THE PATHOGENESIS OF *Campylobacter* DIARRHOEA

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by

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To my mother and father

Therefore, if anyone is in Christ, he is a new creation; the old has gone, the new has come ! (2 Cor. 5:17)

There is nothing like youth. The middle-aged are mortgaged to Life. The old are in life's lumber room. But youth is the Lord of Life. Youth has a kingdom waiting for it. Everyone is born a king, and most people die in exile, like most kings. To win back my youth...there is nothing I wouldn't do - except take exercise, get up early, or be a useful member of the community.

Oscar Wilde. 1854-1900.

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Contents

Abstract

The Pathogenesis of *Campylobacter* Diarrhoea.

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Campylobacter jejuni/coli are the most common cause of acute diarrhoeal disease in man. The disease is worldwide, affects all age groups, and is mostly sporadic although common source outbreaks are frequently reported. The organism characteristically causes infection of the small intestine with extension into the colon and rectum, the disease being an acute enterocolitis. Illness may be inflammatory, with mucosal oedema and polymorphonuclear infiltration and blood in the faeces, or non-inflammatory with watery diarrhoea. The pathogenesis of the disease is unknown.

Bacterial invasion of intestinal mucosa has been proposed as a mechanism of mucosal inflammation causing tissue damage. Investigation of the enterocyte-like Caco-2 and other epithelial cell lines for the ability of clinically characterised strains to adhere and invade showed that strains from colitic illness exhibited a greater tendency to invade than strains from non-inflammatory illness. Colitis and some non-inflammatory strains were also shown to transcytose from the apical to the basolateral cell membrane.

Phosphorylation of mammalian cell proteins (such as ion channels) is important in diarrhoeal illness, mediated by bacterial cells and their secreted toxins. *C. jejuni* bacterial cells and a secreted toxin in culture supernatants caused phosphorylation of Caco-2 cell proteins, effects that mimic protein kinase C phosphorylation of myosin light chain. Culture supernatants increased intracellular calcium, an effect known to mediate fluid secretion. These effects are independent of the cholera-like toxin that is found in small amounts in culture supernatants.

Colitis strains tested in rabbit ileal loops induced similar histological effects to those seen in man, caused fluid secretion, and white cell infiltrate consisting of polymorphonuclear leucocytes and macrophages. Villi were shortened and tissue was oedematous with submucosal bleeding. Tissue damage may prevent effective absorption of fluid and contribute to diarrhoea but biochemical analysis suggests a true secretory component to the diarrhoea. By contrast a non-inflammatory strain showed no histological changes in loops and elicited no fluid secretion.

Large amounts of the host derived secretagogue prostaglandin E2 were induced in infected ileal loops and correlated with the tissue white cell infiltrate (along with leukotriene B4). In the absence of a cholera-like toxin produced by the bacteria PGE2 seems to be responsible for the increase in infected tissue cyclic AMP. PGE2 acts by binding to a cellular receptor and activating cell adenylate cyclase resulting in a rise in cAMP. Thus a host inflammatory mediator may contribute to fluid secretion in *C. jejuni* enterocolitis.

Chapter 1

GENERAL INTRODUCTION

1.1. The genus *Campylobacter*.

The genus name *Campylobacter* was derived from the Greek for curved rod. It was proposed by Sebald and Veron (1963) to include microaerophilic bacteria that were different from the aerobic vibrios in their microbiological characteristics. *Campylobacter* species are motile Gram negative bacteria, 0.5-8 μm long and 0.2-0.5 μm wide, with characteristically spiral, curved, and S shaped cells (Sebald and Veron, 1963). Motility is rapid and darting in corkscrew fashion, a feature by which their presence among other bacteria can be detected by phase contrast microscopy (Karmali and Fleming, 1979). They generally have a single polar unsheathed flagellum (monotrichous) or a flagellum at each end (amphitrichous). Their guanine plus cytosine (G+C) content is low, ranging from 28 to 38 mol % (Smibert, 1984). The genus *Campylobacter* currently includes 13 species. These are *C. cinaedi, C. coli, C. concisus, C. cryoaerophila, C. fennelliae, C. fetus* subsp. *fetus, C. fetus* subsp. *venerealis, C. hyointestinalis, C. jejuni, C. lari, C. mucosalis, C. nitrofigilis, C. sputorum* subsp. *bubulus, C. sputorum* subsp. *sputorum* and *C. upsaliensis*. A convenient practice in the past has been to divide species into two groups on the basis of catalase production, a scheme which originated with veterinary microbiologists (Cottral, 1978), who found that the test differentiated the bovine pathogen *C. fetus* from commensals (now classified as *C. sputorum* subsp. *bubulus*, Karmali and Skirrow, 1984). This grouping has become less relevant recently since the newly described non-pathogenic species *C. cryoaerophila* and *C. nitrofigilis* have also been found to be catalase positive. Other biochemical reactions by which *Campylobacter* species may be differentiated are relatively few because of their inability to ferment or oxidize the usual carbohydrate substrates available in a diagnostic laboratory. They have a respiratory type of metabolism and use amino acids and intermediates of the tricarboxylic acid cycle. They are oxidase positive and reduce nitrates. Because of this lack of discriminatory characteristics descriptions of new species have been difficult, and in some instances species differ more in the degree to which a

characteristic is demonstrable than in the number of different characters.

1.2. Growth characteristics.

With respect to atmospheric conditions, there exists a spectrum from the anaerobic requirements of some species, to the natural oxygen tolerance of another, *C. cryoaerophila* (Neill *et al.*, 1985). Most species are microaerophilic, requiring an oxygen concentration between 3-15% (although a few strains may grow slightly aerobically in 20% oxygen) and a carbon dioxide concentration of 3-5%. Hydrogen at about 6% is required for optimal isolation from clinical material. Some species can grow under anaerobic conditions with fumarate, with or without formate (Smibert, 1984). With small inocula containing few cells, low oxygen tension (3-6%) is needed for growth, but oxygen toxicity can be overcome in solid or liquid media with very large inocula containing large numbers of cells. *C. fetus, C. jejuni*, and *C. coli* have superoxide dismutase activity. Addition of 0.025% each of ferrous ions, sodium metabisulphite and sodium pyruvate to culture media will increase the aerotolerance of cultures of these species and allow growth at oxygen concentrations of 15-20% (George *et al.*, 1987). This mixture destroys hydrogen peroxide and superoxide anions that appear in the medium when exposed to air and light, and to which *C. fetus*, *C. jejuni* and *C. coli* are extremely sensitive. Bovine superoxide dismutase and catalase added to culture media greatly enhance oxygen tolerance. The use of agar media containing blood also enhances the aerotolerance of campylobacters because blood contains catalase and superoxide dismutase.

There is a wide range in the temperatures required for culturing bacteria, extending from 15°C for *C. cryoaerophila* to 42°C for *C. jejuni*, *C. coli*, and *C. lari*. Moreover, species and individual strains vary in tolerance of growth temperatures other than the optimum. Thus, while *C. fetus* subsp. *fetus* grows at 25°C and can be cultured at 37°C, some strains have been isolated at 42°C (Smibert and Von Graevnitz, 1980). *C. jejuni* and *C. coli* have been referred to as the thermophilic group of the genus, but *C. lari* and *C. upsaliensis* are also thermophilic. *C. hyointestinalis* is also considered thermophilic, although it would perhaps be more appropriate to regard this species as thermotolerant because it grows more abundantly at 37°C than

at 42°C (Fennell *et al.*, 1986). In contrast *C. cinaedi* and *C. fennelliae* grow at 37°C but not at 25 or 42°C (Totten *et al.*, 1985).

1.3. Isolation.

Before the development of selective media, enteric campylobacters had been isolated only from normally sterile sites such as the bloodstream, and their association with diarrhoeal illness was merely suspected (King, 1957). Workers in Belgium first demonstrated this association directly (Dekeyser *et al.*, 1972; Butzler *et al.*, 1973) by using filtration techniques that depend on the fact that *Campylobacter* species are smaller than most other bacterial species in the faecal flora. Skirrow (1977) subsequently demonstrated the necessity for selective media for isolation of *C. jejuni* from a complex faecal flora, and confirmed the importance of the organism in diarrhoeal disease. Subsequent work has been aimed at refining selective isolation media in terms of their ability to suppress commensals and to support more luxuriant growth of enteric campylobacters. The most popular of these media are described below, but most clinical laboratories now use the Preston formulation (Bolton and Robertson, 1982).

In most clinical laboratories isolation of *Campylobacter* species is accomplished by two basic methods. The first involves filtration of the cells through membrane filters with pore sizes of 0.45, 0.65 and 0.8 μm, after which the filtrate is streaked onto agar medium or inoculated into broth, and incubated in a microaerophilic atmosphere, for which commercial products are available (Dekeyser *et al.*, 1972; Butzler *et al.*, 1973). The second method requires the use of selective agar media. *C. fetus* subsp. *fetus* and subsp. *venerealis* can be isolated from sheep and cattle using blood agar or Brucella agar containing antibiotics. The selective isolation medium developed by Skirrow (1977), containing vancomycin, polymixin B, and trimethoprim in a blood agar base with lysed horse blood, is still widely used, but efforts have continued to produce media with greater selectivity and greater ability to suppress other bacterial species. Butzler *et al.* (1973) used thioglycolate agar with 15% sheep blood containing bacitracin, novobiocin, cyclohexamide, colistin and cefazolin. Blaser *et al.* (1979) developed a medium referred to as Campy-BAP, consisting of Brucella agar

base with sheep erythrocytes and vancomycin, trimethoprim, polymixin B, amphoteracin, and cephalothin. Moskowitz and Chester (1982) showed that *Pseudomonas* and *Achromobacter* species were most likely to grow as contaminants in the antibiotic containing media. To increase suppression of *Pseudomonas*, Goossens *et al.* (1983) added cefoperazone to a medium containing rifampicin, colistin and amphoteracin B. With the wider objective of increasing isolation efficiency, not only from human specimens but also from non-human sources, Bolton and Robertson (1982) designed a medium (Preston medium) containing polymixin, rifampicin, trimethoprim and cyclohexamide, an antibiotic combination that achieves the aim of high campylobacter yield plus excellent commensal suppression.

The fact that sterile blood for the above media is costly and is not readily available in some developing countries has stimulated interest in developing blood-free isolation media. Taking advantage of findings by George *et al.* (1978) and Hoffman *et al.* (1979) that a supplement of ferrous sulphate, sodium metabisulphite and sodium pyruvate enhanced the growth and aerotolerance of *Campylobacter* species, Bolton *et al.* (1983, 1984) attempted to replace blood with these supplements. As a result of their systematic approach, CCD medium (charcoal, cefazolin, deoxycholate agar) was developed that incorporated a single antibiotic and sodium deoxycholate as selective agents in blood-free agar containing charcoal, casein hydrolysates, ferrous sulphate, and sodium pyruvate. Although CCD medium was less selective than Preston agar, it performed equally well as an isolation medium (Bolton *et al.*, 1984). Karmali *et al.* (1984) used vancomycin, cefoperazone and cyclohexamide to give additional selectivity in a charcoal, haematin and pyruvate supplemented blood-free medium. Indeed this medium is more selective than Skirrow's medium (Skirrow, 1977) and less inhibitory to antibiotic-susceptible strains of *C. coli*. Thus blood-free media represent acceptable alternatives to presently used isolation media that contain blood or blood products. While the use of enrichment media is not recommended for routine isolation of thermophilic campylobacteria (Morris and Patton, 1985) enrichment is essential for isolation of campylobacters from food, and the reported blood-free enrichment media increases rates of recovery significantly (Chan *et al.*, 1987).

Although the use of selective media and incubation temperatures of 41 to 43°C has been successful for isolation of *C. jejuni* and *C. coli*, a single isolation procedure will not recover all pathogenic enteric campylobacters. Ng *et al.* (1985) demonstrated that some thermophilic campylobacters are inhibited by most of the antibiotic combinations used in isolation media so that potential pathogens could be missed in the clinical laboratory. Steele and McDermott (1984) found that *C. jejuni* which failed to grow on antibiotic-containing media could be isolated by filtration. By dark-field microscopy, Paisley *et al.* (1982) demonstrated the presence of spiral bacteria in stools that were culture negative. Tee *et al.* (1987) found that incubation of cultures at 37°C yielded nine atypical campylobacter strains, three of which were *C. jejuni* that grew poorly or not at all at 43°C. These "non-culturable" isolates constitute only a small percentage of faecally derived campylobacters, but represent a larger proportion of those present in the environment.

1.4. Historical background.

The observation of spiral, curved bacteria by McFadyean and Stockman in 1913 and by Levy in 1946 (reviewed in Kist, 1981) are generally assumed to be the first descriptions of campylobacters in animal and human disease respectively. In 1947 Vinzent was successful in growing *Vibrio fetus* from the blood of three pregnant women, two of whom aborted during illness, and ten years later King (1957) first associated diarrhoea in children with the detection of "related vibrios" in blood cultures. It was not until 1972, however, that such microorganisms were isolated from diarrhoeic stool specimens (Dekeyser *et al.*, 1972; Butzler *et al.*, 1973) and the development and introduction of a highly selective solid growth medium by Skirrow in 1977 provided the breakthrough for routine culture. Interestingly, however, a number of older, predominantly German language papers devoted to the role of spiral organisms in human diarrhoea have subsequently come to notice. In 1886, Theodor Escherich described spiral bacteria associated with large intestinal mucous in 16 of 17 children who had died of diarrhoeal disease. Growth on a solid medium was not successful. He also observed spiral bacteria microscopically in stool specimens of 35 of 72 infants suffering from enteric disease, but he

considered that they played a prognostic rather than aetiological role in diarrhoeal disease. In six cases he described "whip shaped" microorganisms which he thought were not associated with acute gastroenteritis. Interestingly, at the same time, he published similar observations on kittens which had died of diarrhoeal disease. In the same year, Pfeiffer described post mortem examination of the large intestine of a nun who had died of a disease strongly resembling classical campylobacter colitis. He found spiral bacteria, but falsely concluded that extensive inflammation of the gut wall would have provided normal cholera vibrios suitable conditions in which to develop spiral forms. In 1892, Fuerbringer described a case of fatal cholera-like disease in which spiral, curved, non-culturable bacteria were found in the small intestine, but cholera vibrios were not detected. In 1893 Kowalski reported delicate spirilla in 11 patients with cholera and 2 patients with cholera-like disease.

In 1923, Broughton-Alcock published the results of an extensive microbiological investigation of 20,000 stool specimens in which he concluded that spiral organisms were not important in Britain as enteric pathogens, but might be endemic in tropical and subtropical areas. In contrast, Nishiyama (1936) published a systematic study of the occurrence of spiral organisms in man, in which they were found in 12% of non-acutely ill children and 31% of adults compared with 53% in a diseased population. Hartman and Lacey (1918) described an actively motile, spirally curved bacteria isolated during illness as well as post mortem from the blood of a 22 year old Italian in Pittsburgh who died of an illness clinically resembling typhoid fever, but accompanied by enlargement of lymph nodes, splenomegaly and destructive changes in bone marrow indicating a systemic disease (possibly Hodgkins disease). Despite the fact that the bacteria isolated underwent morphological changes during subculture, their initial morphological behaviour and growth characteristics closely resembled those of *C. fetus* subsp. *fetus*. From these early papers, the following features suggest that the organisms described were probably *Campylobacter* species: (a) typical morphology, (b) association with enteritis in neonates (infants and kittens), (c) failure to grow on solid media despite microscopic detection, and (d) to date no other bacteria with comparable morphology have been associated with human enteric infections.

In the 1960s and early 1970s, the few successful isolations that were made came from blood or other clean sites that were free from competing organisms (Middlekamp and Wolf, 1961; Darrell *et al.*, 1967). These observers suspected that the organisms were present in the gut, and were not as rare as the few previous reports had suggested, but attempts to prove this were frustrated by the lack of a suitable selective culture technique. Since *Campylobacter* species were already well known in veterinary practice as a cause of infectious infertility and abortion in cattle and sheep, it was not surprising that an appropriate technique eventually came from this quarter. In Brussels, Dekeyser *et al.* (1972) isolated campylobacters from the stools of two patients, and quite independently in Australia, Cooper and Slee (1971) made similar isolations. Although the latter did not pursue their investigations, the Brussels team went on to show that campylobacters were present in the stools of at least 5% of children with diarrhoea (Butzler *et al.*, 1973). Later, Skirrow (1977) confirmed and extended this work by isolating campylobacters from the faeces of 7.1% of both children and adults suffering from acute diarrhoea. Since then, other workers throughout the world have reported similar findings, and in some laboratories campylobacter isolations outnumber those of salmonella and shigella combined.

1.5. Pathogenic *Campylobacter* species.

1.5.1. *C. fetus*. Strains of *C. fetus* are divided into two subspecies, *fetus* and *venerealis*. This classification stems from the report of Florent (1959) who recognised that different disease entities could be attributed to the two subspecies. The major habitat of *C. fetus* subsp. *fetus* is the intestine, and, while isolations from healthy sheep and cattle are not uncommon (Garcia *et al.*, 1983), the prevailing view is that it is only rarely isolated from human intestinal contents (Lauwers *et al.*, 1978). The genital tract of sheep and cattle and the content of placentas and stomachs of aborted sheep and cattle foetuses are also sources of this subspecies (Smibert, 1984). Other animals and birds have been implicated as a source of the organism but only as secondary reservoirs (Garcia *et al.*, 1983).

C. fetus subsp. *fetus* causes sporadic abortion in sheep and cattle. The infection is believed to arise from bacteria in the intestine acquired through

ingestion of food or water contaminated with bacteria from faeces, aborted foetuses, or vaginal discharges of the aborting animals. During the course of a bacteraemic phase, the organisms, which have a particularly high affinity for placental tissues, invade the uterus and multiply in the immunologically immature foetus, which subsequently aborts. If live births occur, the newborn animals may survive for a few hours but certainly not longer than 4 or 5 days (Cottral, 1978). Laboratory diagnosis depends on the isolation and identification of the organisms from the placenta or from organs of the foetus in which they occur in large numbers (Garcia *et al.*, 1983).

Human infection by *C. fetus* subsp. *fetus* is rare and generally limited to septicaemia in patients with predisposing conditions (Guerrant, 1978). Meningitis is the next most frequently diagnosed disease, but infections at other sites may lead to pericarditis, peritonitis, salpingitis, septic arthritis, and abscesses (Rettig, 1979). *C. fetus* subsp. *fetus* was implicated as a cause of premature labour and neonatal sepsis (CDC, 1984), an atypical isolate being recovered from amniotic fluid. Although the incidence of such infections is low and reports are rare in current literature, the tropism exhibited by this organism for foetal tissue can evidently be manifested in the human, and the possibility that human foetal infections may occur should not be overlooked by the clinical laboratory. It was originally believed that *C. fetus* subsp. *fetus* was rarely found in the human intestine and was not a cause of human enteritis (Butzler, 1978). However, the subspecies is susceptible to cephalothin and would therefore not be isolated on medium containing this antibiotic. Moreover, incubation at 42°C inhibits many strains of this subspecies. The extent to which such routine conditions affect the isolation rate cannot be estimated, but faecal strains of *C. fetus* subsp. *fetus* associated with gastroenteritis have been isolated by Harvey and Greenwood (1983), and, in addition, one of the two strains investigated by Edmonds *et al.* (1986) was from a case of diarrhoea. Interestingly most of these strains were capable of growth at 42°C. In contrast to *C. fetus* subsp. *fetus*, subspecies *venerealis* is not associated with human infections (Smibert, 1984). It is, however, the major cause of bovine genital campylobacteriosis, an infectious disease of concern to the cattle industry. It causes infertility but is only rarely associated with abortion.

1.5.2. *C. hyointestinalis*. In 1983, strains isolated from cases of proliferative enteritis in pigs were found biochemically to belong to the genus *Campylobacter* (Gebhart *et al.*, 1983) and the new species *C. hyointestinalis* was formally proposed in 1985 (Gebhart *et al.*, 1985). Recent isolations from human patients with proctitis and diarrhoea (Fennell *et al.*, 1986) clearly implicate the species as an infrequent pathogen of humans as well.

1.5.3. *C. jejuni* and *C. coli*. In the group known as the thermophilic campylobacters (thermotolerant) that prefer to grow at 42°C, *C. jejuni* and *C. coli* were first recognised, and *C. lari* (originally *C. laridis*) was described later. *C. jejuni* is the most important species in terms of numbers of infections, and indeed this species is now appreciated as a major infectious agent of man.

Following the discovery of Butzler *et al.* (1973) that campylobacters could be isolated from children with enteritis, and its confirmation through a systematic study of 803 patients by Skirrow (1977), it was soon established that these organisms were a very frequent, if not the most frequent, cause of human diarrhoea, particularly among paediatric patients, who are more likely to present for medical attention (Karmali and Fleming, 1979). However, no test was available for differentiating between the species until Harvey (1980) found that *C. jejuni* was capable of hydrolyzing hippurate while *C. coli* was not. To date, no other single biochemical test has had as much impact on clarifying the taxonomy of the thermophilic species. Originally the hippurate test was used simply to confirm that faecal isolates from patients with diarrhoea were thermophilic campylobacters, which serves to underline the point that *C. fetus* is neither a common human pathogen nor a normal resident of the intestine.

On the basis of a test for hydrogen sulphide production, Skirrow and Benjamin (1980) were able to show the existence of two biotypes in *C. jejuni*. The development of a sensitive test for DNA hydrolysis (Lior and Patel, 1987) and a rapid test for hydrogen sulphide (Lior, 1984) extended the biotyping scheme for *C. jejuni* and *C. coli* to include *C. lari*. A bacteriophage typing system described by Grajewski *et al.* (1985) also discriminates among

isolates of *C. jejuni* and *C. coli*. This system has potential use in epidemiological studies but has not yet been adopted by reference laboratories.

Interest in serotyping schemes emerged soon after the discovery of the importance of *C. jejuni*. Abbott *et al.* (1980) demonstrated agglutinating antibody to heat labile and heat stable antigens in rabbit antisera prepared against isolates from patients. Penner and Hennessey (1980) proposed a scheme based on extracted thermostable antigens that were later proved to be the LPS somatic O antigens located in the outer membrane (Preston and Penner, 1987). O specificities were determined by titrating each of the serotyping antisera against antigen from the various isolates by passive haemagglutination. Apart from a few cross reactions between *C. jejuni* and *C. coli* O serotypes, each species possesses its own array of antigens. Penner *et al.* (1983) refined the serotyping scheme originally developed for both *C. jejuni* and *C. coli* into seperate schemes for each species, based on 42 antisera for *C. jejuni* and 18 antisera for *C. coli*. Lior *et al.* (1982) described systems for serotyping *C. jejuni* and *C. coli* based on thermolabile antigens. Typing antisera are absorbed with heated cell suspensions of the homologous serostrain to remove antibodies against the thermostable antigens, leaving antibodies directed against determinants of thermolabile antigens. Serotyping on the basis of thermolabile antigens is performed by coagglutination rather than slide agglutination, and has the advantage of requiring considerably less antisera. Serotyping of thermophilic campylobacters has been shown to be of value in numerous epidemiological investigations.

1.5.4. *C. lari*. A group of thermophilic strains isolated predominantly from seagulls was found to be different from *C. jejuni* and *C. coli* on the basis of its resistance to nalidixic acid (Skirrow and Benjamin, 1980). These strains were designated "nalidixic acid resistant thermophilic campylobacters" (NARTC) until 1983, when the species name *C. laridis* was proposed (Benjamin *et al.*, 1983) and later changed to *C. lari*. A consistent feature of *C. lari* appears to be the production of hydrogen sulphide, but sensitive methods are required for its detection (Lior, 1984). Tauxe *et al.* (1985) reported six human infections with *C. lari*, one of which was

bacteraemia in an elderly man with multiple myeloma. In the other five cases, symptoms mimicked those generally described for patients with *C. jejuni* enteritis. All isolates were confirmed as *C. lari* by DNA relatedness tests.

1.5.5. *C. upsaliensis*. Concern about the possible transmission of pathogenic campylobacters from diarrhoeic dogs prompted Sandstedt *et al.* (1983) to undertake a study of canine campylobacters. As expected *C. jejuni* and *C. coli* were observed, but an unusual group of hippurate negative strains were also isolated that grew at 42°C and gave either negative or weak catalase reactions. DNA hybridization tests showed that these strains represented a new species, *C. upsaliensis* (Sandstedt *et al.*, 1983), which is now recognised as a relatively common human diarrhoeal pathogen, perhaps as common as *C. coli*. In immunocompromised patients, bacteraemia and death may occur (Goossens *et al.*, 1990).

1.5.6. *C. fennelli*ae and *C. cinaedi*. The inability of selective media to isolate all strains causing enteritis was highlighted by Fennell *et al.* (1986), who examined the causes of enteritis in homosexual males in Seattle, Washington. In addition to isolations of *C. jejuni* and *C. fetus* subsp. *fetus* they recovered strains with phenotypes quite distinct from described species. These so called CLOs (campylobacter-like organisms) were of three classes; CLO-1 and CLO-2 strains were recognised as two newly described species, *C. cinaedi* and *C. fennelliae* respectively (Totten *et al.*, 1985), while the taxonomic status of CLO-3 strain has not yet been resolved. These organisms grow microaerophilically at 37°C but not at 25 or 42°C. *C. cinaedi* occurs significantly less frquently in asymptomatic than in symptomatic homosexual patients, and bacteraemia with both *C. cinaedi* and *C. fennelliae* was also observed. It has recently been proposed that these species more closely resemble members of the genus *Helicobacter* and should therefore be classified as such.

1.6. Epidemiology.

Campylobacters are common organisms. They have been found wherever they have been sought, from Alaska to New Zealand and places in between (Brieseman, 1984; Christie, 1987). They are present in cows

(Waterman and Bramley, 1984), sheep (Blaser *et al.*, 1983), hens and turkeys (discussed later), seagulls (Fricker *et al.*, 1983), flies (Wright, 1983), pigs (Blaser *et al.*, 1983), pigeons (Skirrow, 1982), cats, turtles (Harvey and Greenwood, 1985) and dogs (Sandstedt *et al.*, 1983). They contaminate the environment, water sources and kitchen floors (De Wit *et al.*, 1979). They pass from one habitat to another, and from animals or the environment to man, but it is not always easy to trace their passage. It is important to stress here the difference between colonization and infection; a cow for example, may excrete a strain of *C. jejuni* for months and yet be completely asymptomatic (Mawer, 1981), while transfer of the same strain to man may cause severe illness, after which the patient on recovery excretes for only a week or two. The cow may have acquired immunity to infection as a calf, or the particular *Campylobacter* strain may be non-pathogenic for cows but pathogenic for man. Alternatively, the organism may have increased in virulence on passage to a new host. In single sporadic cases, the source and route of infection are not known. The source is probably always an environmental one, and when one studies the prevalence of various campylobacter serotypes in the environment, and in the animals and birds in the environment, it is found that there is a tendency for the same few serotypes to be found in the environment and among isolates from human cases (Skirrow and Benjamin, 1980; Jones *et al.*, 1984; Lauwers *et al.*, 1981). Poultry, and less commonly pigs (Lior *et al.*, 1982), may be the source, but birds and insects may also play a part.

Most cases of campylobacter enteritis in humans are single, sporadic episodes with no apparent link with other cases, and usually no clue to the source of infection. On the other hand there are now many reports of outbreaks of campylobacter enteritis where the source of infection has been established with some certainty. Milk, water and poultry are all common sources, other foods are sometimes suspected, and contact with farm animals, household pets, or other non-domestic animals (seagulls, flies, waterfowl, etc.) may also be implicated. There is some risk to laboratory workers (Penner *et al.*, 1983). Person-to-person spread in families or at day care centres is reported, and travel abroad may also frequently lead to contact with campylobacters.

1.6.1. Milk as a source. Some of the largest outbreaks have been due to the drinking of raw, unpasteurised milk (Report, 1981; Skirrow, 1982). The epidemiological picture in these cases is usually the same, strong circumstantial evidence in time and place of an association between drinking of raw milk and human illness, excretion of campylobacters by cows in the dairy farm, and negative cultures from the milk itself. It is interesting to note that while a healthy cow may excrete as many as 10^5 campylobacters per gram of faeces, the dose needed to infect humans may be quite small; for example, 500 campylobacters in a glass of milk was enough to produce illness (Robinson, 1981). Another volunteer swallowed one million organisms (Steele and McDermott, 1978) but the severity of symptoms did not seem to be related to the dosage. Thus even a few grains of heavily contaminated cow faeces reaching a bulk milk tank could be sufficient to produce an infectious dose in a glass of milk, even though it would not be detected in the whole tank. Moreover, the incubation period of campylobacter enteritis in humans is several days, so there is every chance that the milk will no longer be contaminated when samples are taken on the first day an outbreak is notified.

1.6.2. Water as a source. Outbreaks due to contaminated water affected 3000 people in Vermont, USA, in 1978 (Vogt *et al.*, 1982) and 2000 people in Sweden in 1980 (Mentzing, 1981). In the latter case the water supply came from deep ground water and was of generally good quality, filtered but not chlorinated, but it was situated near a large poultry farm and so could have been contaminated by campylobacters. Other causes of contaminated water may be sheep or seagulls, both of which harbour and excrete campylobacters (Fricker *et al.*, 1983; Skirrow, 1982). At a boarding school in England over 200 pupils and staff contracted campylobacter enteritis over a period of 8 weeks. The drinking water came from an open topped water tank which may have been contaminated by birds or bats (Palmer *et al.*, 1983); campylobacters isolated from two samples of water were of the same serotype as isolates from some of the patients (Palmer *et al.*, 1983; Penner *et al.*, 1983).

1.6.3. Birds as a source. Campylobacter infection or colonisation is common in ducks (Leuchtefeld *et al.*, 1980), pigeons (Robinson and Jones,

1981), rooks (Skirrow, 1982), seagulls (Fricker *et al.*, 1983), blackbirds, starlings and many other birds (Smibert, 1969) and may be the source of contamination of streams, rivers and ponds. In one investigation (Leuchtefeld *et al.*, 1980) 35% of 445 healthy ducks were found to be excreting *C. jejuni*. Ducks that fed on mud at the bottom of rivers and ponds were much more often infected (66%) than ducks feeding mainly on green food on the surface (16%). In another investigation (Report, 1979), waterfowl contaminated a riverside meadow with *C. jejuni*, dogs roaming the meadow became infected with the same serotype, and the infection then spread to man (Khan, 1982). Seagulls seem often to be infected mainly with *C. lari* or *C. coli* types (Fricker *et al.*, 1983).

Poultry, especially chickens and turkeys, are often heavily infected or colonized with campylobacters (Bruce *et al.*, 1977; Simons and Gibbs, 1979; Park *et al.*, 1981; Smeltzer, 1981; Shanker *et al.*, 1982; Christenson *et al.*, 1983). For example in Sydney, Australia, 52-100% of carcasses in three poultry plants were infected with campylobacters (Shanker *et al.*, 1982), as were 94% of chickens in an abbatoir in Queensland (Smeltzer, 1981). When the poultry reaches the point of sale in shops and stores, contamination is still present (Simons and Gibbs, 1979; Park *et al.*, 1981; Skirrow, 1982) and when they reach the kitchen the organism can be spread to cutting boards, sinks, dishes, cloths and other items, and also to kitchen workers' hands (Dawkins *et al.*, 1984; De Wit *et al.*, 1979). Kitchen workers who handle raw chicken may be stricken with campylobacter enteritis (Oosterom *et al.*, 1984), and may occasionally become temporary carriers, excreting campylobacters in their faeces (Norkrams and Svedhem, 1982). However, in spite of all the evidence of poultry contamination throughout the world, the number of outbreaks due to eating chicken is relatively small. It is probable that thorough cooking destroys contaminating campylobacters, and only those who handle raw carcasses or eat undercooked chicken are in danger of infection.

1.6.4. Animal carcasses as a source. Campylobacters can usually be isolated in large numbers from animal carcasses in abattoirs (Bolton *et al.*, 1982). In one investigation in northern England, campylobacters were isolated in 32% of cattle, 70% of sheep, and 56% of pig carcasses. When the

survey was extended to include wholesale and retail butchers' premises, however, the picture was quite different (Bolton *et al.*, 1982). 170 swabs at the wholesale butchers' and 100 at 20 retail shops taken weekly for 6 months were all negative. Campylobacters are oxygen sensitive and tend not to survive exposure in cold storage at the abattoir or in butchers' premises. Also, *C. jejuni* is rapidly inactivated at 50°C, so there would be no risk from cooked meat (Gill and Harris, 1982).

1.6.5. Pets as a source. There are many reports of campylobacter enteritis in humans where infected pets seem to have been the source of infection (Morbidity Mortality Report, 1978; Skirrow *et al.*, 1980; Skirrow, 1981; Skirrow, 1982). Dogs (usually puppies) are most commonly found to be infected, but it is often not clear whether the organism is pathogenic to the dog or not. In gnotobiotic puppies, experimental infection with campylobacters caused a mild non-invasive colitis (Holt, 1981). It is also clear that when dogs are kept together campylobacters can spread among them. When infection is traced to a cat, the story is very often of a recently acquired kitten, which suffers from diarrhoea shortly after its arrival in the family circle and after a few days one or more of the family members catch the disease (Blaser *et al.*, 1980; Skirrow *et al.*, 1980; Skirrow, 1982).

1.6.6. Epidemiology in the developing world. Campylobacter infection seems not to spread very readily from person to person in families or institutions, although the evidence varies a little from outbreak to outbreak (Rishpon *et al.*, 1984). In the developing world, where water supplies and sanitation are of a low standard and where living conditions are very crowded, campylobacters may spread more readily than in the developed world. In the Gambia (Billingham, 1981), Nigeria (Olusanya *et al.*, 1983), Bangladesh (Glass *et al.*, 1983) and Zaire (De Mol *et al.*, 1983), campylobacters are reported to be among the commonest organisms causing diarrhoea, particularly affecting the youngest children in the villages. It is less frequent in adults, possibly because they have acquired immunity to the most prevalent strains, but the carrier rate in adults is higher than in the developed world. The differences in clinical and epidemiological features of *C. jejuni* infection in developed and developing countries may be explained by differences in characteristics of the aetiological agent, or of the

populations, or both. In developing countries, the high frequency of asymptomatic infections suggests that transmission of the organism is intense early in life, or that it may not be pathogenic in the population.

According to the review by Christie (1987), evidence for the pathogenicity of *Campylobacter jejuni* isolates from developing countries may be summarized as follows. (1) *C. jejuni* is a frequent cause of traveller's diarrhoea in visitors from developed countries (Speelman and Struelens, 1983), and the characteristics of these infections, including bloody diarrhoea, are similar to those experienced by persons with campylobacter enteritis acquired in developed countries. (2) No biochemical characteristics have been shown to distinguish isolates from persons infected in developed and developing countries. (3) Many of the most common serotypes of *C. jejuni* present in developed countries are also present in developing areas. (4) In two seperate surveys, isolation of *C. jejuni* in children in developing areas was associated with prior diarrhoeal illness (Bokkenheuser *et al.*, 1979; Blaser *et al.*, 1980), especially in children under 9 months old. Therefore, most evidence to date suggests that isolates from the developing and developed countries are similar, implying that the differences in epidemiology and natural history of *C. jejuni* infections may largely be explained by differences in host characteristics.

The most important identifiable host characteristic that might distinguish populations in the two areas is immunity to *C. jejuni*. In developing countries, both the prevalence of infection (Glass *et al.*, 1983a) and the case:infection ratios (Glass *et al.*, 1983b) are inversely correlated with age, suggesting that immunity is being acquired. Shortened duration of convalescent excretion in developing areas also suggests acquisition of immunity, and laboratory evidence indicates that persons in developing countries acquire specific antibodies to campylobacters early in life and that these antibodies persist. Serum antibody responses to *C. jejuni* cell surface antigens were studied in healthy children from Bangladesh and the United States (Blaser *et al.*, 1985). For each age group studied, *C. jejuni*-specific antibody levels were significantly higher in Bangladeshi children and *C. jejuni*-specific serum IgG and IgM levels were already significantly higher in children under 1 year of age than in comparable U.S. children, a

difference which was accentuated in the second year of life. Among the Bangladeshi children, specific IgA levels rose linearly with age, IgG levels peaked in the 2-4 year age group and then fell, and IgM levels reach a plateau level by the 2-4 year age group. In population-based studies in Thailand similar immunological data was observed (Blaser *et al.*, 1986); differences in serum IgA and IgG levels in this study were interpreted as reflecting the effects of gut immunity. Recurrent exposure to *C. jejuni* during childhood in Bangladesh and Thailand may lead to progressive gut immunity as evidenced by high serum levels of specific IgA. Decreased levels of IgG may reflect the fact that gut immunity becomes so complete in children over the age of 5 years that IgG is no longer stimulated. These data support the hypothesis that differences in the clinical and epidemiological features of *C. jejuni* infections in developed and developing countries reflect host rather than organism characteristics. Mild or absent symptoms in *C. jejuni*-infected persons in developing countries may reflect infection in a partially or completely immune host.

1.6.7. Flies as a source. Campylobacters have been cultured from flies; in 2.8% of flies caught in an English country garden (Wright, 1983) but in 28.4% of flies near farms in Norway (Rosef and Kaperud, 1983). Flies are unlikely to be a source of infection when food hygiene is of a reasonable standard, but the presence of campylobacters in flies emphasizes how widespread contamination is. The level of contamination in the environment varies. In one investigation in England, 2% of women attending a city antenatal clinic had antibody against campylobacter in their serum, in a more rural area the percentage was 5, but in meat and poultry plants 18% of veterinary assistants had antibody and between 27 and 68% of regular workers (Jones and Robinson, 1981).

1.6.8. Risk for travellers. The environment or exposure to it varies in different parts of the world and travel abroad seems to be a hazard. In Finland (Pitkanen *et al.*, 1983) and in Sweden (Norkrans and Svedhem, 1982) most patients with campylobacter enteritis were apparently infected when on holiday abroad. The same is true of some patients in England (Skirrow, 1977). In most parts of Europe and America the infection rate is highest in summer (Blaser *et al.*, 1979), but in Hong Kong (Pitkanen *et al.*,

1983) and Israel (Shmilovitz *et al.*, 1982) it is highest in the winter months. Why this should be so is not known.

1.6.9. Epidemiology in neonates. Anders *et al.* (1979) reported that 8 full-term normal infants passed blood in the stools between 2 and 11 days after birth but had no abdominal tenderness, were afebrile and all recovered. *C. jejuni* was recovered from five of the mothers, though only one had a history of diarrhoea five weeks earlier. Infection apparently spread from mothers to infants rather than in the nursery (Anders *et al.*, 1979). In cases where campylobacters have been implicated as the cause of neonatal meningitis, all seem to have been infected perinatally. Intrauterine infection may also occur (Vinzent *et al.*, 1947; Gilbert *et al.*, 1981, Gribble *et al.*, 1981). For example, blood cultures from a baby delivered by caesarian section from a mother with diarrhoea were positive for *C. jejuni*, suggesting intrauterine infection. The baby had diarrhoea on the third day of life and *C. jejuni* was isolated from the faeces of mother and child. Campylobacter infection early in pregnancy may cause death of the foetus (Gribble *et al.*, 1981).

1.7. Clinical manifestations of *C. jejuni/coli* infection.

As with other intestinal pathogens, infection with campylobacters does not always produce symptoms (Butzler and Skirrow, 1979); asymptomatic excreters and mild cases can be found among the close contacts of affected people. At the other extreme, deaths have been reported, but these have usually been in elderly or debilitated patients. Severe illness may occur in otherwise healthy young people, usually of a self-limiting, if unpleasant attack of acute diarrhoea lasting for a few days. The incubation period is normally 3-5 days (rather longer than with other enteric infections), but may be as little as 1.5 and as long as 7-10 days. The source and timing of infection can seldom be pinpointed, however, so these figures are estimated largely on circumstantial evidence or volunteer studies (Robinson, 1981; Steele and McDermott, 1978).

In about half of all patients diarrhoea is preceeded by a febrile prodromal period with some or all of the following symptoms: malaise, headache, dizziness, backache, myalgia, abdominal pain, and sometimes

rigors. Body temperature is commonly raised to 40°C, and if very high there may be confusion or delirium (Rettig, 1979). Central abdominal pain may occur at any time during this prodromal period, which lasts typically from a half to one day, but ranges from a few hours to a few days. Eventually the abdominal pain becomes colicky and this heralds the onset of diarrhoea. The stools become liquid, foul smelling, often bile stained and then watery. Fresh blood sometimes appears in the stools after a day or two, and most acute samples that are examined microscopically contain inflammatory cellular exudate. Nausea is common but vomiting occurs only in a minority of patients and is seldom protracted. Many patients experience a feeling of urgency or incontinence, sometimes precipitated by sudden changes in position, such as turning over.

The acute diarrhoea lasts for about 2 to 3 days, by which time the patient is somewhat dehydrated and feels exhausted (Christie, 1987). Bowel actions gradually become fewer and the stools semi-formed, but characteristically the abdominal pain and discomfort persist and in some cases may be distressing. The patient is usually anorexic and only able to take fluids. Sometimes relapses occur, but they are usually milder than the first attack. The faeces of patients who are not given chemotherapy remain culture positive for about 3-5 weeks after an attack of campylobacter enteritis. Mild cases may excrete the organism for only a few days, and a few cases excrete for much longer.

Children tend to be less severely affected than adults, but they sometimes suffer from a relapsing or persistent type of disease (Karmali and Fleming, 1979). A notable feature is the presence of blood in the stools usually 2-4 days after the onset of symptoms. Most children over the age of 3 months have fever and some have abdominal pain without diarrhoea which may persist for up to six weeks. In hot climates dehydration may also be a feature of the disease.

The intensity of abdominal pain coupled with abdominal tenderness can bring the patient, particularly young adults or older children, to a surgical bed with a diagnosis of acute peritonitis. A few have acute appendicitis secondary to congestion and oedema of the bowel caused by

campylobacter infections, but most do not. Those that proceed to surgery are found to have acute inflammation of the ileum, jejunum and associated mesenteric lymph nodes. These appearances can be very striking, so much so that in two reported instances they led to a mistaken diagnosis of typhoid fever (Butzler and Skirrow, 1979). Such patients are not helped by surgical intervention. Sometimes babies with campylobacter enteritis pass blood in the stools without having diarrhoea. This has led to a mistaken diagnosis of intussusception, resulting in laparotomy. Other complications of *C. jejuni* infection include cholecystitis (Daring *et al.*, 1979), pancreatitis (Castilla-Hiuero *et al.*, 1989), hepatitis (Reddy *et al.*, 1983), peritonitis (Pepersack *et al.*, 1982), urinary tract infection (Davies and Penfold, 1979), haemolytic uraemic syndrome (Chamovitz *et al.*, 1983), thrombotic thrombocytopenic purpura (Morton *et al.*, 1985), urticaria (Pitkanen *et al.*, 1983), erythema nodosum (Eastmond and Read, 1982), osteitis (Pedler and Bint, 1984), carditis (Pitkanen *et al.*, 1980), Guillain-Barre syndrome (Rhodes and Tattersfield, 1982), reactive arthritis, (Kosunen *et al.*, 1981; Pitkanen *et al.*, 1980, Bengtsson *et al.*, 1983), abortion (Gilbert *et al.*, 1981), meningitis (Goossens *et al.*, 1986), and septicaemia (Mascart and Gottignies, 1979).

1.8. Pathology.

In people who have died of campylobacter enteritis and septicaemia, the macroscopic pathology consists of haemorrhagic inflammation, and congestion of the jejunum and first half of the ileum. Oedema and inflammation of the ileum have also been described at laparotomy for suspected acute appendicitis. In a significant proportion of patients, stools contain fresh blood, pus, or mucus, and this suggests that colitis and colorectal inflammation are not uncommon in campylobacter infection. Although infection seems to start in the small intestine, the disease is really an acute enterocolitis and spread into the colon and rectum is usual (Skirrow, 1984). Laparotomy, sigmoidoscopy, colonoscopy, and post mortem examinations carried out on patients who have succumbed to the disease have shown that mucosal damage may occur during the course of infection. The pathological features may vary from small changes in the mucosa (Lambert *et al.*, 1979) to inflammation and oedema of the full thickness of the intestinal wall (Skirrow, 1977), haemorrhagic lesions or frank necrosis

(Evans and Dadwell, 1967), gangrene and perforation (Stephenson and Cotton, 1985).

The microscopic histology of mucosal biopsies from patients with campylobacter enteritis is indistinguishable from that of salmonellosis or shigellosis (Skirrow, 1986). Tissue injury is observed in the jejunum, ileum and colon, with similar microscopic pathology. Van Spreeuwel *et al.* (1985) concluded that campylobacter infections produce the histological picture of acute infectious colitis and demonstrated invasive organisms in the colonic mucosa. There is an acute inflammatory reaction consisting of focal collections of polymorphonuclear leukocytes in the lamina propria and lumen of mucosal capillaries, often with crypt abscess formation. Mucosal oedema is seen as a separation of the crypts of Lieberkuhn and a widening of the gap between crypts and the muscularis mucosae. The epithelium may be flattened and sometimes eroded. Late in the disease the presence of chronic inflammatory cells gives rise to histology indistinguishable from ulcerative colitis (discussed in Chapter 5). The clinical picture and the propensity of campylobacter infection for young adults makes differential diagnosis with ulcerative colitis (Price *et al.*, 1979) and Crohn's disease (Loss *et al.*, 1980) difficult. Histological examination of rectal biopsies from *C. jejuni*-infected patients indicates a range from normal to inflammatory changes, suggestive of acute infectious colitis or inflammatory bowel disease (Lambert *et al.*, 1979).

1.9. Treatment and resistance.

The corner stone of treatment of all acute diarrhoeal illnesses is replacement of faecal volume losses (Nalin *et al.*, 1979). The current consensus is that antibiotic therapy (for 5-7 days) is indicated in patients who are acutely ill with enteritis and have persistent fever, bloody diarrhoea, more than eight bowel movements per day or significant volume loss, and in patients with symptoms that persist for more than one week (Blaser, 1989; Butzler and Skirrow, 1979). Patients with mild symptoms need not be treated.

A large number of *C. jejuni* and *C. coli* isolates have been tested *in vitro* for susceptibility to antimicrobial agents (Vanhoof *et al.*, 1978).

Virtually all organisms are susceptible to clinically achievable concentrations of aminoglycosides, chloramphenicol, furazolidone and carbenicillin. *C. coli* strains are often resistant to erythromycin, clindamycin and tetracycline whereas *C. jejuni* are usually susceptible (Wang *et al.*, 1984). Trimethoprim-sulphamethoxazole and ampicillin should not be used for empiric treatment since many strains are resistant.

The agent likely to be most useful for *C. jejuni* enteritis is erythromycin; this has the advantage of a narrow spectrum of activity and low toxicity so that high enough serum concentrations can be achieved to enable the antibiotic to act on organisms in the tissues. Erythromycin stearate, which is acid resistant, is widely used because some is converted to the active base in the duodenum (i.e. before absorption), where it exerts a direct effect on the infecting organism (Butzler and Skirrow, 1979; Christie, 1987). Tetracyclines are also effective for treatment of enteritis. Bacteraemia and other systemic infections should also be treated with antibiotics. Erythromycin has been used with success in such cases, but because of the problem of resistance in a minority of strains it is advisable to give an antibiotic with more certain coverage in terms of pharmacology. Gentamicin has been used successfully in this context; the fact that it has to be given parenterally is of no disadvantage since this route would be preferred in such a patient. Chloramphenicol is also effective in this setting.

1.10. Host defence and Immunity.

C. jejuni is susceptible to low pH, and in volunteer studies ingestion of organisms with sodium bicarbonate was associated with a higher rate of illness than ingestion with milk (Black *et al.*, 1988). Severe or fatal campylobacter enteritis in patients receiving antimotility agents indicates the importance of peristalsis as a non-specific host defence (Smith and Blaser, 1985). Disturbances in ileal myoelectric activity in rabbits infected with *C. jejuni* suggest that the organism may also induce alterations in gut motility and disrupt normal peristalsis (Sninsky *et al.*, 1985). *C. jejuni* isolates are generally susceptible to bactericidal activity due to complement and natural antibody of normal human serum (Pennie *et al.*, 1986; Blaser *et al.*, 1985), although isolates from extraintestinal sites exhibit greater serum

resistance than gastrointestinal isolates (Blaser *et al.*, 1986). Bacteraemia with serum sensitive organisms occasionally occurs, particularly in debilitated hosts (Blaser *et al.*, 1986). Convalescent sera from patients infected with *C. jejuni* demonstrate greater bactericidal activity against homologous organisms than non-immune sera, but fail to exhibit enhanced activity against heterologous isolates (Pennie *et al.*, 1986).

1.10.1. Antigens recognised in infection. *C. jejuni*-specific serum IgA, IgM, and IgG levels rise and remain elevated for several weeks following acute infection (Blaser and Duncan, 1984). A 44 kDa porin protein, a 62-63 kDa flagellar protein and a 29kDa surface protein are consistently recognised by convalescent human sera (Blaser *et al.*, 1984; Blaser and Duncan, 1984; Nachamkin and Hart, 1985). The 29 kDa and 62-63 kDa proteins in particular have high ratios of antigenicity to molar representation in the outer membrane, and thus may be immunodominant antigens (Nachamkin and Hart, 1985). IgA, IgG and IgM antibodies are detectable in sera from healthy as well as convalescent persons (Blaser *et al.*, 1984), which suggests that large segments of the population have been exposed to *C. jejuni* or related organisms, or that antigenic determinants are shared by *C. jejuni* and other commonly encountered Gram negative organisms. *C. jejuni* infection also induces a serologic response to both homologous and heterologous LPS (Perez-Perez and Blaser, 1987); the heterologous response may be due to shared core LPS antigen. Humoral immune responses to enterotoxin have been reported in Mexican and Japanese patients (Ruiz-Pallacios *et al.*, 1985; Honda *et al.*, 1986), but antibody responses to enterotoxins or cytotoxins have not been demonstrated in infected persons with inflammatory *C. jejuni* enteritis in the USA (Perez-Perez *et al.*, 1987). Rising titres of intestinal IgA have also been described (Winsor *et al.*, 1986) particularly directed against the 62-63 kDa flagellar antigen, the 44 kDa porin and a 58 kDa antigen (Winsor et al., 1986).

1.10.2. Epidemiological evidence for immunity. Several epidemiological studies suggest that immunity against *C. jejuni* is acquired as a result of one or more infections. For example, Glass *et al.* (1983) demonstrated a progressive decrease in the illness/infection ratio with

increasing age among children in Bangladesh, and Black *et al.* (1985) reported similar results among children in Peru. The development of immunity was also suggested by Blaser *et al.* (1985) who conducted an age-stratified analysis of antibodies to *C. jejuni* and found elevated levels in healthy children from Bangladesh compared to those from the USA. They also showed that persons who drank raw milk regularly, and therefore presumably have multiple exposures to *C. jejuni*, have persistent elevation of anti-campylobacter IgG levels and little or no illness compared with persons exposed for the first time (Blaser *et al.*, 1987). Evidence for the acquisition of immunity as a result of natural infections was confirmed in volunteer studies (Black *et al.*, 1985).

1.10.3. Antibody as the mediator of immunity. Several lines of evidence suggest that specific antibodies are an important host defence against *C. jejuni* infection. Severe, recurrent, prolonged, or extraintestinal infections with *C. jejuni* have occurred in persons with congenital or acquired hypogammaglobulinaemia (Johnson *et al.*, 1984). Genetic influences on ability to respond to *C. jejuni* infection may also be present (Panday and Blaser, 1986). Prolonged excretion also occurs in patients with IgA deficiency, which suggests a primary role for humoral immunity against campylobacter infection (Johnson *et al.*, 1984; Melamed *et al.*, 1983). Antibodies in serum are formed against flagella and other surface antigens that may be involved in colonisation. Short term homologous reinfection immunity in human volunteers rechallenged with *C. jejuni* was associated with elevated *C. jejuni*-specific intestinal IgA (Perez-Perez *et al.*, 1987). In a study by Mascart-Lemone *et al.* (1985), IgA antibody to *C. jejuni* in serum samples from almost 400 healthy people and approximately 60 patients presenting with acute enteritis was examined. In healthy individuals, the frequency of detection of IgA antibodies in serum increased significantly with age, from less than 10% in children under 1 year to 37% in 20 year old adults and 75% in elderly people. In those healthy people, anti-campylobacter IgA was mainly monomeric IgA1. In patients, however, polymeric IgA1, the form synthesized primarily in the intestinal lamina propria, was predominant. In addition, analysis of an outbreak of *C. jejuni* enteritis associated with raw milk indicated that chronic raw milk consumption and elevated levels of *C. jejuni* specific serum antibodies were

related to immunity to symptomatic infection (Blaser *et al.*, 1987).

C. jejuni is not a common pathogen among patients with the acquired immune deficiency syndrome (AIDS) indicating that cell-mediated immunity may be a less important host defence against *C. jejuni* than against *Salmonella* species. Persistent *C. jejuni* infection in AIDS patients has been associated with a defective humoral response to the organism (Perlman *et al.*, 1988).

1.10.4. Animal models of immunity. Rabbits challenged using the RITARD (removable intestinal tie adult rabbit diarrhoea) procedure (Caldwell *et al.*, 1983) with 10^4-10^5 *C. jejuni* organisms exhibited increased IgG, IgM, and secretory IgA. On rechallenge, levels of all these immunoglobulins rose. The size of the inoculum seems to influence the triggering of different immune responses. Low and high doses confer protection against the second homologous strain, whilst high doses trigger an intense systemic and local response. In this model secretory-IgA seems to be involved in immunity and T cell clones can be analyzed for the bacterial components ability to induce helper T cells.

Infant Balb/c mice were protected from colonisation by *C. jejuni* by an anti-flagellar antiserum (Newell and Dolby, 1985). Also in the Balb/c mice model, polymorphs seem to be important for translocation of *C. jejuni* in the blood stream (Bar and Hewel, 1985). Opsonization with immune sera or monoclonal antibodies enhanced phagocytosis. *C. jejuni* is not killed by polymorphonuclear leukocytes in the presence of heat inactivated normal serum (Blaser *et al.*, 1989), but in the presence of immune serum some strains are killed (Pennie *et al.*, 1986). *C. jejuni* also appear to survive and multiply within mononuclear phagocytes in the absence of immune serum. Phagocytosis may thus facilitate survival of *C. jejuni* (Kiehlbach *et al.*, 1985).

1.11. Aims of the project.

The mechanisms of virulence, especially with regard to the diarrhoeal process caused by enteric campylobacters, are not known, and the aims of this project were to identify bacterial virulence factors involved in the

disease and to start to characterise these determinants at the molecular genetic level. The initial aim was to make a gene library of selected strains in a cosmid vector, and screen the library with DNA probes for the presence of a cholera-like enterotoxin. The bacterial factors responsible for resistance to erythromycin were also to be investigated using a genetic approach. However, the difficulty of cloning *C. jejuni* DNA into vectors primarily derived from *E. coli*, and the lack of expression of such DNA halted this approach and was abandoned. An alternative was to investigate the pathophysiology of the disease, using *in vitro* and *in vivo* models. Such models would prove to be essential in screening any genetically defined mutants that could be generated subsequently. It would also help to define the interaction of the organism with the host, and try to identify disease mechanisms, at the time not known. Also by defining phenotypes of pathogenic strains, mutants could be screened for alteration of defined phenotypes. Other workers in our group are now in the process of generating mutant *C. jejuni* strains by polymerase chain reaction generated fragments of campylobacter genes. *C. jejuni/coli* strains with different clinical characterisation were assessed for the ability to colonise and invade cell lines as an indicator of the potential for tissue damage seen in the natural disease. Thus strains are characterised for their ability to initiate infection and to look for distinct bacterial characteristics that may cause a clinical presentation of colitis or watery diarrhoea.

The rabbit ileal loop model was used as a source of infected tissue in order to study the diarrhoeal response and the biochemical mediators of secretion activated by bacterial products or host secretagogues within infected tissue. In particular bacteria alone were assessed for their ability to raise cAMP and cGMP, possible mediators of the diarrhoea seen in the disease.

In order to investigate changes in infected cells exposed to viable whole bacteria or to bacteria-free supernatants, a role for protein phosphorylation of cellular ion channels or other proteins was assessed, along with investigations into a possible role of increasing intracellular calcium as a diarrhoeal mechanism.

The role of host secretagogues, particularly prostaglandin E2, in the diarrhoeal process and inflammation induced by infecting *C. jejuni* was also investigated.

Chapter 2

Adherence to and invasion of cultured cells by *Campylobacter jejuni* and *Campylobacter coli.*

2.1. INTRODUCTION

This chapter assesses the correlation of bacterial adhesion with disease symptoms, using the previously described HeLa cell association model (Fauchere *et al.*, 1986). It also investigates the use of the differentiated cell line Caco-2 (Grasset *et al*, 1985; Pinto *et al.*, 1983) as a relevant model for investigating *C. jejuni* interaction with enterocytes, with emphasis on adhesion, invasion and bacterial transcytosis (Mostov and Simister, 1985; Finlay *et al.*, 1988) through the host cell.

2.1.1. Adherence. Adherence to target cells is a key factor for disease initiation in pathogenic bacteria (Mims, 1982) and the evidence for *C. jejuni* cell adherence is summarised below. The literature is confused, but the general consensus has revealed adherent strains in the cell culture systems used. Flagella, lipopolysaccharide (McSweegan and Walker, 1986), and surface proteins of 28 kDa and 32 kDa appear to be important in adherence of *C. jejuni* to HEp-2 epithelial cells *in vitro* (De Melo and Pechere, 1990). Only the 32 kDa protein was associated with cell invasion. HeLa cell adherent strains have been isolated more frequently from patients with fever and diarrhoea than from patients without these symptoms (Fauchere *et al.*, 1986). This study demonstrated that bacteria were internalised in endocytic vacuoles within the cell. In a study using the intestinal cell line INT 407 (McSweegan and Walker, 1985), two classes of strain, adherent and low adherent, were recognised. Treatment with proteases reduced adherence by 50-60%. Adherence was also inhibited by fucose, mannose and rabbit mucus gel. HeLa cell studies also revealed associative and non-associative phenotypes among isolates of which 40% were correlated with infectious diarrhoea (McSweegan and Walker, 1986). HeLa cell adherence requires a host cell active contribution, is inhibited by 2,4-dinitrophenol and cytochalasin B, and leads to cytotoxic effects, although toxin production was not determined (Rosenau *et al.*, 1987). Bacteria were internalised but did not multiply. Association with HeLa cells involves a protein adhesin,

variously described as 25, 28 to 31 or 32 kDa, which elicits an immunological response in infected patients (Skirrow, 1986). Fibronectin amino and 120/140 kDa carboxy terminals mediate the attachment of *C. jejuni* to epithelial cells, and laminin and types 1, 3 and 5 collagen mediate adherence to extracellular matrices (Moran *et al.*, 1989). Workers using various cell systems, have studied the role of flagella in adherence (Newell and Pearson, 1984). Flagella and the major outer membrane protein (MOMP) were needed for adherence and invasion in HeLa cells, and studies using INT 407 cell lines concluded that these cells express a receptor for an adhesin present on the flagella. This attachment was inhibited by glucose, galactose, fucose, mannose and N-acetyl-glucosamine.

2.1.2. Invasion and translocation. The clinical and pathological characteristics of *C. jejuni* enterocolitis in developed countries and the occurrence of bacteraemia in occasional cases suggest that tissue invasion could be a mechanism by which *C. jejuni* causes disease (Cover and Blaser, 1989). Tissue and bloodstream invasion have been demonstrated in several animal models (Fox, 1982). Histologic evidence of human tissue invasion is sparse (Price *et al.*, 1984) but was demonstrable in one study (Van Spreeuwel *et al.*, 1985). *C. jejuni* fails to cause conjunctivitis in the Sereny test (Manninen *et al.*, 1982; Sereny, 1957). *C. jejuni* can translocate to mesenteric lymph nodes in gnotobiotic mice (Youssef *et al.*, 1985), a property associated with invasiveness.

In a study of invasion of HEp-2 cells growing on Sepharose beads (Soto *et al.*, 1989), it was concluded that lipopolysaccharide may play a role in moderation of adherence and outer membrane proteins may be important for invasion. De Melo *et al.* (1989) also demonstrated invasion of HEp-2 cells; bacteria were seen in endocytic vacuoles in which phagosome-lysosome fusion occurred with a decrease in bacterial viability. Oelschlaeger *et al.* (1991), investigating invasion of Henle 407 cells, concluded that uptake was reduced by inhibitors of coated pit formation, and by calmodulin inhibitors such as stelazine. Preventing *de novo* bacterial protein synthesis using chloramphenicol also inhibited cell entry. In contrast with other invasion systems, *C. jejuni* uptake is not dependent on microfilaments (Oelschlaeger *et al.*, 1991). Invasion of HeLa cells (Moyen *et al.*, 1985; Manninen, 1982)

depends on temperature of incubation and the viability of the organisms. Synergistic interaction between *C. jejuni* and other enteropathogens such as *S. typhimurium, E. coli*, and *Shigella* spp facilitates invasion of cell cultures (HEp-2 and A549) by *C. jejuni* (Bukholm and Kaperud, 1987).

2.1.3. Role of flagella in adherence and invasion. There has been much debate about whether bacteria adhere to or merely colonize mucus in the course of natural infection (Lee *et al.*, 1983). In a mouse model, *C. jejuni* colonises the mucus layer and crypts of the intestinal mucosa. Motility was found to be important along with oxygen tension. Therefore colonization of mucus was thought to be important in disease initiation, while specific attachment was thought to be unnecessary. *C. jejuni* spontaneously undergoes bidirectional transition between flagellated and aflagellate phenotypes (Caldwell *et al.*, 1985). Growth in culture favours emergence of the aflagellate phenotype at a rate of about 10^{-3} per cell generation, whereas growth in rabbit intestine strongly favours emergence of the flagellated phenotype (Caldwell *et al.*, 1985). Aflagellate clinical isolates of *C. jejuni* are rare, and stool cultures from volunteers fed a mixture of flagellated and aflagellate strains yielded only flagellated organisms (Black *et al.*, 1988). Motile, flagellated strains colonize the gastrointestinal tracts of experimentally infected mice more successfully than aflagellate strains (Newell and McBride, 1985), which further indicates the importance of flagella *in vivo*. The role of flagella in adhesion to, and penetration into eukaryotic cells was investigated using homologous recombination to inactivate the two flagellin genes *flaA* and *flaB* of *C. jejuni* (Wassenaar *et al.*, 1991). Mutants in which *flaB* but not *flaA* were inactivated remained motile, while a defective *flaA* gene lead to immotile bacteria. Invasion studies showed that mutants without motile flagella did not penetrate human intestinal cells *in vitro*.

2.1.4. Transcytosis. Methods have been devised to grow epithelial cell lines on permeable filters as impermeable polarised monolayers (Simons and Fuller, 1985), with a basolateral surface (adherent to the filter) and a nonadherent apical surface separated by tight junctions. Caco-2 cells form polarised monolayers with well defined brush borders, and domes of cells which actively transport fluid from the apical to the basolateral surface

mimicking human intestinal epithelium (Pinto *et al.*, 1983). Interaction of *Salmonella choleraesuis* and *S. typhimurium* with Caco-2 cells has been shown to be similar morphologically to epithelial infection in animal models (Finlay and Falkow, 1990), suggesting that this *in vitro* system may be useful to study pathogens that interact with and penetrate human intestinal epithelia. The ability of bacteria to pass from a cells apical to its basolateral surface is termed transcytosis (Mostov and Simister, 1985). In this chapter Caco-2 cells grown on semi-permeable filters are used to demonstrate transcytosis by *C. jejuni*.

2.2. MATERIALS AND METHODS

2.2.1. Bacteria. *C. jejuni* and *C. coli* strains used in this study were isolated from stools during routine bacteriological screening by staff at the Department of Microbiology, St Pieters University Hospital, Brussels. 21 strains were isolated from children with inflammatory diarrhoea. Colitis was confirmed by recto-colonoscopy and by light microscopic examination of biopsy specimens taken from the colon and/or rectum; in all cases mucosal hyperaemia was observed on recto-colonoscopy, and moderate to severe polymorphonuclear lymphocyte infiltration was detected in biopsy samples, with or without oedema and cryptitis. 23 strains were isolated from children presenting with apparent non-inflammatory diarrheoa; absence of colitis was confirmed either by normal recto-colonoscopy, or by failure to detect leucocytes or erythrocytes in Giemsa stained stool specimens. All faecal cultures were negative for *Salmonella*, *Shigella*, *Yersinia enterocolitica*, and enterotoxigenic or enteropathogenic *Escherichia coli*. *Campylobacter* strains were biotyped according to the Lior biotyping scheme (Lior, 1984). Isolates were stored both as freeze dried samples and at -70^{o}C in 10% glycerol broth; a new aliquot was used for each experimental procedure.

2.2.2. Growth media. Mueller-Hinton (MH) broth and agar (Oxoid) were used to prepare logarithmic cultures and for subculturing bacteria. Bacteria were incubated at 37^{o}C in an atmosphere of 6% hydrogen, 5% carbon dioxide, 5% oxygen and 84% nitrogen in a variable atmosphere incubator cabinet, or at 42^{o}C in an anaerobic jar with a nickel palladium

catalyst and a Campylobacter microaerophilic generating envelope (Oxoid).

2.2.3. Cell culture. HeLa cells (Flow Laboratories) were maintained in Modified Eagles Medium (MEM) with Earles salts containing 1% non-essential amino acids, 10% foetal calf serum (FCS) and glutamine without antibiotics. The Caco-2 (human colon carcinoma) cell line (Rousset *et al.*, 1984) was maintained in MEM with 10% or 20% FCS without antibiotics. Cells were routinely grown in flasks at 37°C in a 5% carbon dioxide humidified atmosphere. Confluent stock cultures were trypsinized and new stock cultures seeded at approximately 10^5 cells/ml.

2.2.4. Adherence to Hela cells. Glass coverslips were seeded with HeLa cells and grown to 75% confluence. Bacteria were harvested from logarithmic phase broth cultures, and resuspended in Hanks solution or MEM containing 10% FCS to a density of approximately 10^7 organisms/ml; exact titres were determined retrospectively on MH agar plates. Bacterial suspensions (0.5 ml) were added to coverslips in plastic trays, three coverslips for each strain tested, and incubated for 6 h to allow bacterial adherence. Monolayers were then washed with MEM containing 1% FCS and stained with carbol fuchsin or Giemsa stain. The association index (AI, adherent bacteria/cell) for each strain was determined according to the method of Fauchère *et al.* (1986). Association index values for 1 h incubations were determined by Dr. Herman Goossens, St Pieters University Hospital, Brussels.

2.2.5. Adherence to Caco-2 cells. Caco-2 cells grown on membrane filters (0.4 μm pore size) in a Transwell unit (Costar) were used at approximately 10-12 days post confluence. At this time the cells were fully differentiated, forming a polarised monolayer which exhibits defined apical and basolateral surfaces separated by tight junctions and with a transepithelial electrical resistance (Pinto *et al.*, 1983). Light microscopic analysis of bacterial adherence to Caco-2 cells was difficult because of lack of contrast in stained preparations. Therefore the method of Finlay *et al.* (1988), in which radiolabelled bacterial cultures were used to quantify cell adherence, was modified to screen the collection of *C. jejuni/coli* isolates. Bacteria in the mid-logarithmic growth phase were washed in phosphate buffered saline (PBS), and suspended in methionine assay medium (MAM,

Difco). Bacterial suspensions were incubated microaerophilically at 37°C for 30 to 60 min, pelleted by centrifugation and suspended in MAM containing 1.85 MBq/ml [^{35}S]methionine (Amersham, specific activity >29.6 TBq/mmol). After incubation for a further 60 min, bacteria were again harvested, washed thoroughly, and suspended to a density of 10^7 organisms/ml in MAM.

Aliquots of bacterial suspensions (5 µl) were added to polarised Caco-2 cell monolayers, either living to permit quantitation of adherent and invasive bacteria, or fixed with 3% glutaraldehyde in phosphate buffer so that bacterial invasion was inhibited, giving a measure only of adherence. After 6 h incubation with bacteria, monolayers were washed thoroughly in cold PBS, and the radioactivity associated with each filter was determined in Optiphase (LKB) scintillant in a Packard Tri-Star liquid scintillation spectrometer.

2.2.6. Cultured cell invasion. Confluent monolayers of Hela or fully differentiated Caco-2 cells in 24-well plastic tissue culture plates (Nunc) were infected with approximately 10^7 bacteria. After incubation for 6 h to allow adherence and invasion, monolayers were washed with MEM, and incubated for 2 h in MEM containing 200 µg/ml gentamicin to kill extracellular bacteria. Monolayers were again washed thoroughly before lysis of cells with 0.5% sodium deoxycholate (Difco) in PBS, and serial dilution for viable counts of bacteria on MH agar.

2.2.7. Caco-2 cell transcytosis. The ability of *C. jejuni/coli* to pass through confluent polarised Caco-2 cell monolayers grown on semi-permeable filters in Transwell units (Costar) was determined as a measure of penetration of the host epithelial cell barrier (Finlay *et al.*, 1988). 10^7 log phase organisms in MEM were added to the top of the monolayers, incubated for 6-8 h, and the presence of bacteria in the wells beneath the filters was detected by culturing on MH agar. *E. coli* K-12 strain DH5α (Finlay *et al.*, 1988) was used as a non-penetrating control organism. Tight junctions in polarised monolayers were disrupted by incubation in calcium free medium (Flow), after which DH5α cells could be detected in the medium beneath the filter (in Transwells of 3 µm pore size only).

2.2.8. Statistical analysis. Distribution of values within groups of

strains was analyzed by the χ^2 test; $P < 0.05$ was considered as statistically significant.

2.2.9. Outer membrane protein preparation. Strain F132 was grown for 48 h in MH broth in a microaerophilic atmosphere. Bacteria were harvested by centrifugation at 10,000 x *g* for 20 min at room temperature, resuspended in 10 ml of 100 mM Tris.HCl (pH 7.8) containing 10 mM magnesium chloride, and disrupted by sonication. Non-disrupted organisms were removed by centrifugation at 9000 x *g* for 10 min at 4°C, and the supernatant fraction subjected to centrifugation at 100,000 x *g* for 1 h at 4°C. Pellets containing the total membrane fraction were resuspended in 1 ml of Tris-$MgCl_2$ buffer containing 2% (v/v) Triton X-100 to dissolve inner membrane proteins, and again subjected to centrifugation at 100,000 x *g* for 1 h at 4°C. Pellets containing outer membranes were resuspended as before, incubated at room temperature for 30 min and again centrifuged at 100,000 x *g* for 90 min at 4°C. Pellets were resuspended in 50 mM sodium phosphate buffer (pH 7.2).

2.2.10. Polyacrylamide gel electrophoresis (PAGE). Membrane proteins were boiled for 5 min in 20% glycerol-20% (w/v) sodium dodecylsulphate (SDS)-2.5% β-mercaptoethanol (Sigma) containing 2.5% (v/v) bromothymol blue (solubilisation buffer) in a final volume of 40 µl. Electrophoresis was performed on a 16% (w/v) gradient polyacrylamide separating gel with a 4% (w/v) stacking gel. Electrophoresis was at constant current (25 mA) at room temperature, and gels were visualised by staining with Coomassie brilliant blue.

2.2.11. Western blotting. Proteins separated by PAGE were electroblotted onto nitrocellulose paper (Cellulose nitrate E, Schleicher and Schuell) by the method of Towbin *et al.* (1979). Nitrocellulose was soaked overnight at 4°C in PBS containing 0.05% (v/v) Tween 20, incubated with primary antiserum (convalescent serum) diluted 1:200 in PBS-Tween for 30 min at room temperature and then with secondary antiserum (Sigma, rabbit anti-human IgG, IgM, IgA coupled to horseradish peroxidase) diluted 1:500 in PBS-Tween for 30 min at room temperature. After thorough washing, nitrocellulose was stained for 2 min in 30 mg 4-chloro-1-naphthol in 20 ml methanol to which 50 ml of 50 mM-Tris.HCl (pH 7.6) and 100 µl of 30% (v/v)

hydrogen peroxide had been added immediately before use.

2.2.12. Fluorescence and confocal microscopy. Caco-2 cells on glass coverslips were extensively washed with PBS, infected with *C. jejuni* strains (F132, C119, L115, N82, O81, P71 or NCTC 12189) at an inoculum of approximately 10^8 organisms/ml, and incubated in 5% CO_2 for 2, 4, 6, or 8 h. Monolayers were subsequently washed, fixed in 3% (v/v) formaldehyde in PBS, and permeabilised in 0.1% (v/v) Triton X-100 in PBS. After thorough washing, human convalescent or hyperimmune rabbit sera were added for 30 min at room temperature, followed by rabbit anti-human or goat anti-rabbit IgG respectively, coupled to fluorescein isothiocyanate (FITC, Sigma) for a further 30 min and washed again. Coverslips were mounted in glycerol-PBS and examined with a Zeiss AXIOPHOT 10 photofluorescence microscope and a MRC-600 confocal laser scanning microscope.

2.3. RESULTS

Detailed data for each *Campylobacter* strain are shown in Table 2.1, while Table 2.2 gives composite data for the two diagnostic groups.

2.3.1. HeLa cell association and invasion. Using AI values greater than 3.0 after 1-h infections as the criterion for HeLa cell association (Fauchere *et al.*, 1986), a statistically significant difference was observed between the two groups of strains (Table 2.2). Taking an association index of 3.0 as a seemingly arbitrary criterion to distinguish adherent from non-adherent strains is of little predictive value, since the majority of isolates from all clinical sources apparently did not adhere to HeLa cells in 1 h. Even taking an association index of 1 as the criterion for adherence (on the assumption that intracellular proliferation can result from invasion by a single bacterium), 30% of our colitis strains would still be considered non-adherent. Moreover, in this case there was no significant difference between the two groups of isolates (Table 2.2). Preliminary time course studies of infection indicated, in contrast with previously published data (Fauchere *et al.*, 1986), that adherence of the majority of strains tested increased to a maximum at about 6 h, at which time there was no significant difference between the two groups of strains (Table 2.2). Indeed, 12 of the 25 strains that were apparently non-adherent after 1 h (six *C. jejuni* from each diagnostic group, see table 2.1) were in fact observed to

adhere upon longer incubation. Conversely, five strains (P71, E116, V214, O69 and M233) that were positive for adherence at 1 h were negative at 6 h, perhaps because the ability to adhere is a reversible process, or because rapid adherence and subsequent invasion prevents further adherence by down-regulation of receptor proteins.

A statistically significant difference was observed between colitis strains and strains from non-inflammatory diarrhoea with regard to invasion of HeLa cell monolayers, as determined by recovery of gentamicin-protected (internalised) bacteria after 6 h incubation (Table 2.2). Among the non-inflammatory strains that did invade, however, viable counts of recovered bacteria were similar to the range observed for colitis strains (Fig. 2.1, panel a). In the majority of cases of invasion, less than 1% of the original inoculum was internalised (Table 2.1).

2.3.2. Caco-2 cell association. The human colonic carcinoma line Caco-2, which differentiates to give microvillated polarised monolayers (Pinto *et al.*, 1983), was investigated as an alternative model for the study of campylobacter adherence and invasion. Radioactively labelled bacteria were used for this test, primarily because adherent bacteria are difficult to visualise directly in stained preparations, but also because the method permits independent determination of adherence and invasion with gluteraldehyde-fixed and living monolayers respectively. All *C. jejuni/coli* strains in our collection adhered to Caco-2 cells (Table 2.1). Colitis isolates generally gave higher counts than non-inflammatory strains, but levels of adherence varied considerably between strains, and there was no significant difference in the mean levels of adherence between the two diagnostic groups with either gluteraldehyde-fixed or living cells (Fig. 2.1, panel b). With fixed cells, in which association represents surface attachment of bacteria only, the average cell-associated radioactivity was 474 cpm for colitis strains compared with 375 cpm for isolates from non-inflammatory diarrhoea. With living Caco-2 cells, associated counts were generally lower than with fixed cells (154 and 100 cpm for colitis and non-inflammatory strains, respectively). This may reflect changes in cells due to the process of fixation, or the dynamic process of transcytosis (see below) permitting release of labelled bacteria from the monolayers. Kinetic studies revealed that adherence to Caco-2 cells occurred within 4 h of infection, depending on

the strains used (data not shown).

2.3.3. Caco-2 cell invasion. A statistically significant difference was observed between colitis and non-inflammatory strains with regard to invasion of Caco-2 cell monolayers, as determined by recovery of gentamicin-protected bacteria after 6 h incubation (Table 2.1). Among the non-inflammatory strains that did invade, however, viable counts of recovered bacteria were similar to the range observed for colitis strains (Fig. 2.1, panel c). The proportion of the original inoculum internalised was somewhat greater than with HeLa cells, but there is a strong correlation between the extent of invasion of the two cell types (Fig. 2.1, panel d). Kinetic studies with representative strains indicated that invasion of Caco-2 cells occurred between 2 and 4 h (data not shown).

2.3.4. Transcytosis through Caco-2 monolayers. There was a statistically significant difference between the ability of colitis and non-inflammatory isolates to pass through intact Caco-2 cell monolayers from the apical to the basolateral surface (Table 2.2). Bacteria were recovered in the medium below the filter in numbers from 10^2 to greater than 10^4/ml, 4 to 6 h after infection (Fig. 2.2). However, since culture conditions in this test are suboptimal for campylobacter growth and survival, exact figures were not determined for each strain. Instead, simply the presence of bacteria in the lower compartment was taken as a qualitative indicator of transcytosis. It should be noted that six strains (B415, E230, H104, K105, O69 and D265, see Table 2.1) capable of penetrating the polarised Caco-2 cell monolayer were found by other criteria to be non-invasive. Further experiments are required to establish whether these strains pass directly through cell junctions rather than by an intracellular pathway.

2.3.5. Western blotting. To investigate the possibility of using immunological techniques to determine bacterial association with cultured cells, outer membrane proteins of strain F132, isolated from a patient with inflammatory diarrhoea, were separated by polyacrylamide gel electrophoresis and Western blotted using convalesent serum from the same patient. Among the proteins recognised were the flagella protein (67 kDa), major outer membrane protein (45 kDa) and the recognised adhesin of 29 kDa (Fig. 2.3.). A large number of other proteins were also recognised but

Footnotes

[a] Association indices (bacteria per cell) determined at 1 and 6 h (numbers are averages from at least three independent experiments).

[b] Viable bacteria recovered after gentamicin treatment of infected HeLa cells (numbers are averages from at least three independent experiments).

[c] Radioactivity (cpm) associated with monolayers infected with radiolabelled (^{35}S) bacteria (numbers are averages from at least three independent experiments).

[d] Viable bacteria recovered after gentamicin treatment of infected Caco-2 cells (numbers are averages from at least three independent experiments).

[e] Recovery of bacteria beneath polarised Caco-2 monlayers (experiments were performed at least three times).

[f] n.d., not done.

Table 2.1. Characteristics of individual clinical *C. jejuni* and *C. coli* isolates.

Strain number	HeLa association[a] 1 h	6 h	HeLa invasion[b]	Caco-2 association[c] live	fixed	Caco-2 invasion[d]	Caco-2 transcytosis[e]
(i) colitis							
C. jejuni biotype I							
A259	3.3	3.8	2.8×10^3	126	265	2.8×10^4	+
D246	1.6	3.1	2.1×10^3	170	538	1.4×10^4	+
H132	1.3	4.7	9.0×10^4	513	331	1.1×10^6	+
K85	0.1	3.9	2.4×10^3	137	318	5.3×10^3	+
L115	3.2	4.2	2.3×10^4	116	232	8.3×10^5	-
M288	1.4	4.5	2.1×10^2	85	108	2.1×10^2	+
P71	3.8	2.6	1.7×10^3	77	227	2.3×10^4	+
R143	0.6	3.8	1.5×10^2	91	617	1.0×10^3	+
T82	0.1	4.4	4.4×10^3	136	1147	7.3×10^3	+
C. jejuni biotype II							
A143	4.6	4.2	7.8×10^1	90	122	7.3×10^2	+
B404	2.3	1.8	1.4×10^4	99	628	1.4×10^4	+
C119	4.3	4.1	9.0×10^3	266	168	1.2×10^4	+

Table 2.1 (continued).

Strain number	HeLa association 1 h	HeLa association 6 h	HeLa invasion	Caco-2 association live	Caco-2 association fixed	Caco-2 invasion	Caco-2 transcytosis
D217	4.3	3.0	1.0×10^4	105	137	1.4×10^4	+
E116	4.4	1.2	1.3×10^4	102	234	1.3×10^6	+
K131	0.1	1.2	2.2×10^3	63	172	3.6×10^2	+
O81	0.1	0.3	1.1×10^3	30	420	1.2×10^3	-
V214	4.1	0.0	2.2×10^4	78	30	1.9×10^4	-
V221	4.4	3.5	1.5×10^4	100	249	1.3×10^4	+
C. coli biotype I							
D272	3.0	3.8	8.6×10^3	179	1768	1.9×10^5	+
M175	n.d.[f]	3.6	4.5×10^4	134	913	3.6×10^6	+
C. coli biotype II							
M254	3.0	3.2	3.4×10^2	84	1716	1.0×10^4	+

Table 2.1 (continued)

Strain number	HeLa association 1 h	HeLa association 6 h	HeLa invasion	Caco-2 association live	Caco-2 association fixed	Caco-2 invasion	Caco-2 transcytosis
(ii) non-inflammatory diarrhoea							
C. jejuni biotype I							
A365	1.5	3.4	2.3×10^3	93	840	6.0×10^2	-
B415	0.1	1.8	0.0	48	215	0.0	+
B378	0.1	0.2	0.0	75	171	0.0	-
E178	0.4	0.1	1.2×10^4	n.d.	n.d.	8.5×10^5	-
E206	3.1	3.4	0.0	96	859	0.0	-
E228	1.1	0.5	2.5×10^4	79	135	7.3×10^4	-
E230	0.0	0.0	0.0	28	42	0.0	+
J75	13.9	3.6	2.2×10^2	91	160	1.7×10^3	-
J78	0.1	0.4	7.2×10^2	145	728	1.7×10^3	-
H104	0.0	0.4	0.0	72	143	0.0	+
G136	0.9	3.1	5.4×10^2	85	884	4.0×10^3	-
K105	1.0	4.2	0.0	263	705	0.0	+
N82	0.0	1.5	3.2×10^4	62	134	1.0×10^5	+

Table 2.1 (continued)

Strain number	HeLa association 1 h	HeLa association 6 h	HeLa invasion	Caco-2 association live	Caco-2 association fixed	Caco-2 invasion	Caco-2 transcytosis
R169	0.3	5.2	3.2×10^4	28	163	3.4×10^6	+
T117	1.2	3.7	3.0×10^4	190	419	2.5×10^3	-
V161	n.d.	2.5	3.8×10^2	44	118	1.5×10^3	-
C. jejuni biotype II							
A293	2.6	1.4	8.7×10^2	95	210	1.5×10^3	+
C139	0.6	4.0	4.5×10^4	38	428	5.2×10^5	-
O69	3.7	2.8	0.0	101	186	0.0	+
C. coli biotype I							
D265	3.5	6.2	0.0	99	488	0.0	+
M233	4.3	0.0	2.7×10^3	24	575	9.9×10^2	+
O73	0.5	0.1	3.5×10^4	202	85	2.4×10^4	-
C. coli biotype II							
A320	0.3	3.2	1.0×10^2	112	579	1.3×10^3	+

Table 2.2. Summary data

Property	Colitis (n=21)	Non-inflammatory (n=23)	
HeLa cell association			
1 h, AI>3.0[a]	11[a] (55%)	5[b] (23%)	χ^2=4.6, p=0.03
1 h, AI>1.0	15 (75%)	10[b] (45%)	χ^2=3.7, p=0.05
6 h, AI>3.0	15 (71%)	10 (43%)	χ^2=2.0, p=0.15
HeLa cell invasion	21 (100%)	15 (65%)	χ^2=7.4, p=0.005
Caco-2 cell invasion	21 (100%)	15 (65%)	χ^2=7.4, p=0.005
Caco-2 cell transcytosis	18 (86%)	11 (48%)	χ^2=7.0, p=0.008

[a] data of Dr. H. Goossens.

[b] one strain in each group was not tested

Fig.2.1 Association of campylobacter with cultured human epithelial cell lines. Panels a and c show viable counts/ml of intracellular bacteria recovered from gentamicin-treated HeLa and Caco-2 cells respectively. Horizontal bars indicate mean values, excluding strains unable to invade. Panel b shows the association of radioactively labelled bacteria to live (open symbols) or glutaraldehyde-fixed (closed symbols) Caco-2 cells. Horizontal bars indicate mean cpm for each group. C, colitis isolates; NI, isolates from non-inflammatory disease. Panel d is a direct comparison of invasion (determined as viable counts/ml of intracellular bacteria recovered from gentamicin-treated monolayers) of HeLa and Caco-2 cells by each campylobacter isolate (○ , colitis; ● , non-inflammatory).

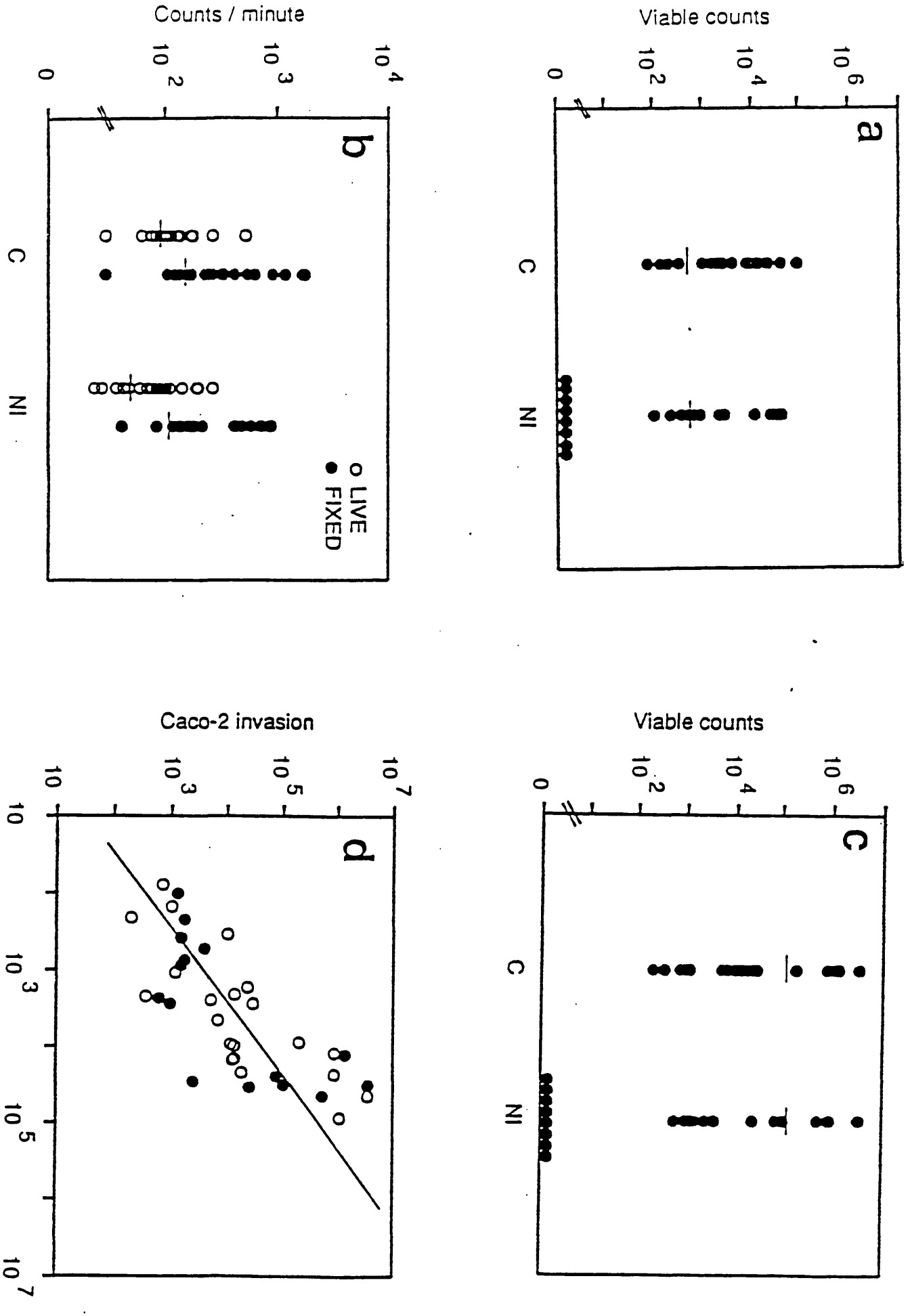

Viable counts
a
C
NI
Counts / minute
b
o LIVE
● FIXED
Viable counts
c
C
NI
Caco-2 invasion
d
HeLa invasion

Fig. 2.1

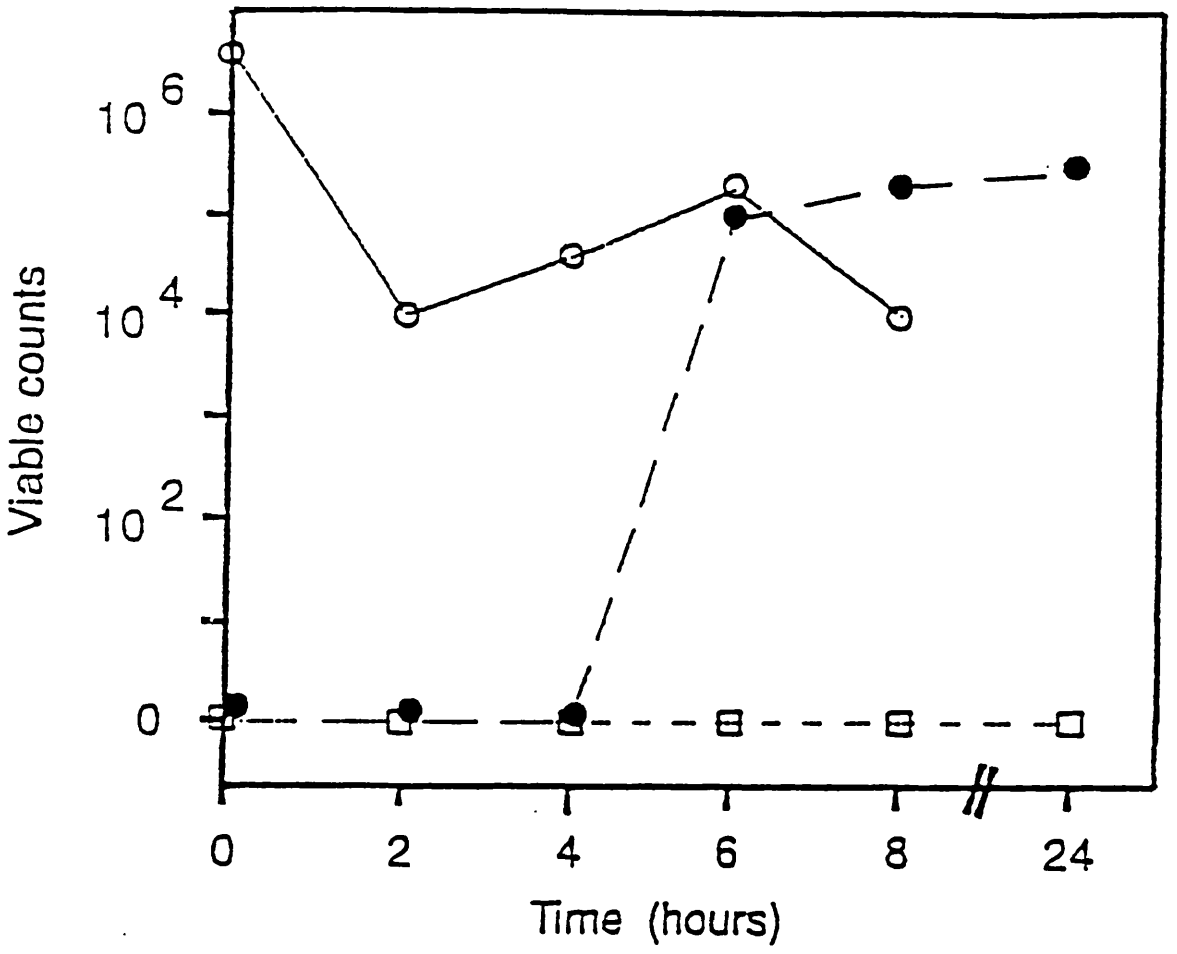


Fig.2.2 Time course of transcytosis of strain E116 through polarised Caco-2 cell monolayers. ○, viable counts/ml recovered from monolayers by deoxycholate lysis (adherent and internalised bacteria). ●, viable counts/ml recovered from the medium below the support filter. Strain DH5α (□) was used to confirm integrity of monolayers.

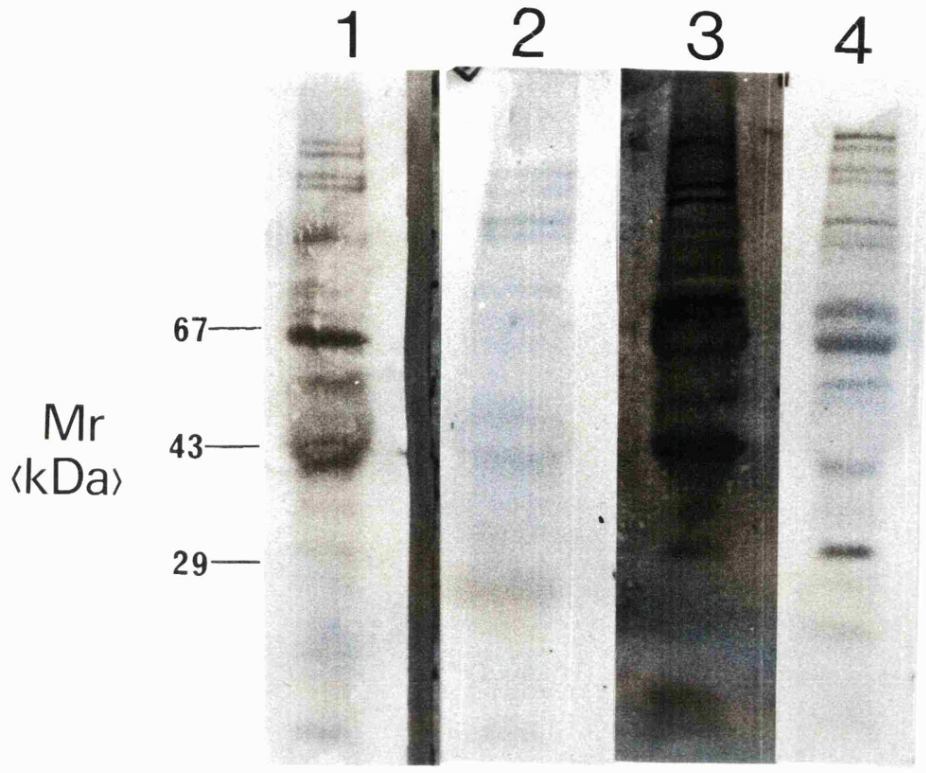
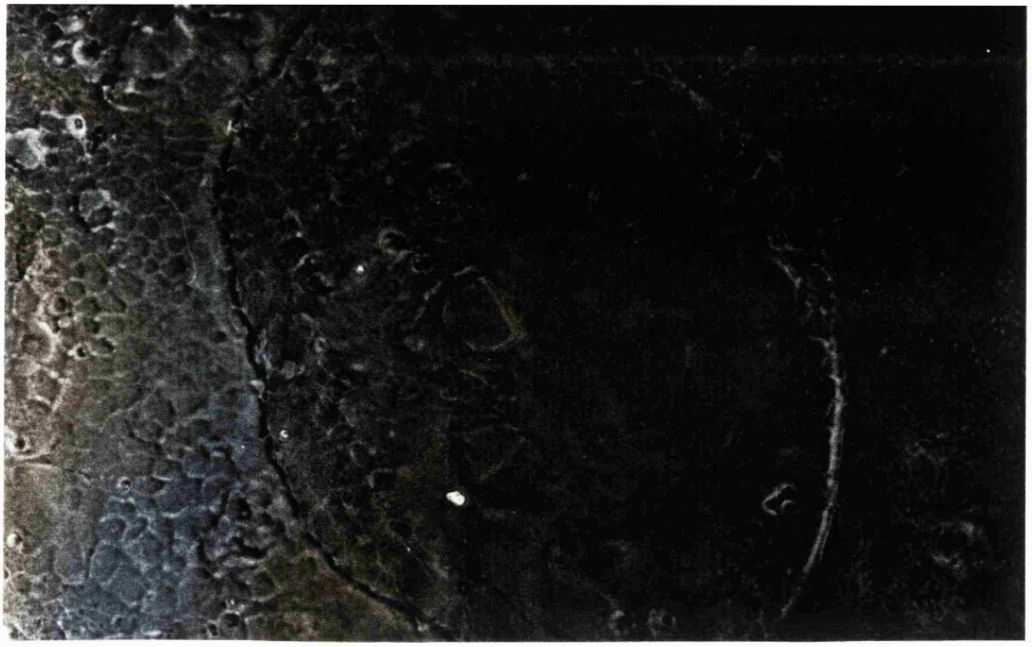


Fig. 2.3. Western blot analysis of *C. jejuni* strain F132 outer membrane proteins probed with convalescent sera. Lane 1. Ponceau stain of *C. jejuni* outer membrane proteins seperated by PAGE and electroblotted to nitrocellulose. Blotted proteins were treated with convalescent serum and then with anti-human IgA (Lane 2), anti-human IgG (Lane 3) or anti-human IgM (Lane 4). Mobility of marker proteins bovine plasma albumin (67 kDa), egg albumin (43 kDa) and carbonic anhydrase (29 kDa) are indicated.

Figure 2.4. (a). Phase contrast microscopy of Caco-2 dome infected with *C. jejuni* strain F132 after 6 h (b). Phase and fluorescence microscopy (using convalescent serum against the bacteria and FITC-conjugated anti-human IgG) of same dome. Bacteria can be visualised within the structure of the dome. (c) Fluorescence microscopy of the infected dome (x 10, low power).



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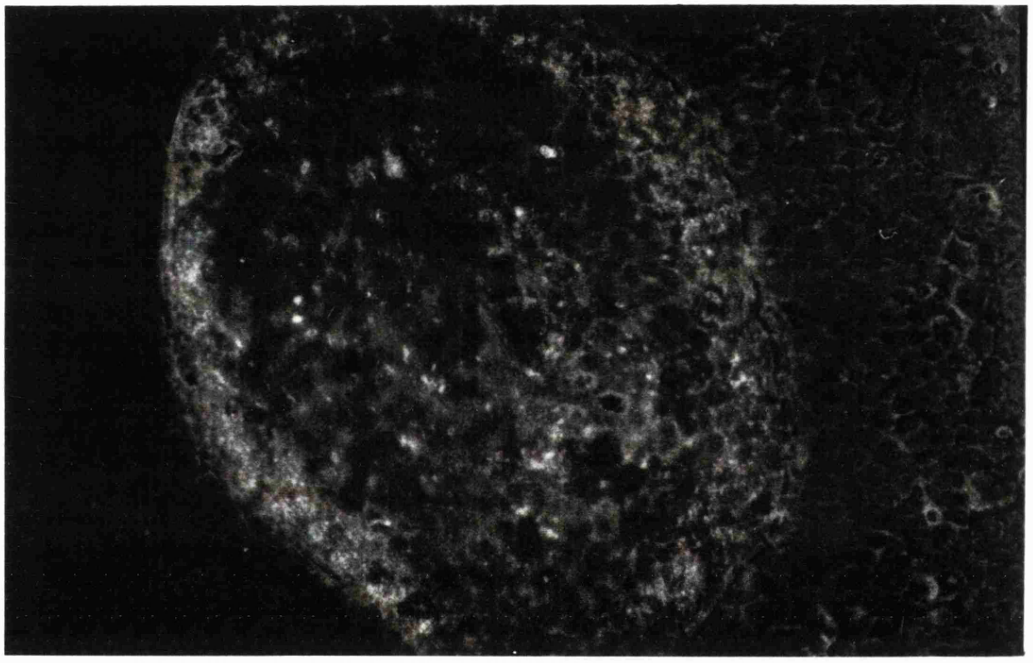
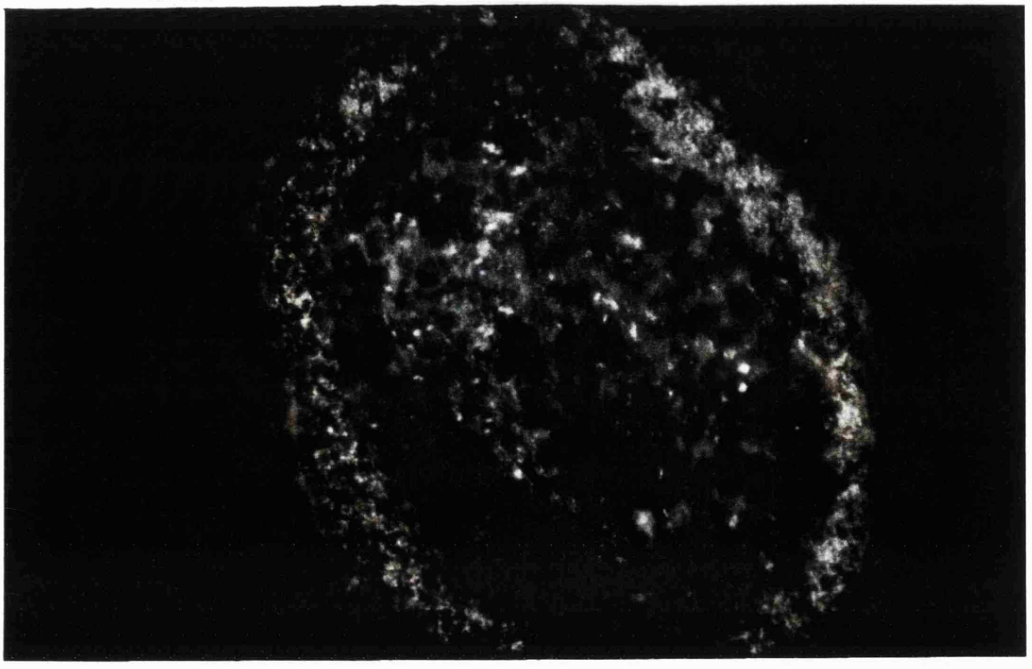
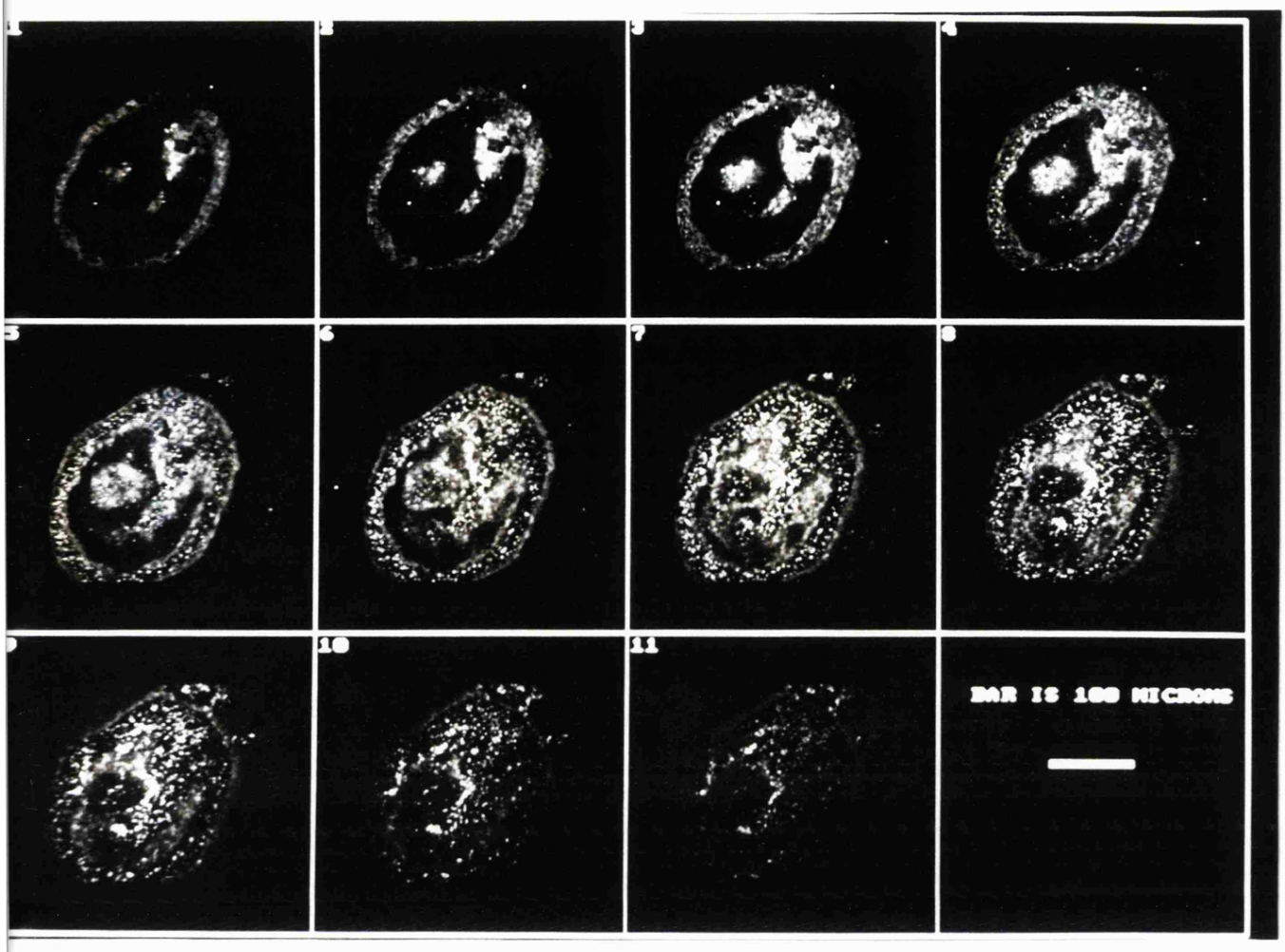


b



c

Figure 2.5. (a) Series of photographs through an infected dome using confocal microscopy, from the apical surface to the basolateral surface. Bacteria, exposed to convalescent antisera and then labelled with a FITC conjugate against the primary antiserum, are seen at all planes within the infected dome showing bacterial invasion within the transporting structure. (b) Reconstruction of infected dome. Confocal microscopy reconstructs planes through the infected dome to give a sideways projection of the structure. Again bacteria can be seen as fluorescence throughout the dome.



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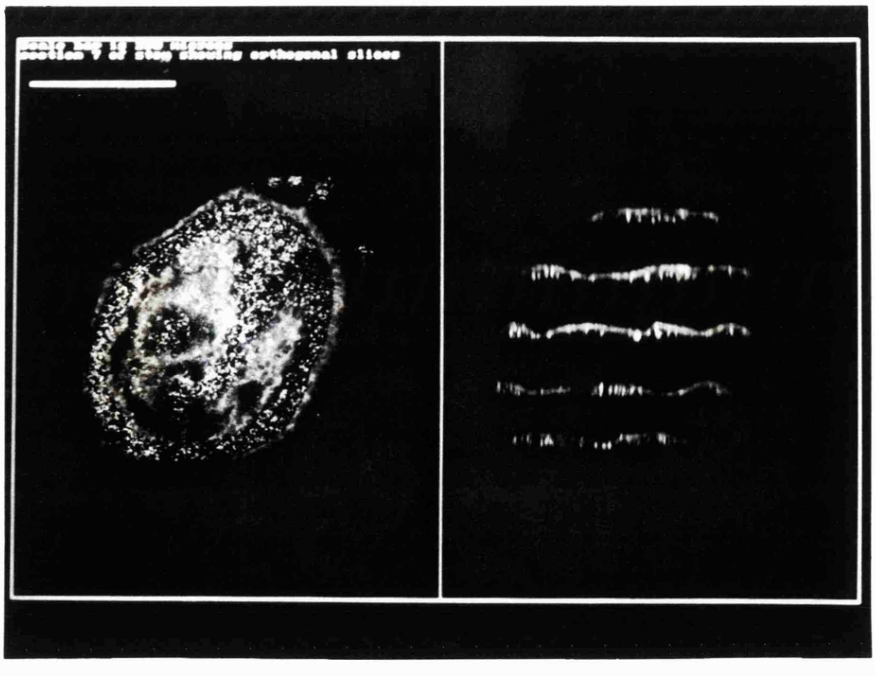
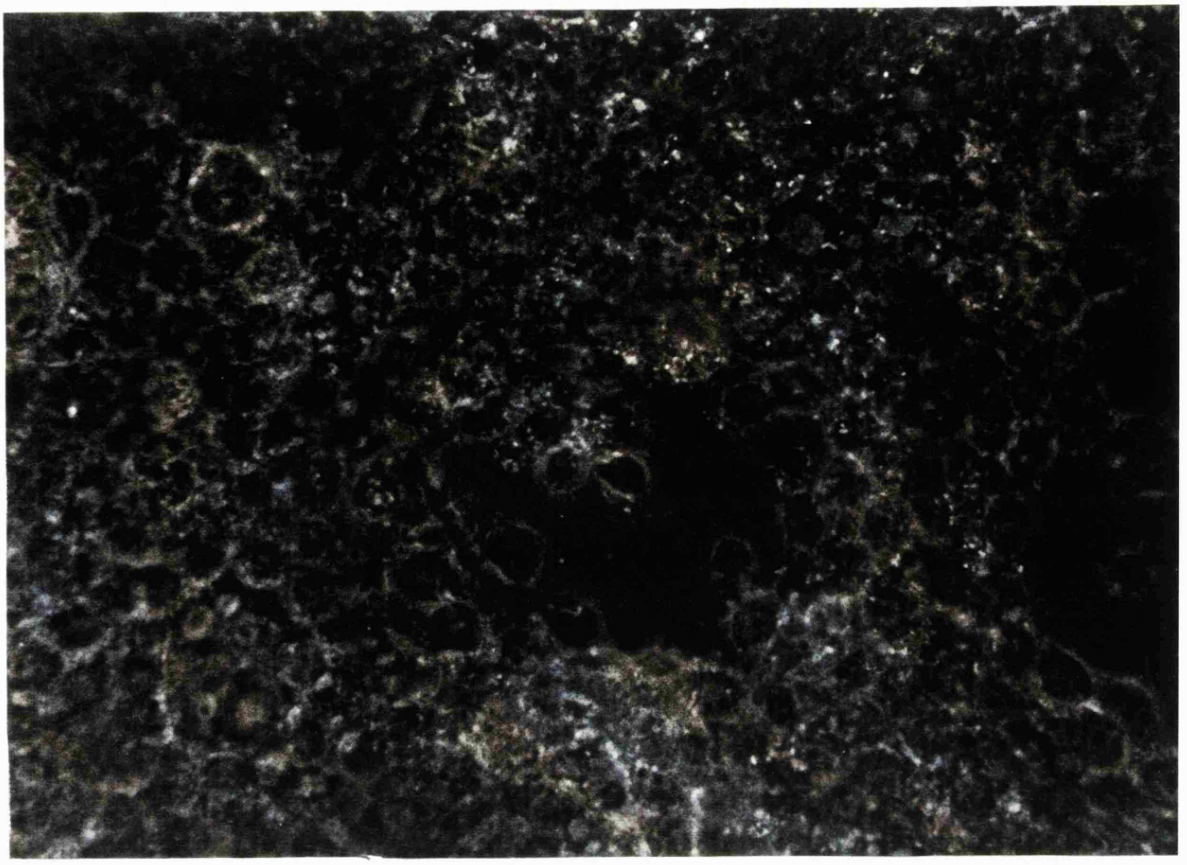


b

Figure 2.6. Fluorescence microscopy of (a) uninfected Caco-2 monolayer, and (b) Caco-2 cells infected with strain L115 (using rabbit serum against the bacteria and FITC-conjugated anti-rabbit IgG), showing bacteria covering the entire monolayer (x 40 high power).



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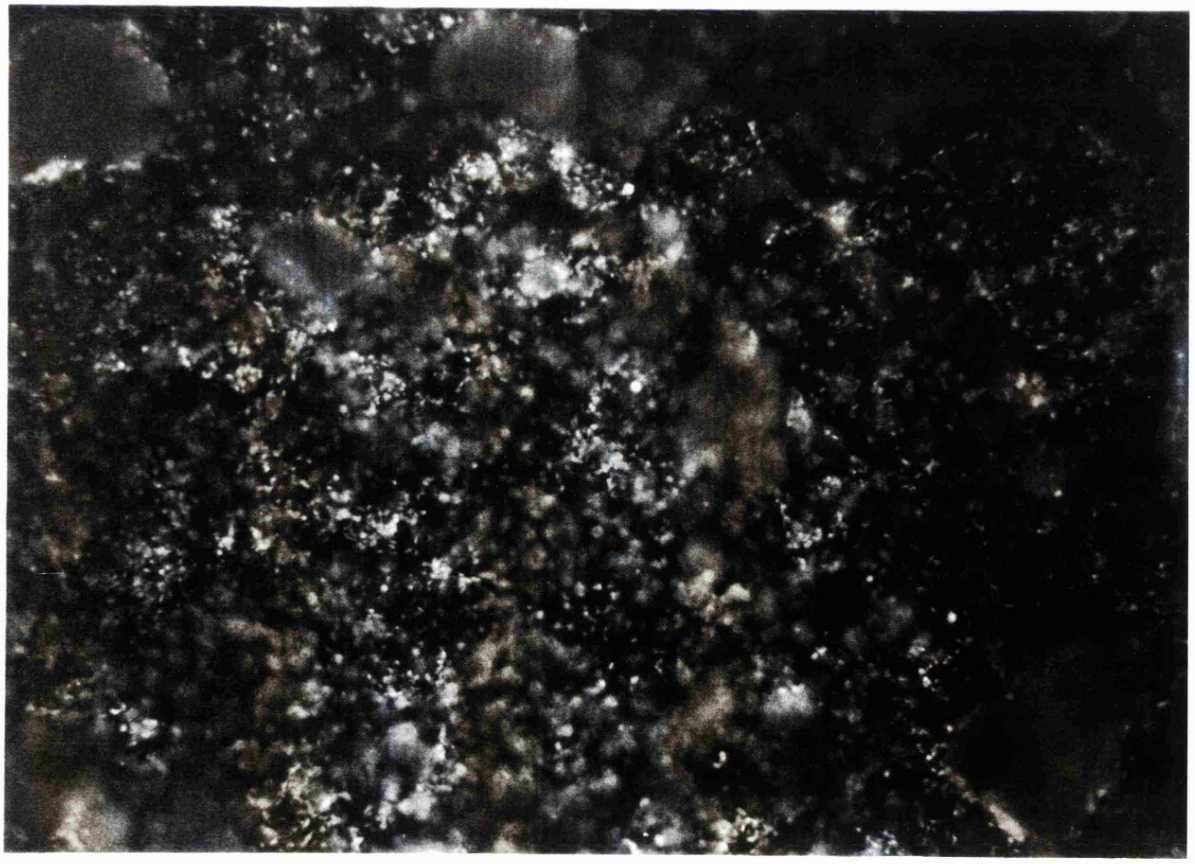


b

Figure 2.7. Fluorescence microscopy of a Caco-2 monolayer infected with strain P71 (using rabbit serum against the bacteria and FITC-conjugated anti-rabbit IgG). Out of focus area represents area of dome (x 40 high power).

their identity and role in disease are not known. Because of the strong recognition of several bacterial surface proteins in Western blots, this serum was considered useful for fluorescence studies of the interaction of F132 with Caco-2 cells. Strain F132 was the only strain for which specific antiserum was available; other strains cross reacted in fluorescence studies with antisera against F132, and also with a rabbit serum raised against a number of other strains, but to a lesser extent.

2.3.6. Fluorescence microscopy of infected Caco-2 cells. Areas of intense fluorescence in infected monolayers correspond to the formation of domes within the monolayer, and represented bacteria (strain F132) adhering to, and invading cells within the dome structure (Fig. 2.4. a-c). These structures, which were randomly distributed over the monolayer in numbers varying from 10 to 40/cm^2, comprise actively transporting cells able to transfer fluid from the apical to the basolateral surface. Large numbers of bacteria were seen at all time points attached to the apical surface of dome cells, and confocal microscopy revealed intracellular bacteria within all planes of the dome structure and beneath the monolayer (Fig. 2.5, panels a and b). Bacteria were seen attached to surrounding cells, but in much smaller numbers.

Strain F132, however, may not be typical; other strains tested showed different patterns of adherence to Caco-2 cells, including adhesion of bacteria all over the monolayer, or adhesion to cells other than areas of dome formation (Fig. 2.6 and 2.7). As yet no clear pattern of adherence has emerged for strains from different clinical groupings, but work is continuing in an attempt to characterise adherence patterns.

2.4. DISCUSSION

Forty four clinical isolates of *Campylobacter jejuni/coli* from children with colitis (21 strains) or watery diarrhoea (23 strains) were characterised with respect to cell adhesion and invasion. Of these isolates, 37 were *C. jejuni* and 7 were *C. coli*; there was no obvious association of species or biotype with clinical symptoms.

The pathophysiological characteristics of campylobacter enterocolitis

suggest that mucosal invasion is an important component of pathogenicity. Penetration of cultured HeLa cells by *Campylobacter* strains has been demonstrated by several groups (Manninen *et al.*, 1982; Newell *et al.*, 1985; Newell and Pearson, 1984; Fauchere *et al.*, 1986), and suggested as a convenient model system for discriminating between isolates from different sources. The data presented here, however, indicate that for systematic analysis of adhesion, presumably the essential first step in invasion, the HeLa cell model suffers from variability inherent in the fact that a time dependent process is observed at a single arbitrarily chosen experimental time point. A statistically significant difference was observed between colitis and non-inflammatory isolates with respect to invasion of HeLa cells. However, the fact that several strains that were invasive as defined by gentamicin protection would be classified as non-adherent or low adherers (AI<3) by the criteria of Fauchere *et al.* (1986) gives further cause for concern.

Caco-2 cells are a better model for studying interactions of campylobacter with eukaryotic cells because of their enterocyte-like characteristics, features that are more likely to be encountered during natural infections of the gut. Caco-2 cells differentiate to give a microvillous brush border characteristic of intestinal enterocytes, tight junctions and formation of domes typical of transporting epithelial monolayers (Grasset *et al.*, 1984; Grasset *et al.*, 1985; Rousset, 1986; Rousset *et al.*, 1985). All isolates in the strain collection adhered to Caco-2 cells as determined by radioactive labelling experiments. Strains that invaded HeLa cells were also able to invade Caco-2 cell monolayers. Transcytosis through Caco-2 cell monolayers was a feature of 18 of the 21 colitis strains, compared with only 11 of the 23 isolates from non-inflammatory diarrhoea. Six strains of the latter type were found to be non-invasive; transcytosis by these strains may be due to disruption of tight junctions and paracellular passage across the monolayer but further experiments are needed to establish this. The ability of campylobacter to penetrate and pass through epithelia is likely to be important in the intestinal damage and occasional bacteraemia observed in enterocolitis. Transcytosis of *Salmonella choleraesuis* through a canine kidney cell line (Madin Darby Canine Kidney) and Caco-2 cells has been described (Finlay *et al.*, 1988; Finlay and Falkow, 1990) but *C. jejuni* was

reported to be unable to penetrate MDCK cells (De Melo and Pechere, 1990), possibly because the necessary receptors for attachment were not present on the cell surface. MDCK cells obviously do not represent the normal target cells of *C. jejuni*, and in this respect the use of Caco-2 cells in the study of transcytosis is more representative of invasion steps in natural infection.

Strains classified as non-inflammatory that were able to invade cultured cells or pass through polarised monolayers may have been isolated from cases of colitis in which inflammatory cells were missed, or in which characteristic symptoms of the disease were absent at the time of diagnosis. Host factors may also critically influence the progress of an infection and contribute to difficulties in defining isolates purely on the basis of symptoms. It may be that the density of invading organisms in the mucosa is an important factor in the type of disease produced. In the case of *Salmonella*, the most severe histological damage in rabbit ileal loops was associated with the highest bacterial counts in the tissue (Wallis *et al.*, 1989). Thus in disease due to campylobacter, colitis strains may invade in greater numbers, exerting a higher bacterial load in the tissues, and initiating the inflammatory response and white cell infiltration characteristic of colitic illness. On the other hand, some non-inflammatory strains, although capable of invading cells, may not be able to increase bacterial density in tissue above a threshold level needed to initiate inflammation. Alternatively a bacterial toxin, or an outer membrane or cell wall component may trigger the host inflammatory response, and the clinical groupings may reflect differences in the expression of such components.

The ability of convalescent serum to recognise a large number of bacterial proteins suggests that these are important *in vivo*. The proteins recognised vary depending on which class of antibody is used to probe the nitrocellulose filter. Interestingly the 29 kDa adhesin protein is more strongly recognised by IgM and IgG fractions, IgM being an important component of the initial immune response against invading organisms. It would be expected that an adherence component of the bacterial cell envelope would be a crucial protein expressed by bacteria early in infection in its interaction with an enterocyte, perhaps accounting for the strong IgM component against this protein in the serum. Convalescent and

hyperimmune rabbit serum are therefore useful reagents to look at the interaction of F132 and six other strains (used in the rabbit ileal loop studies, Chapter 4) with Caco-2 cells using immunofluorescence. Visualisation of the interaction of strain F132 with Caco-2 dome cells shows invasion at all levels of the dome and is direct evidence for transcytosis. F132 is the only strain so far screened that has this characteristic pattern of adherence, and in this respect F132 seems to be atypical, in that other strains tested adhere all over the monolayer (Fig. 2.6) and not only to the dome cells. Indeed, another isolate, P71, seems not to stick to dome cells but only to the non-transporting cells within the monolayer (Fig. 2.7). Investigation of these isolates and patterns of adhesion is in progress in an attempt to identify both cell surface receptors and bacterial adhesins.

Work in this chapter demonstrates techniques for distinguishing between organisms isolated from colitic and watery diarrhoea, and indicates that the range of symptoms observed in campylobacter infections is, at least in part, a reflection of strain differences between causative organisms. It is suggested that Caco-2 cells provide a useful model for the study of enteric pathogenic bacteria, such as *Campylobacter* species, because in differentiated form they have the characteristic features of enterocytes and in this respect permit detailed study of the interaction of campylobacter with target cells.

Chapter 3

Protein phosphorylation and calcium elevation in *C. jejuni*-infected Caco-2 cells.

3.1. INTRODUCTION

It is clear that bacterial enteric pathogens can cause diarrhoea by release of toxins that mediate phosphorylation of ion channels leading to fluid loss. The mechanism of diarrhoea in non-inflammatory *C. jejuni* illness has indeed been postulated to be the result of toxin-mediated effects (Ruiz-Palacios *et al.*, 1983) (see Chapter 4). This chapter investigates the phosphorylation events in Caco-2 cells induced by infection with two non-inflammatory *C. jejuni* isolates, and the ability of these and other strains to increase intracellular calcium levels. In addition calcium has other effects on cytoskeletal rearrangements, and kinase activities involved in protein phosphorylation can be calcium dependent (Rieker and Collins, 1987). Demonstration of such intracellular events may give the first clues to second messenger-controlled events involved in fluid loss in *C. jejuni* disease.

3.1.1. Mammalian cell signal transduction and diarrhoeal disease. Studies of signal transduction in mammalian cells have revealed the crucial role of cyclic nucleotides, calcium and phospholipid metabolites (diacylglycerol, inositol triphosphate) as second or third messengers linking hormone receptors to their physiological targets. In intestinal epithelium cyclic AMP (cAMP), cGMP and calcium have all been implicated in the action of neurohumoral agents and microbial toxins on transepithelial transport of electrolytes and water (Frizzell *et al.*, 1979; Rao and Field, 1983). An additional mechanism is activation of ion channels by guanosine triphosphate (GTP) binding proteins (de Jonge and Rao, 1990), although a direct physiological role remains to be demonstrated. High affinity receptors for cAMP and cGMP in intestinal epithelium have been identified as the regulatory moieties of cyclic nucleotide-dependent protein kinases (de Jonge, 1981). This strongly supports a model, analogous to the control of some key metabolic enzymes, in which ion transport systems in the surface membrane of the enterocyte are activated or inactivated by cAMP and cGMP dependent phosphorylation of a protein component of the ion pump or

channel. An increase in the intracellular concentrations of any of the above mentioned mediators results in a decrease in absorption and/or an increase in secretion, the net effect being fluid accumulation in the lumen. Such a mechanism would allow the transport responses to be fast, and reversible due to the action of protein phosphatases.

3.1.2. Protein kinases. Protein kinases catalyse the transfer of the terminal phosphate groups of ATP to the hydroxyl group of serine, threonine or tyrosine residues to form a phosphomonoester bond. Phosphoproteins are dephosphorylated by specific hydrolytic phosphatases which may be regulated by second messengers. At physiological pH phosphoaminoacids are ionized with two negative charges, leading to conformational changes which may alter protein activity (Rao, 1989). Substrates may be phosphorylated at multiple sites by the same or different kinases. Thus a single transport-related protein could be regulated by multiple second messengers acting through specific protein kinases. The second-messenger regulated phosphoprotein could be the transporter itself or a modulator of its activity.

Protein kinases exhibit specificity in terms of both their regulators and their substrate sites, and the levels of these enzymes vary with the species, tissue and state of differentiation (Cohen, 1982). Cyclic nucleotides bind to protein kinases, and at least two classes of calcium dependent kinases (calcium-calmodulin and calcium phospholipid) with wide substrate specificities are known. These kinases phosphorylate serine or threonine residues. A class of protein kinases which phosphorylate tyrosine residues has been identified among a number of receptors for growth regulators and oncogene products, and a role for these kinases in growth regulation is suggested (Ullrich *et al.*, 1985).

The cAMP-dependent protein kinases exist as a tetramer with two identical regulatory (R) and catalytic (C) subunits. Two isozymes of the enzyme with common C but different R subunits are known. cAMP binds to R causing dissociation of the RC complex, free C being the catalytically active component (Beavo and Mumby, 1982). Evidence suggests that R may possess topoisomerase activity (analogous with the bacterial cAMP-binding protein catabolite activator protein, CAP) and may directly regulate gene function by binding to DNA (Constantinou *et al.*, 1985). Although there are

homologies between the two cyclic nucleotide dependent kinases, the cGMP-dependent enzyme exists as a dimer, and the C and R components are not dissociated upon activation (Kuo and Shoji, 1982). Two major classes of calcium-dependent protein kinases, the calmodulin-dependent and the phospholipid-dependent have been identified. At least five subtypes of calcium-calmodulin kinases are known; myosin light chain kinase, phosphorylase kinase and the calcium-calmodulin kinase III are highly substrate specific (Nairn *et al.*, 1985). The second class of calcium-dependent kinases, C kinase, requires phospholipid, especially phosphatidylserine, for its activation. Diacylglycerol is a potent endogenous activator of the enzyme; tumour promoting phorbol esters activate the enzyme by binding to it and by-passing the action of diacylglycerol, and so are useful tools for studying C kinase-mediated events. Cyclic AMP, cGMP, and both calcium-calmodulin and calcium-phospholipid specific protein kinases and substrates have been identified not only in small intestinal tissues from a variety of species but also in brush border and microvillus preparations (Rao *et al.*, 1982; de Jonge and Lohman, 1985; Donowitz *et al.*, 1984). There are also two isozymes of cGMP-dependent kinases, one of which appears to be restricted to the brush border membrane of the mammalian intestine (de Jonge, 1981).

3.1.3. Protein phosphorylation. The major problem in studying the regulation of ion transport in epithelia is that, in order to demonstrate functionality, intact basolateral and apical membrane systems which have retained structural and functional differences are required. Although all mediators act on common ion-transport mechanisms in the rabbit ileum, preliminary evidence indicates that they do not appear to do so by altering the phosphorylation of a common substrate (Rao *et al.*, 1982). Proteins have been identified whose phosphorylation is regulated by two of the three mediators, calcium and cAMP in rabbit ileum (Donowitz *et al.*, 1984), calcium and cGMP in flounder intestine (Rao *et al.*, 1984) and cAMP and cGMP in rat brush border (de Jonge and Lohmann, 1985). In the mammalian intestine, the best characterized protein is the 86 kDa cGMP-specific protein localized to the ileal microvillus membrane. This is a larger isozyme (type II) of the soluble cGMP-specific protein kinase (type I) found in other tissues. More recently a 25 kDa acidic proteolipid, which is phosphorylated by both cAMP and cGMP-dependent protein kinases, but

which is a poor substrate for calcium-dependent protein kinases, has been found in rat ileal microvillus membranes (de Jonge and Lohmann, 1985). Phosphorylation has been demonstrated when rat microvillus membranes were entrapped with cAMP and MgATP, and cAMP stimulation of chloride conductance in vesicles was ATP-dependent. In separate experiments, similar concentrations of cAMP increased the phosphorylation of five substrates in the membrane (van Dommelen and de Jonge, 1984). In the presence of ATP, calcium-calmodulin increased the phosphorylation of six specific rabbit microvillus membrane proteins (137, 116, 77, 58, 53, and 50 kDa) which are specifically inhibited by low concentrations of promethazine (8 μM). At similar concentrations promethazine inhibited net chloride absorption in stripped rabbit ileum (Cohen *et al.*, 1986). A correlation between the respective second messenger mediated effects on ion transport and the relevant phosphoproteins remains to be demonstrated for many systems as do the interactions, if any, of the calcium, cAMP and cGMP-specific phosphoproteins detected thus far and their role in ion transport. It is highly likely that such mediator-specific phosphoproteins are modulators of transport proteins rather than being transporters *per se*, and may be located on the membrane or on membrane-associated sites such as cytoskeletal elements (Rao, 1989). Stimulation of chloride secretion in T84 cells involves recruitment of intracytoplasmic vesicles to the membrane, which suggests that cytosolic phosphoproteins might potentially be transport-related. There are at least four candidate cytosolic phosphoproteins in T84 cells (Cohn, 1987), and at least one in Caco-2 cells (Burnham and Fondacaro, 1989). Although the cyclic nucleotide-regulated phosphoproteins relevant to transport have not been identified, circumstantial evidence indicates that there is a definite role for cyclic nucleotide-dependent phosphorylation in the modulation of intestinal ion transport. A role for non-kinase mediated actions of cyclic nucleotides in the intestine does not seem to be apparent from preliminary experiments (de Jonge *et al.*, 1989).

To prove conclusively the role of second messenger-specific protein phosphorylation in a physiological process, a series of stringent criteria must be met, and these have been fully characterised in a very few systems (Cohen, 1982). The most important criterion is to establish that the

phosphoprotein bears a functional relationship to the physiological process, and undergoes a reversible step due to dephosphorylation. Although an ion-transport related phosphoprotein remains to be fully characterised, a role for second messenger stimulated protein phosphorylation in ion transport has been best demonstrated in the regulation of the slow inward calcium current in heart cells (Osterreider *et al.*, 1982) and of the anomalous rectified K current in neurons (Lemos *et al.*, 1984).

3.1.4. Role of calcium in cell processes. In enterocytes, as in most cells, the concentration of free cytosolic calcium is in the submicromolar range (usually around 100 nM). An increase in intracellular calcium concentration can be induced in at least three ways. First, agents such as the neurotransmitter substance P can increase calcium permeability of the plasma membrane (Chang *et al.*, 1986). Second, occupation of muscarinic cholinergic and serotoninergic receptors (Akhtar, 1987; Snider *et al.*, 1986) can activate phosphatidylinositol metabolism resulting in the formation of inositol triphosphate, which causes the release of calcium from calcium-rich intracellular organelles (Berridge, 1984).

Cyclic nucleotides can cause persistent increases in intracellular calcium concentration by a unknown mechanism, even in the absence of extracellular calcium (Semrad and Chang, 1987). Calcium modulates intracellular processes by a variety of mechanisms, either alone or in conjunction with other mediators. It acts as an allosteric activator of proteins including enzymes and ion pumps, it regulates protein phosphorylation via specific kinases or phosphatases, it modulates cytoskeletal elements such as spectrin, and it influences cyclic nucleotide metabolism by regulating cyclase and phosphodiesterase activities (Nishizuka, 1986). A number of calcium-regulated events are mediated by the association of calcium with the specific heat and acid stable binding protein, calmodulin (Means *et al.*, 1982). Calmodulin may directly alter the conformation of certain plasma membrane transport proteins.

Inositol phospholipid breakdown results in the formation of diacylglycerol, which is always associated with activation of protein kinase C. C kinase plays a role in stimulus response systems involving phospholipid turnover; for example hormonal stimulation may activate phosphotidylinositol turnover with release of diacylglycerol and inositol

phosphates. The latter can trigger calcium mobilization from intracellular stores. Alternatively hormone receptor coupling could also directly activate calcium gating. Calcium with or without diacylglycerol can activate C kinase and cause a biological response (Nishizuka, 1986). A role for GTP-binding proteins in the phosphoinositide cascade has been implicated (Cockcroft and Gomberts, 1985).

Calcium signalling mechanisms are key elements in the regulation of intestinal electrolyte transport. Increases in calcium inhibit sodium and chloride absorption and stimulate chloride secretion (Field *et al.*, 1989). It is generally accepted that calcium transport mechanisms located both on the plasma membrane and on intracellular membranes can be invoked to raise calcium levels transiently.

3.2. MATERIALS AND METHODS

3.2.1. Bacterial strains. *C. jejuni* strain H104 and *C. coli* strain D265, isolated from cases of non-inflammatory diarrhoea, were used in phosphorylation experiments. H104 produces a cholera-like enterotoxin but no cytotoxic activity, while D265 is enterotoxin negative, but produces a cytotoxin. The strains were grown in Mueller-Hinton broth (Oxoid) at 42°C in a microaerophilic atmosphere, and bacteria in early or mid log phase were used to infect Caco-2 cell monolayers. In order to ascertain the effect of bacterial culture supernatants on cellular phosphorylation events, bacteria were pelletted by centrifugation, and the supernatants collected and diluted 1:2 in Dulbeccos modified Eagle medium (DMEM) (GIBCO) before addition to Caco-2 cells. Calcium experiments were performed using the six strains tested in rabbit ileal loops (see Chapter 4), L115, C119, N82, P71, O81 and NCTC 12189, together with H104 and D265. Strains were incubated overnight with shaking at 42°C in a microaerophilic atmosphere in DMEM containing 20% foetal calf serum (FCS). Bacteria were used in mid log phase; cell free culture supernatants were added to cells as described.

3.2.2. Cell culture. Caco-2 cells were routinely grown in DMEM containing 10 or 20% (vol/vol) FCS at 37°C under 5% CO_2 in air. The medium was replaced every 2 days until monolayers were confluent and differentiated. Cells for phosphorylation were used undifferentiated at 5

days post-confluence and differentiated at 20 days post-confluence. HEp-2 and CHO cells were grown in DMEM containing 10% FCS and used for calcium assays 3 days post confluence.

3.2.3. Phosphoprotein preparation and analysis. Confluent Caco-2 cell monolayers were maintained for 4 h immediately before use in 2 ml of phosphate free DMEM containing 0.5% FCS and 100 μCi of carrier free ^{32}P (Amersham) to label intracellular ATP pools. After treatment with bacterial suspensions (10^8 organisms in 0.5 ml) for the times indicated, the cells were rapidly washed three times in ice cold phosphate buffered saline (PBS) to remove unincorporated ^{32}P. They were then suspended in 35 μl aliquots of 50 mM Tris.HCl (pH 5.0) buffer containing 50 mM benzamidine hydrochloride, 50 mM sodium fluoride, 2.5 mM sodium inorganic pyrophosphate, 5 mM β-glycerophosphate, 2 mM EDTA, 20 μg/ml pepstatin A, 1.5% (vol/vol) Triton X-100, and 0.1% (wt/vol) sodium dodecyl sulphate (SDS), and rapidly frozen. Cell suspensions were sonicated, cytoplasmic and membrane fractions separated by centrifugation and frozen. Thawed cell suspensions were disrupted by sonication on ice, and the proteins were solubilized by the addition of an equal volume of 62.5 mM Tris.HCl buffer (pH 6.8) containing 4% (wt/vol) SDS, 20% (vol/vol) glycerol, 10% (vol/vol) 2-mercaptoethanol, and 0.001% (wt/vol) bromophenol blue. Solutions were boiled for 5 min. Cell debris was pelleted by centrifugation, and solubilized phosphoproteins were analyzed by electrophoresis on 10 or 12.5% (wt/vol) polyacrylamide gels containing SDS by the method of Laemmli (1970). The gels were subsequently stained with Coomassie brilliant blue, destained, and dried. Radiolabelled proteins were detected by autoradiography.

3.2.4. Qualitative demonstration of intracellular calcium. Confluent Caco-2, CHO or HEp-2 cells grown on coverslips were infected with bacterial strains (10^8 organisms/ml) for various times as specified. One hour before the end of the incubation period, cells were washed in PBS and loaded with 50 μM FLUO-3/AM, the acetoxymethyl ester derivative of 9-{4-bis-(carboxymethyl) amino-3-[2 -(2-bis(carboxymethyl)amino-5methyl-phenoxy) ethoxy]phenyl} -2,7-dichloro-6-hydroxy-3H-xanthin-3-one (FLUO-3). FLUO-3/AM loads readily into mammalian cells where it is cleaved by endogenous esterases to yield the free dye (Kao *et al.*, 1989) which has visible excitation and emission wavelengths (490 and 530 nm respectively).

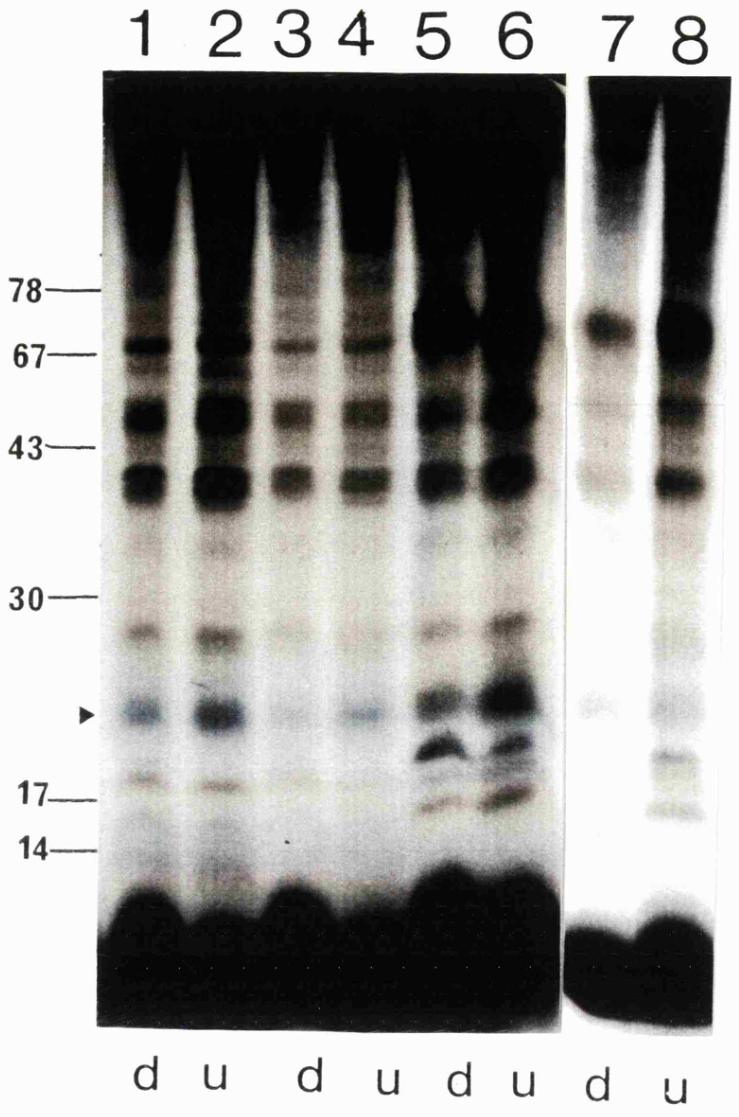


Figure 3.1. Autoradiograph of phosphorylated membrane proteins in differentiated (d) and undifferentiated (u) Caco-2 cells induced by *C. jejuni* whole bacteria and by bacteria free supernatants. Lanes 1 and 2, infection with strain H104. Lanes 3 and 4, treatment with strain H104 culture supernatant. Lanes 5 and 6, infection with strain D265. Lanes 7 and 8, treatment with strain D265 culture supernatant. Arrowhead indicates protein at 21 kDa. Molecular weight markers are carbonic anhydrase (30 kDa), egg albumin (43 kDa) and bovine plasma albumin (67 kDa).

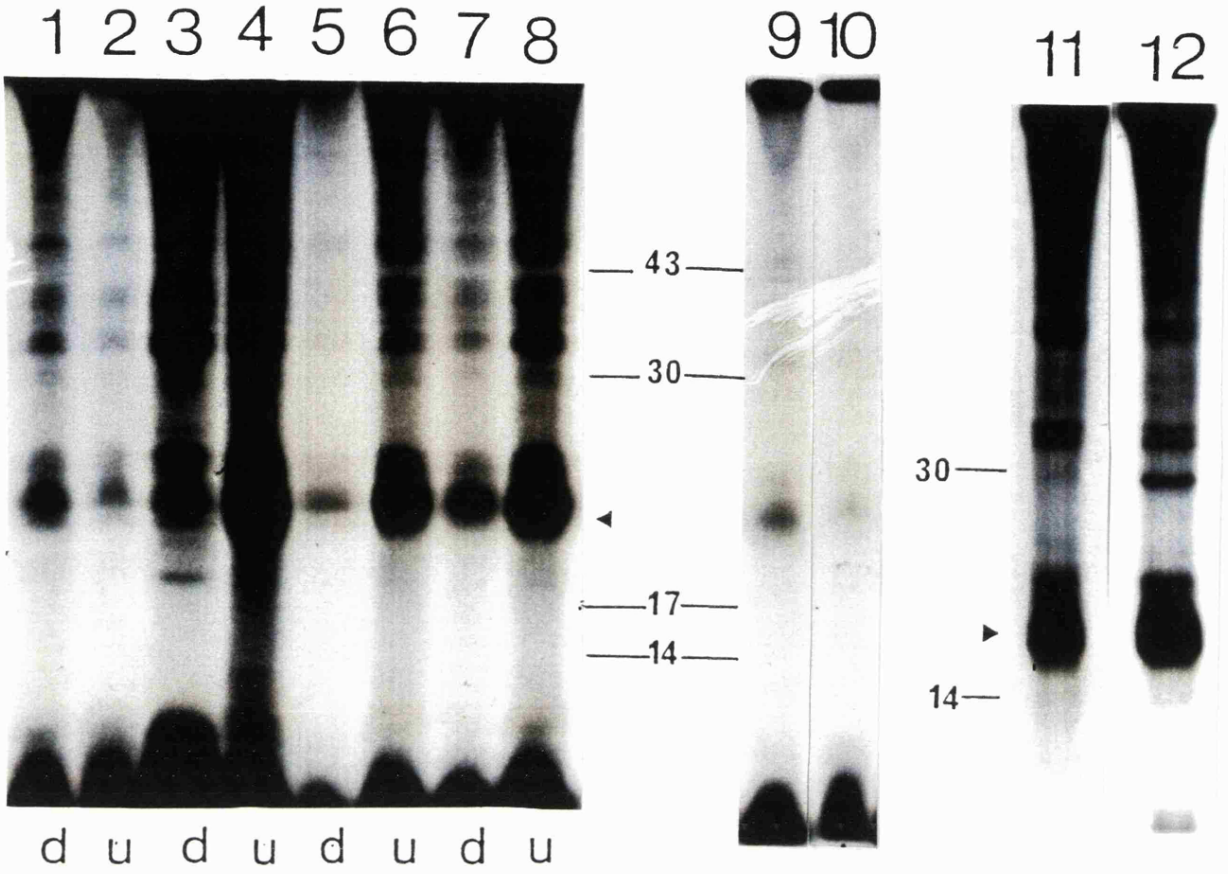
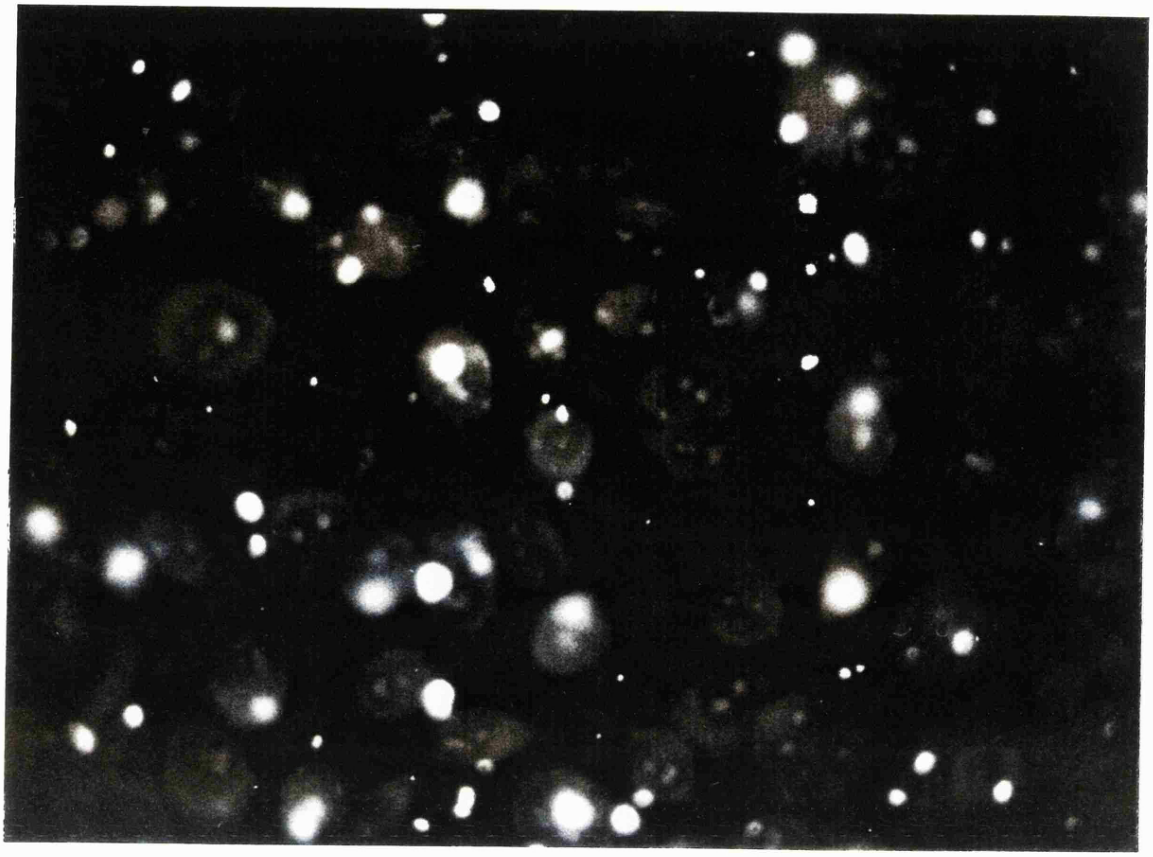


Figure 3.2. Phosphorylation of cytoplasmic fractions of differentiated (d) and undifferentiated (u) Caco-2 cells induced by *C. jejuni* whole bacteria and culture supernatants. Phosphoproteins resolved using PAGE and autoradiography. Lanes 1 and 2, infection with strain H104. Lanes 3 and 4, treatment with strain H104 culture supernatant. Lanes 5 and 6, infection with strain D265. Lanes 7 and 8, treatment with strain D265 culture supernatant. Lane 9, control uninfected undifferentiated Caco-2 cells, cytoplasmic fraction. Lane 10, Control uninfected undifferentiated Caco-2 cells membrane fraction. Lane 11, total cell phosphoproteins treated with 10 nm TPA for 2 h. Lane 12, phosphoproteins prepared from Caco-2 cell monolayers incubated for 3 h with enteropathogenic *E. coli* strain 2036-80 (data of Tom Baldwin). Arrowhead indicates protein of 21 kDa. Control differentiated uninfected Caco-2 cell fractions show no phosphoproteins. Markers are indicated in Fig. 3.1.

Figure 3.3. Elevation of intracellular calcium levels: HEp-2 cells exposed to *C. jejuni* culture supernatants. Cells were loaded with FLUO-3 as described. (a) Untreated HEp-2 cells (b) cells exposed to *C. jejuni* strain C119 supernatants showing fluorescence due to calcium elevation.



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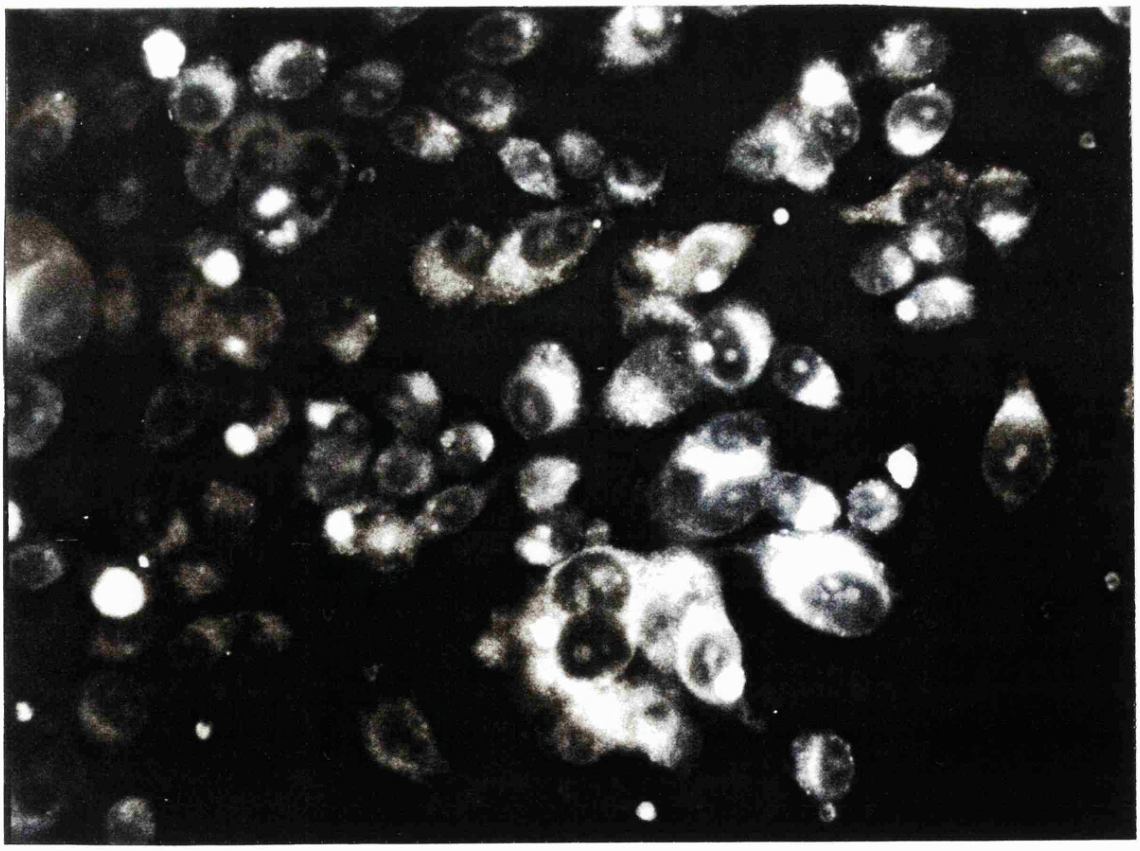


b

Figure 3.4. Confocal microscopy picture of HEp-2 cells exposed to *C. jejuni* supernatants and loaded with FLUO-3. Increased localised fluorescence suggests calcium increase in intracellular compartments.

Table 3.1. Qualitative rise in intracellular calcium in HEp-2 and CHO cells

strain	enterotoxin	cytotoxin	FLUO-3 dependent fluorescence due to elevation of calcium induced by:- whole bacteria	culture supernatants
L115	+	+	-	-
C119	+	+	-	+
O81	+	+	-	+
N82	+	-	-	+
P71	-	+	-	-
NCTC 12189	+	+	+	+
H104	+	-	-	+
D265	-	+	-	+

Table 3.2 Uptake of ^{45}Ca by Caco-2 cells treated with *C jejuni* supernatants

strain	cpm/minute
uninfected	2073
NCTC 12189	4856
F132	7904
C119	8142
L115	4971
P71	8522
O81	6268
N82	11,632
H104	8397
Total count	1798938

Average of 8 assays per strain

After incubation the cells were again washed well, and coverslips were wet mounted and examined for changes in calcium levels in infected cells using conventional fluorescence microscopy, and also by laser scanning confocal microscopy using a Zeiss AXIOPHOT 10 fluorescence microscope in conjunction with an MRC 600 laser scanning confocal microscope. To minimize fluorescence quenching all manipulations were carried out in subdued light.

3.2.5. Measurement of intracellular calcium in cells exposed to *C. jejuni* supernatants. The fluorescent indicator dye fura-2 was used for quantitative estimation of intracellular calcium concentration, for which commercially available software has been developed (Perkin-Elmer). The excitation wavelengths of the free dye are 340 and 380 nm, with emission at 509 nm. Confluent Caco-2 cells grown on coverslips were washed in HEPES buffered saline and treated with bacterial culture supernatants for 6 h. At the end of this time cells were again washed, and coverslips were placed into a Perkin-Elmer LS5B spectrofluorimeter. 5 μM of the acetoxymethyl ester fura-2/AM in HEPES buffered saline was added, and intracellular calcium levels of supernatant treated cells and untreated controls were calculated from the ratio of fluorescence at 340/380 nm excitation wavelengths where R_{max} and R_{min} were determined with 0.1% Triton X-100 and 3 mM EGTA respectively.

3.2.6. Radioactive calcium uptake. Caco-2 cells grown on coverslips were exposed to culture supernatants of *C. jejuni* for 6 h at 37°C in 5% CO_2 in the presence of 50 μl of ^{45}Ca. At the end of the incubation period cells were washed well in HEPES buffered saline, placed in 10 ml Optiphase (LKB) scintillant and counted in a Packard Tri-Star liquid scintillation spectrometer.

3.3. RESULTS

3.3.1. Phosphorylation of Caco-2 cell proteins. Incubation of Caco-2 cell monolayers for various times with whole bacteria and culture supernatants of strains H104 and D265 increased phosphorylation of several Caco-2 cell membrane and cytoplasmic proteins (Figs. 3.1 and 3.2). Proteins of 21, 23 and 35-50 kDa were phosphorylated in membrane

fractions; heavily phosphorylated proteins were also seen in the cytoplasmic fractions of Caco-2 cells. Phosphorylation of Caco-2 cell proteins by *C. jejuni* was time-dependent, increasing in intensity up to 18 h. Cytoplasmic proteins were phosphorylated at 18 h by bacteria free supernatants of strain H104, and by whole bacteria and culture supernatants in strain D265. Heavily phosphorylated proteins were the same in differentiated and undifferentiated cells. Phosphorylation was strain specific, with different phosphoproteins induced by supernatants and whole cells of the two strains. H104 whole bacteria induced phosphoproteins in the membrane fractions of differentiated and undifferentiated cells at 18 h. D265 whole cells and supernatants also affected phosphorylation of proteins in the membrane fraction. Treatment of *C. jejuni* with ^{32}P in the absence of Caco-2 cells showed no significant phosphorylation of bacterial proteins. Uninfected differentiated cells showed no protein phosphorylation, while control undifferentiated cells showed weak bands at 21, 23, 31 and 43 kDa in the membrane fraction and 20, 21, 35, 40 and 68 kDa in the cytoplasmic fraction. From these experiments it seems that a soluble factor (possibly a toxin) produced in bacterial exponential growth mediates this phosphorylation. Figure 3.2 includes panels showing phosphorylation of Caco-2 cell proteins by enteropathogenic *E.coli* (data of Tom Baldwin) and TPA, a known activator of PKC.

3.3.2. Qualitative demonstration of intracellular calcium. Chinese hamster ovary (CHO) and HEp-2 cells loaded with FLUO-3 showed low level fluorescence when treated only with tissue culture medium. Whole cells treated with culture supernatants of strains H104, D265, C119, N82, O81, P71 and NCTC 12189 showed significantly increased fluorescence, indicating a significant rise in intracellular calcium (Fig. 3.3). Cells exposed to culture supernatants of strains L115 and P71 showed fluorescence comparable to uninfected control cells. The only strain for which the presence of whole bacteria resulted in elevated intracellular calcium levels was NCTC 12189 (Table 3.1). Confocal microscopy of HEp-2 cells exposed to culture supernatants showed an increase in cytoplasmic calcium but also demonstrated increased calcium in cellular organelles presumably within the endoplasmic reticulum or mitochondria (Fig. 3.4). Caco-2 cells were difficult to load with FLUO-3 and results were difficult to interpret, possibly

due to the thickness of the cells.

3.3.3. Measurement of intracellular calcium. The fluorescent calcium indicator fura-2 was used to confirm that increased calcium levels were concentrated in intracellular organelles. At the concentrations used fura-2 does not penetrate intracellular compartments and therefore will only measure free calcium in the cytoplasm. Measurements using fura-2 failed to show quantitative increases in intracellular calcium levels in response to treatment with bacterial supernatants. This is in contrast with qualitative data using FLUO-3 that clearly indicate an increase in supernatant-treated cells.

3.3.4. Radioactive calcium uptake by Caco-2 cells. Culture supernatants of all strains tested induced uptake of ^{45}Ca compared to untreated cells (Table 3.2). This may be due to the presence of a pore forming toxin allowing calcium to enter the cell, an undescribed toxin mediating calcium release from intracellular stores, or a bacterial product inducing cell calcium uptake.

3.4. DISCUSSION

Protein phosphorylation is important in the control of a variety of cellular processes including the regulation of ion transport across biological membranes (de Jonge and Lohmann, 1985). For example, enteropathogenic bacteria such as *V. cholerae* and enterotoxigenic *E. coli* secrete soluble toxins that can activate specific host cell kinases; these are presumed to be important in the diarrhoeal process because they phosphorylate membrane proteins that transport ions from the cell (Field, 1976 and 1981). Supernatants of *C. jejuni* strain H104 and *C. coli* strain D265 contain an activity which stimulates cellular protein kinases in Caco-2 cells, causing phosphorylation of several proteins. The major phosphorylated protein (21 kDa) is probably a substrate for PKC, since a protein of this size was also phosphorylated in response to treatment with the known activator of PKC, the phorbol ester TPA. The same Caco-2 cell proteins were phosphorylated on infection with live bacteria suggesting that a stable soluble factor (possibly a toxin) is produced by live bacteria in contact with cells in this *in vitro* system.

Activation of protein kinases and the phosphorylation of cellular proteins are probably independent of the cyclic nucleotide cAMP. Secretagogues acting via cAMP (vasoactive intestinal peptide, prostaglandin E2) activate chloride-specific channels in the apical membrane and alter the phosphorylation of several soluble proteins in enterocytes (Cohn, 1987). In Caco-2 cells, increased membrane permeability to chloride induced by secretagogues that increase cAMP (such as PGE2) is associated with enhanced phosphorylation of a 42 kDa protein (Burnham and Fondacaro, 1990). However, no significant phosphorylation of a 42 kDa protein was observed in Caco-2 cells exposed to whole bacteria or supernatants, suggesting that increased cAMP levels alone cannot account for diarrhoea seen in campylobacter disease.

The possibility that one or more toxins, exist in these strains is supported by the fact that supernatants increased intracellular calcium concentrations in a variety of cell lines. It is possible that a toxin that increases cAMP levels and a toxin that elevates intracellular calcium concentrations may be synergistic in inducing secretion. A persistent increase in intracellular calcium, even in the absence of extracellular calcium, can occur in response to cyclic nucleotides by an unknown mechanism (Semrad and Chang, 1986, 1987; Lehninger, 1975). Confocal microscopy using FLUO-3 showed compartmentalisation of calcium within cell organelles at concentrations far higher than those normally seen. To confirm this effect, fura-2 was used at concentrations too low to allow penetration into these compartments; thus fura-2, in contrast to FLUO-3, apparently showed no enhancement of intracellular calcium. High levels of calcium in organelles is also likely to cause cell death, and so viability of supernatant-treated cells should be investigated in future studies. Experiments with radioactive calcium showed accumulation of label from the extracellular medium. This may be due to the activity of a new toxin or cytotoxin of *C. jejuni* strains, but evidence that this factor is distinct from toxins already described comes from studies using strain N82, which has no measurable cytotoxic activity but still increases cell calcium. On the other hand calcium increases in intracellular compartments might stimulate compensatory uptake of calcium from the surrounding medium. Toxin mediated increase in intracellular calcium concentrations may help to

explain the pathogenesis of watery diarrhoea in *C. jejuni* infection. In almost all studies of enterocytes, regardless of species, an increase in intracellular calcium inhibits Na and Cl absorption, stimulates anion secretion, and/or modulates either the apical or basolateral membrane K conductance (Donowitz and Welsh, 1987). As in the case of the cyclic nucleotides, this results in net luminal fluid accumulation.

In addition to the already well described kinase activation and protein phosphorylation in response to bacterial toxins (de Jonge and Lohmann, 1985), enteropathogenic *E. coli* (EPEC) cause phosphorylation of several cellular proteins, including the major protein species of 21 kDa, in HEp-2, Caco-2 cells (Fig. 3.2), and *in vivo* mediated not by a soluble toxin but by intimate bacterial attachment to cell membranes (Baldwin *et al.*, 1990). The 21 kDa protein phosphorylated by EPEC infection of Caco-2 cells is presumably the same protein observed in cells treated with campylobacter supernatants. The 21 kDa protein has been identified as a myosin light chain (Manjarrez-Hernandez *et al.*, 1992), and phosphorylation is thought to regulate the extensive cytoskeletal rearrangements observed in the characteristic lesion formed by EPEC on both gut tissue and cultured epithelial cell monolayers. Myosin light chain is a substrate for both PKC and the calcium activated myosin light chain kinase, but phosphorylation by the two enzymes has different results. PKC gives a phosphorylated protein which becomes cytoplasmic. After EPEC infection, however, phosphorylated myosin light chain is associated with the membrane cytoskeletal fraction in complex with actin. Phosphoamino acid analysis of the latter type, shows activity of myosin light chain kinase, an enzyme that has been identified in enterocyte brush border preparations (Rieker and Collins, 1987). The role of myosin in the function of the intestinal epithelium has yet to be established, although involvement in the regulation of paracellular water and electrolyte movement has been proposed (Broschat *et al.*, 1983). The finding that EPEC causes an increase in intracellular free calcium has raised the intriguing possibility that EPEC activates cellular signal transduction mechanisms which cause release of calcium from intracellular stores. This in turn leads to calcium-dependent kinase activation and ion channel regulation resulting in cell death and fluid loss. Another class of diarrhoeagenic *E. coli*, enteroaggregative *E. coli* (EAEC), produce a soluble haemolysin-like toxin

that increases intracellular calcium by influx from the external medium; the same patterns of phosphorylation as EPEC were observed (Baldwin *et al.*, 1992).

Protein phosphorylation and increased intracellular calcium levels in cells treated with *Campylobacter jejuni/coli* supernatants can therefore be interpreted in the light of the EPEC and EAEC data. Treatment with campylobacter supernatants results in a major phosphoprotein in the 21 kDa region similar to EPEC and EAEC treated cells, increases intracellular calcium levels, and induces morphological changes in the cells, from normal pavement morphology to rounding up. The rounded cells are proposed to be due to the cytotoxin produced by campylobacters and cannot be neutralised by antibodies against LPS lipid A. The cell morphological change is associated with the increases in intracellular calcium observed in epithelial cells incubated with culture supernatants (demonstrated using FLUO-3, and also by uptake of radioactively labelled calcium). An increase in intracellular calcium may activate calcium-dependent kinases, such as PKC; the major 21 kDa myosin light chain phosphoprotein induced by treatment with campylobacter culture supernatants is located predominantly in the cytosolic fraction, an effect similar to TPA activation of PKC. As mentioned above myosin light chain is thought to be a regulator of cytoskeletal rearrangements leading to change in cell morphology. TPA induces intestinal secretion by activation of PKC, and *C. jejuni* supernatants mimic TPA-induced phosphorylation of Caco-2 cell proteins. Secretion induced by campylobacter infection may therefore be due to activation of PKC by a bacterial toxin. In this respect campylobacter induced phosphorylation of cell proteins is more like EAEC than EPEC.

In summary, phosphorylation of ion channels, mediated by second messengers activated by bacterial products, is important in the pathogenesis of diarrhoeal disease. *C. jejuni* supernatants mediate phosphorylation of proteins in the enterocyte-like Caco-2 cell line, at least one of which is a cytosolic component and some of which may be ion channels. It should be noted, however, that as yet the functional significance of any phosphorylated protein in disease is not known. The pattern of phosphorylation is consistent with an increase in intracellular calcium, induced by a soluble bacterial product and increased intracellular calcium is

associated with cytoskeletal changes and anion secretion.

Chapter 4

Rabbit ileal loop model of *C. jejuni* diarrhoea.

4.1. INTRODUCTION

Tissue culture cells make useful *in vitro* models for the study of bacterial and host cell interaction but only by the use of a relevant animal model can disease mechanisms be elucidated. Animal models are relevant in the study of bacterial pathogenicity because the use of a model that can mimic human pathology will reveal basic mechanisms of disease. Such understanding can hopefully lead to effective treatment based on testing in these model systems.

4.1.1. Animal models. Many animal models have been used to study the pathogenesis of campylobacter enteritis, but progress has been hampered by the absence of a simple model. However several are notable in that they seem to mimic human infection. Prescott (1981) fed gnotobiotic beagle puppies with *C. jejuni*, and they subsequently developed mild diarrhoea with colitis consisting of neutrophil infiltration of the lamina propria, exfoliation of the surface epithelium and loss of goblet cells with hypertrophy of the glands. Fitzgeorge *et al.* (1981) used young rhesus monkeys which also developed disease very similar to that seen in humans. Although the symptoms were mild, the animals had associated bacteraemia and prolonged intermittent excretion of organisms in their faeces. After recovery, animals challenged with the same strain remained asymptomatic, had no associated bacteraemia and excreted the organism for only three days. In *Macaca nemestrina,* challenge with *C. jejuni* produced acute diarrhoea with bloody stools and faecal leucocytes lasting 7-10 days (Russell *et al.*, 1989), with colitis and cellular infiltration along with an increase in specific IgA, IgM and IgG. In this case also, prior infection protected against subsequent challenge.

No established small mammal model exists that mimics human disease in the absence of previous treatment or surgical proceedure. Simple intragastric inoculation of adult mice (Field *et al.*, 1984) and hamsters (Humphrey *et al.*, 1985) results in transient colonization without symptoms.

This colonization can be prolonged in adult mice by pretreatment with antibiotics which alters the normal colonic flora (Field *et al.*, 1984). Blaser *et al.* (1984) showed that oral infection of mice does not induce disease, but is associated with colonization and bacteraemia. An infant mouse model is presently the simplest of the *in vivo* systems that have been developed (Kazmi *et al.*, 1984). Of 42 infant mice challenged intragastrically with one of three strains of *C. jejuni*, 36 developed severe diarrhoea.

Caldwell *et al.*, (1983) showed that the removable intestinal tie adult rabbit diarrhoea (RITARD) procedure (Spira *et al.*, 1981; Spira and Sack, 1982) can be used for *C. jejuni*. After a surgical procedure that results in temporary small bowel obstruction, rabbits develop mucous diarrhoea, with or without bacteraemia, and abnormal intestinal histology very similar to that described in man. The RITARD procedure is useful for studies of pathogenic mechanisms and immune responses, but is not suitable for screening large numbers of strains for differences in virulence factors.

The rabbit ileal loop test (RILT) has been used as a relevant model for study of intestinal fluid secretion and tissue histology in a variety of infectious diarrhoeal diseases (Sedlock and Deibel, 1978; Leitch, 1988; Wallis *et al.*, 1989). The technique involves tying off blind loops of ileum or colon and infecting with the bacterium under test. It has the advantage that fluid and tissue can be collected for later biochemical and histological examination. The experiments in this chapter were performed in order to collect infected tissue with similar histological profile to that seen in human disease, in order that the role of inflammatory mediators (Chapter 5) and second messengers (Chapters 3 and 6) in the pathophysiology of the disease could be investigated, with particular regard to fluid secretion. The RILT model fulfils such criteria (although it does not serve as a model for initiation of illness by colonisation via the oral route) and, according to a preliminary report at the 2nd International Conference on *Campylobacter* Infections, has been used for the study of *C. jejuni* ileal fluid secretion and histology (McCardell *et al.*, 1983). To date no full description of this work has appeared in the literature, and this chapter seeks to characterise selected isolates with regard to their ability to cause to fluid secretion in the RILT and to their effects on intestinal histology.

4.2. MATERIALS AND METHODS

4.2.1. Bacteria. *C. jejuni* strains from the collection studied in Chapter 2 were were selected on the basis of endoscopic and histologic data in man and toxin profile (Table 4.1). A non-colonising mutant strain NCTC 12189 was included as a negative control strain. Bacteria were grown in Mueller-Hinton (MH) broth overnight at 42°C, adjusted to approximately 10^8 bacteria/ml, centrifuged and resuspended in 1 ml of phosphate buffered saline (PBS) for loop injection.

4.2.2. Serum sensitivity. The six strains were tested for their susceptibility to the bactericidal action of human and rabbit serum before inoculation into rabbit ileal loops, and after subsequent isolation. Gelatin veronal buffer plus Mg^{2+} and Ca^{2+} at pH 7.5 (GVB^{2+}), prepared according to Taylor (1985), was used to assess serum bactericidal activity because it provides essential divalent cations for complement activity, does not deleteriously affect the viability of Gram negative bacteria and provides an environment in which high rates of serum killing can be achieved. GVB^{2+} was prepared from the following stock solutions:- (a) 5 x veronal buffer: 41.2 g of sodium chloride (BDH) and 5.095 g sodium 5,5 diethyl barbiturate (Sigma) were dissolved in 700 ml distilled water, adjusted to pH 7.35 with 1 N hydrochloric acid (BDH) and made up to 1 l; (b) stock metals: equal volumes of 2 M magnesium chloride (BDH) and 0.3 M calcium chloride (BDH); (c) working solution: 1 g of gelatin (Oxoid) was dissolved in 600 ml of hot water and cooled to room temperature. 200 ml of 5 x veronal buffer and 1 ml of stock metals were added, made up to 1 l with water and sterilised by membrane filtration. A 100 mM solution of magnesium ethylene glycol bis-(*p*-aminoethyl ether)-tetra acetic acid (MgEGTA, Sigma) in 0.85% (w/v) saline containing 0.1 M magnesium chloride ($MgCl_2.6H_20$), was prepared by heating and addition of 5 N NaOH until the EGTA went into solution, and titrated to pH 7.45 with 1 N HCl (Fine *et al.*, 1972). When required, 0.1 ml of MgEGTA solution was added to 0.9 ml of serum to give a final concentration of 10 mM.

Bactericidal assays were performed as described by Taylor and Kroll (1983). Logarithmic phase cultures were diluted in warm GVB^{2+} to a density of 10^7 organisms/ml, and 0.25 ml aliquots were incubated at 37°C with 0.5

Table 4.1. Characteristics of *C. jejuni* strains tested in the RILT.

strains	Endoscopy[a]	Histology[b]	Cyto-toxin[c]		Entero-toxin[d]	Caco-2 invasion[e]	Transcytosis[e]
			son	sup			
Colitis							
L115	3	3	+	+	+	+	-
C119	3	3	+	+	+	+	+
O81	2	2	+	+	+	+	+
P71	1	1	+	-	-	+	+
Non inflammatory							
N82	ND	ND	-	-	+	+	+
NCTC 12189	ND	ND	ND	ND	ND	ND	ND

[a] Endoscopic observations: 1, moderate hyperaemia; 2, micronodular ulceration, moderate oedema and hyperaemia; 3, large nodular ulceration, severe oedema and hyperaemia with exudate of mucous and pus.
[b] Histological observations: 1, moderate infiltration of polymorphonuclear leucocytes and lymphocytes; 2, marked infiltration of polymorphonuclear leucocytes and lymphocytes with oedema; 3, marked infiltration of polymorphonuclear leucocytes and lymphocytes with oedema and cryptitis.
[c] Cytotoxic activity of bacterial sonicates (son) or culture supernatants (sup) by CHO assay; data of Dr Herman Goossens.
[d] Enterotoxin activity of supernatants using CHO assay; data of Dr Herman Goossens.
[e] Data from chapter 2.
ND, not done.

ml of undiluted serum. Samples were taken at 0, 30, 60, 120, and 180 min for viable counting on MH agar after microaerophilic incubation at 42°C. Control normal serum inactivated by heating at 56°C, and a buffer control were run in parallel with every serum tested. MgEGTA selectively inactivates the classical pathway of complement killing by chelating divalent cations required for the classical but not the alternative pathway (Roberts and Phillips, 1981). Thus the activity of the alternative pathway was selectively investigated by comparing the results of bactericidal assays with or without MgEGTA-chelated serum. Serum killing activity was defined as recommended by Taylor (1983); greater than 80% survival after 180 min indicated failure of the serum to kill the organism under test, while serum sensitivity was indicated by less than 20% survival at 180 min.

4.2.3. Rabbit ileal loop surgery. Since the susceptibility of rabbits to enteric pathogens is age dependent (Pazzaglia *et al.*, 1990), the age of the animals used in this study was chosen for maximum susceptibility. 7-9 week-old specific pathogen free (SPF) New Zealand White rabbits, not more than 2.0 kg in weight, were used. Two animals were used for each bacterial strain studied; each animal had 2 test loops, a negative (PBS) control loop, and a positive (cholera toxin, 2 μg/ml) control loop .

Surgery was performed by Dr. Paul Sibbons at the Institute of Child Health, Great Ormond Street, under licence by standard procedures. Animals were anaesthetised with halothane over oxygen and nitrous oxide. The abdomen was shaved from the lower rib margin to the level of the iliac fossa. Skin was prepared using chlorhexidine followed by iodine. Laparotomy was performed from the lower liver margin to the level of the iliac fossa using diathermy. The distal ileum and the ileo-caecal junction were elevated and kept moist with warm sterile saline. The distal end of each loop (4 per animal) was tied with 4/5 metric Mersilk, proximal from the ileo-caecal junction, 10 cm apart. The ligatures for proximal ends of loops were passed 5 cm proximal to distal ties. Loops were injected with test bacteria, cholera toxin (positive control, 2 μg/ml) or negative control (0.5 ml PBS); the needle was passed into the lumen at the proximal end of the loop, and the ligature was tightened as the needle was withdrawn. All loops were checked for loss of integrity in the form of leaks, bleeding or tissue damage. Loops were replaced into the peritoneal cavity in their original position and

the peritoneum was closed with 2/0 Dexon Plus in a continuous mattress stitch using an atraumatic needle. The skin was closed with 2/0 prolene with a cutting needle using an interrupted mattress stitch. Animals were allowed to recover and observed. At 18 h post-infection, animals were anaesthetized using halothane and laparotomy was performed using the original incisions. The loops were removed separately and intact, and then weighed. Any fluid in the loop was removed to a sterile container.

4.2.4. Enterotoxin. *Campylobacter* strains were grown in Brucella broth (Difco) supplemented as previously described (Goossens *et al.*, 1985). Cell-free culture supernatants (20 ml) were added to 400 ml Modified Eagles Medium (MEM) with Earles salts containing 1% FCS and 100 μg/ml gentamicin in individual wells of a Lab Tek 8 chamber tissue culture plate containing CHO monolayers. The cells were incubated for 16-18 hours, fixed with methanol and stained with 1% Giemsa solution (Merck). Elongated CHO cells indicative of enterotoxin activity were those with a length to width ratio greater than 3:1. At least 200 cells were counted per well. All assays were performed by Dr Herman Goossens, St Pieters Hospital, Brussels.

4.2.5. Cytotoxic activity. Strains were grown in a biphasic culture system composed of MH agar (Difco) as the solid phase and medium 199 (Gibco) with Hanks salts and L-glutamine as the liquid phase. Incubation was at 42°C for 48 h in a microaerophilic atmosphere. Bacteria were pelleted by centrifugation, and the supernatants filter sterilized. Pellets were resuspended in a tenth volume of medium 199, disrupted by sonication, centrifuged and filter sterilized. Dilution series of culture supernatants and sonicates were added to CHO monolayers in MEM with Earles salts and 10% FCS in 96-well microtitre plates (Nunc), and incubated for 6 days. Morphological changes were observed by phase contrast microscopy; cytotoxic activity (which was not neutralized by antiserum against purified Shiga toxin) was indicated by rounding and death of CHO cells. All assays were performed by Dr Herman Goossens at St Pieters Hospital, Brussels.

4.2.6. GM-1 enzyme linked immunosorbent assay (ELISA). Since cholera-like toxin activity has been attributed to *C. jejuni*, rabbit ileal loop fluids and homogenised tissue extracts were tested using

monosialganglioside GM-1 ELISA. All assays were performed by Mr Robin Leece in this department. Loop fluids and tissue extracts were tested neat and diluted in PBS containing 0.1% (w/v) bovine serum albumin (BSA, Sigma). 96-well microtitre plates (Nunc) were coated with 100 µl/well GM-1 in PBS (1.5 µg/ml) overnight at room temperature. Plates were washed twice in PBS, and 200 µl of 0.1% (w/v) BSA in PBS were added to each well and incubated at 37°C for 30 min, after which the plates were again washed thoroughly in PBS. Samples were added as a doubling dilution series in PBS containing 0.1% BSA in a final volume of 100 µl. Cholera toxin (Sigma, 1 µg/ml) was used as a standard. Plates were left at room temperature for 1 h and then washed 3 times with PBS; the primary antibody, a 1/100 dilution of anti B subunit of cholera toxin in 0.1% BSA in PBS (100 µl/well) was added, incubated at room temperature for 60 min, and washed 3 times in PBS containing 0.05% Tween 20. The secondary antibody, a 1/5000 dilution of rabbit anti-goat IgG-horseradish peroxidase conjugate in 0.1% BSA and 0.05% Tween 20 in PBS, was added (100 µl/well), again incubated at room temperature for 60 min, and again washed 3 times with PBS containing 0.05% Tween 20. The hydrogen peroxide substrate was 1 mg/ml o-phenylenediamine dihydrochloride (OPD) in 0.1 M citrate buffer (pH 4.5) to which 0.4 µl of 30% hydrogen peroxide/ml was added immediately before use. 100 µl of the solution were added, and absorbance at 450 nm was recorded after 15 min incubation at room temperature on a Biorad microplate reader model 3550. The reaction was stopped by addition of 100 µl of 1 M sulphuric acid (BDH).

4.2.7. Blood culture. Blood cultures were taken 4 h post-operation and again just before post mortem. A portion of freshly taken blood was lysed using 0.5% deoxycholate (Sigma), diluted, and plated onto MH agar to give an exact count of the numbers of organisms/ml present in the bloodstream. The remainder was inoculated into MH broth (Oxoid) and incubated in a microaerophilic atmosphere at 37°C. The enriched culture was then subcultured onto MH agar after 24 h incubation.

4.2.8. Viable counts. Loop fluid was diluted in PBS and plated onto MH agar; an average of 3 counts were performed for each fluid. Loop tissue was weighed, homogenised in a small volume of PBS and then diluted for viable counting as above. At post-mortem internal organs were also cultured

for the presence of *C. jejuni*.

4.2.9. Histology. Tissue samples (4 per loop) for light microscopy were washed in PBS, and placed in 3% formaldehyde. Thin sections were cut with a microtome, stained in haematoxylin and eosin and examined using a Zeiss AXIOPHOT microscope. [Note: tissue was also analysed for cyclic AMP, cyclic GMP and prostaglandin E2 (results presented in Chapters 5 and 6)].

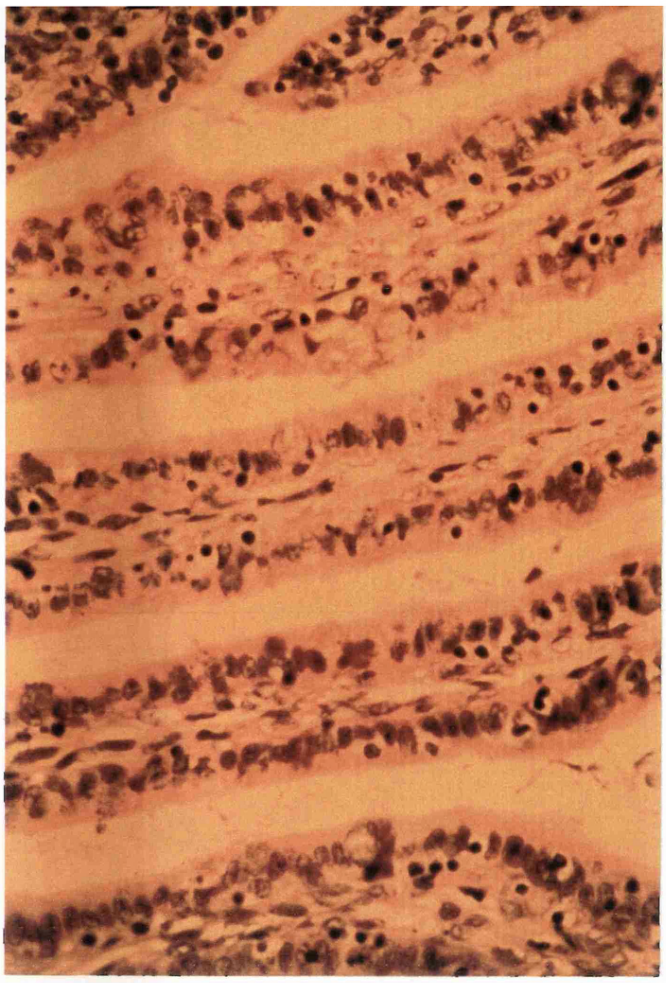
4.2.10. Fluid biochemical analysis. Fluid from infected and positive control loops were examined biochemically using a Howe ABL-2 acid-base laboratory analyser (Radiometer, Copenhagen). pH, haemoglobin (g/l), bicarbonate (mmol/l) and total protein (g/l) were measured for all fluids. Loop fluids were examined by light microscopy for the presence of red and white blood cells. [Note: fluids were also tested for the presence of prostaglandin E2 and leukotriene B4 (results presented in Chapter 5)].

4.3 RESULTS

4.3.1. Rabbit ileal loop tests (RILT). There were obvious similarities between the severity of histology observed in human patients (Table 4.2) and in ligated rabbit ileal loops (Fig. 4.1) infected with the four strains from human colitis; all gave inflammatory reactions with fluid accumulation in rabbit ileal loops (Tables 4.2 and 4.4). In contrast, a strain from a case of watery diarrhoea (N82), that was shown to produce enterotoxin (by CHO elongation), elicited neither histological changes to the mucosa nor fluid accumulation.

Histological responses ranged from flattened villi with cell damage, large numbers of white cells and submucosal bleeding, to completely normal mucosa. C119 elicited white cell infiltration of the mucosa, with villi resembling Christmas trees, a response similar to that associated with cholera toxin-mediated fluid secretion (Ketley *et al.*, 1987). This strain also caused the most fluid secretion. In all cases of fluid secretion macroscopical or microscopical blood was observed in the fluid, fluid and tissue polymorphonuclear leucocytes (PMNL) were present, and some histologic sections showed eosinophils and macrophages. These observations also correlate with the inflammatory type of illness seen in humans, where blood

Figure 4.1. Histology of *C. jejuni* infected ileal loops. (a) Normal villi from negative control loop (x 40 high power objective). (b) Cholera toxin treated villus (x 40 high power objective). (c) Tissue from loop infected with colitis strain O81 showing shortening of villi, and distortion of villous architecture (x 10 low power objective). (d) Tissue from loops infected with strain C119 showing "Christmas tree"-like villi similar to those treated with cholera toxin (x 40 high power objective). (e) Submucosal white cell infiltrate and tissue oedema in loop infected with colitis strain O81 (x 10 low power objective). (f) High power (x 40) detail of white cell infiltrate in previous picture showing predominant polymorphonuclear leukocyte response. (g) High power (x 40) detail of submucosal bleeding. (h) Severe tissue oedema in O81 infected loop (x 10 low power objective).



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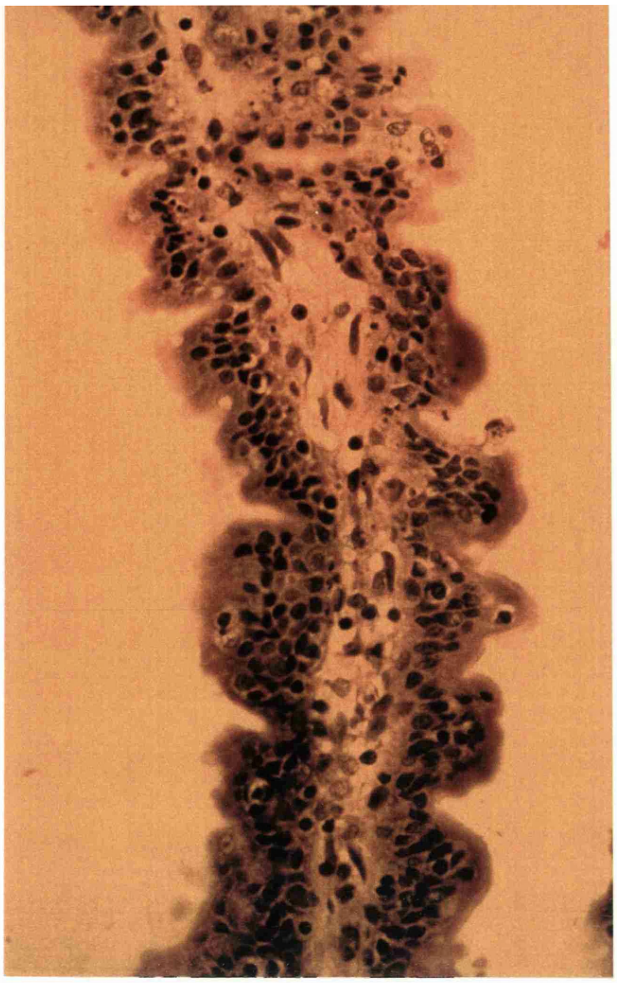

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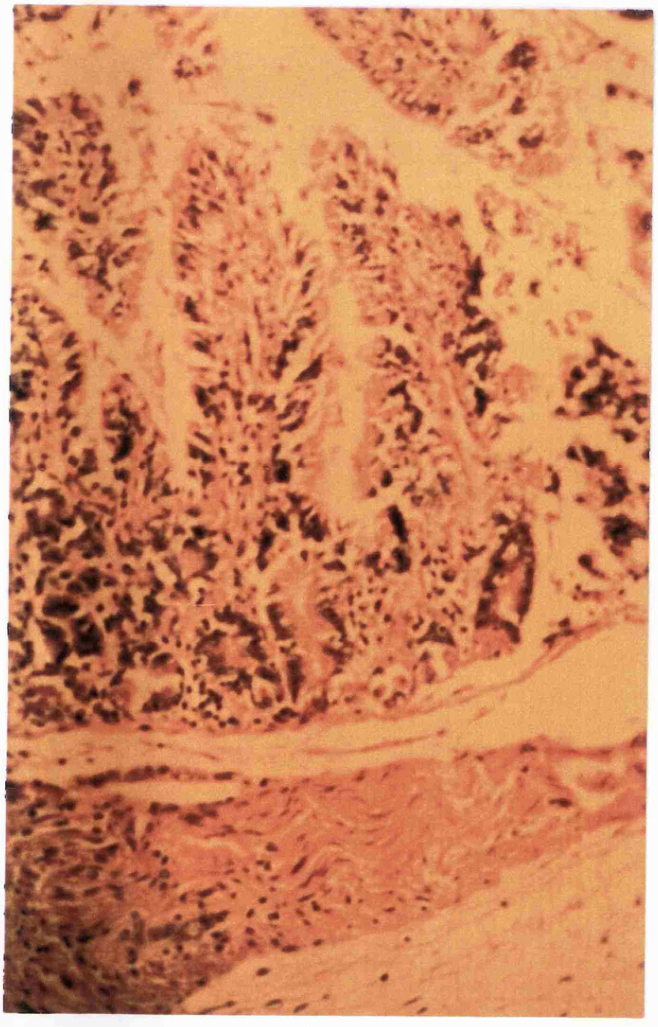

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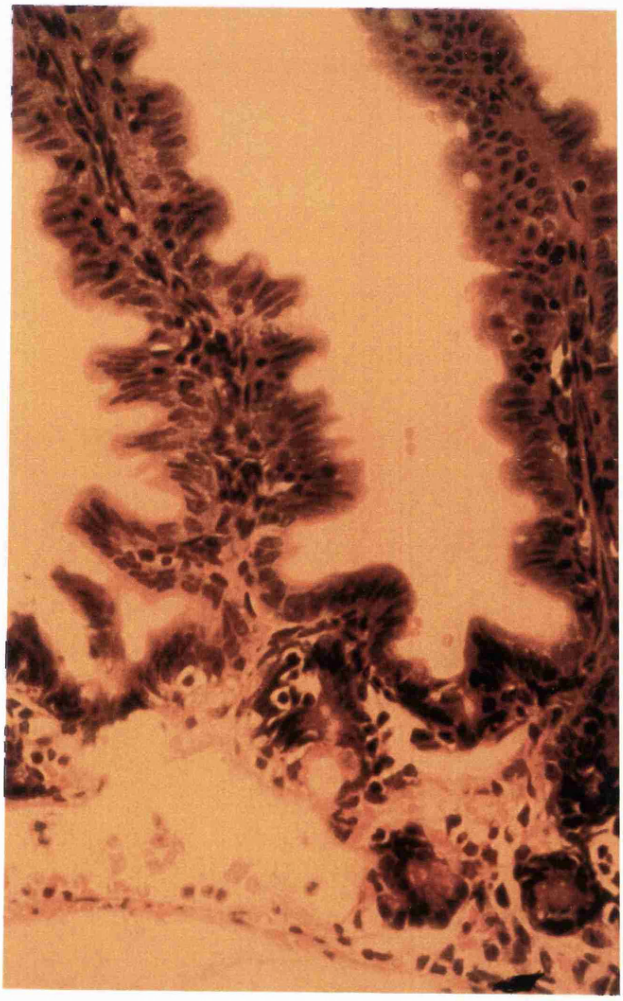

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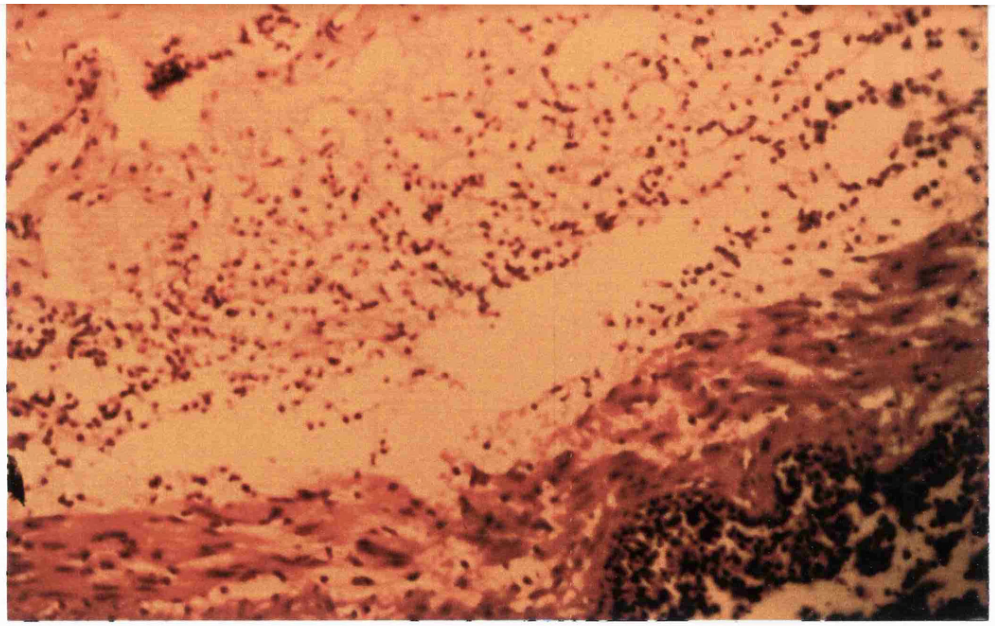

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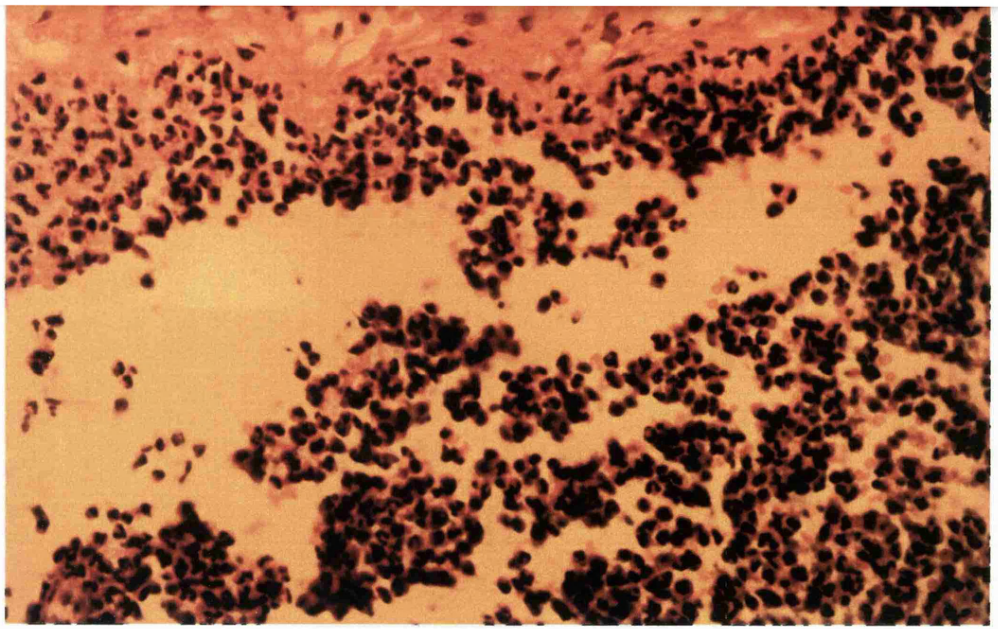

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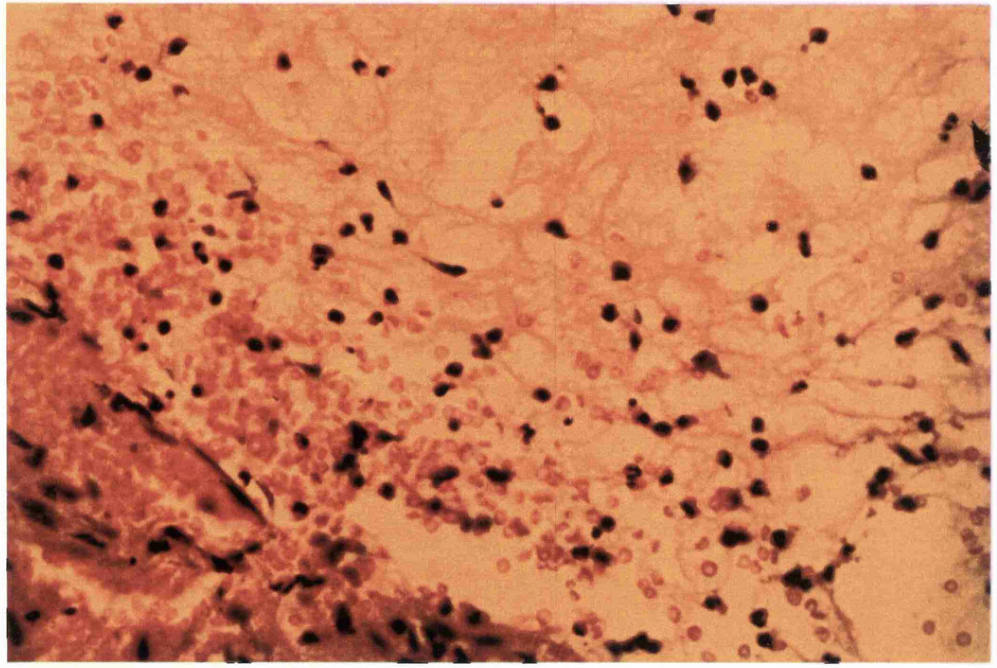

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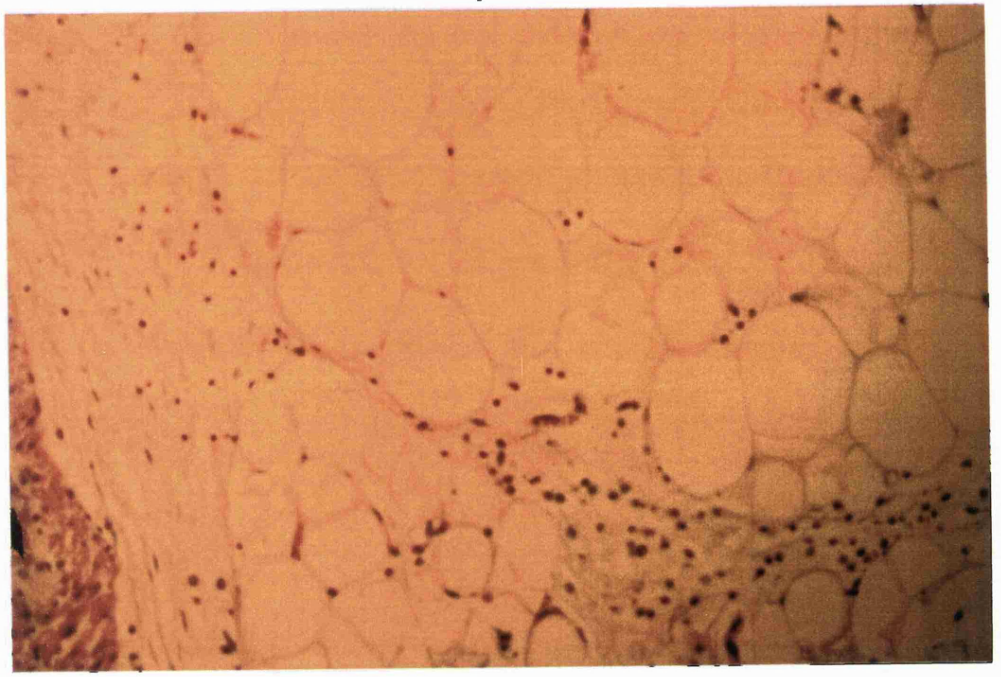

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Fig. 4.2a

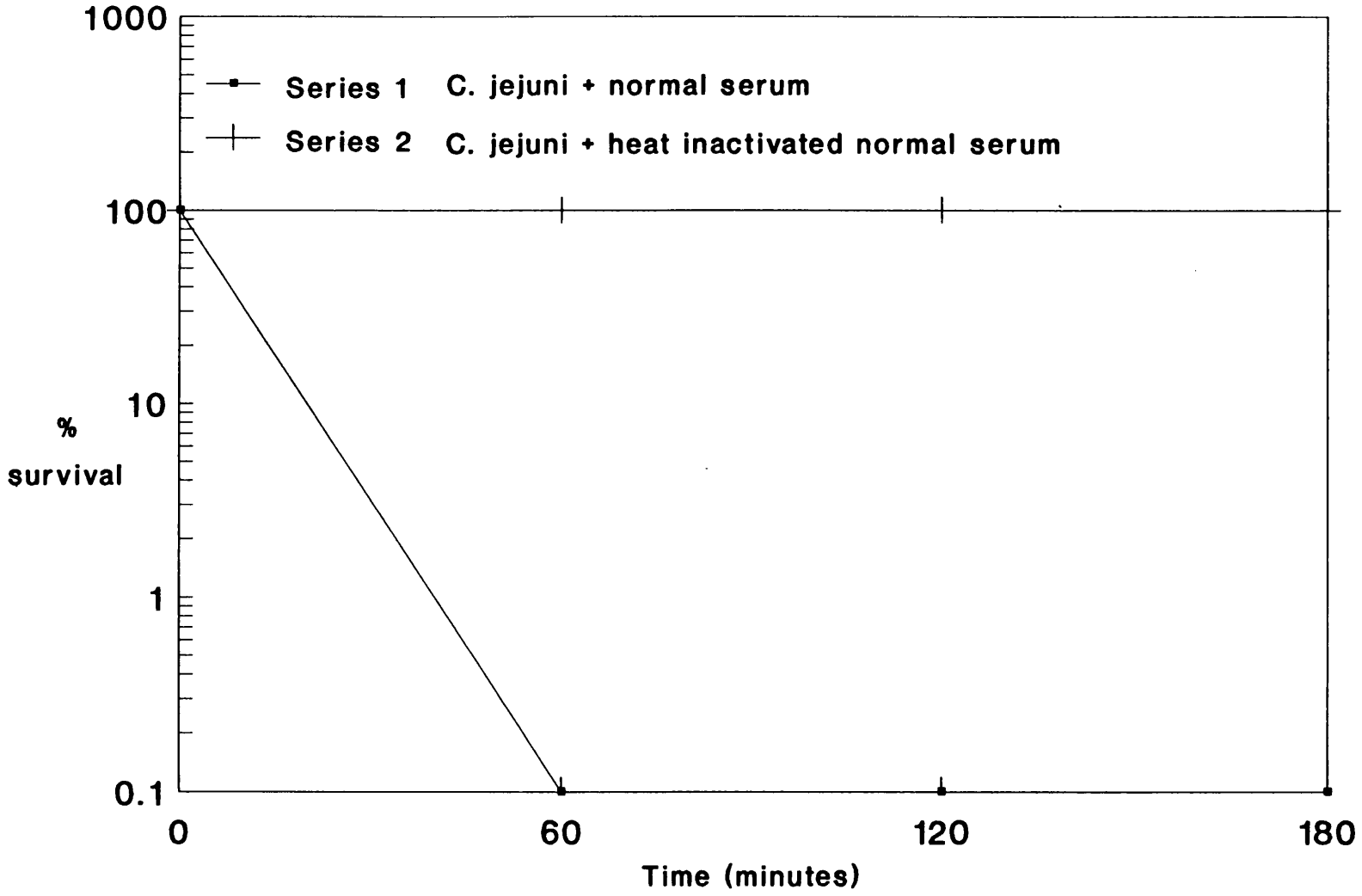

1000
100
10
1
0.1
%
survival
Series 1 C. jejuni + normal serum
Series 2 C. jejuni + heat inactivated normal serum
0
60
120
180
Time (minutes)

Fig. 4.2b

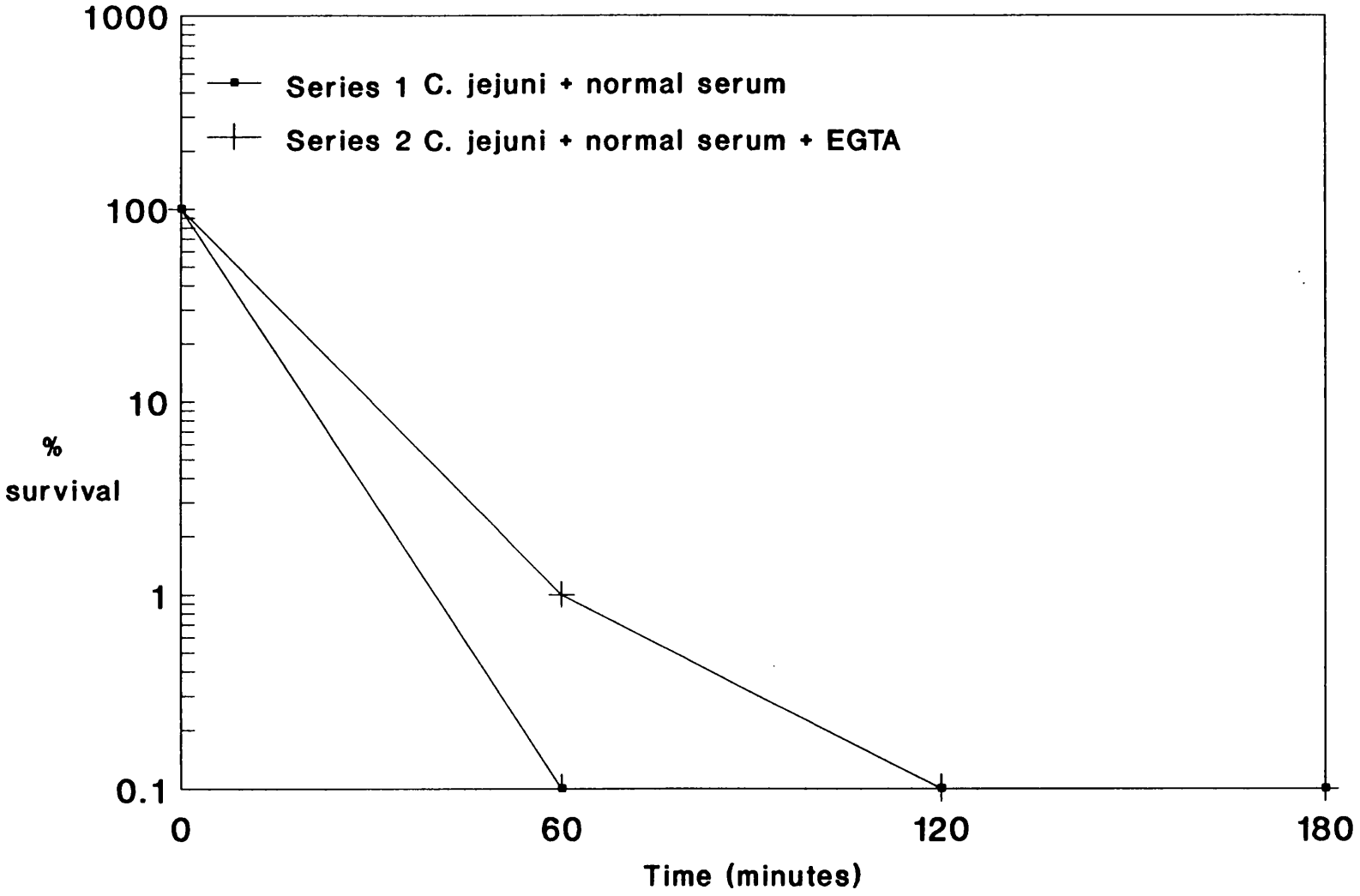

1000
100
10
1
0.1
%
survival
Series 1 C. jejuni + normal serum
Series 2 C. jejuni + normal serum + EGTA
0
60
120
180
Time (minutes)

Table 4.2. Histology in RILT compared to endoscopy and histology in man.

strain	Endoscopy	Histology in man	Histology in RILT
L115	large nodular ulceration, severe oedema, hyperaemia, mucous and pus.	tissue oedema, cryptitis, marked infiltrate of PMNL[a].	blood stained mucosa, flattened villi, cell damage, moderate infiltrate of PMNL, submucosal bleeding.
C119	large nodular ulceration, severe oedema, hyperaemia, mucous and pus.	tissue oedema, cryptitis, marked infiltrate of PMNL.	blood stained mucosa, "Christmas tree" villi, submucosal oedema and bleeding, moderate PMNL infiltrate.
O81	micronodular ulceration with moderate oedema and hyperaemia.	tissue oedema; marked infiltration of PMNL.	haemorrhagic mucosa, oedema and bleeding into submucosa, damaged villi, marked PMNL infiltrate.
P71	moderate hyperaemia	moderate PMNL infiltration	blood stained mucosa, flattened villi, light PMNL infiltrate, red cells in submucosa.
N82	ND	ND	villi and submucosa normal.
	ND	ND	villi and submucosa normal.
cholera toxin			most villi and submucosa normal, some "Christmas tree" villi.
PBS			villi and submucosa normal.

[a] PMNL = polymorphonuclear leucocytes.
ND, not done.

Table 4.3. Microbiological investigation of *C. jejuni*-infected ileal loops

Strain	Average viable count(cfu/ml)[a]		blood culture		Organ
	fluid	tissue	4 h	PM	culture
L115	$1.5x10^{11}$	$2.7x10^{11}$	+	+	peritoneum,liver, gall bladder
C119	$1.1x10^{11}$	$2.9x10^{10}$	-	+	peritoneal fluid
O81	$3.9x10^{10}$	$4.9x10^{10}$	-	+	peritoneal fluid
P71	$6.0x10^{7}$	$4.0x10^{7}$	-	+	peritoneal fluid
N82	-	$5.0x10^{10}$	+	+	-
NCTC 12189	-	$8.2x10^{7}$	-	-	-
CT	-	-	-	-	-
PBS	-	-	-	-	-

[a] viable count includes bacteria attached to surface mucosa and invading the tissues

Table 4.4. Fluid production in *C. jejuni* infected loops

Strain	No. of loops with fluid	Av. weight of loop (g)	Av. volume of fluid (ml)	Av. weight to length ratio[a]
L115	4/4	6.0	3.0	0.6
C119	4/4	13.4	8.2	1.6
O81	3/4	5.1[b]	2.0	0.4[b]
P71	1/4	6.2[c](9.6)	5.0	1.0
N82	0/4	4.0	0.0	0.0
NCTC 12189	0/4	3.6	0.0	0.0
CT	12/12	20.3	4.0	2.8
PBS	0/12	4.1	0.0	0.0

[a] positive value considered to be greater than or equal to 0.5.

[b] average for loops showing fluid secretion

[c] average for all loops. Bracketed figure for loop showing fluid secretion

Table 4.5. Fluid biochemical analysis

strain	pH	Haemoglobin (g/l)	Bicarbonate (mmol/l)	Protein (g/l)
L115	7.83	0.1	48.3	2.0
C119	7.92	0.06	66.5	2.0
O81	7.65	0.35	30.5	2.5
P71	8.0	0.6	54.1	5.0
N82	-	-	-	-
NCTC 12189	-	-	-	-
CT	8.05	<0.01	89.7	0.3
PBS	7.25	0.0	6.8	0.0

All values are averages of 4 determinations

Table 4.6. Mucosal appearance and fluid cell type in *C. jejuni* infected loops

Strain	Macroscopic appearance of mucosa	Fluid RBC[a]	Fluid WBC[a]	White cell type in fluid
L115	blood stained	+++	+++	polymorphs
C119	blood stained	+	+	polymorphs
081	haemorrhagic	+++	++	polymorphs
P71	blood stained	+++	+	polymorphs
N82	normal	no fluid	no fluid	-
NCTC 12189	normal	no fluid	no fluid	-
CT	normal	-	-	-
PBS	normal	-	-	-

[a] +++,>20 cells/high power field (hpf), ++,10-20 cells/hpf, +, 5-10 cells/hpf

and polymorphs are seen in faecal samples from patients with *C. jejuni* enterocolitis (Butzler and Skirrow, 1979). Some loops were macroscopically damaged with obvious mucosal haemorrhage, and two loops infected with strains O81 and P71 were perforated. This has also been seen in human disease, but is very rare (Skirrow, 1986).

Tissue and (where applicable) fluid viable counts were high (Table 4.3), reflecting mucosal colonisation as well as probable tissue invasion. Strain N82 gave high tissue viable counts but showed no fluid accumulation; strains L115 and C119 gave high counts and elicited fluid in all infected loops (Table 4.4). Blood cultures were positive at 4 h for two strains (L115 and N82) and at post mortem for all 5 clinical strains. However, viable bacterial counts in blood were very low (10-12 bacteria/ml of blood) and all strains were serum sensitive before and after loop inoculation, including N82, which gave no histological damage or fluid secretion, despite being shown to invade and transcytose tissue culture cells (Chapter 2). NCTC 12189 was negative at both time points. Therefore the presence of bacteria in blood for the five clinical isolates probably represents true bacteraemia as a result of a pathological process. Other organs were shown to be culture positive at post mortem, probably reflecting seeding via the bloodstream.

Fluid biochemical analysis (Table 4.5) revealed the presence of large amounts of haemoglobin in campylobacter-infected loop fluids (av. 0.25 g/l) compared with CT-induced fluid (0-0.01 g/l). In general fluid pH correlated with the presence of bicarbonate; fluid from *C. jejuni* infected loops was generally of slightly lower pH (and bicarbonate) level than CT-induced fluid. This is a reflection of the smaller fluid volume in *C. jejuni*-infected loops since the more fluid present, the higher the bicarbonate level in the fluid. Protein levels were very high in *C. jejuni* infected loop fluids (av. 2.6 g/l) compared with fluids from CT-treated tissue (0-0.3 g/l).

The measurement of haemoglobin in fluid recovered from infected loops, is a reflection of the numbers of red cells present, microscopically visible upon examination of fluid (Table 4.6). The tissue white cell infiltrate is reflected by microscopically visible polymorphonuclear leucocytes in loop fluid, typical of inflammatory diarrhoea.

4.3.2. Serum sensitivity. All six strains tested were serum sensitive

in human and rabbit sera as judged by the criteria of Taylor *et al.* (1985), both before animal inoculation and after recovery from infected animals. A typical killing curve is shown in Fig. 4.2a. Bacteriostatic effects were observed even after heat treatment of sera, probably due to the presence of the serum iron binding protein, transferrin (Taylor *et al.*, 1985). Upon addition of MgEGTA, serum killing of bacteria was slightly delayed (Fig. 4.2b.), but the inoculum was still reduced by >90% in 1 h. This effect is solely due to the action of the alternative pathway of complement, since MgEGTA inactivates the classical pathway. It is likely, therefore, that both alternative and classical pathways of complement activation contribute to killing *C. jejuni* in the bloodstream.

4.3.3. GM-1 ELISA on fluid and tissue. CT-like activity could not be demonstrated in *C. jejuni* infected loop fluids or tissue by GM-1 ELISA. CT treated loop fluids had detectable toxin levels but tissue toxin levels were no different from PBS treated negative control loops. CHO enterotoxin and cytotoxin assay results are presented in Table 4.1.

4.4. DISCUSSION

The rabbit ileal loop model was used in order to obtain *C. jejuni* infected material that mimicked the histological effects of the disease in man. This was important because the effects of inflammatory mediators could be investigated (Chapter 5), along with the intracellular mediators of secretion (Chapter 6). Strains varied in their effects in this model, those causing the most severe disease in humans, as judged by endoscopy and histology, also caused the most severe histological damage in rabbit ileal loops. In general, loops infected with human colitis strains showed shorter villi than uninfected controls, white cell infiltration and bleeding into the mucosa. In contrast, N82, an isolate from non-inflammatory (watery) diarrhoea caused no pathology and elicited no fluid secretion, suggesting that the rabbit ileal loop is not a good model for the pathogenesis of these strains. It should be remembered, however that *C. jejuni* illness is clinically classified as an enterocolitis (Skirrow, 1986), with both ileal and colonic involvement observed in the few studies that have looked for such pathology (Skirrow, 1986). Therefore any future studies should investigate colonic

loops, using strains from both clinical groupings.

Quantitative differences in inflammatory response were observed for different strains. It may be that the ability of a strain to elicit such an inflammatory response is the deciding factor in the type of disease produced, and this may be linked to the ability of a strain to invade enterocytes (Chapter 2). However invasion alone is not enough to elicit secretion in salmonella-infected loops (Wallis *et al.*, 1989); salmonellae invaded ileal mucosa but did not elicit fluid secretion and caused an intermediate inflammatory response. It may be that in campylobacter-mediated diarrhoea the ability to invade and elicit significant histological inflammation, as opposed to the ability to invade alone, may explain secretion. In individual strains the ability to provoke inflammation may be a property of the bacterial envelope or a secreted toxin. The cytotoxin of *C. jejuni* may be a candidate for this role.

The amount of fluid in *C. jejuni* infected loops was less than in CT treated loops. GM-1 ELISA revealed no CT-like toxin in *C. jejuni* infected loop tissue or fluids. This may be a reflection of the very low levels of toxin produced by the strains, or of the possibility that toxin bound to, or inside cells was not released by the homogenisation process. Histological damage to mucosa causes fluid to accumulate in the gut lumen by leakage of serum components through damaged epithelium. Thus the high protein content of fluid in *C. jejuni* infected loops compared with CT loops (in which fluid accumulates solely by active secretory diarrhoea) probably represents a serum component from blood leakage from a damaged mucosa. This does not, of course, rule out a secretory component, especially for strain C119, which elicited up to 20 ml of almost clear fluid (although it contained macroscopic blood) in two of the loops. The large volume was also reflected in the bicarbonate loss in the fluid, again typical of secretory diarrhoea (Field, 1981). The secretory component may be due to a bacterial toxin (although toxin levels in these strains were very low, see Chapter 6), or to a secretagogue produced by the host (see Chapter 5). Certainly mucosal colonisation alone does not explain the fluid secretion seen in loops. Thus similar bacterial counts were observed for strains P71 and N82 even though the former elicited fluid secretion and mucosal damage while the latter did not. Strain differences in interaction with mucosa may explain differences in

fluid secretion and histology, rather than colonisation of mucosa alone.

Blood cultures were positive for all strains except NCTC 12189, reflecting true bacteraemia, rather than operational trauma. However, all strains were sensitive to both the classical and alternative pathways of complement killing, as previously shown by Blaser *et al.* (1985), and the number of viable bacteria in the bloodstream was extremely low. The removal of blood and dilution of the killing component of serum may explain why isolation was possible. Alternatively the organisms may be present in blood phagocytes, particularly monocytes, reflecting the process of translocation (Wells *et al.*, 1988). *C. jejuni* can survive in monocytes (Kielbach *et al.*, 1985) and so would be protected from the bactericidal action of serum. Lysis would release organisms from such cells and result in bacterial isolation. Bacteraemia is probably under-reported in *C. jejuni* enteritis (Skirrow, 1986), due to the fact either that blood cultures are not often taken in diarrhoeal illness, or that bloodstream invasion is an early event in the illness before presentation to a physician (Skirrow, 1986). Septicaemia due to *C. jejuni* is rare, because most strains are sensitive to the bactericidal action of normal serum. Patients with septicaemia usually have an underlying serum defect.

Chapter 5
Eicosanoids and the pathogenesis of infective diarrhoeal disease

5.1. INTRODUCTION

C. jejuni causes an enterocolitis similar histologically to acute ulcerative colitis and inflammatory bowel disease (Skirrow, 1986). Eicosanoids are associated with the inflammation seen in inflammatory bowel disease. Secretion of water and electrolytes is associated with prostaglandin-induced diarrhoea, and physiological as well as pharmacological doses of several prostaglandins produce secretion in the small intestine and colon (Rampton and Hawkey, 1984). A role for these mediators in infective campylobacter diarrhoea involving inflammation is postulated.

5.1.1. Eicosanoids as secretagogues. Eicosanoids are unsaturated lipids derived from arachidonic acid or similar polyunsaturated fatty acid precursors via the cyclooxygenase or lipoxygenase metabolic pathways (Donowitz, 1985). This group of compounds includes prostaglandins (PGs), thromboxanes, leukotrienes (LTs), lipoxins, hydroperoxyeicosatetranoic acids (HPETEs) and hydroeicosatetranoic acids (HETEs). The lipoxygenase and cyclooxygenase pathways are not equally used in all tissues. For instance, in monocytes free arachidonic acid is oxygenated by both pathways (Rouzer *et al.*, 1977), whereas in polymorphonuclear leukocytes the lipoxygenase pathway is the predominant and almost exclusive pathway (Lewis and Austin, 1981). PGs of the E and F type are quantitatively more important in the gastrointestinal tract and may be considered to be important as physiological local regulators of fluid and ion transport in the small intestine and colon. PGs of the E type decrease active sodium and chloride absorption and increase fluid secretion in both the small intestine (Rask-Madsen and Bukhave, 1981) and the colon (Racusen and Binder, 1980). In addition, products of the lipoxygenase pathway, such as 5-HPETE and 5-HETE, can induce secretion in some species at low concentrations (Musch *et al.*, 1982).

PGE2 alters active electrolyte transport in the small intestine and

colon by activation of adenylate cyclase. It has therefore been speculated that PGs might be mediators of the secretory effects of cholera toxin (Duebbert *et al.*, 1985), although this has been disputed by other workers (Kimberg *et al.*, 1974). Certainly inhibitors of PG synthesis, such as aspirin and indomethacin, decreased or inhibited the secretory effect of cholera toxin, but it was considered that the mechanism by which PGs elicit active secretion in the intestine depends on cyclic AMP (cAMP) (Rask-Madsen, 1986), and that the role of PGs is secondary rather than primary. However, *in vitro* evidence of secretory effects can be obtained with PG concentrations 100-1000 times lower than those required to affect adenylate cyclase activity, provided that the formation of endogenous PGs is suppressed by indomethacin (Rask-Madsen, 1986). Thus PGE2 can alter active electrolyte transport before causing a detectable change in adenylate cyclase activity. It is also possible that PGE2 affects transport via some other second messenger (Donowitz, 1985). Intestinal secretion is induced by 5-hydroxytryptamine (5-HT) and cholinergic agonists, both of which stimulate PG synthesis and raise intracellular calcium levels without affecting cAMP (Beubler *et al.*, 1986).

5.1.2. Control of arachidonic acid metabolism. Receptors for control of intestinal secretion can be divided into two major classes. One triggers the production of cAMP. Another initiates a cascade which includes inositol phospholipid turnover, calcium mobilization, and frequently arachidonate release and cGMP production (Rask-Madsen, 1986; Nishizuka, 1984).

Inositol phospholipids represent only a small percentage of cellular phospholipids, but their turnover rate is rapid. The intracellular calcium-activated phospholipid-dependent protein phosphorylase protein kinase C (PKC) has a central role in signal transduction for a variety of extracellular stimuli. This enzyme is activated by diacylglycerol (DG), an early product of receptor-linked breakdown of phosphatidylinositol (PI) within the cellular membrane, while inositol 1,4,5-triphosphate (IP3) is released into the cytosol to function as a second messenger for mobilizing intracellular calcium. In addition, IP3 undergoes catabolism sequentially to inositol 1-4-biphosphate (IP2), inositol 1-phosphate (IP) and finally free inositol, which

can then be resynthesized into PI. When cells are stimulated, DG is only transiently produced, probably due both to its resynthesis to inositol phospholipids and to its further conversion to arachidonate for eicosanoid formation. The first route involves phosphorylation of DG by DG kinase to form phosphatidic acid, which serves as a precursor for the resynthesis of PI (Nishizuka, 1984). The second involves the action of diglyceride lipase leading to the production of arachidonate. In contrast, tumour promoting phorbol esters, when intercalated in the cell membrane, may substitute for DG and permanently activate PKC and arachidonate metabolism.

A connection between PI metabolism, cytosolic calcium levels, electrogenic anion secretion, electroneutral sodium/hydrogen exchange activity, intracellular pH and sodium pump rate has been revealed (Macara, 1985). Increases in intracellular free calcium activate calmodulin-dependent kinases, and responses to phorbol esters suggest several prospective targets for PKC, two of which are the electrogenic anion secretion system (Chang *et al.*, 1985) and the electrically silent sodium/hydrogen exchange system controlling intracellular pH (Macara, 1985). A secondary consequence of the rise in intracellular sodium levels following activation of sodium/hydrogen exchange is an increase in sodium/potassium-ATPase activity. Exploration of the roles of PI breakdown and arachidonate metabolism is crucial for the understanding of acute (including infectious) and chronic secretory diarrhoea, and for obtaining insight into the mechanism of abnormal mucosal cell growth and chronic diarrhoea in inflammatory bowel disease (Rask-Madsen, 1986).

5.1.3. Prostaglandins and bacterial diarrhoeal disease. PGs of the E and F type elicit fluid and electrolyte secretion in the small intestine *in vivo* following oral (Misiewicz *et al.*, 1969), intra-jejunal (Matuchansky and Bernier, 1973) and parenteral administration (Milton-Thompson *et al.*, 1975) in humans. In attempting to determine the pathophysiology of the abnormal active electrolyte transport seen in inflammatory bowel disease, analogies to other disease states with mucosal inflammation are likely to be useful. The histology of infectious colitis and acute exacerbations of ulcerative colitis are identical (Skirrow, 1986) and in the absence of any obvious bacterial virulence factors in the latter that could cause secretion,

the underlying physiological process causing diarrhoea could be similar in both illnesses. Indeed inflammatory bowel disease (IBD), viewed as a chronic inflammatory process, is to some extent histologically and functionally a prolonged acute inflammatory response which correlates with the presence of neutrophils in the mucosa (Stenson, 1990). The pattern of arichodonate metabolism seen in IBD mucosa is not specific, but is probably common to all forms of intestinal inflammation with an acute component (Stenson, 1990).

PG release by inflammatory cells (polymorphonuclear leucocytes, PMNL and macrophages) has been suggested as a mechanism of secretion arising from PMNL-*Salmonella* interaction (Stephen *et al.*, 1985). Three lines of evidence have suggested this association. First, indomethacin (a known potent inhibitor of PG synthesis) abolished secretion induced by *Salmonella typhimurium* in rabbit ileal loops, but in the same system only partially inhibited ileal secretion induced by *Shigella flexneri*, *Vibrio cholerae* and cholera toxin, despite no change in the pattern of invasion and inflammatory response (Gots *et al.*, 1974). Second, Gianella *et al.* (1975) showed that indomethacin rapidly enhanced absorption in the jejunum, ileum and colon of normal monkeys and reversed the secretion induced by *S. typhimurium* in both small and large intestines. Gianella *et al.* (1979) successfully used nitrogen mustard in the rabbit ileal loop model to inhibit fluid secretion at doses which depressed the influx of PMNs but which had no effect on ileal morphology or relevant enzyme activities, or on responsiveness to cholera toxin or *S. typhimurium*, as judged by the kinetics of invasion and properties of organisms re-isolated from rabbit ileal loops. Differences in intensity of PMN influx suggested a threshold level of PGs below which no response was evoked. Stephen *et al.* (1985) showed that the onset of fluid secretion in salmonella-infected ileal loops occurred about 8 h post infection, at which time significant numbers of PMNs had been recruited to the infected villi and were interacting with invading salmonellae; in PMNs, PG release equates with synthesis consequent upon phagocytosis of bacteria. Fluid secretion was not observed in the absence of PMNs and it was postulated that interaction between PMNs and a relevant bacterial phenotype provoked release of an enterotoxic factor from the salmonellae, or release of PGs from PMNs, or both. The histology of

campylobacter enteritis, salmonellosis and shigellosis is identical, and, as mentioned above, these diseases share identical histology with acute exacerbations of inflammatory bowel disease and ulcerative colitis (Skirrow, 1986). It may be that these diseases share a common diarrhoeagenic mechanism which may be due to prostaglandins, enterotoxin, or both.

Duebbert and Peterson (1985) used rabbit intestinal loops to show that challenge with *S. typhimurium* or *V. cholerae* resulted in significant elevation of mucosal cAMP and PG concentrations. This effect could be reproduced *in vitro* by exposing either isolated epithelial cells from normal rabbits or chinese hamster ovary cells to purified cholera toxin or cell free lysates of salmonella-challenged loops. PG concentration was increased in crypt epithelial cell fractions, and in isolated intestinal epithelial cells exposed to purified cholera toxin *in vitro*. These data suggest that PGs synthesized by the epithelial cells are involved in the pathogenesis of both experimental cholera and salmonellosis. It was postulated that the data are consistent with an enterotoxin-mediated mechanism for both diarrhoeal diseases and might argue against the role of inflammatory cells as the source of elevated cAMP and PGs appearing in the intestinal mucosa during experimental salmonellosis.

Colonic inflammation in shigellosis, salmonellosis, and ulcerative colitis all appear, at least superficially, to be similar in animal models (Rout *et al.*, 1978; Gianella *et al.*, 1975). A monkey model exists for colonic involvement in shigellosis (Rout *et al.*, 1978) but colonic adenylate cyclase activity has not been reported in either shigellosis or salmonellosis (Donowitz, 1985), and increased colonic adenylate cyclase activity could not be detected in patients with shigellosis (Rachmilewitz *et al.*, 1983). It may be that the shigella biopsy specimens reported were not comparable with those from ulcerative colitis either in the amount of inflammation or in the stage of disease activity. It may also be that the diseases differ in the nature of the inflammatory mediators involved and the primary signal activating arachidonate metabolism, or that the reactions may act via two different pathways.

This chapter seeks to measure the amount of inflammatory mediators

PGE2 and LTB4 produced in response to *C. jejuni* infection, using material from infected ileal loops (Chapter 4), and in the interaction of *C. jejuni* with Caco-2 cells and the macrophage-like cell line U937.

5.2. MATERIALS AND METHODS

5.2.1. Treatment of Caco-2 cells. Caco-2 cells were infected with inflammatory strain D217 and systemic strain L1/1 (10^8 bacteria/ml), and also treated with the calcium ionophore A23187 (12.5 μM) or with arachidonic acid (50 μM) in order to stimulate eicosanoid synthesis.

5.2.2. U937 cell infection. The macrophage-like cell line U937 was used because eicosanoid synthesis in tissue has been associated with tissue inflammatory cells including neutrophils and macrophages (Powell, 1985). Assays were performed in Hanks balanced salt solution (HBSS) plus 0.1% gelatin (Oxoid) (van Furth and van Zwet, 1973) using strains D217 and L1/1 at a concentration of 1 x 10^8 bacteria/ml. 0.2 ml of the cell suspension was added to 0.2 ml of convalescent serum or foetal calf serum (both heat-treated to inactivate complement) and placed in a water bath at 37°C for 2 h. At the end of this time the mixture was centrifuged and supernatants collected for PGE2 radioimmunoassay. Cells were washed well and incubated with HBSS containing 200 μg/ml gentamicin for a further 2 h, after which they were again washed well, lysed with 0.5% sodium deoxycholate and plated on MH agar for viable counting.

5.2.3. Tissue and cell extraction. Extraction of PGs and LTs from homogenised tissue and cells was performed according to Powell (1988). Tissue was homogenised in sodium acetate buffer (pH 3.5) and centrifuged, and the supernatant fraction was passed down a C8 Bond-elute column (Analytichem International) to concentrate eicosanoids. The column was prepared by adding 1 ml of methanol and allowing it to pass down the column under pressure, 1 ml of distilled water was added (pH 3.5) and finally the sample was loaded in a total volume of 5 ml. The column was then washed in 1 ml of distilled water followed by 1 ml of 10% (v/v) ethanol and 1 ml of hexane (BDH), and the sample was eluted from the column in 2 ml of methanol. Samples were dried under oxygen-free nitrogen (BOC) and

reconstituted in Tris-gelatin (TG) buffer (1 g of gelatin, 6.08 g of sodium chloride, 10.84 g of potassium hydrogen phosphate, 1.73 g of potassium dihydrogen phosphate, and 0.73 g of sodium azide per litre of distilled water) for radioimmunoassay.

5.2.4. Radioimmunoassay for PGE2. PGE2 standards of 10, 15, 20, 30, 40, 50, 70, 100, and 200 pg/100 µl were prepared from a 1 mg/ml stock solution in TG buffer; 100 µl of TG buffer was used as a blank. 200 µl TG buffer were placed into tubes in duplicate in order to ascertain the total count of radioactivity added to the samples. 100 µl of each standard, or 100 µl of each test sample were placed in appropriate tubes. 100 µl of anti-prostaglandin E2 antibody (Sigma) (diluted 1:8 in TG buffer) were placed in all except the total count and blank tubes. 100 µl of [^{3}H]prostaglandin E2 (5 µCi/ml) (Amersham) were added to all tubes, mixed carefully, and left at 4^{o}C overnight. Dextran-coated charcoal (1.25 g of charcoal and 0.125 g of dextran dissolved in 500 ml of TG buffer) was added to all tubes with the exception of the tubes used to determine the total count of radioactivity. Tubes were incubated at 4^{o}C for 12 min, and centrifuged at 3600 rpm for 15 min. Supernatants were placed in 10 ml of Optiphase scintillant (LKB) and radioactivity was detected using a Packard Tri-Star liquid scintillation spectrometer. Test samples were quantitated with reference to a standard curve.

5.2.5. Radioimmunoassay for LTB4. Fluid from *C. jejuni*-infected and cholera toxin-treated rabbit ileal loops was tested for the presence of LTB4 using a radioimmunoassay kit from Amersham. Standards were prepared in assay buffer to final concentrations of 1.6, 3.1, 6.2, 12.5, 25, 50, 100 and 200 pg/100 µl. Total counts were determined as for the PGE2 assay. 100 µl of each standard or sample were placed in appropriate assay tubes. 100 µl of [^{3}H]LTB4 (5 µCi/ml) were added along with 100 µl of anti-LTB4 serum (Amersham) to all except total count and blank tubes. All tubes were incubated for 2 h at room temperature, after which 200 µl dextran-coated charcoal were added and the mixtures were centrifuged at 2000 x *g* for 10 min at 4^{o}C. The supernatants were placed in 10 ml Optiphase scintillant and counted as described above.

5.3. RESULTS

5.3.1. PGE2 production by Caco-2 cells. PGE2 production by Caco-2 cells was not detectable after treatment with the calcium ionophore A23187 or with arachidonic acid. This is consistent with other work (Smith *et al.*, 1982) which has shown that the source of most eicosanoids in the gut is inflammatory cells (polymorphs, macrophages) rather than intestinal epithelia. Infection of Caco-2 cells with *C. jejuni* strains D217 and L1/1 also failed to induce detectable PGE2 production.

5.3.2. PGE2 production by macrophage cell line U937. Treatment of U937 cells with arachidonic acid produced detectable PGE2 in the medium, but infection with the two *C. jejuni* strains did not. This may be explained by the possibility that two stimuli are needed to induce PGE2 production by macrophages, namely phagocytosis of bacteria and interleukin 1 (IL-1). IL-1 produced by macrophages in response to bacterial infection may stimulate other macrophages to produce PGE2 (Male *et al.*, 1991).

5.3.3. PGE2 and LTB4 in loop fluids. Fluids taken from *C. jejuni* infected and CT treated rabbit ileal loops were tested for the presence of PGE2 (Table 5.1). Fluids from *C. jejuni*-infected loops contained higher PGE2 concentrations than those from CT-treated loops, a reflection of the increased levels of inflammatory cell infiltration of the tissues and then of the intestinal lumen.

A statistically significant correlation was observed between the numbers of polymorphs in loop fluids and tissue and the amount of LTB4 present in fluid (Mann Whitney U Test $p=<0.001$) (Table 5.1). This is consistent with the fact that LTB4 induces chemotaxis of neutrophils and macrophages. CT-treated loops showed no white cell infiltrate, also consistent with the non-inflammatory histological picture of cholera. LTB4 levels were low in CT-treated loops.

5.3.4. PGE2 in tissue. PGE2 in infected rabbit ileal loop tissue treated with strains from human colitis was significantly increased compared to uninfected and CT treated loops (Mann Whitney U test, $p=<0.001$). Tissue infected with strains able to induce fluid accumulation

Table 5.1 Comparison of fluid white cells, LTB4 and PGE2

Strain	Loop No.	Animal No.	polymorphs[a]	PGE2[b] (ng/ml)	LTB4[c] (ng/ml)
L115	1	1	++	1.4	0.9
	2	1	++	2.1	4.2
	3	2	+	1.5	0.38
	4	2	+	5.0	0.52
C119	5	3	+	2.2	0.38
	6	3	+	1.7	0.1
	7	4	+	0.9	0.45
	8	4	+	2.1	0.41
O81	9	5	+++	5.6	4.4
	10	5	+++	4.2	4.2
	11	6	+++	5.7	5.0
P71	17	10	++	5.2	5.0

All values are averages of duplicate assays
[a] +++ = >20 cells/high power field (hpf), ++ = 10-20 cells/hpf, + = 5-10 cells/hpf.
[b] CT-induced fluid gave average PGE2 levels of 0.63 ng/ml (range 0.5-0.78).
[c] CT-induced fluid gave average LTB4 levels of < 0.05 ng/ml

Table 5.2. Prostaglandin E2 in tissue

Strain	Loop No.	PGE2[a]	Animal No.	PGE2 in controls a
L115	1	30.8	1	CT = 15.5
	2	16.8	1	PBS = 15.0
	3	24.7	2	CT = 17.0
	4	38.9	2	PBS = 16.4
C119	5	37.7	3	CT = 26.2
	6	59.0	3	PBS = 15.3
	7	38.0	4	CT = 31.0
	8	40.0	4	PBS = 33.1
O81	9	34.1	5	CT = 38.6
	10	19.1	5	PBS = 33.1
	11	38.3	6	CT = 24.0
	12	40.3	6	PBS = 17.0
P71	13	53.0	7	CT = 36.7
	14	19.2	7	PBS = 39.5
	15	40.3	8	CT = 12.5
	16	33.5	8	PBS = 17.0
N82	17	18.3	9	CT = 30.2
	18	11.3	9	PBS = 34.5
	19	51.4	10	CT = 30.2
	20	45.4	10	PBS = 35.5
NCTC 12189	21	15.4	11	CT = 12.0
	22	18.4	11	PBS = 19.0
	23	31.6	12	CT = 40.8
	24	39.4	12	PBS = 40.3

a ng/g of tissue; all samples were tested in duplicate

Fig. 5.1 PGE2 assay of loop tissue infected with inflammatory strains and controls

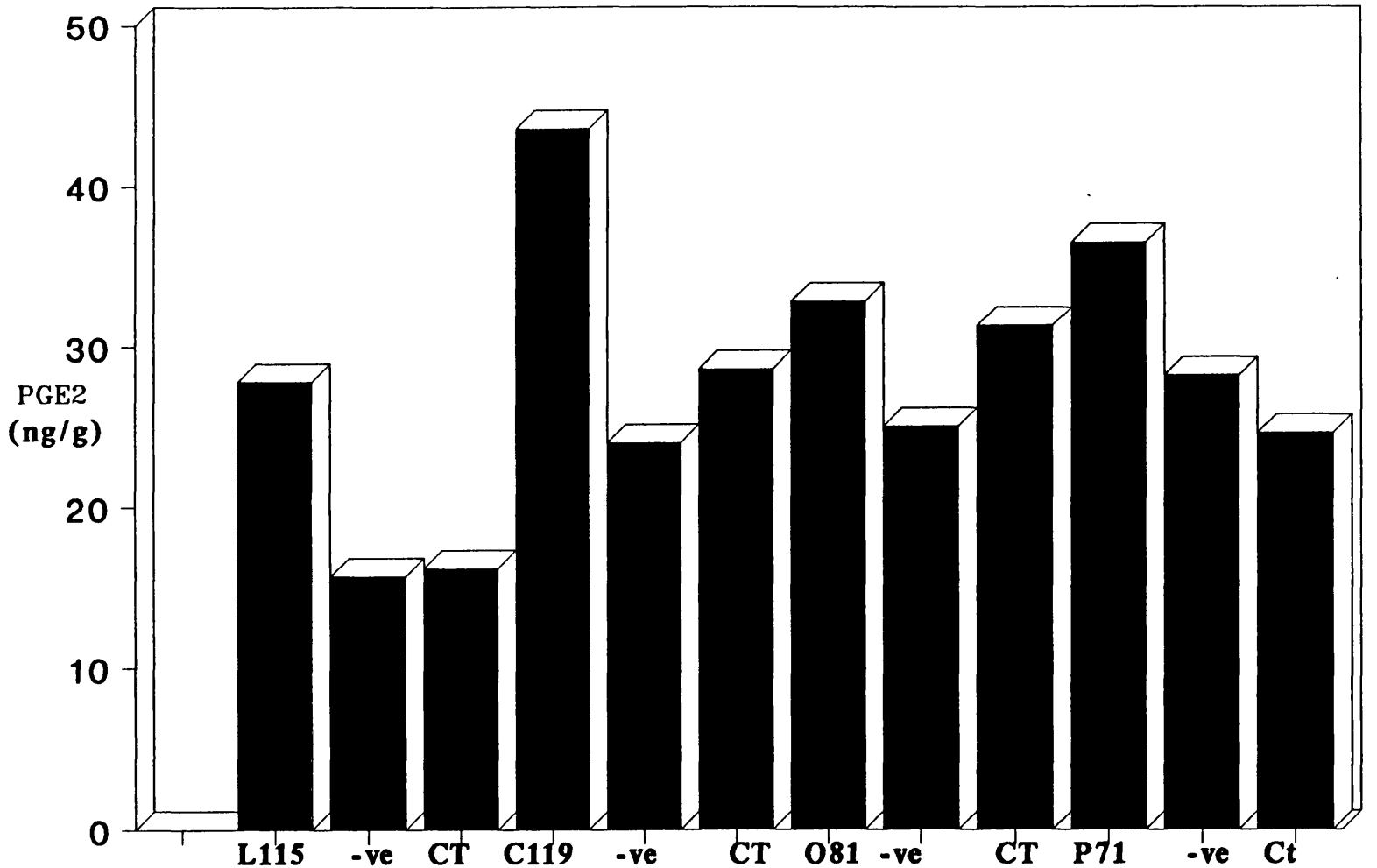
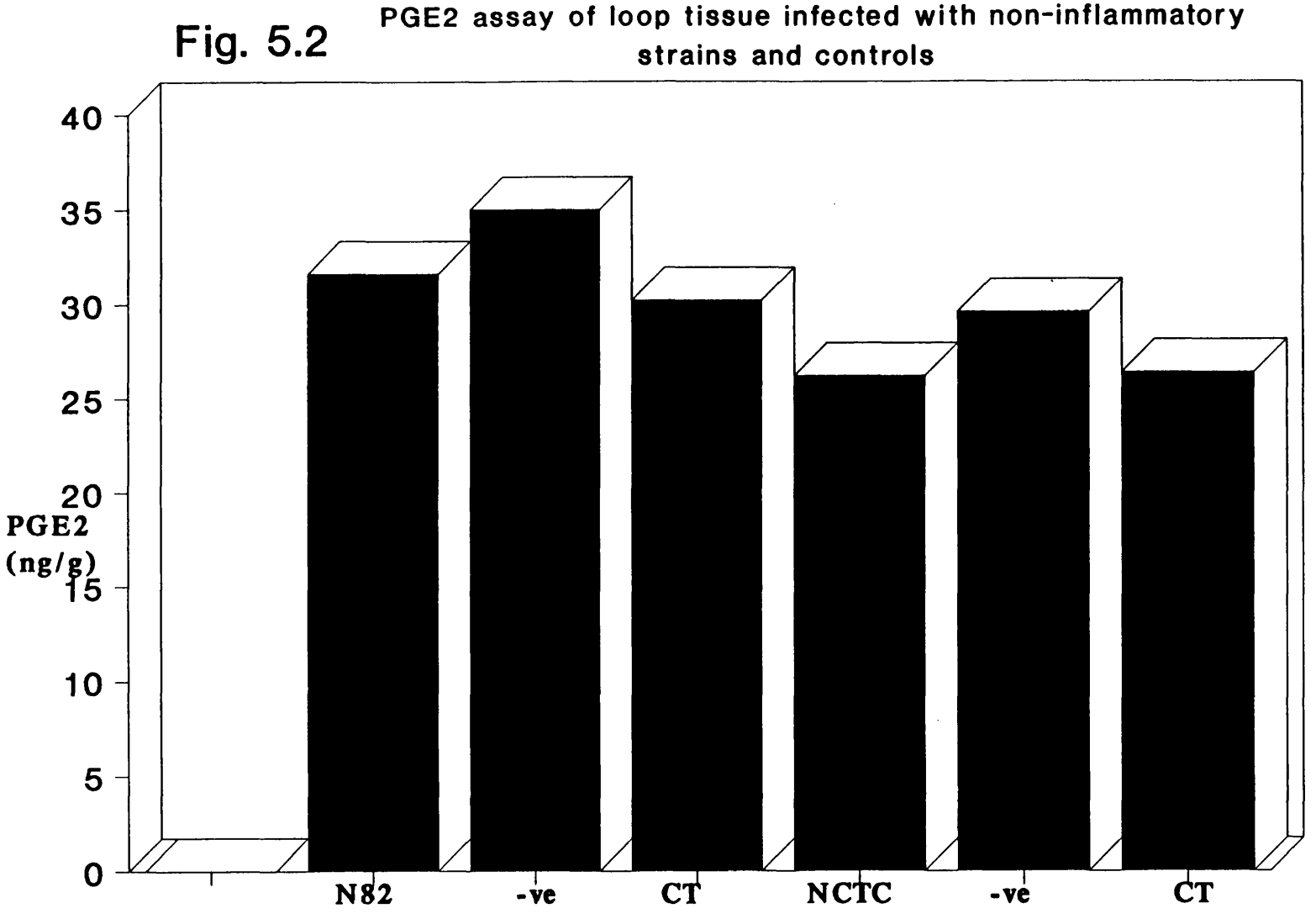


Fig. 5.2


PGE2 assay of loop tissue infected with non-inflammatory strains and controls
PGE2 (ng/g)
40
35
30
25
20
15
10
5
0
N82
-ve
CT
NCTC
-ve
CT

contained higher PGE2 levels, compared with controls in the same animal, than strains causing no fluid accumulation (p=0.023) (Table 5.2 and Fig. 5.1). Tissue from loops challenged with non inflammatory isolates showed no difference from controls (Fig. 5.2).

PGE2 is known to enhance the chemotactic activity of LTB4 (Stenson, 1990) and levels of PGE2 also correlated with white cell infiltration into tissue (p=0.02).

5.3.5. Summary of results. *C. jejuni* isolated from patients with colitis increased mucosal PGE2 by 14.5 ng/g (median, range 2.6-22.8 ng/g, p=0.008) and accumulated fluid of 1.5 ml (median, range 0-18 ml) compared with negative control loops. Accumulated fluid contained 2.2 ng/ml PGE2 (median, range 1.6-5.2 ng/ml) and 0.71 ng/ml LTB4 (range 0.1-5.0 ng/ml). Neutrophil infiltration of the mucosa from these loops correlated with fluid LTB4 and PGE2 levels. By contrast loops inoculated with non-inflammatory or non-colonising mutant strains showed no neutrophil infiltrate, no fluid accumulation and had no effect on PGE2 production compared to uninfected controls.

5.4. DISCUSSION

C. jejuni strains from cases of inflammatory diarrhoea induce histological inflammation, white cell infiltrate, and large amounts of PGE2 and LTB4 compared to uninfected controls and non-inflammatory strains. These isolates also increase tissue cAMP in infected loops (Chapter 6). PGE2 is a known secretagogue (Rask-Madsen and Bukhave, 1981), causing an increase in adenylate cyclase activity and a corresponding increase in cAMP in affected cells and tissues, and could be a candidate for these effects seen in the ileal loops. Work presented in Chapter 6 shows that PGE2 in fluids induced by inflammatory strains is capable of increasing enterocyte cAMP more than infecting strains alone. By implication, PGE2 may therefore be responsible for some of the fluid accumulation present in infected loops. There was no correlation between the amount of PGE2 in infected tissue and the volume of fluid in the loop, or the amount of cAMP in the tissue. These observations may be explained by the fact that mucosal invasion by large numbers of bacteria leads to white cell infiltrate and

mucosal inflammation, with the liberation of PGE2 by inflammatory cells. Free PGE2 binds to corresponding receptors on enterocytes, increasing cAMP within the cells, in turn leading to fluid secretion. However at the time of animal sacrifice cellular cAMP is likely to have decreased from an earlier peak, or cells may have been shed from an infected mucosa, so that PGE2 may not necessarily correlate with cAMP at the sampled time point. If the animal had been sacrificed at an earlier time, rising cAMP may have been demonstrated. A large amount of PGE2 present may not be important if all cell receptors for the secretagogue were saturated. If this were the case only a threshold level of PGE2 would need to be present to cause increases in cAMP, not a vast excess. This may cause an increase in cAMP leading to secretion, but prevent any further binding of PGE2 due to receptor blockade.

It is not known what contribution inflammatory mediators alone make to tissue damage, but white cell products can cause tissue injury and cell death, and white cell infiltrate correlates with release of PGE2 and LTB4. Therefore white cell infiltrate in infectious and ulcerative colitis correlates with tissue damage, and tissue damage in the gut has significant effects on its absorptive ability. This will be discussed again later. Resolution of white cell response by treatment with anti-inflammatory drugs, therefore decreasing LTB4 levels, correlates with resolution of symptoms of bloody diarrhoea, fever and abdominal pain in ulcerative colitis. Similarly in infectious colitis removal of the inflammatory stimulus, the infecting organism, eventually but not immediately results in resolution of the white cell infiltrate, fever, abdominal pain and diarrhoea. Anti-inflammatory drugs bring about resolution of symptoms in ulcerative colitis, and antibiotics remove the infectious stimulus in campylobacter colitis. However, antibiotics do not produce relief of the immediate symptoms of the disease, and may indeed prolong or exacerbate these symptoms, although this is at present controversial (Reid, 1992). It is known that anti-inflammatory drugs (drugs that inhibit eicosanoid synthesis) prevent diarrhoea in ulcerative colitis, caused by inflammatory secretagogues such as PGE2, 5-HPETE, and 5-HETE. From this study, it is clear that PGE2 is induced by *C. jejuni* in the RILT model, and it may be that these other mediators may also be induced. Further work is needed to confirm this. The implication by analogy is that eicosanoids responsible for disease symptoms

produced in large amounts in ulcerative colitis, are also produced in infectious colitis, and that drugs used in controlling one disease may help to relieve symptoms in the other. The PG synthesis inhibitor indomethacin almost completely abolished fluid secretion in response to *S. typhimurium* infection (Gianella *et al.*, 1975), and significantly reduced secretion mediated by *S. flexneri*, *V. cholerae* and cholera toxin (Gots *et al.*, 1974). Mucosal invasion by *S. typhimurium* was an essential prerequisite for the secretion of ileal fluid; after indomethacin treatment, histology and marked mucosal inflammation was unaltered.

The fact that PGs appear to be locally synthesized suggests that they may serve an important physiological role in the regulation of intestinal ion transport, either directly or by modifying specific stimuli. In support of this is the observation that intestinal secretion can be stimulated by physiological PG concentrations (10^{-11}-10^{-7} M) (Bukhave and Rask-Madsen, 1980). In addition, PGs have been shown to increase the propulsive activity of the gut (Chang and Fedorak, 1985) which could contribute to diarrhoea by decreasing contact time of intestinal fluids with the absorptive surface. Recently it has also been shown that certain prostanoids, synthesized from arachidonate through the action of lipoxygenase, are secretory stimuli in the colon (Musch *et al.*, 1982). The effective lipoxygenase products do not include the leukotrienes, but they do include the leukotriene precursors 5-HPETE and 5-HETE; these leukotriene precursors are produced in large quantities in leucocytes (Chang and Fedorak, 1985), but it is not known if they are also synthesized in enterocytes. The intracellular mediator for 5-HPETE-induced secretion is also not known, but it does not appear to be cyclic nucleotides or exogenous calcium. All the above observations suggest a mechanism by which active secretion is stimulated in acute and chronic inflammation of the intestine, and may have particular relevance to diarrhoea associated with inflammatory bowel disease or to acute infectious diarrhoeas.

This work has shown that in the RILT model of *C. jejuni* inflammatory diarrhoea, eicosanoids are induced by the bacterial stimulus that are able to induce fluid secretion. It may be that in campylobacter colitis, and indeed in other forms of infectious colitis, diarrhoeal symptoms are induced at least in part by the inflammatory response of the host.

Chapter 6

cAMP as a mediator of secretion in *C. jejuni* enterocolitis.

6.1 INTRODUCTION

The pathogenesis of *C. jejuni* diarrhoeal illness implicates a cholera like-enterotoxin (McCardell *et al.*, 1984; Ruiz-Palacios *et al.*, 1983) which may stimulate secretion through activation of adenylate cyclase, increasing cellular cAMP. Experiments in this chapter measure cyclic nucleotides in infected tissue, and look at effects of whole bacteria and bacteria free culture supernatants on Caco-2 cells with regard to increases in cellular cAMP. Intestinal fluid induced by *C. jejuni* in rabbit ileal loops was also tested on Caco-2 cells for its ability to increase intracellular cAMP. These fluids contained no detectable cholera-like toxin activity (by GM-1 ELISA) but contained large amounts of the secretagogue PGE2 (by radio-immunoassay) which can cause secretion by increasing cAMP. *C. jejuni* strains were also tested by GM-1 ELISA for their ability to produce a CT-like enterotoxin.

6.1.1. Toxins. One important mechanism by which bacterial entero-pathogens induce diarrhoea is through the production of potent toxins. *C. jejuni* produces heat labile enterotoxin (CJT), but the proportion of strains that produce it and the quantities produced vary considerably from study to study. Increasing the iron concentration of the growth medium increases CJT yield (McCardell and Madden, 1985). Although the toxin has not yet been fully characterised, it is known to be a large protein with a molecular weight in the range 60,000-70,000 (McCardell *et al.*, 1984). It is completely inactivated after 1 h at 56°C, or after 10 min at 96°C. It is partially inactivated at pH 4, and completely destroyed at pH 2 and pH 8. Daikoku *et al.* (1990) have partially purified CJT from culture supernatants. A 68-kDa polypeptide has an immunological relationship with cholera toxin (CT), and 68 and 54-kDa polypeptides seem to be responsible for recognition of gangliosides. These fractions enhanced adenylate cyclase activity in HeLa cell membranes 1.5-fold over untreated control cells. CJT induces elongation or rounding of cultured CHO and Y1 mouse adrenal cells (McCardell *et al.*, 1984). It also causes increased intracellular cAMP concentrations in

cultured cells (Ruiz-Palacios *et al.*, 1983), and increased intraluminal fluid accumulation in ligated rat (Klipstein and Engert, 1984) and rabbit ileal loops (McCardell *et al.*, 1984). The B subunits of CJT, *E. coli* heat labile toxin (LT) and cholera toxin all bind GM1 ganglioside (Klipstein and Engert, 1985), and are immunologically related, CJT B being more closely related to LT B than to CT B (Klipstein and Engert, 1984). However DNA probes for the A and B subunit genes of LT and CT fail to hybridise consistently with *C. jejuni* genomic or plasmid DNA (Baig *et al.*, 1986). Indeed, some workers have reported that partial DNA sequence homology exists between LT and CT genes and the genomic DNA of both toxigenic and non-toxigenic strains of *C. jejuni* (Fernandez *et al.*, 1989).

C. jejuni also produces extracellular toxins that injure cultured CHO, HeLa and Vero cells (Guerrant *et al.*, 1987). The cytotoxic activity of some strains is neutralisable by anti-Shiga toxin antibodies, by monoclonal antibody prepared against the B subunit of *E. coli* Shiga-like toxin 1 (Moore *et al.*, 1988), and by antisera to the cytolysin of non-O1 *Vibrio cholerae*. However, Shiga-like toxins produced by *C. jejuni* are genetically distinct from *E. coli* Shiga-like toxin 1 (Moore *et al.*, 1987). A cytotoxin has been isolated and characterised by Mahajan and Rodgers (1990); a 68 kDa protein in culture supernatants caused rounding and death of chicken embryo fibroblast cells, CHO cells and Int407 cells. Its physical properties were distinct from CJT, and cell binding was mediated by a protein or glycoprotein-like receptor on cell membranes.

Several studies have attempted to correlate the toxigenic profile of *C. jejuni* isolates with clinical illness. Enterotoxin producing strains were isolated more frequently from symptomatic patients than from asymptomatic carriers in Mexico (Ruiz-Palacios *et al.*, 1985), but the proportion of enterotoxin producing strains did not differ significantly between symptomatic and asymptomatic Indian children (Mathan *et al.*, 1984). In another study, watery diarrhoea without faecal leucocytes was associated with enterotoxin producing strains, whereas bloody diarrhoea was associated with cytotoxin producing strains (Klipstein *et al.*, 1985). The pathogenic significance of *C. jejuni* toxins therefore remains to be established (Cover and Blaser, 1989). Titres of toxins produced by *C. jejuni*

are low in comparison with those produced by organisms such as *Clostridium difficile, V. cholerae*, enterotoxigenic and enterohaemorrhagic *E. coli*, and *Shigella dysenteriae* type 1. Moreover the lack of detectable anti-toxin antibody response in large numbers of tested subjects (Perez-Perez *et al.*, 1987) suggests either that *C. jejuni* enteritis is not solely a toxin-mediated disease, or that *in vitro* testing methods may be inadequate to demonstrate *C. jejuni* toxins activity consistently (Cover and Blaser, 1989).

6.1.2. Electrolyte and fluid secretion. The following changes are observed when tissue or tissue culture cells are exposed to secretagogues: (i) potential difference and short circuit current across the tissue increase, detectable in Ussing chambers (Frizzell *et al.*, 1979), (ii) net chloride absorption changes to net chloride secretion, due to a decrease in mucosal to serosal flux and an increase in serosal to mucosal flux, (iii) the paracellular chloride conductance of the mucosal border increases (Powell, 1974), (iv) net fluid and sodium absorption are diminished and sometimes net secretion is observed *in vitro* (Nellans *et al.*, 1974), and (v) the paracellular spaces and submucosal space collapse over the entire epithelial surface and simultaneously the electrical resistance of the tissue rises (Corbett *et al.*, 1977). A model of electrolyte secretion which accounts for the observed changes in intestinal ion transport in the presence of secretagogues (Rao and Field, 1983) suggests that separate changes occur in crypt and villus cells. In crypt cells, intracellular chloride is higher than its electrochemical equilibrium as a result of co-transport of NaCl across the basolateral membrane. As the final response to secretory stimulus is an increase in anion conductance across the apical membrane, this induces increased chloride leakage from the cell down its electrochemical potential gradient and accounts for the observed increase in chloride secretion by isotopic fluxes and increased short circuit current. In villus cells, on the other hand, the secretagogue is thought to act by reducing NaCl absorption across the mucosal border. An alternative model (Naftalin and Simmons, 1979) has been proposed to account for the changes in unidirectional ion fluxes across the mucosal and serosal borders and paracellular pathway on exposure to secretagogues and suggests that, following the action of secretagogues, there is an increase in mucosal membrane chloride conductance. Although the tight junction sodium conductance is high, there is normally little leakage of

NaCl across the mucosal surface because the chloride conductance of the mucosal surface is low. However, when the secretagogue dependent increase in Cl conductance is activated, there is a net leakage of both ions across the mucosal surface from the lateral intercellular space because of current circulation between the anion and cation conducting pathways. Since the backflux of sodium is electrically coupled to chloride, there will be a 1:1 stoichiometry in the reduction of NaCl absorption. This model requires no localization of the secretory processes to the crypt cell and no inhibition of NaCl influx across the mucosal surface. While neither model offers a complete explanation for all intestinal secretory phenomena, they have many common features. The main difference is the absence of any requirement for localized chloride secretion within the crypts in the latter model.

6.1.3. cAMP and cGMP as intracellular mediators. cAMP is generated from ATP through the action of adenylate cyclase, a membrane-bound enzyme that is coupled through stimulatory (Gs) and inhibitory (Gi) G proteins to a number of hormone receptors (Anon, 1987). In enterocytes, adenylate cyclase is located exclusively on the basolateral membrane (Walling *et al.*, 1987) and is stimulated by the activation of the Gs by vasoactive intestinal peptide (VIP) (Cristophe *et al.*, 1986). In contrast alpha-2-adrenergic agonists activate Gi, thereby inhibiting adenylate cyclase activity (Limberd, 1988). Three agents are commonly used to investigate cAMP effects in cells, cholera toxin which causes persistent activation of adenylate cyclase activity (Cassel and Pfeuffer, 1978) resulting in profuse watery diarrhoea characteristic of cholera, *Bordetella pertussis* toxin which prevents inhibitory effects upon adenylate cyclase activity (Katada and Ui, 1980), and forskolin which is a specific activator of adenylate cyclase activity (Seamon and Daly, 1981). In enterocytes cAMP levels are raised by agents including CT (Schafer *et al.*, 1970), PGs (Martens et al., 1985), LT producing *E. coli* (Field, 1976), and VIP (Schwartz *et al.*, 1978).

Guanylate cyclase exists in cells in particulate and soluble forms. The colon contains both forms, whereas the small intestine contains only the particulate form, most of which is in the brush border membrane (Rao *et al.*,

1980; de Jonge, 1975). There is also a descending gradient of guanylate cyclase activity from the tip of the villus to the crypts (de Jonge, 1975). The heat stable toxin of *E. coli* and probably other peptides activate particulate guanylate cyclase in intestinal preparations.

Apart from the activation of retinal sodium channels by cGMP (Koch and Kaupp, 1985), all known effects of cyclic nucleotides are mediated by protein kinases (Blackshear *et al.*, 1988). A number of intestinal membrane proteins are phosphorylated by cyclic nucleotide dependent protein kinases, but, as discussed in Chapter 3, it is not known which of these proteins have roles in ion transport.

6.2. MATERIAL AND METHODS

6.2.1. Toxin assays. The ability of the six strains used in the RILT (Chapter 4) to produce a cholera-like enterotoxin was tested by GM-1 ELISA, using the methodology described in Chapter 4. Bacteria were grown in MH broth, Brucella broth, or Dulbeccos' modified Eagles medium (DMEM) containing 10% foetal calf serum (FCS), with shaking for 24 h in a microaerophilic atmosphere. Bacteria were harvested by centrifugation. Bacterial sonicates and bacteria-free supernatants were tested for enterotoxin-like activity.

6.2.2. cAMP assay. cAMP was measured using a commercial [^{3}H]radioimmunoassay system (Amersham). The following samples were tested: (i) infected rabbit ileal loop tissue, and CT and PBS treated control loops, (ii) Caco-2 cells infected with viable *C. jejuni* strains or bacteria free supernatants, (iii) Caco-2 cells exposed to fluid collected from *C. jejuni*-infected ileal loops, passed down a Bond Elute extraction cartridge (Analytichem International) to concentrate prostaglandins, (iv) Caco-2 cells exposed to unextracted fluid collected from *C. jejuni*-infected ileal loops, neat, or diluted (after centrifugation to remove bacteria) in DMEM containing 10% FCS, and (v) Caco-2 cells treated with fluid from *C. jejuni*-infected loops in the presence of antibodies againstcholera toxin or antibodies against PGE2.

cAMP was extracted with ethanol, allowing easy concentration of

samples if very low levels of cAMP were present. Tissue and cells were homogenised in 2 ml of absolute ethanol, incubated at room temperature for 5 min, centrifuged, and the supernatant collected. The pellet was washed in 1 ml of ethanol-water (2:1) and again centrifuged. Supernatants were combined and evaporated to dryness under a stream of nitrogen, and the residue was dissolved in 0.5 ml of TE buffer (0.05 M Tris, pH 7.5, containing 4 mM EDTA). Standards for the assay were prepared to give solutions of 16, 8, 4, 2, and 1 pmol of cAMP/tube. Assay tubes were placed in an ice-water bath, and blanks containing 150 µl of TE buffer, tests and standards containing 50 µl of sample, and total count tubes with 50 µl of TE buffer were prepared in duplicate. 50 µl of [8-^{3}H]adenosine 3,5-cyclic monophosphate (5 µCi/ml) were added to all tubes. 100 µl of the cAMP binding protein were added to test, total count and standard tubes, and vortexed. Tubes were placed on ice for 2 h, after which 100 µl of charcoal were added to all tubes, mixed and centrifuged. 200 µl of the supernatants were added to 2 ml of Optiphase scintillant (LKB), and radioactivity was determined in a Packard Tri-Star liquid scintillation spectrometer. A standard curve was constructed, and the tissue and cell samples determined. Results were expressed as pmol of cAMP/g of tissue.

6.2.3. Fluid extraction. Fluid from infected loops (Chapter 4) was extracted to concentrate any prostaglandins or leukotrienes present. The method of extraction is detailed in Chapter 5.

6.2.4. cGMP assay. Tissue from *C. jejuni*-infected rabbit ileal loops, as well as tissue from CT and PBS-treated loops, were tested for increased cellular cGMP. Tissue extraction was performed as for the cAMP assay, and cGMP was tested using a [^{3}H]radioimmunoassay system (Amersham) and a protocol similar to that for the cAMP assay except for the use of a specific antiserum against cGMP, [8-^{3}H]guanosine 3,5,-cyclic phosphate (5 µCi/ml), and 60% saturated ammonium sulphate instead of charcoal reagent. Counts were determined and results expressed as pmol of cGMP/g of tissue.

6.3. RESULTS

6.3.1. Enterotoxin assays. Strains grown in the three different media produced very small amounts of a cholera-like enterotoxin that cross reacted with the B subunit of cholera toxin, as detected using GM-1 ELISA (Table 6.1). This small amount of toxin is responsible for CHO elongation testing as previously described in Chapter 4. In the same assay a strain of *Vibrio cholerae* gave over 100 μg/ml toxin.

6.3.2. cAMP in Caco-2 cells infected with live bacteria. Infection of Caco-2 cell monolayers with clinical isolates of *C. jejuni* induced increases in cellular cAMP up to 3 times the negative uninfected control (Table 6.2). Only the NCTC 12189 strain increased cAMP levels in Caco-2 cells markedly (12 times the negative control). This compares with a 400-fold increase in cAMP levels in the presence of 2 μg/ml of CT, and a 16 times increase with 10 ng/ml PGE2 (Table 6.5).

6.3.3. cAMP in Caco-2 cells treated with culture supernatants. Culture supernatants from growing organisms added to Caco-2 cell monolayers gave an increase in cAMP concentrations 1.4 to 2.2 times those in the untreated control monolayer after 4 h (Table 6.3). Values at later time points were difficult to determine because supernatants had toxic effects, seen as cell rounding and detachment from the plastic support. Detachment was inhibited by antibody against *E. coli* lipopolysaccharide (Sigma) and was therefore assumed to be due to the effects of endotoxin, probably lipid A. Cell rounding, however, was not inhibited, and probably reflects the activity of a *C. jejuni* cytotoxin, as described for its effects on other cell lines (Mahajan and Rodgers, 1990).

6.3.4. cAMP and cGMP levels in infected tissue. 11/16 (69%) tissue samples from loops infected with inflammatory strains L115, C119, O81 and P71 showed elevation of cAMP (Table 6.4). There was a statistically significant correlation between fluid volume in the loop (Chapter 4) and increased loop tissue cAMP (RS=0.44, p=0.019). Some strains showed increased levels of tissue cAMP over the CT treated control at the time of animal sacrifice. This is probably a reflection of the fact that cellular cAMP in CT-treated loops had decreased significantly by this time point. Strains eliciting no fluid secretion (N82 and NCTC 12189) gave

Table 6.1 Enterotoxin assay of supernatants and sonicates using GM-1 ELISAa

	Brucella broth	MH broth	DMEM
Culture supernatants			
L115	0.26	0.8	0.8
C119	0.4	0.72	0.4
O81	0.59	0.27	0.5
P71	0.09	0.27	0.4
N82	1.4	1.6	0.5
NCTC 12189	0.33	0.22	0.4
V. cholerae	100.o	120.0	ND
Bacterial sonicates			
L115	3.9	0.4	0.08
C119	3.0	1.3	0.5
O81	0.46	0.0	0.0
P71	3.6	0.0	0.0
N82	2.4	0.56	0.9
NCTC 12189	3.5	0.0	0.21

a Enterotoxin levels (ng/ml) were determined in conjunction with Robin Leece
ND, not done

Table 6.2. cAMP levels in Caco-2 cells infected with live bacteria

Strain	Incubation time (h) 2	4	6	8	18	36
CT 2 µg/ml	307·0[a]	1899.6	1306.0	439.8	185.7	160.5
UNINFECTED	1.5	2.5	5.2	5.5	5.8	5.6
L115	1.2	7.8(x3)	5.5	3.3	4.2	3.8
C119	1.15	5.1	10.1(x2)	3.1	4.5	0.8
O81	1.8	1.6	1.5	1.4	3.6	0.8
P71	1.9	1.1	6.9(x1.3)	1.6	7.7	5.1
N82	0.97	2.8	8.6(x1.6)	5.4	4.8	0.6
NCTC	18.27 (x12)[c]	11.6	10.2	12.2	9.4	7.5

[a] cAMP concentration expressed as pmol/g of cells. Values are averages of at least three experiments for each strain; numbers in parentheses indicate maximum increase over uninfected control values

Table 6.3. cAMP levels in Caco-2 cells treated with bacterial culture supernatants[a]

strain	Incubation time (h)		
	4	8	18
CT (2 μg/ml)	2322.2	456.0	186.0
UNINFECTED	4.22	7.48	5.8
L115	8.84 (x2.2)	4.89	0.9
C119	8.0 (x2)	6.2	0.2
O81	7.0 (x1.6)	7.21	0.8
P71	6.27 (x1.4)	1.9	0.9
N82	6.96 (x1.6)	6.04	0.56
NCTC 12189	6.36 (x1.5)	5.11	0.5

[a] cAMP concentrations expressed as pmol/g of cells. Values are averages of at least of 3 experiments; numbers in parentheses indicate maximum increase over uninfected control values

Table 6.4. cAMP and cGMP in infected tissue

Animal No.	Strain	cAMP (pmol/g)				cGMP (pmol/g)			
		controls		test loops		controls		test loops	
		CT	PBS	1	2	CT	PBS	1	2
1	L115	4.1	2.4	0.4	1.5	0.8	0.8	0.09	0.3
2	L115	4.6	2.8	10.6	6.7	0.8	0.8	0.4	0.3
3	C119	8.0	4.7	5.5	3.9	1.0	0.6	0.15	0.22
4	C119	2.1	0.7	4.5	5.0	0.4	0.2	0.3	0.7
5	O81	1.8	1.2	5.5	7.6	1.3	0.8	0.1	0.75
6	O81	5.4	2.2	0.5	0.2	1.0	0.5	0.57	0.8
7	P71	6.8	3.5	5.0	4.0	0.77	1.4	0.4	0.8
8	P71	1.9	1.7	6.8	2.4	0.67	0.6	0.8	0.68
9	N82	5.2	2.6	2.8	2.8	0.94	0.6	0.29	0.28
10	N82	4.2	2.1	0.7	1.6	0.8	0.8	1.03	1.3
11	NCTC	5.1	5.0	2.0	4.0	1.0	2.0	0.9	1.2
12	NCTC	0.8	0.3	0.4	0.2	0.2	0.4	0.5	0.04

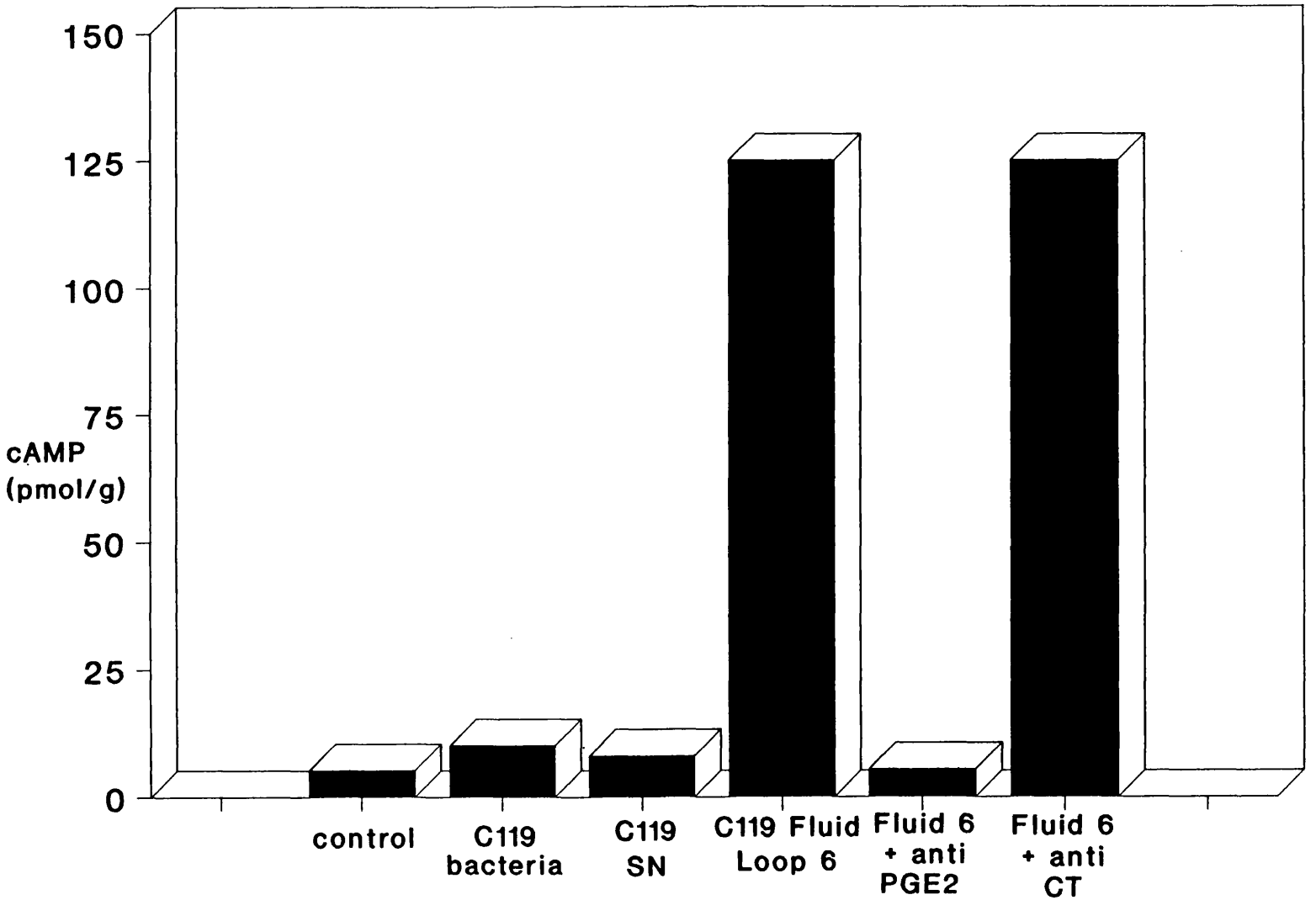
All assays were performed in duplicate.

Table 6.5. cAMP levels in Caco-2 cells treated with unextracted loop fluids

sample	Incubation time (h)				
	2	4	6	8	18
CT (2 µg/ml)	380.0	1900.0	1306.0	439.8	186.0
CT + ANTI-CT	4.3	3.5	4.1	3.6	4.6
untreated	1.5	2.5	5.2	5.5	5.8
PGE2 (2ng/ml)	4.1	5.7	5.7	8.9	14.7
PGE2 (5ng/ml)	2.2	3.3	5.9	23.4	13.5
PGE2 (10ng/ml)	8.1	12.8	34.8	78.9	6.6
C119 FLUID 5	5.8	6.4	7.6	9.8	5.3
C119 FLUID 6	5.3	125.0	36.6	33.3	10.6
C119 FLUID 6 + ANTI-PGE2	3.6	5.4	10.0	19.0	10.1
C119 FLUID 6 + ANTI-CT	5.4	125.0	36.0	34.0	9.5
L115 FLUID 4	8.3	9.0	9.1	32.0	10.6
L115 FLUID 4 + ANTI-PGE2	6.2	7.5	7.1	10.5	8.4
L115 FLUID 4 + ANTI-CT	8.3	9.0	9.4	32.6	10.6
O81 FLUID 11	24.3	32.7	11.1	7.8	7.3
O81 FLUID 11 + ANTI-PGE2	10.0	14.2	8.4	5.1	4.2
O81 FLUID 11 + ANTI-CT	24.0	34.0	11.0	8.6	5.4
P71 FLUID 17	16.6	11.3	8.6	7.9	3.6

cAMP expressed as pmol/g cells; minimum of 3 experiments per sample

Fig.6.1 cAMP assay of Caco-2 cells treated with strain C119, culture supernatants, loop fluids and fluids with anti-Ct or anti-PG



essentially no increase in tissue cAMP compared to the uninfected control loop. All cGMP levels were extremely low, and there was no significant difference between infected and control loops (Table 6.4).

6.3.5. cAMP in Caco-2 cells treated with extracted loop fluids. Fluid from infected loops was prepared as described in Materials and Methods to concentrate prostaglandins and leukotrienes. No increase in cAMP was detected in Caco-2 cells exposed to the concentrated loop extracts. Large amounts of PGE2 were shown to be present in this fluid (Chapter 5), and so it was concluded that extraction inactivates biological activity (but not immunological detection).

6.3.6. cAMP in Caco-2 cells treated with unextracted loop fluids. Caco-2 cells were exposed to untreated loop fluids and assayed for cellular cAMP (Table 6.5). Activity was higher (as much as 25 fold) in cells treated with loop fluid than in those infected with bacteria or exposed to bacteria free supernatants, but not as high as in cholera toxin treated cells. Fluids contained no detectable cholera-like toxin, and enhancement of cAMP levels was not inhibited by anti-cholera toxin antiserum. The activity was, however, reduced by an antiserum against PGE2, indicating that this eicosanoid is responsible for increasing cAMP in Caco-2 cells, and probably also in infected loop tissue (Fig. 6.1). Activity was not completely abolished, suggesting that other eicosanoids may also be involved in increasing cAMP.

6.4. DISCUSSION

The *C. jejuni* strains used in this study produce a cholera-like toxin with homology to the B subunit of CT, but in very low quantities *in vitro*. It is possible, of course, that toxin production is important in diarrhoea and is greatly enhanced *in vivo*, perhaps controlled by a two component regulatory system (DiRita and Mekalanos, 1989) which senses an environmental stimulus that cannot be mimicked *in vitro*, or that invasive bacteria release a small amount of toxin directly inside the host cell. However there is no evidence from the experiments described here that this is the case, since toxin was not detected in loop fluids or in homogenised tissue, although it may be the case that homogenisation was insufficient to disrupt bound toxin. The quantities of toxin recoverable from culture supernatants and

bacterial sonicates also raises questions about the relevence to disease of cholera-like toxin production in these strains. In human studies the absence of neutralising antibody in convalescence argues against a role in disease, but it may be that the detection system for toxin production is inadequate if the degree of homology to CT is not as great as is generally thought. The recent successful partial purification of CJT (Daikoku *et al.*, 1990) should enable better systems for detection to be developed and its relevence to disease determined.

Bacteria or culture supernatants increased cAMP levels in cultured Caco-2 cells 1.3-12 times those in uninfected controls. The consequences of raised cAMP within cells in the intestine is that chloride and then water leaves the cells. Increasing cAMP also inhibits absorption within absorptive villus cells (Donowitz and Welsh, 1987). The increases seen in Caco-2 cells presumably reflect the small amount of toxin detected by GM-1 ELISA. Toxin production does not seem to increase when bacteria are in contact with cells, although cultural conditions are suboptimal for the bacteria in this system. Increases in cAMP have been seen in cultured cells exposed to *Shigella* species (Clerc *et al.*, 1988), and the authors speculate from these experiments that increased cAMP is associated with cell death.

C. jejuni causes increases in tissue cAMP related to fluid volume collected in the gut lumen. It is unlikely that these increases are due to detectable toxin since toxin could not be demonstrated in loop fluids and increases in cAMP were not abolished by antibody against CT. An alternative candidate for a secretagogue increasing tissue cAMP is PGE2, which was present in large amounts in the loop fluids induced by the bacteria; enterocytes have PGE2 receptors (Rousset, 1986). Other eicosanoids may also be involved. From experiments in Chapter 5 it was demonstrated that Caco-2 cells cannot be induced to make PGE2, so that any that is present must have come from an exogenous source. Addition of anti-PGE2 antibodies to loop fluid inhibited induction of cellular cAMP from levels demonstrated with fluid-treated cells. Increases were not as large as seen with CT but exceed those of bacteria alone. Campylobacter diarrhoeal fluid loss is seldom as severe as in cholera, and cAMP rises are presumably not as great in magnitude, or as prolonged in affected cells. From these experiments PGE2 seems the most likely candidate for increased cAMP in

C. jejuni-infected tissue. The effects of this increase are fluid secretion in crypt cells and inhibition of absorption in mature villous cells.

Chapter 7

GENERAL DISCUSSION

The main findings of the work presented in this thesis may be summarised as follows. (i) A model of *C. jejuni* and *C. coli* adhesion, invasion and transcytosis using an enterocyte-like cell line (Caco-2) has revealed significantly larger numbers of invasive and trancytosing strains amongst organisms isolated from colitic patients, compared with isolates from patients with watery diarrhoea. (ii) *C. jejuni* supernatants stimulated phosphorylation of cytosolic and cytoskeletal membrane proteins in Caco-2 cells, suggesting the activity of a secreted toxin. The phosphorylation events are consistent with an increase in intracellular calcium, but there was no evidence for phosphorylation due to enterotoxin-induced increases in cAMP levels. (iii) The rabbit ileal loop test is a good model of *C. jejuni* inflammatory diarrhoea in that it mimics the histology of disease in man. Biochemical analysis of secreted fluid indicated a high protein content indicative of fluid loss due to tissue damage as well as bicarbonate typical of secretory diarrhoea. (iv) cAMP levels are elevated in tissue infected with *C. jejuni* strains causing inflammation. (v) A bacterial enterotoxin could not be demonstrated to cause the increases in cAMP in infected tissue. Levels of the host inflammatory mediator PGE2, were increased in infected tissue, and are postulated to be the cause of cAMP-mediated secretion.

Initially, this project concentrated on two groups of clinically well characterised *C. jejuni* and *C. coli* isolates. These were defined as colitis (or inflammatory) or non-inflammatory strains based on observed clinical symptoms, microscopic analysis of inflammatory cells, histology, endoscopy and laboratory isolation of the infecting organism. Strains from both clinical groupings invaded Caco-2 cells, but colitis strains had a greater propensity to invade the cell monolayer compared to non-inflammatory isolates. In inflammatory disease, the ability to invade and initiate an inflammatory response presumably depends on a bacterial outer membrane component or toxin. In other enteric infections, bacterial numbers in tissue correlate with the level of tissue damage and reduction in the absorptive capacity (Rout *et al.*, 1974). A model of the proposed pathogenesis of inflammatory diarrhoea

caused by *C. jejuni* is presented in Figure 7.1. After bacterial invasion has occurred, bacterial products induce white cells, polymorphs and macrophages, into the tissue by chemotaxis; presumably the greater the bacterial insult, the larger the polymorphonuclear leucocyte infiltrate within the tissue. Campylobacter diarrhoea resembles salmonellosis and yersiniosis in that mucosal lesions are usually less severe than those caused by shigella but the organisms are more likely to invade underlying tissues and gain access to mesenteric lymph nodes. Pain in campylobacter enterocolitis is more severe and lasts longer than in salmonellosis (Skirrow, 1988). Pain leads to laparotomy but the appendix is rarely inflamed. In abnormal cases, mesenteric adenitis, inflammation of the ileum and sometimes bacteraemia may be present. The overall invasive process can be considered as the integration of two stages, invasion of individual epithelial cells, and connective tissue invasion (Sansonetti *et al.*, 1988). Macrophages represent the major line of defence in the lamina propria (UCLA conference, 1987), and are a potent source of eicosanoids. Tissue damage and inflammation result from the production and release of prostaglandins and leukotrienes by white cells in the tissue and their subsequent effects.

Campylobacter culture supernatants and whole bacteria are chemotactic for PMNLs (Krausse and Ullmann, 1987). Bacterial formylated peptides are potent chemoattractants for inflammatory cells, and their ability to induce leucocyte infiltration *in vivo* results in inflammation. One such compound, the tripeptide n-formyl-methionyl-leucyl-phenylalanine (fMLP), is a major product of bacteria. fMLP induces neutrophil chemotaxis and adherence, as well as degranulation and production of both oxygen radicals and pro-inflammatory eicosanoids (Nast and LeDuc, 1988). In the intestine fMLP has multiple effects. It has been reported to release both prostaglandins and leukotrienes from normal and inflamed rabbit colon (Zipser *et al.*, 1987; LeDuc and Nast, 1990), to increase mucosal permeability in rabbit distal ileum (Von Ritter *et al.*, 1988), and to induce colonic inflammation in rats and mice (Chester *et al.*, 1985). In contrast bradykinin caused the release of PGE2 in a rabbit model but colitis was not present (Zipser *et al.*, 1987); presumably a bacterial product is needed to initiate tissue inflammation. It has been suggested that *C. jejuni* cytotoxin may be a candidate for initiating inflammation and tissue damage (Yeen *et*

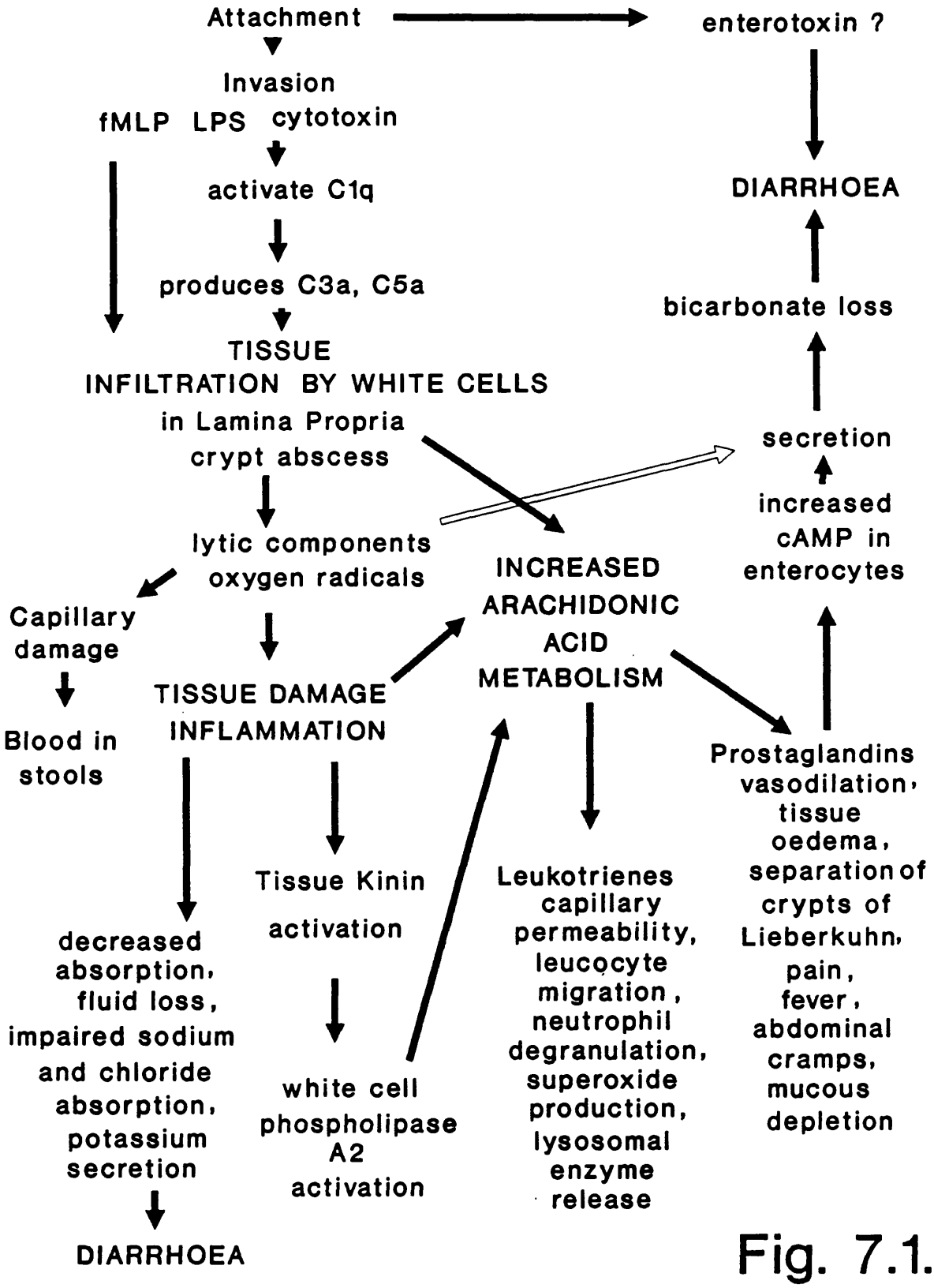

Attachment
enterotoxin ?
Invasion
fMLP LPS cytotoxin
activate C1q
DIARRHOEA
produces C3a, C5a
bicarbonate loss
TISSUE
INFILTRATION BY WHITE CELLS
in Lamina Propria
crypt abscess
secretion
increased
cAMP in
enterocytes
lytic components
oxygen radicals
INCREASED
ARACHIDONIC
ACID
METABOLISM
Capillary
damage
TISSUE DAMAGE
INFLAMMATION
Blood in
stools
Prostaglandins
vasodilation,
tissue
oedema,
separation of
crypts of
Lieberkuhn,
pain,
fever,
abdominal
cramps,
mucous
depletion
Tissue Kinin
activation
Leukotrienes
capillary
permeability,
leucocyte
migration,
neutrophil
degranulation,
superoxide
production,
lysosomal
enzyme
release
decreased
absorption,
fluid loss,
impaired sodium
and chloride
absorption,
potassium
secretion
white cell
phospholipase
A2
activation
DIARRHOEA

Fig. 7.1.

al., 1983). In the study presented in this thesis, cytotoxin production correlated with the ability of strains to cause colitis, but the majority of non-inflammatory isolates also produced a cytotoxin.

The role of PMNLs in phagocytosis and killing ingested bacteria is well recognised. Less well known is their function as secretory cells, releasing a variety of potent inflammatory mediators into the surrounding tissue (Dale and Foreman, 1987). The resting neutrophil has within its cytoplasm numerous granules, containing a variety of biologically active constituents. These include proteolytic enzymes (such as elastase, collagenase, myeloperoxidase and cathepsin G), acid hydrolases, several highly cationic proteins, lactoferrin and vitamin B12-binding proteins. These granular constituents are extruded from the neutrophil during activation by a variety of stimuli including phagocytosis, antigen-antibody complexes, activated complement components, and soluble stimulants such as leukotrienes and specific peptides (Tracey *et al.*, 1987). Upon activation by phagocytic or soluble stimuli, both neutrophils and macrophages undergo a respiratory burst, characterised by an increase in oxygen consumption, activation of the hexose monophosphate shunt, and generation of reactive oxygen-derived free radicals, superoxide and hydrogen peroxide. Several other reactive oxygen-derived metabolites are also produced, including hydroxyl radicals and hypochlorous acid. Although most of the oxygen free radicals produced during the respiratory burst are localised within the phagolysosomes, significant amounts also appear extracellularly, and are toxic to endothelial cells, erythrocytes and platelets, as well as damaging to non-cellular proteins (Fantone and Ward, 1982). Neutrophils can damage endothelial cells during inflammation (Smedley *et al.*, 1986), and may account for the presence of blood in rabbit intestinal loops infected with colitis strains. The earliest event in inflammation is adherence of neutrophils to the vascular endothelium (Harlan, 1983). The alteration of the endothelial surface which permits adherence of leucocytes can be induced by interleukin 1, complement components or leukotriene B4 (Harlan, 1983), and may serve to bring inflammatory mediators released from neutrophils into close proximity with the endothelium. Neutrophils stimulated by C5a or specific peptides can lyse endothelial cells (Smedley *et al.*, 1986). The endothelial cell damage is enhanced in the presence of

endotoxin, and is probably mediated by both oxygen free radicals and proteolytic enzymes released from the neutrophil (Smedley *et al.*, 1986).

Increased vascular permeability to albumin and other plasma proteins explains the high protein content in loop fluids, and oedema in infected tissue. Several mechanisms may cause an increase in vascular permeability, including endothelial cell destruction, opening of inter-endothelial spaces and damage to the basement membrane mediated by serotonin, leukotrienes, proteolytic enzymes or the kinin pathway (Anderson *et al.*, 1979). Cationic proteins released from inflammatory cells may bind to and neutralise the negative charge on endothelial cells and basement membranes, thereby reducing the capillary wall negative charge and increasing permeability to albumin (Wight, 1980). Fluid was elicited within infected loops by all colitis strains tested, by a mixture of true secretion from the mucosa and exudation or transudation of serum components through damaged mucosa as assessed by tissue histology. Such tissue damage results in decreased area for fluid absorption. Thus, mucosal damage to the colon may be associated with zero water transport or net water secretion, both resulting in diarrhoea (Rask-Madsen and Jensen, 1973).

When the inflammatory process is limited to a single colonic segment, mucosal absorptive function may be sufficiently impaired to produce a clinically significant increase in faecal water excretion. In human colon the rate of water absorption is directly related to the rate of sodium absorption (Devroede and Phillips, 1969) which possesses both electroneutral and electrogenic components. Electrogenic sodium transport generates a high lumen negative transmucosal potential difference throughout the colon, but mucosal inflammation leads to a decrease in transmucosal potential difference, gross impairment of sodium and chloride absorption, enhanced potassium secretion, and a decrease in net water absorption (Rask-Madsen and Jensen, 1973). Defects in the biophysical properties of colonic epithelial cell membranes are likely to be important in the pathogenesis of diarrhoea in inflamed tissue (Sandle *et al.*, 1990). Thus the tissue inflammation induced by *C. jejuni* infection is directly responsible for cell damage resulting in decreased area for fluid absorption.

The importance of inflammation in the pathogenesis of the diarrhoeal

illness caused by *C. jejuni* was confirmed in human volunteer challenge studies (Black *et al.*, 1988). These showed that fever preceeded the onset of diarrhoea, all ill persons had faecal leucocytes, and rectal biopsies showed inflammatory cells and oedema. A classic enterotoxin alone would not explain these clinical features. In the RILT, tissue histology after treatment with *C. jejuni* strains derived from cases of human colitis correlated well with the histology seen in humans, while one non-inflammatory strain from a case of watery diarrhoea (N82) elicited no fluid secretion and no change in tissue histology. This model may not therefore be appropriate for non-inflammatory strains, although more should be assessed for confirmation. Peroxides and oxygen radicals formed by macrophages and granulocytes also stimulate fluid secretion (Powell, 1990). The secretory response to prostaglandins, histamine, and oxygen radicals, as well as those in intestinal anaphylaxis, can be partially inhibited by neurotoxins and cholinergic antagonists (Powell, 1990).

Eicosanoids produced by white cells in response to antigens and products of bacteria can be induced by kinins, small peptides that have diverse biological actions (Gaginella and Kachur, 1989). Kinins are liberated from plasma components by serine proteases called kallkreins, precursors of which (prekallikreins) have been localised in a number of organs including the intestine (Schachter *et al.*, 1983). Plasma components can be liberated into tissue by endothelial damage by bacterial invasion. Plasma kallikreins release bradykinin from a high molecular weight kininogen. Leukokinins are also released from leukocytes at sites of inflammation (Zachariae *et al.*, 1968). Kinins directly or indirectly activate phospholipase A2 and phospholipase C and they also stimulate cell populations (fibroblasts, mast cells, leukocytes) to produce prostaglandins, leukotrienes, and lipoxygenase intermediates in the sub-epithelium. The high PG synthetic capacity of the sub-epithelium compared with enterocytes has led workers independently to conclude that elements in the lamina propria, including fibroblasts and vascular endothelial cells, are the major source of kinin-generated PGs (Lawson and Powell, 1987; Warhurst *et al.*, 1987). Some prostaglandin effects may be neurally mediated. For example PGE2 caused atropine-sensitive chloride secretion in the rat descending colon, but the inhibitory effect of atropine only occurred in a preparation

containing sub-mucosal neurons (Diener *et al.*, 1988). The authors concluded that secretion induced by these prostaglandins in the rat colon is due to both a direct effect on epithelial cells, consistent with the data presented here, and the release of acetylcholine. Macrophages are the major source of PGE2 in the gut (Male *et al.*, 1991; Rask-Madsen *et al.*, 1990). PGs enhance vasodilation and oedema, acting synergistically with other mediators of inflammation, whereas leukotrienes promote leukocyte migration and stimulate aggregation and degranulation of neutrophils and the release of lysosomal enzymes and superoxide production (Stenson, 1990). LTB4 is the most potent chemotactic eicosanoid (Marcus, 1985); it induces neutrophil chemotaxis *in vitro* at concentrations of 3 ng/ml (Goetzl and Picket, 1980), comparable to the concentrations seen in *C. jejuni* infected loops.

PGs modulate electrolyte transport in the intestine and are candidates for therapeutic intervention in inflammatory gastrointestinal infections. The diarrhoea of inflammatory intestinal disease may be mediated at least in part by the release of eicosanoids from the inflammatory cells that characteristically infiltrate the mucosa in these disorders. Supporting this suggestion is the ability of prostaglandin inhibitors such as aspirin and indomethacin to reduce the intestinal secretion evoked in animal models by *Salmonella* (Gianella *et al.*, 1977) and *Shigella* (Gots *et al.*, 1974). No clinical studies to date have examined the effects of non-steroidal anti-inflammmatory drugs specifically in infectious inflammatory enteritis, but it is of interest that the beneficial influence of sulphasalazine in ulcerative colitis has been attributed to its ability to block the synthesis of the eicosanoids 5-HETE and 5-HPETE (Stenson and Lobos, 1982). The other well known class of anti-inflammatory drugs, the glucocorticoids, has also been shown to enhance intestinal fluid absorption. It is unclear if this is due to a direct effect on the apical epithelial membrane or to the enhanced production of macrocortin (lipomodulin), a protein that inhibits one of the enzymes (phospholipase A2) involved in deacylating phospholipids to release more membrane arachidonate for eicosanoid synthesis. The glucocorticoids have too slow an onset of action and are associated with too many side effects to warrant their use in infectious enteritis (Kandel and Donowitz, 1989).

In infectious diseases there is usually a certain amount of direct microbial damage to host tissues, and bacteria release products that are themselves inflammatory, or that induce the host to respond by releasing mediators of inflammation (Mims 1982). In recent years it has become clear that morbidity and mortality in some bacterial diseases are the result of the intense host inflammatory response to infection (Tarlow, 1991). Further advances in the control of inflammation depend on a more detailed understanding of how this response develops, and will enable development of treatments to correct the disordered physiological state. Along with antibiotics to reduce the initiating cause of inflammation, anti-inflammatory drugs could therefore be used to reduce associated morbidity.

In contrast with the basic mechanisms of tissue inflammation and associated diarrhoea, the pathogenesis of watery diarrhoea in invasive disease is not well understood for *Shigella* or *Salmonella* species (Sansonetti *et al.*, 1988), or for the *Campylobacter* strains described in this thesis. However, the ability to invade cells and the ability to initiate inflammation should be distinguished. Some of the non-inflammatory *Campylobacter* strains do invade cell cultures, and in animal models of salmonellosis organisms can be invasive but elicit no fluid secretion (Stephen *et al.*, 1985, Wallis *et al.*, 1989). For one strain (C119) tested in the RILT model, the infected villi resembled those in loops treated with cholera toxin, and tissue also contained some inflammatory cells. However a CT-like toxin could not be detected in fluid or tissue. Moreover a strain (P71) shown to produce no toxin using CHO cell assay and ELISA did cause fluid secretion in the loops. Thus CT like toxin does not seem to be the mediator of secretion in the strains tested using RILT, although the possibility that these organisms produce another toxin having little homology to CT cannot be excluded. Like CT, PGE2 causes an increase in cAMP levels and may explain the similar effects observed. PGE2 present in the loop fluid can act directly on enterocytes to increase cAMP, which may result in fluid secretion from the mucosa or inhibition of absorption from villus tip cells.

Colonisation of the upper small intestine may be a prerequisite for the initiation of watery diarrhoea. In a rhesus monkey model of *Shigella flexneri* diarrhoea (Rout et al., 1975), watery diarrhoea occurs first and correlates with fluid secretion in the proximal jejunum and with the

presence of bacteria in the lumen but not within epithelial cells. The dysenteric phase occurs later when the organisms invade colonic epithelial cells, causing inflammatory colitis. *C. jejuni* illness may follow a similar course (Skirrow, 1986). Jejunal secretion has also been seen in the rhesus monkey model of salmonellosis in the absence of invasion and inflammation (Rout *et al.*, 1974). In human volunteer studies of *C. jejuni* infection, organisms multiply in the presence of bile (Blaser *et al.*, 1980), and colonise the upper small intestine early in the course of infection (Black *et al.*, 1988). Perhaps this non-invasive colonisation process may be related to the causation of watery diarrhoea when it is the sole manifestation of illness, bacteria elaborating a mixture of toxin-like components. Mucosal lesions of the jejunum and ileum have certainly been reported in campylobacter infection (Tomkins, 1983). The overall effect of any mucosal lesion is decrease in surface area, and malabsorption often occurs in the acute and/or recovery phase of the illness. There are more immature enterocytes on the villus than normal and the effect of these immature crypt secretory cells is that net absorption can be turned into net secretion even without the presence of an enterotoxin. Bacterial culture filtrates perfused into rat intestine can reduce lactase activity of the mucosa, indicating microvillous damage (Tomkins, 1983). Campylobacter culture supernatants stimulate phosphorylation of Caco-2 cell proteins and increase intracellular calcium in this and other cell lines. Intracellular calcium may be raised by the activity of a pore forming toxin, such as the haemolysin of uropathogenic *E. coli* and the recently described toxin of enteroaggregative *E. coli* (Baldwin *et al.*, 1992). In the case of *C. jejuni*, the cytotoxin may be the mediator of such effects. Morphological changes of Caco-2 cells occur after treatment with culture supernatants, independent of endotoxin. Production of this toxin in ileum could invoke the mucosal lesions seen, leading to loss of absorptive function and villi covered in crypt secretory epithelium leading to watery diarrhoea. Confocal microscopy indicates that *C. jejuni* supernatants affect cells by causing an increase in intracellular stores of calcium and this is associated with loss of absorptive function and enterocyte secretion (de Jonge and Rao, 1990). Enteropathogenic *E. coli* have been shown to increase intracellular calcium in epithelial cells and it is this cellular increase that may eventually lead to loss of the cells absorptive capacity (Baldwin *et al.*,

1991). From these observations a speculative model of *C. jejuni* watery diarrhoea is presented in Figure 7.2.

Diarrhoea can be looked at from the point of view of the host or of the infecting organism. On the one hand diarrhoea is a very effective way of dispersing *C. jejuni* into the environment, where they may find another host for further disease and dissemination. Alternatively, via animal feeds or manuring of crops *C. jejuni* may colonize the intestines of chickens and other farm animals where it lives as a commensal, a lifestyle for which it is better adapted. On the other hand, diarrhoea, inflammation, gut motility and shedding of infected cells are beneficial to the host because the faster bacteria are removed and the inflammatory stimulus lessens, the faster the disease symptoms will resolve. In terms of non-specific host defence mechanisms therefore, it is not surprising that substances that speed up motility and cause fluid secretion should be initiated in enteric infections. Gut motility and diarrhoea that help rid the host of offending pathogens may therefore be analogous to coughing in pulmonary infections as mechanisms to expel pathogens (Guerrant, 1979).

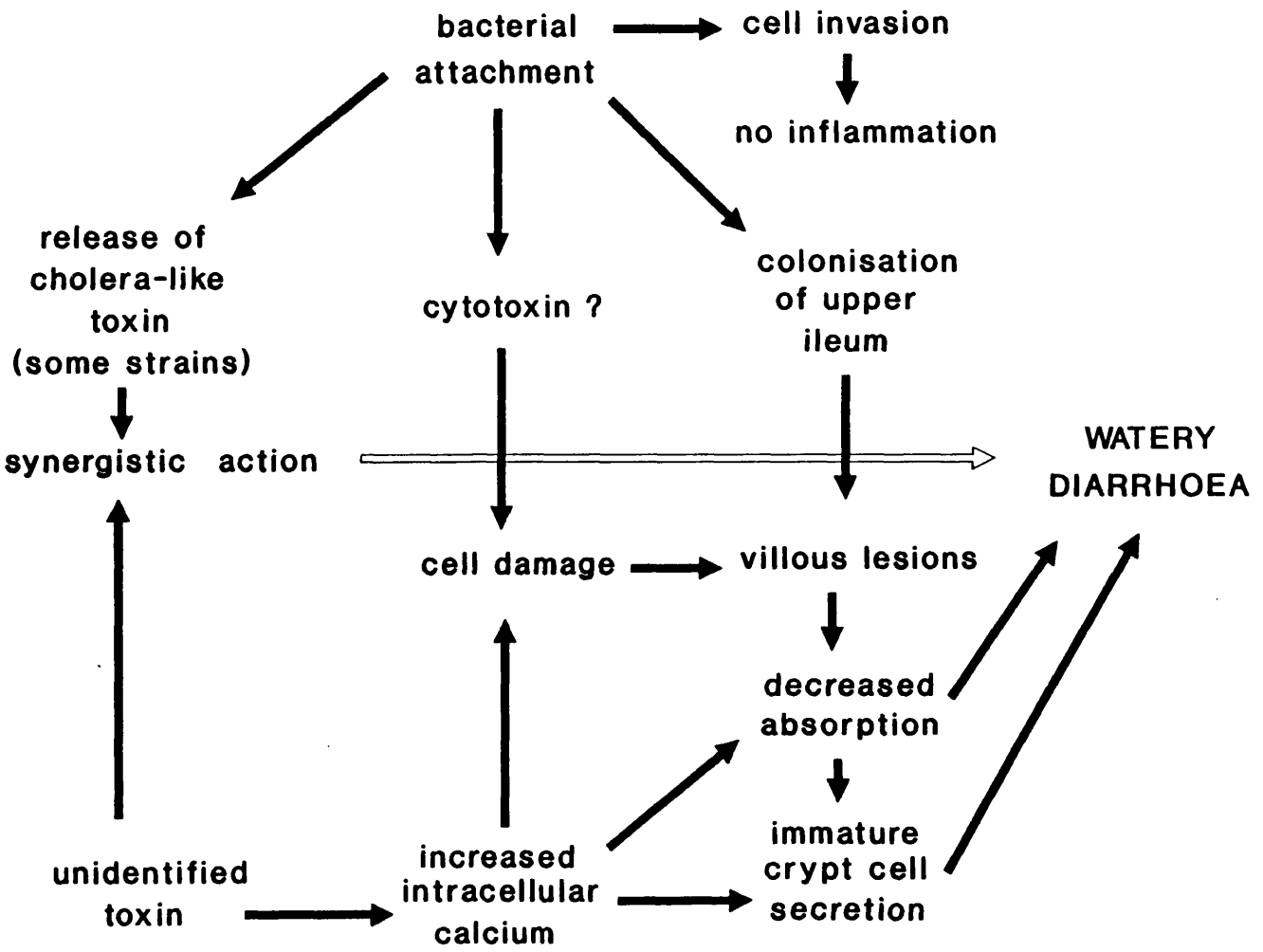

bacterial attachment
cell invasion
no inflammation
release of cholera-like toxin (some strains)
cytotoxin ?
colonisation of upper ileum
synergistic action
WATERY DIARRHOEA
cell damage
villous lesions
decreased absorption
immature crypt cell secretion
unidentified toxin
increased intracellular calcium

Fig. 7.2.

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