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Characterization of a cytotoxin from Campylobacter jejuni and its role in pathogenicity

Sangeeta Mahajan
University of New Hampshire, Durham

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Characterization of a cytotoxin from *Campylobacter jejuni* and its role in pathogenicity

Mahajan, Sangeeta, Ph.D.

University of New Hampshire, 1990

CHARACTERIZATION OF A CYTOTOXIN FROM

CAMPYLOBACTER JEJUNI

AND ITS ROLE IN PATHOGENICITY

BY

SANGEETA MAHAJAN

B. Sc. GOVERNMENT COLLEGE FOR GIRLS,   1983

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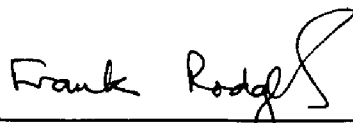
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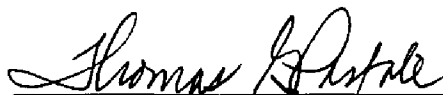
_____
Dissertation director, Dr. Frank G. Rodgers
Associate Professor of Microbiology

_____
Dr. William R. Chesbro, Professor of Microbiology

_____
Dr. Andrew P. Laudano, Assistant Professor of Biochemistry

_____
Dr. Thomas G. Pistole, Professor of Microbiology

_____
Dr. Roger E. Wells, Associate Professor/Pathologist
Animal and Nutritional Sciences

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TO RICKY WITH LOVE

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Abstract

CHARACTERIZATION OF A CYTOTOXIN FROM

CAMPYLOBACTER JEJUNI

AND ITS ROLE IN PATHOGENICITY

by

Sangeeta Mahajan

University of New Hampshire, May 1990

Campylobacter jejuni is a major cause worldwide of human gastroenteritis. In this study, the pathogenicity of the organism was examined in fertile hens' eggs and the mortality data and histopathological findings induced by both the organism and bacteria-free filtered broth were similar. The absence in chick embryo tissues of either bacteria or an inflammatory infiltrate suggested a toxic etiology. A protein was obtained from the filtrate by gel electroelution and this was sensitive to heat, pH changes and trypsin treatment and was lethal for the fertile hen's egg. It showed cytotoxic effects in primary chick embryo fibroblast (PCEF), Chinese hamster ovary (CHO) and intestinal 407 (Int 407) cells. Subsequently, a 68 kilodalton protein was isolated by polyacrylamide gel electrophoresis from the eluted toxic protein (ETP). A monoclonal antibody (CETPMAb$_4$) raised to the ETP abolished toxicity and bound only to the 68 kilodalton protein. No homology between C. jejuni ETP and Vibrio cholerae toxin and their respective antisera was observed. In enzyme-linked immunosorbent assay (ELISA) tests, the ETP did not bind to GM$_1$ ganglioside. Binding of ETP to PCEF and Int 407 host cell membranes was maximal after 2 h and this adherence was significantly reduced by prior treatment of the

cells with proteolytic enzymes, neuraminidase or glutaraldehyde but not with ß-galactosidase, lipase, Nonidet P-40 or sodium metaperiodate. In competitive binding assays, sugars (except N-acetyl neuraminic acid), lectins or GM_1 ganglioside did not adversely influence uptake of the ETP by these cells. Electron microscopic examination of the Int 407 cells treated with either C. jejuni or ETP showed similar ultrastructural damage. Western blot and ELISA tests of bacterial sub-components probed with $CETPMAb_4$ indicated that the toxin was associated with the outer membranes of the organism. Immunoassays on ETP treated eukaryotic host cells demonstrated that the toxin was attached only to the cell membranes and was not internalized. The present study indicates that the production of a membrane adhering cytotoxin that binds to protein or glycoprotein receptors on host cell membranes may be an important virulence factor responsible for the inflammatory diarrhea caused by many C. jejuni strains.

INTRODUCTION

1.1 Historical Perspective:

The members of the genus _Campylobacter_ (Greek meaning 'curved rod') are associated with a wide variety of diseases in humans and animals. These vary from enteritis to ulcers. Not only are these organisms one of the leading causes of gastroenteritis, but they are also the main etiologic agents of enzootic sterility in cattle and abortion in various domestic animals. The potential of these vibrio-like organisms to cause disease was first reported in 1913 by McFadyean & Stockman (95), who found them to be the cause of abortion in cattle and sheep. Smith (135) isolated similar organisms from an aborted bovine fetus. They were characterized by Smith & Taylor (136) and given the name _Vibrio fetus_. In 1931, Jones et al. (59) reported an association of these organisms with winter dysentery in cattle. To investigate the reproducibility of the disease, they fed pure cultures of these vibrios isolated from diseased animals to healthy cattle. The jejunum was the first site in the intestinal tract to be infected and they proposed the name _V. jejuni_ as it resembled _V. fetus_ sufficiently to be regarded as a closely related group. Doyle (31) linked these organisms to another disease after isolating them from the colonic mucosa of hogs with swine dysentery. The name _V. coli_ was proposed.

Levy (83) reported the first case of human infection and raw milk was suspected as the source of the outbreak. As the cultures were lost on subculture, it was Vinzent et al. (156) who reported the first definitive human infection by these vibrios. The organism, subsequently named _V. fetus_ , was isolated from the blood and placenta of a pregnant woman suffering from fever, headache, influenza-like symptoms who delivered a still born fetus. King (67) differentiated two distinct groups of vibrios from blood cultures of infected humans based on their ability to grow at different temperatures. She noted that _V._

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fetus grew at 25°C and 37°C but not at 42°C whereas the other group of organisms which were identical to the V. jejuni of Jones et al. and the V. coli of Doyle could not grow at 25°C but only at 37°C or 42°C.

Surprisingly after this very few cases of related vibrio infections were reported until 1972. The major problem was the lack of a selective culture technique for the isolation of these organisms from fecal samples. The breakthrough came when Dekeyser et al. (29) developed a procedure to isolate these microaerophilic vibrios from stool samples. The genus by then had been renamed by Sebald & Véron (129) as Campylobacter. Their technique involved filtration of the stool suspensions through a 0.65 μm membrane filter. Butzler et al. (21) used this technique but the isolation rate was low. However, Skirrow (130) isolated Campylobacter vibrios, using a selective culture medium. He plated the stool samples directly on Blood agar base 2 supplemented with 7% lysed horse blood, 10 mg/l vancomycin, 2500 IU/L polymyxin B and 5 mg/L trimethoprim. George et al. (40) reported that 0.25% each of ferrous sulfate, sodium metabisulfite and sodium pyruvate (FBP) added to the broth or agar increased the aerotolerance of Campylobacter. Karmali and Fleming (63) developed a method to isolate these organisms from stools based on the principle of Fortner. They streaked a rapidly growing Proteus species on one half of the agar plate which reduced the oxygen tension, enabling Campylobacter to grow on the other half. Butzler and Skirrow (22) and Gilchrist et al. (41) developed other selective media containing different concentrations of vancomycin, trimethoprim, polymyxin B and cephalothin for the isolation of Campylobacter. The result was that even small laboratories were able to perform routine isolations of this organism and this led to much work on the disease potential of these bacteria. Subsequently it was shown that these organisms were isolated from diarrheal stool samples at a rate comparable to the salmonellae.

1.2 Disease:

a)   **Clinical Presentation:** Campylobacter jejuni, an enteropathogenic organism, is

now recognized as the leading cause of diarrhea worldwide. The symptoms of C. jejuni infection are summarized in Table 1. At the mild end of the spectrum, the symptoms only last for 24 h and are then indistinguishable from those of viral gastroenteritis. However, in severe cases, the patients may have an elevated temperature leading to confusion and delirium. This prodromal state may last for 2 days followed by nausea and abdominal cramps. These symptoms are rapidly followed by profuse diarrhea which may be watery or may have fresh blood. Inflammatory exudates containing polymorphs have been observed in some fecal samples examined microscopically. Vomiting, though rare, may occur in some cases. The acute diarrhea lasts for approximately 3 days. Several reports have shown that profuse watery diarrhea is a common feature in patients in developing countries. In some cases this leads to sufficient dehydration and electrolyte imbalance to require hospitalization of the patient. However, the illness usually does not last longer than one week. The presence of blood, pus and mucus in the stools of patients suggests colorectal inflammation. Colgan et al. (26) reported 10 of 11 patients they studied had colitis while Lambert et al. (78) found that 70% of their patients had colon inflammation and Blaser et al. (17) reported colitis in all of their patients. Campylobacter infections can lead to several severe complications including inflammatory bowel disease (102). Some patients develop erythema nodosum (77) or reactive arthritis following Campylobacter enteritis. Urinary tract infection (28) and the Guillain-Barré syndrome (61) due to C. jejuni have also been reported. This organism may also cause meningitis (44, 103, 151).

b)   **Treatment**: Use of chemotherapy for diarrhea is somewhat controversial. For most intestinal infections, antimicrobial agents are considered ineffective and may be disadvantageous. Campylobacter enteritis is usually a mild self-limiting disease. The patient recovers in 1 or 2 days when given antibiotics while in the absence of therapy, the stools can be positive for 2-5 weeks. The drug of choice for C. jejuni infection is erythromycin (33). This agent is relatively non-toxic, has a narrow spectrum and shortens

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TABLE 1   CLINICAL PRESENTATION OF CAMPYLOBACTERIOSIS CAUSED BY C. JEJUNI

Symptoms	% incidence[a]	
	Adults	Children
watery diarrhea or loose stools	61	84
bloody stool	3.6	52
fever and malaise	47	76
severe abdominal pain	47	58
rectal pain	10	51
nausea or vomiting	2.6	32
reactive arthritis	38	2

a : data compiled from references number 14, 18, 23 and 112 and 157.

the fecal excretion of C. jejuni (113). However, strains resistant to erythromycin and tetracycline have been reported (145, 147, 150).

In cases of illness accompanied with severe abdominal pains or complications, antibiotics are necessary. Fluid and electrolyte replacement is often required for infants and children. In vitro, the most active drugs are furazolidone, doxycycline, chloramphenicol and aminoglycosides but the organism is resistant to penicillin G, cephalosporin and trimethoprim (150, 154). Aminoglycosides are recommended for septicemic patients while the use of chloramphenicol is limited due to the serious side effects of this drug. In developing countries, Campylobacter enteritis is a more serious infection compared with developed countries and factors such as low socioeconomic status and malnutrition might explain the severity of infection.

1.3 Epidemiology of C. jejuni:

a)   Reservoirs: C. jejuni occurs as a commensal in the intestinal tract of a wide variety of wild and domestic animals. It has been reported in cattle (57), sheep (134), poultry (45, 107), several species of wild birds (88), and pets including dogs (16, 99) and cats (16, 140). In the U. S., a majority of chicken carcasses sold at retail markets are contaminated with C. jejuni (45, 107) and contamination of raw poultry products can be as high as 89% and seems to be seasonal with a peak in the summer months (52, 108). This organism appears to be a normal commensal of cows but is an important cause of epizootic infectious abortions in sheep (134), although occasionally it may exist as a long term intestinal commensal without morbidity. Studies on non-human primates have shown that Campylobacter infection is common (125, 153). In short, most animals in frequent contact with humans including those used for food or those that are domestic pets are reservoirs for Campylobacter. Besides a wide variety of living hosts, this organism is often found in water and its presence may be due to fecal contamination by wild or domestic animals and

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birds. In United States, it has been isolated from streams, rivers (70) sea water (71) as well as mud and sewage sludge (106).

b) **Incidence & Prevalence**: C. jejuni is an important cause of diarrhea in developed as well as developing countries of the world. The magnitude of infection is difficult to assess as the fecal samples obtained are only a fraction of the people actually having acute diarrheal illnesses. Campylobacter infections have been reported to the Communicable Disease Surveillance Center in England since 1977. For the year 1980, the reported annual isolation rate was about 21/100,000 and for 1981, with approximately 12,500 Campylobacter isolations made, this rate rose to 28/100,000 (65). A demographic survey in 1987 of Campylobacter, Salmonella and Shigella infections in England made by Skirrow (131) showed the maximum isolation rate for the campylobacteria was 5.5% while that for the salmonellae was 3.4% and shigellae was 0.8%. In these studies it was shown that infection with this organism showed a peak incidence in children under 5 years of age and adults between the ages of 15 to 24 years (65, 131). In a study done in France, 39,000 stool specimens were examined for C. jejuni over a two year period (112) and a total of 231 strains of C. jejuni were isolated from 172 patients. Infected symptomatic patients had diarrhea (54%), abdominal pain (32%), nausea (15%) and rectal pain (18%).

In United States, the national reporting started in the early 1980's. Studies conducted in different localities showed that infection by this organism was as common as Salmonella or Shigella infections. In a five year survey conducted from 1982 to 1986 by the Center for Disease Control (142) 1,343 isolates of Campylobacter were reported of which 99% were represented by C. jejuni (Fig 1). A two year study of 2670 fecal samples from patients with gastro-intestinal illness in Denver showed an isolation rate of 4.6% for Campylobacter. The rate of stool positivity was highest during summer and lowest in winter (13). It is the most common enteric pathogen reported among travellers returning

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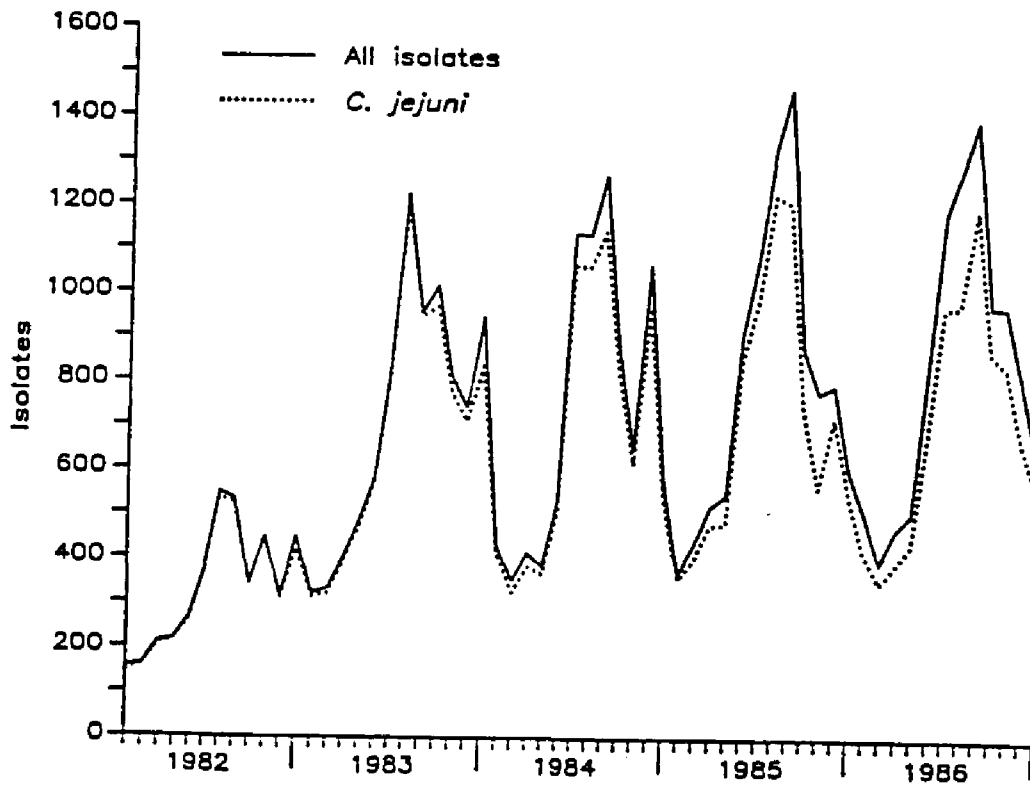
Figure 1: Average incidence of Campylobacter jejuni cases reported to the Center for Disease Control from 1982-1986 (142). Note the higher incidence during the summer months of each year.

with acute diarrheal illness from developing countries (138). In developed countries, it appears that there is a bimodal distribution of Campylobacter infection by age with the highest incidence in infants and in persons 15-24 years of age. However, in developing countries where hygiene conditions are poorer, the prevalence of infection is highest in young children (14, 18, 115). In an epidemiologic study conducted in Bangladesh in 1980 (14), C. jejuni was isolated from 14% of the patients registered in the hospital surveillance system. It was isolated more frequently than Shigella species (12%) and was the third highest detected pathogen after enterotoxigenic Escherichia coli (20%) and rotavirus (19%). However, among the people registered in the village surveillance system, 54% were found to have C. jejuni infection. It seems that infection in Bangladesh is endemic and the organism is found commonly both in patients with diarrhea and in healthy control subjects. In a survey of healthy people in South India in 1981, the infection was found to be prevalent in all age groups (115). In Mexico during the year 1988, C. jejuni infections had a peak annual incidence of 3.5 episodes/child for individuals between the ages of 12-17 months and this incidence declined to 0.6 episodes/child for 5 year olds (23).

c)   Modes of transmission: C. jejuni may be transmitted from its animal reservoir to humans in a number of ways but the most common route is through the ingestion of contaminated food or water. Raw or undercooked poultry is perhaps the major vehicle for food-borne Campylobacter enteritis (139). In different parts of the world, ingestion of poorly cooked chicken, hamburgers, turkey and even cake icing have resulted in illness (11, 13). In England and Wales, unpasteurized milk is the most frequent source of C. jejuni. Between 1978 and 1980, 13 outbreaks due to consumption of unpasteurized milk were reported (60, 114, 118, 119). Robinson (117) reported that consumption of 500 bacterial cells in milk was enough to cause illness and the organisms have been found to be viable in milk for up to 3 weeks (15). The major source of this organism is thought to be

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from fecal contamination of milk by the cow, though bovine mastitis cannot be ruled out (79).

Several large outbreaks of enteritis have been traced to drinking water. In 1978, an outbreak of Campylobacter involving 3000 people or 19% of the residents occurred in Bennington, Vt (157). Another outbreak in Sweden involved an estimated 2000 people (97) and the source of contamination was found to be surface water that had entered the municipal water system which had been insufficiently chlorinated. This organism has been isolated from both salt and fresh water sources in places where humans have acquired the infection (71). C. jejuni can remain viable for weeks in water maintained at 4°C (15).

Person to person transmission of this organism is not common. Most infections due to C. jejuni are associated with mild enteritis but bacteremia in pregnant women may lead to a severe systemic infection in the fetus (46). Neonatal meningitis has been reported in a 12 day old boy and C. jejuni was isolated from cerebrospinal fluid (151). The mother had diarrheal illness 6 weeks before delivery.

1.4 Pathogenesis of the organism:

C. jejuni, recently established as an agent of human gastroenteritis, is recognized as one of the most frequently isolated bacterial species causing infectious diarrheal disease (10, 81, 138). Although the disease has a world-wide distribution, it is particularly severe in the developing countries (14, 23, 115). Due to lack of a suitable animal model for the study of this organism, the mechanisms of pathogenesis have yet to be established. Current evidence suggests that disease follows either colonization, tissue invasion and/or toxin production by C. jejuni strains (158). Indeed, the many factors may account for the wide spectrum of symptoms following infection with this organism.

Recovery of vast numbers of bacteria from stools indicates that it efficiently colonizes and multiplies in the human gut. Organisms have been observed adjacent to the

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mucosal epithelium and the crypts of the small intestine in chicken (122) and mice (37). Most strains show poor adhesion to epithelial cell lines like HeLa and Intestinal 407 cells (92). However, using a mouse cecal model, Lee et al. (82) suggested that mucus colonization in the absence of adhesion is a prelude to intestinal infection. Systemic invasion of the blood and extraintestinal organs has been documented in humans (8, 69, 81) though the incidence is low. In experimentally infected calves (2) and chickens (22, 122), there is a transient systemic spread of the organism. An invasive nature for the organism has been shown by various workers (35, 36, 81, 101); however, strains are uniformly negative in the Sereny test (92) suggesting a lack of invasiveness.

The induction of secretory diarrhea points towards the production of toxin(s) by this organism. Ruiz-Palacios et al. (124) were among the first to report the production of an enterotoxin by C. jejuni and since then reports of an enterotoxin (68, 84, 94) and a cytotoxin (48, 111, 161) have been made. Various eukaryotic cell lines including MRC-5, Hep 2, Chinese hamster ovary (CHO) and Vero cells as well as a number of animal model systems have been used for toxigenicity studies (5, 48, 58, 161). The nature and mode of action of the toxin(s) remains controversial.

A further mechanism characteristic of Salmonella and Yersinia species, by which this organism is thought to cause disease, is termed translocation. In this, the organism penetrates the intestinal mucosa but results in minimal damage and proliferates in the lamina propria and mesenteric lymph nodes (22, 130). It has been suggested by workers (158) that translocation might be the mechanism by which this organism causes disease and the bloody dysentery and watery diarrhea may represent the two ends of the clinical presentation. The prevalence and significance of bacteremia associated with C. jejuni enteritis is controversial. Kiehlbauch et al. (66) demonstrated that C. jejuni can survive intracellularly in blood monocytes in vitro for up to 7 days, leading to the suggestion that

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phagocytosis may facilitate the survival of this organism.

It remains to be determined whether the variability in the disease presentation is due to inherent differences among different strains of C. jejuni or to the host responses. However, it is likely that variations in symptoms may result from different pathogenic mechanisms predominating for different strains, such as is seen for the various types of E. coli.

1.5 Immune response to C. jejuni infection:

The relative importance of humoral and cell-mediated immunity in resistance to campylobacteriosis has not yet been delineated; however various epidemiological studies suggest that immunity against C. jejuni is acquired as a result of infections in humans. In Bangladesh, the age-related increase in anti-campylobacter serum IgA titers noted among children and young adults appears to be the best indicator of C. jejuni immunity (9, 42). The evidence for acquisition of immunity has been substantiated in studies using human volunteers where it was shown that the individuals who were previously challenged with the organism did not become ill on subsequent exposure to C. jejuni (8). Blaser et al. (12) reported that raw milk drinkers have elevated levels of IgG antibody but normal levels of IgM and IgA antibodies to C. jejuni. On the other hand, patients with diarrhea have high antibody titers for all three types but while the IgA titer falls to normal levels rapidly, the titers of IgG and IgM antibodies remain high reaching normal levels only after about 90 days. Individuals with known IgA immunodeficiencies have been reported to have problems recovering from Campylobacter enteritis (91).

Similar experiments in animals have resulted in protection against the organism after rechallenge (20, 123). Both Burr et al. (20) and Ruiz-Palacios et al. (123) found that animals that had been challenged by the removable intestinal tie adult rabbit diarrhea (RITARD) process were protected during the second RITARD challenge. Abimiku and

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Dolby (1) demonstrated that immune mammary secretion strongly protects against intestinal colonization by this organism in natural and fostered infant mice. A high concentration of IgG antibody specific for C. jejuni was found in the serum and mammary secretion of vaccinated dams. However, the duration of this protective immunity to Campylobacter species and its association to antibody response is yet to be reported in both animals and humans.

1.6 Taxonomy and nomenclature of the genus Campylobacter:

Nowhere in the area of campylobacteria work is there more confusion than in its nomenclature. Until 1963, when Sebald and Véron (129) proposed the name Campylobacter, these microaerophilic organisms were included in the genus Vibrio. However, based on the microaerobic nature of the bacteria, their inability to ferment or oxidize carbohydrates and difference in their G+C ratio (133) as compared with the Vibrio species, they were assigned to a different and new genus.

This genus comprises 14 species, recently reviewed by Penner (110). For many years it had been convenient to divide this genus into pathogenic and non-pathogenic species based on catalase production (54, 64). However, this grouping became less relevant after the discovery of non-pathogenic, catalase positive species, C. cryaerophila and C. nitrofigilis. Catalase negative or weak strains were first isolated from dogs by Sandstedt et al. (127) and DNA hybridization studies showed that they belonged to a separate species. Although yet to be officially designated, they have been called C. upsaliensis (121, 127).

C. fetus contains two subspecies due to the high degree of relatedness by DNA hybridization and identical protein profiles; subsp. fetus and subsp. venerealis (51, 65). C. jejuni is considered a separate species rather than a subsp. of C. fetus (134). Recently hippurate positive, nitrate negative isolates of Campylobacter have been recovered from

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adult human gastric biopsy and feces of children with diarrhea and are considered a subsp. of *C. jejuni* (137). It is suggested that the species should be subdivided into *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei*. It is the only hippurate positive species of this genus (53) and can be differentiated from *C. coli* based on this test, though the two are currently grouped together. These two can be further divided into several biotypes based on the scheme proposed by Lior (85) (Table 2). *C. hyointestinalis* was isolated from lesions of proliferative ileitis in pigs (39). It closely resembles *C. fetus* except that it produces H_2S in TSI medium and can grow anaerobically in 0.1% trimethylamine N-oxide hydrochloride. *C. laridis* was isolated from sea gulls in 1980 and differs from *C. jejuni* by its resistance to nalidixic acid (132). It was originally referred to as nalidixic acid resistant, thermophilic *Campylobacter* till the species name *C. laridis* was proposed in 1983 by Benjamin *et al.* (6). *C. concisus* was isolated from human periodontal disease in 1981 (141).

C. sputorum, a non-pathogenic species, has been of little or no clinical significance and is comprised of three biovars or biotypes, biovar *sputorum*, biovar *bubulus* and biovar *fecalis*. The oxidase positive, catalase negative species *C. mucosalis* was for sometime considered a subsp. of *C. sputorum* but due to differences in DNA relatedness it was assigned to a separate species (121). The discovery of *C. pylori* may prove one of the most exciting developments in microbiology in the last decade. Although not yet conclusive, there is evidence that these urease positive bacteria are responsible for gastric ulcers. There is much controversy with regard to its nomenclature as it seems to be more closely related to the genus *Wolinella* than to *Campylobacter* (120). It was recently proposed that *C. pylori* be transferred to a new genus *Helicobacter* as the organism differs in ultrastructure, morphology, fatty acid composition and growth characteristics from the genus *Wolinella* (43).

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TABLE 2   BIOTYPING SCHEME FOR CAMPYLOBACTER JEJUNI

AND CAMPYLOBACTER COLI[a]

Tests	C. jejuni				C. coli	
	I	II	III	IV	I	II
Hippurate hydrolysis	+	+	+	+	-	-
Rapid H$_2$S test	-	-	+	+	-	-
DNA hydrolysis	-	+	-	+	-	+

[a] : Proposed by Lior in 1984 (85).

According to the Bergey's Manual of Systematic Bacteriology, the genus _Campylobacter_ comprises only 5 species. However, following the isolation of newly recognized organisms, which has led to a rapid expansion in the number of putative members of this genus, the taxonomy and nomenclature of this group is in flux. Those organisms within the group are listed in Table 3 along with proposed alternative nomenclature.

1.7 Morphological characteristics of C. jejuni:

The organisms belonging to this genus are slender, nonsporing S-shaped rods approximately 0.2 to 0.5 μm wide and 1.5 to 3.5 μm long with tapering ends (80, 134). Electron microscopic studies have revealed that different morphological forms exist within different parts of the same colony (76). At the periphery cells are spiral while in the center the cells are mostly coccus-shaped. These coccoid cells are usually found to be non-viable on subculture. The organisms are motile with their characteristic darting corkscrew-like motion by means of a single polar flagellum (134). The unsheathed flagella are approximately 21 nm in diameter and range from 2.6 to 3.9 μm in length (109). A recent study by Han et al. (50) reported the presence of sheathed flagella in C. cinnaedi and C. fenneliae which makes them closely related to C. pylori (H. pylori). Other adherence appendages like fimbriae and pili have not been observed on C. jejuni. Virtually all members of this genus are oxygen sensitive and require oxygen tension to grow.

The outer cell membrane has a wave-like morphology and is a highly asymmetric lipid bilayer that loosely fits over the cell wall. SDS-PAGE profiles reveal that the outer membrane of C. jejuni contains 7 major polypeptide bands (86) of which the 43-kilodalton protein comprises almost 70% of the membrane protein content and is thought to be a porin. The 68-kilodalton protein has been found to be temperature dependent and its molecular weight increases when the incubation temperature of the organism is altered from 37°C to 42°C (93). The LPS is of low molecular weight and lacks long repeating O-

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TABLE 3   TAXONOMY AND BIOHEMICAL CHARACTERISTICS OF THE GENUS CAMPYLOBACTER

Species	Catalase	H₂S on TSI	Nitrate	Hippurate	Nalidixic acid	Cephalothin	Growth (°C) 25	37	42	G+C content (mol %)
C. jejuni										
spp. jejuni	+	-	+	+	S	R	-	+	+	30-32
spp. doylei	+/-	-	-	+	S	S	-	+	+/-	29-30
C. coli	+	-	+	-	S	R	-	+	+	31-33
C. fetus										
spp. fetus	+	-	+	-	R	S	+	+	-	33-34
spp. venerealis	+	-	+	-	R	S	+	+	-	33-34
C. sputorum										
biovar sputorum	-	+	-	-	S	S	-	+	+	31-32
biovar bubulus	-	+	+	-	R	S	-	+	+	31-32
biovar fecalis	+	+	+	-	R	S	-	+	+	32-33
C. concisus	-	+	+	-	R	R	-	+	+	38-39
C. hyointestinalis	+	+	+	-	R	S	+	+	+	35-36
C. laridis	+	-	+	-	R	R	-	+	+	31-33
C. mucosalis	-	+	+	-	R	S	+	+	+	38-39
C. cryaerophila	+	-	+	-	V	R	+	+	-	29-30
C. nitrofigilis	+	?	+	-	S	S	+	+	-	28-29
C. upsaliensis	-	-	+	-	S	S	-	+	+	35-36
C. pylori*	+	-	V	-	R	S	-	+	+	36-37
C. cinaedi*	+	-	+	-	S	I	-	+	-	37-38
C. fennelliae*	+	-	-	-	S	S	-	+	-	37-38

+ : Positive reaction/growth; - : Negative reaction/ no growth; +/- : 10% to 90% strains are positive;
? : not known; V : Variable reaction; R : Resistant; S : Sensitive; I : intermediate zones of inhibition

* = A new genus, Helicobacter has been proposed for the three strains C. pylori, C. cinaedi and C. fennelliae .

antigen side chains (86). It contains L-glycero-D-manno-heptose, glucose, galactose, and glucosamine. The fatty acids present are mainly 3-hydroxy-tetradecanoic acid, 3-deoxy-C-manno-octulosonic acid and n-hexadecanoic acid (98). In some Gram-negative species, the outer membrane is released during growth in the form of 'blebs' or vesicles. Electron microscopic examination of _C. jejuni_ has revealed distinct outer membrane blebbing (80, 109). Protein profiles of the blebs showed them to be similar but not identical to the outer membrane; however, the major outer membrane protein is present (80).

A loose fitting cell wall encloses the asymmetrically electron-dense cell membrane. The periplasm separates the outer and cell membrane. The cytoplasm is a compact matrix consisting of evenly dispersed ribosomes and a fine skein of nuclear material. The cytoplasm is occasionally traversed by dark staining clefts and membrane-bound osmiophilic granules or laminated structures approximately 50 to 75 nm in diameter are found in the cytoplasm of some cells (80). In _C. fetus_ a 97-kilodalton glycoprotein forms a microcapsule which may afford some protection to the organism from phagocytosis (160). No such antiphagocytic surface protein is seen in _C. jejuni_. The organisms belonging to this genus are unable to accumulate PHB granules in the cytoplasm, which differentiates them from the genus _Spirillum_.

1.8 Biochemical properties of the organism:

C. jejuni is a non spore forming, microaerophilic, Gram-negative, non-acid fast organism. It is unable to utilize carbohydrates and has a strictly respiratory type metabolism (134). These organisms derive energy chiefly from amino acids and intermediates of the tricarboxylic acid (TCA) cycle. Hoffman et al. (55) found this organism to respire with formate, succinate, lactate, malate, glyoxylate, glutamate, oxaloacetate, pyruvate, acetate, fumarate and α–oxoglutarate, though oxidation of formate

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and hydrogen are thought to be the prime sources of energy and growth. Both formate dehydrogenase and hydrogenase are located in the periplasm (56). Respiratory activities determined with membrane vesicles are 50 to 100 fold higher with formate and hydrogen than with succinate, malate, or NADH as substrate. The stoichiometry of respiratory driven proton translocation (H+/O) is 2 for all substrates except hydrogen (H+/O = 3.7) and formate (H+/O = 2.5) (56). Addition of nitrate, aspartate or fumarate does not allow strains of C. jejuni to grow anaerobically as they do for the subspecies of C. fetus. Even though C. jejuni produces menaquinones in the same manner as bacteria capable of respiring nitrate or fumarate, the organism is unable to use this electron carrier to transfer electrons to nitrate or fumarate under anaerobic conditions (155). They do not possess lipase and cannot hydrolyze gelatin, urea, casein, ribonucleic acid, deoxyribonucleic acid or esculin (133). The presence of cytochromes in Campylobacter species has been demonstrated and these are chiefly of the b- and c- type, being associated with a complex terminal oxidase system (24, 55, 56). The respiratory chain of C. jejuni is partially sensitive to inhibitory effects of 2-heptyl-4-hydroxyquinone-N-oxide (HQNO), which is used to determine the entry point of electrons into the electron transport chain (55).

A microaerobic environment is required for the growth of C. jejuni and a concentration of 5% oxygen and 10% carbon dioxide is determined as optimal, while 21% oxygen is bacteriostatic (134). The microaerophilic nature of this organism is due to a high sensitivity to exogenously generated superoxide anions and hydrogen peroxide even though superoxide dismutase and catalase are present in the cell (55). Carbon monoxide has little inhibitory effect on respiration except under very low oxygen tension (56). George et al. (40) described an FBP supplement that neutralizes the toxic derivatives of oxygen if added to the basal medium. Moreover, these iron salts and dihydroxy phenyl compounds, known to bind iron, are assumed to alleviate the iron deficiency of this

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organism. All enzymes essential for activity in the presence of oxygen contain or require iron for their activity. Norepinephrine, a dihydroxy phenyl compound has been shown to stimulate iron uptake (55).

1.9 Physico-chemical properties of C.jejuni:

The growth of C. jejuni in laboratory culture media is restricted to temperatures above 32°C and below 45°C. The optimal temperature for growth is 42°C though it grows well at 37°C. In foods this organism survives better at 4°C than at room temperature (15). Christopher et al. (25) reported that C. jejuni cells do not survive heating in skim milk at 60°C for 1 min. Hence a typical pasteurization process of heating at 62°C for 30 min is sufficient to free the food products of this organism. The optimum pH for growth is 7.3 - 7.5 though it can grow in media with pH between 6.0 to 8.0 (25). The organism can grow in the presence of 1.5% sodium chloride but 2.0% is inhibitory (32).

1.10 Genetics of the organism:

One of the powerful methods for understanding the pathogenesis of a microorganism is the genetic analysis of the determinants that contribute to its virulence. The relatively recent recognition of C. jejuni as a pathogen and its microaerophilic nature have limited our knowledge in this area. Plasmids have been shown to specify virulence traits like adherence, invasiveness, antimicrobial resistance and toxin production in a variety of pathogens. Although workers have shown plasmids in C. jejuni, till now only antimicrobial resistance has been found to be plasmid encoded (3, 19). Erythromycin and ampicillin resistance in C. jejuni has been shown to be specified by chromosomal genes, while tetracycline resistance is plasmid encoded (143, 145, 147, 148). Tetracycline resistance is transmissible within the genus Campylobacter but all attempts to transconjugate it into E. coli have failed (146, 148). However, the tet gene has been cloned and introduced into several strains of E. coli (150). Using a guinea pig model,

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Taylor and Bryner (144) showed that pathogenicity in C. jejuni and C. coli is not plasmid mediated.

Enterotoxins from E. coli have been shown to be plasmid coded (49), but the *tet* resistance plasmid does not encode either cytotoxin or enterotoxin activity in C. jejuni as originally thought (149). Although toxin production may be encoded on some plasmid or the chromosome, the other virulence factor studied with respect to genetic control is the flagellum. Newell et al. (101) fed mice with mixtures of Fla+ and Fla- strains and recovered only Fla+ strains from the feces. In a similar study Black et al. (8) observed that in human volunteers fed with both flagellated and non-flagellated strains, only the flagellated strains were isolated from their stool samples. A bidirectional switch in the expression of flagella has been observed in some strains.

A group of researchers working with an independently isolated kanamycin tetracycline resistant plasmid has suggested that their kanamycin resistance determinant may be translocated in C. jejuni (73). Identification of a transposon in Campylobacter would definitely have a great impact on the genetic studies of this genus.

MATERIALS AND METHODS

Techniques used to study pathogenesis of Campylobacter jejuni

2.1 Origin and maintenance of the bacterial strains:

Seven strains of Campylobacter jejuni isolated from fecal samples of patients with acute gastroenteritis or diarrhea (Table 4) were passaged once on buffered charcoal yeast extract (BCYE) agar medium at 37°C for 48 h in an atmosphere of 10% CO_2 and 5% O_2. These were identified as C. jejuni based on morphological and biochemical tests including Gram stain, motility, catalase, oxidase, hippurate hydrolysis, oxidation of carbohydrates and growth at 25°C and 42°C (Table 3). They were subsequently maintained at -70°C in 10% serum with 1% sorbitol. These strains were biotyped by the method described by Lior (85) which separates the organism into different types based on three tests: hippurate hydrolysis, rapid H_2S production and DNA hydrolysis (Table 2).

2.2 Production and concentration of crude toxin from Campylobacter jejuni:

Thawed samples of the seven strains were used to inoculate 100 ml of thioglycolate broth (pH 7.3) and these were grown microaerobically at 37°C to a density equivalent to approximately 5 x 10^7 to 4 x 10^8 colony forming units (cfu) /ml. Cell-free filtrates were obtained by filtration through a 0.22 μm-pore-size membrane filter.

For further purification of the crude toxin, C. jejuni strain WD 2483 was used to inoculate thioglycolate broth in 1-liter batches and these were grown microaerobically as described. The final bacterial concentration ranged between 5 x 10^7 and 4 x 10^8 cfu/ml. The bacteria-free supernatants were obtained by centrifugation of broth grown organisms at 15,300 x g using a Beckman J2-21M Induction drive centrifuge (Beckman Instruments, Inc., Palo Alto, CA) for 10 min at 4°C followed by filtration through a 0.22 μm pore-size

TABLE 4   SOURCE AND HISTORY OF THE STRAINS USED IN THE STUDY

Strains	Specimen	Clinical condition	Origin
WD 2483	fecal sample	gastroenteritis	Wentworth Douglass Hospital, Dover, NH
WD 2914	fecal sample	gastroenteritis	Wentworth Douglass Hospital, Dover, NH
WD 2920	fecal sample	gastroenteritis	Wentworth Douglass Hospital, Dover, NH
WD 2939	fecal sample	gastroenteritis	Wentworth Douglass Hospital, Dover, NH
MA 1	fecal sample	diarrhea	Mt. Auburn Hospital, Boston, MA
MA 2	fecal sample	diarrhea	Mt. Auburn Hospital, Boston, MA
BS 1	fecal sample	gastroenteritis	Bay State Medical Center, Springfield, MA

membrane filter. The filtrate was concentrated 50-fold in a rotary evaporator (model R110, Brinkmann Instruments Inc., Westbury, NY). The concentrate was dialyzed against phosphate buffered saline (PBS) pH 7.3 using 6 to 8 kilodaltons molecular mass cut off Spectrapor # 1 cellulose dialysis tubing (Spectrum Medical Industries, Inc., Los Angeles, CA) and further concentrated to one-sixth its volume using polyethylene glycol (molecular weight 15 to 20 kilodaltons) (PEG) by the method of Whitby and Rodgers (159). The sample was then dialyzed in PBS overnight at 4°C and the protein concentration determined by the method of Lowry et al. (87).

2.3 Egg type, and determination of LD_{50}:

Antibiotic-free, fertile White Leghorn hens' eggs were obtained from the UNH Poultry Farm, and incubated in a humid atmosphere at 36°C. Thawed samples of strain WD 2483 were grown on the bacteriological media and the bacteria were washed from the surface of the agar and suspended in 5 ml of phosphate buffered saline (PBS), pH 7.3, to give an optical density equivalent to 10^9 cfu/ml as determined by a Klett Summerson photoelectric colorimeter. Bacterial density data were confirmed by colony counts on BCYE agar.

Organisms were introduced into eggs by four inoculation routes: allantoic cavity and chorio-allantoic membrane at 10 days of embryo incubation, amniotic and yolk sac at 6 days of embryo incubation (Fig 2 and 3). Serial 10-fold dilutions of C. jejuni strain WD 2483 were made in PBS, with organism number equivalents ranging from 10^1 to 10^7 cfu/ml. Samples of 0.1 ml of each dilution were inoculated into 10 eggs by each of the inoculation routes. Eggs were incubated and candled twice daily to record embryo viability. In order to develop a reproducible assay for pathogenesis as determined by the induction of disease, the LD_{50} for each inoculation route was determined by the method of Reed and Muench (116). Experiments for each route were done three times and the LD_{50}
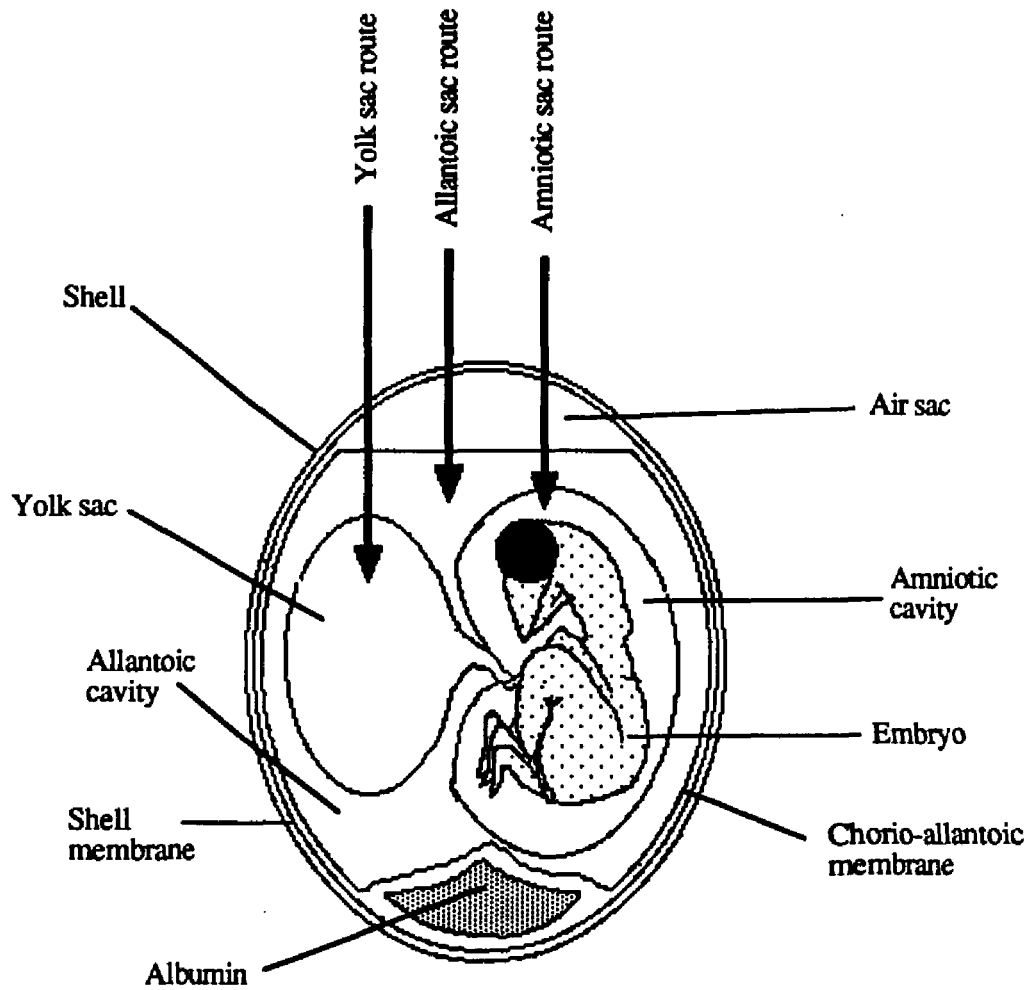
Figure 2: Schematic diagram of developing chick embryo showing the allantoic, amniotic and yolk sac routes of inoculation.

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Chorio-allantoic membrane route

False air sac

Air displaced
from sac
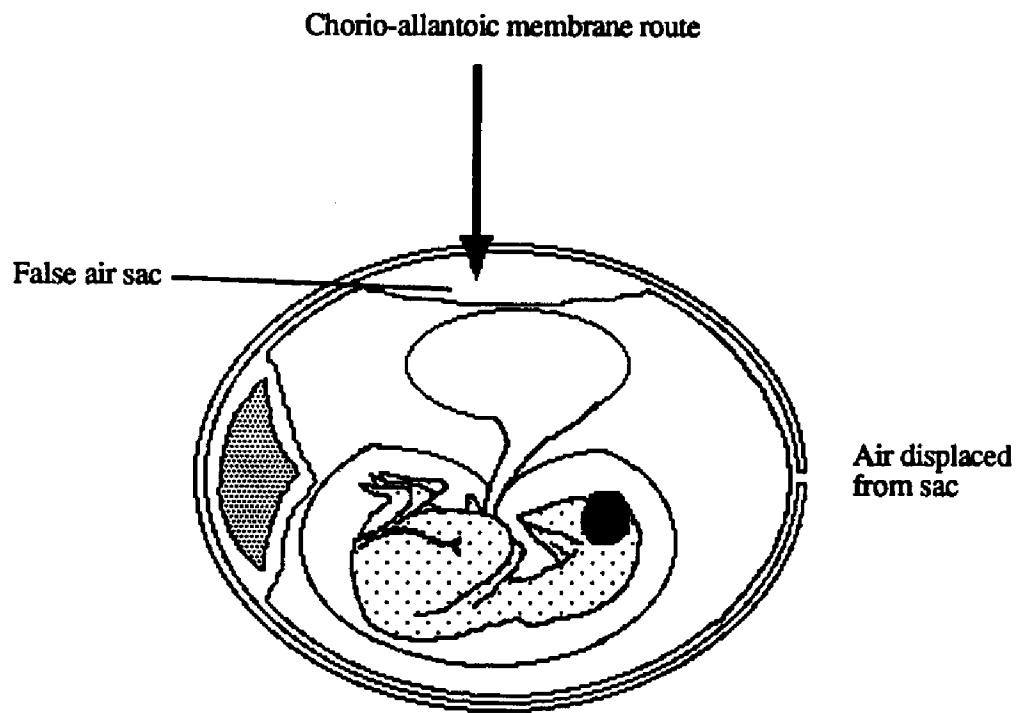
Figure 3: Schematic diagram of the chorio-allantoic membrane route of inoculation in the chick embryo. (For labels see figure 2).

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calculations averaged.

Similar 10-fold dilutions of the unconcentrated bacterium-free filtered broth, originally containing approximately 5×10^7 to 4×10^8 cfu/ml of the seven strains prior to organism removal, were made and 0.1 ml injected into the yolk sacs of fertile eggs. For each dilution 10 eggs were used and these were candled twice daily and embryo mortality was recorded.

2.4 Preparation and maintenance of cell cultures:

The cell culture media used are described in detail in Appendix 2. All media were warmed to 37°C before use and the cells were incubated in 5% CO_2 at 37°C. The three cell cultures used in this study were primary chick embryo fibroblast (PCEF) cells, human intestinal 407 (Int 407) cells and chinese hamster ovary (CHO) cells.

a) <u>Primary chick embryo fibroblast (PCEF) cells</u>: Embryos from 10 day old fertile hens' eggs were removed aseptically and placed in a sterile petri dish. The head, wings and legs were removed and the torso was rinsed in Hank's balanced salt solution (HBBS). The torso was chopped with sterile scissors into approximately 1 mm^3 cubes and the minced tissue was aseptically transferred to a trypsinizing flask containing a stirring bar and 10 ml of a 0.1% trypsin solution. Trypsinization proceeded at room temperature for 10 min and the supernatant was poured through a sterile silk filter into a 50 ml centrifugation tube containing a few drops of calf serum. The cell suspension was maintained cold by keeping the tube in crushed ice. Care was taken not to lose any of the minced tissue. A further 10 ml of trypsin was added and trypsinization allowed to proceed for another 20 min. The trypsin-tissue mixture was filtered into the centrifugation tube as described and subjected to centrifugation for 10 min at 175 x g. The supernatant fluid was decanted and the pellet resuspended in 2 ml of nutrient media (Eagle's minimum essential media (MEM) supplemented with 200 mM glutamine (10 ml/L), 7.5% sodium bicarbonate (29.4 ml/L)

and 10% fetal bovine serum). Cells were seeded in T-25 cm^2 flasks (Costar Corporation, Cambridge, MA) at a density of 10^4 cells/ml and grown to confluent monolayers. The monolayers were trypsinized using trypsin-EDTA (0.05%-0.02%) and the cells redistributed at a density of 10^4 cells/well in 6 well plates (Costar, Cambridge, MA), each well of which contained a glass coverslip. When the fibroblast cells formed an almost confluent monolayer, the growth media was replaced with maintenance media containing 1% serum and the plates were ready for inoculation.

b)   **Human Intestinal 407 (Int 407) cells**: Epithelial Int 407 cells, derived from the jejunum and ileum of a two month old Caucasian embryo, were obtained from the ATCC (Rockville, MD) and were maintained in Eagle's MEM supplemented with 7.5% sodium bicarbonate (29.4 ml/L), 200 mM glutamine (10 ml/L) and 10% new born calf serum. The cells were grown in T-75 cm^2 flasks at 37°C in a CO_2 incubator to give confluent monolayers. These were trypsinized using 2 ml of trypsin-EDTA (0.05%-0.02%) and the cells were redistributed in 96 well plates (cell density 10^2 cells/well) and 6 well plates containing glass coverslips (cell density 10^4 cells/well) and grown to give confluent monolayers for subsequent use in binding, IFA, and cytotoxic studies. Prior to all inoculations, the growth media was replaced with maintenance media.

c)   **Chinese hamster ovary (CHO) cells**: CHO cells, an epithelial cell line derived from the ovary of an adult chinese hamster, were obtained from ATCC and grown in T-75 cm^2 flasks in Eagle's MEM supplemented with 200 mM glutamine (10 ml/L), 7.5% sodium bicarbonate (29.4 ml/L) and 10% new born calf serum at 37°C in a CO_2 incubator. Confluent monolayers were resuspended using 2 ml of trypsin-EDTA and distributed in 6 well plates containing glass coverslips (cell density 10^4 cells/well). When cells were in confluent monolayers the growth media was replaced with maintenance media containing 1% serum and they were used to establish the nature of the C. jejuni toxin.

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2.5 Polyacrylamide gel electrophoresis (PAGE) and immunoblotting:

Gel electrophoresis was used for the isolation and purification of the toxin as well as for the analysis of protein profiles of the cellular fractions of strain WD 2483 and the Int 407 cells. The reagent formulations used are given in Appendix 5. Preparative, non-denaturing, discontinuous, PAGE was performed using a modification of the procedure by Davis (27). A 10% separating gel and a stacking gel containing 5% acrylamide in 0.125 M tris (pH 6.8) was used. Electrophoresis was performed at 30 mA. For identification, the protein bands were stained with 0.1% Coomassie Blue R 250 (Sigma Chemical Co., St. Louis, MO.).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions by the procedure previously described by Laemmli (75). The separating gel contained 10% acrylamide solution and 0.2% SDS on 0.35 M Tris hydrochloride buffer (pH 8.8) while the stacking gel contained 5% acrylamide solution and 0.1% SDS in 0.125 M Tris hydrochloride buffer (pH 6.8). The running buffer was 0.025 M Tris hydrochloride-0.192 M glycine with 0.1% SDS. Electrophoresis was performed at 30 mA. The proteins were silver stained by the method of Oakley (105). Each gel contained molecular weight standards (BioRad, Richmond, Ca).

Electrophoretic transfer of the proteins from polyacrylamide gels on to nitrocellulose paper was accomplished by Western blot technique (152). Typical transfer conditions were 100 mA constant current for 18-24 h in a Trans-Blot transfer cell (BioRad). Nitrocellulose membranes were then equilibrated in Tris buffered saline pH 7.5 (TBS) for 30 min. Unoccupied sites on the nitrocellulose were blocked by incubating the blots in 3% gelatin-TBS for 30-40 min. Immunoblots were washed twice in TBS and then incubated for 1 h at room temperature in the primary antisera (mouse monoclonal or rabbit polyclonal antibodies, see section 2.11). After washing in TBS, the blots were incubated in either goat anti-mouse or goat anti-rabbit-horseradish peroxidase conjugate (Organon

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Teknika Corp., Malvern, PA) as appropriate, each diluted 1:2000 in 1% gelatin-TBS.

All immunoblots were developed in 4-chloro-1-naphthol (BioRad). Substrate buffer was made by dissolving 60 mg of 4-chloro-1-naphthol in 20 ml of ice cold methanol which was then added to TBS containing 0.015% H_2O_2. The reaction was stopped by rinsing the blots in distilled water followed by TBS.

2.6 Enzyme-linked immunosorbent assay (ELISA):

ELISA was used for the various binding studies and was performed in 96 well plates. The preparation and formulation of the various reagents used in ELISA are described in Appendix 4. The wells were coated with the GM_1 ganglioside, cell monolayers, or cellular fractions depending on the study involved. They were washed with PBS and post coated with 0.3 ml of 3% bovine serum albumin. These were washed three times with 0.5% Tween 20 in PBS (T-PBS) and 100 µl of primary antibody (monoclonal or polyclonal) was added to each well. The plates were incubated at 37°C for 2 h and after three washes with T-PBS, 100 µl of goat anti-mouse or goat anti-rabbit-horseradish peroxidase conjugate was added for 1 h. After three washings with T-PBS, 3, 3', 5, 5'-tetramethyl benzidine substrate was added to each well and the reaction stopped with 2 M sulphuric acid. The color intensity was read using an EIA reader (Whittaker MA bioproducts, Walkersville, MD) at 405 nm.

2.7 Indirect immunofluorescence assay (IFA):

After histological processing of infected chick embryo organs (see section 2.9), deparaffinized tissue sections and tissue homogenates as well as eukaryotic cell monolayers that had been treated with bacteria or toxin for binding assays were fixed in 10% buffered formalin for 2 h and washed in PBS. They were subsequently treated with a 1:10 dilution of polyclonal antibody raised to C. jejuni strain WD 2483 or the monoclonal antibody to the ETP for 1 h at 37°C. Unbound globulin was removed with three washings in PBS. Cells or tissues were then treated with a 1:200 dilution of goat anti-rabbit or goat anti-

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mouse fluorescein isothiocynate (FITC) conjugated antibody (Organon Teknika Corp.) for 1 h. Unbound globulin was removed by washing in PBS and the tissue sections, homogenates or the cell monolayers on coverslips were mounted in 1% 1, 4 - diazobicyclo (2.2.2) octane (DABCO) (Sigma) and viewed with an Olympus BH-2 epifluorescence microscope.

2.8 Electron microscopy:

The reagents used for electron microscopy, both transmission and scanning, are described in detail in Appendix 6.

a) **Preparation of samples:** After treatment with $\underline{C}$. $\underline{jejuni}$ strain WD 2483 or its isolated toxin Int 407 cells were washed twice in HBSS and fixed in 5% (v/v) glutaraldehyde in 0.05 M sodium cacodylate containing 10 mM $MgSO_4$ for 1 h. Fixed monolayers were rinsed ten times in cacodylate buffer, scraped from the plastic surface with a rubber policeman, collected by centrifugation and pre-embedded in 2% (w/v) Noble agar. Cubes of $1mm^3$ thickness were cut and post fixed in 1% osmium tetroxide (OsO_4) in cacodylate buffer for 1 h.

b) **Resin embedding:** Post fixed samples were washed in cacodylate buffer followed by a ethanol dehydration series for 5 min in each of 50%, 70%, 90%, and 95% ethanol in cacodylate buffer and twice for 10 min in absolute alcohol. Care was taken to prevent the samples from drying during these steps. After alcohol dehydration, samples were rinsed in propylene oxide. The resin used for embedding was an Epon-Araldite mix. To allow complete resin infiltration, samples were placed in a 2:1 mixture of propylene oxide and resin for 1 h and then in 1:2 mixture of propylene oxide and resin for another hour. This was replaced with complete resin for 1 h and then by degassed resin for another hour. Specimens were transferred to embedding blocks using a wooden swab stick sharpened at one end and the blocks filled with degassed resin. Great care was taken to

prevent the introduction of air bubbles in the resin. Blocks were polymerized in an oven at 60°C for 24 h and left at room temperature for a further 24 h to allow complete hardening to occur.

c)   **Thin sectioning**: Blocks were trimmed using an LKB Ultratome III ultramicrotome with glass knives made on an LKB knife maker. Thin sections were cut with a diamond knife using the thermal advance on the ultramicrotome. Ribbons of grey and silver sections (approx. 30-90 nm thickness) were picked up on 400 mesh copper grids and the grids with sections on them were blotted dry using a piece of filter paper. All materials in contact with the sections including the knife, water, and grids were stringently cleaned so as to completely exclude dust and grease.

d)   **Staining**: Sections were stained with 5% uranyl acetate for 1 min and washed thoroughly with boiled double distilled water using 20 ml per grid. The grids were then stained with 0.4% lead citrate for 20 sec and washed again with 40 ml of boiled double distilled water. Sections were blotted with filter paper and allowed to dry. Stains were made fresh for each use and filtered through a 0.2 μm filter before use. Sections were examined by transmission electron microscopy (TEM) in a Hitachi H 600 electron microscope at 75 kV and photographed.

e)   **Scanning Electron Microscopy (SEM)**: For SEM, confluent cell monolayers grown in 6 well plates with glass coverslips were similarly inoculated with C. jejuni strain WD 2483 or its toxin and the samples were treated in the same way as described above up to and including the dehydration in the graded ethanol series. The coverslips with the monolayers were further rinsed in absolute alcohol and critical point dried using liquid CO_2 in a Samdri-790 (Tousimis Research Corp., Rockville, MD). Prior to examination, samples were coated with 12 nm of gold palladium with a target to specimen distance of 5 cm at a current of 15 mA for 4 min in a Hummer V Sputter Coater.

The Organism

2.9 Histopathology of C. jejuni in chick embryo:

Inoculum doses of strain WD 2483 equivalent to 10, 100 and 1000 times the yolk sac LD_{50} were made in PBS. Each multiple dose LD_{50} was inoculated into the yolk sacs of fertile eggs (30 eggs per multiple dose) and the embryos were harvested daily up to 8 days post-inoculation. Organs of the control as well as the experimentally treated eggs including liver, heart, kidney, spleen and the gastro-intestinal (GI) tract were removed and fixed in buffered 10% formalin. Fixed organs were embedded in paraffin wax in a Lab Tek tissue processor (Miles Laboratories Inc., Naperville, Ill.) and trimmed using a razor blade. Four micron thick sections were cut with an American Optical rotary microtome, deparaffinized and stained with hematoxylin and eosin as described in Appendix 1. Organs from similarly treated eggs were removed aseptically for bacterial colony counts. They were rinsed in PBS, weighed, homogenized in a Sorval Omni Mixer, serially diluted and 0.1 ml portions were plated on BCYE agar. Deparaffinized tissue sections and formalin fixed tissue homogenates were incubated with rabbit anti-C. jejuni serum and FITC-conjugated goat anti-rabbit serum for immunofluorescence studies as described earlier.

Pathological changes induced by the cell-free filtered C. jejuni strain WD 2483 were assayed by inoculation of 0.1 ml of a 1:100 dilution of the filtrate originally containing 5 x 10^7 cfu/ml into the yolk sac of the eggs. As described previously, organs of the inoculated eggs were harvested and the different tissue sections were analyzed for any histological changes. Samples of filtrates from all seven C. jejuni strains were subjected to a 15-min exposure to 60°C or 100°C, changes in pH using 1 N HCl or 1 N NaOH, or treated with 0.5 mg/ml each of trypsin XI, protease XIV and lipase I for 30 min. The residual toxic activity was assayed after these treatments by inoculation into the yolk sac of fertile hens' eggs.

2.10 Isolation and biochemical characterization of the toxin from C. jejuni:

Approximately 2 ml of the concentrated, dialyzed bacterium-free filtrate (described in section 2.2) together with an equal volume of sample buffer (10% glycerol in 0.08 M tris, pH 6.8) was loaded on a 1.5 mm thick preparative, non-denaturing, discontinuous polyacrylamide gel. A 10% separating gel and a 5% stacking gel were used as described in section 2.5. For identification, the protein bands were stained with 0.1% Coomassie Blue R 250 (Sigma) and similar unstained bands were cut from the gels and individually eluted in an Elutrap apparatus (Schleicher & Schuell). Each eluant was filter sterilized and tested for toxicity by inoculation into the yolk sacs of 6 day old fertile Leghorn hens' eggs. The eluted toxic protein (ETP) was subjected to SDS-PAGE and the gel was silver stained (105) to reveal the proteins. Each gel contained molecular weight standards (BioRad).

As for the bacterium-free filtrate, the ETP was also subjected to 15 min treatments of 60°C or 100°C, to changes in pH, to freeze-thawing, or to treatment with 0.5 mg/ml each of trypsin, protease and lipase for 30 min. The toxic activity of the ETP was assayed after these treatments by inoculation into the yolk sac of fertile hens' eggs.

2.11 Preparation of antibodies:

Specific antibodies are needed for the various assays performed in this study like ELISA, IFA, immuno-electron microscopy, Western blots and the neutralization assays. For this reason polyclonal antibody against C. jejuni strain WD 2483 and monoclonal antibody against its toxin were raised as described.

a)   Polyclonal antibody: C. jejuni polyclonal antibody to strain WD 2483 was prepared in white New Zealand rabbits. The organisms (10^8 cfu/ml) were fixed in 1% buffered formalin containing some sterile egg yolk. These formalin-fixed organisms were mixed with an equal volume of Freund's incomplete adjuvant and injected into the flanks of rabbits by 30 sub-dermal injections using a 21-gauge needle. Further immunizations were given after two and four weeks. Two weeks after the final inoculations, the rabbits were

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anesthetized with 50 mg of Pentobarbital Sodium (Nembutal) injection in the peripheral vein of the ear and exsanguinated by cardiac puncture . Serum was examined for antibody specificity by both neutralization of chick embryo lethality assays and immunofluorescence of whole organisms using goat anti-rabbit FITC conjugate (Organon Teknika Corp.).

b)   Monoclonal antibody (MAb): MAb was raised against the ETP from *C. jejuni* strain WD 2483 using a modification of the procedure by Galfré and Milstein (38). The media formulation used in the preparation is given in Appendix 3. Two Balb/c mice were primed by intraperitoneal injection with 1 to 5 µg of ETP in increasing doses in Freund's incomplete adjuvant (Sigma) over eight weeks. The spleens of the primed mice were removed aseptically, washed in serum free media and cut into small fragments. The spleen suspension was then aspirated and expelled using a 20 ml syringe with a 18-gauge needle to break up clumped cells. NS-1 mouse myeloma cells were used for fusion with spleen cells and were grown as confluent monolayers in T-75 cm^2 flasks. A total of 6 x 10^7 NS-1 cells were fused with 10^8 spleen cells using warm polyethylene glycol. The fused cells were suspended in RPMI-1640 cell culture media containing 10% hypoxanthine, 1% aminopterin and 10% thymidine (HAT medium) and 0.1 ml was distributed into 96 well plates at a concentration of 5 x 10^4 spleen cells/well. The plates were incubated at 37°C in 5% CO$_2$. The medium in the plates was changed after 4 days and care was taken not to allow the medium to evaporate. Positive wells were identified using a microtiter mirror. They appeared as white "spotches" in the bottom of a well.

Clones producing ETP specific MAb were selected by incubating 100 µl of the medium from each well with ETP at a protein concentration of 10 ng. After incubation at 37°C for 30 min the mixtures were inoculated into fertile hens' eggs for lethal assay. The ability of the MAb to neutralize the toxic potential of the toxic filtrates from the other 6 strains was similarly assayed by egg inoculation. The specificity of the MAb for the ETP

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was further confirmed by transfer of the ETP to nitrocellulose by Western blot techniques, as described in section 2.5.

2.12 Cytopathic affect of C. jejuni on eukaryotic cells:

Thawed samples of C. jejuni strain WD 2483 were harvested from BCYE agar plates. They were washed in HBSS and suspended in maintenance media that contained 1% serum at a concentration of approximately 5×10^8 cfu/ml. The confluent monolayers of PCEF cells in individual wells of 6 well plates ($2\text{-}3 \times 10^6$ cells/well) and were inoculated with the bacterial suspension at a multiplicity of infection (MOI) of 100 bacteria to 1 eukaryotic cell for up to 36 h. These experiments were repeated using Int 407 cells at a similar cell density and bacterial inoculum. The monolayers were examined twice daily for any cytopathic and cytolytic affects.

2.13 Ultrastructural changes in Int 407 cells induced by C. jejuni:

Int 407 cells inoculated with C. jejuni strain WD 2483 at a MOI of 100:1 were harvested at 2, 6, 12 and 24 h post infection and prepared for electron microscopic examination. Infected and control cell monolayers were examined using electron microscopy as described in section 2.8. Briefly, samples were rinsed in 0.05 M cacodylate buffer, fixed in 5% glutaraldehyde, post fixed in 1% OsO_4, dehydrated in a graded ethanol series, embedded in epon-araldite, thin sectioned and the sections examined in a Hitachi H 600 electron microscope.

For SEM, the cell monolayers were similarly treated as far as dehydration in ethanol. Samples were then critical point dried, sputter coated and viewed in a Hitachi Temscan electron microscope.

2.14 Adherence of C. jejuni to eukaryotic host cells:

For a consistent population of organisms in all experiments C. jejuni strain WD 2483 were harvested from BCYE agar plates and inoculated into thioglycolate broth to give

approximately 10^5 organisms/ml and incubated microaerobically at 37°C until the culture concentration had reached $4-5 \times 10^8$ cfu/ml as determined by viable counts. Bacteria were harvested by centrifugation at 3000 x g, washed in HBSS and resuspended in HBSS prior to adherence assays. Six well plates with a confluent monolayer of either PCEF or Int 407 cells ($2-3 \times 10^6$ cells/well) were inoculated with the bacterial suspension at a multiplicity of infection (MOI) of 100 bacteria to 1 eukaryotic cell and incubated for up to 36 h. The number of organisms bound to each cell type was assayed by viable counts and immunofluorescence. At each time interval (1, 2, 4, 6, 12, 24, 36 h) the monolayers were washed with HBSS and the washing plated on the BCYE agar to assay the number of unbound bacteria. Cells of the washed cell monolayers were lysed by adding 1.0 ml of sterile Milli-Q water and 0.1 ml of this lysate was serially diluted and plated on the agar plates. This gave the number of adhered as well as internalized organisms. To assay for internalized bacteria only, at each time interval the infected monolayers were treated with 250 µg/ml gentamicin for three hours to kill the extracellular organisms. Monolayers were washed thoroughly to remove gentamicin and cells were lysed by adding 1.0 ml of sterile Milli-Q water. To assay solely for adherence, 1 µg/ml of cytochlasin B was added along with the bacterial inoculum, to prevent phagocytosis and the washed monolayers were lysed, serially diluted and plated as above.

Nonspecific binding of organisms to plastic culture plates were determined for each experimental trial. For every well viable count estimations of unbound organisms contained in washes made following the various incubation periods for adherence assays were determined. This value was added to the corresponding lysate value for the appropriate well and subtracted from the inoculum to give bacteria bound to the plastic.

Int 407 cells inoculated with C. jejuni strain WD 2483 at an MOI of 100:1 were harvested at 2, 6, 12 and 24 h post infection and also examined for adhering or internalized

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organisms by electron microscopy. Infected and control cell monolayers were rinsed in 0.1 M cacodylate buffer, fixed in 5% glutaraldehyde, post fixed in 1% OsO$_4$, dehydrated in a graded ethanol series and prepared for SEM and TEM as described in section 2.8

The Toxin

2.15 Localization of the toxin in the organism:

The C. jejuni toxin isolated in this study is secreted by the organism and the treatment of the bacterial cells with polymyxin B does not increase the toxin concentration in the broth suggesting that the toxin is not localized in the periplasm. The localization of this toxin in the organism prior to its secretion was studied by the various techniques described below.

a)   **Cellular fractionation of strain WD 2483**: The method described by Schnaitman (128) was used to separate outer membrane proteins, cytoplasmic membrane proteins, and soluble (cytoplasm and periplasm) proteins as outlined in Fig 4. C. jejuni cells were suspended in HEPES buffer (pH 7.0) with 1 mM phenyl-methyl-sulfonyl-fluoride (PMSF) and broken mechanically using glass beads in a Sorval Omni Mixer. The intact cells were removed by sedimentation at 5000 x g for 5 min. Broken cells were centrifuged at 200, 000 x g for 60 min in a Beckman L8-70 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA). Sediment 1 was removed and the supernatant incubated at 21°C for 30 min to allow tiny membrane lipid fragments to aggregate. Failure to include this incubation step would prevent these small fragments from sedimenting in ultracentrifuge. After incubation, supernatant 1 was centrifuged at 200, 000 g for 60 min. Supernatant 2 was collected which contained the cytoplasmic and periplasmic proteins. The sediment 2 was combined with sediment 1 and suspended in HEPES buffer at 10 mg of protein per ml. Triton X-100 was then added at a final concentration of 20 mg/ml protein. This was centrifuged at 30, 000 x g for 60 min and the sediment and supernatant

LYSED C. JEJUNI CELLS

centrifuged at 200, 000 x g
for 60 min

SUPERNATANT 1   SEDIMENT 1

incubated at 21° C
for 30 min and centrifuged
at 200, 000 x g for 60 min

SUPERNATANT 2   SEDIMENT 2 + SEDIMENT 1
(cytoplasm and periplasm)

centrifuged at 200, 000 x g
for 60 min

SUPERNATANT 3   SEDIMENT 3
(cytoplasmic membrane)   (outer membrane)
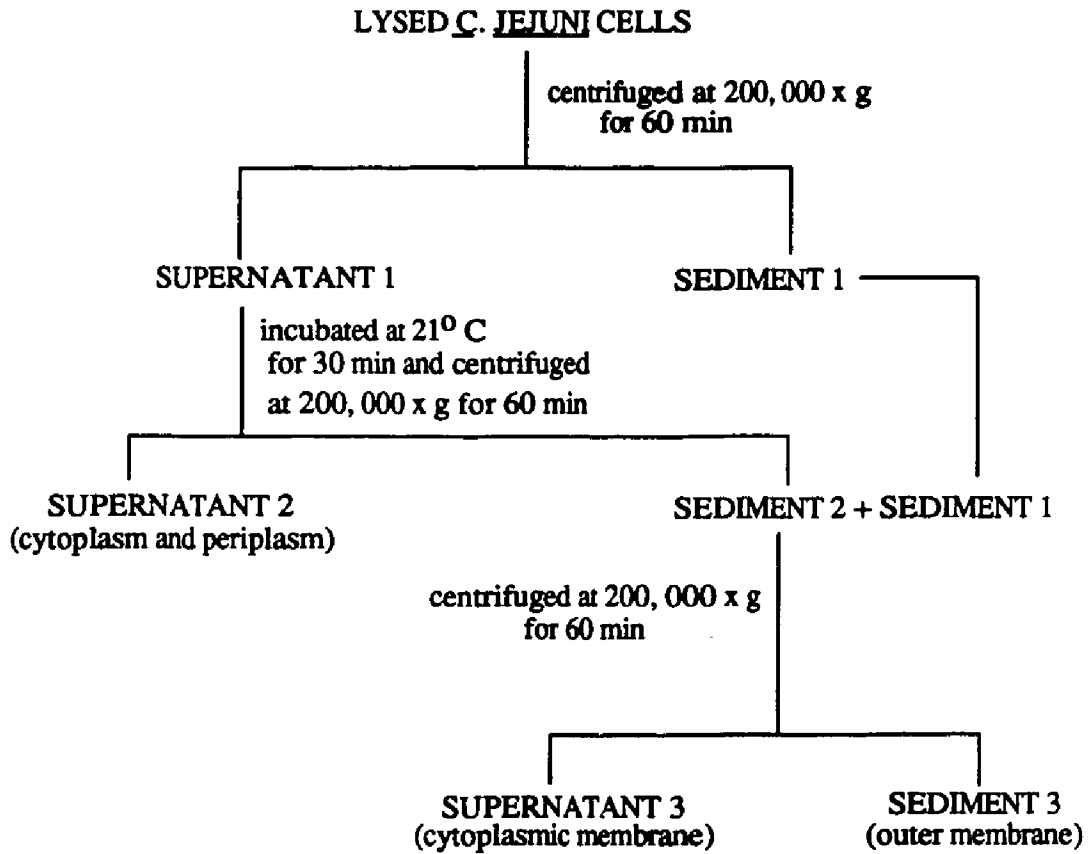
Figure 4: Scheme followed to separate the different cellular components of lysed
Campylobacter jejuni cells. The cells were lysed using glass beads and the intact cells were
removed by centrifuging at 5000 x g for 5 min.

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were both collected. The supernatant 3 contained the cytoplasmic membrane fractions while the sediment 3 contained the outer membrane fraction of the organism.

The relative activity of succinic dehydrogenase (SDH), an integral enzyme of the cytoplasmic membrane (30), and the concentration of 2-keto-3-deoxyoctonate (KDO), a constituent of outer membrane lipopolysaccharide, were used as indices of the purity of cell fractions (62).

b) **SDS-PAGE analysis and Immunoblotting:** The protein concentrations of the cell fractions obtained from the above described techniques were determined by the method of Lowry (87) and 8 µg of protein of each of the cellular components was loaded in separate wells of the polyacrylamide gel and separated by SDS-PAGE. All cell fractions were loaded in duplicate. After electrophoresis, the gel was cut in half. One-half was silver stained to reveal the proteins while the other half was transferred onto a nitrocellulose membrane following the Western blot technique (152). Transferred nitrocellulose blots were incubated in the MAb (CETPMAb4) to the C. jejuni ETP, washed in TBS, reincubated in goat anti-mouse-horseradish peroxidase and the immunoblots were developed in 4-chloro-1-naphthol as described in section 2.3.

c) **ELISA:** ELISA experiments were performed with 96 well plates coated with the separated cell fractions of strain WD 2483. Equal concentrations of protein (50 µg/well) were added to the 96 well plates and left overnight at room temperature. Excess protein was removed by washing with T-PBS, after which the plates were post-coated with 0.3 ml of 3% bovine serum albumin for 30 min. All plates were washed with T-PBS and 100 µl of CETPMAb4 was added to each well for 2 h followed by incubation in 100 µl of goat anti-mouse-horseradish peroxidase conjugate (section 2.4). Similarly coated plates were also incubated with polyclonal antiserum to the whole organism and subsequently incubated in goat anti-rabbit-horseradish peroxidase conjugate to serve as positive control. The color intensity was read using an EIA reader at 405 nm.

d)   **Immuno electron microscopy**: Thawed samples of strain WD 2483 were grown on BCYE agar plates microaerobically at 37°C and the organisms were harvested in 5 ml of PBS. They were washed twice and centrifuged at 3000 x g for 5 min. The resultant pellet was lightly fixed in 0.5% glutaraldehyde for 10 min, suspended in 10% DMSO for 30 min and immediately freeze-thawed at -70°C. The thawed sample was thoroughly washed in PBS to remove all traces of DMSO and was then incubated in a 1:10 dilution of CETPMAb$_4$ for 2 h at 37C° with continuous shaking. After two washings in PBS to remove unbound globulin, the samples were incubated in a 1:10 dilution of gold-conjugated goat anti-mouse serum (Organon Teknika Corp.) at room temperature. Unbound globulin was removed by washing and the samples were fixed in 5% glutaraldehyde and embedded for electron microscopy (section 2.6).

2.16 Nature of the toxin:

The nature of the toxin isolated from strain WD 2483 was determined by : a) the CHO cell assay; b) GM$_1$ ganglioside binding assay and c) the cytopathic effects induced in the PCEF and Int 407 cells.

a)   **CHO cell assay**: As described by Guerrant et al. (47), the CHO cell assay was performed with the ETP to establish the nature of the toxin. Cell monolayers were grown on 6 well plates with glass coverslips (section 2.2). The percentage of elongated cells were calculated after incubating the cells with the ETP (0.01, 0.1 and 30 μg/ml) for 24 h at 37°C. Assays were repeated using the bacterial cell-free filtrates of the remaining 6 strains of C. jejuni at a protein concentration of 30 μg/ml. Heat labile enterotoxins were obtained in similar fashion from E. coli strain H10407 (Dr. Stanley Falkow, Stanford Univ., CA) and Vibrio cholerae strain 2868 (Dr. Rita Colwell, Univ. of Maryland, MD) grown overnight in tryptic soy broth to a density of approximately 5 x 10^7 cfu/ml. The broth was filtered through a 0.2 μm filter and the cell-free filtrate was added to CHO cell monolayers

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at protein concentrations of 0.01, 0.1 and 30 µg/ml as positive controls

b)   **Binding to GM₁ ganglioside:** It is a characteristic feature that E. coli and V. cholerae enterotoxins bind to the monosialoganglioside (GM₁) and the use of GM₁ as a specific sorbent for enterotoxins has been an alternative approach for their identification (4, 126). Therefore, similar ELISA experiments were performed in which wells of the microtiter plates were each coated with 100 µl of a 1.5 µM solution of GM₁ (Sigma) and the binding of ETP and the 6 cell-free filtrates to the ganglioside were assayed. V. cholerae enterotoxin was used as a positive control in these binding studies. The antitoxin to V. cholerae was kindly provided by Dr. James Kaper (Center for Vaccine Development, Univ. of Maryland, Baltimore, MD).

c)   **Cell culture assays:** Int 407 cells and PCEF cells were used to determine the cytotoxic activity of the ETP. Cells were grown in 6 well plates as confluent monolayers, incubated with ETP (100 ng/ml protein) or the remaining six bacterial cell-free filtrates (30 µg/ml) and observed for 36 h for any cytotoxic or cytolytic effects. For neutralization of the toxic activity, the ETP and the six filtrates were incubated at 37°C for 30 min with a 1:10 dilution of CETPMAb₄. These were added to the Int 407 or PCEF cells in 6 well plates and examined for cell changes.

2.17 Toxin binding studies:

Most toxins are known to adhere with specific receptors on the host cell surface prior to the initiation of their toxic response. Hence, the binding of the ETP from C. jejuni strain WD 2483 was assessed using two cell cultures, PCEF and Int 407 cells.

a)   **Maximal binding assay:** ELISA was used to determine the binding of ETP to the Int 407 and PCEF cells. To determine the time for maximum binding, the cell monolayers were grown in 96 well plates (10^5 cells/well) and incubated with ETP (10 ng protein) for various time intervals up to 36 h. The cell monolayers were washed three

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times with T-PBS and for each time interval 0.1 ml of CETPMAb4 and subsequently 0.1 ml of goat-anti mouse-horseradish peroxidase conjugate was added. The color intensity was read using an EIA reader at 405 nm. To determine the amount of ETP required to saturate the receptors on the host eukaryotic cells, similar monolayers in 96 well plates were incubated with different concentrations of the ETP for 2 h and the binding measured by ELISA as described above.

b) **Characterization of binding by blocking experiments:** The role of eukaryotic surface proteins, glycoproteins, lipid and sugar moieties in the toxin binding process was examined. The type, treatment, concentration and mode of action of the agents used to modify the surface of the eukaryotic host cells are shown in Table 5. With the exception of glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) all agents were obtained from Sigma. Cells were treated with the agents for the time indicated, washed three times with HBSS and the binding of ETP assayed by ELISA after the various treatments.

Competitive blocking studies were conducted in the presence of mono- and disaccharide sugars commonly found on mammalian mucosal surfaces, lectins and GM_1 ganglioside. The type, treatment time and concentration of these agents are shown in Table 6. The agents were prepared at the specified concentration in HBSS and added to the cells 10 min prior to the addition of the ETP. After the binding period, the wells were washed three times with HBSS and adherence was assayed by ELISA. HBSS was used in place of the agents as control.

2.18 **Interaction of the toxin with host cells:**

a) **Electron microscopy of toxin treated Int 407 cells:** Cell monolayers of Int 407 cells were grown in 6 well plates and treated with the ETP for 2, 6, and 12 h. After the end of each time period, the monolayers were lightly fixed with 0.05% glutaraldehyde

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TABLE 5 ENZYMES AND OTHER AGENTS USED IN THE EUKARYOTIC CELL TREATMENT PRIOR TO TOXIN BINDING

Agents	Concentration	Time (min)	Function
Sodium metaperiodate	5 mM	10	oxidation of CHO moieties
Nonidet P40	0.005%	60	action on membrane lipids
Glutaraldehyde	0.1%	10	immobilization of protein moieties on membrane
Trypsin (type XI)	50 units/ml	30	
Protease (type XIV)	0.005 units/ml	30	degradation of proteolytic,
Chymotrypsin (type II)	0.1 units/ml	30	lipid, galactose or
Pepsin	100 units/ml	30	neuraminic acid-like
Neuraminidase (type VI)	1.0 units/ml	30	receptors on
Lipase (type I)	100 units/ml	30	eukaryotic cells
ß-galactosidase	100 units/ml	30	

TABLE 6 AGENTS USED IN THE COMPETITIVE BINDING OF THE TOXIN TO EUKARYOTIC CELLS

Agents	Concentration	Time (hour)
Wheat germ agglutinin	100 μg/ml	2
Concanavalin A	100 μg/ml	2
GM_1 ganglioside	100 μg/ml	2
N-acetyl glucosamine	100 mM	2
N-acetyl galactosamine	100 mM	2
N-acetyl neuraminic acid	100 mM	2
α-D (+) - Fucose	100 mM	2
D (+) - Mannose	100 mM	2
ß - D (+) Glucose	100 mM	2
D (+) Galactose	100 mM	2
D (-) - Arabinose	100 mM	2
ß - D (-) Fructose	100 mM	2
Maltose[a]	100 mM	2
Sucrose[b]	100 mM	2

[a] : 4 - O - α - D - Glucopyranosyl - D- Glucose
[b] : α - D - Glucopyranosyl ß - D - Fructofuranoside

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for 10 min. Similarly 12 h treated monolayers were incubated in 10% DMSO for 30 min and then freeze-thawed at -70°C. Thawed monolayers were washed in cacodylate buffer to remove DMSO, scraped from the plastic surface by means of a rubber policeman and transferred to separate Eppendorf tubes. They were gently centrifuged at 200 x g for 5 min and the pellets were incubated in 1:10 dilution of CETPMAb₄ for 2 h at 37°C with continuous shaking. After two washings in cacodylate buffer to remove unbound globulin, the samples were incubated in 1: 10 dilution of gold-conjugated goat anti-mouse serum (Organon Teknika Corp.) at room temperature. Unbound globulins were washed and the samples fixed in 5% glutaraldehyde and embedded for electron microscopy (section 2.8).

Similar cell monolayers of Int 407 cells grown in 6 well plates with cover slips were treated with the ETP for 2, 6, and 12 h and prepared for SEM as described in section 2.8 to look for toxin induced surface changes on the eukaryotic host cells.

b) **Fractionation of Int 407 cells:** The method described by Krueger et al. (74) was used to separate the lysed Int 407 cells into different fractions. Cell monolayers of Int 407 cells were treated with ETP for 12 h and the cells were scraped off the flasks with a rubber policeman. They were suspended in PBS and pelleted by low speed centrifugation. The cell pellets were resuspended in 10 ml of buffer containing 10 mM KCl, 20 mM tris (pH 7.0), 0.1% 2-mercaptoethanol, 1 mM EDTA and 250 μg/ml PMSF (Sigma) and allowed to swell on ice for 10-20 min. Swollen cells were homogenized by 50 strokes with a tight-fitting Dounce homogenizer. As judged by phase contrast microscopy, all cells were lysed but the nuclei were still intact.

Cellular fractionation was performed by a series of centrifugation steps (Fig 5). The cell lysates were centrifuged at 1000 x g for 5 min and obtained a pellet enriched in the nuclei. The resultant post nuclear supernatant was centrifuged at 12,000 x g for 10 min and a pellet of the cellular membranes was obtained. Further centrifugation of the supernatant at 200,000 x g for 45 min yielded as a pellet the small granular fraction while

HUMAN INTESTINAL 407 CELLS

|
treated with ETP
for 12 h
|

LYSED IN A DOUNCE HOMOGENIZER

|
centrifuged at 1000 x g
for 5 min
|

PELLET   POST-NUCLEAR FRACTION
(nuclei fraction)

|
centrifuged at 12,000 x g
for 15 min
|

PELLET   SUPERNATANT
(membrane fraction)

|
centrifuged at 200,000 x g
for 45 min
|

PELLET   SUPERNATANT
(small granular fraction)   (soluble cytoplasmic fraction)

Figure 5: Scheme followed to separate the different cellular components of the eukaryotic human intestinal 407 (Int 407) cells.

the supernatant contained the soluble cytoplasmic protein fraction. The relative activity of SDH, an integral enzyme of the plasma membrane was used to establish the purity of the cell fractions (30).

c)   **SDS-PAGE analysis and Immunoblotting**: The protein concentration of the cell fractions was determined by the method of Lowry (87) and 8 μg of protein of each of the cellular components was loaded in different wells and separated by SDS-PAGE. The cell fractions were loaded in duplicate on the same gel. After electrophoresis, the gel was cut in half and one-half silver stained to reveal proteins while the other half was transferred onto a nitrocellulose membrane by Western blot technique (152). Transferred nitrocellulose blots were incubated in the MAb (CETPMAb₄) to the C. jejuni ETP, washed in TBS, reincubated in goat anti-mouse-horseradish peroxidase and the immunoblots were developed in 4-chloro-1-naphthol as described in section 2.3.

d)   **ELISA**: ELISA experiment were performed with 96 well plates coated with the separated cell fractions of Int 407 cells treated with ETP for 12 h. Equal concentration of proteins (50 μg/well) was added to the 96 well plates and these were incubated overnight. Excess protein was removed by washing each well in T-PBS, after which 100 μl of a 1:10 dilution of CETPMAb₄ was added for 2 h and this was followed by incubation in 100 μl of goat anti-mouse-horseradish peroxidase conjugate for 1 h. After washings with T-PBS, TMB substrate was added to each well and the reaction stopped with 2 M sulfuric acid. The color intensity was read using EIA reader at 405 nm.

e)   **Indirect Immunofluorescence assay**: Int 407 cells were grown as confluent monolayers in 6 well plates with glass coverslips. They were treated with ETP (100 ng/well) for 12 h and subsequently fixed with either 1 ml of 0.5% glutaraldehyde in cacodylate buffer or methanol. Fixed monolayers were washed and treated with polyclonal antiserum to strain WD 2483 or the monoclonal antibody, CETPMAb₄ followed by goat

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anti-rabbit or goat anti-mouse FITC and viewed using an Olympus epifluorescent microscope. Similar fixed monolayers were treated in duplicate with 0.2 mg/ml of trypsin for 10 min to remove any bound toxin, one set of monolayers was lysed while the other set of cells was left intact and prepared for fluorescence microscopy (section 2.7).

RESULTS

3.1 Identification and biotyping of the strains

All seven clinical strains were confirmed as Campylobacter jejuni based on the morphological and biochemical tests outlined in Table 7. These strains were Gram-negative with a spiral S- shaped morphology and were motile with a characteristic darting corkscrew-like motion. They were oxidase and catalase positive and hydrolysed hippurate. According to the biotyping scheme of Lior (85) shown in Table 2 all the strains belonged to biotype 1 as none produced H_2S or DNase.

3.2 Histopathological observations in the chick embryo

The LD_{50} values for C. jejuni strain WD 2483 introduced into the chick embryo by different routes of inoculation were determined and are shown in Table 8. The number of organisms required to induce lethality in eggs ranged from 2.1×10^2 cfu/ml for organisms inoculated into the amniotic cavity to 9.5×10^4 cfu/ml for allantoic sac. The LD_{50} results demonstrated that inoculation of relatively small doses of organisms into the immediate environment or into the nutritional source of the embryo were sufficient to kill embryos as compared to the dose required for more peripheral routes.

Examination of embryo organs such as liver, heart, kidney, spleen and the GI tract after yolk sac inoculation with multiple doses of the yolk sac LD_{50} are summarized in Table 9 and illustrated in Plates 1 to 5. The control tissue sections from 14 day old embryos are shown in Plates 1a to 5a. The liver parenchyma was observed in a continuous lattice with radially disposed central veins throughout the tissue (Plate 1a). The contractile myocardial muscle fibers forming a network were easily discernable in the heart tissue (Plate 2a). Section through the kidney showed the well spaced proximal and distal tubules and

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glomeruli with dispersed red blood cells (Plate 3a). The spleen tissue appeared organized with the white pulp scattered throughout the red pulp (Plate 4a) and the GI tract showed the columnar epithelial cells and the crypts of Lieberkuhn extending into the lamina propria typical of their structure (Plate 5a). The embryo organs inoculated with 10, 100 or 1000 times the yolk sac LD_{50} of C. jejuni strain WD 2483 showed congestion of the blood vessels, slight tissue damage due to edema, and small amounts of hemorrhage (Plates 1b to 5b). Cell breakdown or necrosis were not evident. The lack of an inflammatory infiltrate was striking and was observed in all organs examined irrespective of the inocula used or the time post-inoculation. The heart was highly congested and the tissue was disrupted as a result of edema (Plate 2b). In the kidney tissue the convoluted tubules were condensed and the glomeruli were not clearly visible (Plate 3b). The congestion in spleen tissue was less severe than in liver, heart or kidney (Plate 4b). Surprisingly, the GI tract looked similar in appearance to the control tissues throughout the study (Plate 5b). The chorio-allantoic membrane did not show any visible pocks or pathological changes. The histological changes in different organs did not appear till day 3 post-inoculation and were most severe from day 5 to day 8 post-inoculation. The tissues from day 1 and day 2 post-inoculation appeared normal.

Inoculation of multiple doses of yolk sac LD_{50} resulted in the growth and multiplication of the organism only in the organ of inoculation, yolk sac. There was a 3 log increase in the number of organisms by day 5. The bacterial colony counts of aseptically removed homogenized embryo organs showed no bacteria irrespective of time post-inoculation. Indirect immunofluorescence studies on deparaffinized tissue sections and formalin fixed homogenates also showed that dissemination of the organisms had not occurred in any of the embryo organs.

Inoculation of the cell-free filtrate of the strains of C. jejuni intra-yolk sac in 6 day

old eggs induced lethality. Filtrate from strain WD 2483 (originally containing 5×10^7 cfu/ml) was found to be most toxic as it retained toxicity for the eggs when diluted up to 10^{-4} while strain WD 2914 (originally containing 6.8×10^7 cfu/ml) and BS 1 (originally containing 8.0×10^7 cfu/ml) retained toxicity only up to 10^{-2} (Table 10). Inoculation of yolk sacs with bacteria-free filtered broth obtained from strain WD 2483 induced within 24 h histopathological changes similar to those produced by organisms (Plates 1c to 5c). The liver, heart, kidney and the spleen showed congestion, edema and hemorrhage while the GI tract appeared as the controls.

Toxicity of these filtrates for embryos was destroyed by exposure to heat at 60°C or 100°C for 15 min, pH 3.0, pH 9.0, trypsin, or protease but not to lipase or long term storage at 4°C. There was no effect on the amount of toxin produced when the bacterial cells were treated with 1 mg/ml of polymyxin B to release the periplasmic proteins.

3.3 Electrophoretic pattern of the concentrate from Campylobacter jejuni

The preparative PAGE of the concentrated dialyzed cell-free filtrate from C. jejuni strain WD 2483 showed four bands after staining with Coomassie blue (Plate 6, lane A). After each of these bands were separately electroeluted and injected into the yolk sac of eggs, only one of the protein bands proved to be lethal to the embryos. Pieces of acrylamide were electroeluted in a similar fashion and 0.1 ml injected into eggs as controls. No mortality was observed. The SDS-PAGE of this eluted toxic protein (ETP) yielded two bands, one at 68 kilodalton and the other at 65 kilodalton (Plate 6, lane B and C). When similar unstained bands were eluted out of the reducing gel and injected into eggs, no mortality was observed. The lethality of the ETP for the fertile hen's egg was abolished by treatment with 0.5 mg/ml of trypsin and protease, freeze-thawing, heating at 60°C or 100°C, but not with lipase or storage at 4°C for up to 4 weeks (Table 11).

Monoclonal antibody : While screening the various monoclonal antibodies raised

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against the toxin of C. jejuni, forty clones were found to be specific for the ETP. ELISA readings of greater than 0.15 at 405 nm were achieved when Int 407 cells previously treated with the ETP for 2 h were probed with the supernatant of each. When 100 µl of the globulin containing medium from these clones was incubated with the ETP for 30 min and injected into the yolk sac of 6 day old eggs, one clone was found that destroyed the toxicity of the ETP for the eggs. This clone was further tested for specificity by immunoblotting using the standard Western blot technique described in Section 2.5. Immunoperoxidase probes of ETP resolved by SDS-PAGE and transferred to nitrocellulose showed both the 65 and 68 kilodalton protein bands when the primary antibody used was the polyclonal antiserum raised against C. jejuni strain WD 2483 (Plate 6, lane D). However, the supernatant from the clone (CETPMAb$_4$) which abolished lethality for eggs reacted with only the 68 kilodalton band (Plate 6, lane E).

3.4 Pathogenesis of Campylobacter jejuni on host eukaryotic cells

PCEF and Int 407 cells inoculated with C. jejuni strain WD 2483 at an MOI of 100:1 were observed for cytopathic and/or cytolytic affects. No significant visible changes were seen until 6 h post-inoculation. However, by 12 h up to 50% of the cells were rounded. There was extensive cellular vacuolation resembling fatty degeneration and a number of refractile cells were observed (Plates 7 and 8). By 24 h a majority of the cells had lysed and were no longer attached to the bottom of the wells.

Bacterial binding to PCEF and Int 407 cells of C. jejuni stain WD 2483 at a MOI of 100:1 was assayed for up to 36 h and the resultant adherence kinetics data are shown in Fig 6 and 7. At this inoculum level, marked non-specific binding of the organisms to the plastic of the culture dishes was observed. Approximately, 3-4 x 10^6 cfu/well bound non-specifically when the inoculum was 2 x 10^8 cfu/well. Viable count assay of the lysate of cell monolayers treated with 250 µg/ml gentamicin showed no internalized bacteria,

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irrespective of the time post-inoculation. The maximum adherence was at 4 h post-inoculation in both cell lines which corresponds to an average cfu/eukaryotic cell ratio of 1 per 10 cells. IFA equivalent ratios were 1 bacterium per 20 cells for Int 407 and 1 bacterium per 25 PCEF cells. The data indicated that 0.1% of the inocula bound to the cells while 1.6 -2.0% of it bound to the plastic showing approximately a twenty times higher efficiency of bacterial binding to the plastic than the host eukaryotic cells.

Ultrastructural observations of Int 407 cells inoculated with strain WD 2483 and incubated for 2, 6, 12 and 24 h are shown in Table 12 and illustrated in Plates 9 to 13. The control Int 407 cells had cell membranes rich in microvilli. Within the cytoplasm the golgi apparatus could be clearly seen (Plate 9) and the mitochondria with complex membrane configuration were typical in appearance. The Int 407 cells had large nuclei. Cell monolayers inoculated with the organisms at an MOI of 100:1 and incubated for 2 h appeared, in general, similar to the controls except for the presence of membrane enclosed amorphous-staining bodies observed in all cells (Plate 10). By 6 h post-inoculation mitochondrial damage along with initial stages of cytoplasmic clearing and membrane lysis were obvious (Plate 11). At 12 h post-inoculation there was breakdown of cytoplasmic and nuclear membranes as well as clearing of the cytoplasm of affected cells. Apparently empty mitochondria were seen. Cells showed margination of the chromatin material with nuclear clearing and this along with dilation of the perinuclear region and ballooning of the nuclear membranes was the most common feature (Plate 12). By 24 h post-inoculation most cells were lysed and in the few surviving cells ghost mitochondria, cytoplasmic clearing with chromatin margination and ballooning of the nuclear membrane were observed consistently (Plate 13). At no time through out the study were internalized or adherent bacteria observed.

Scanning electron microscopy of these infected cell monolayers at 2, 6, 12 and 24 h post-inoculation confirmed the cellular damage observed by TEM and this is illustrated in

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Plates 14 and 15. The degree of cell damage increased with time. The untreated control

cells appeared as relatively uniform monolayers with occasional clumped cells showing

those cells undergoing mitosis (Plate 14). The Int 407 cells treated with C. jejuni strain

WD 2483 for 2 h appeared similar to the control (Plate 15a). Cell damage was evident at 6

h post-inoculation by which time the monolayer had lost its integrity and many rounded

clumps of cells were seen (Plate 15b). Numerous blebs were observed on the surface of

infected cells and cell lysis was apparent. The damage to the cells increased progressively

through 12 and 24 h post-inoculation (Plate 15 c and d). At no time throughout the study

were any bacteria observed adhering to or penetrating the host eukaryotic cells.

3.5 Localization of the toxin in the organism

Electrophoretic profile of the three cellular fractions; outer membrane, cytoplasmic

membrane as well as cytoplasm and periplasm of strain WD 2483 and their Western blot

subsequently derived from these are shown in Plate 16. The molecular weight markers are

located in lane a. The banding patterns of the proteins from the outer membrane,

cytoplasmic membrane and soluble protein (cytoplasm and periplasm) fractions respectively

are shown in lanes b, c and d. Approximately 10 protein bands could be seen in the outer

membrane profile of the organism and these bands ranged in molecular weight from 18 to

68 kilodaltons. The OMP profile was dominated by 3 protein bands at 25.7, 43 and 68

kilodaltons. The 43-kilodalton protein is thought to be a porin. Lane e, f, g and h are the

immunoblots of the soluble proteins, cytoplasmic membrane, outer membrane and the

molecular weight markers probed with CETPMAb4. The monoclonal antibody showed

antigenic specificity for five bands in the outer membrane fraction and these corresponded

to bands with approximate molecular weights of 20, 23, 35, 43 and 68 kilodaltons. No

protein bands in the cytoplasmic membrane or the cytoplasmic and periplasmic fraction

were recognized by the monoclonal antibody. The percent purity of the cell fractions

analyzed by succinate dehydrogenase (SDH) activity showed 82% of the SDH activity in

the cytoplasmic membrane fraction with 10% in the cytoplasmic & periplasmic fraction and 8% in the outer membrane fraction. With respects to the concentration of 2-keto-3-deoxyoctonate (KDO), approximately 80% occurred in the outer membrane with 13% in the membrane fraction and 7% in the cytoplasmic and periplasmic fraction of strain WD 2483.

These cellular fractions were coated onto 96 well plates overnight and ELISA was performed using CETPMAb$_4$ and goat anti-mouse horseradish peroxidase. The absorbance at 405 nm of the different fractions is shown in Fig 8. The monoclonal antibody bound to proteins in the outer membrane fraction with an absorbance value 0.194 as compared to almost negligible absorbance values for the cytoplasmic membrane or cytoplasmic and periplasmic fractions. However, the cellular fractions probed with the polyclonal antibody to strain 2483 showed specificity for all three fractions.

C. jejuni organisms probed with the monoclonal antibody, CETPMAb$_4$ and gold conjugated antiserum are illustrated in Plate 17. The gold probe was seen attached only to the outer membrane in small amounts. On average only one or two sites on the outer membrane with gold particles occurring singly or in clumps were observed per cell. Control organisms that were treated with PBS instead of CETPMAb$_4$ and the conjugated antiserum showed no probe (Plate 17a). In freeze-thawed samples the organisms were seen almost deprived of cytoplasm and most were lysed. In these lysed and deformed bacterial cells the probe was seen attached to the outer membrane (Plate 17b). At no instance was the gold probe seen inside the organisms. Normal washed C. jejuni organisms probed with the monoclonal antibody and the conjugated antiserum again showed the gold particles attached to the outer membrane only (Plate 17c).

3.6 Nature of the toxin

The effect of the ETP, cholera and E. coli enterotoxins on CHO cells are illustrated

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in Plate 18. The ETP caused rounding of about 70-80% of the CHO cells at 24 h post-inoculation (Plate 18b) and of these rounded cells 72% were non-viable by trypan blue assay. The enterotoxins of $\underline{V}$. cholerae and $\underline{E}$. coli showed elongation in approximately 75% of the CHO cells by 24 h (Plate 18c and 18d). When the ETP was incubated with CETPMAb₄ for 30 min and added to the CHO cells, no cytotoxic activity was observed. However, the monoclonal antibody did not neutralize the cholera or $\underline{E}$. coli enterotoxin and cholera antitoxin had no effect on the ETP as determined by cell culture and fertile hen's egg lethality assay. The cell-free filtrate of the remaining 6 strains of $\underline{C}$. jejuni showed rounding of 68-82% of the CHO cells and their cytotoxic activity was neutralized when 0.1 ml of the filtrate was incubated with 1:10 dilution of the monoclonal antibody for 30 min. None of these crude filtrates shared any immunologic resemblance with the cholera toxin in that the antisera against cholera toxin did not neutralize their toxic activity (Table 13).

Binding of the various toxins to GM_1 ganglioside as measured by ELISA are illustrated in Fig 9. The cholera and $\underline{E}$. coli enterotoxins avidly bound the ganglioside while the ETP or the crude toxic filtrates from the other 6 $\underline{C}$. jejuni strains showed no binding. The results illustrated are an average of three separate trials.

The effect of the ETP on PCEF and Int 407 cells were observed by inverted microscopy and are illustrated in Plates 19 and 20. No significantly visible changes were seen prior to 2 h post-inoculation but some granular cells were observed after 6 h. However by 12 h approximately 80% of the cells in both the cell lines had rounded up. Of these rounded cells 78% were non-viable in trypan blue exclusion assays and all the detached cells floating in the medium were dead. There was cellular vacuolation resembling fatty degeneration observed in the affected cells. A number of refractile cells were also seen . The cytopathic effects induced on the cell lines were similar to those induced by the organism. By 24 h most of the cells had lysed and were no longer attached to the wells.

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3.7 Binding specificity of the toxin

The binding curves of ETP to PCEF and Int 407 cells are summarized in Fig 10 and 11. As observed, the ETP bound avidly to both the cell lines and this was maximal after 2 h incubation at 37°C. The maximum binding values of the toxin obtained by ELISA were 0.378 for the PCEF cells and 0.412 for the Int 407 cells. The adherence subsequently decreased and by 24 h most of the cells were rounded or lysed. The saturation curves for the toxin receptors on both PCEF and Int 407 cells are shown in Fig 12 and 13. In both cell cultures, 10 ng of ETP saturated all the receptors on eukaryotic host cell monolayers in 96 well microtiter plates and there was no significant difference observed in toxin binding on increasing the protein concentration to as high as 10 µg/well.

The binding of toxin to host cell membranes is a complex process and experimental procedures could lead to contradictory results. For these reasons treatments that reduced binding by 50% or more as compared to the untreated controls are considered significant. In this study similar inhibition levels were used to measure the significance of the various treatments for the binding of ETP to PCEF or Int 407 cells. Fig 14 and 15 illustrates the percent reduction in toxin binding following modification of the host eukaryotic cell surfaces with the agents listed in Table 5. Proteolytic enzymes, neuraminidase and glutaraldehyde reduced adherence of the ETP by more than 50% in both cell lines while treatment with ß-galactosidase, lipase, sodium metaperiodate and Nonidet P-40 had no significant affect.

Coincubation of PCEF and Int 407 cells with sugars, lectins and GM$_1$ ganglioside listed in Table 6 are shown in Fig 16 and 17. Of the lectins, GM$_1$ ganglioside or the sugars tested only N-acetyl neuraminic acid (NurNAc) significantly reduced toxin binding. NurNAc reduced adherence by 50% and 56.5% in Int 407 and PCEF cells respectively. The remaining mono- and disaccharide sugars each accounted for less than 7% reduction in toxin binding.

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3.8 Fate of the toxin after interaction with Int 407 cells

Ultrastructural observations on Int 407 cells treated with ETP for 2, 6 and 12 h and probed with monoclonal antibody and gold-conjugated goat anti-mouse serum are summarized in Table 14 and illustrated in Plates 21 to 25. The effect of the toxin was clearly visible at 2 h and the mitochondria showed the most damage (Plate 22). Initial stages of cytoplasmic clearing accompanied by some fatty degeneration was observed. Breakdown of cell membranes with ballooning of the nuclear membrane by 6 h was very striking and consistent throughout the study (Plate 23). The mitochondria were severely damaged with degeneration of these organelles and at later stages apparently empty mitochondria were seen. The microvilli were fewer and blunted as compared to the normal cells. By 12 h there was breakdown of cytoplasmic and nuclear membranes and clearing of the cytoplasm in cells. Dilation of the perinuclear space with ballooning of the nuclear membrane was seen (Plate 24). A majority of the cells had lysed and a few surviving cells showed margination of the chromatin material with nuclear clearing. At this stage, the microvilli had almost disappeared (Plate 25).

SEM analysis of the Int 407 cells treated with ETP confirmed the cell damage observed by TEM and is illustrated in Plates 26 and 27. Compared to the untreated control Int 407 cells (Plate 26), the cell damage induced by the toxin in 2 h was marked (Plate 27a). The monolayers were stretched and appeared to be split apart. By 6 h the effect of the toxin was quite pronounced with cells showing numerous blebs on the surface and the integrity of the monolayers was lost (Plate 27b). At 12 h post-treatment 80% of the cells had rounded up with lysed surfaces as shown in Plate 27c and 27d.

The gold probe was seen at all times and was only attached to the cell membranes. At no instance was the probe seen in the cytoplasm. Interestingly, it seemed that the probe was found attached mostly to the microvilli in cells treated with the ETP for 2 and 6 h. In freeze-thawed toxin treated cells, no probe was seen at any time (Plate 25). However, the

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toxic effects of the ETP were clear in the freeze-thawed cells.

Electrophoretograms of each soluble fraction of Int 407 cells treated with the ETP for 12 h are shown in Plate 28. Lanes a, b, c and d contain the soluble cytoplasmic fraction, membrane fraction, nuclei fraction and the small granule fraction respectively. Lane e has the molecular weight markers. In each of these fractions, more than 60 proteins are evident including several major proteins with molecular weights ranging from 20 to 90 kilodaltons. The percent purity of the cell fractions analyzed by succinate dehydrogenase (SDH) activity showed most of the enzymatic activity in the membrane fraction (79%) with 7% in the small granule fraction, 2% in the soluble cytoplasmic fraction and 12% in the nuclei fraction. Immunoblots of these cellular fractions and the molecular weight markers probed with the monoclonal antibody, CETPMAb$_4$ are shown in Plate 28, lanes f to j in the same order as above. The monoclonal antibody showed antigenic specificity for four bands in the membrane fraction at approximate molecular mass of 32, 43, 54 and 63 kilodaltons. No other protein bands in any of the remaining three fractions were positive on probing with the monoclonal antibody.

These cellular fractions were coated onto 96 well plates overnight and ELISA was performed using CETPMAb$_4$ and goat anti-mouse horseradish peroxidase. The absorbance values at 405 nm of the different fractions are shown in Fig 18. The monoclonal antibody bound to proteins in the membrane fraction with an absorbance value of 0.163 as compared to almost negligible absorbance values for the other three fractions of the Int 407 cells.

Immunofluorescence results of Int 407 cells incubated with the ETP for 12 h after various treatments probed with either the polyclonal antibody or the monoclonal antibody, CETPMAb$_4$ are summarized in Table 15 and illustrated in Plates 29 to 31. Positively fluorescing cells were observed after fixation with either glutaraldehyde or methanol (Plates

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29a and 30a). No fluorescence was seen in cells which were trypsin treated to remove cell-membrane bound toxin (Plates 29b and 30b). Similar fluorescence was observed in lysed cells which were not trypsin treated (Plate 31a) but cells that were trypsin treated and subsequently lysed showed no binding of the FITC-conjugated antiserum (Plate 31b).

TABLE 7   MORPHOLOGICAL AND BIOCHEMICAL
CHARACTERISTICS OF THE STRAINS

- non spore forming

- Gram negative spiral S- shaped

- microaerophilic

- darting cork screw like motility

- oxidase (+)

- catalase (+)

- nitrate reductase (+)

- H_2S production on TSI (-)

- oxidation of carbohydrates (-)

- hippurate hydrolysis (+)

- growth at 25°C (-)

- growth at 42°C (+)

- biotype 1

TABLE 8   LD$_{50}$ ENDPOINTS OF C. JEJUNI STRAIN WD 2483 FOR THE FOUR ROUTES OF INOCULATION IN THE FERTILE HENS' EGGS

Routes	Colony forming units/ml[a]	
	Average	Range[b]
Allantoic cavity	**9.5 x 10^4**	8.8 x 10^4 - 9.9 x 10^4
Amniotic sac	**2.1 x 10^2**	1.9 x 10^2 - 3.8 x 10^2
Yolk sac	**3.3 x 10^2**	2.0 x 10^2 - 5.6 x 10^2
Yolk sac (washed)	**1.0 x 10^3**	8.8 x 10^2 - 3.1 x 10^3
Chorioallantoic membrane	**8.0 x 10^3**	6.5 x 10^3 - 9.5 x 10^3

a : data represents an average of three separate experiments
b : the lowest and highest LD$_{50}$ of the three experiments for each inoculum site.

TABLE 9   HISTOPATHOLOGICAL OBSERVATIONS IN EMBRYO
ORGANS INOCULATED WITH 10, 100 OR 1000 TIMES
THE YOLK SAC LD$_{50}$ OF C. JEJUNI STRAIN WD 2483[a]

Changes in embryo tissues[b]	Days post-inoculation		
	1 2	3 4	5 6 7 8
congestion of blood vessels	-	++	+++
edema	-	+	++
hemorrhage	-	+	++
inflammatory response	-	-	-
necrosis	-	-	-

[a] : inoculation intra-yolk sac at 6 days of development
[b] : liver, heart, kidney and spleen showed identical results. Gastrointestinal tract appeared similar to controls throughout the study period
- = damage not found; + = slight; ++ = moderate; +++ = severe

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TABLE 10 ACTIVITY OF THE CELL FREE FILTRATES FROM C. JEJUNI

Strain	cfu/ml[a]	Dilution[b]
WD 2483[c]	5.0×10^7	10^{-4}
WD 2914	6.8×10^7	10^{-2}
WD 2920	1.0×10^8	10^{-3}
WD 2939	1.2×10^8	10^{-3}
MA 1	1.5×10^8	10^{-3}
MA 2	4.0×10^8	10^{-3}
BS 1	8.0×10^7	10^{-2}

a : Strains were grown in thioglycolate broth microaerobically at 37°C for 48 h, assayed
   for viable counts as cfu/ml and prepared as bacterial cell-free filtrates
b : Highest dilution of C. jejuni cell-free filtrate inducing death in eggs. Dilutions of
   filtrate tested were from 10^{-1} to 10^{-7}
c :   strain used in subsequent characterization studies

TABLE 11 PHYSICO-CHEMICAL CHARACTERIZATION OF THE ETP ISOLATED FROM C. JEJUNI STRAIN WD 2483

Treatment	Time	Lethality in fertile hens' eggs
0.5 mg/ml trypsin	30 min	-
0.5 mg/ml protease	30 min	-
0.5 mg/ml lipase	30 min	+
pH 3.0	30 min	-
pH 9.0	30 min	-
60°C	15 min	-
100°C	15 min	-
freeze-thaw	10 min	-
storage at 4°C	4 weeks	+

+ = Induced death in eggs
- = No mortality

TABLE 12   ULTRASTRUCTURE CHANGES IN INT 407 CELLS AFTER INOCULATION WITH C. JEJUNI STRAIN WD 2483 AT AN MOI OF 100:1

Cell damage or feature	Hours post-inoculation			
	2	6	12	24
break down of cell and nuclear membranes	-	+	+++	+++
cytoplasmic clearing	-	-	+++	+++
fatty degeneration	-	-	+++	+++
ballooning of nuclear membrane	-	-	+++	+++
mitochondrial damage	-	+	+++	+++
margination of chromatin	-	-	+++	+++
membrane bound amorphous inclusions	++	++	++	++

Presence of C. jejuni organisms

adherent to cell membranes	-	-	-	-
intracellular	-	-	-	-

- = Cell damage or feature not found; + = Slight ; ++ = Moderate ; +++ = Extensive

TABLE 13   CYTOLOGICAL CHANGES IN CHO CELLS DUE TO THE TOXINS OF C. JEJUNI, V. CHOLERAE OR E. COLI AFTER INCUBATION WITH ANTISERA

Toxin	Percent effect on cells after incubation with[a]		
	no antiserum	CETPMAb4	Cholera antitoxin
C. jejuni			
ETP (0.1 μg/ml)	80	4	75
ETP (0.01 μg/ml)	70	2	68
Filtrates from 6 additional strains (30 μg/ml)	68-82	<10	68-82
V. cholerae (30 μg/ml)	75	72	9
V. cholerae (0.1 μg/ml)	62	68	0
V. cholerae (0.01 μg/ml)	10	8	0
E. coli (30 μg/ml)	75	72	15
E. coli (0.1 μg/ml)	60	68	0
E. coli (0.01 μg/ml)	15	10	0

[a] : Incubation of toxins and antisera was at 37°C for 30 min and cytopathic changes recorded after 24 h. Numbers represent % cell rounding for C. jejuni toxin and % cell elongation for the toxins of V. cholerae and E. coli.
These results were generated from three separate experiments.

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TABLE 14 ULTRASTRUCTURAL OBSERVATIONS IN INT 407 CELLS AFTER INOCULATION WITH THE ETP FROM C. JEJUNI STRAIN WD 2483

Cell damage	Hours post-inoculation		
	2	6	12
breakdown of cell and nuclear membranes	-	+	+++
fatty degeneration	+	++	+++
cytoplasmic clearing	+	++	+++
ballooning of nuclear membrane	-	++	+++
mitochondrial damage	++	+++	+++
margination of chromatin	-	+	++
vesiculation	-	++	++
blunted microvilli	-	++	+++

- = No visual damage ; + = Slight ; ++ = Moderate ; +++ = Extensive

Gold probe was seen attached to the membrane of toxin treated cells irrespective of the time post treatment.

TABLE 15   LOCATION OF THE ETP AFTER INTERACTION WITH INT 407 CELLS BY IMMUNOFLUORESCENCE USING POLYCLONAL OR MONOCLONAL ANTIBODIES

Treatment[a]	Rationale	Fluorescence
fixed with glutaraldehyde	to immobilize cells	+
fixed with methanol	to make the membranes leaky	+
fixed with glutaraldehyde or methanol and treated with trypsin	to remove extracellular bound toxin	-
fixed with glutaraldehyde or methanol and lysed	to probe for both extracellular and intracellular toxin	+
fixed with glutaraldehyde or methanol, treated with trypsin and lysed	to probe for only intracellular toxin	-

[a]: cells were treated with the ETP for 12 h, fixed as indicated and either exposed to trypsin and/or lysed
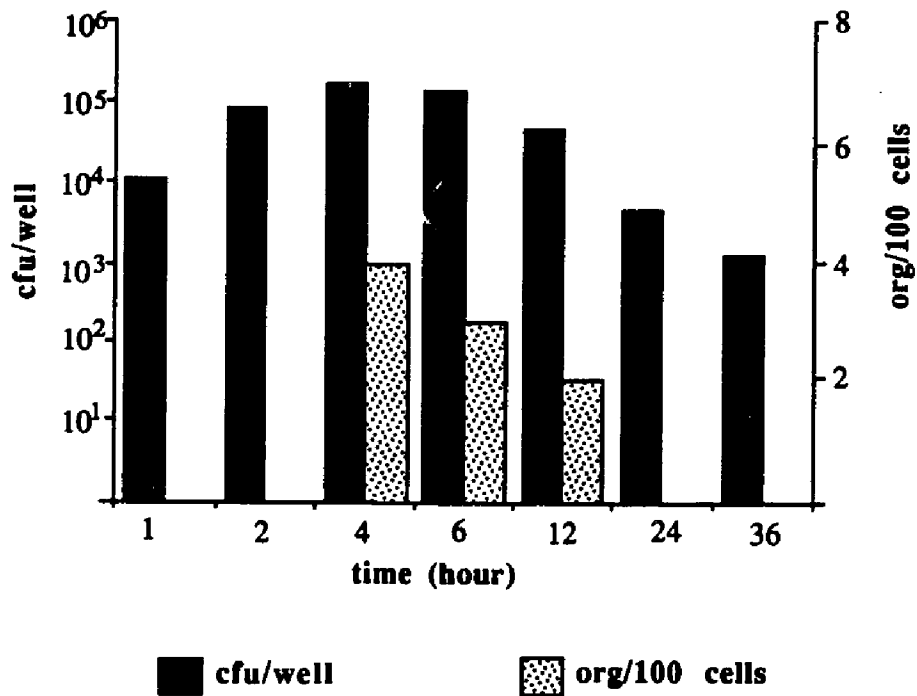
Figure 6: Bacterial binding of C. jejuni strain WD 2483 to PCEF cells as measured by viable counts of organisms released from lysed cells/well and by IFA of adherent organisms/100 cells. For these experiments the average cell number was 2.0×10^6 cells/well. Each well was inoculated with 2.0×10^8 cfu (or at a multiplicity of 100 cfu/cell). The maximum adherence was observed 4 h post-inoculation by both assays and corresponded to a ratio of 1 cfu/10 eukaryotic cells as measured by viable counts and 1 org/25 eukaryotic cells by IFA. The nonspecific binding of the organisms to the plastic was $3-4 \times 10^6$ cfu/well compared to the 2×10^5 cfu/well that bound to the cells. The data showed that 0.1% of the inoculum bound to the cells while 1.6-2.0% bound to the plastic. This indicated that bacterial binding to the plastic occurred at approximately twenty times higher efficiency than to the host eukaryotic cells. The results are an average of three separate trials.

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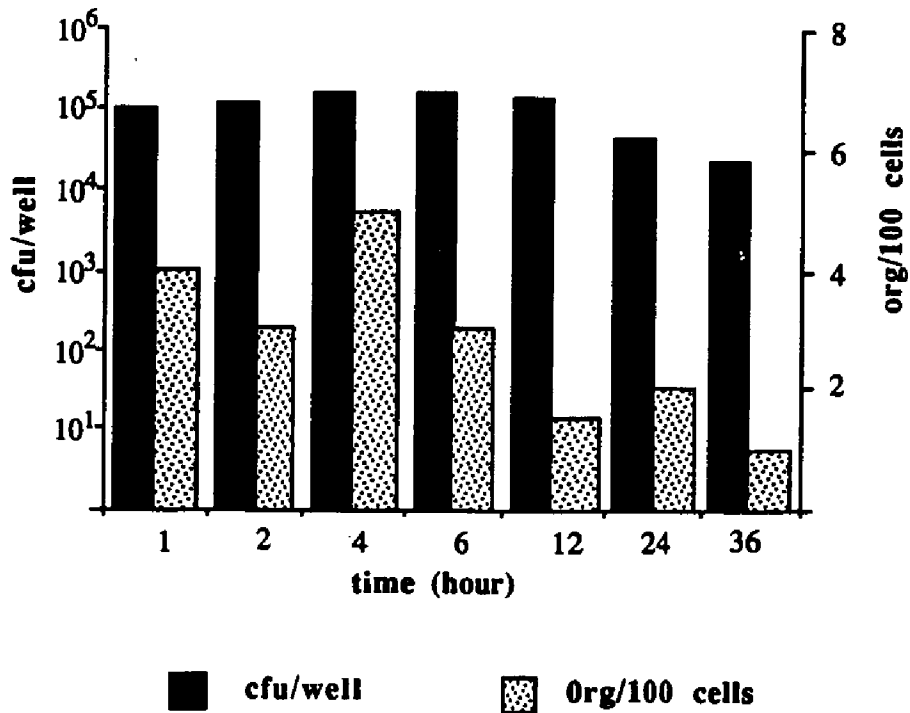
Figure 7: Bacterial binding of C. jejuni strain WD 2483 to Int 407 cells as measured by viable counts of organisms released from lysed cells/well and by IFA of adherent organisms/100 cells. For these experiments the average cell number was 2.0×10^6 cells/well. Each well was inoculated with 2.0×10^8 cfu (or at a multiplicity of 100 cfu/cell). The maximum adherence was observed 4 h post-inoculation by both assays and corresponded to a ratio of 1 cfu/10 eukaryotic cells as measured by viable counts and 1 org/20 eukaryotic cells by IFA. The non-specific binding of the organisms to the plastic was $3-4 \times 10^6$ cfu/well compared to the 2×10^5 cfu/well that bound to the cells. The data showed that 0.1% of the inocula bound to the cells while 1.6 -2.0% bound to the plastic. This indicated that bacterial binding to the plastic occurred at approximately twenty times higher efficiency than to the host eukaryotic cells. The results are an average of three separate trials.

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a = outer membrane

b = cytoplasmic membrane

c = cytoplasm + periplasm
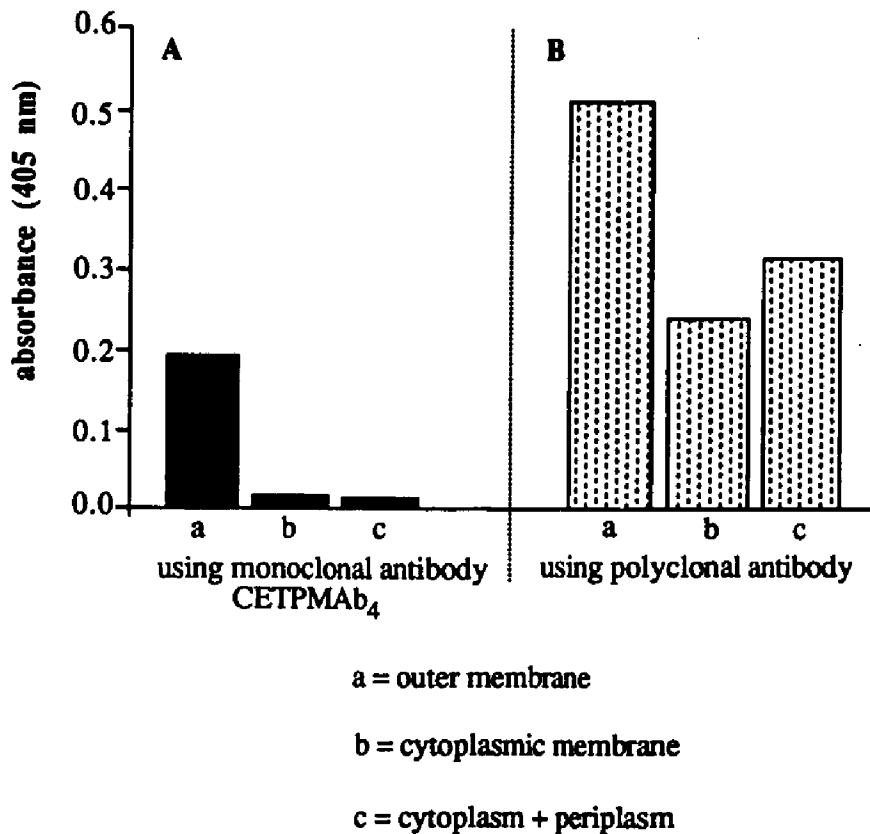
Figure 8: Location of the ETP in different fractions of C. jejuni strain WD 2483. The outer membrane (a), cytoplasmic membrane (b) and cytoplasm & periplasm (c) fractions were allowed to adhere to the wells of 96 well microtiter plates and probed with either the monoclonal antibody, CETPMAb4 specific for the ETP or the polyclonal antibody to strain WD 2483. The binding of the respective antibodies to the cellular fractions was measured by ELISA. As is shown in A, CETPMAb4 mainly bound to the outer membrane fraction whereas the polyclonal antibody showed specificity for all the three fractions as illustrated in B.

72
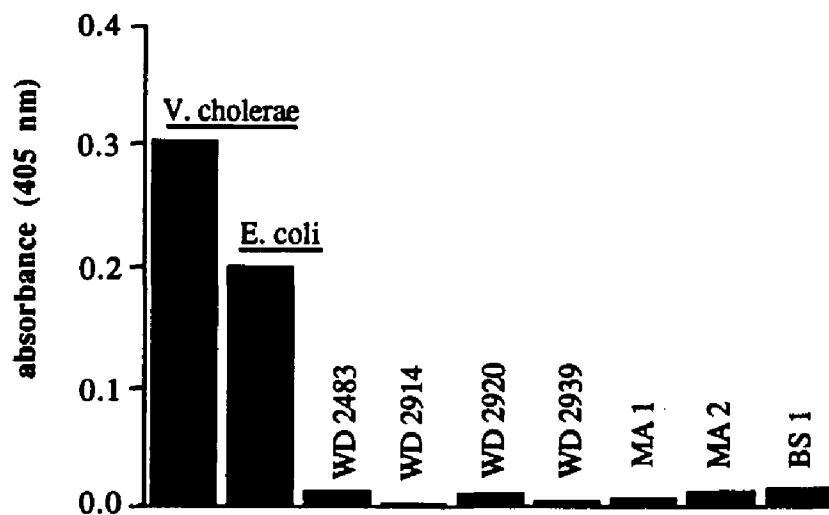
Figure 9: Toxin binding of C. jejuni strains to GM$_1$ ganglioside as measured by ELISA. The binding of Vibrio cholerae strain 2868 and Escherichia coli strain H10407 are illustrated as positive controls.
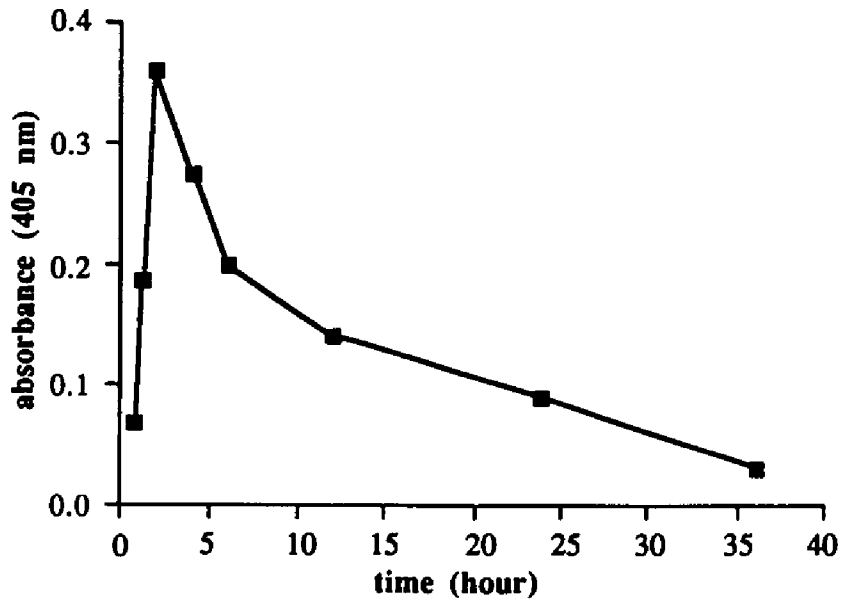
Figure 10: Binding of C. jejuni ETP to PCEF cells as shown by ELISA. The maximum binding of the toxin to the eukaryotic cells occurred after 2 h incubation. The results are an average of three separate trials.
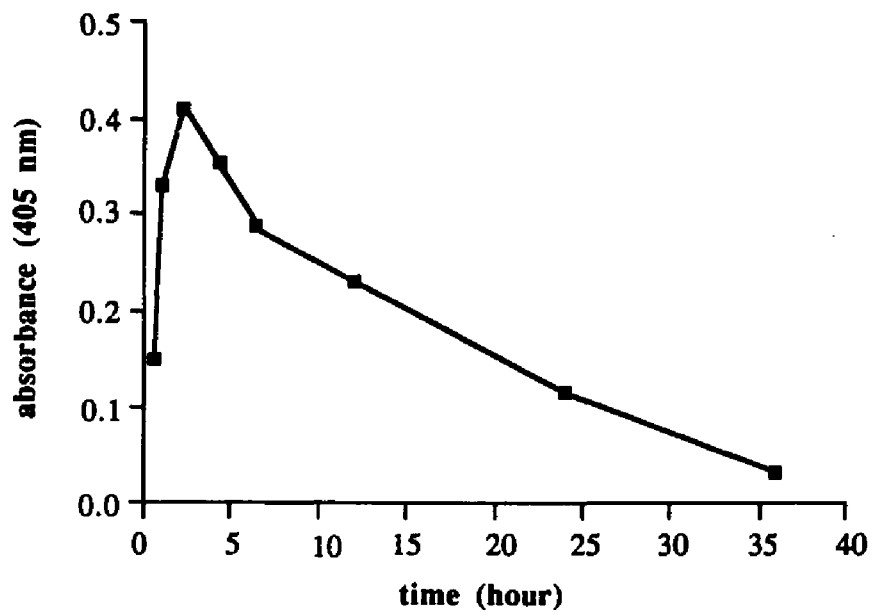
Figure 11: Binding of C. jejuni ETP to Int 407 cells as shown by ELISA. The maximum binding of the toxin to the eukaryotic cells occurred after 2 h incubation. The results are an average of three separate trials.
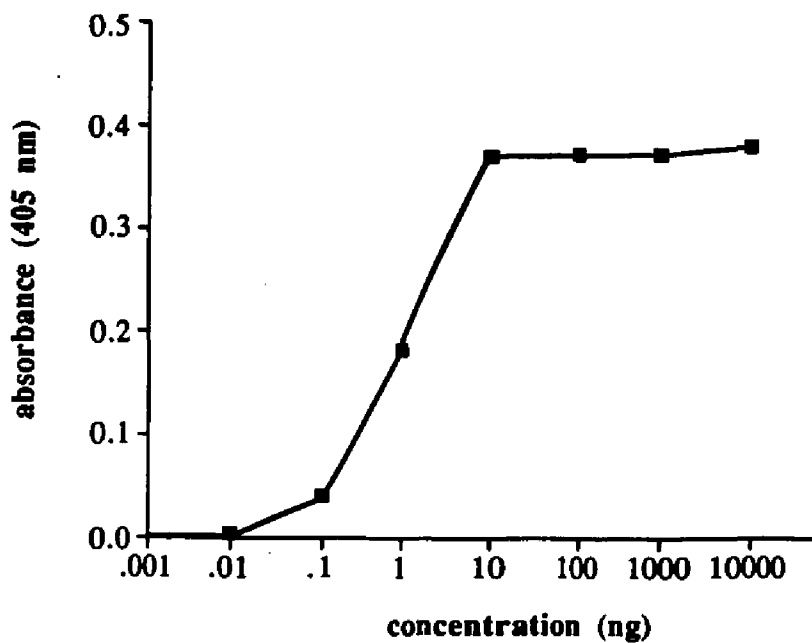
Figure 12: Saturation curve for the toxin binding receptors on PCEF cells. The cells were grown as confluent monolayers in 96 well microtiter plates and incubated with increasing concentrations of the ETP for 2 h. The saturation of the receptors was obtained with 10 ng of the toxin.
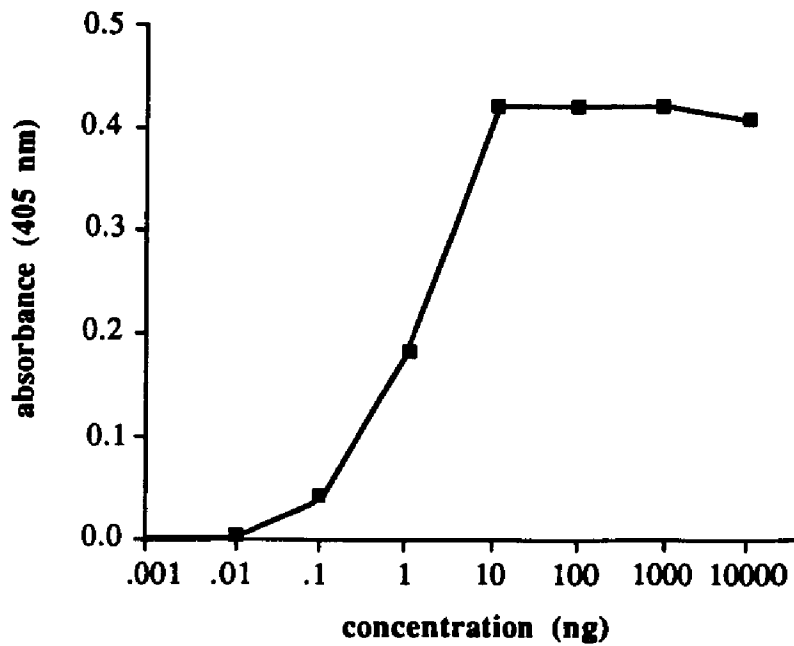
Figure 13: Saturation curve for the toxin binding receptors on Int 407 cells. The cells were grown as confluent monolayers in 96 well microtiter plates and incubated with increasing concentrations of the ETP for 2 h. The saturation of the receptors was obtained with 10 ng of the toxin.

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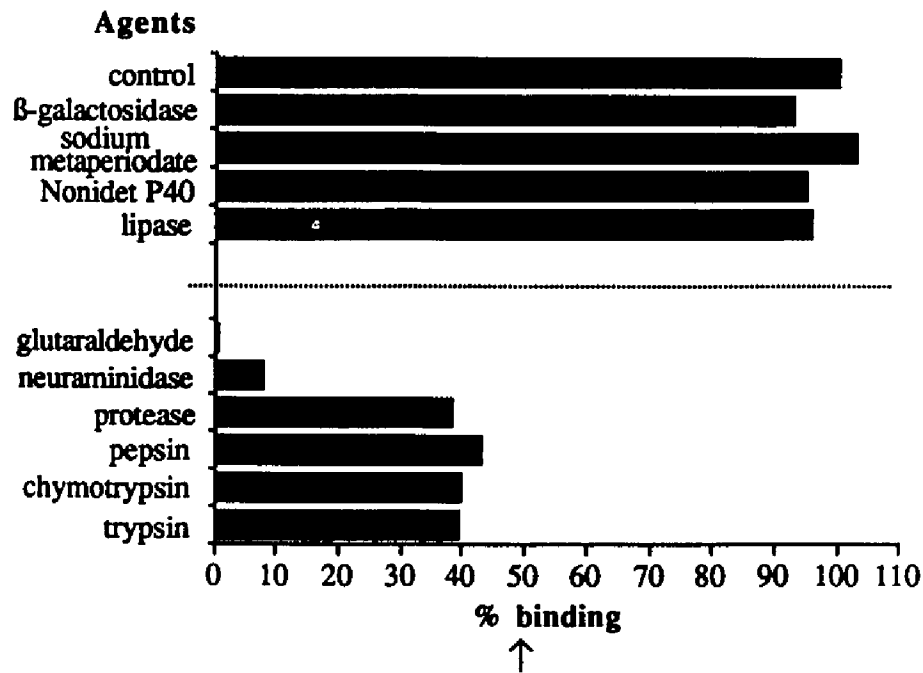
Agents

Figure 14: Percent binding of C. jejuni ETP to PCEF cells following eukaryotic treatments as measured by ELISA. Untreated cells gave 100% binding figures as controls. The 50% reduction point (shown by arrow) was selected as significant. Those agents having no influence on toxin binding included lipid modifiers, lipase and Nonidet P-40; the carbohydrate modifiers, sodium metaperiodate and ß-galactosidase (given above the dotted line). The proteolytic enzymes trypsin, chymotrypsin, pepsin and protease as well as glutaraldehyde and neuraminidase (given below the dotted line) all effected a greater than 50% reduction in binding.

78
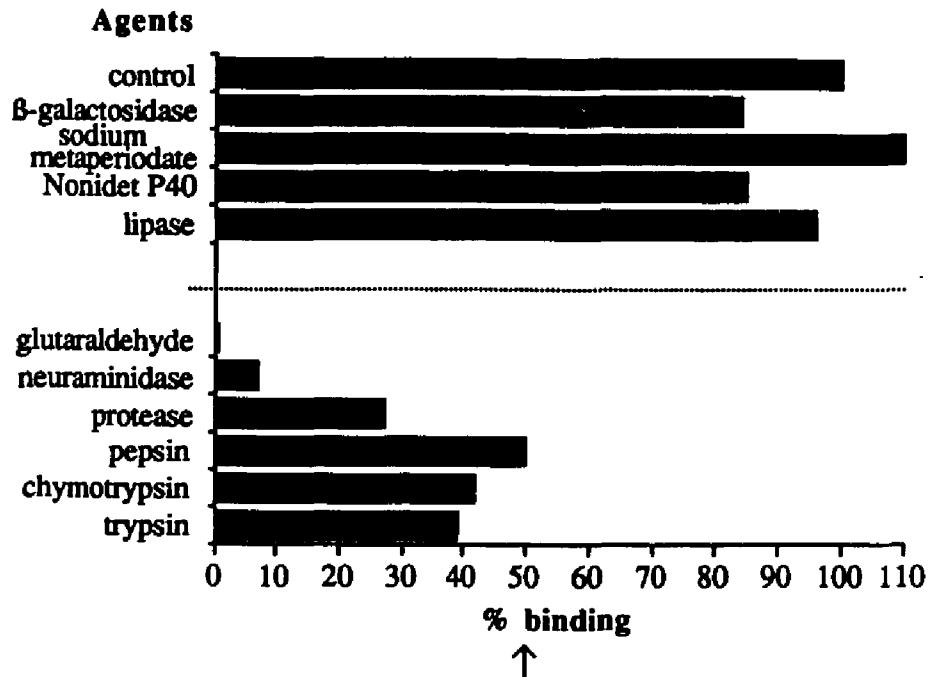
Figure 15: Percent binding of C. jejuni ETP to Int 407 cells following eukaryotic treatments as measured by ELISA. Untreated cells gave 100% binding figures as controls. The 50% reduction point (shown by arrow) was selected as significant. Those agents having no influence on toxin binding included lipid modifiers, lipase and Nonidet P-40; the carbohydrate modifiers, sodium metaperiodate and ß-galactosidase (given above the dotted line). The proteolytic enzymes trypsin, chymotrypsin, pepsin and protease as well as glutaraldehyde and neuraminidase (given below the dotted line) all effected a greater than 50% reduction in binding.

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Agents

control
fructose
maltose
sucrose
mannose
fucose
arabinose
galactose
glucose
GlNAc
GaNAc
WGA
Con A
GM$_1$

NurNAc

0 10 20 30 40 50 60 70 80 90 100

% binding

↑
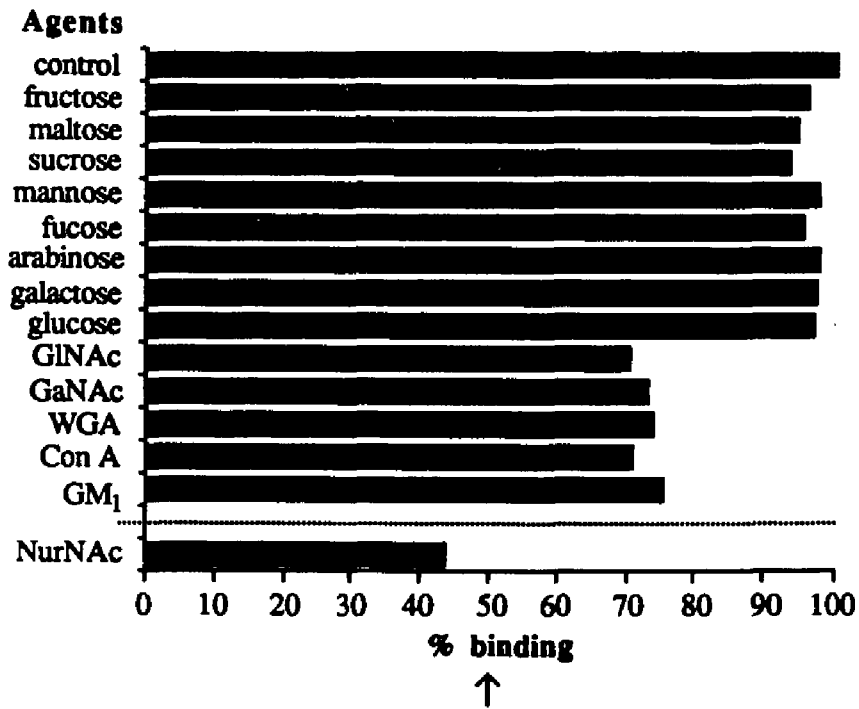
Figure 16: Competitive binding of C. jejuni ETP to PCEF cells following coincubation with sugars, lectins and GM$_1$ ganglioside as measured by ELISA. Untreated cells gave 100% binding as controls. The 50% reduction point (shown by arrow) was selected as significant. Except for NurNAc (given below the dotted line), no agent tested significantly affected toxin binding. The results are an average of three separate trials.
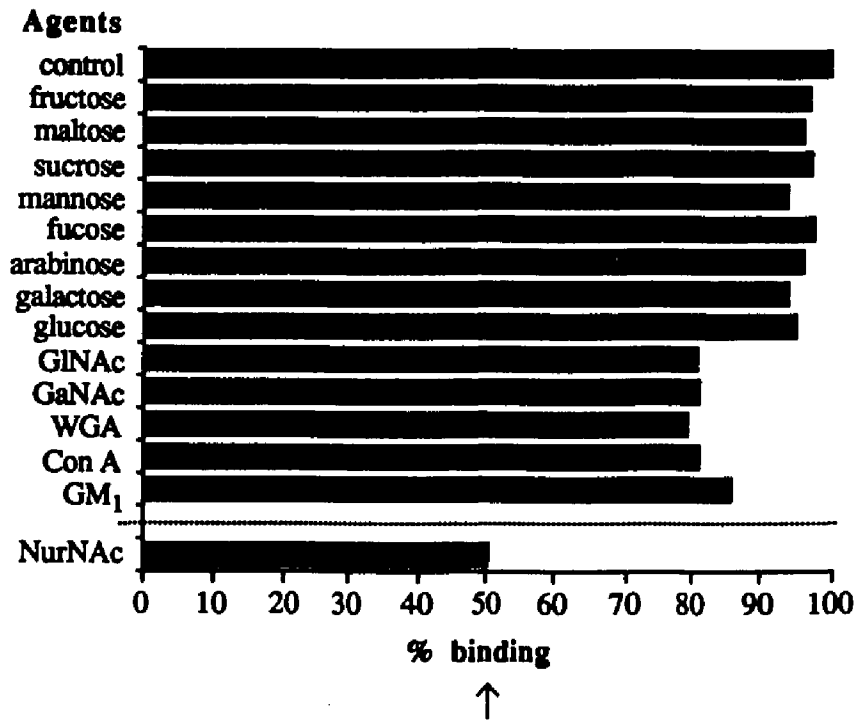
Figure 17: Competitive binding of C. jejuni ETP to Int 407 cells following coincubation with sugars, lectins and GM_1 ganglioside as measured by ELISA. Untreated cells gave 100% binding as controls. The 50% reduction point (shown by arrow) was selected as significant. Except for NurNAc (given below the dotted line), no agent tested significantly affected toxin binding. The results are an average of three separate trials.
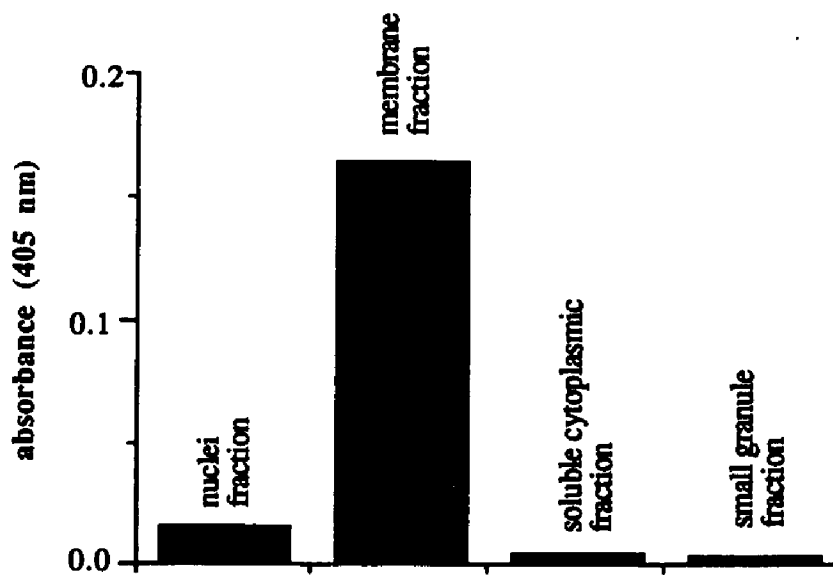
Figure 18: Location of the toxin in the four cellular fractions of Int 407 cells treated with the ETP for 12 h. The toxin was detected using the monoclonal antibody, CETPMAb$_4$ in ELISA tests. The ETP bound to the membrane fraction of the eukaryotic host cells.

Plate 1 Histological sections of liver tissue from the chick embryo. Hematoxylin and eosin stain (x 165)

a) normal tissue of 14-day old embryo with dispersed red blood cells; b) liver tissue 5 days post-inoculation with 100 times the yolk sac LD_{50} of $\underline{C}$. $\underline{jejuni}$ strain WD 2483; c) liver tissue 1 day post-inoculation intra-yolk sac with bacteria-free filtrate originally containing 5×10^7 cfu/ml of strain WD 2483. Note the congestion (closed arrow) and slight edema with an absence of inflammatory cells in b and c.

LIVER

CONTROL

ORGANISM

TOXIN

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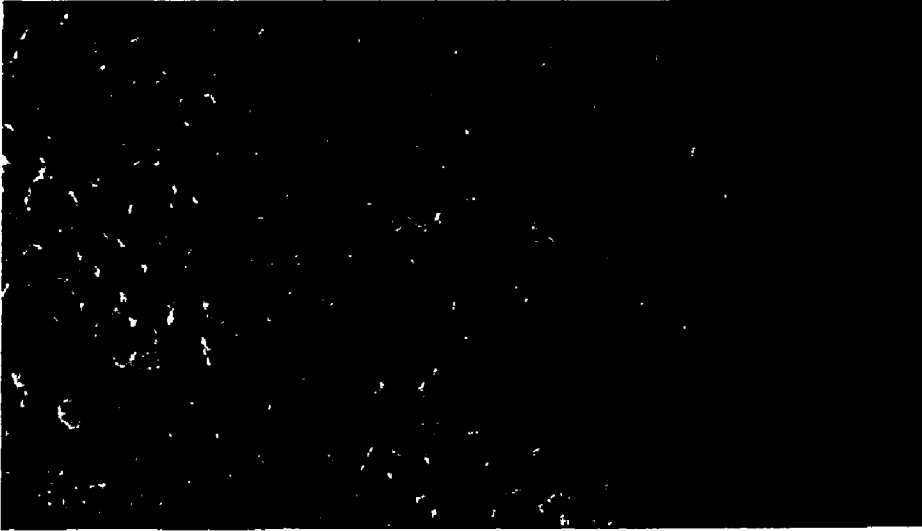
Plate 2 Histological sections of heart tissue from the chick embryo. Hematoxylin and eosin stain (x 165)
a) normal tissue of 14 day old embryo; b) heart tissue 5 days post-inoculation with 100 times the yolk sac LD_{50} of C. jejuni strain WD 2483; c) heart tissue 1 day post-inoculation intra-yolk sac with bacteria-free filtrate originally containing 5×10^7 cfu/ml of strain WD 2483. Note the vascular congestion (closed arrow) and edema (open arrow) with an absence of inflammatory cells in b and c.

HEART

CONTROL

ORGANISM

TOXIN

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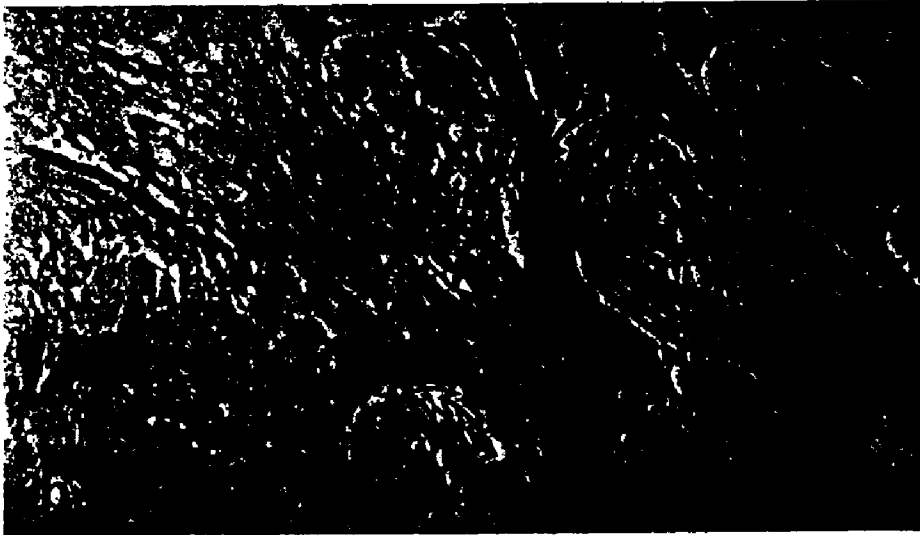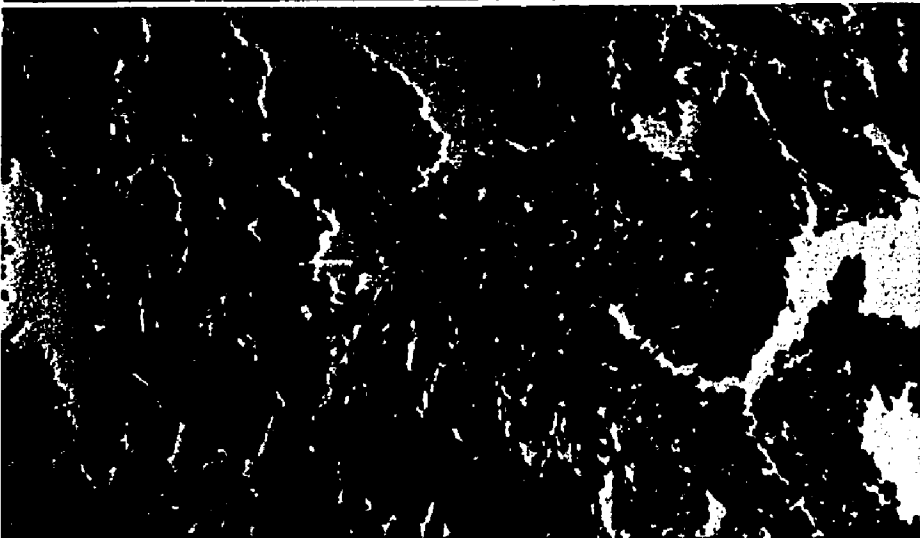
Plate 3 Histological sections of kidney tissue from the chick embryo. Hematoxylin and eosin stain (x 165)

a) normal tissue of 14 day old embryo showing the well spaced convoluted tubules, glomeruli and the circulating red blood cells; b) kidney tissue 5 days post-inoculation with 100 times the yolk sac LD_{50} of C. jejuni strain WD 2483; c) kidney tissue 1 day post-inoculation intra-yolk sac with bacteria-free filtrate originally containing 5×10^7 cfu/ml of strain WD 2483. Note the congestion of the blood vessels (closed arrow), slight edema (open arrow) and hemorrhage (long arrow) in b and c. An absence of glomeruli in the infected tissues and damaged, apparently condensed convoluted tubules were striking.

KIDNEY
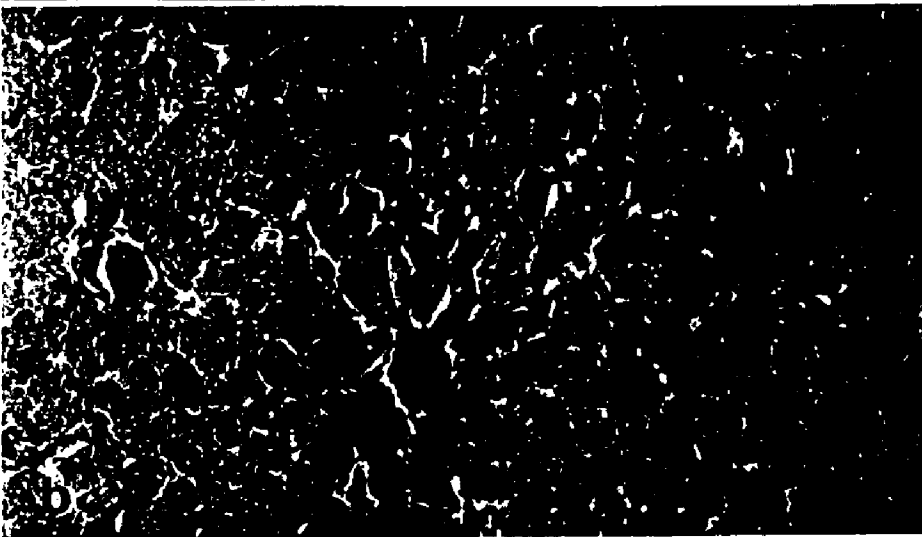
CONTROL

ORGANISM

TOXIN

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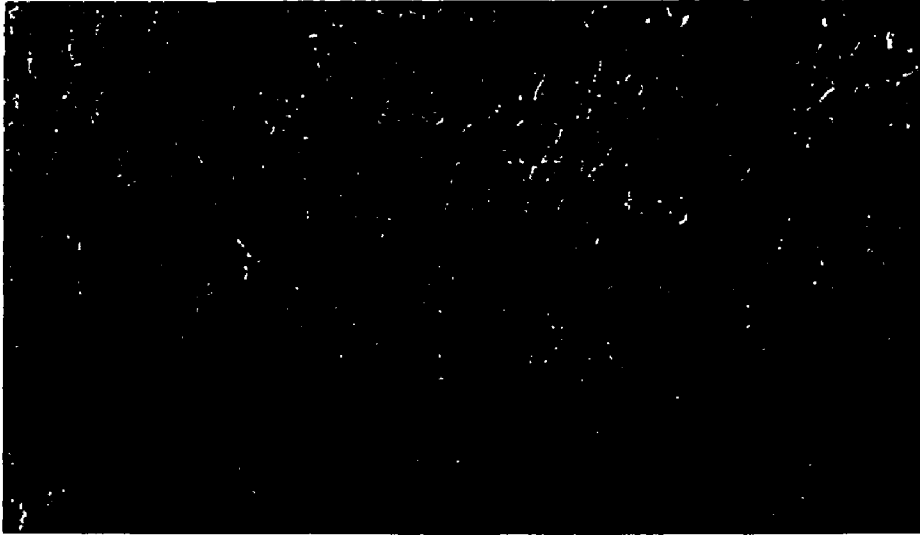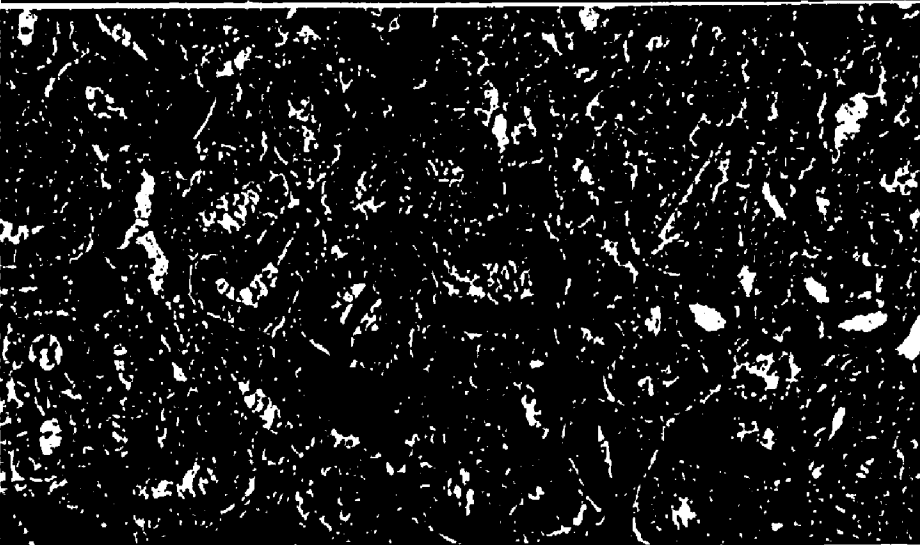
Plate 4 Histological sections of spleen tissue from the chick embryo. Hematoxylin and eosin stain (x 165)

a) normal tissue of 14 day old embryo with dispersed red blood cells; b) spleen tissue 5 days post-inoculation with 100 times the yolk sac LD_{50} of C. jejuni strain WD 2483; c) spleen tissue 1 day post-inoculation intra-yolk sac with bacteria-free filtrate originally containing 5×10^7 cfu/ml of strain WD 2483. Note in b and c the congestion (closed arrow), hemorrhage (long arrow) and an increase in red blood cells dispersed within the tissue. There was an absence of inflammatory cells in b and c.
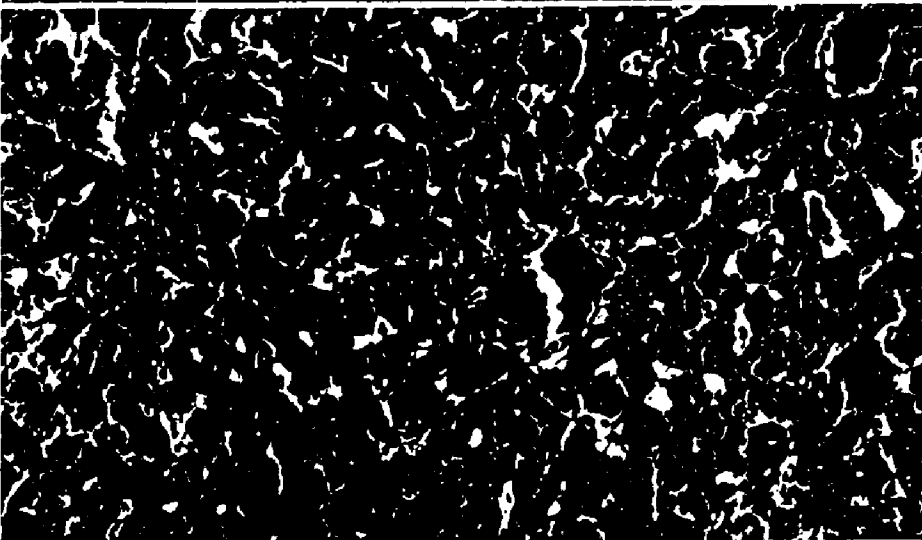
89

SPLEEN
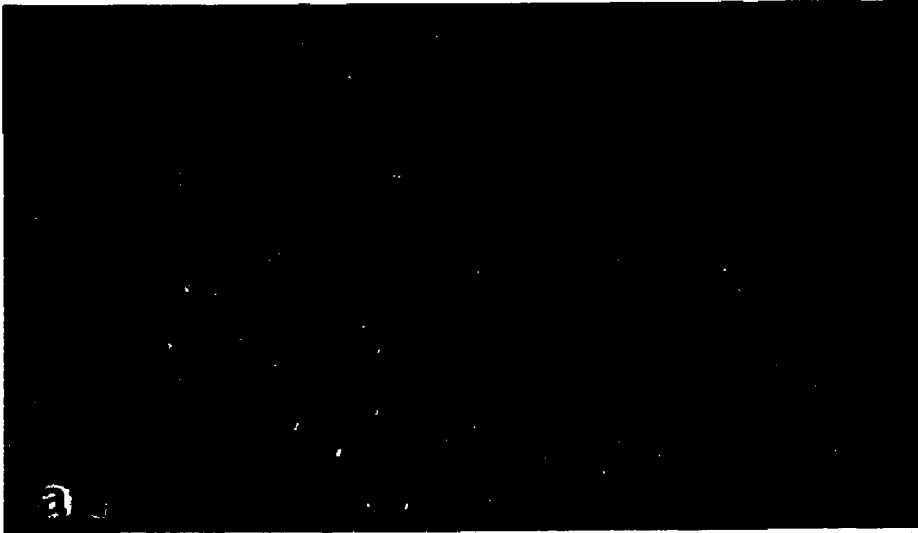
CONTROL
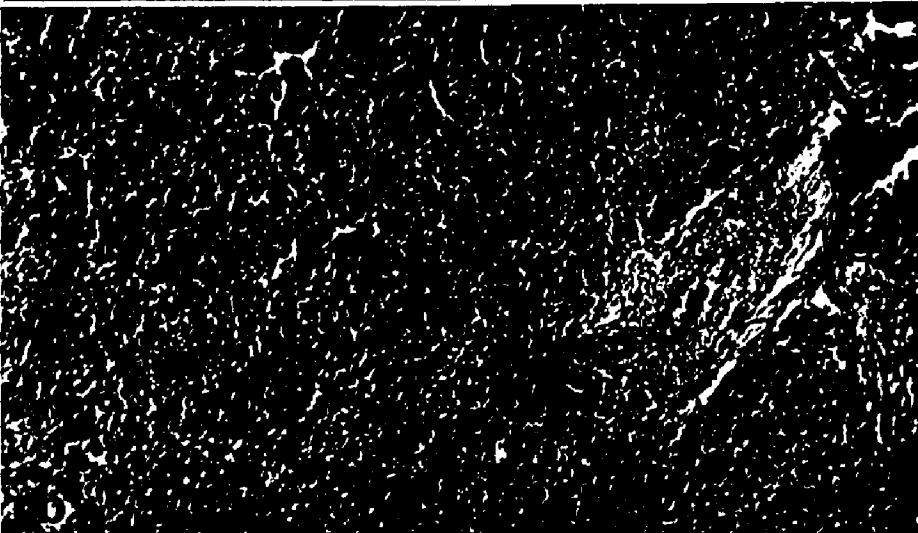
ORGANISM
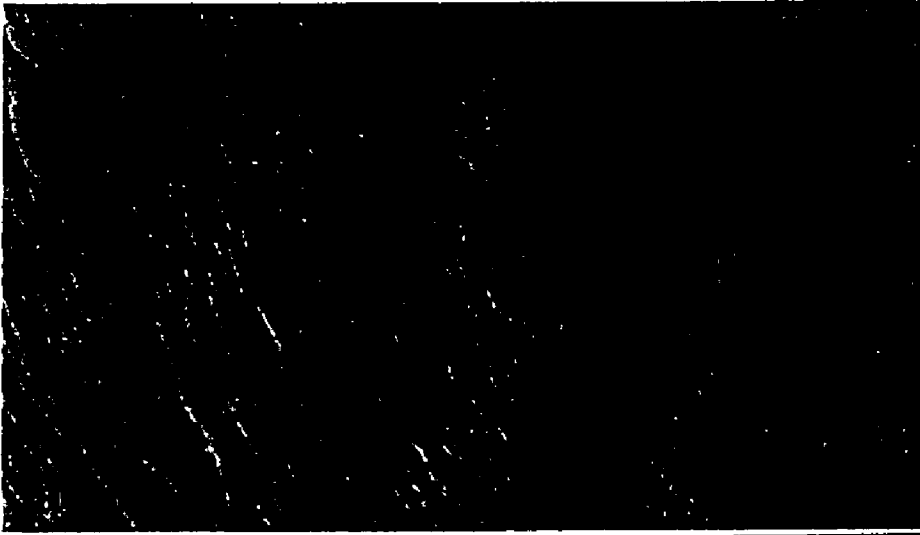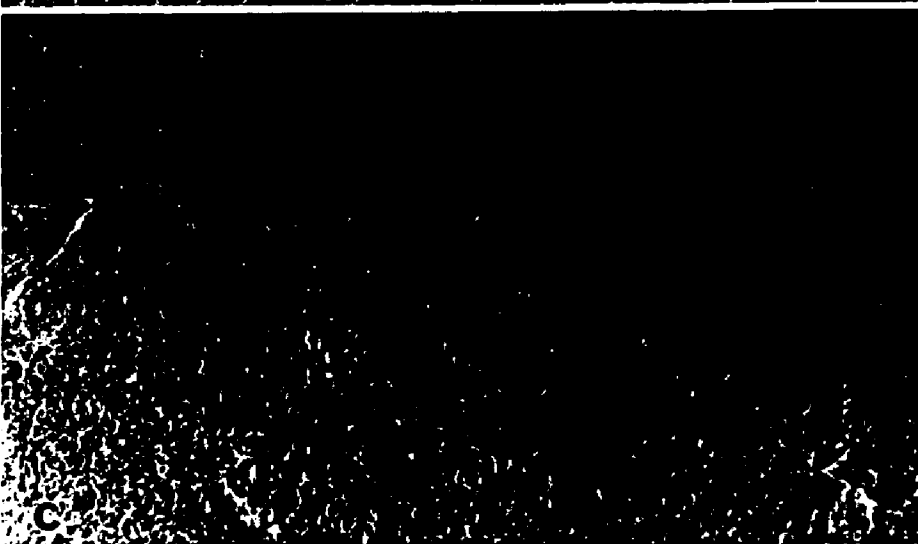
TOXIN

Plate 5 Histological sections of GI tract from the chick embryo. Hematoxylin and eosin stain (x 165)

a) normal tissue of 14 day old embryo; b) section of the GI tract 5 days post-inoculation with 100 times the yolk sac LD_{50} of $\underline{C}$. $\underline{jejuni}$ strain WD 2483; c) section of the GI tract 1 day post-inoculation intra-yolk sac with bacteria-free filtrate originally containing 5×10^7 cfu/ml of strain WD 2483. Note the absence of congestion, edema or hemorrhage in the infected tissue. The treated sections appeared as controls.

GI TRACT

CONTROL

ORGANISM

TOXIN

92
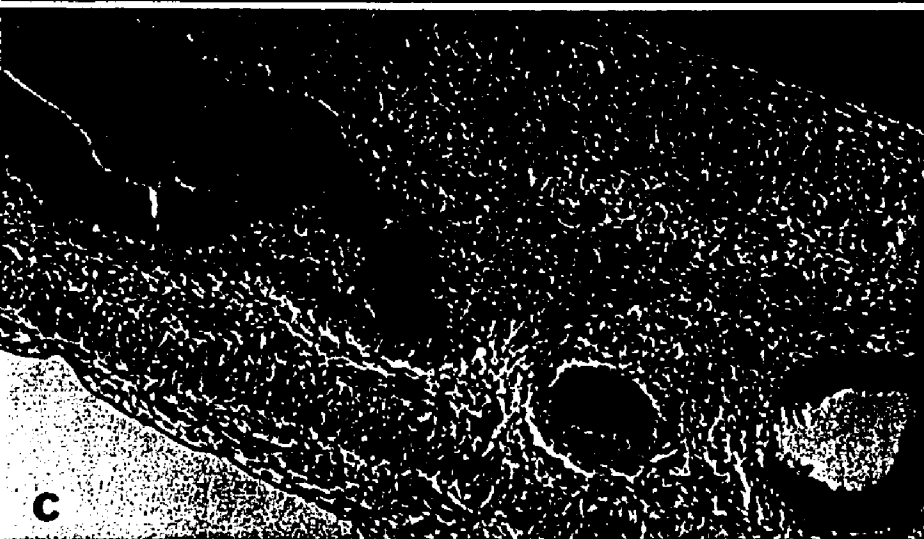
Plate 6 **Lane A**: non denaturing, discontinuous PAGE of the concentrated dialyzed bacterial cell-free filtrate of C. jejuni strain WD 2483 showing the eluted toxic protein (ETP); **Lane B**: 10% SDS-PAGE of the ETP showing two bands at 65 and 68 kilodaltons; **Lane C**: molecular weight markers at 14.3, 25.7, 43, 68, 97.4 and 200 kilodaltons; **Lane D**: western blot of the ETP profile from lane B treated with polyclonal antibody to strain WD 2483; **Lane E**: western blot of the ETP profile from lane B treated with the monoclonal antibody, CETPMAb$_4$.
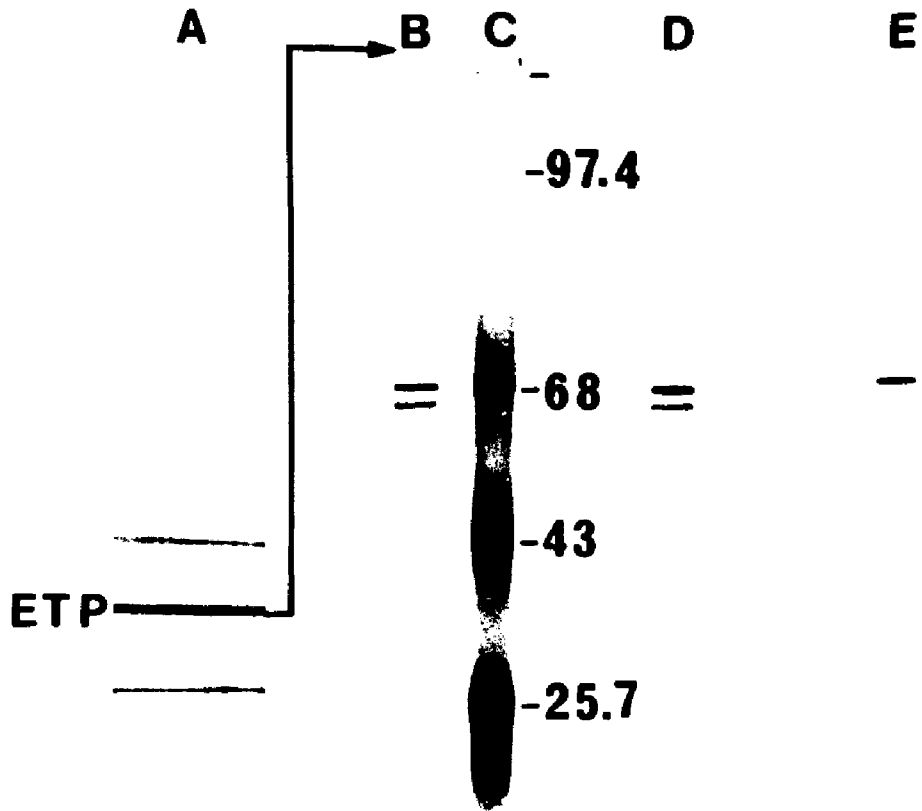
Plate 7 Phase contrast microscopic examination of primary chick embryo cells. (x 5280)
a) untreated cell monolayer; b) cells treated with C. jejuni strain WD 2483 at a multiplicity
of 100 cfu/cell for 12 h. Note the cell rounding, lysis and cellular vacuolation resembling
fatty degeneration.
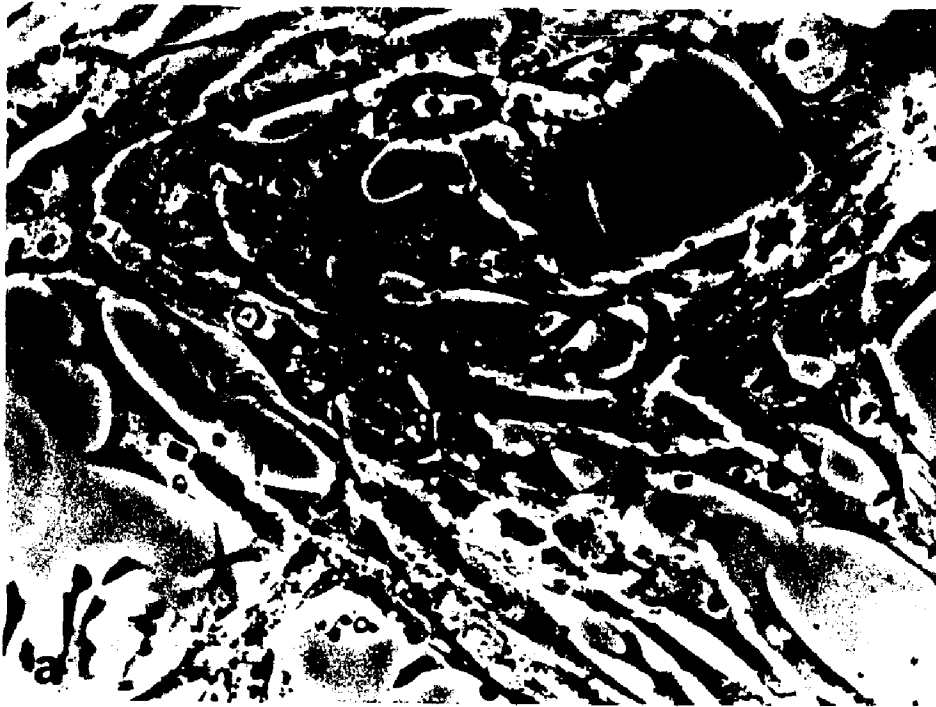
CONTROL

INFECTED

Plate 8 Phase contrast microscopic examination of Human Int 407 cells. (x 5280)
a) untreated cell monolayer; b) cells treated with C. jejuni strain WD 2483 at a multiplicity
of 100 cfu/cell for 12 h. Note the cell rounding, lysis and cellular vacuolation resembling
fatty degeneration.

CONTROL

INFECTED

98
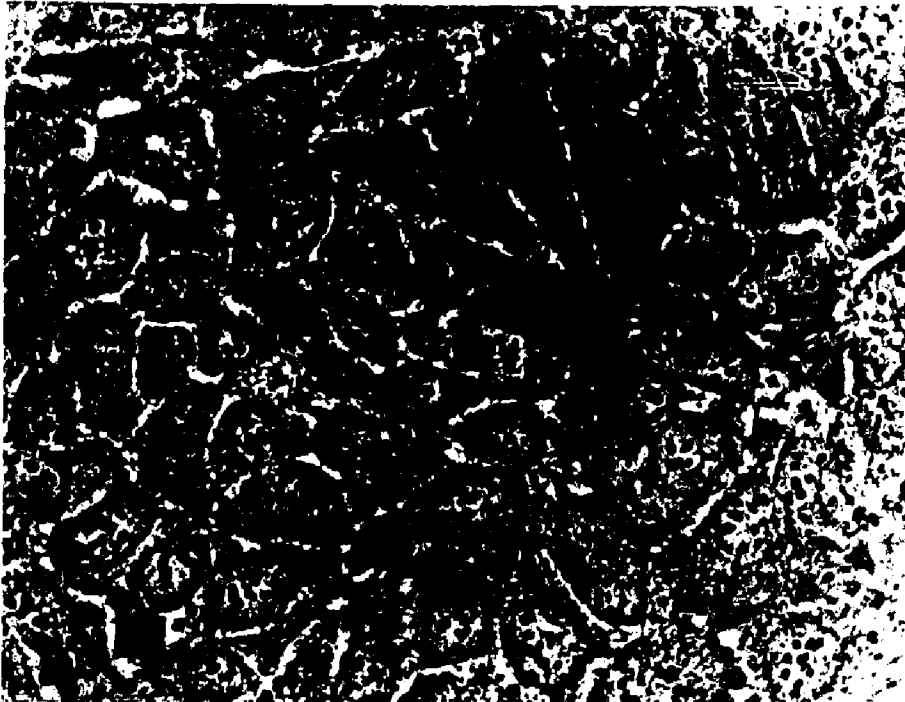
Plate 9 Electron micrograph of untreated control Int 407 cells illustrating the principal organelles in the eukaryotic host cell. Note the prominent golgi complex (arrow) in the cytoplasm along with numerous mitochondria and a large nucleus.
(x 24,000)   Bar = 1 μm .
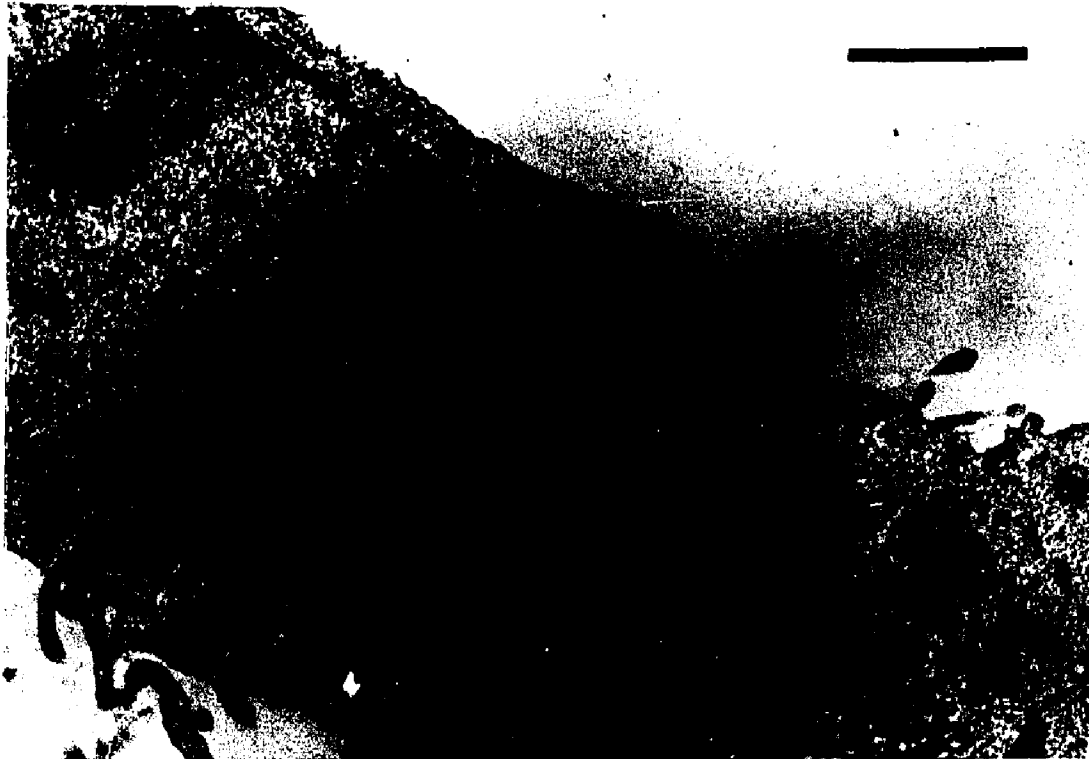
Plate 10 Electron micrograph of Int 407 cells inoculated with $\underline{C}$. $\underline{jejuni}$ strain WD 2483 at a multiplicity of 100 cfu/cell and incubated for 2 h. Other than a number of membrane enclosed amorphous-staining bodies (arrow) present in the cytoplasm, the cytology of these cells appeared relatively normal. Organisms were not evident. (x 27,000)   Bar = 1μm.

102
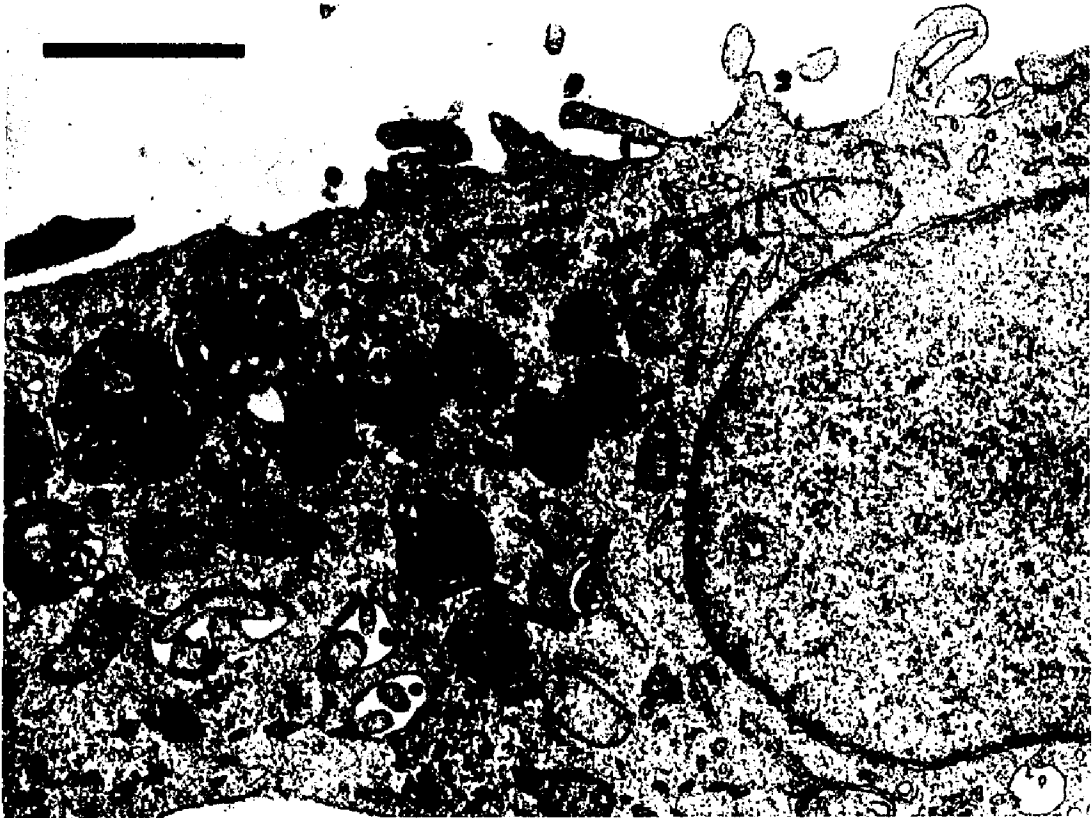
Plate 11 Electron micrograph of Int 407 cells inoculated with C. jejuni strain WD 2483 at a multiplicity of 100 cfu/cell and incubated for 6 h. A number of membrane enclosed amorphous-staining bodies occurred in the cytoplasm. Note the membrane lysis (arrow) and initial damage to the mitochondria. Organisms were not evident. (x 30,600)   Bar = 0.5 μm.

104
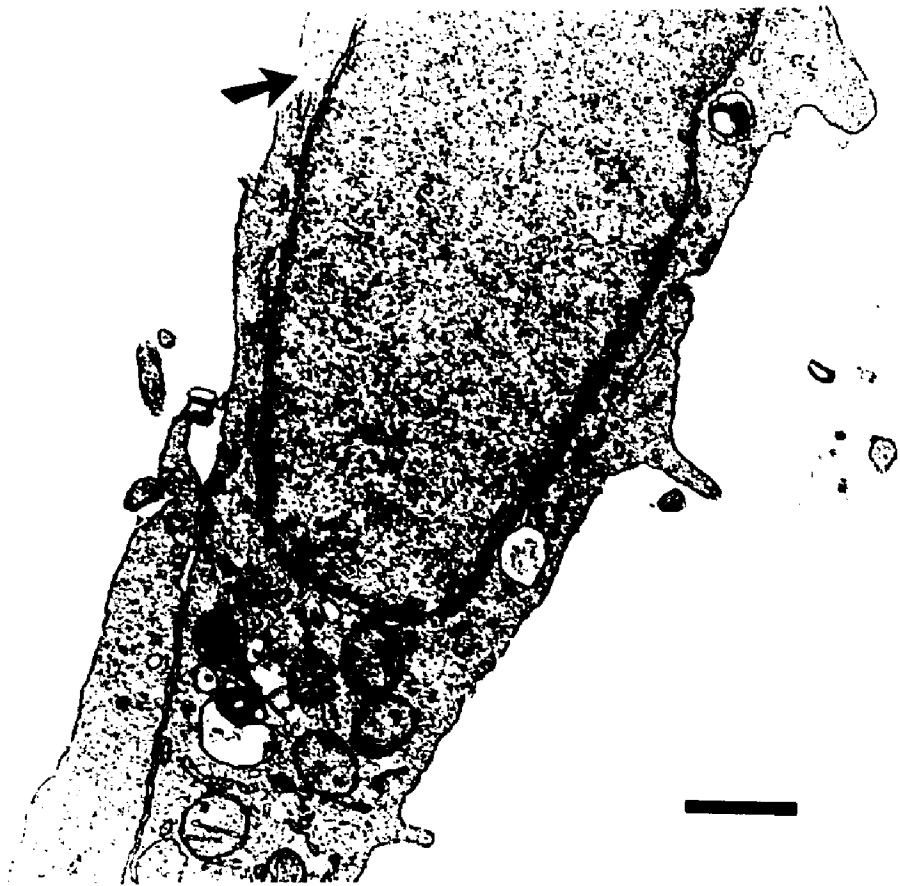
Plate 12 Electron micrographs of Int 407 cells inoculated with C. jejuni strain WD 2483 at a multiplicity of 100 cfu/cell and incubated for 12 h. Cell lysis, cytoplasmic clearing and margination of the chromatin are shown in both a and b Note an absence of organelles in a and ballooning of the nuclear membrane (closed arrow) and blunted microvilli (open arrow) in b. Organisms were not evident.
(a x 28,500; b x 22,800)   Bars = 1 μm.
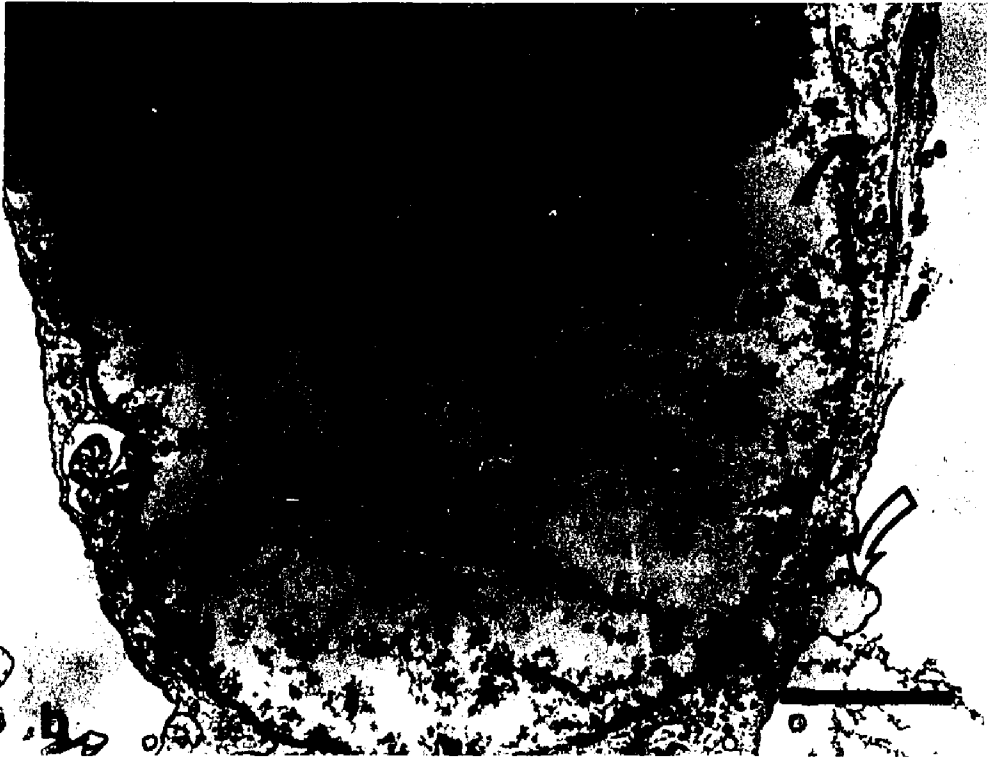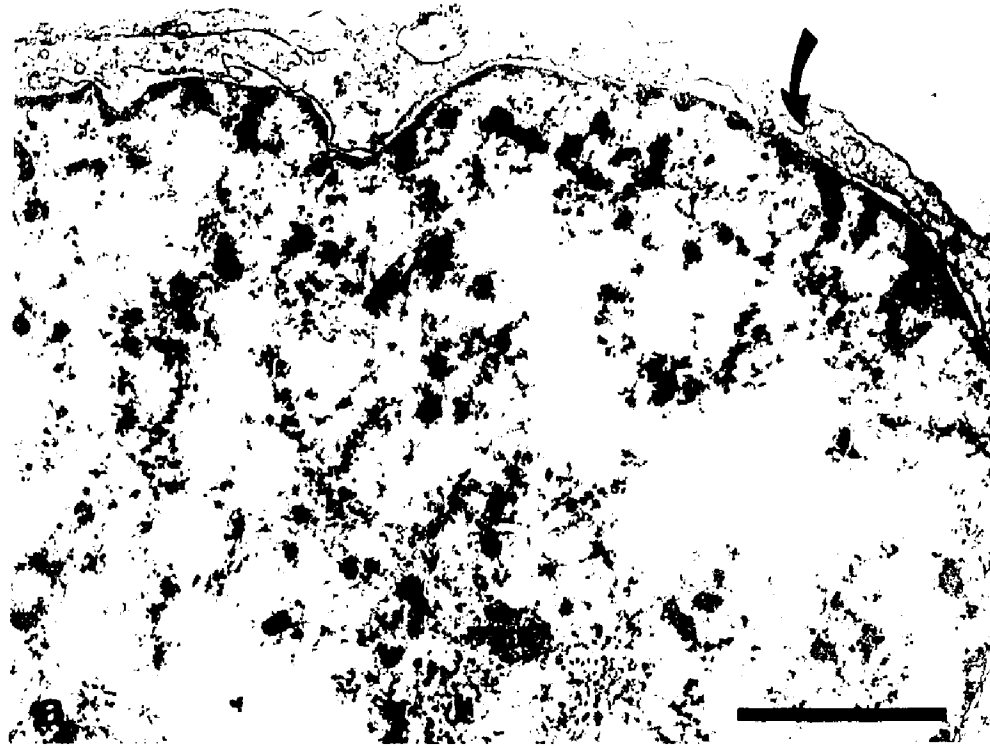
Plate 13 Electron micrographs of Int 407 cells inoculated with C. jejuni strain WD 2483 at a multiplicity of 100 cfu/cell and incubated for 24 h. Note cell lysis, cytoplasmic clearing, margination of the chromatin, ghost mitochondria and extensive ballooning of the nuclear membrane (closed arrow) in **a**. A majority of the cells were lysed as shown in **b**. Organisms were not evident.
(**a** x 12,000; **b** x 40,000)   Bars = 0.5 μm.

a

b

108
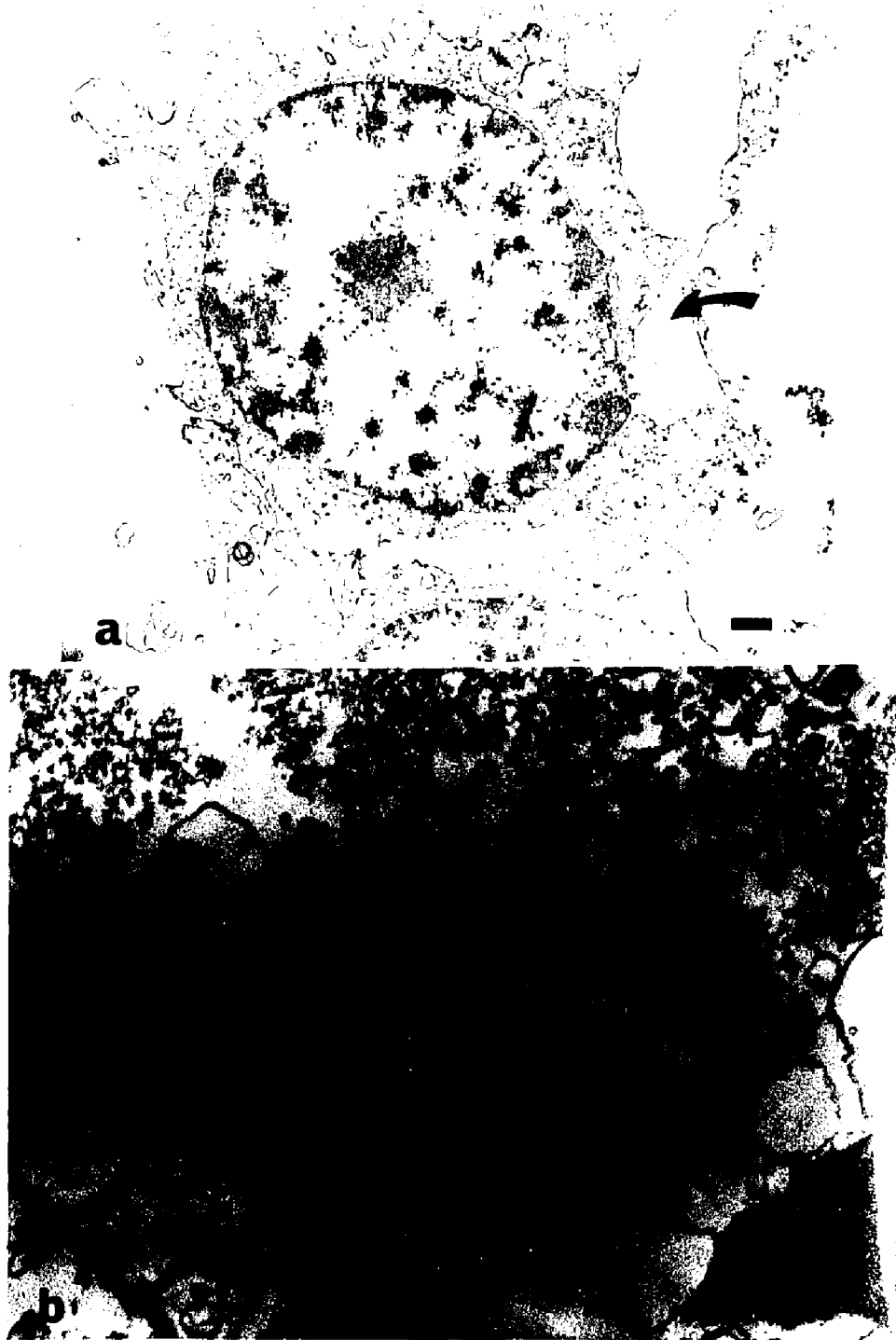
Plate 14 Scanning electron micrograph of untreated control Int 407 cells showing the "normal" surface of the cells. A few clumped cells are those fixed at a stage in cell division.
(x 2000)   Bar = 5 μm.

110

Plate 15 Scanning electron micrographs of Int 407 cells treated with C. jejuni strain WD 2483 at a multiplicity of 100 cfu/cell.
a) cells treated for 2 h with the organism; b) cells treated for 6 h with the organism; c) cells treated with 12 h with the organism; d) cells treated with 24 h with the organism. Note the progressive changes in the surface of the infected cells with time as the monolayer loses its integrity. Numerous blebs were seen on the cell surface and cell lysis was apparent. At no stage of infection were adherent organisms observed.
(a x 1500; b x 2500; c x 2500; d x 2500)   Bars = 5 μm.

a   — b

c   — d

112
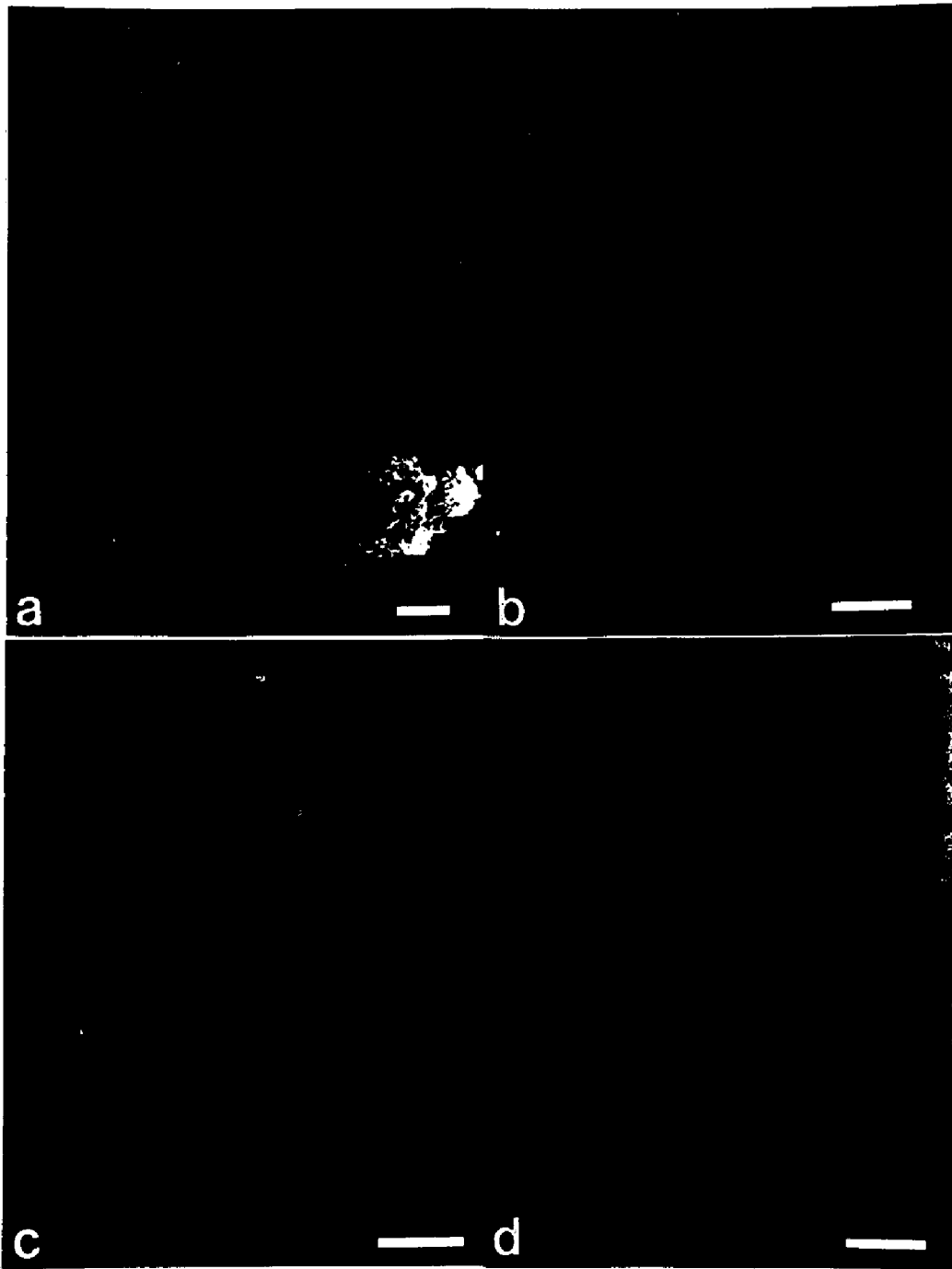
Plate 16 SDS-PAGE (10%) and Western blot transfer of the cellular fractions of C. jejuni strain WD 2483.
Lane a: molecular weight markers in kilodaltons; **Lane b**: outer membrane fraction showing ten discernable protein bands. The outer membrane protein profile is dominated by three bands at molecular weights of 25.7, 43 and 68 kilodaltons; **Lane c**: protein profile of the cytoplasmic membrane; **Lane d**: protein profile of the soluble proteins (cytoplasmic and periplasmic) showing more than 20 bands; **Lane e-h**: western blot transfer of soluble proteins, cytoplasmic membrane, outer membrane and molecular weight markers probed with the monoclonal antibody, CETPMAb4. Five bands in the outer membrane fraction of approximate molecular weights 20, 23, 35, 43 and 68 kilodaltons reacted with the antibody. Interestingly, the monoclonal antibody did not show any antigenic specificity for the proteins in the cytoplasmic membrane or the soluble protein fraction.

97.4 —
68 —
43 —
25.7 —
18.4 —
14.3 —

a b c d e f g h
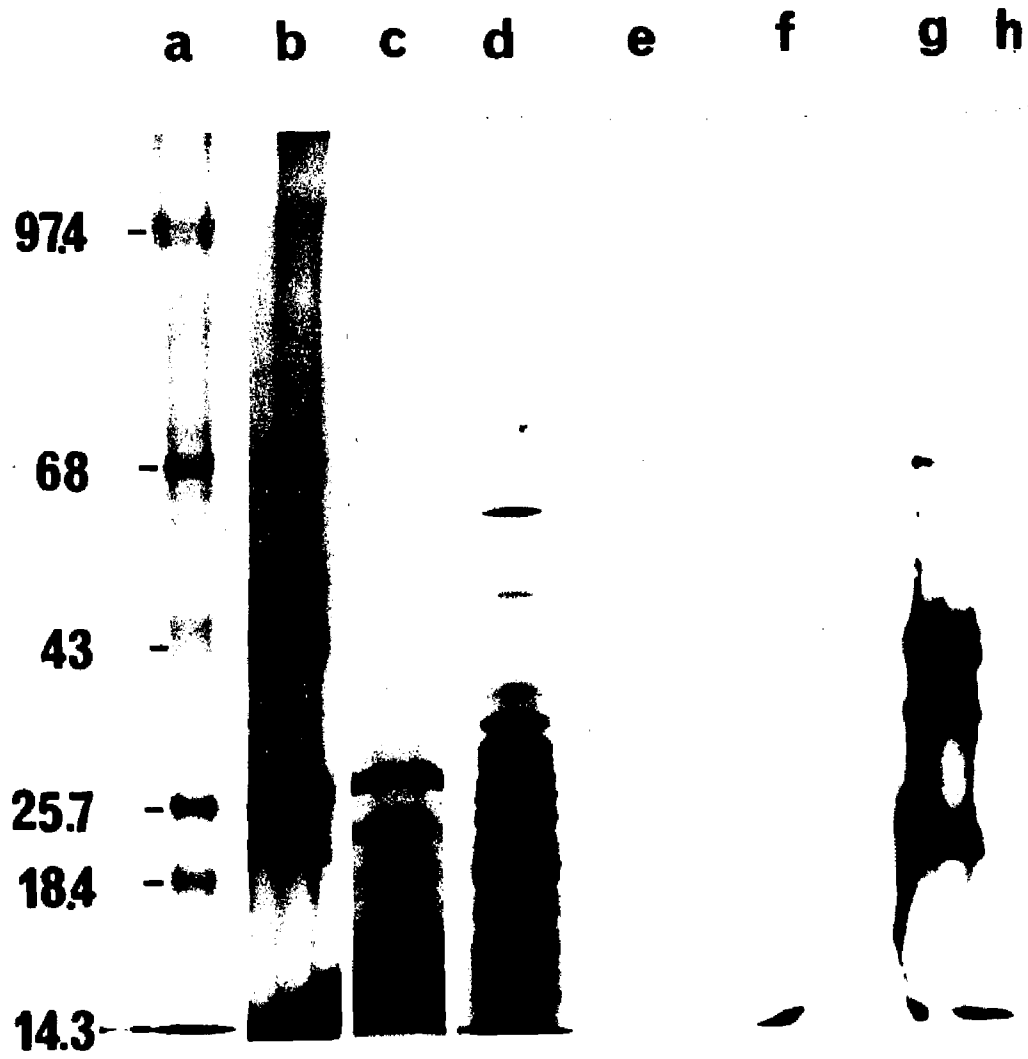
Plate 17 Electron micrographs of C. jejuni strain WD 2483 probed for the ETP using immunogold label.
a) control cells treated with PBS and the gold-conjugated goat anti-mouse serum. No gold probe particles are evident; b) freeze-thawed C. jejuni cells treated with the monoclonal antibody, CETPMAb$_4$ and probed with conjugated goat anti-mouse serum. The organism was very sensitive to the freeze-thaw procedure and gold particles were seen attached to the outer membrane only; c) S-shaped C. jejuni organism treated with CETPMAb$_4$ and the gold conjugated antisera showing the probe attached on the outer membrane.
(a x 80,000; b x 52,500; c x 52,500)   Bars = 0.25 μm.

115

a

b

c

116
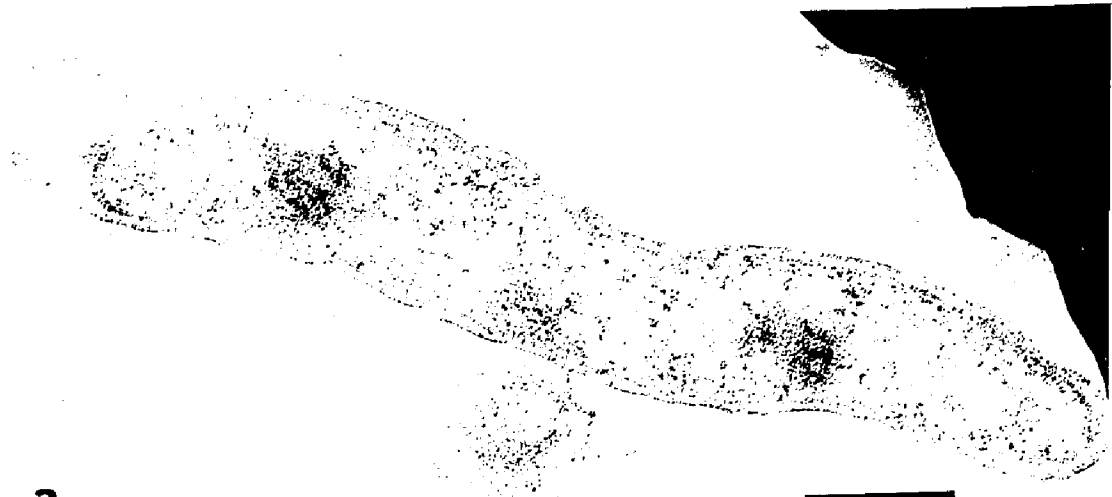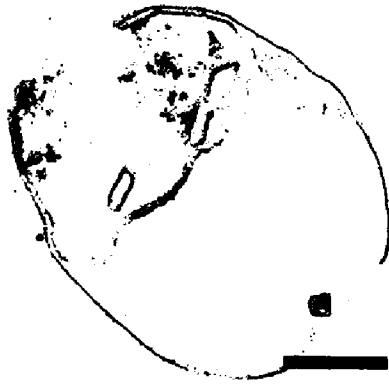
Plate 18 Toxic effect on CHO cells treated with ETP and enterotoxins from V. cholerae and E. coli (100 ng/ml) for 24 h as observed by phase contrast microscopy. (x 3960) a) untreated control showing confluent cell monolayer; b) cells treated with ETP. Note the rounding and lysis of affected cells; c) cells exposed to V. cholerae enterotoxin; d) cells exposed to E. coli enterotoxin. In contrast to the activity of the ETP, cell elongation was the characteristic feature due to treatment with the enterotoxins of V. cholerae and E. coli.

CONTROL

C. JEJUNI

V. CHOLERAE

E. COLI

Plate 19 Cytotoxic effect of the ETP on primary chick embryo fibroblast cells observed by phase contrast microscopy (x 5280)
a) untreated control cells; b) cells treated with the ETP for 12 h. Note the cell rounding, cell lysis and cellular vacuolation resembling fatty degeneration.

CONTROL

TOXIN

120
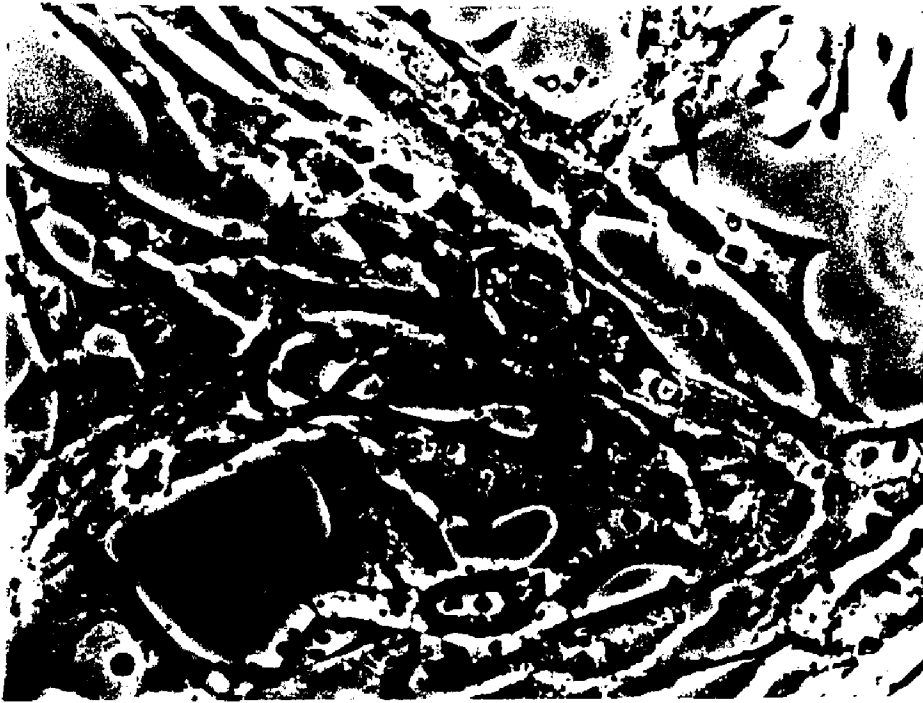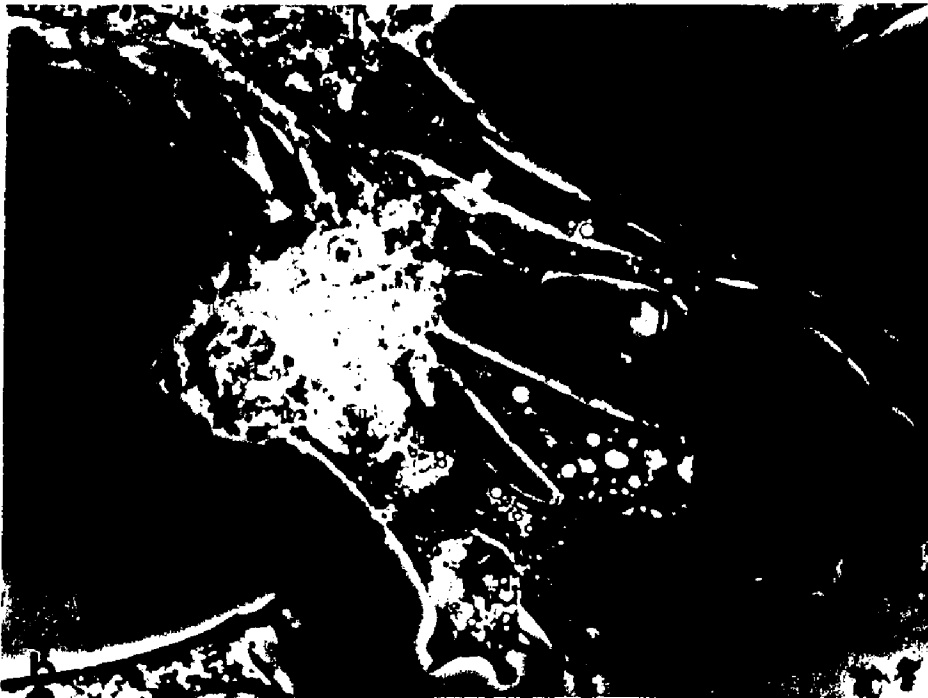
Plate 20 Cytotoxic effect of the ETP on Human Int 407 cells observed by phase contrast microscopy (x 5280)
a) untreated control showing confluent cell monolayer; b) cells treated with the ETP for 12 h. Note the cell rounding and extensive cell lysis.
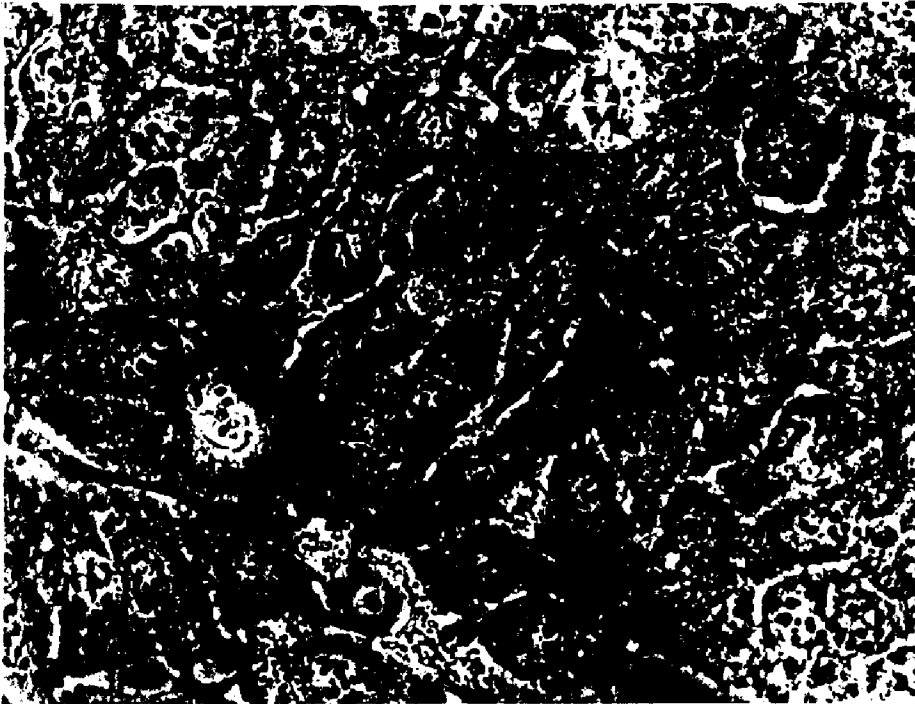
CONTROL

TOXIN

Plate 21 Electron micrograph of untreated control Int 407 cells. Note the mitochondria with their complex membrane structure (closed arrow) and the flattened cisternae of the endoplasmic reticulum oriented in parallel fashion (open arrow).
(x 24,000)   Bar = 1 μm.
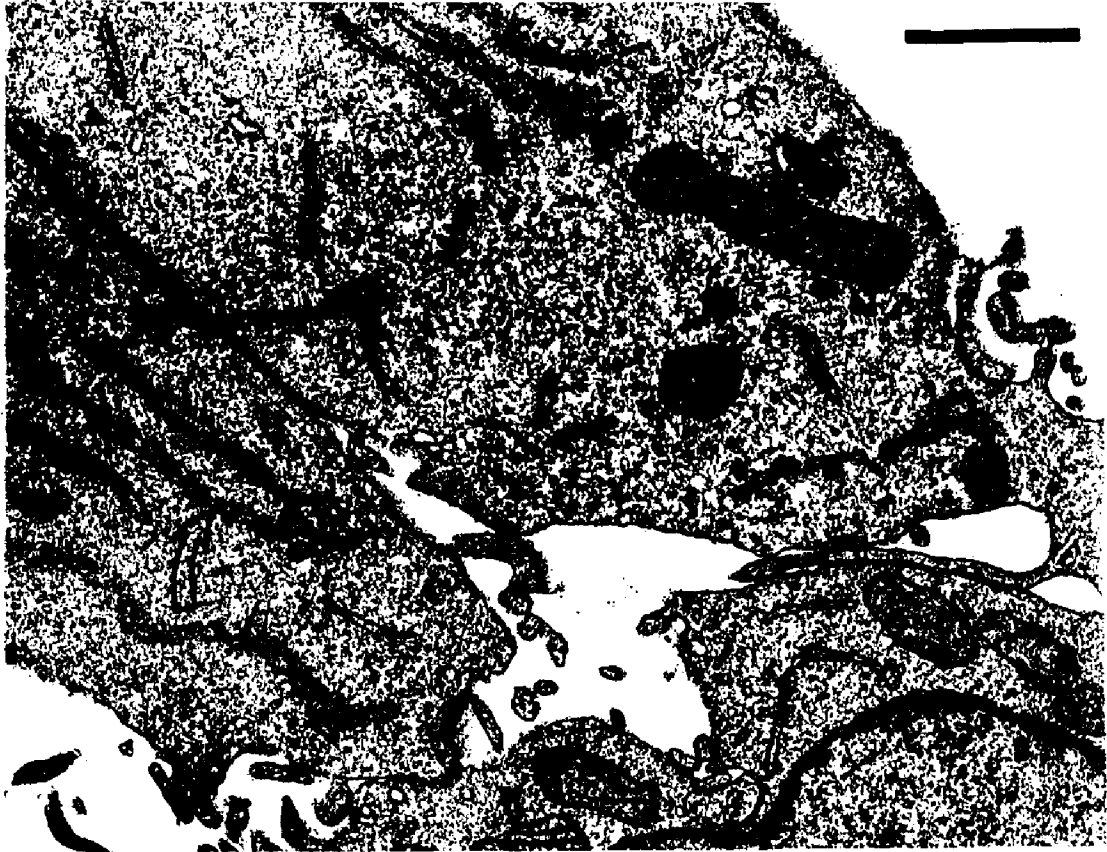
Plate 22 Electron micrographs of Int 407 cells treated with the ETP for 2 h. The cells were subsequently treated with the monoclonal antibody, CETPMAb$_4$ and probed with gold conjugated goat anti-mouse serum.
Note the damage to the mitochondria (open arrow) in both **a** and **b**. The initial stages of cytoplasmic clearing (closed arrow) are apparent in **a**. The gold particles were attached only to the cell membrane in the region of the microvilli as shown in **b** (long arrow).
(**a** x 32,000; **b** x 19,200)   Bars = 0.5 μm.

126
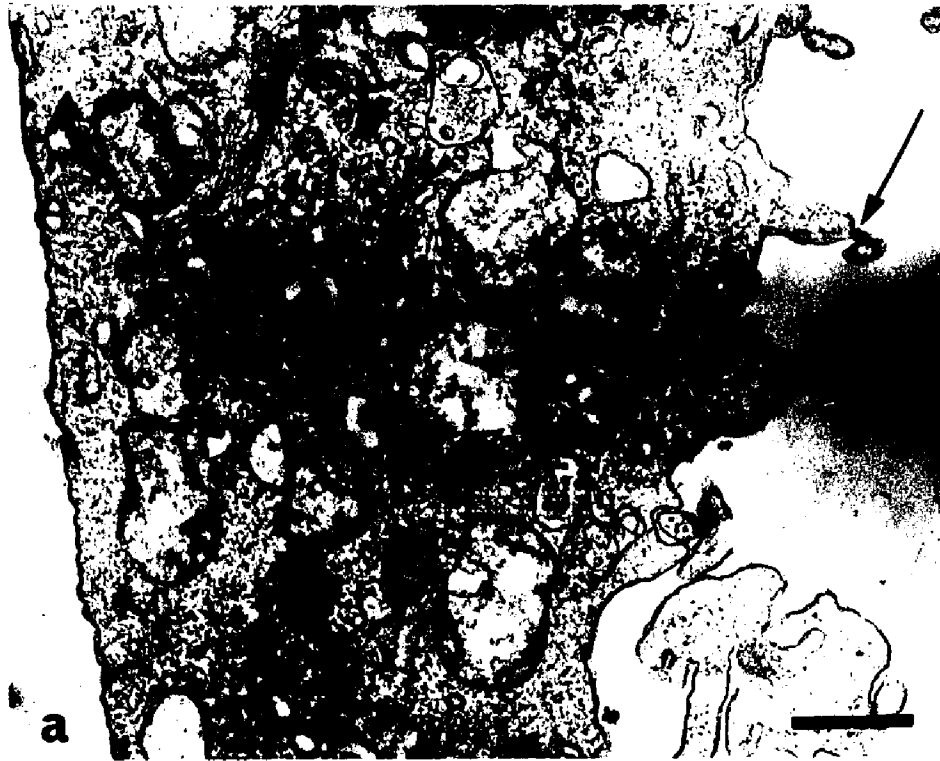
Plate 23 Electron micrographs of Int 407 cells treated with the ETP for 6 h. The cells were subsequently treated with the monoclonal antibody, CETPMAb₄ and probed with gold conjugated goat anti-mouse serum.
Note the extensive damage to the mitochondria, as well as membrane lysis with extrusion of the cytoplasm (open arrow) in both a and b. Cytoplasmic clearing and ballooning of the nuclear membrane (closed arrow) are evident in a and empty mitochondria in b. The gold probe was seen attached to the membrane surfaces only (long arrow).
(a x 18,000; b x 18,000)   Bars = 1 μm.

a

128
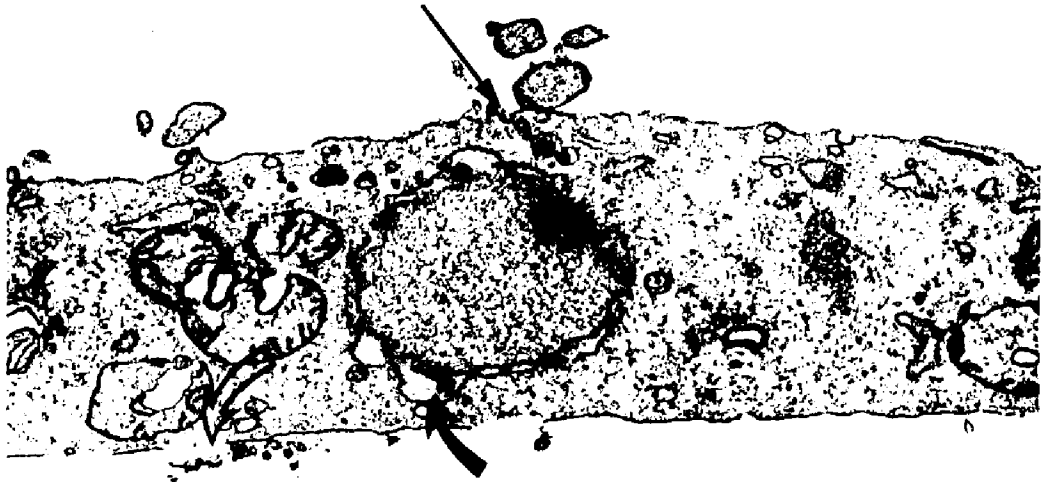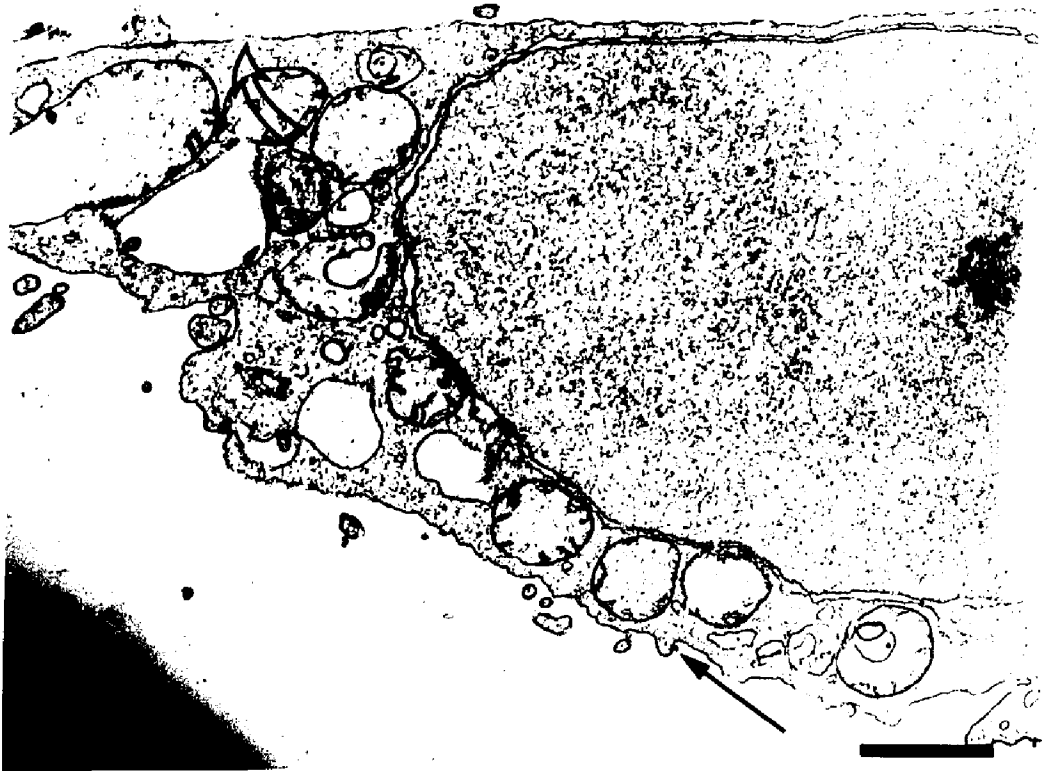
Plate 24 Electron micrographs of Int 407 cells treated with the ETP for 12 h. The cells were subsequently treated with the monoclonal antibody, CETPMAb4 and probed with gold conjugated goat anti-mouse serum.
Few surviving membrane bound cells were observed. As shown in a extensive ballooning of the nuclear membrane (closed arrow), cytoplasmic clearing with empty mitochondria are evident. A majority of the cells were lysed (b) and no longer membrane bound (c). Note the extrusion of the cytoplasm (open arrow) and ballooning of the nuclear membrane (closed arrow) along with extensively damaged mitochondria in b. The gold particles were still seen to be membrane bound and not associated with cytoplasmic content (long arrow).
(a x 21,600; b x 15,000; c x 21,600)   Bars = 1 μm.

a

b
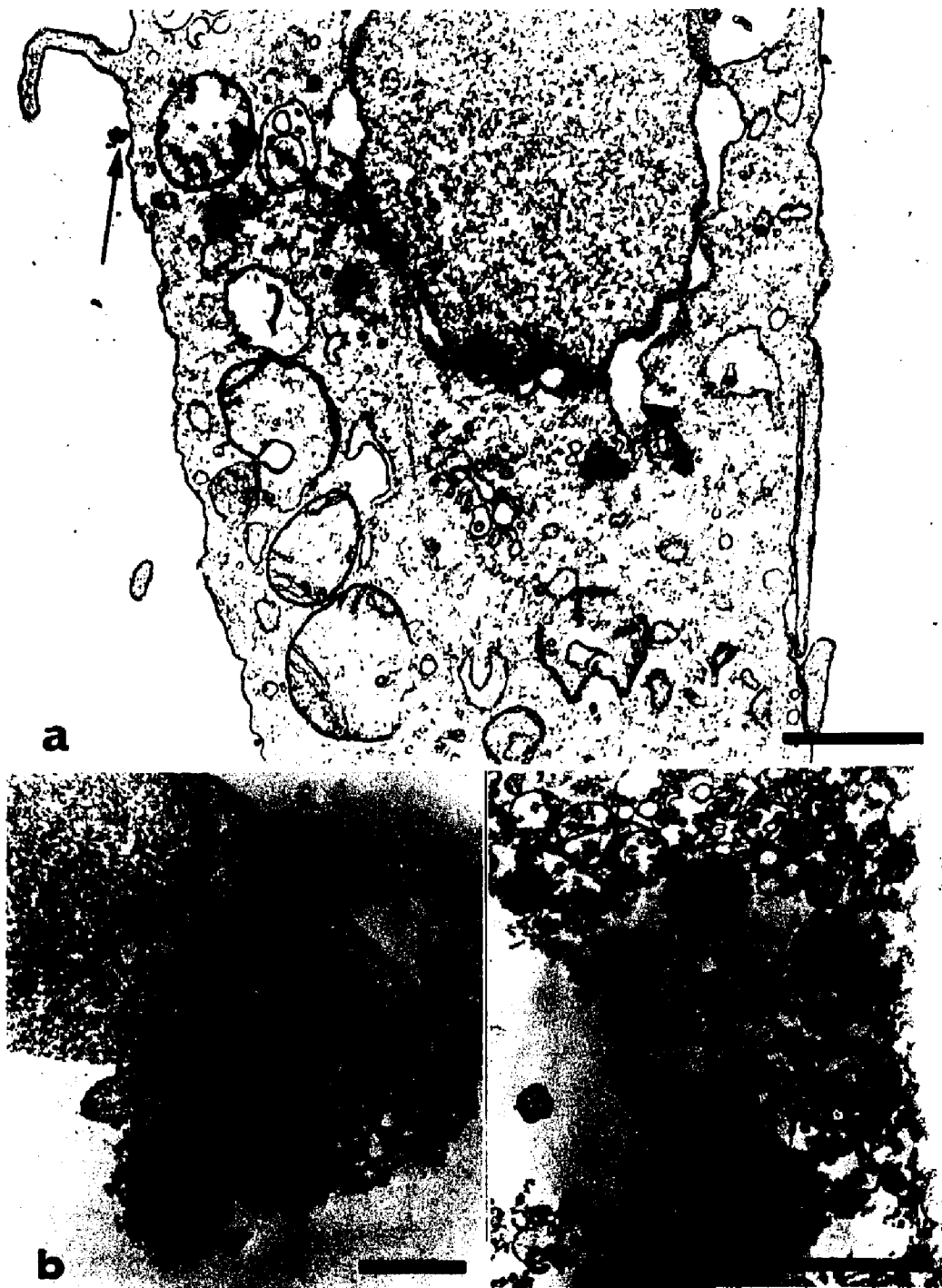
Plate 25 Electron micrographs of Int 407 cells treated with the ETP for 12 h and freeze-thawed. The cells were subsequently treated with the monoclonal antibody, CETPMAb$_4$ or PBS and probed with gold conjugated goat anti-mouse serum.
a represents ETP treated cells which were incubated with PBS (no monoclonal antibody) b represents ETP treated cells probed with CETPMAb$_4$ and the second conjugated antibody. Note the membrane lysis, cytoplasmic clearing, margination of the chromatin, empty mitochondria (open arrow) and ballooning of the nuclear membrane (closed arrow) in both a and b. At no time were any gold particles seen on either of these cells, either membrane bound or in the cytoplasm.
(a x 14,400; b x 14,400)   Bars = 1 μm.

a
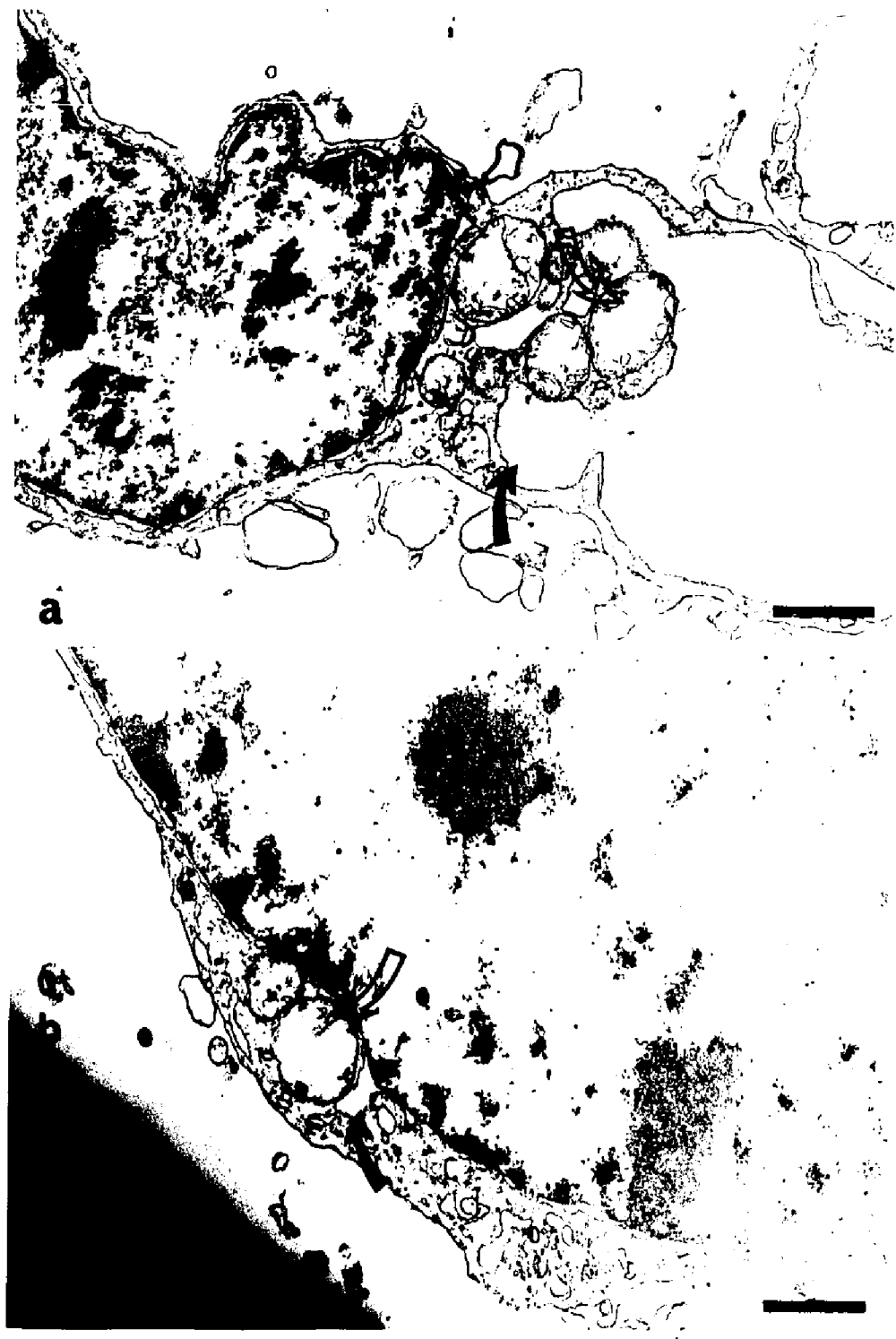
Plate 26 Scanning electron micrograph of untreated control Int 407 cells showing the "normal" surface of the cells. A few clumped cells are those fixed at a stage in cell division.
(x 2000)   Bar = 5 μm.

134
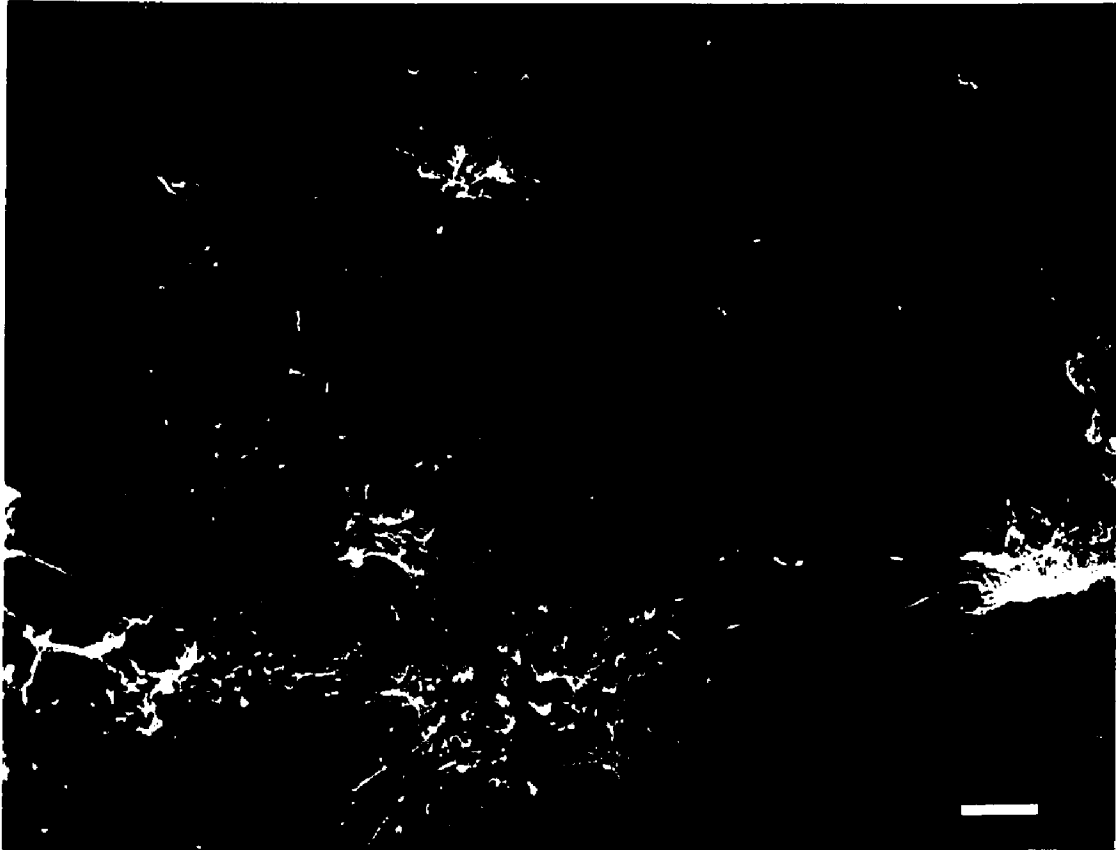
Plate 27 Scanning electron micrographs of Int 407 cells treated with the ETP for different time periods.
a) cells treated for 2 h with the toxin; b) cells treated for 6 h with the toxin; c) & d) cells treated for 12 h with the toxin. Note the progressive changes in the surface of the infected cells with time as the monolayer looses its integrity. The toxic effect was evident even at 2 h but by 12 h a majority of the cells were in clumps. Numerous blebs were seen on the cell surface and cell rounding and lysis was apparent.
(a x 1500; b x 1500; c x 2500; d x 2500)   Bars = 5 μm.
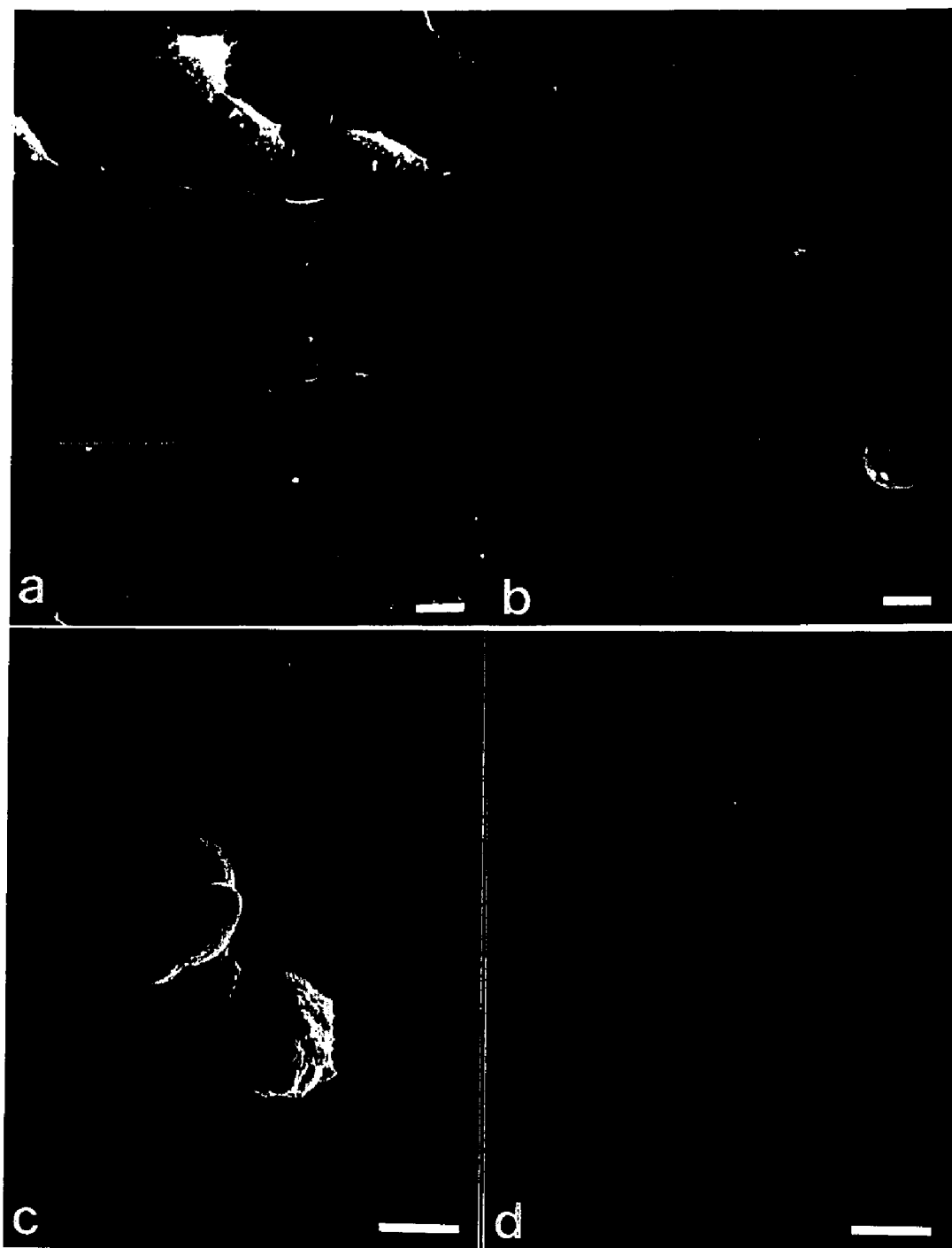
Plate 28 SDS-PAGE (10%) and Western blot transfer of the cellular fractions of Int 407 cells treated with the ETP for 12 h.
Lane a: protein profile of the soluble cytoplasmic fraction; Lane b: protein profile of the membrane fraction; Lane c: protein profile of the nuclei fraction; Lane d: protein profile of the small granule fraction; Lane e: molecular mass markers in kilodaltons; More than 60 protein bands were present in each of these fractions. Lane f-j: western blot transfer of the soluble cytoplasmic, membrane, nuclei, small granule fractions and molecular weight markers probed with the monoclonal antibody, CETPMAb$_4$. Four bands in the membrane fraction of approximate molecular weights 32, 43, 54 and 63 kilodaltons reacted with the antibody. The monoclonal antibody showed no antigenic specificity for the proteins in the other three fractions.

a b c d   e   f g h i   j

200

97.4

68

43

25.7
18.4

14.3

138
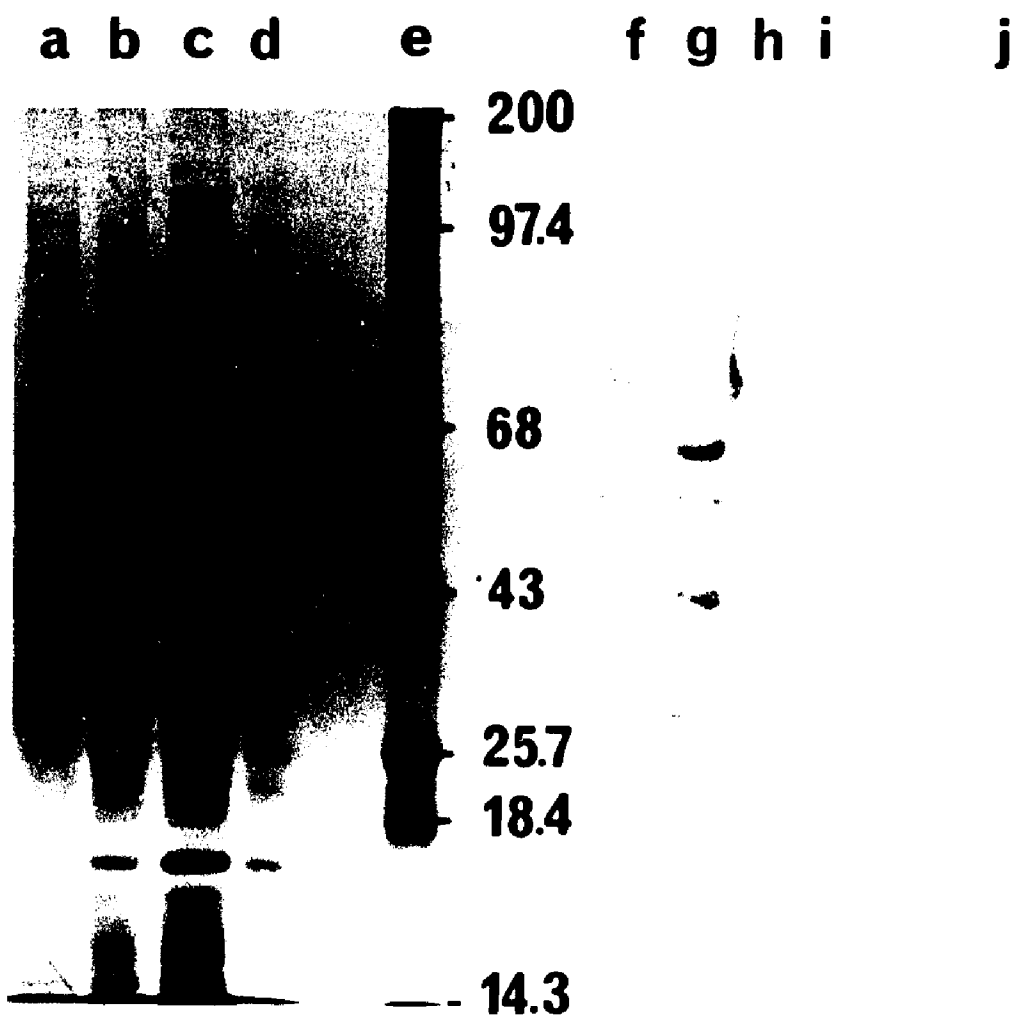
Plate 29 Location of the toxin on Int 407 cells by indirect immunofluorescence.
Glutaraldehyde fixed ETP treated cells were subsequently incubated with monoclonal
antibody CETPMAb4 and labelled with goat anti-mouse FITC and showed positive
fluorescence as shown in a. Cells treated with ETP, subsequently treated with trypsin to
remove extracellular bound toxin and probed with the primary and secondary antibody
showed no fluorescence shown in b. (x 660)
Similar results were observed in cells probed with the polyclonal antibody.

TOXIN

TOXIN
+
TRYPSIN

140
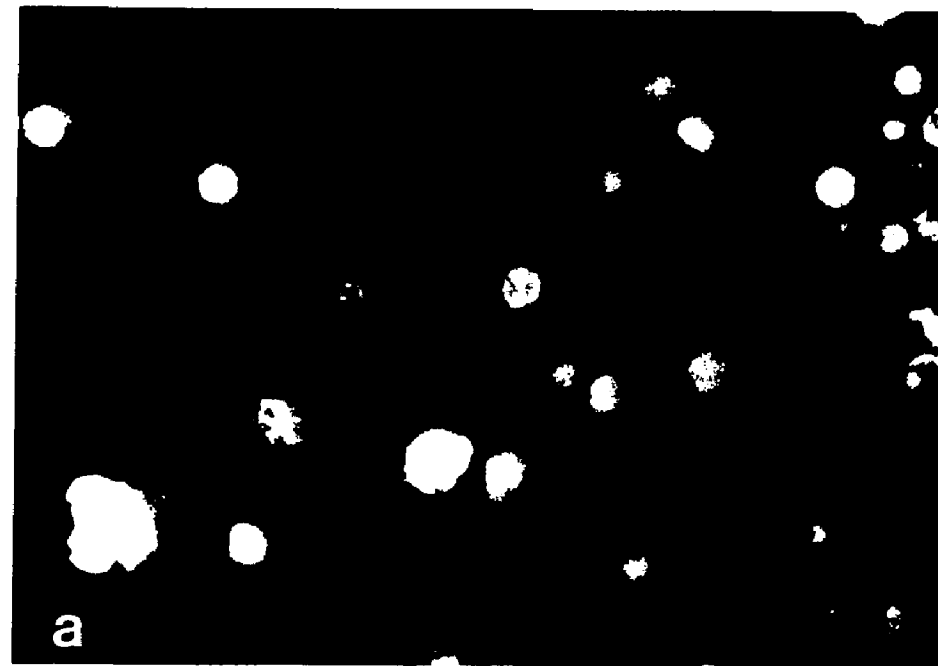
Plate 30 Location of the toxin on Int 407 cells by indirect immunofluorescence. Methanol fixed ETP treated cells were subsequently incubated with polyclonal antibody to strain WD 2483 and labelled with goat anti-rabbit FITC and showed positive fluorescence as shown in a. Cells treated with ETP, subsequently treated with trypsin to remove extracellular bound toxin and probed with the primary and secondary antibody showed no fluorescence shown in b. (x 660)
Similar results were observed in the cells treated with CETPMAb$_4$

TOXIN

TOXIN
+
TRYPSIN

142
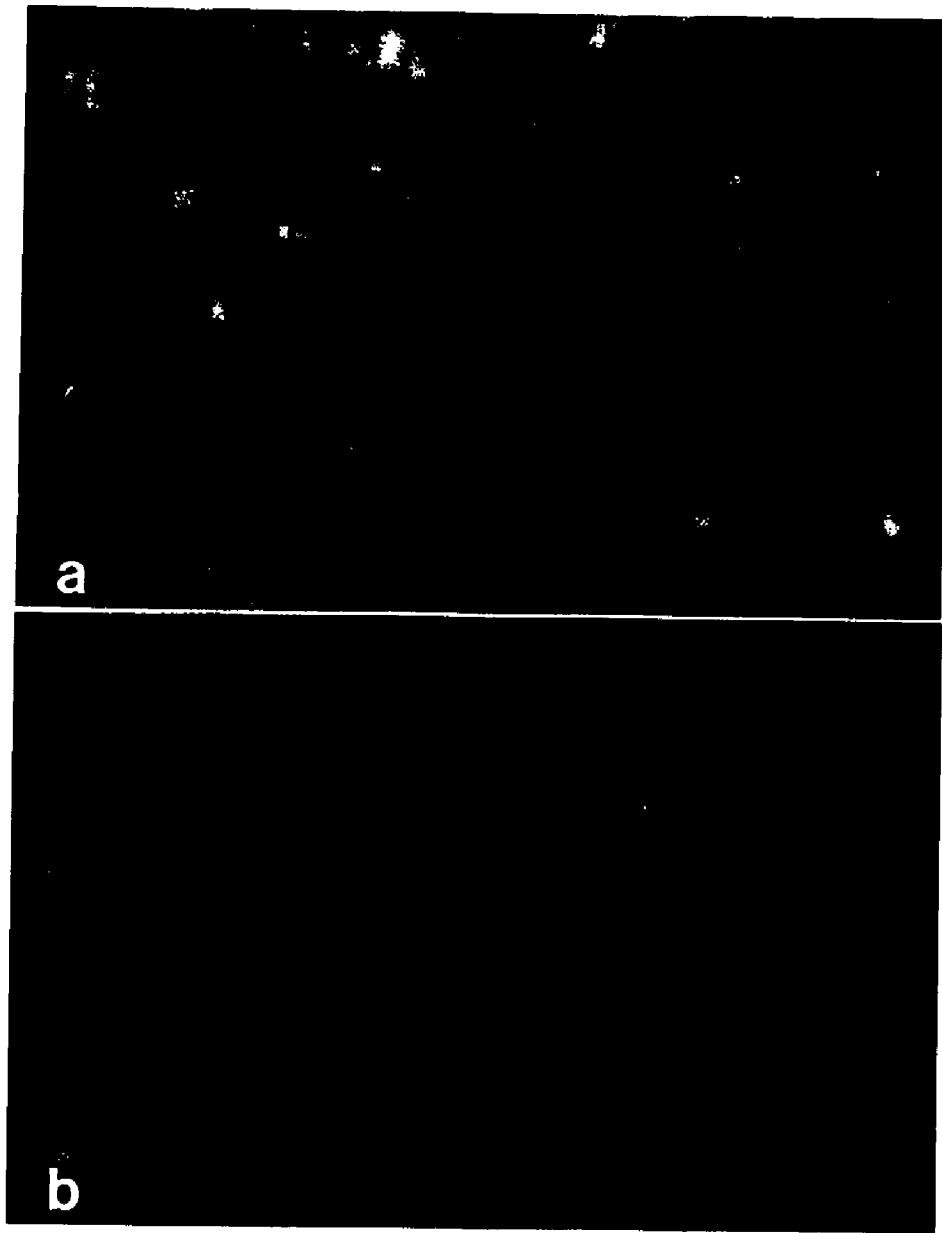
Plate 31 Location of the toxin on lysed Int 407 cells by indirect immunofluorescence. Methanol fixed ETP treated cells were lysed, incubated with polyclonal antibody to strain WD 2483 and labelled with goat anti-rabbit FITC and showed positive fluorescence as shown in a. Cells treated with ETP, subsequently treated with trypsin to remove extracellular bound toxin, lysed and probed with the primary and secondary antibody showed no fluorescence as shown in b. (x 660)
Similar results were observed for cells treated with CETPMAb$_4$

TOXIN

TOXIN
+
TRYPSIN

144
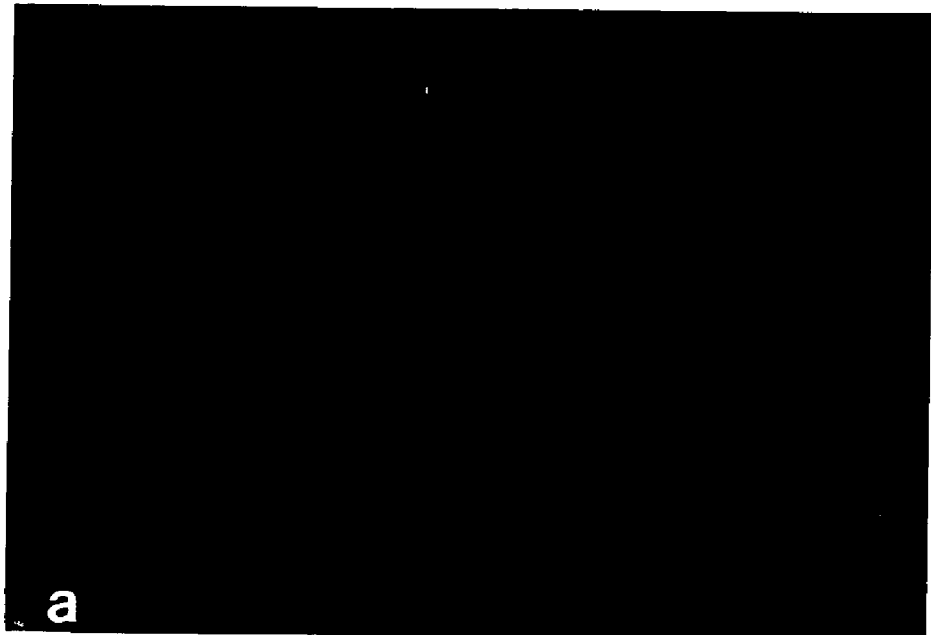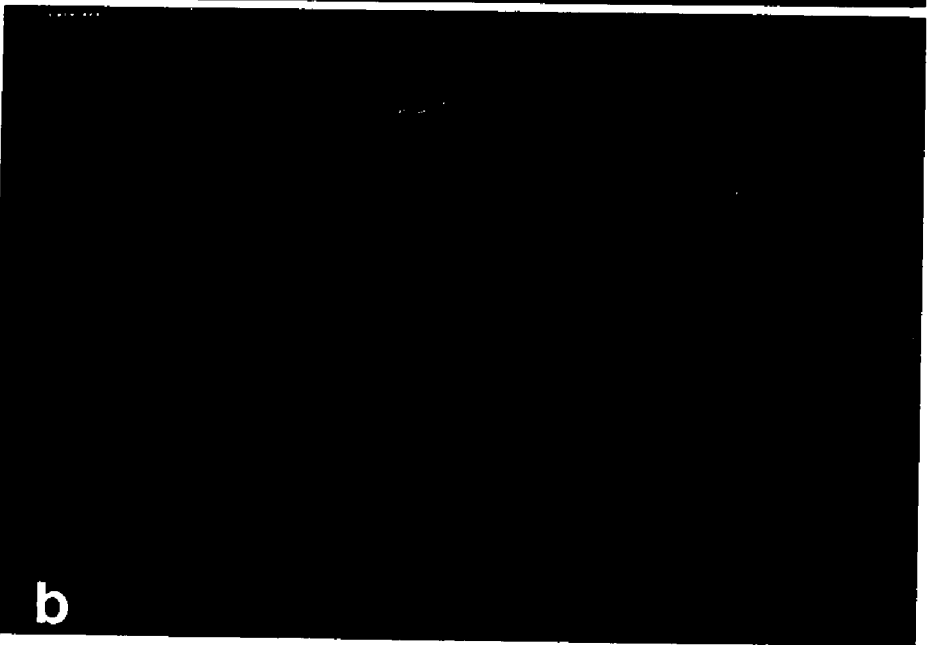
DISCUSSION

The pathogenesis of Campylobacter jejuni has been widely investigated but the nature of the bacterial virulence factors and the mechanisms by which this organism initiates enteric disease are not clear. The organism is responsible for the secretory form of diarrhea seen particularly in children in developing countries but in United States and other developed countries most cases of Campylobacter enteritis manifest as an inflammatory process accompanied with fever (26, 42, 78, 100). The presence of non-pathogenic campylobacteria in the environment may account for the high incidence of asymptomatic carriage in domestic and wild animals (101) and in humans in developing countries (115).

To date, there has been no suitable animal model for the investigation of the pathogenic strategies of C. jejuni. Studies using chickens, mice, pigs, monkeys and calves have failed to demonstrate a reproducible infection (92, 122). Many of these species appear to be healthy intestinal carriers of C. jejuni and also constitute reservoirs of the infection for humans (22, 45, 107). A suitable model of enteritis would involve animals that are not asymptomatic carriers but develop signs of the disease after oral inoculation in similar fashion to humans. With the current pressures to reduce the use of vertebrate animals in research, it is difficult to get an accurate assessment with large vertebrate animals as a significant number cannot be tested easily. The fertile hen's egg offers an alternative useful tool for studying the pathogenesis of this organism at the tissue and cellular levels. Fertile hens' eggs are relatively easy to handle, inexpensive and can be used in statistically significant numbers and no specialized facilities are required.

The LD_{50} studies revealed a high degree of pathogenicity of C. jejuni for the chicken embryo. Relatively few numbers of bacteria were required to achieve these mortality data and this depicted the highly virulent nature of the organisms for the chick

embryo. Histopathological examination of the liver, heart, kidney and the spleen following inoculation with multiple doses of C. jejuni strain WD 2483 revealed congestion of the blood vessels with slight edema and hemorrhage and a lack of inflammatory cells. No organisms were detected in any of these organs throughout the course of study. In contrast to the findings of Field et al. (36), the data in this study indicated that C. jejuni caused a non-invasive fatal infection in the chicken embryos. The wide range in the LD_{50} of C. jejuni strains in fertile hens' eggs reported by Field et al. (36) indicated that invasiveness alone cannot account for the entire spectrum of virulence shown by the organism in this system. The relative paucity of C. jejuni isolates from outside the GI tract (142) and the uniformly negative response of the strains in Sereny test (92) suggest a lack of invasiveness for this species. Mortality data and histopathological findings in the embryos were similar for the organism and the bacteria-free broth filtrates. These findings together with a lack of organisms in viable counts and IFA studies in the chick embryo organs suggest a toxic etiology (89). Even though the chick embryo does not mimic the infection in humans it has proved to be an important tool for assaying the virulence of the organism and the potency of its toxin.

The role of toxins in the pathophysiology of C. jejuni induced diarrhea is still not established but it can be speculated that the two pathogenic mechanisms now recognized, invasion and enterotoxin production, may account in part for the variable clinical pattern of diarrheal disease caused by this bacterium. However production of a cytotoxin by this organism has been reported by some workers (48, 111, 161) but its contribution in the disease process is not clear. Production of a cholera-like enterotoxin by C. jejuni first reported by Ruiz-Palacios et al. (124) has gained importance and enterotoxin production undoubtedly causes the mucus and blood free watery diarrhea often observed in C. jejuni infections; this would be particularly so for infections caused by strains lacking invasive properties. This enterotoxin is thought to behave like the cholera and heat-labile E. coli

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enterotoxins in that it is capable of altering cyclic nucleotide function of intestinal mucosal cells resulting in pronounced release of fluids into the lumen. On the other hand, the cytotoxin may be responsible for the inflammatory diarrhea.

Belbouri and Megraud (5) reported 64% of the strains tested to have enterotoxin-like activity while Lindblom et al. (84) reported only 32% of the strains isolated from humans with acute gastroenteritis and healthy egg laying hens to be toxigenic. It is possible that failure to identify toxin production in apparently non-toxigenic strains may reflect variations in storage and growth conditions prior to assay. It has been observed in other toxin producing organisms that, after several in vitro passages, strains revert to non-toxigenic forms, especially if the toxin production is plasmid-mediated. This may be the case for C. jejuni.

At 68 kilodalton, the molecular mass of the toxin isolated from C. jejuni strain WD 2483 was lower than that of cholera toxin (84 kilodalton) or the E. coli heat labile enterotoxin (91.5 kilodalton), though it approximates the single 70-kilodalton band reported by McCardell et al. (94) for C. jejuni. The 65-kilodalton protein band also present in the ETP lacked toxic activity. The flagellar band at 63 kilodalton demonstrated by many researchers has been shown to consist of one or several charge trains in 2D electrophoresis depending on the species (34). It could be quite possible that the protein band observed at 65 kilodalton is the flagellar protein. From embryo protection studies, the toxic activity of the ETP was neutralized when incubated with CETPMAb4 which showed antigenic specificity only for the 68-kilodalton protein. Furthermore, the degree of ETP binding to Int 407 cells as measured by ELISA was the same whether CETPMAb4 or the polyclonal antibody to strain WD 2483 was used. Given that the toxicity of the ETP resided solely with the 68-kilodalton protein, which did not further cleave in the presence of ß-mercaptoethanol, this C. jejuni toxin, unlike those of V. cholerae and E. coli, did not possess subunits. The activity of the ETP was sensitive to heat, acid, alkali and proteases

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but not to lipase suggesting it to be protein in nature (90).

The ability of organisms to adhere to host cells is well documented and it is generally believed that in enteric pathogens, adherence forms a prelude to initiation of the disease processes. A number of bacterial components including fimbriae and pili have been shown to mediate adherence of the bacterium to eukaryotic host cells. The adherence data of strain WD 2483 using the two cell cultures PCEF and Int 407 cells indicated that a specific binding interaction between the organism and the host cells either did not exist or occurred only at very low levels. Bacterial binding of 1 cfu/10 eukaryotic cells by viable count and approximately 1 org/25 eukaryotic cells for PCEF and 1 org/20 eukaryotic cells for Int 407 cells by IFA was considered too low to be significant. A high non-specific binding of the organism to the wells of plastic plates was observed at all times. At a multiplicity of 100 cfu/cell, 0.1% of the inocula bound to the cells while 2% bound non-specifically to the plastic indicating that the bacterial binding was twenty fold higher for the plastic than for the eukaryotic cells. No internalized bacteria were found in either cell type, further confirming the non-invasive nature of strain WD 2483. In contrast to these findings, McSweegan and Walker (96) reported a high degree of binding of this organism to Int 407 cells. The cytopathic effects on both PCEF and Int 407 cells were similar in that the organism caused cell rounding and lysis but the initial stages of the effect were not observed until 6 h post-inoculation. The organism elicited severe cellular and subcellular damage to Int 407 cells as revealed by electron microscopy. The ultrastructural damage was evident at 6 h post-inoculation thus supporting the cytopathic observations made in Int 407 cells using the inverted microscope. The damage to the mitochondria was of a type typical for toxin treated cells. It is quite possible to assume that this organism resembles E. coli, in that some strains are enteroinvasive and some toxic in nature with virulence a multifactorial process.

Using a mouse cecal model, Lee et al. (82) reported that mucus colonization and not

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adherence to the intestinal cells was a determinant for pathogenicity. They did not report bacterial adherence in the intestinal crypts and wet preparations examined by phase contrast microscopy showed actively moving C. jejuni organisms in the mucus. Whether organisms adhered to mucus or not was not determined. Mucus penetration in human infection is undoubtedly facilitated by the spiral shape and darting motility of this organism. The present data are in accordance with the suggestion of Lee et al. (82) that adherence and invasiveness of this organism may not be as important a virulence factor as previously thought. It could be conjectured that in non-adhering or non-invasive strains colonization of the gut plays a more important role in virulence. As a result of the close association between the bacteria and the mucosal wall the toxin that the organism produces may bind to the intestinal cell membranes and thereby exert its activity. Such cytological changes would then lead to the diarrhea associated with infections due to this organism.

Most bacterial toxins are localized in the periplasm of organisms that produce them. Studies by Klipstein and Engert (68), McCardell (94) and Guerrant et al. (48) showed an increase in toxin production after the cells were treated with polymyxin B which caused a release of the periplasmic proteins. Interestingly, the Western blots of the different cellular components of strain WD 2483 probed with the monoclonal antibody CETPMAb4 failed to show any antigenic specificity for proteins in the cytoplasmic membrane or to the cytoplasmic and periplasmic fractions. However, 5 bands from the outer membrane reacted with the monoclonal antibody, and of these one was a major outer membrane protein previously reported to be a porin (86) and had an approximate molecular mass of 43 kilodalton. The 68-kilodalton protein could be the cytotoxin characterized in this study and it is quite possible that the toxin is associated with the outer membrane before finally being secreted into the medium. The other proteins recognized were at 20, 23 and 35 kilodaltons.

The Western blot results indicated that the CETPMAb4 recognized a very conserved sequence of the toxin which is exposed in the outer membrane. This sequence may be

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hidden when the toxin is in the cytoplasm or periplasm due to its specific configuration and is exposed and recognized by the monoclonal antibody only when the toxin is secreted and attached to the outer membrane due to a change in the spatial arrangement of the toxic protein molecule. However, it is quite possible that some of the C. jejuni outer membrane proteins share a conserved sequence with its cytotoxin which is recognized by this monoclonal antibody. Production of more monoclonal antibodies to different sequences of the toxin in similar experiments will further elucidate the location of the toxin in the organism.

The ELISA tests of the cellular components further support the evidence that a conserved sequence recognized by the CETPMAb$_4$ is present in the outer membrane fraction of the organism only. However, the polyclonal antibody to strain WD 2483 showed specificity for all the fractions. C. jejuni organisms probed with CETPMAb$_4$ and gold-conjugated goat anti-mouse antiserum showed the gold probe attached only in a few sites in the outer membrane of the organism. In freeze-thawed organisms in which some bacterial cells were subjected to lysis, the probe was only seen attached to the membrane and at no time inside the cell which confirmed the antigenic specificity of the monoclonal antibody to protein sequences located either only or preferentially in the outer membrane.

The GM$_1$-ELISA for the detection of enterotoxins from E. coli and V. cholerae offers a reliable and reproducible method for laboratories without cell culture facilities (4, 126). It is a practical, simple and inexpensive test to detect enterotoxins closely related to those of E. coli and V. cholerae. Since this assay depends on both the attachment of the toxin to the ganglioside and binding of the antitoxin, preparations positive in these tests have both the ability to bind to the ganglioside as well as antigenic specificity for the particular antitoxin used. In other words, if an unknown toxin tests positive in GM$_1$-ELISA when probed with cholera antitoxin then it can be assumed that the unknown toxin

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was immunologically related to the cholera toxin and may have toxin binding receptors on the host eukaryotic cells similar to those for cholera toxin. Moreover, the enterotoxin from V. cholerae and E. coli produced similar elevations of cyclic AMP and parallel characteristic morphological changes in CHO cells (47). Studies on the enterotoxin of C. jejuni by McCardell et al. (94) and Johnson and Lior (58), have shown elongation of the CHO cells similar to that for V. cholerae or E. coli. In contrast to these, the ETP from C. jejuni strain WD 2483 did not bind to the GM_1 ganglioside and induced rounding and lysis of the CHO cells (90). When the PCEF and Int 407 cells were treated with the ETP, it caused lysis and rounding of the cells and the cytopathic effect was similar to that observed in the two cell types treated with strain WD 2483. However, the effect was much faster with the toxin and was observed at 2 h post treatment compared with similar findings at 6 h post-inoculation with the organism. From these data it would appear that the toxin isolated from strain WD 2483 differed markedly from the enterotoxins of V. cholerae, E. coli and that reported for C. jejuni by McCardell et al. (94), Ruiz-Palacios et al. (124) and Klipstein and Engert (68). Furthermore, their lack of immunological relatedness was confirmed by the absence of cross neutralizing reactivity between ETP and cholera toxin and their respective antisera. C. jejuni enterotoxin has been reported to be immunologically and biologically similar to the cholera toxin (94, 124). Indeed, the characteristics of the toxin isolated from strain WD 2483 demonstrated that it was a cytotoxin rather than an enterotoxin (90).

Koga et al. (72) recently reported leukocidal effects of C. jejuni cell-free filtrates on rabbit leukocytes in vitro. Similarities between the cytotoxin isolated in this study and the crude cytotoxic filtrates previously reported (48, 58, 161) include molecular weight, heat and trypsin sensitivity and CHO cell rounding. However, the toxicity of the ETP was lost on freeze-thawing but was retained for up to 4 weeks at 4°C and was unaffected by treatment with polymyxin B.

Most toxins are known to interact with specific receptors on susceptible host cell

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membranes as a prelude to cytoplasmic uptake or initiation of their toxic responses. Others actually damage the cell membranes. The parameters for both an effective toxin binding assay and a rationale for determining binding were established. These criteria were used to characterize the nature of "receptors" on host cells involved in the binding of this cytotoxin. The toxin binding curves indicated that both PCEF and Int 407 cells carry receptors on their membranes specific for the cytotoxin and showed maximal binding after 2 h of treatment followed by rounding and lysis of the host cells with a concomitant decrease in toxin binding by 24 h. Toxin binding receptors may be delineated on eukaryotic host cells using specific blocking agents. The process of investigating function through a loss of function was used to assess the reduction or loss in adherence of the cytotoxin due to inhibitory agents.

Degradation or immobilization of the eukaryotic cell surface proteins with enzymes and fixative treatments significantly reduced toxin binding to both the cell types. That lipase, Nonidet P-40 and ß-galactosidase treatments of the eukaryotic cells did not significantly reduce binding implied that lipid and lactose moieties do not appear to be important components in the binding process and therefore an glycolipid-like receptor does not seem likely. Likewise, treatment of the cell surfaces with sodium metaperiodate did not adversely affect toxin binding, rather a 5% increase in binding to PCEF cells and a 10% increase in adherence to Int 407 cells were observed. Even though these values are much below the 50% mark considered as significant, this observation was very interesting. Of the mono- and disaccharides tested, none had any effect on toxin binding except for NurNAc, a parent acid of a family of amino sugars. It accounted for a 56.5% reduction in binding to PCEF and a 50% reduction in binding to Int 407 cells. Treatment of cells with neuraminidase, a receptor destroying enzyme that liberates N-acetyl neuraminic acid residues from the cell membranes reduced binding by greater than 90%. However, commercially available neuraminidase has residual protease activity and this might explain

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the higher reduction in toxin binding as compared to the treatment with protease alone.

From these data it would appear that a simple, single receptor system for the cytotoxin on the surface of PCEF and Int 407 cells does not occur. A consistently observed slight increase in binding subsequent to periodate treatment indicated possible stearic hindrance by a carbohydrate moiety. It is possible that configurational changes after the oxidation of this moiety led to greater or full exposure of the toxin receptor on the cell surface thus leading to a slight increase in toxin binding as seen in this study. The compiled data point towards a surface protein or glycoprotein-like receptor for the cytotoxin. It may be postulated that the amino-terminal end of NurNAc acts as a receptor for the C. jejuni cytotoxin per se. Alternatively, it is possible that the toxin receptor is a surface protein either linked directly to or acting in consort with a neuraminic acid moiety (90). Coincubation of the cytotoxin with NurNAc or treatment of the host cell surface with neuraminidase released the NurNAc residues leading to competitive binding of the cytotoxin with the free and bound NurNAc moieties. McSweegan and Walker (96) have shown that the LPS molecules of C. jejuni are involved in the adhesion of the organism to Int 407 cells and the adherence was reduced in the presence of fucose or mannose. The presence of these two monosaccharides had no significant effect on the binding of the cytotoxin. Although the present study does not support the adherence of C. jejuni to PCEF or Int 407 cells, it is possible that strain variations exist and some strains possess the ability to bind to such cells. In cases in which the organisms adhere to eukaryotic cells and also produce a cytotoxin that binds to host cells, it does not seem unreasonable that the organism and its toxin would have developed an adherence process using different cell receptors and thereby avoid competition for the same host cell binding sites. This could explain differences between the binding process for adherence positive, cytotoxin negative strains versus adherence negative, cytotoxic positive organisms.

However, one has to be careful in extrapolating the results derived in these

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experimental conditions to those in the clinical situation. Although the Int 407 cells are epithelial cells derived from the jejunum and ileum of a caucasian embryo, it is a transformed cell line. Moreover, the environmental and nutritional differences that exist between cells propagated in vitro as compared to in vivo conditions may well effect the expression of eukaryotic host cell receptors. Further studies using purified receptors with known sequences in similar binding assays could further characterize the specific components responsible for mediating toxin binding. Elucidation of the receptors and factors that contribute to the binding of this cytotoxin to host cells will increase our understanding of the mode of action of this potential virulence trait that may be involved in the inflammatory diarrhea caused by many C. jejuni isolates.

Ultrastructural damage observed in Int 407 cells treated with the toxin was similar to the cellular damage observed in the organism treated cells as described previously. However, the toxin induced damage was much more rapid and the disintegration of the mitochondria was clearly evident by 2 h post treatment with the toxin as opposed to that seen after 6 h in the organism treated cells. It is perhaps not surprising to detect a lag period between the induction of cell damage by organisms, should the histopathologic changes induced be a direct function of the toxin. By 12 h post-treatment, the few cells that survived the toxic action of the ETP showed ballooning of the nuclear membrane along with membrane lysis and clearing of the cytoplasm at the ultrastructural level and confirmed the potency of this cytotoxin.

Int 407 cells treated with the toxin for various time periods and probed with the gold-conjugated anti-mouse serum showed irregularly spaced single or small clusters of particulate gold label only on the cell membranes. At no time was the probe seen inside the cell or attached to any other cell organelle. Unlike the cytotoxin of Pseudomonas aeruginosa in which the cytotoxin labelled with colloidal gold attaches to the nuclei and cell membrane (7), the action of C. jejuni cytotoxin was restricted solely to the cell membranes.

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From the extreme levels of sub-cellular damage, it may be speculated that the response was due, at least in part, to the lysosomal enzymes released by the action of the cytotoxin. Western blots of the cellular fractions of Int 407 cells treated with the cytotoxin for 12 h and probed with the monoclonal antibody showed antigenic specificity for 4 bands in the membrane fraction only. This further confirmed the membrane binding nature of the cytotoxin which was further supported by ELISA tests on the cellular fractions. IFA results on the toxin treated Int 407 cells probed with the polyclonal antiserum to strain WD 2483 indicated that this cytotoxin did not nick itself into A and B subunits as in many other bacterial toxins. In these the B subunit is responsible for binding while the A subunit enters the host cell and is responsible for the ultimate toxic effects. If the whole toxin molecule or a part of it had entered the cells then a negative IFA result using the CETPMAb$_4$ and polyclonal antibody as probes would not be observed with cells treated with trypsin which removed the adherent toxin but had no effect on the intracellular toxin molecules. The compiled data demonstrated that the cytotoxin bound to specific receptors on the host cell membrane, nicked itself and elicited its toxic response by damaging the membrane as such. This would appear to be the logical conclusion given that the molecular mass of this cytotoxin was 68 kilodalton but the molecular masses of the proteins in the membranes to which it was bound were lower than the molecular mass of the whole toxin. This reduction in weight is possible only if the toxin nicked itself into smaller fragments, some or all of which retained toxic activity; otherwise the protein bands observed in the membrane fraction should have a molecular mass higher than 68 kilodalton.

In brief, the results from the present study indicated that the production of a membrane adhering cytotoxin that bound to protein or glycoprotein-like receptors on host cell membranes was an important virulence factor in non-invasive strains and may be responsible for the inflammatory diarrhea caused by many C. jejuni strains. The cytotoxin did not elevate the cyclic adenosine monophosphate content in affected cells as observed

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from the CHO cell assay. It may be postulated that the action of C. jejuni cytotoxin was similar to that of Shiga toxin (104) in that it also bound to specific receptors on the absorptive cells of the intestine and ultimately led to cell death. As a result diarrhea would be due to inhibition of absorption rather than active secretion. The loss of microvilli in the Int 407 cells treated with the cytotoxin in the present study supports this hypothesis. However, active secretion in the lumen of the intestine is the mode of action for the well studied enterotoxins of V. cholerae, E. coli, and is now also the proposed mechanism for the C. jejuni enterotoxin induced diarrhea.

Acute diarrheal disease continues to be a major cause of morbidity and mortality among children in the developing countries as well as a cause of sickness worldwide. Antimicrobials are not recommended for the alleviation of uncomplicated diarrhea because of the risk of developing bacterial resistance. Currently, vaccines are being developed for the other two major enteric pathogens, rotavirus and enterotoxigenic E. coli. These together with C. jejuni are principally responsible for diarrheal diseases in developing nations and, if Salmonella species are added, in developed countries as well. In the case of C. jejuni, it seems that toxigenic strains are a significant cause of diarrhea and toxoid-type vaccines might provide some degree of protection against the enteric disease caused by them.

Much remains to be determined concerning the precise role of C. jejuni cytotoxin in disease. The wide range of clinical symptoms associated with Campylobacter infections in humans and animals suggests that there are differences in the response of the host. Even though the presence of toxins may represent the principal determinant of virulence and the cause of disease, they may not be the principal determinant of infectivity. Bacteria that cause disease as a direct result of toxin secretion are usually avirulent when the toxin gene(s) are removed. In this study the production of a cytotoxin by C. jejuni indicates that it is an important virulence factor which may be responsible for influencing the severity of

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Campylobacter enteritis. The location of the genes responsible for toxin production and the effect of mutants lacking these particular genes on virulence would lead to a better understanding of the contribution of the cytotoxin in the disease process and ultimately the organisms.

At present, a variety of methods used for cytotoxin detection include cell cultures and ELISA tests which are convenient assays for laboratories. However, for large scale epidemiological surveys, the use of DNA probes may prove to be appropriate. Currently, it is unknown whether the toxin is plasmid associated or chromosomally determined. Molecular cloning of the toxin genes along with the development of highly specific monoclonal antibodies would provide powerful tools for the analysis of the structure of the cytotoxin and the relationship between structure and receptor binding activity as well as the mechanism(s) for the mode of action of the toxin. Genetically engineered strains of C. jejuni that differ only with respect to their toxigenicity or non-toxigenicity would determine whether the toxin is an essential or accessory virulence factor for primary pathogenesis. Characterization of the structural genes of the toxin as well as the nucleotide and corresponding amino acid sequences of their translational products will provide information on the extent of homology with other known cytotoxins like Shiga toxin and Pseudomonas exotoxin. Furthermore, the precise post-binding location and orientation of the toxin within the host eukaryotic cell membranes would eventually decipher its mode of action at the cellular and molecular levels and eventually lead to the elucidation of the role this potent cytotoxin plays in the pathogenesis of infection.

Possible future studies:

This work has revealed a variety of interesting problems, the investigation of which would extend the available data base on the pathogenesis of C. jejuni :

1) sequencing of the 68-kilodalton cytotoxin and raising monoclonal antibodies recognizing different sequences would further elucidate the location of the toxin within the organism and its post binding orientation within the host eukaryotic cell membranes;

2) a recycling fermentor could be usefully employed to maximize the production of the toxin for use in characterization studies, to develop diagnostic detection systems and as a potential for toxoid vaccine development;

3) location of the gene(s) responsible for cytotoxin production in C. jejuni and the relationship of this toxin to virulence of the organism following mutation or deletion of these genes;

4) molecular cloning of the toxin producing gene(s) in appropriate bacterial hosts along with the effect the expression of these cloned genes has on the virulence of the new host organism;

5) comprehensive characterization of the purified toxin binding receptor on the eukaryotic cells and the number of specific receptors present on the host cells (especially of intestinal origin);

6) immune response in an animal model to the heat inactivated toxoid of the cytotoxin for development of a possible vaccine;

7) role of humoral and cell-mediated immunity in the chick embryo to C. jejuni infection in the face of bacterial challenge;

8) role of enterotoxin production, cytotoxin production and invasiveness of the different strains of the organism in the overall pathogenesis of disease.

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APPENDIX 1

1. Reagents used in histological studies

1.1 Hematoxylin solution

Hematoxylin crystals	5 g
Absolute alcohol	50 ml
Aluminum ammonium sulfate	100 g
De-ionized water	1000 ml
Mercuric oxide	25 g

The hematoxylin crystals were dissolved in alcohol and mixed rapidly with aluminum ammonium sulfate dissolved in water and brought to the boil. Mercuric oxide was then added slowly and the mixture reheated for 20 min. This was transferred to glass coplin jars ready for use.

1.2 Eosin solution

10 grams of eosin was added into a liter of de-ionized water and 2 ml of glacial acetic acid was added to the mixture. This stock solution was stored at 20°C. A working solution was made by mixing 50 ml of the stock solution with 120 ml absolute alcohol, 30 ml de-ionized water and 1 ml glacial acetic acid.

1.3 Acid alcohol solution

This was made by adding 70% alcohol (710 ml of 95% alcohol in 290 ml de-ionized water) to 10 ml concentrated hydrochloric acid. The solution was stored at 20°C.

1.4 Saturated lithium carbonate

4.5 grams of lithium carbonate was dissolved in 450 ml of de-ionized water and stored in a stoppered bottle at room temperature.

1.5 Buffered formalin solution

A 10% formalin solution was made in PBS by adding 27 ml of formalin (37%) to 173 ml of PBS and stored in stoppered bottles.

1.6 Procedure for hematoxylin and eosin staining

Histoclear (2 changes)	2 min
100% alcohol (2 changes)	1 min
95% alcohol (2 changes)	1 min
De-ionized water	3 dips
Hematoxylin	6 min
Running water	2 min
Acid alcohol	8 dips
De-ionized water	3 dips
Lithium carbonate	7 dips
Running water	15 min
Eosin	4 min
95% alcohol (2 changes)	15 sec
100% alcohol (2 changes)	15 sec

APPENDIX 2

2. Media used in cell cultures

2.1 De-ionized water

The water used in cell culture studies was obtained from a Millipore Milli Q

filtration unit. Ten megaohm water was collected, filter sterilized through a 0.2 μm pore

size filter and stored in sterile bottles at 4°C.

2.2 Sodium bicarbonate (NaHCO₃)

A 7.5% solution of sodium bicarbonate (Sigma) was made by dissolving 7.5 grams

of $NaHCO_3$ in 100 ml of de-ionized water and filter sterilized applying positive pressure

through a 0.2 μm filter. The solution was stored in sterile bottles at 4°C.

2.3 Phosphate buffered saline (PBS)

PBS was made by dissolving 8 grams of NaCl, 0.2 grams of KCl, 0.2 grams of

KH_2PO_4 and 0.15 grams Na_2HPO_4 in a liter of de-ionized water. It was sterilized by

autoclaving and stored at 4°C.

2.4 Glutamine

The 100x stock solution of glutamine (200 mM) (Sigma) was made by dissolving

2.92 grams of glutamine in 100 ml of de-ionized water and filter sterilized. It was stored in

sterile bottles at -20°C.

2.5 Hank's Balanced Salt Solution (HBSS)

A 10x stock solution of HBSS with phenol red was obtained from Irvine Scientific.

The stock solution was diluted ten fold under aseptic conditions with sterile de-ionized

water. Sterile $NaHCO_3$ (0.35 g/l) was added and the solution stored in sterile bottles at

4°C.

2.6 Minimum essential media (MEM)

A 10x stock solution of MEM with non-essential amino acids was obtained from

Irvine Scientific. The concentrated solution was diluted ten fold with sterile de-ionized water and stored at 4°C.

2.7 Trypsin-EDTA

Trypsin-EDTA solution was made by dissolving 0.5 grams of trypsin (Sigma) and 0.2 grams of EDTA (Sigma) in a liter of PBS. The solution was filter sterilized through a 0.2 μm filter and stored in sterile bottles at 4°C.

2.8 Serum

New born calf serum (Sigma) and Fetal bovine serum (Irvine Scientific) were distributed in 50 ml aliquots in sterile plastic tubes and stored at -20°C.

2.9 Growth medium

MEM	450 ml
Serum	50 ml
NaHCO$_3$ (7.5%)	10 ml
Glutamine (200 mM)	0.5 ml

2.10 Maintenance medium

The maintenance media was prepared the same way as the growth media except that the serum concentration was reduced to 1%.

APPENDIX 3

3. Media and procedure used for preparing Monoclonal antibodies

3.1 Serum free medium (SFM)

RPMI 1640 with NaHCO$_3$ (Irvine Scientific)	990.0 ml
L - Glutamine (200 mM)	10.0 ml

The media was stored in sterile glass bottles at 4°C.

3.2 HAT medium

100x thymidine (1.6 mM) was made by dissolving 38.8 mg of thymidine in serum free medium.

1000x aminopterin (0.08 mM) was made by dissolving 3.5 mg of aminopterin in 100 ml of sterile double distilled water.

100x hypoxanthine (10 mM) was made by dissolving 136 mg hypoxanthine in 100 ml of serum free medium. The solution was put in a shaking water bath at 70°C for 3 h. All three solutions were filter sterilized and kept in sterile bottles.

Serum free medium	879.0 ml
Fetal bovine serum (heat inactivated)	100.0 ml
100 x hypoxanthine	10.0 ml
1000 x aminopterin	1.0 ml
100 x thymidine	10.0 ml

The media were made under aseptic conditions and gloves were worn when handling this media. It was stored in sterile bottles at 4°C.

3.3 Polyethylene glycol (PEG) preparation

PEG (mol. wt. 4000) was obtained from Sigma. 10 grams of it was placed in a screw cap tube and autoclaved for 20 min. Serum free medium (18.4 ml) was warmed to 56°C and 1.6 ml of DMSO was added to it. This mixture was filter sterilized. The autoclaved PEG was cooled to 70°C and the Serum free medium-DMSO mixture was

added to it. This was stored at 4°C until needed.

3.4 2x Phosphate buffered saline (PBS)

PBS was made by dissolving 8 grams of NaCl, 0.2 grams of KCl, 0.2 grams of KH_2PO_4 and 0.15 grams Na_2HPO_4 in 500 ml of de-ionized water. It was sterilized by autoclaving and stored at 4°C.

3.5 Macrophage collection

Two female Balb/c female mice were injected intraperitoneally with 2 ml of sterile thioglycolate broth. After 4 days, the mice were cervically dislocated and their abdomens were sprayed with 70% alcohol. With a 18 G needle, 10 ml of SFM was injected into the abdomen of each mouse. The SFM was expelled and aspirated carefully out of the abdomen three times to collect as many peritoneal cells as possible. The cell suspension was placed in a 50 ml tube and centrifuged at 400 x g for 5 min. The pellet was resuspended in 0.3 ml of SFM and 5 ml of sterile de-ionized water was added to lyse the red blood cells. Immediately, 5 ml of 2x PBS was added and the suspension was centrifuged again at 400 x g for 5 min. The pellet was suspended in 10 ml of HAT medium and the macrophage concentration adjusted to 3 x 10^5 cells/ml. Using 96 well plates, 0.1 ml of the macrophage preparation was dispensed into the wells a day before the fusion.

3.6 Spleen preparation

The spleen from the primed mouse was removed aseptically and placed in a sterile petri dish containing 3 ml of SFM. The connective tissue was removed and the spleen was injected with SFM using a 25 G needle so as to remove the red blood cells from the sinusoids. The spleen was then transferred to another petri dish with 2 ml of SFM and cut into small fragments. The suspension was aspirated and expelled using a 18 G needle to get individual cells of the spleen tissue. The spleen suspension was then transferred into a sterile 50 ml conical tube and centrifuged at 450 x g for 5 min. The pellet was resuspended in 0.3 ml of SFM and 5 ml of sterile de-ionized water was added to lyse the red blood cells.

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Immediately, 5 ml of 2x PBS was added and the suspension was centrifuged again at 450 x g for 5 min. The pellet was then suspended in 10 ml of SFM and centrifuged again at 450 x g for 3 min. The resultant pellet was again suspended in 5 ml of SFM and the number of cells counted. The spleen cells were now ready for fusion.

3.7 NS-1 cell preparation

The NS-1 cells were obtained from the freezer at -70°C two weeks before the fusion. They were thawed at 37°C in a water bath and transferred to a 75 cm² flask containing the SFM with 10% fetal bovine serum. The flask was incubated at 37°C in a CO_2 incubator. When a confluent layer was formed the cells were split into three flasks and reincubated. On the day of the fusion 8 flasks of NS-1 cells were ready to be fused with the spleen cells. The cells were suspended in SFM in a 50 ml tube and centrifuged at 450 x g for 3 min. The pellet was resuspended in SFM and washed three times by centrifuging. After the washing the cells were suspended in SFM and counted.

Approximately 6×10^7 NS-1 cells were mixed with 10^8 spleen cells and centrifuged at 450 x g for 5 min.

3.8 Fusion

The PEG preparation was warmed in a 37°C water bath. Using a 1 ml pipette, 1 ml of PEG prep was added to the pellet (containing both cell types) over a 1 min period with stirring. Next a 25 ml pipette was filled with SFM and 1 ml was added slowly over a 1 min period, 3 ml over the next minute and the remaining 16 ml was added over the course of another minute. The suspension was centrifuged at 450 x g for 5 min. The supernatant was removed and 10 ml of HAT medium was added to wash the residual PEG slowly so as not to disturb the pellet. The media was removed and the pellet was suspended in 1 ml of HAT medium over a period of 1 min and slowly another 9 ml were added. The suspension was added in a T-75 cm² flask containing 10 ml of HAT medium and incubated

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at 37°C for 2 h. The fusion mixture was then diluted 1:10 and 0.1 ml was distributed carefully in the 96 well plates which were coated with the macrophage feeder layer, a day before. The final cell concentration was approximately 5×10^4 cells/well.

The plates were left undisturbed in the incubator for 4 days post fusion. At this time the medium in the wells was changed. Thereafter, fresh medium was added every 2-3 days and the cells were maintained in HAT medium for 18 days. Positive wells were identified as white 'spotches' in the bottom of a well, using a microtiter mirror. The wells positive for antibody production were transferred to 24 well plates, then 6 well plates and subsequently to T-75 cm² flasks. The hybridomas were then frozen at -70°C.

4. Reagents used in enzyme-linked immunosorbent assay (ELISA)

4.1 10x Phosphate buffered saline (PBS)

Sodium Phosphate (monobasic)	7.73 g
Sodium Phosphate (dibasic)	20.44 g
Sodium chloride (0.15 M)	87.66 g
Distilled water	1000 ml

The stock solution was stored at room temperature. Before use, it was diluted ten-folds in distilled water.

4.2 T-PBS (washing buffer)

To a 1x solution of phosphate buffered saline (pH 7.3), 0.5 % (v/v) of Tween-20 (BioRad) was added to make the washing buffer. It was made fresh before each use.

4.3 Post Coat Buffer

Bovine Serum Albumin (BSA)	30.0 g
1x PBS (pH 7.3)	1000 ml

The post coat buffer was aliquoted in 50 ml tubes and stored at -20°C.

4.4 Horseradish peroxidase conjugate

The conjugates, both goat anti-rabbit and goat anti-mouse were obtained from Organon Teknika Corp. For ELISA these were diluted to 1:8000 in 1x PBS (pH 7.3) and 0.1 ml added to each of the wells of 96 well plates for 1 h at 37°C.

4.5 Peroxidase substrate buffer

Sodium acetate (trihydrate)	13.6 g
Distilled water	1000 ml

The pH was adjusted to 4.9 by adding solid citric acid, monohydrate. The buffer was stored in glass bottles at 4°C.

4.6 3,3', 5,5'- Tetramethyl Benzidine (TMB) solution

TMB (42 mM)	100 mg
Methyl Sulfoxide	10 ml

Both TMB and methyl sulfoxide were obtained from Sigma. The solution was stored in the dark at room temperature.

4.7 TMB Substrate

The substrate mixture was prepared by adding 1.0 ml of TMB solution dropwise with gentle swirling to 100 ml of peroxidase substrate buffer followed by 14.7 μl of 30% H_2O_2. The substrate was prepared immediately before use. The reaction was stopped by adding 0.1 ml of 2 M sulfuric acid.

APPENDIX 5

5. Reagents used in polyacrylamide gel electrophoresis and Immunoblotting

Non-denaturing discontinuous gels

5.1 Lower tank buffer (pH 7.47)

Tris (63 mM)	30.27 g
1N HCl (50 mM)	200 ml
De-ionized water	4000 ml

5.2 Upper tank buffer (pH 8.89)

Tris (37.6 mM)	4.56 g
Glycine (40 mM)	3.0 g
De-ionized water	1000 ml

5.3 Separating gel buffer (pH 8.48)

Tris (1 M)	11.47 g
1N HCl (0.289 mM)	28.92 ml
De-ionized water	100 ml

5.4 Monomer solution for separating gel (40%T 5%C)

Acrylamide	38.00 g
Bis	2.00 g
De-ionized water	100 ml

The monomer solution was kept in a screw capped bottle wrapped with aluminum foil at 4°C

5.5 Catalyst

Ammonium persulfate	0.06 g
Riboflavin phosphate (0.02%)	10 ml
De-ionized water	100 ml

The catalyst was kept in a screw capped bottle wrapped with aluminum foil at 4°C

5.6 Separating gel (10%T 5%C)

Monomer solution	7.50 ml
Separating buffer	7.50 ml
De-ionized water	3.75 ml
Catalyst	3.75 ml
TEMED	20 µl

The mixture was degassed in a 50 ml side arm vacuum flask in the dark before adding TEMED. The gel was polymerized by illuminating them with a light box.

5.7 Stacking gel (5%)

The stacking gel was made the same way as for SDS gels (given in 3.15) except that the SDS was not incorporated in the mixture.

Sodium dodecyl sulfate (SDS) gels

5.8 Laemmli running buffer (10x)

Tris	30.0 g
Glycine	144.2 g
SDS	10.0 g
De-ionized water	1000 ml

The buffer was stored in glass bottles at room temperature and was diluted ten fold with de-ionized water before use.

5.9 Laemmli acrylamide solution

Acrylamide (30%)	60 g
Bis (0.8%)	1.6 g
De-ionized water	200 ml

The solution was filtered through a 0.45 µm filter and stored in a screw capped glass bottle wrapped with aluminum foil at 4°C. Acrylamide is neurotoxic and was always handled with care.

5.10 1M Tris (pH 6.8)

A one-molar solution of tris was made by dissolving 121.1 grams of tris in 800 ml of de-ionized water and the pH was adjusted to 6.8 with HCl. The volume was then brought up to 1000 ml with water and stored in glass bottles at room temperature.

5.11 1M Tris (pH 8.8)

A one-molar solution of tris was made by dissolving 121.1 grams of tris in 800 ml of de-ionized water and the pH was adjusted to 8.8 with HCl. The volume was then brought up to 1 liter with water and stored in glass bottles at room temperature

5.12 SDS (20%)

A solution of 20% SDS was made by dissolving 50 grams of SDS in 250 ml of de-ionized water. It was filtered through a 0.45 μm filter and the solution was stored in a glass bottle at room temperature.

5.13 Ammonium persulfate (10%)

The solution was made by dissolving 50 mg of ammonium persulfate in 500 μl of distilled water. It was stored at 4°C and made fresh every four to five days.

5.14 Separating gel (10%)

1 M Tris (pH 8.8)	11.2 ml
20% SDS	150 μl
Acrylamide	10.0 ml
De-ionized water	8.7 ml
10% ammonium persulfate	100 μl
TEMED	20 μl

The mixture was degassed for 20 min before adding ammonium persulfate and TEMED

5.15 Stacking gel (5%)

1 M Tris (pH 6.8)	1.25 ml
20% SDS	50 μl

Acrylamide	1.67 ml
De-ionized water	7.03 ml
10% ammonium persulfate	50 μl
TEMED	10 μl

The mixture was degassed for 20 min before adding ammonium persulfate and TEMED.

5.16 Non-denaturing non-reducing sample buffer

1 M Tris (pH 6.8)	8 ml
Glycerol	10 ml
De-ionized water	82 ml

The buffer was stored at 4°C in a sterile screw cap glass bottle.

5.17 Non-reducing sample buffer

1 M Tris (pH 6.8)	8 ml
Glycerol	10 ml
20% SDS	10 ml
De-ionized water	72 ml

The buffer was stored at 4°C in a sterile screw cap glass bottle.

5.18 Reducing sample buffer

1 M Tris (pH 6.8)	8.0 ml
Glycerol	10.0 ml
20% SDS	10.0 ml
ß-mercaptoethanol	4.8 ml
De-ionized water	67.2 ml

The buffer was always made in a fume hood and stored at 4°C in a sterile screw cap glass bottle.

Solutions for Immunoblotting

5.19 10x Tris buffered saline (TBS)

Tris (20 mM)	24.2 g
NaCl (500 mM)	292.4 g
De-ionized water	1000 ml

Before use the stock solution was diluted ten fold with water.

5.20 Tween 20-TBS (T-TBS)

It was made by adding 0.5 ml of Tween-20 to 1 liter of a 1x solution of TBS fresh before use.

5.21 Blocking solution

Gelatin	3.0 g
TBS (1x)	100 ml

5.22 Antibody buffer

Gelatin	1.0 g
TBS (1x)	100 ml

5.23 HRP color development solution

It was made fresh just before use. 60 mg of HRP color development reagent (4-chloro-1-naphthol) (BioRad) was added to 20 ml of ice cold methanol. This mixture was added to 60 μl of H_2O_2 (30%) in 100 ml of TBS at room temperature and was ready for use.

APPENDIX 6

6. Reagents used in Electron Microscopy

6.1 Grids

Copper grids (400 mesh) were used and were supplied by Electron Microscopy Sciences (EMS).

6.2 Cacodylate buffer

A 0.1 M buffer was made by dissolving 21.4 grams of sodium cacodylate (EMS) in a liter of double distilled water while 0.05 M was made by dissolving 10.2 grams in a liter of double distilled water The pH was adjusted to 7.3 using hydrochloric acid and the buffer was filtered through a 0.2 μm filter and was stored at 4°C.

6.3 Glutaraldehyde

Glutaraldehyde was obtained from EMS grade 50% (v/v) aqueous solution. A working solution of 5% (v/v) was made in cacodylate buffer as required.

6.4 Osmium tetroxide

A 2% (w/v) stock solution of osmium tetroxide (Stevens Metallurgic Corporation) was made by dissolving a 1 g ampule in 50 ml of cacodylate buffer. It was always handled in a fume hood. The solute dissolved after standing overnight at 4°C and the solution was stored at the same temperature in a tightly capped bottle covered with aluminum foil. The working solution (1%) was made in cacodylate buffer before use.

6.5 Ethanol series

A graded ethanol series was made by mixing appropriate amounts of absolute alcohol and double distilled water. The 50% and 70% ethanol was stored at 4°C while the 90% and 95% were stored at room temperature. Absolute alcohol was stored in a desiccator at room temperature.

6.6 Epoxy propane (propylene oxide)

This was obtained from EMS and supplied ready for use. It was always kept in

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tightly stoppered containers.

6.7 Epon-Araldite resin mixture

The resin mixture was made from the following components in the following proportions

Epon 812 (resin)	5 parts
Dodecenyl succinate anhydride (DDSA)	11 parts
Araldite (resin)	3 parts
Dibutyl phthalate (plasticizer)	0.8 parts
DMP-30 (accelerator)	0.4 parts

The components were added in the above order with the exception of DMP-30 and shaken vigorously. The accelerator was added just before the resin was to be used and properly mixed.

Stains for thin sections

6.8 Uranyl acetate

A 5% (w/v) solution of uranyl acetate was made by dissolving 1 g in 20 ml of double distilled water. The solution was left overnight at room temperature to dissolve the uranyl acetate. It was filtered through a 0.2 μm filter and stored in a dark stoppered bottle at room temperature.

6.9 Lead citrate

A pellet of sodium hydroxide was dissolved in 15 ml of boiled cooled double distilled water and 0.1 g of lead citrate was added to it. It was mixed to dissolve the lead citrate and distilled water (boiled cooled) was added to bring the volume to 25 ml. The stain was stored in tightly screw capped bottle at room temperature.

Embedding of specimens for electron microscopy

Procedure	Time
1. Wash in cacodylate buffer	15 min
2. Fix in 5% glutaraldehyde in cacodylate buffer	60 min
3. Wash in cacodylate buffer (ten changes)	45 min
4. Post-fix in 1% osmium tetroxide	60 min
5. Wash in cacodylate buffer	5 min
6. Dehydrate in ethanol series	20 min
7. Complete dehydration in absolute alcohol and propylene oxide	40 min
8. Resin / epoxy propane mixture	120 min
9. Resin (two changes)	120 min
10. Polymerization	24 h
Total time required	Approximately 32 h

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