other obligate intracellular bacteria (Doughri et al., 1972; Moulder, 1985; Prozesky and Du Plessis, 1987). Bacteria seen entering crypt cells in this study may have originated from other infected crypts which had completed their life-cycle and released bacteria.

Although attachment and bacterial entry into cells was observed, specific immunolabelling of the organism with monoclonal antibody IG4 appeared to be site specific; only those that were present in the cytoplasm were specifically labelled. In contrast, both extracellular and intracellular bacteria were labelled in in vitro, centrifuged and non-centrifuged IEC-18 cells infections (Chapters 5 and 6). This leads to the conclusion that *Ileobacter* could undergo antigenic change (variation) in vivo which may be influenced by different environmental

conditions. Similar findings have been observed by McOrist et al. (1989) in gnotobiotic pigs and conventional hamsters infected with the bacteria directly extracted from the lesions of PE in pigs.

Antigenic variation is defined as the ability of a single strain of a microbe to alter the antigenic character of its surface components. This involves rapid on and off switching of bacterial genes expression associated with adaptation of the bacteria to various environments (Seifert and Magdalene, 1988). Different environments are believed to have different "environmental signals" (eg. iron, temperature, pH, calcium) which regulate the expression of bacterial genes. It was suggested by Mekalanos (1992) that the expression of virulence determinants in bacterial interaction with a host cell (adherence, invasion and resistance to host defence mechanisms) depend on "environmental signals" that regulates the expression of genes controlling the virulence determinant (Mekalanos, 1992). The two systems of infection are different; one is the intact living animal and the other, cells in continuous culture. The environment in the natural host is

competitive unlike that of cultured cells. The burden upon the pathogen that follows the alimentary route is considered substantial. The organism must tolerate life outside the host for variable periods of time prior to infection, after ingestion, the bacteria are exposed to higher temperatures, extremes of pH, digestive enzymes, bile salts, different nutrients, peristaltic movements, the epithelial mucus barrier and competitive commensal bacterial populations. Different environments in the two systems of infection may influence the type of signals present and therefore initiate the alteration of antigens in *Ileobacter*.

Antigenic change can be a reversible event; *Ileobacter* express the 25-27k antigen when present in the intracellular location but antigenic expression may be reduced or altered when the bacteria are exposed to the extracellular environment. It is also possible that the site specific labelling of *Ileobacter* in the hamsters is an artefact of fixation or the preparative techniques for electron microscopy. However, this is less likely because all samples were treated equally and immunolabeling of the

bacteria in the intracellular location was light but consistent. Other possible explanation is that the bacteria in the crypt lumina including those attached and entering the enterocytes are not *Ileobacter*, and as such are not labelled.

Entry into endocytic vacuoles is followed by escape of the bacteria free into the cytoplasm which may be preceded by limited vacuolar multiplication. Endocytic vacuoles containing several bacteria, some of which were seen to coalesce to form larger endocytic vacuoles were noted occasionally. The numbers of bacteria in the endocytic vacuoles suggests that multiplication of the bacteria in this intracellular compartment had occurred. Entry vacuoles containing single or several bacteria showed vacuolar membrane breakdown. Bacteria may therefore produce a membrane lytic agent that mediates escape from the vacuole and the lytic activity may also result in cytoplasmic disruption.

The presence of several bacteria in endocytic vacuoles is not a recognised feature of the established disease in

pigs or hamsters (Johnson and Jacoby, 1978; McOrist et al., (1989). I. intracellularis may have evolved more than one adaptive mechanism of survival in the intracellular environment (Moulder, 1985). However, the presence of several bacteria in a vacuole may merely reflect a difference between the ability of pig-derived bacteria to completely initiate the disease in hamsters. The significance of electron-dense material in some endocytic vacuoles is discussed in Chapter 8.

Some bacteria in the cytoplasm were observed with a thickened outer membrane when close to the mitochondria and rough endoplasmic reticulum. The close association of bacteria with the cytoplasmic matrix, mitochondria and the rough endoplasmic reticulum could facilitate the direct transfer of nutrients and may indicate an inability of these organisms to synthesize ATP and an adaptation to the use of host ATP. Membrane modification in *Ileobacter* may aid in energy transfer from the host cell. Chlamydia are unable to synthesize high energy compounds like ATP and therefore rely on the host cell to obtain this essential metabolic requirement (Weiss and Wilson, 1969; Hatch,

1975). Ethidium bromide which blocks mitochondrial RNA synthesis of cultured human amnion cells prevents multiplication of Chlamydia (Becker and Asher, 1972). Because mitochondria are numerous and located in the apical cytoplasm it is difficult to prove this association, perhaps the preferred location of *Ileobacter* in the apical cytoplasm emphasizes the same point.

The mechanisms that underlie hyperplasia are unknown. It is believed that the lesions produced are both a consequence and necessity of infection. Intracellular parasitism in PE is always associated with hyperplastic lesions and crypt epithelial cells that are hyperplastic fail to differentiate. There are many possible explanations. The bacteria may produce growth factors capable of stimulating mitotic activity or may render crypt epithelial cells receptive to the natural growth factors, such as epidermal growth factor (Playford, Woodman, Clark, Watanapa, Vesey, Deprez, Williamson and Calam, 1993; Poulsen, 1993). Epidermal growth factor is secreted into the gut lumen by the salivary glands, Brunner's glands of the duodenum and the small intestinal Paneth cells

(Poulsen, 1993). Receptors for this growth factor are present on the basolateral and intestinal microvillus membranes of intestinal epithelial cells (Thompson, 1988; Sheving, Shiurba and Nguyen, 1989) and release of the factors produces a local effect on growth regulation.

Continuous induction of crypt epithelial cell proliferation by *Ileobacter* will upset the normal balance between immature and mature enterocyte populations. A reduced mature population will result in failure of the normal feedback mechanism that controls crypt proliferation (Jacoby and Johnson, 1981).

The possible S-layers were not observed in *Ileobacter* in infected hamsters in comparison to in vitro infections. Specific immunolabelling with the monoclonal antibody showed gold particles on S-layers of extracellular and intracellular bacteria in in vitro infection. Gold particles were only visualised on the outer membrane of intracellular *Ileobacter* in affected hamsters. The sample preparation techniques could have influenced the visualisation of this layer (Chapters 5 and 6).

This study has shown that I. intracellularis derived from pigs is pathogenic in hamsters and capable of reproducing the characteristic PE lesions on its own and therefore this work largely clarifies the aetiology of the disease.

CHAPTER 8
GENERAL DISCUSSION

GENERAL DISCUSSION

8.1 Introduction

It has been almost 63 years since porcine proliferative enteropathy was first reported (Biestler and Schwarte, 1931); until today the most important question has remained the aetiology of the disease. This study has provided a substantial insight of the aetiology and pathogenesis of the disease both in an in vitro and in vivo model of infection.

In recent years, evidence has accumulated to suggest that the cultivated Campylobacter spp. are not the cause of the disease. There has been renewed interest following the suggestion that another microorganism which at that time had not yet been cultivated (Lawson and McOrist, 1993) and designated as I. intracellularis (Gebhart et al., 1993) was a possible candidate agent of the disease. However, before the aetiological significance of this organism could be determined, cultivation of this agent in a cell culture system was a first priority. Much of the early work concentrated on achieving bacterial multiplication in cell

culture. Techniques which allowed passage of infection and enhanced yields of bacteria remained to be established.

This study has shown that the intracellular bacteria can be grown and the infection maintained in the IEC-18 cell line. The attributes of a successful intracellular bacteria are the ability of the bacteria to attach and gain entry into a host cell, evade the host defence mechanism, multiply and then exit from a host cell in order to transfer the infection to another host (Moulder, 1985; Finlay and Falkow, 1989; Falkow, 1991). Cell culture has been shown to be an important laboratory model and of use in the investigation of the host-bacterial interaction. The present study was initiated to investigate the interaction of *Ileobacter* with the host cell using IEC-18 cells as an in vitro model of infection. The model has successfully elucidated for the first time, the morphological events in the pathogenesis of *Ileobacter* infection in intestinal epithelial cells in vitro.

Centrifugation has been shown to be an important method of assisting infection, capable of enhancing infection of

IEC-18 cells by the intracellular bacteria. Similar benefits have been observed in many in vitro infections by various microorganisms (Hughes, 1993). Ultrastructural observation demonstrated the rapid attachment and entry of the intracellular bacteria in IEC-18 cells, immediately after centrifugation.

Centrifugation was not essential to initiation of infection in IEC-18 cells and comparison was made of the morphological events of infection in both in vitro systems at different time intervals after infection. It was important to establish whether the morphological events in the two systems were similar and there is no necessity that the pathways of cell entry are identical under different experimental conditions. Many observations have suggested that centrifugation can modify the natural events of infection (Pearce and Ainsworth, 1981). Centrifugation may alter the properties of host cell membranes, overcome electrostatic repulsive forces, alter the functions of cells so that they cannot inhibit bacteria in the intracellular location or alternatively speed up the entry process and thus reduce the effect of antibiotic on the

intracellular bacteria. Entry therefore could be a specific or non-specific interaction involving or not the complementary interaction of bacteria-host cell ligands, in addition some of the entry processes may be associated with the participation of host microfilaments (Silverstein et al., 1977; Beachey, 1981). Minor ultrastructural differences of the entry phase were detected in comparisons between centrifuged and non-centrifuged in vitro infection and hamster infection. Therefore, it is uncertain whether the cellular events of centrifuged infections are comparable and can be regarded as a suitable model for comparison with in vivo infection.

Despite the reservations expressed above, ultrastructural observation showed a similar sequence of cellular events in both centrifuged and non-centrifuged infections. Although spontaneous interaction of the intracellular bacteria with the host cell is more likely to resemble the natural events of infection in the animal host it is still a highly artificial event. Successful ultrastructural observation of the cellular events in both types of infection, however, requires high organism to cell

infection levels, without which visualisation of the cellular events are not possible. The numbers of bacteria entering enterocytes in in vivo infection may not approach those required to make ultrastructural observation of in vitro infection possible. Additionally, IEC-18 cell lines in these experiments were not grown in circumstances which would encourage cell structural orientation.

Since the significance of *Ileobacter* in relation to the disease, proliferative enteropathy, was still unknown, hamsters were used as an in vitro model to test for the first time the pathogenicity of this intracellular bacteria. This experiment was based on the work by McOrist and Lawson (1987) who showed that hamsters were susceptible to the intracellular bacteria extracted directly from the lesions in pigs. At that time the intracellular bacteria had not yet been cultivated; the finding therefore could only be substantiated by immunological means and the crude inocula could have contained a multiplicity of microorganisms. Proof of pathogenicity of obligate intracellular bacteria demands infection of experimental animals with the bacteria grown in a cell associated

culture system shown to be free of other agents. This study has proven that hamsters are indeed susceptible to I. intracellularis, when infected with this organism grown in cultured cells, thus demonstrating that the intracellular bacteria derived from pigs with proliferative enteropathy are pathogenic in hamsters. This organism by itself was shown capable of producing cell proliferation characteristic of PE. Viral or Chlamydial agents were not detected either by cytopathic effect in cell culture, by ultrastructural examinations of cell lysates of Ileobacter infected IEC-18 cells, or at any stages of cell culture infection or in hamster infections. Specific immunological search for Chlamydial agents in IEC-18 cells infected with the intracellular bacteria purified from the lesions of PE proved negative. Cultural examination of IEC-18 cells infected with the intracellular bacteria in conventional media did not reveal any other bacteria; these results provide substantial evidence that infection employed a pure culture of the agent and support the opinion that it is solely responsible for the lesions produced.

The ultrastructural characteristics of this bacteria

were also studied and the morphology of the cell culture grown bacteria has for the first time been fully described. This include the first description of the presence of a possible S-layer in the intracellular bacteria.

In addition to the successful reproduction of the disease with morphological evidence of the expected lesions in hamsters, a comparison is now possible between the pathogenic events of infection of hamsters by this organism and of other intracellular bacteria in their hosts. These observations also form a basis for the comparison of the cellular events of infection observed in the in vitro cell model with that which occurs in the hamsters. It may also be useful to compare the results of infection of cultured cells by *Ileobacter* and other intracellular bacterial pathogens, such an analysis may provide evidence for the mechanisms involved in cell entry and bacterial proliferation.

8.2 Comparison of the ultrastructure of cell culture infection with *Ileobacter intracellularis* and other intracellular bacterial parasites

The main aim of host infection by intracellular bacteria is to reach the intracellular location of the host cell. The preferred intracellular location differs between intracellular bacteria. Life inside the host cell does not only provide the essential nutrients for growth and multiplication of the bacteria but protection from competitive microflora and defence mechanisms at the cell surface (McNabb and Tomasi, 1981; Moulder, 1985). However, life in the intracellular location is also not without dangers to microbial existence (Moulder, 1985). Different bacteria have evolved different mechanisms designed to allow bacterial proliferation in the intracellular environment and as a result of these differences have a diverse range of life-cycles. Intracellular bacteria either escape from the entry vacuole free into the cytoplasm thus avoid being killed by lysosomal fusion or remain in the entry vacuole throughout their life-cycle either by resistance to the results of lysosomal fusion or inhibiting the fusion of entry vacuoles with lysosomes. Without the ability to persist or multiply in the chosen location, intracellular bacteria are unable to produce disease.

The in vitro model of cell infection by *Ileobacter* developed in this study, has revealed that this organism, like many other intracellular bacteria, initiate infection by attachment and entry into endocytic vacuoles (Moulder, 1985; Williams and Vodkin, 1987). These events are generally termed endocytosis (Moulder, 1985). The organism then escapes from this intracellular compartment, possibly by lysing the membrane of the endocytic vacuole into the cell cytoplasm. Escape from the entry vacuole may be a prerequisite for bacterial multiplication. In the cytoplasm the bacteria have free access to the available nutrients either directly from the cytoplasm or possibly by associating with host cell organelles such as mitochondria. The outer membrane of the Chlamydial elementary body alters, during the change from this form into the reticulate form (Peterson and De La Maza, 1988). This alteration is believed to promote nutrient transfer. The observed modification of the bacterial outer membrane in *Ileobacter* which become thickened at certain stages of development may also facilitate transfer of nutrients from the cytoplasm. The energy derived from the host enables an important morphological event to occur, that is,

multiplication of the bacteria. *Ileobacter* may have also prepared for their multiplication free in the cytoplasm by inducing a close association with host organelles, mainly mitochondria. Similar types of association are seen in the Chlamydia where metabolically active reticulate forms are closely associated with the phagosomal membrane and mitochondria (Peterson and De La Maza, 1988).

This study has shown that *Ileobacter* have evolved a survival tactic which prevents death and destruction of the bacteria which occurs when lysosome fuses with the entry vacuole with subsequent release of the lethal lysosomal constituents; thus the bacteria are able to achieve their preferred intracellular location and multiply. Many other intracellular bacteria have a similar strategy for evading the defence mechanism of the host cell. Some *Rickettsia* spp., *S. flexneri* and *L. monocytogenes* are also found free and multiplying in the cytoplasm after escape from the entry vacuole. The effector mechanism for escape is the production of membrane damaging agents such as phospholipase or listeriolysin (Ewing et al., 1978; Sansonetti et al., 1986; Gaillard, Berche, Richard and

Sansonetti, 1987; Winkler and Turco, 1988). The morphological changes suggest that *Ileobacter* produce a membrane lytic enzyme similar in function to those described in *L. monocytogenes* and *S. flexneri*.

Other intracellular bacteria such as *Chlamydia spp.*, *Mycobacterium tuberculosis*, *C. burnetti*, *S. typhimurium* and *Y. enterocolitica* do not usually escape from the entry vacuole after host cell entry but multiply mainly in this intracellular location throughout their life-cycle. It is therefore obvious that this intracellular location can still provide the essential nutrients for these bacteria, possibly from the cytoplasm by diffusion through the vacuolar membrane.

Chlamydia spp. and *M. tuberculosis* have evolved the capability to avoid lysosomal killing by inhibiting the fusion of the entry vacuole with lysosomes. Such bacteria may produce fusion inhibiting factors or lack fusion promoting factors. It has been suggested that *M. tuberculosis* produces sulfatides or ammonia which blocks the ability of lysosomes to fuse with entry vacuoles

containing this organism (Goren, D'Arcy Hart, Young and Armstrong, 1976; Gordon, D'Arcy Hart and Young, 1980).

S. typhimurium, Y. enterocolitica and C. burnetti, unlike *Ileobacter* and other intracellular bacteria, readily allows lysosomes to fuse with their entry vacuole (phagolysosome) and are able to resist the bactericidal effect of this fusion (Carrol, Jackett, Aber and Lowrie, 1979; Hackstad and Williams, 1981; Finlay and Falkow, 1989). However, variations occur between intracellular bacteria that have evolved this method of intracellular survival. The acidic environment of the phagolysosome is required to trigger the multiplication of *Coxiella* (Hackstad and Williams, 1981) but not *Yersinia* or *Salmonella* (Finlay and Falkow, 1988).

Bacterial multiplication is followed by their exit (release) from the host cell and these events are usually associated with host cell lysis. Exit from the host cell is possibly a bacterial directed activity associated either with microbial produced lytic enzymes or due to intolerance to the mechanical and osmotic stress that results from

unrestricted bacterial multiplication (Moulder, 1985). Intracellular bacteria can either be released by cell lysis or by direct transfer to neighbouring cells; differences in the method of release exist between or within bacterial genera or species. Cell to cell and host to host transmission are made possible by bacteria which have been released and initiate a new cycle of infection.

The in vitro model of *Ileobacter* infection supports the view that transfer of infection occurs unexposed to the extracellular environment mainly via host cell division. *Ileobacter* multiply to large numbers to fill the cytoplasm and are apparently released by extrusion from degenerate cell cytoplasm that may protrude into spherical-shaped structures (cytoplasmic protrusion), or which may be pinched off from the cell surface of degenerate host cells. This release of *Ileobacter* from the host cell is considered to be non-specific. Heavily infected cells have altered membrane function and detach from the monolayer, whether the cell protrusions are a further manifestation of the same effect is at the present time not clear, but possible. Bacteria were seen in cells in mitosis and furthermore, the

dynamics of cell culture infection support the opinion that this is the main method of spread within infected monolayers. Infected foci of cells often remain surrounded by uninfected cells and prolonged incubation does not result in an increase in infected cells. The detachment of *Ileobacter* infected cells is possibly the in vitro event that mimics the natural event of apoptosis and cell shedding in vivo (Iwanaga, Han, Adachi and Fujita, 1993).

Chlamydia are released free or within intact entry vacuoles, also by extrusion from spherical-shaped cell cytoplasmic protrusion (De La Maza and Peterson, 1982; Todd and Caldwell, 1985; Schachter, 1988). Release of C. psittaci is associated with the release of host lysosomal enzyme and host cell destruction (Todd and Storz, 1975).

Rickettsia spp. are released either free (ie. R. rickettsii; Walker and Cain, 1980), enclosed in host membranes (ie. R. tsutsugamushi; Ewing et al., 1978; R. rickettsii; Silverman and Wisseman, 1979; Silverman, 1984), or within thin and long cell projections which may have distinct breaks at the distal end adjacent to bacteria and

closely opposed to the surface of adjacent cells (R. rickettsii; Schaechter, Bozeman and Smadel, 1957; Walker and Cain, 1980). It has been suggested that phospholipase plays a role during release of Rickettsias which result in cell lysis (Winkler and Miller, 1980, 1982). It has also been postulated that host cell cytoskeletal protein (ie. actin). assists transfer of R. rickettsii from one cell to the other (Heinzen, Hayes, Peacock and Hackstad, 1993).

L. monocytogenes and S. flexneri are released unexposed to the extracellular environment by remaining in the cell cytoplasm which elongates and project a cytoplasmic tube that penetrates deeply into adjacent cells and contains the bacteria within a double membrane. The bacteria then lyse the double layered vacuole membrane and escape free into the cytoplasm to begin a new cycle of infection. The process of transfer of these organism is aided by the cytoskeletal protein, actin, and possibly lysis of the vacuolar membrane by phospholipase (Goldberg and Sansonetti, 1993; Tilney and Tilney, 1993).

Transfer of infection is a necessary event since single

infected cells are unable to support the expanding bacterial population. Competition of bacteria with host cell for nutrients will occur, host cell nutrients are exploited to meet the demand of bacterial growth which deprives the host cells of their own basic needs for survival. Exit from the host cell is therefore believed to be a mechanism not only to disseminate infection but on occasion to preserve host cell function (Moulder, 1985). In order to survive, an obligate intracellular bacterium must either have a mechanism that arrests organism multiplication at certain critical bacterial numbers or alternatively readily transfer to adjacent cells or a new host.

Exit from the host cell is not without risk of exposure to host defence mechanisms. In addition to adapting to hostile life inside the cell, intracellular bacteria have also evolved a method of extracellular survival. Chlamydia structurally adapts to survive inter-host transit whilst *Ileobacter* may do so by their ability to accumulate and utilise energy reserves which are morphologically evident as cytoplasmic granules in the bacterial protoplasm (Dawes

and Senior, 1973; Shively, 1974; Scanlan, 1988). Only occasionally were membrane changes identified in *Ileobacter* released from host cells.

8.3 Comparison of pathogenesis of cell culture and hamsters infection

The pathogenic events of infection were observed when hamsters were orally dosed with the intracellular bacteria grown in cell culture. The pathogenic events were similar to those observed in cell culture infection.

Infection begins by attachment of the bacteria to the cell membrane of enterocytes at the crypt lumen of the intestine followed by single organism entry into endocytic vacuoles. Disruption of the membrane of the endocytic vacuole took place and bacteria escaped free into the cytoplasm. Neither infection showed marked alteration to the host cell membrane with the exception that some bacteria in centrifuged infection adhered by a type of electron-dense cap. Lysosomal fusion was absent indicating that the organism employs similar mechanisms of survival

from the bactericidal effect of this fusion. Similarly, in both systems numerous bacteria were found free in the cytoplasm identifying this as the preferred site for multiplication of the bacteria and that escape from the entry vacuole is a prerequisite for such events. It is believed that this move facilitates the transfer of nutrients from the cytoplasm. *Ileobacter* were also observed to exhibit thickened outer membranes in infected hamsters.

However, multiplication in endocytic vacuoles may also occur. Multiplication in and escape from this intracellular compartment, was a strikingly similar and rare feature in both cell culture (ie. non-centrifuge infection) and hamster infection. The ability to multiply and escape from endocytic vacuoles has never been reported in other intracellular bacteria which usually enter and escape singly from the endocytic vacuole.

Ground substance similar in morphology to the cytoplasmic matrix (in cell culture infection) and electron-dense material (in infected hamsters) was observed

in endocytic vacuoles containing several or single *Ileobacter*. The nature of this material is unknown. It may be suggested that the enzyme responsible for bacterial release is not highly biologically active in that cell membranes remain undamaged until heavily parasitised; bacterial phagosomal proliferation may represent those instances where there is delay in synthesis or release of such enzymes. Electron-dense material has been observed adjacent to endocytic vacuoles containing *Cowdria ruminantium* and it has been suggested by Jongenjan et al. (1991) as a mechanism which blocks lysosomal fusion. *C. ruminantium*, is a bacterial parasite which multiplies in endocytic vacuoles throughout its life-cycle and survives by preventing the fusion of entry vacuoles with lysosomes. While rapid escape from endocytic vacuoles allows ample time for *Ileobacter* to escape into the cytoplasm before lysosomal fusion could occur, the bacteria may also produce an factors inhibitory to lysosomal fusion, thus are not killed and are able to multiply before escape into the cytoplasm.

Multiplication of the intracellular bacteria in the

hamsters crypt enterocytes results in hyperplasia of these cells and the infection is believed to be transferred by host cell division analogous to that which occur in vitro. This is a survival tactic that the bacteria have evolved allowing them to remain within the host cell to obtain nutrients, whilst at the same permitting spread of infection within the dividing host cell. However, no clear evidence of infected cell proliferation has been obtained from the in vitro model of infection. The proportion of infected cells increases as infection progresses suggesting that these cells are dividing. Extended periods of incubation of infected cells results in loss of infected cells into the supernatant fluid which confuses assessment. There is no general proliferation of cells in infected culture (Lawson et al., 1993).

While transfer of infection takes advantage of host cell division, release of bacteria via extrusion from degenerate enterocytes is also observed in infected hamsters. Highly infected cells burst open to release bacteria into the intestinal lumen. Cell to cell or host to host transmission may occur via organisms released by

this method. The in vitro model of Ileobacter release is closely similar to that observed in infected hamsters.

Intracellular bacteria must be able to exist in the extracellular environment. This may require the ability to accumulate and utilise energy reserves during this phase of infection. Bacteria in the faeces of infected animals are likely to be the major source of infection (Jones, Ward, Gebhart, Murtaugh, and Collins, 1993a; Jones et al., 1993b; Jones, Ward, Murtaugh, Rose and Gebhart, 1993c); such released extracellular bacteria may possess special features that allow transit between hosts.

From observations of the disease in the hamsters and cell culture, it is possible to suggest the important pathogenic events of the disease (Fig. 98).

8.4 General conclusion

This study has shown that the intracellular bacteria of PE can be cultured and passaged in the IEC-18 cell line and that the in vitro model of infection is a relevant model of

Fig. 98 Pathogenesis of porcine proliferative enteropathy

SOURCE OF INFECTION

I. intracellularis in the faeces



TRANSMISSION OF INFECTION

faecal-oral-route



EXTRACELLULAR PHASE OF INFECTION

- I. Bacteria in intestinal lumen
- II. Bacteria in crypt lumen
- III. Attachment to crypt enterocytes



INTRACELLULAR PHASE OF INFECTION

- I. Entry into endocytic vacuole
- II. Escape from endocytic vacuole
- III. Multiplication free in the
cell cytoplasm
- IV. Spread through the epithelium
by dividing cells
- V. Infected cells continue

to divide



SHEDDING OF INFECTION

- I. Release of infected cells, extrusion
- II. Extrusion of bacteria from cytoplasm

in vivo infection, in hamsters. Both models show similar life-cycles and stages in the pathogenesis of infection.

8.5 Future research

An understanding of the many aspects of bacterial parasite interaction with the host cell are not an "overnight achievement" but involves years of research. The study of Chlamydia is an excellent example; much of the pioneering work of the study of intracellular parasitism concentrated on the cultivation and establishment of cell associated infection systems, observation of bacterial morphology, fundamental understanding of cellular events of infection, observation of the disease in the natural host and comparison of in vitro and in vivo systems of infection (Storz, 1971). These early studies provided an outline of the mechanisms involved in Chlamydial multiplication and were a great impetus for further research (Moulder, 1991).

The study of intracellular infection by *Ileobacter*, have been initiated and successfully achieved. Along with these achievements, this study has provided a sound

foundation for further cell culture research and certainly, the most important of all it has established for the first time the pathogenicity of the organism derived from pigs.

Attention should be directed towards reproduction of the disease in the natural host with pig-derived intracellular bacteria obtained from infected cell culture. This has been done and the disease has now been reproduced both in conventional health status and gnotobiotic pigs with a defined bacterial flora (McOrist et al., 1993; McOrist, Mackie, Neef, Aitken and Lawson, 1994).

In this work, the lesions of PE reproduced in hamsters were observed at one point in time (day 21 post-infection), further investigation of the pathogenesis of the disease should be attempted. Hamsters are normally observed with lesions at this time after exposure to infection (McOrist and Lawson, 1987; McOrist et al., 1989). In this first attempt to reproduce the disease with cell culture derived bacteria, necropsy was therefore carried out at the point in time that lesions were likely to be maximal.

Bacterial entry was observed in this study and is believed to be re-entry of bacteria that had been released by extrusion from other infected cells. Some workers have reported intracellular bacteria in the cytoplasm of hamster enterocytes as early as five days following exposure to infection with diseased mucosa. Lesions could only be visualised five days later at which time bacteria were absent from the cell surface (Johnson and Jacoby, 1978). The presence of entering and intracellular bacteria in proliferating adjacent cells suggests that the former bacteria were derived from bacteria extruded or released, whether locally or elsewhere in the intestine is not clear.

Study of the experimental disease in pigs has mainly observed intracellular bacteria in developed lesions (McOrist et al., 1989). The chance of observing the events of entry is limited as it is transient and after entry into the cells there no longer remains a bacterial surface population. Moreover, escape of the bacteria free into the cytoplasm from the entry vacuole has been shown to be rapid.

Further study should include the study of the development of lesions by analysing infection at different time intervals. However, this study is not likely to be without difficulty as early entry may not be associated with bacterial multiplication or the development of lesions. The presence of bacteria could possibly be assessed by screening using conventional staining or more likely immunological assays to detect areas of intestines for ultrastructural examination. Application of similar studies to the pathogenesis of the disease in pigs could then be developed.

The intracellular bacteria may also be capable of producing lesions in the stomach. In future in vivo experiments, detailed analysis for the presence of lesions should include the stomach as well as the traditional intestinal sites and samples taken. Rationale of this is the first report of the presence of intracellular bacteria in proliferating gastric epithelial cells in a dog (Leblanc, Fox, Le Net, Masson and Picard, 1993). The gastric mucosa showed thickening which appeared closely similar to the lesions normally seen in the intestines of

pigs and other animals with PE (Rowland and Lawson, 1992). More interestingly, the bacteria reacted with the specific monoclonal antibody developed against pig-derived *Ileobacter*. Leblanc and co-workers (1993), however, did not observe lesions of PE in the dog's intestines. Examination of samples from the stomach may yield information that can lead to further understanding of the pathogenesis of the disease.

It would be useful to define other aspects of the events of infection. The mechanism of entry of the organism could be investigated in an in vitro model. Morphologically identical events of infection do not necessarily indicate an identical mechanism of entry. The use of drugs can determine the mechanism of entry. Cytochalasins B and D disrupt host cell microfilaments and blocks bacterial entry, results of the effect of such drugs may therefore suggest microfilament-dependant entry mechanism. Entry which can be prevented by drugs such as monodansylcadaverine and amantadine (Ward and Murray, 1984) suggest receptor mediated endocytosis which is a non-microfilament dependant entry mechanism (Silverstein et

al., 1977). The involvement of the host cell microfilament could be proven by staining with a fluorescence probe such as phalloidin-FITC which enables direct visualisation of polymerised actin (Barak, Yocum, Nothnagel and Webb, 1980).

Such fluorescence staining could also allow investigation of the possibility of *Ileobacter* employing the host cytoskeletal system in intracellular movement as observed in *S. flexneri*, *L. monocytogenes* and some *Rickettsial spp.* (Silverstein et al., 1977; Bernardini et al., 1989; Tilney and Portnoy, 1989 ; Mounier, Coquis-Rondon and Sansonetti, 1990; Teyssere, Chiche-Portiche and Raoult, 1992; Heinzen et al., 1993).

Although this study has shown that *Ileobacter* escapes from entry vacuoles that do not fuse with host cell lysosomes, the identity of the entry vacuole (whether phagosome or phagolysosome) could be confirmed by staining with the specific lysosomal probe such as thorium dioxide (Kielian and Cohn, 1980; Kielian, Steinman and Cohn, 1982). The mechanism of *Ileobacter* escape from entry vacuole may be novel. The bacteria may allow fusion of lysosome with

entry vacuole, resist the lethal effect of this fusion and only then finally escape free into the cytoplasm. Confirmation of this postulate depends on the identity of the ground substance seen in some entry vacuoles containing the bacteria. *Ileobacter* which escape from such vacuoles are apparently viable.

Assessment of the dynamics of cell entry should utilise methods which differentiate between extracellular and intracellular bacteria possibly by double fluorescence assay pre and post-fixation of infected cells. Such technique would provide a better assessment of the location of bacteria around the time of entry of the bacteria into the cell. Enumeration by electron microscopy does not provide a satisfactory assessment of host cell infection rates, due to the limitation of ultrastructural examination, but adequately identifies the intracellular location of the bacteria. Despite, its limitation the technique has provided an excellent understanding of the ultrastructural details of bacterial-host cell interaction that cannot be resolved by conventional light microscopy.

The study of PE has been revolutionised by the use of cell culture and the study of intracellular parasitism. The present achievements are a starting point to further understanding of the pathogenesis and the causative agent of the disease.

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APPENDIX

Appendix A

I) Buffers/Washing solutions

0.1M sodium cacodylate buffer

Stock solution 0.2M

4.28g of sodium cacodylate buffer was added to 0.69ml of 1N hydrochloric acid and made up to 100ml of distilled water.

Working solution 0.1M

Stock solution was diluted 1:1 in distilled water and brought to pH 7.4.

Locke's balanced salt solution

9.0g of sodium chloride, 0.42g of potassium chloride, 0.24g of calcium chloride and 0.20g of sodium hydrogen carbonate per litre of double deionised water. Sterilised by autoclaving at 15ibs pressure for 15 minutes.

Phosphate buffered saline

Stock solution - PBS x10

400.0g of sodium chloride, 10.0g of potassium chloride, 10.0g of disodium hydrogen orthophosphate and 57.50g of potassium dihydrogen orthophosphate per 5 liter of double deionised water. Sterilised by autoclaving at 15ibs pressure for 15 minutes.

Working solution - PBS x1

1 part of PBS x10 diluted in 9 parts of double deionised water and brought to pH 7.2.

Phosphate buffered saline with azide

Stock solution - PBS x10 with azide

32.50g of sodium azide was added to PBS x 10 (stock solution) and mixed in a fume cupboard.

Working solution - PBS x 1 with azide

One part of PBS x 10 with azide diluted in 9 parts of double deionised water and brought to pH 7.2.

Immunoperoxidase buffer

0.08% vol/vol Tween 80 (Polyoxyethylene sorbitan mono oleate, Sigma, U.K.) and 0.1% (wt/vol) Bovine serum albumin in PBS x1.

3,3 Diaminobenzidine tetrahydrochloride (DAB) solution

DAB tablet (10mg), Sigma, U.K., was placed in 20ml of PBS x1 and 40ul of hydrogen peroxide added.

Tris-casein buffer

0.242g of tris-hydroxymethyl methylamine, 0.13g of sodium nitrite, 0.90g of sodium chloride and 0.1g casein per 100ml of distilled water, filter sterilised (0.65um) (Millipore, U.K.) and brought to pH 8.2.

Young's buffer

44.0ml of acetic acid (0.2M) was added to 6.0ml acetate (0.2M), made up to 100ml with distilled water and brought to pH 3.8.

II) Media

Skirrow's medium

40g of Blood agar base no.2 (Oxoid CM271) was suspended in

one litre of distilled water, dissolved by boiling and autoclaved at 15lbs pressure for 15 minutes. The agar was allowed to cool and 8% lysed horse blood, 3% nystatin (10 000units/ml) (Sigma, U.K.) and 1% Skirrow's growth supplement and antibiotics (Gibco, U.K.) was added and mixed before pouring into plates.

Brucella semi-solid broth

2.8g wt/vol Brucella broth (Difco 0495), 0.3% (wt/vol) yeast extract (L-21), 0.15% (wt/vol) Bacteriological agar (Oxoid L33) and 1% (vol/vol) FBP supplement and autoclaved at 15lbs pressure for 15 minutes. FBP supplement is 5.0g ferrous sulphate, 5.0g sodium pyruvate, 5.0g sodium metabisulphate in 100ml distilled water.

Columbia blood agar

Columbia agar base (Oxoid CM 331) with 7% defibrinated horse blood prepared as for Skirrow's medium but without supplements.

PUBLICATIONS ARISING FROM WORK IN THIS THESIS

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- 1) Lawson, G.H.K., McOrist, S. Jasni, S. and Mackie, R.A. (1993). Intracellular bacteria of porcine proliferative enteropathy: Cultivation and maintenance in vitro. J. Clin. Microbiol. 31: 1136-1142.
- 2) S.Jasni, Lawson, G.H.K., McOrist, S. and Mackie, R.A. (1993). Culture and investigation of the intracellular bacteria from porcine proliferative enteropathy. Assoc. Vet. Teachers and Research Workers Meeting, Scarborough, England. Abstract. pp 5.
- 3) S.Jasni, McOrist, S. and G.H.K. Lawson (1994). Experimentally induced proliferative enteritis in hamsters: an ultrastructural study. Res. Vet. Sci. 56: 186-192.
- 4) S. Jasni, McOrist, S. and G.H.K. Lawson (1994). Reproduction of proliferative enteritis in hamsters with a pure culture of porcine-derived Ileal symbiont Intracellularis. Vet. Microbiol. In press.
- 5) S. Jasni, Lawson, G.H.K., McOrist, S. and Mackie, R.A. Life-cycle of lleal Symbiont Intracellularis in cultured rat enterocytes. J. Gen. Microbiol. In preparation.

Experimentally induced proliferative enteritis in hamsters: an ultrastructural study

S. JASNI, S. McORIST, G. H. K. LAWSON, *Department of Veterinary Pathology, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Veterinary Field Station, Easter Bush, Midlothian EH25 9RG*

Hamsters, three weeks old, were dosed orally with suspensions of intracellular bacteria grown in rat enterocyte tissue culture cells IEC-18, which had been infected with suspensions of intracellular bacteria derived from the lesions of proliferative haemorrhagic enteropathy occurring naturally in two pigs. Each bacterial strain, identified as Ileal symbiont intracellularis, was passaged in the cell lines once, twice or five times, collected with the cells and used as inocula. Ten of 16 hamsters dosed with 916/91 passaged one or five times developed lesions of proliferative enteritis. In these 10 hamsters, marked hyperplasia of ileal enterocytes associated with numerous intracellular curved bacteria was detected. An ultrastructural study of epithelial cells in the ileum of affected hamsters showed numerous intracellular bacteria in the cytoplasm. Similar bacteria were not seen in unaffected animals. Intracellular bacteria were usually seen in groups and could appear as electron dense or in a more electron lucent form. These bacteria were clearly seen to enter cells from the intestinal lumen, via endocytic vacuoles at the brush border. There was rapid breakdown of the entry vacuoles, leaving bacteria free in the cytoplasm where division was usually observed. These bacteria were often seen in close association with normal or distended mitochondria and rough endoplasmic reticulum.

PROLIFERATIVE enteropathy or enteritis presents as a similar clinical and pathological syndrome in pigs and hamsters, with hyperplasia of crypt epithelial cells, primarily in the ileum and colon (Rowland and Lawson 1974, Frisk and Wagner 1977). Natural disease in hamsters has not

been reported in the UK, only from the USA, whereas the condition in pigs occurs worldwide.

The disease in both species is consistently associated with the presence of intracellular curved bacteria in the cytoplasm of the hyperplastic crypt epithelial cells. The presence of these intracellular bacteria early in the course of natural and experimentally induced disease in pigs and hamsters suggests that they are involved in the aetiology (Frisk and Wagner 1977, Jacoby and Johnson 1981, McOrist et al 1989). *Campylobacter mucosalis*, *C. hyointestinalis* and *C. jejuni* isolated from pigs with proliferative enteropathy do not colonise the intestines of hamsters and do not cause any proliferative lesions in hamsters or pigs (Boosinger et al 1985, McOrist and Lawson 1987).

Immunological, cultural and DNA studies of the intracellular bacteria in the lesions in pigs have indicated that they are obligate intracellular bacteria belonging to a new taxonomic group (Lawson and McOrist 1993). Sequencing of the 16S rDNA portion of the genome of these intracellular bacteria, derived from lesions in pigs, established that this taxonomic group was distinct from all known bacteria and are currently designated ileal symbiont *intracellularis* (Gebhart et al 1993). The transmissibility of the disease in hamsters with cultures infected with bacteria derived from naturally affected hamsters has been confirmed recently (Stills 1991). In hamsters, ultrastructural study of the naturally occurring disease, and disease induced by oral dosing with homogenates of affected mucosa from natural disease in hamsters has indicated that most of the intracellular bacteria lie free in intestinal epithelial cell cytoplasm, with evident bacterial multiplication (Frisk and Wagner 1977, Johnson and Jacoby 1978). Similar findings

were reported in hamsters dosed with tissue cultures infected with bacteria derived from hamster lesions (Stills 1991), although the identity of the bacteria used in that study was not clear. Subsequent work indicated that the tissue cultures used had been contaminated by a *Chlamydia* species (Stills et al 1991).

Ultrastructural study of lesions in hamsters by oral dosing with homogenates of affected mucosa from natural disease in pigs again indicated intracellular bacteria free in the cytoplasm and some possible entry mechanisms (McOrist et al 1989). However, the exact method of entry into epithelial cells by the bacteria and possible pathogenesis inside the cell remain unexplored. This study illustrates the association of proliferative enteritis in hamsters dosed with identified intracellular bacteria of porcine origin grown in tissue culture.

Materials and methods

Hamsters

Hamsters (*Mesocricetus auratus*) were obtained from a closed colony which was naturally infected with *C jejuni*, maintained in a stable laboratory animal facility, and weaned and dosed at three weeks old. The control and inoculated hamsters were placed in separate cages after dosing.

Pig-derived bacteria

The initial bacteria used for inoculation of tissue culture cells were derived from the intestines of two pigs naturally affected with proliferative haemorrhagic enteropathy designated as 916/91 and 1482/89. The method of preparation of pure suspensions of intracellular bacteria from the lesions has been described previously (McOrist et al 1987). These initial bacterial suspensions were diluted, filtered and resuspended finally in sucrose potassium glutamate (Bovarnick et al 1950) containing 20 per cent v/v fetal calf serum in 1 ml vials. Prepared vials of both original cultures were stored frozen at -70°C for three to four months before they were used.

Inocula

IEC-18 cells (rat enterocytes), European cell culture collection number 88011801, were grown to a monolayer covering 20 to 30 per cent of 25 cm² plastic flasks in 24 hours in Dulbecco's

modification of Eagle's medium (DMEM) supplemented with L-glutamine, amphotericin (Fungizone; Squibb) and 10 per cent v/v fetal calf serum. Infection of these cells by intracellular bacteria derived from pig intestines is described in detail elsewhere (Lawson et al 1993).

Briefly, after rapidly thawing, a vial of pig-derived bacteria was added to 14 ml of warm DMEM with the supplements already described but with 7 per cent v/v fetal calf serum. The diluted suspension was then added to the IEC-18 cell monolayers. Infection of cells was assisted by centrifugation of the flasks at 2000 g for 30 minutes. The flasks were incubated microaerophilically (8 per cent oxygen, 8.8 per cent carbon dioxide, 82 per cent nitrogen) for three hours, then fed with the same media containing neomycin and vancomycin and further incubated. Infected cell monolayers were passaged on day 6 after inoculation by treatment with potassium chloride (Lawson et al 1993), followed by the removal of the cells in each flask by a cell scraper. The scraped cells were then ruptured by passage six times through a 19 G syringe needle before they were used to inoculate fresh monolayers of IEC-18 cells.

The new infected cells were grown for a further six days before being prepared for inoculating hamsters or being passaged again. Six and 10 hamsters were dosed with 916/91 inoculum of passages 1 and 5, respectively, and 14 hamsters received 1482/89 inoculum of passage 2. Cells from each passage used were removed from the flasks with a scraper, homogenised for 15 seconds, and the hamsters were dosed orally with 2 ml each via a 16 G blunt-ended needle.

The control hamsters were either not dosed ($n = 20$) or dosed orally with non-infected IEC-18 cells ($n = 4$). Confluent monolayers of these cells were detached by trypsinisation by standard methods (Paul 1975), then homogenised for 15 seconds and each hamster was dosed with 2 ml.

Monitoring of inocula

To monitor the quantity of infection in each hamster inoculum, parallel inoculation of IEC-18 cells on glass coverslips cultured in identical media in small vials were conducted in addition to the infected flasks set up for tissue-culture derived inocula. On day 6 after inoculation, the relevant coverslips were collected at the same time as the infected flasks, washed in Locke's salt solution,

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. Antibody IG4 is known to be specific for the intracellular organism of proliferative enteropathy (McOrist et al 1987).

In addition, 10 µl of each bulked inoculum used to dose the hamsters was smeared on to a glass slide, air-dried, fixed in acetone, and stained by an indirect immunofluorescence assay using the same primary antibody IG4 followed by a fluorescein conjugated sheep anti-mouse antibody step.

Necropsy procedure

A full necropsy was conducted 21 days after dosing and samples of small and large intestines taken for light and electron microscopy as described previously (McOrist and Lawson 1987, McOrist et al 1989). Briefly, sections of formalin-fixed intestines (duodenum, jejunum, ileum, caecum, colon) were stained with haematoxylin and eosin or Young's silver impregnation stain. At necropsy, further samples of terminal ileum, caecum and proximal colon from all the hamsters were trimmed into 1 mm cubes and fixed overnight in 2.5 per cent glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4). After washing twice in the buffer alone, they were processed for routine transmission electron microscopy and examined under a Philips EM400 microscope. Immunogold staining of further selected ultrathin sections from each group of hamsters was performed as described previously (McOrist et al 1989) with monoclonal antibody IG4 as the primary antibody and anti-mouse IgG conjugated with 15 nm particles (BioCell Research Labs, Cardiff) as the secondary antibody.

Results

All the hamsters were clinically healthy throughout and appeared normal at necropsy. Grossly evident thickening of the intestinal mucosa was not detected in any of the hamsters. Immunoperoxidase staining of infected IEC-18 cells on coverslips harvested in parallel to the flasks used for preparation of inocula for the hamsters, and immunofluorescence staining of smears of the prepared inocula from the infected flasks



FIG 1: Electron micrograph of epithelial cells in ileum of hamster dosed with 916/91. Numerous curved bacteria free in the cytoplasm. $\times 12,500$

showed numerous curved bacteria reactive with the primary monoclonal antibody. The 916/91 inoculum consistently contained over 100 organisms per high power field, while the 1482/89 inoculum had only five to 10 such organisms.

Tissue culture derived inocula

Microscopic lesions consistent with proliferative enteritis were seen in only 10 of the 16 hamsters dosed with inoculum 916/91. All the affected hamsters showed marked hyperplasia of crypt epithelial cells in the terminal ileum and three of these hamsters also had similar lesions in the jejunum. Affected crypts were enlarged, had reduced numbers or total absence of goblet cells and many mitotic figures. Silver stains of affected intestines showed numerous curved bacterial forms within the apical cytoplasm of crypt cells.

Ultrastructural examination showed numerous intracellular bacteria in the cytoplasm of crypt epithelial cells in the terminal ileum of the affected hamsters. Such bacteria were not seen in the control hamsters in which intestinal cells appeared normal.

The intracellular bacteria were most frequently observed in groups free in the apical cytoplasm (Fig 1); they were seldom seen singly. The bacteria

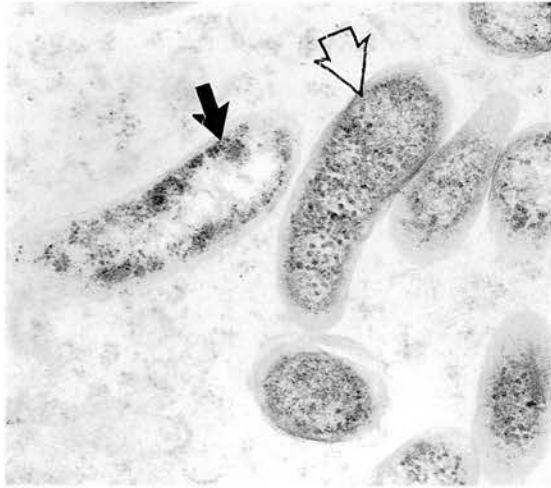


FIG 2: Electron micrograph of epithelial cells in ileum of hamster dosed with 916/91. Electron-dense (open arrow) and electron lucent bacterial forms (closed arrows). $\times 41,160$

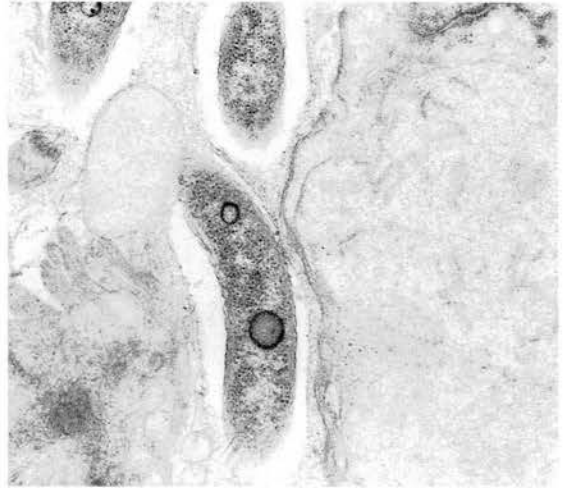


FIG 4: Electron micrograph of epithelial cells in ileum of hamster dosed with 916/91. Bacterium with electron dense globule at an advanced stage of entry into the cell and vacuole formation in the cell. $\times 24,600$

had a wavy trilaminar outer membrane and a cytoplasmic membrane separated by a periplasmic space. Curved to straight rods, ovoid or comma shapes, were evident. Some bacteria had electron dense protoplasm and others a more electron lucent protoplasm. The internal structure of the former consisted of an amorphous and homogenous elec-

tron dense material and many granules, presumably ribosomes, whereas the latter appeared reticulate with fewer granules (Fig 2). Electron lucent forms predominated in the cytoplasm.

Bacteria at the brush border, some containing either pale or electron dense 'globules', appeared to enter the cell singly via endocytic vacuoles. These structures appeared as depressions of slightly thickened membrane in the luminal surface of epithelial cells, with an associated bacterium in the depression. There was loss and disruption of microvilli in adjacent areas of the brush border (Fig 3). Each vacuole and associated bacterium extended into the cell (Fig 4). Two further events were apparent after bacterial entry. In a few instances single vacuoles appeared to coalesce into larger endocytic vacuoles containing several bacteria (Fig 5). In most instances, there was breakdown of the entry vacuoles with most bacteria of both forms appearing free in the cytoplasm. Bacterial division was observed only in electron lucent forms either free in the cytoplasm or within vacuoles.

In most infected cells, there was marked distension and disruption of some mitochondria and rough endoplasmic reticulum. The terminal web adjacent to groups of bacteria was usually disrupted, disorganised and increased in electron density. Many free bacteria appeared to be very closely associated with normal and distended mitochondria and rough endoplasmic reticulum

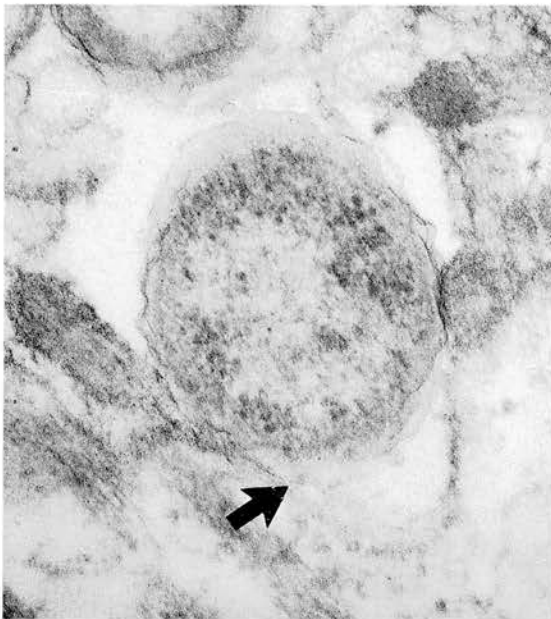


FIG 3: Electron micrograph of epithelial cells in ileum of hamster dosed with 916/91. Single Bacterium attached to disrupted brush border and initial vacuole formation (arrow). $\times 100,000$



FIG 5: Electron micrograph of epithelial cells in ileum of hamster dosed with 916/91. Several bacteria in an endocytic vacuole. $\times 35,500$

(Fig 6). Some bacteria had evidence of moderate focal thickening of portions of their outer membranes when free in the cytoplasm and close to the rough endoplasmic reticulum (Fig 7).



FIG 6: Electron micrograph of epithelial cells in ileum of hamster dosed with 916/91. Group of intracellular bacteria associated with distended mitochondria. $\times 46,000$

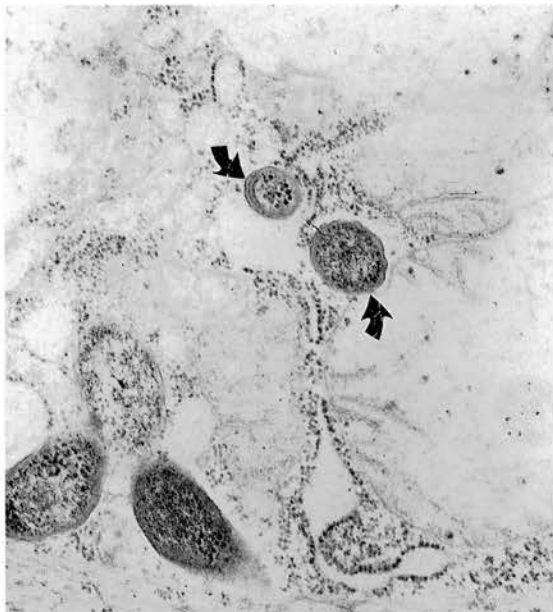


FIG 7: Electron micrograph of epithelial cells in ileum of hamster dosed with 916/91. Two bacteria (arrows) associated with distended mitochondria and rough endoplasmic reticulum. Note the moderate, focal thickening of bacterial membranes. $\times 35,500$

Neutrophils and macrophages evident in the mucosa contained many bacteria at various stages of degeneration and lysis in phagolysosomes.

Occasional crypt lumina of both affected and control hamsters contained numerous curved bacteria. Approximately 30 per cent of the luminal bacteria and 10 per cent of the intracellular bacteria had a pale or electron dense 'globule' within their cytoplasm.

Immunogold staining showed intracellular bacteria reactive to the monoclonal antibody IG4 only in the affected crypt cells. Immunogold particles were seen on the outer membrane of the intracellular bacteria. Similar bacteria in the crypt lumina and those just entering showed no associated gold particles. No particles were evident in sections from the control hamsters, stained in an identical manner.

Discussion

This study in hamsters suggests that the intracellular bacteria, currently known as Ileal symbiont intracellularis, characteristic of proliferative enteritis can enter crypt epithelial cells from the crypt lumen by attachment to the microvillus brush border, followed by ingestion in endocytic

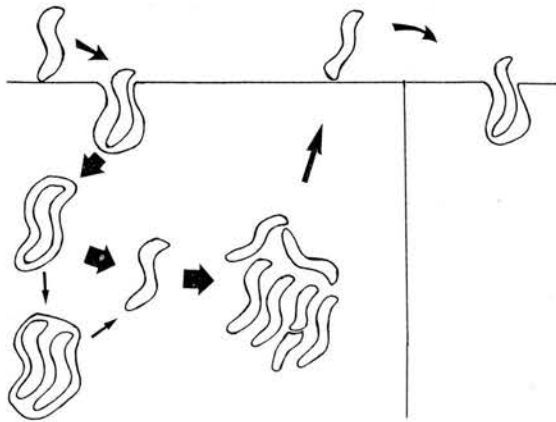


FIG 8: Diagrammatic representation of possible life cycle of ileal symbiont intracellularis in experimentally induced proliferative enteropathy in the hamster

vacuoles, and final release into the cell cytoplasm. Released bacteria then multiply freely in the cytoplasm. This parasitism is associated with the characteristic lesions. This study is consistent with previous studies of the early lesions of proliferative enteritis in pigs and hamsters which have postulated a similar sequence of events, but which have not involved experimentally induced lesions produced with a single cultured agent. A hypothetical model of the purported life cycle of Ileal symbiont intracellularis in hamsters is shown in Fig 8.

As the hamsters in this study were examined three weeks after dosing, it is possible that the bacterial entry observed may be due to crypt cell reinfection from released bacteria derived from other crypt cells which have completed their life cycles. Initial bacterial entry in proliferative enteritis is believed to occur as early as five days after infection (Johnson and Jacoby 1978), with the onset of hyperplasia occurring shortly after entry, being clearly visible 10 days after infection (Johnson and Jacoby 1978, McOrist et al 1989). Bacterial release and host cell reinfection is a common feature in the life cycles of other obligate intracellular bacteria (Doughri et al 1972, Moulder 1985, Prozesky and DuPlessis 1987). To survive as a successful intracellular pathogen, obligate intracellular bacteria must exit from an infected host cell following their replication to initiate a fresh infection in a new susceptible host and transfer bacterial progeny from one host cell to another.

Coalesced groups of bacteria in larger endocytic vacuoles were noted occasionally in the present study, but these are not a recognised feature of

natural disease in pigs or hamsters (Johnson and Jacoby 1978, McOrist et al 1989). Many other intracellular bacteria, such as some *Rickettsia* species, *Shigella flexneri* and *Listeria monocytogenes*, are found lying free in the cytoplasm after escaping from entry vacuoles; probably by the production of membrane damaging agents such as phospholipase or listeriolysin (Ewing et al 1978, Sansonetti et al 1986, Gaillard et al 1987, Winkler and Turco 1988). Other intracellular bacteria, such as *Chlamydia* species and *Coxiella* species do not escape from the phagosomic vacuoles following internalisation but undergo an intracellular growth in this compartment (Williams and Vodkin 1987). It is possible that Ileal symbiont intracellularis has evolved more than one adaptive mechanism of survival in the intracellular environment, as intracellular bacteria have a diverse range of life cycles (Moulder 1985). In the natural disease it appears that early escape from vacuoles is of greater survival benefit, while the authors' finding of bacteria in vacuoles may reflect a difference between the ability of pig-derived bacteria to completely initiate the disease in hamsters.

The close association of bacteria with the cytoplasmic matrix and organelles such as the mitochondria and the rough endoplasmic reticulum could help the direct transfer of nutrients to meet the bacterial requirements. Other intracellular bacteria, such as chlamydia, show similar modifications of the outer membranes when close to host cell mitochondria and this is believed to aid energy transfer (Peterson and De La Maza 1988). Chlamydia are unable to synthesise high energy compounds like adenosine triphosphate (ATP) and therefore rely on the host cell to obtain this essential metabolic requirement (Weiss and Wilson 1969). This may indicate an inability of these intracellular bacteria to synthesise ATP, with an adaptation to use host ATP.

The identity of the intracellular bacteria in the original pig lesions, the infected cell cultures, the inocula and in the hamster lesions produced by morphological and immunological tests was assessed. The positive reactions of all these bacteria with the specific monoclonal antibody IG4 indicates that they are identical to the intracellular organisms present in pig lesions as these were purified and used to prepare that antibody (McOrist et al 1987). Subsequent work established that those organisms had distinctive immunological and DNA profiles, and sequencing of their 16S

rDNA confirmed that they belong to a new taxonomic group named Ileal symbiont intracellularis (McOrist et al 1990, Gebhart et al 1993). The ability to grow these organisms in vitro in tissue culture cell lines has only recently been demonstrated (Lawson et al 1993). This study represents the first ultrastructural study of proliferative enteritis in hamsters using pure cultures of an agent derived from pigs. Similar results have been reported with tissue culture derived inocula prepared from hamsters (Stills 1991).

The doses used were considered to be relatively small, but 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 similar reduction in virulence on repeated subculture (Moulder 1985). It is possible that some of the mechanisms necessary for bacterial adherence, entry and intracellular survival in vivo become redundant when grown in cell culture systems. Any ability of strain 1482/89 to initiate lesions was not demonstrated, although extensive trials were not used. Differences in the rates at which strains of intracellularis become less virulent in culture may occur.

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

G. H. K. LAWSON,* S. McORIST, S. JASNI, AND R. A. MACKIE

Department of Veterinary Pathology, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Veterinary Field Station, Easter Bush, Midlothian EH25 9RG, United Kingdom

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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 (PHF).

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 CRI 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 $\mu\text{g}/\text{ml}$). 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 bijoux 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

* Corresponding author.

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 re-filtering (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

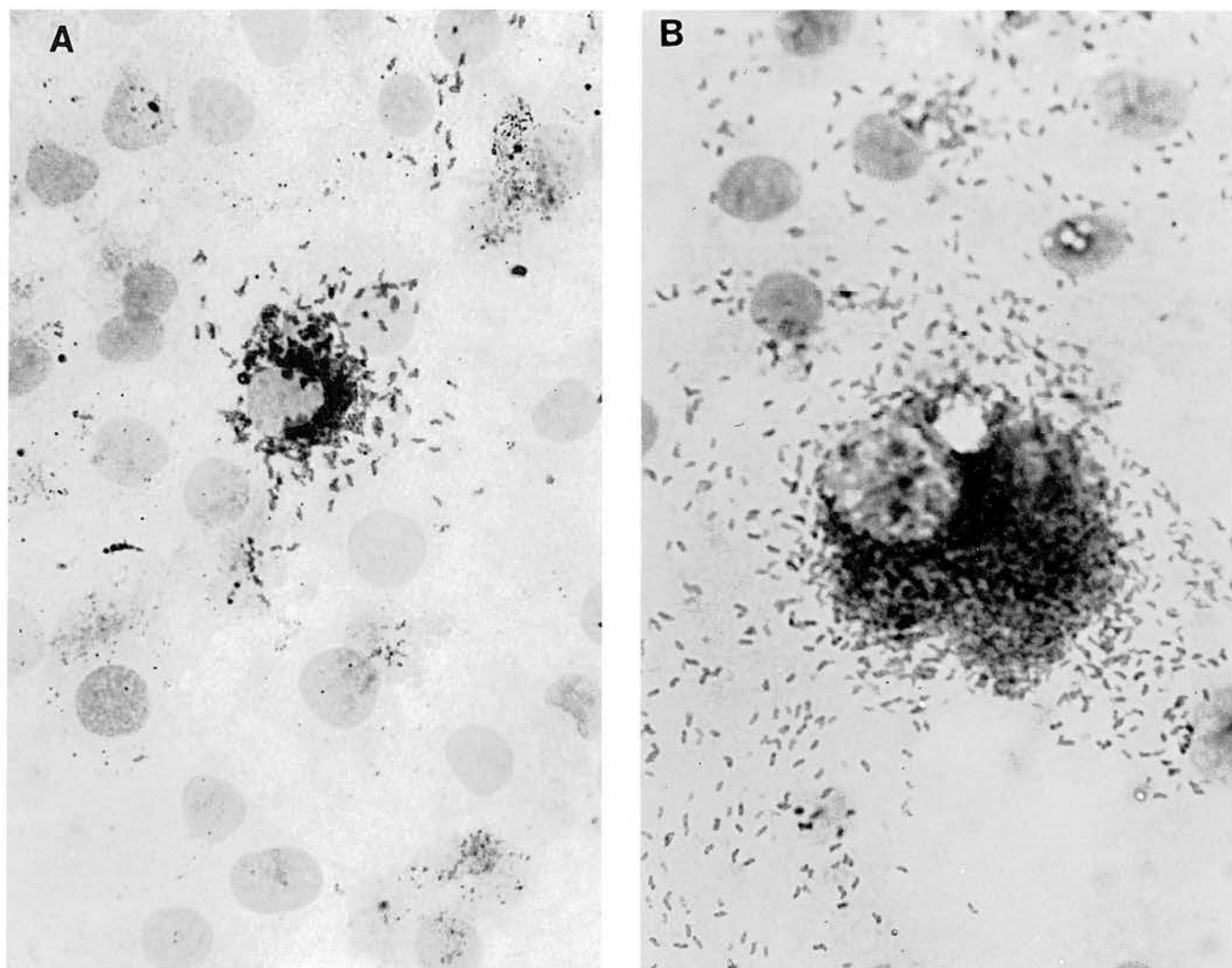


FIG. 1. IEC-18 cells infected with IO derived from cases of proliferative enteropathy were immunoperoxidase stained by employing anti-IO monoclonal antibodies and hematoxylin counterstain. (A) Isolated HIC. The cell cytoplasm is full of SIO. Magnification, $\times 576$. (B) HIC, with many hundreds of 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

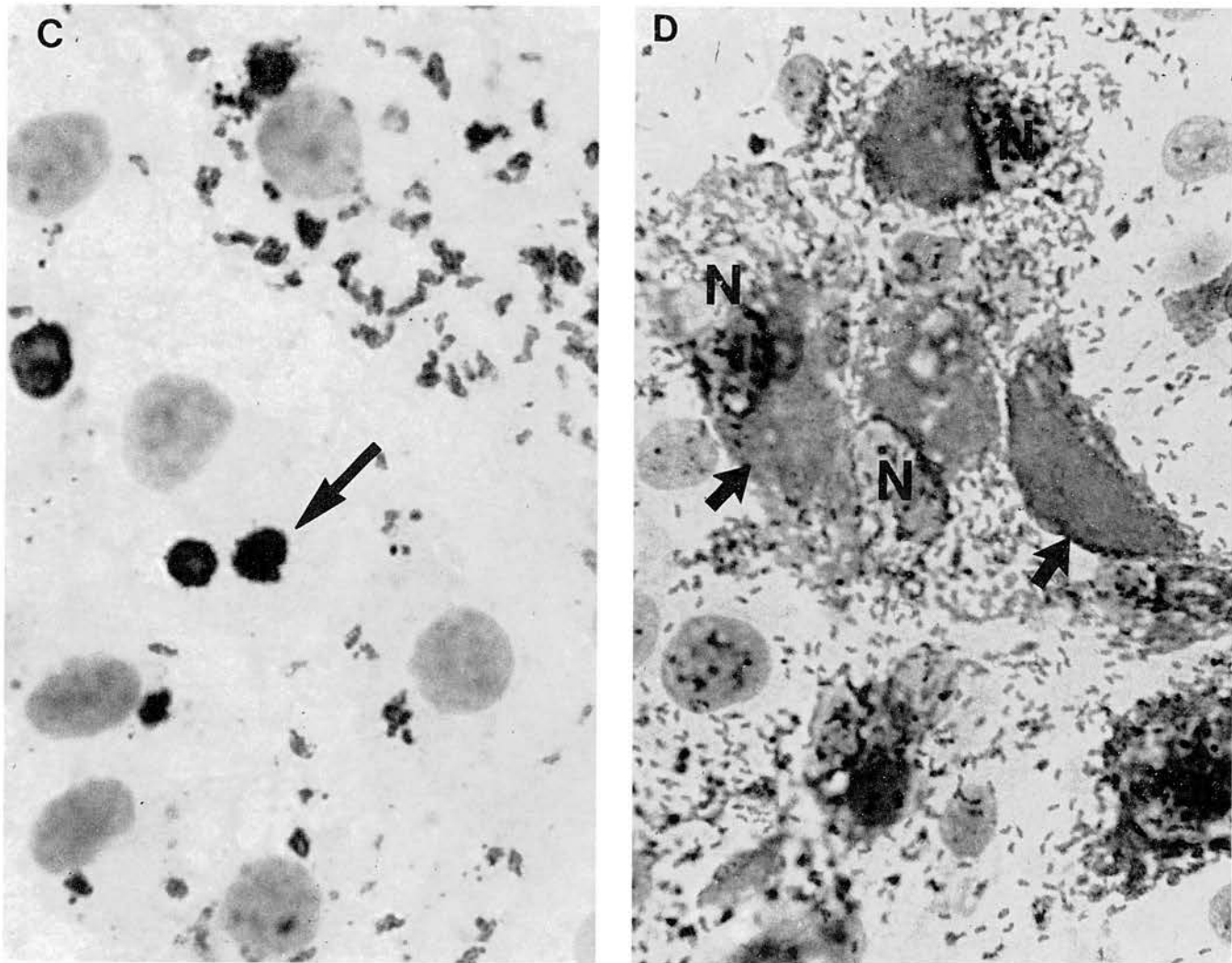


FIG. 1—Continued.

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-

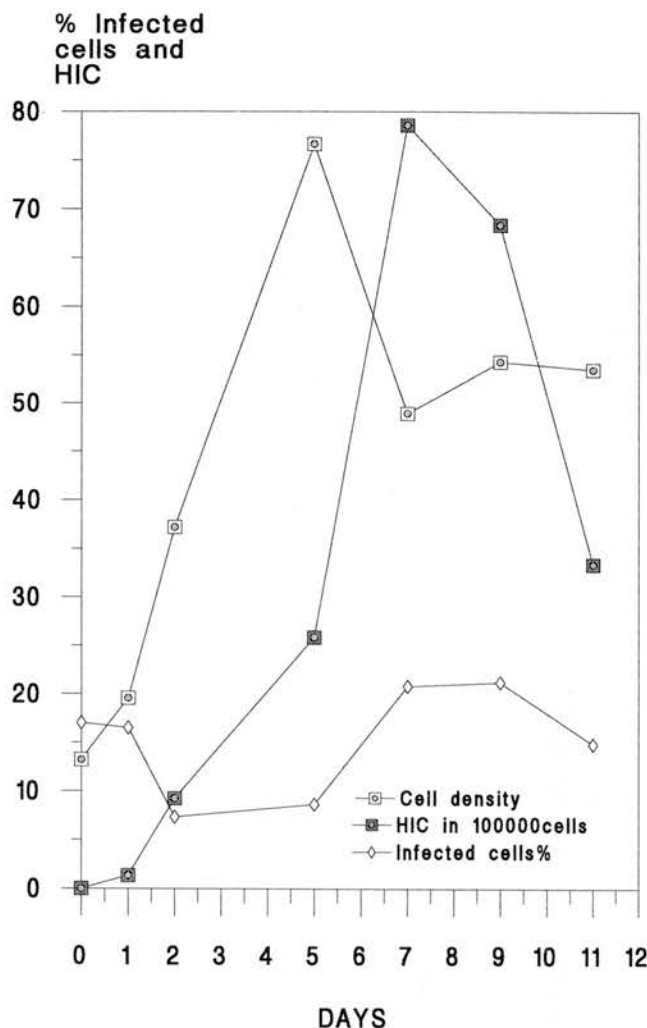


FIG. 2. IEC-18 cells exposed to infected supernatant fluid from IEC-18 cells infected with IO of strain 916/91 (passage 12). Infected cells were assessed by counting 200 cells; the HIC density was calculated from the total number of HIC; cell density was derived from nuclear counts in five areas of 0.0672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

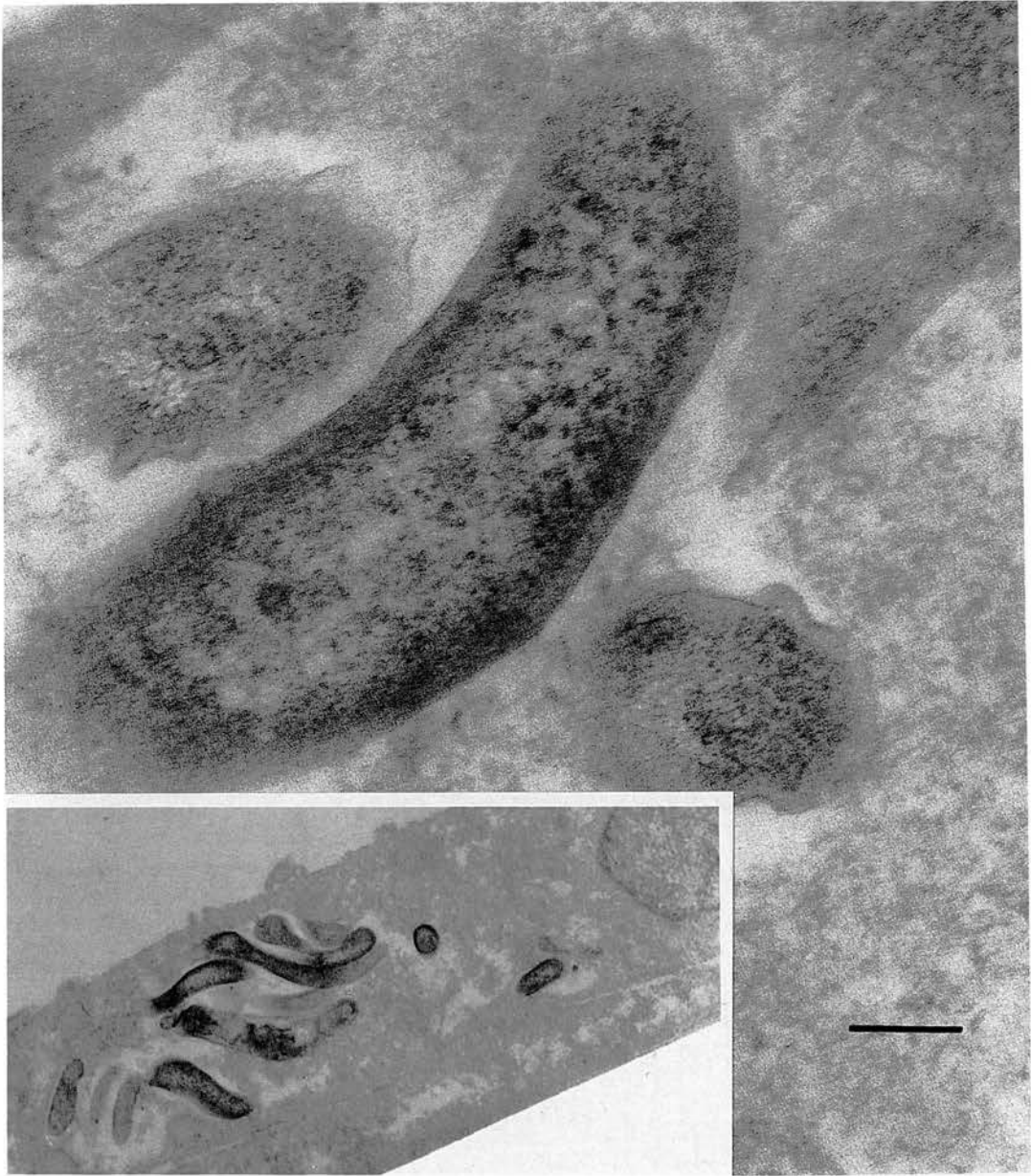


FIG. 3. Ultrastructure of IEC-18 cells infected with IO derived from cases of proliferative enteropathy at day 6 p.i. The cells contain numerous IO. Bacteria lie freely within cell cytoplasm, with no evidence of a cell membrane, as shown by uranyl acetate-lead citrate staining. Magnification, $\times 77,000$. 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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