CAMPYLOBACTER INFECTION IN INTESTINAL ORGAN CULTURES

A thesis presented in partial
fulfilment (50%) of the requirements
for the degree of Master of Philosophy
in Veterinary Pathology at Massey University
Palmerston North
New Zealand

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1987

ABSTRACT

Six different media (T199 medium , T199 + 10% foetal calf serum , T8 medium, T8 + 10% foetal calf serum, RPMI medium and RPMI + 10% foetal calf serum) were tested for their ability to maintain foetal lamb intestine in organ culture. T199 medium + 10% foetal calf serum was chosen because it gave more consistent results in maintaining the foetal intestine for a period of six days in culture.

Two groups of foetal lamb intestine were cultured, a control group and a group infected with Campylobacter jejuni. The effects of the microorganisms on the intestinal culture were assessed at 6 hours, 13 hours and 15 hours post-culture.

Light, Transmission and Scanning Electron Microscopy were used to study the pathogenicity of C.jejuni at the cellular level. Light microscopic studies showed that C.jejuni were attached and colonised the tips of the villi and the crypt epithelium of the intestinal cultures at 6 hours, 13 hours and 15 hours. The epithelial cells showed marked necrosis at the tips of the villi. The microorganisms also invaded the cytoplasm of epithelial cells of the villi and the intestinal crypt.

Transmission Electron Microscopy revealed degeneration of the microvilli in the infected cultures. The microorganisms were found attached to the tips of the microvilli of the villous epithelial cells by pilus-like structure. Microorganisms were present within phagolysosomes of macrophages in the lamina propria. Various cytoplasmic changes were observed at 6, 13 and 15 hours post-infection.

Scanning Electron Microscopy confirmed the different changes in the morphology of the infected epithelial cells. The microorganisms were observed adhering to the surface of the epithelial cells at 6 , 13 and 15 hours post-culture.

ACKNOWLEDGEMENTS

I would like to place on record my deep appreciation to the IRAQI government for supporting my studies at Massey.

My appreciation also to Massey University, the Department of Veterinary Pathology and Public Health and Professor B.W.Manktelow for my training and studies. I would like to acknowledge the help of my supervisor Dr A.C.Johnstone. A very special mention to Mr S.G.Fenwick who devoted much of his time to the final preparation of this thesis.

I would like to thank many others who contributed to the preparation of this thesis. Skilled assistance was provided by: Mrs P.Slack and Mrs P.Davey in the preparation of histological sections. Mr R.Holdaway for the preparation of Campylobacter jejuni and Mrs J.Schrama in the microbiological studies. Mr T.Law for the printing of the photographs and Mr P.Wildbore for supplying the equipment. Mrs L.Denby for the preparation of organ culture media. The Feilding and Longburn abattoirs for supplying the foetuses. The Electron Microscopy unit of DSIR. The Computer centre of Massey University whose assistance in producing the scripts and final print of this thesis is also acknowledged with warm gratitude.

I also wish to thank the staff of the Massey University Library for their efficient assistance in obtaining references.

Last but not least, I would like to thank my friends for their support and encouragement.

I also wish to express my appreciation to my mother and my brothers for their support during the whole course of this work.

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INTRODUCTION

The first reports on the Campylobacters were published about 74 years ago (McFadyean and Stockman, 1913). However their association with animal and human diseases has only been appreciated recently. It is now recognised that they cause diarrhoea in man (Butzler and Skirrow, 1979) and diarrhoea and abortion in a wide range of animal species [Moon et al., 1974; Hoorens et al., 1977; Smibert,1978; Vandenberghe and Hoorens, 1980; Field et al., 1981; Firehammer and Myers, 1981; Lomax et al., 1982; Pearson et al., 1982; Stephens, 1983; Stephens et al., 1984; Vandenberghe et al., 1985].

There has been a renewed interest in the genus Campylobacter in the past decade, and improvements in the isolation and identification of the organisms, together with changes in the taxonomy of the genus have led to a better understanding of the epidemiology of Campylobacter infections in humans and animals (Veron and Chatelon, 1973; Smibert, 1974, 1984).

Early research was carried out independently by workers in the medical, veterinary and biological fields which led to a lot of information being gathered about the organisms and the diseases they caused. However, this has changed, and the various aspects of Campylobacter infections, such as pathogenesis, pathogenicity, serology , epidemiology, molecular biology, biotyping, taxonomy, growth requirements, and host resistance factors are now better understood. More emphasis will be put in this study on aspects of the pathogenesis and pathogenicity of the Campylobacters.

Current investigations look at the nature, development and mechanisms of Campylobacter infections. Areas of investigation which have been covered are natural infections, experimental infections and the use of'in vivo' models to elucidate the pathogenic mechanism(s) and pathogenesis of Campylobacter infections.

Many reports of natural infection are present in the literature and refer to a range of host animals: man (Butzler and Skirrow, 1979; Duffy et al., 1980; Manninen et al., 1982); rats and mice (Field et al., 1981; Newell and Pearson, 1984; Vandenberghe et al., 1985); lambs (Hoorens et al., 1977; Vandenberghe and Hoorens, 1980; Firehammer and Myers,1981); pigs (Staley et al., 1969; Love and Love, 1979; Lomax et al., 1982); Mink (Hunter et al., 1986); cattle (Firehammer and Myers, 1981; Taylor, 1982); rabbits (Moon et al., 1974); dogs (Prescott et al., 1981; Fox et al., 1985); monkeys (Bryant et al., 1983).

The ubiquitous nature of Campylobacter infections must be pointed out. The comparative aspects of these infections have not been determined yet, but Campylobacter infections are usually associated with enteric disease. The significance of multiple infections in Campylobacter enteritis is still unclear.

A number of animals have been infected experimentally with Campylobacter species: calves (Al-Mashat and Taylor, 1980; Firehammer and Myers, 1981);lambs (Firehammer and Myers, 1981); mink (Hunter et al., 1986); pigs (Taylor,1982); dogs (Macartney et al., 1982); mice (Merrell et al., 1982; Field et al., 1981); rhesus monkeys (Fitzgeorge et al., 1982); hamsters (Humphrey et al., 1985,1986); chickens (Welkos, 1984) . The results obtained have varied considerably. Generally there is a colonisation of the gastrointestinal tract, however the reported sites of infection are quite conflicting.

Gnotobiotically reared animals have been used for the study of Campylobacter species including dogs, pigs, and chickens (Prescott et al., 1981). There are no published reports on the use of organ culture or foetal organ culture to study the interaction between the host tissue and the microorganisms. It can be argued that such models would provide advantages such as the absence of an immune system, hormonal factors and nutritional factors. Foetal organ cultures would have the additional advantage of a complete absence of commensal bacteria.

It is clear from a review of the literature on Campylobacter infections that the pathogenicity of Campylobacter species is still unclear. Several hypotheses suggest that colonisation,attachment, penetration and the production of toxins are possible mechanisms of pathogenesis (Butzler and Skirrow,1979; Firehammer and Myers, 1981; Prescott et al., 1981). Further useful studies would be the relationship between the attachment of the microorganisms to the cell, invasion and cytotoxicity. There is evidence to suggest that Campylobacter species invade the cells but the mechanism of entry has not been reported yet.

The aim of the present study was to investigate the possibility of using ovine foetal intestinal organ culture as a model for the study of Campylobacter infections, with emphasis on the pathogenesis and pathogenicity of Campylobacter jejuni.The reasons for using foetal intestinal organ culture are discussed in the text.The study covered two aspects, which are thought to be closely related to the successful use of intestinal organ culture. In the first experiment, several media were used, with or without serum, and the viability of the foetal intestinal tissues in these media was assessed over time. Light Microscopy , Transmission Electron Microscopy and Scanning Electron Microscopy techniques were used to assess the changes in the architecture of the tissues over time. The medium which gave the most satisfactory results, based on the above criteria, was chosen (T199 medium + 10% foetal calf serum) and used in Experiment II.

In a second experiment ,ovine foetal tissues were infected with Campylobacter jejuni and the changes observed at 6, 13 and 15 hours post-infection. The changes were studied at the cellular level, using the three microscopic techniques described above, and compared with control tissues at corresponding times. An attempt was made to study the pathogenesis of the microorganisms on the foetal ovine intestines and the possible pathogenic mechanism(s).

CHAPTER 1

REVIEW OF THE LITERATURE

1.1 ORGAN CULTURE

The term organ culture has been defined as the maintenance or growth of tissues,organ primordia,or the whole or parts of an organ *in vitro*, in a way that allows differentiation and preservation of the architecture and /or the function of that organ (Federoff, 1967; Sharp, 1977; Schaffer, 1979).

Although cell division takes place,the study of growth,in the sense of cell multiplication,is seldom the primary object of the technique; rather, the method is designed to provide an environment which permits differentiated tissues to exercise their normal functions under the closely controlled conditions obtainable. Once this has been achieved for a given tissue, many experiments become possible, which could not be undertaken *in vivo* (Fell 1976).

Harrison (1907) demonstrated for the first time the possibility of growing tissues outside the body. He observed that embryonic tissue of the frog, when transplanted into coagulable lymph, developed normally. In another experiment, he found that the central nervous system of a frog embryo,when immersed in fluid from the lymph sac of an adult frog, produced long nerve fibres. Carrel and Burrows (1911);Thomson (1914) and Maximow (1925) further developed the technique, distinguishing between uncontrolled(unorganized or histiotypic) and controlled (organised or somatic) growth. The general characteristics of the former resemble the processes of wound healing in the body. Amoeboid cells "wander"from the cut edges of the explant into the culture medium, where they divide actively, forming a broad halo of new tissue around the original fragment. The histological structure of the unorganised growth is simplified in the zone of outgrowth, which consists of " de-differentiated " cells. The unorganised

growth takes place at the margin of the original fragment. This phenomenon can only take place from injured surfaces where there is no basement membrane. Organised,controlled growth corresponds more closely to normal growth in the body, with the tissue enlarging as a whole. The normal histological structure of the tissue is preserved and if the explant is at an early stage of development it usually continues to differentiate histologically and sometimes anatomically also. Organised (controlled) growth frequently occurs in the interior of an explant.

A major advance occurred in the history of organ culture in 1926, when Strangeways and his colleagues began a series of experiments which showed that many embryonic organs could be cultured and be seen to develop normally in vitro (Fell,1940). Trowell (1961) published a method for culturing a mature organ in a synthetic medium which has provided the basis for nearly all subsequent work in this field. He successfully preserved many adult tissues from rats up to ten days of age but he did not attempt to culture intestinal tissue because he considered the problem of asepsis would be too great.However, Browning and Trier(1969), successfuly applied Trowell's technique for organ culture to adult human small intestinal mucosa. Their results encouraged other workers to use organ culture of the human small intestine to study normal and abnormal structures and functions of the gastrointestinal tract.

1.1.1 Types of Organ Culture

Organ culture can be subdivided into two main types,namely: embryonic and mature organs.

1.1.1.1 Culture of Mature Differentiated Organs

The culture of mature differentiated organs provides a useful technique for a wide range of nutritional, metabolic, functional and pathological studies (Moscona et al., 1965). Such cultures have a steady metabolic state which makes it possible to maintain

a culture for several days, instead of a few hours, without it undergoing substantial morphological change. In contrast to embryonic cultures, mature organ cultures obtain most of thier energy by respiration, and adequate oxygen supply is essential for their survival. For this reason they must always be cultured on the surface of the medium (Moscona et al., 1965). To maintain the organ culture size at 1.5-2mm without central necrosis, it is essential, with most tissues, to use an oxygen-gas-phase (Trowell, 1959,1961,1962). An exception to this is the retina which is poisoned by oxygen concentrations of 60% or more (Lucas and Trowell, 1958).Small cultures can be maintained in air, with a limiting size of approximately 0.5-1.0 mm diameter, although spherical organs cultured in air can be about 2mm in diameter. Entire organs from young rats,adult mice or adult bats such as the ovary,adrenal,pituitary,pineal, thyroid, prostate, seminal vesicle, spinal ganglia, and lymph nodes can be cultured. In addition, long narrow tubular organs such as the uterus, ductus deferens, bile duct and arteries can be cultured in infinite length, and thin sheets such as the retina, skin, diaphragm and mesentery in infinite widths. For some organs which are too large to be cultured, e.g: the liver, kidney,lung, and thymus,it is necessary to cut them into small pieces. According to Trowell (1959), cut pieces of organs usually survive as well as whole organs.

1.1.1.2 Culture of Embryonic Organs

The embryonic rudiments such as limbs,bones,teeth,eyes, gonads and various glands are usually cultured in entirety. The organ enlarges as a whole without diffuse spreading of the tissues and develops as an intact organ.In addition embryonic tissues are more resistant than mature tissues to anoxic conditions as they obtain most of their energy by glycolysis (Moscona, Trowell and Coillmer, 1965).

1.1.2 Technical Development of Organ Culture

The major historical developments in organ culture techniques may be summarized as follows:

1.1.2.1 The Hanging Drop Method (Harrison, 1907)

In this method, small pieces of tissue are embedded in clotted plasma to which has been added embryonic extract from an 11 day old fowl. It 1s unsatisfactory for most organ systems, because the tissues digest the clotted plasma and slowly sink into a pool of liquefied plasma which eventually impairs the oxygen supply.

Chemically defined media cannot be used in this system and the medium cannot be changed without transplanting the cultures. The medium cannot be removed for analysis, during the culture period and in addition, there is a high risk of contamination.

1.1.2.2 Maximow's Double Cover Slip Method (Maximow, 1925)

In this method the culture is explanted onto a small round cover slip. This is then attached to the centre of a large square cover slip by means of the surface tension exerted by a drop of sterile saline solution spread between the two. Both cover slips are inverted over the cavity in a depression slide, and the edges of the large cover slip are sealed on to the slide with wax. When the culture is to be fed, the large cover slip is detached from the slide, and the smaller one carefully removed. After the medium has been replenished the small cover slip carrying the culture is then reattached to a fresh, sterile large cover slip. In this way, contamination is avoided, and hanging drop preparations may,with reasonable care, be maintained for long periods.

The advantage of this method is that the components are simple and cheap to obtain (Maximow, 1925). The cultures can readily be examined with the low power of a microscope. However the method is not suitable for a detailed study of the living cells at a higher magnification. The disadvantage of this method is that the volume of the medium in the hanging drop is small and must be replenished frequently.It is therefore time-consuming to maintain more than a few of these cultures (Maximow, 1925).

1.1.2.3 Roller Tube Method (Gey, 1933)

This method was described by Gey in 1933. Several organ explants are arranged in a row on the inner surface of a test tube, and covered with a plasma clot. A few millilitres of liquid medium are then added and the tube is sealed with a sterile, non-toxic rubber stopper. A number of such tubes are then inserted into a slowly rotating drum, within an incubator maintained at 37C. As each tube is carried round in the drum the explants are alternately immersed in the liquid medium and then exposed to the air within the tube. The movement of the liquid medium ensures uniform distribution of its components.

A disadvantage of this method is the poor optical quality of the curved test-tube wall, which makes examination of the cultures difficult. A risk of infection occurs with this technique when the rubber stopper is removed to renew the medium. The technique can be improved by attaching the explant to a rectangular cover slip, thus allowing easy removal of the cultured tissue for examination. The advantages of this method are that it is a convenient way of maintaining considerable numbers of explants at a minimal cost in time and money and that sample medium can be conveniently altered.

1.1.2.4 Watch Glass Method (Fell and Robinson, 1929, Fell, 1951)

This method is the basis of the technique in general use today and was used by Fell and Robinson (1929) and again by Fell,(1951). A watch glass which has the convex surface painted black to facilitate microscopic observation,is placed on a layer of moistened, absorbent cotton wool in a petri dish. The explant is then stuck by a plasma clot to the concave surface and medium added. The culture is maintained in a humid environment, preventing the evaporation of water from the culture medium.

The disadvantage of this method lies in the necessity to detach the explants and transplants to a new supporting plasma clot when medium is removed.

1.1.2.5 Trowell's Method (1959)

Trowell (1959), used a square piece of fine wire gauze,with the edge bent over to form short legs, and stood this in a shallow glass or plastic dish. A piece of very thin, soft tissue paper was laid on the grid and the culture was "planted" on the paper which had been moistened with the fluid medium by capillary action. The dish was then placed in a thick-walled Perspex (Lucite) chamber of 30 ml capacity.

There are a number of advantages to Trowell's method. A large number of specimens can be cultured in each chamber. Samples of medium can be removed for analysis and additional nutrients or drugs can be added at any stage of the experiment.The medium can be changed and the cultures can be washed without disturbing their position. Reinsertion of the supporting material (grid and paper) which may retain or adsorb constituents of the medium, is reduced to a minimum. Synthetic fluid media of known chemical composition can be used, and the gaseous environment is also under complete control.

1.1.3 The Application of Organ culture in Virology, Microbiology and Toxicology.

For studies using organ cultures to be meaningful, at least two criteria must be met. Firstly, the cultured host tissue must be able to be maintained in a state of normal structure and functional activity throughout the duration of the experiment. Secondly, the response to the infectious agent by the cultured tissue *in vitro* must be substantially the same as the response of the target tissue in the natural disease *in vivo* (Fell,1976).

The employment of organ culture to study the effects of infectious agents on host tissue was first used in 1957, when Barski,*et al.,* (1957 and 1959), used cultures of human and simian tissues from the bronchial and tracheal lining to study the effects of poliovirus and adenovirus on respiratory ciliated epithelium. Subsequently, organ cultures have been used to investigate the interrelationships of viruses,mycoplasma and bacteria with their host tissues. For example, some viruses apparently have strict metabolic requirements that can be supplied only by the specific cell which is their natural host *in vivo*.These viruses appear unable to survive in the environment of cell culture.In this situation organ culture has proved invaluable. Human strains of corona virus causing upper respiratory tract disease were isolated using cultures of human embryonic trachea and nasal tissue(Tyrrell and Bynoe, 1965; McIntosh *et al.,* 1967).It has also been demonstrated that strains of human rhino virus, which are difficult to isolate in tissue culture, can be established in organ culture of human foetal respiratory tract epithelium (Dolin *et al.,* 1971); Clarke *et al.,*(1972) . Higgins *et al.,* (1973) isolated parvoviruses from intestinal organ culture.

Organ culture has also been used in studies of immunogenesis in different animals. Organ culture methods have been of value in the study of the mechanisms of formation and / or secretion of local antibody, and for the demonstration of latent viral

infection.(Barski et al., 1957; Heuschele and Easterday 1970; Finkelstein et al., 1972 and Schmidt and Maassab 1974). Heuschele et al., (1970) used organ culture to demonstrate antibody formation and persistence of virus in the tracheae of chickens infected with Newcastle disease virus (NDV) after an aerosol exposure to the infectious agent. The tracheae of the chickens (killed by cervical dislocation and exsanguination) were cut into rings approximately 1.5 mm wide and cultured in 5 ml T199 maintenance medium supplemented with 3% foetal bovine serum. It was found that tracheal organ cultures from chickens after aerosol exposure to B1 strain (Lentogenic Blasksburg Strain) of NDV were producing plaque-neutralising antibodies and were resistant to challenge with the GB strain (Velogenic Gilbert- Bony Strain) of NDV. It was observed that cultures persistently infected with B1 strain consistently resisted infection when inoculated with the GB strain. The persistence of the NDV virus and the production of antibodies were shown to occur for up to 120 days after exposure by aerosol to the B1 and G1 strains of NDV.

Organ cultures are well suited to histological and cytological studies of the pathological effects of viruses on organ epithelium.Thus, Hoorn et al.,(1965) and Hoorn et al., (1969) found that the enteroviruses and echovirus II multiplied in organ cultures of human ciliated epithelium and rapidly destroyed the ciliated cells. Collier and Clyde (1971),and Butler, and Ellaway(1971) studied the effect of Mycoplasma mycoides var.capri and Mycoplasma gallisepticum on human and chicken tracheal organ cultures.They observed the destruction of the ciliated epithelium and alteration of the tracheal cartilage, characterized by rounded cells with densely stained nuclei, and the matrix which had lost its capacity to retain the stain.

Fleming, Brown and Balls (1975); Balls et al., (1975); and Brown, Pryor and Balls (1975a,b) studied the hepatotoxic effect of paracetamol on cultured adult amphibian liver and observed a loss of transaminases and lactate from the tissue to the medium during the first few days in culture, followed by a rapid depletion of

the hepatocyte glycogen stores.

1.1.4 Reasons for the use of Organ Culture

The aim of explanting tissue *in vitro* is to study the behaviour and fate of cells, tissues, or organs separated from the whole organism. The main reasons for using organ culture are:

a) Organ cultures have no circulation and are probably not able to react immunologically to bacteria or viruses (Reed, 1969).

b) The metabolic rate of the culture is probably slower than normal and many of the responses which normally affect growth of organisms *in vivo* are absent, making it possible to study the direct effect of the organisms on the tissue . However organ culture can tell us little about the systemic effects of an infectious agent (Tyrrell and Byone, 1965).

c) Cells in organ cultures, similar in structure and function to that of the intact host,may provide a convenient experimental model for studying some aspects of the pathogenesis of certain microorganisms. (Tyrrell and Bynoe 1965).

1.2 INTESTINAL ORGAN CULTURE

The study of explant culture systems of mammalian gastrointestinal tissues has been limited by the rapid necrosis and degeneration of the mucosal epithelial cells (Autrup, 1980). It is possible to maintain open intestinal segments or everted rings and sacs of intestinal tissue in oxygenated buffer solutions for periods of two hours or less (Wilson and Wisman, 1954; Parson, 1968), but epithelial cell necrosis occurs if incubation of intestinal tissue is prolonged much beyond this time,(Browning et al., 1969). Trier, (1976) cultured material derived from biopsies of human gastrointestinal tissue and demonstrated marked differences in the ability of the epithelia at different levels to sustain metabolic activities in vitro. He observed that the gastric fundus degenerated rapidly, whereas gastric antrum, small intestine, colon and rectum were well maintained. This was also found to be the case with rabbit gastrointestinal tract. However, the small intestinal mucosa of rat , mouse and hamster cannot be maintained under the same cultural conditions (Trier,1976; (Harty et al., 1977).

1.2.1 Research Applications of Intestinal Organ Culture.

Intestinal organ culture techniques have been used principally to study human gastrointestinal tract disorders. Coeliac disease, ulcerative colitis and cystic fibrosis have been the most widely studied of these . In addition, the mechanisms of carcinogenesis and viral infection have been examined by the techniques.

1.2.1.1 Coeliac Disease

The histogenesis of the intestinal lesions in patients with coeliac sprue, and the likely mechanisms by which noxious glutens may damage the small bowel mucosa, have been studied by intestinal organ culture. The toxic glutens damage the surface absorptive cells of the intestinal villi and reduce the lifespan of these

cells, causing them to be shed prematurely into the intestinal lumen. Trier and Browning (1970) showed that diseased coeliac mucosa reverted to normal after only 24 hours of culture in a gluten- free environment. The epithelial cells became more columnar and less vacuolated. Thymidine incorporation into the crypt cells demonstrated that untreated coeliac mucosa had enhanced epithelial cell proliferation compared with normal tissue. The excessive proliferation was decreased in tissue derived from patients treated with a gluten- free diet. This finding was supported by Falchuk et al (1974), who observed that the addition of gluten peptides to the culture medium prevented the expected improvement in surface cell morphology. Intestinal organ culture was also used by Trier and Browning (1970); Townly et al., (1973); Falchuck et al., (1974); Jos et al.,(1975) and Hauri et al., (1978) to study the effects of some toxic agents such as the cereal glutens in wheat and barley. The absorptive cells showed severe degenerative changes of the attenuated microvilli and the formation of large lipid vacuoles in the cytoplasm of the undamaged epithelial cells.

1.2.1.2 Ulcerative Colitis

Organ cultures of rectal mucosa from patients with active ulcerative colitis have been shown to convert increased amounts of labelled glucosamine into glycoprotein and to secrete labelled glycoproteins more rapidly than those of healthy controls. By using this technique Rachmilewitz et al., (1978) and Hawkey et al., (1981),found that the inflamed rectal mucosa from patients with ulcerative colitis produced increased amounts of prostaglandin E2.

1.2.1.3 Cystic Fibrosis

Intestinal organ culture has been used to study in vitro the physiological activities and mucus secretion of normal intestinal and rectal mucosa from patients with cystic fibrosis (Johansen,et.al., 1968). He demonstrated an imbalance between

ions and water in the glycoprotein secreted from the intestinal epithelial cells in cystic fibrosis. In addition the viscosity of mucus secretion was increased in the lumen at the site of the goblet cells and the intestinal crypts. It appears that this is due to inhibition of the movement of water across the secretory epithelial cells resulting in increased levels of sodium and chloride ions and a decreased level of bicarbonate ions. The ions have been examined in intestinal organ culture by Neutra et al., (1977). He observed that the goblet cells in cystic fibrosis were more prominent than normal and that the crypt lumens often appeared distended. This was taken as evidence of hypersecretion of mucus (Parkins et al., 1963). Neutra et al.,(1977) suggested that the condition was either due to a defect in the intracellular regulation of secretion or to high levels of glycosyltransferase activity. In addition, the epithelial cells from cystic fibrosis patients were shown to accumulate lipid and glycogen in organ culture, whereas controls did not (Neutra et. al., 1978).

1.2.1.4 Studies of Carcinogenesis

The technique of intestinal organ culture was used in the study of the effects of carcinogens on cultured mammalian colon(Autrup,1980). It was found that cultured human and rat colons were able to manufacture procarcinogens from various classes of chemicals, such as polynuclear aromatic hydrocarbons, N-nitrosamines, dialkylhdrazine, and aflatoxin B1. Autrup,(1980) also demonstrated that the mean level of binding of aflatoxin B1 and 1,2-dimethylhydrazine (DMH) induced colon neoplasia in rats at a higher rate than in man. He suggested that the ability of colonic tissue to metabolize benzo [a]pyrene (BP) into ultimate carcinogenic forms could indicate an individual's susceptibility to colonic carcinoma caused by BP. When the effects of two different carcinogens,BP and DMH, were compared in cultured colonic tissue from the same patient, a positive correlation was observed. This indicated that the same activation system was involved in carcinogens of different chemical classes.

1.2.1.5 Viruses

Intestinal organ culture has been used to cultivate viruses such as parvovirus which cannot grow in other tissue culture or cell culture systems (Dolin et al., 1971). Dolin et al., (1971) and Clark et al., (1972), used intestinal organ culture to isolate the Norwalk agent which causes acute, infectious, non-bacterial gastroenteritis in humans. Dolin et al., (1970) noted that intestinal organ culture was a more sensitive system than conventional tissue culture for the isolation of an occult virus (for example Parvovirus) having the intestine as the target organ. Dolin et al., (1972) found that intestinal organ culture could support the growth of viruses such as Herpes Simplex Virus, Newcastle disease virus, and Epidemic Diarrhoea of Infant Mice (E.D.I.M) up to titers comparable to those obtained by other established tissue culture techniques.

The E.D.I.M. virus has not been grown in vitro, but intestinal organ culture infected with virus developed acute degenerative changes in the intestinal epithelium and the virus particles were seen in the cytoplasm of the cells (Rubenstein et al., 1971). Adams and Kraft (1967), and Banfield,Kasnic and Blackwell,(1968) observed that mucosal sections from intestinal organ culture infected with E.D.I.M. showed progressive flattening of the villi, with the eventual replacement of the single columnar cell pattern by pseudostratified mucosa, leaving only small islands of epithelial cells.

Human foetal intestinal organ culture was used in the search for viral agents involved in gastrointestinal diseases (Mitus et al., 1970; Stenhous, 1970; Dolin et al., 1972, . Wyatt et al., 1974 cultivated a virus-like agent from gastroenteritis in infants and children. They found that virus particles infected some of the epithelial cells lining the villi of the intestinal explant,while the rest of the epithelial cells were not susceptible to the virus infection. In addition, Dolin et al., (1972) found that human foetal intestinal organ cultures infected

with Herpes Simplex Virus showed reduced numbers of villi and denuding of the epithelium. They also observed that the changes in the cytoarchitecture of human foetal intestinal organ culture caused by Herpes Simplex Virus began in the epithelial cells and spread to the lamina propria and submucosa.

1.2.2 Techniques Used for Intestinal Organ Cultures

Numerous techniques have been used to maintain intestine in organ culture. Different media (both solid and liquid) have been used to explant intestinal organs. Many modifications have been made to the techniques. The most commonly used method at present is the Trowell modified technique (1954,1959) and that the obvious advantages of this method have contributed to its extensive use (See section 1.1.2.5). Wolf (1952) used agar jelly both as a medium and as a support for an intestinal explant.Trowell (1953) cultured the intestine on the surface of cotton wool soaked in fluid culture medium. Fell and Martinovitch (1953) used a different technique by culturing the organs on the surface of a solid medium, namely a plasma clot. The solid medium constituted both the solid substrate carrying the explanted tissue, and the nutrient medium. A synthetic liquid medium was used by Trowell (1954,1959) and by Barker et.al., (1964). The intestinal fragment was maintained in the medium on the surface of stainless steel mesh. Manax et.al., (1965) and Momose et.al., (1968) cultured large segments of canine distal ileum by using a vascular perfusion technique. Immediately after removal of the ileal segment by abdominal laparotomy, it was perfused through the arterial stump with cold (5C-8C) 5% dextran in a balanced salt solution, buffered to a pH of 7.4 (with bicarbonate and trome-thamine). Perfusion continued with water until the effluence from the vein was clear. After perfusion, the ileal segment was placed in a precooled bath (0-4 C) within a small hyperbaric chamber (1-3 atmospheric oxygen tension). The intestinal segment was successfully stored for up to 72 hours under these conditions. One serious limitation of this technique is the need for large segments of fresh tissue. Folkman and

French (1968) used three different techniques to culture the small intestine from germ-free rats, to evaluate the viability of the Peyer's patches in order to use these to study large organized populations of lymphocytes in vitro. Included were, the Rocker tube , Spinner flask and Perfusion chamber methods. By using these techniques , it was observed that the Peyer's patches remained viable throughout the period of culture. Browning and Trier (1969) used a technique similar to tissue culture technique by immersing the intestine in a tissue culture medium consisting of Eagle's basic salt solution . The liquid medium was kept oxygenated by agitating it in a metabolic shaker after gassing it with 95% O2 and 5% CO2.

Table 1.1 shows other techniques used by other workers for intestinal organ culture, the types of medium used, the period of survival, and the type of species investigated.

Table 1.1 Other techniques that have been used for intestinal organ culture.

Species	Technique	Medium	Survival Period	Reference
Mouse	Stationary; membrane filter on grid	Waymouth's MB 752/1 FCS + 10%	several weeks	Defries and Frank (1977)
Hamster (foetal)	Stationary; fibrin foam	Leibovitz's L-15 Waymouth's MB 752/1 + 10% FCS	up to 3 weeks	Schiff, (1975)
Rat	Rocking	CMRL 1066 + 5% BSA	up to 4 weeks	Autrup et al. (1978)
Rat	Rocking	CMRL 1066 + 10% FCS	up to 9 weeks	Shamsuddin et al., (1978)
Rabbit	Stationary; steel grid	Trowell's T-8 + 10% FCS	up to 3 days	Mak and Chang (1978)
Human	Stationary; steel grid	Trowell's T-8 + 10% FCS	up to 24 hours	O'Gorman and La Mont (1978)
Human	Rocking; gelatin sponge	CMRL 1066 + 5% BSA	up to 22 days	Autrup et al., (1978a)

Source Autrup, H. (1980)

1.2.3 Reasons for using Foetal Lamb Intestinal Organ Culture

Much of the published work on intestinal organ culture has been performed on human tissues using both normal (Browning *et al.*, 1969 ;James *et al.*, 1971; Beeken *et al.*, 1974 and Falchuk *et al.*, 1974;) and diseased human intestines (Croft *et al.*, 1965; Kedinger *et al.*, 1974 and Jos *et al.*, 1975) . Other researchers have used rabbits,mice,rats and hamsters (Erchholz, 1967 ; Schlegel *et al.*, 1972; Billington *et al.*, 1975, 1976; and Schiff,1975) but there are no reports of these techniques being used to study the direct effect of microorganisms on the sheep intestine *in vitro*.

The object of the study reported in this thesis was to study the effect of *C.jejuni* on the mucosa of foetal lamb intestinal organ culture. The advantage of using an *in vitro* system is that the environment of the tissues under study can be controlled more precisely than when *in vivo* models are used. Factors such as intestinal content, circulating humoral factors and neurogenic stimuli are eliminated. It is possible to study the effects of microorganisms on the cells and tissues and to better determine the role of the individual's defence reactions (Trier, 1976).

1.3 CAMPYLOBACTERIOSIS

Campylobacter species have long been recognised as pathogens in animals.Jones et al., (1931) reported that the organism Vibrio jejuni (the original name of C.jejuni) caused jejunitis in calves and dysentery in cattle. Recognition of the importance of C.jejuni as a major cause of diarrhoea and enteritis in both animals and humans has only occurred within the last few years. Infection has been reported in calves (Smibert,1974; Firehammer and Myers,1981; Prescott,et.al., 1981); lambs and adult sheep (Russell,1955; Hoorens et al., 1977; Jopp and Orr 1980; Vandenberghe et.al., 1980; Firehammer and Myers, 1981; Prescott, et al., 1981; Stephens, 1983,1984); chickens (Ruiz-Palacios et al., 1981); rabbits (Moon et al 1974); dogs and cats (Bruce, 1980 ;Collins and Libal,1983); pigs (Roland and Lawson,1974); Lomax et al., 1982), and humans (Butzler and Skirrow, 1979; Karmali and Fleming,1979; Manninen et al., 1982).

A review of the literature reveals that the pathogenesis of C.jejuni infections is still unclear. However, several hypotheses have been made, based on clinical observations, regarding the colonisation,attachment and invasiveness of the microorganisms (Butzler and Skirrow, 1979; Firehammer and Myers, 1981; Prescott et al., 1981).

The work conducted up to now has prompted the need to find a suitable animal model in which C.jejuni occurs naturally and in which it can be produced experimentally. Several animals have been used as models: mink (Hunter et al., 1986); calves (Al-mashat and Taylor, 1980; Firehammer and Myers, 1981); lambs (Firehammer and Myers, 1981); gnotobiotic dogs (Prescott et al., 1981); chickens (Ruiz-Palacios et al., 1981); and mice (Welkos, 1982).

There are no reports in the literature regarding the use or validity of intestinal organ culture as a model for studying the pathogenic mechanisms of C.jejuni.

1.3.1 Significance of C.jejuni infection in sheep in New Zealand

Sporadic cases of C.jejuni enteritis have been reported in sheep in New Zealand (Russell, 1955; Jopp and Orr, 1980). A morbidity rate of 10% and a mortality rate of approximately 5% have been recorded (Jopp and Orr,1980). No indication was given on the possible source of infection. Jopp and Orr, (1980) reported that the most consistent gastroenteric lesion was a distension of mucus glands, occuring at all levels of the intestinal tract.

1.3.2 Pathogenesis of Campylobacter species Infections

The results reported in this section were derived from either natural or experimental enteric infections with Campylobacter species. It should be noted that there are conflicting opinions in the literature regarding the location of the lesions in infected animals.

Table 1.2 Shows the association of various Campylobacter species with clinical symptoms in different animal species.

1.3.2.1 Sheep

C.jejuni is capable of causing pathological changes in the intestinal tract of lambs. Firehammer and Myers,(1981) found intermittent flecks of blood and mucus production in the faeces of lambs after oral adminstration of C.jejuni, but failed to observe any diarrhoea. Stansfield et al., (1986) reported that C.jejuni type 1 was responsible for a sudden outbreak of gastroenteritis in fattening lambs. The abomasal mucosa showed small haemorrhages, intense congestion and hyperaemia and the abomasal contents were fluid. The most severe lesions were seen in the caecum and to a lesser extent the colon. Stephens et al., (1984) reported that 30% of sheep died in a naturally occurring outbreak caused by C.jejuni, characterised by soft fluid faeces. There was a marked increase in the fluidity of the colon contents. Microscopic lesions were confined to the large intestine. The inoculation of

Table 1.2. Association of various Campylobacter species with different animal species, the clinical symptoms and site of infection.

Host	Campylobacter species	Clinical symptoms and site of infection	References
Human	C. jejuni	Enteritis and diarrhoea (jejunum and ileum)	Veron and Chatelain (1973) and Skirrow (1977)
Human	C. coli and C. fetus-subsp fetus	Enteritis and diarrhoea (jejunum)	Smibert (1978)
Pig	C. sputorum subsp mucosalis	Proliferative enteritis (small intestine)	Lomax et al. (1982)
Pig	C. coli	Enteritis, Colitis (ileum and colon)	Taylor, (1982)
Pig	C. hyointestinalis	Proliferative ileitis (ileum)	Gebhart, et al. (1983)
Cattle	C. fecalis and C. jejuni	Enteritis, diarrhoea and dysentery (jejunum and ileum)	Jones, et al. (1931) and Al-mashat, et al. (1980)
Sheep	C. jejuni	Enteritis and diarrhoea (jejunum and ileum)	Russell, (1955) and Firehammer, et al. (1981)
Sheep	C. sputorum	Proliferative regional ileitis (ileum)	Hoorens et al. (1977) and Vandenberghe, et al. (1980)
Sheep	C. jejuni and C. intestinalis	Colitis and Nephrosis (colon and kidney)	Jopp and Orr (1980)

Table 1.2. cont.

Dog	C. jejuni and C. coli	Enteritis (jejunum and ileum)	Macartney et al. (1982)
Cat	C. jejuni	Enteritis (small intestine)	Bruce et al. (1980)
Foals	C. jejuni and C. coli	Enteritis, diarrhoea, Colic and tympany (small intestine and caecum)	Atherton, et al. (1980)
Patas Monkeys	C. jejuni	Chronic mucohaemorrhaic diarrhoea (jejunum and ileum)	Bryant, et al. (1983)
Rabbit	C. jejuni	Acute Typhlitis (colon)	Moon, et al. (1974)
Poultry	C. jejuni	Enteritis and diarrhoea (caecum)	Prescott, et al. (1981) and Ruiz-palacios et al. (1981)
Turkey, domestic and wild birds	C. jejuni	Enteritis and diarrhoea (caecum)	Butzler, et al. (1979)

C.jejuni isolated from clinical cases into experimental sheep resulted in diarrhoea . Focal necrosis of the colonic mucosa and the presence of numerous dilated glands filled with necrotic debris were noted.

Campylobacter-like bacteria were demonstrated by electron microscopy in the epithelial cells of lambs showing clinical signs of terminal ileitis (Hoorens et al., 1977) and regional enteritis (Vandenberghe and Hoorens, 1980). C.jejuni and C.intestinalis were isolated from sheep showing combined colitis and nephrosis (Jopp and Orr, 1980). Russell,(1955) claimed that Vibrio species were associated with diarrhoea and high mortality in sheep in New Zealand . However, Reid,(1976), suggested that Campylobacter species are not a common cause of diarrhoea in sheep. A strain of Campylobacter (C.fetus subsp.jejuni) was recovered from an outbreak of ovine abortion (Duffell and Skirrow, 1978). The still born foetuses showed cholangitis and lymphoreticular hyperplasia (Shaw and Ansfield, 1982).

1.3.2.2 Calves

Al-Mashat and Taylor, (1980), produced diarrhoea and dysentery experimentally in calves by oral administration of C.jejuni and found both macroscopic and microscopic changes. Enteritis varied from diffuse catarrhal to severe haemorrhagic. In addition they observed hyperaemia and thickening of the mucosa of the jejunum and the ileum, and swelling of the mesenteric lymph nodes due to oedema. Necrosis of the superficial luminal epithelium, stunting and fusion of villi, dilation of the crypts, and thickening of the lamina propria with oedema and congestion were all noted. Firehammer and Myers (1981) concluded that C.jejuni is well adapted to the intestinal tract of the calf, causing enteritis, and that the severity of the infection is more acute in colostrum-deprived animals.

1.3.2.3 Pigs

C.jejuni and C.coli have been isolated from the ilea of naturally infected piglets suffering from diarrhoea (Taylor, 1982). Roland and Lawson, (1974) and Lomax et al., (1982) isolated C.sputorum subspecies mucosalis from the ileal mucosa of pigs suffering from proliferative enteritis. The gross lesions associated with the infection included thickening and erythema of the mucosa,particularly in the distal part of the ileum, and enlargement of the mesenteric lymph nodes.

Histological changes which have been observed include: stunting of the villi, accumulation of plasma cells and polymorph nuclear cells in the lamina propria, crypt abscesses and massive hypertrophy of the submucosal lymphoid tissue. All these changes were associated with high numbers of Campylobacters.

1.3.2.4 Dogs

In dogs, a moderate, superficial erosive colitis was caused by strains of C.jejuni of both human and canine origin (Prescott et al., (1981). However, the pathological picture of the disease in dogs appears milder than its human counterpart, being limited to the caecum and colon (Prescott et al., (1981).

Gross lesions which have been found include: patchy congestion of the colonic mucosa with oedema of the villi, thickening, corrugation and slight congestion of the mucosa , and congestion of the colonic lymph nodes.

Histological findings were limited to the caecum and colon. The superficial epithelium become cuboidal or low columnar with domed superficial cell margins. Mild oedema ,haemorrhage, congestion and neutrophil infiltration of the lamina propria and submucosa were observed. There was a decrease in the numbers of goblet cells in the intestinal glands and moderate hyperplasia of the local lymph nodes, with slightly active germinal centres.

Crypt abscesses were not present.

1.3.2.5 Humans

C.jejuni is recognised as a significant cause of enteritis in humans (Skirrow, 1977; Prescott and Munro, 1982). The principal sites of infection in humans appear to be the jejunum and ileum (Lambert, et al., 1979). Evans and Dadswell (1967) and Duffy et al., (1980) reported that C.jejuni was responsible for focal necrosis and ulceration of the mucosal surface of the ileum together with congestion of the caecum. Prescott, et al., (1981) observed a generalised oedema of the lamina propria with neutrophils in the crypt wall of the infected intestine. There was also a decrease in the number of goblet cells and intestinal glands.

1.3.3 Pathogenicity of Campylobacter species

There are several ways in which enteropathogenic bacteria may cause disease including attachment and colonisation, toxin production and invasion. Different animal models have been used to study the pathogenesis of acute enteritis caused by C.jejuni. Microscopic and biochemical studies have been performed on the infected tissues and appear to indicate that attachment and penetration of C.jejuni into the epithelial cells and the production of toxins by the microorganisms are possible virulence factors . It is well recognised that the establishment of an enteric infection involves complex interactions between host and parasite. The interaction involves various host factors, which function to prevent bacterial colonisation and various bacterial virulence factors which function to overcome the host's defences. Little is known about the host defence mechanisms against C.jejuni.

1.3.3.1 Attachment and colonisation

Electron microscopic studies have shown that the Campylobacters colonise and attach to the microvilli of the infected intestinal epithelial cells (Vandenberghe, et.al., 1980; Blaser et al., 1982; Mc Bride and Newell, 1983; Naess, et al., 1983; Newell et al., 1983; Welkos, 1982; Stephens, et al., 1984; and Humphrey,et al., 1986).

Newell et al., (1983) and Newell et al., (1985) reported that an aflagellate variant of C.jejuni colonised the gut poorly, and concluded that the presence of flagellae may be necessary for pathogenicity. In contrast, Mcbride and Newell, (1983) found that the aflagellate strain of C.jejuni attached significantly better to epithelial cells than the flagellate strain and postulated the presence of adhesins on the aflagellate strains. This theory is supported by Lastovica,(1983), who described the presence of several different adhesins in the cell wall of C.jejuni.

Electron microscopic studies have shown Campylobacter-like bacteria adhering to the colonic epithelium of sheep suffering from colitis (Stephens, et al., 1984) . Naess et al., (1983), using scanning electron microscopy, also showed that C.jejuni had colonised the brush borders of pigs intestine. Lee et al., (1983) reported that scanning electron micrographs of infected mice revealed sheets of C.jejuni on the colonic surface. This led to the suggestion by the authors that C.jejuni is a mucosa-associated bacterium, specifically adapted to the mucus environment of the intestinal surface, and that the colonisation of mucus is an essential step in the pathogenesis of Campylobacter infection.

1.3.3.2 Toxins

Several investigators have recently attempted to establish the possibility of classical enterotoxin and/or cytotoxin-mediated diarrhoea (Guerrant et al., 1978 ; Prescott et al., 1981 ; Ruiz-palacios et al., 1981; Johnson and Lior, (1983); Mccardell

et al., (1983); and Newell,(1984).

1.3.3.2.1 Endotoxins

Campylobacters, like other gram negative bacteria, possess a lipopolysaccharide in the cell wall (L.P.S) with endotoxic properties,(Blazer et al., 1981). Endotoxin-induced anaphylactic shock has been produced in cattle by the intravenous inoculation of broth cultures of Campylobacter jejuni and supernatant (Osbourne et al., 1962). Winter(1966) found that the lipopolysaccharide (L.P.S) of Campylobacters had a lethal effect on mice, and produced a biphasic febrile response and a generalized Shwartzman reaction in rabbits. Fumarola et al., (1982), observed that suspensions of heat-killed strains of Campylobacter species were highly reactive in the Limulus clotting assay, the suspension producing a Limulus activity equivalent to 16-32 ug/ml endotoxin compared with 128 ug/ml endotoxin by an E.coli suspension . Table 1.3 shows the endotoxin concentrations found in suspensions of Campylobacter species and E.coli using the Limulus gelatin assay.

Table 1.3 Limulus gelatin assay and endotoxin concentration in suspensions of *Campylobacter* species and *E.coli*.

Organism	Bacteria/ml	Species	Limulus assay	Endotoxin ug/ml
C.sputorum	1×10^9	sheep	+	16
C.jejuni 1	1×10^9	human	+	32
C.jejuni 2	1×10^9	human	+	16
C.coli	1×10^9	human	+	32
C.coli	1×10^9	sheep	+	32
E.coli	1×10^9	human	+	128

Source : Fumarola *et al.*, (1982)

1.3.3.2.2 Enterotoxins

The frequent occurrence of profuse watery stools in many cases of campylobacteriosis suggested that enterotoxin was also involved in the pathogenesis (W.H.O. Report, 1984). Gubinas et al., (1982) found that a heat-labile enterotoxin isolated from three strains of C.fetus subspecies fetus and two strains of C.jejuni recovered from the cerebrospinal fluid of patients with meningitis, caused morphological changes in cultures of mouse adrenal cells, similar to those caused by E.coli. However, no heat-labile nor heat-stable enterotoxic activity was demonstrated by other scientists,in Y-I, INT407, MRC-5, or CHO cell culture systems, (Newell, 1984; Campbell et al., 1982); in the suckling mouse test (Firehammer et al., 1982); or in the intestinal loops of pigs (Manninen et al., 1982); lambs, (Firehammer et al., 1982); calves (Manninen, et al., 1982) or rabbits (Guerrant et al., 1978).

1.3.3.2.3 Cytotoxin.

Cytotoxin damage to vero cells has been demonstrated using filtered culture supernatants from more than 60% of C.jejuni strains grown in a biphasic medium.This cytotoxicity is due to a heat-labile toxin (Johnson et al., 1983). Drake et al., (1981) could not demonstrate any cytopathic effect of C.jejuni on human cells. Guerrant et al., (1978) detected the cytotoxic activity of filtrates by using Chinese hamster ovary cells . Prescott et al., (1981) and Ruiz-Palacios et al., (1981) suggested that a cytotoxic enzyme might be involved in the destruction of cell membranes allowing penetration of the organisms into the intestinal mucosa. However, the significance of cytotoxic activity in the invasiveness of C.jejuni requires clarification.

1.3.3.3 Invasion

It has been postulated that an important factor in the pathogenicity of Campylobacter jejuni is the direct invasion of

the mucosa of the gastrointestinal tract (Duffy et al., 1980). Although the mechanism of penetration of the mucosa in Campylobacter infections remains unclear, it is currently accepted that invasion is the principal pathogenic mechanism involved in Campylobacter gastroenteritis (Tomita et al., 1968 and Jacoby et al., 1975) .

There are many reports in the literature to suggest that Campylobacter species invade the infected cells (Hampton and Rosario, (1965); Reimann, (1965); Staley et al., (1969); Moon et al., (1974); Butzler and Skirrow, (1979); Love and Love, (1979); Duffy et al., (1980); Vandenberghe and Hoorens, (1980); Field et al., (1981); Firehammer and Myers, (1981);Taylor, (1982); Lomax et al., (1982); Manninen et al., (1982); Newell and Pearson (1984); Stephens et al., (1984); Vandenberghe et al., (1985) and Hunter et al., (1986). Duffy et al., (1980) reported a case of Campylobacter enteritis in humans with ulcerative colitis and crypt abscesses seen in a rectal biopsy, and suggested that the pathogenesis of Campylobacter enteritis involved direct mucosal invasion. Vandenberghe et al., (1985) found Campylobacter-like organisms lying free in the cytoplasm of adenomatous epithelial cells in colonic proliferative and neoplastic changes in rats. Welkos, (1984) reported that Campylobacter-like microorganisms had invaded the intestinal epithelium and lamina-propria of chickens experimentally infected with Campylobacter jejuni. Humphrey et al., (1985,1986) similarly observed the presence of Campylobacter-like organisms in the epithelial cells and the lamina-propria of the small intestine and caecum of hamsters. However, Prescott et al., (1981) reported that there was no invasion of the mucosa of puppies by C.jejuni. Fox et al., (1985) failed to observe any invasion of the epithelial cells of adult beagles, with C.jejuni only adhering to the surface of the cells. Merrell et al., (1981) observed a fibroid meshwork surrounding C.fetus subspecies jejuni, and suggested that this might represent a penetration mechanism. Thus controversy still exists as to whether Campylobacter species are invasive organisms and neither the mechanisms involved nor the

portal of entry have yet been determined.

CHAPTER II

MATERIALS AND METHODS

2.1 COLLECTION OF FOETUSES

Lamb foetuses were obtained from local abattoirs. Each foetus, still inside the placental membrane, was brought to the laboratory within an hour of the slaughter of its dam. Foetuses obtained were of 29 cm to 30 cm crown-rump length and aged approximately 98 days (Evans and Sack, 1973).

2.2 RECOVERY OF FOETAL LAMB INTESTINE FOR ORGAN CULTURE

Explants for intestinal organ culture were prepared within one hour of removal from the ewe.The foetuses were placed on a sterile plastic tray and the placental membranes were swabbed with 70% alcohol before removal of the foetus using sterile techniques. A five to ten cm incision was made midline in the foetal abdominal wall and approximately nine cm of the small intestine was removed with fine forceps and scissors. The intestine was then placed into a sterilized plastic petridish (90x15mm)* containing phosphate buffered saline (PBS) (Figure 2.1) at room temperature, (pH 7.4) and transferred to a class 2 biohazard cabinet.

2.3 CULTURE MEDIA

Preliminary work compared modified T199 ,RPM1 1640** (without L-glutamine) and Trowell's(T8)(without L-glutamine)*** maintenance media for the effective culture of foetal lamb intestine.

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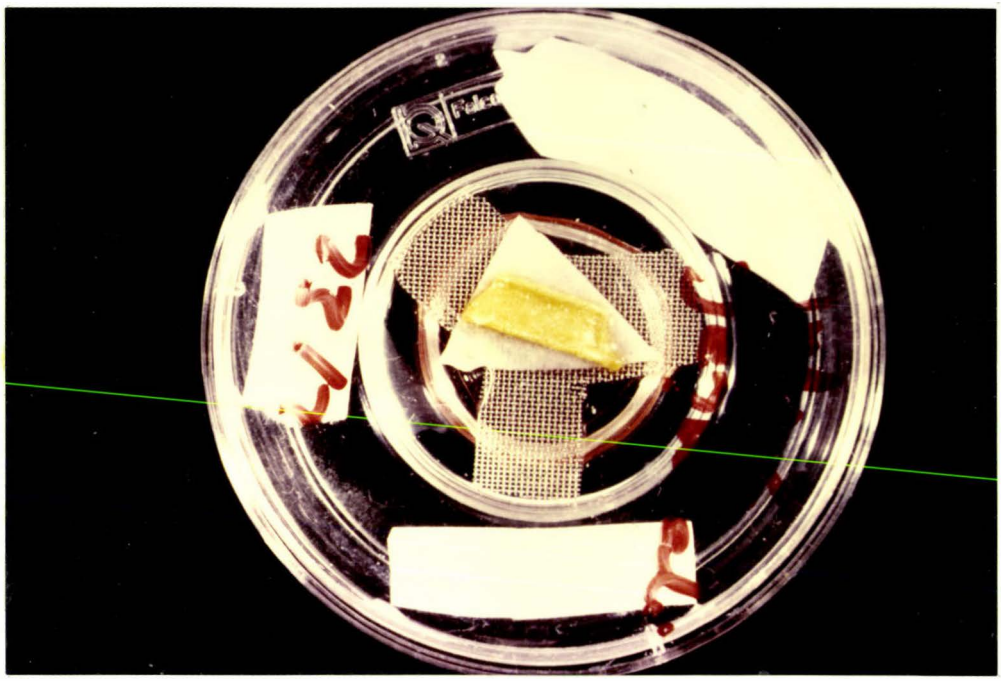
*** Gibco Laboratories, 2392 industrial St., Burlington, Ontario L7P 1AS, Canada.

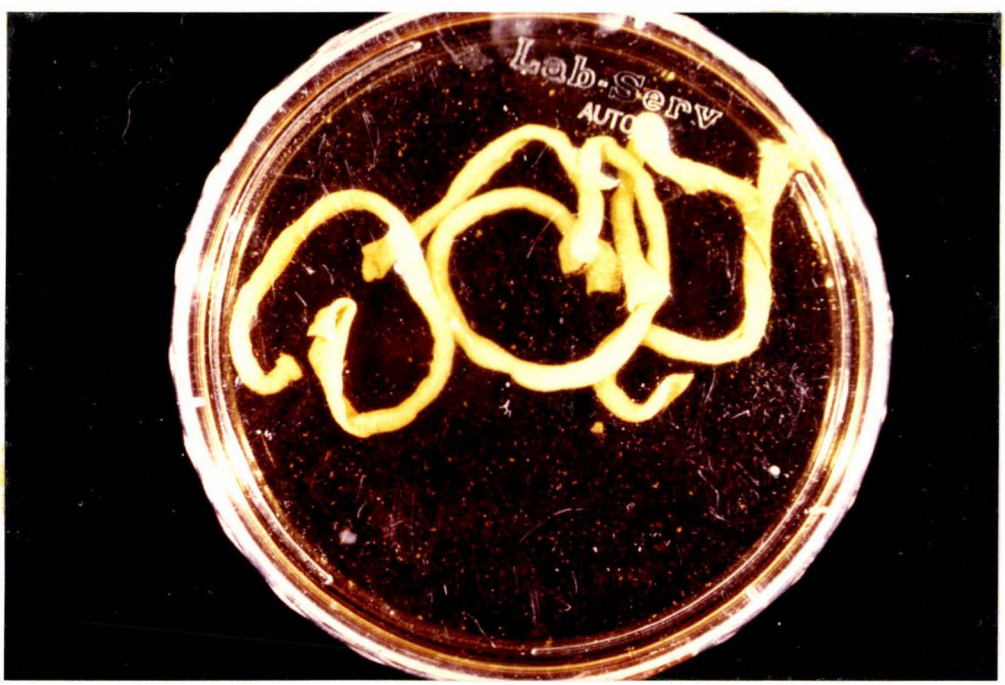
Figure 2.1

Sterilised plastic petridish (90 X 15 mm) with phosphate buffered saline (PBS) (pH 7.4) at room temperature, containing pieces of foetal lamb intestine prepared for organ culture.

Figure 2.2

Organ culture dish (60 X 15 mm) with a piece of foetal lamb intestine.




Lab-Serv

Each medium was used with or without the addition of 10% foetal calf serum. The modified T199 medium contained Earle's salts with L-glutamine, without NaHCO3, and was used without antibiotics, but with fungizone (5 ug/ml) (Squibb). T199 medium was selected for further work on the basis of the survival of the organ culture (Chapter III).

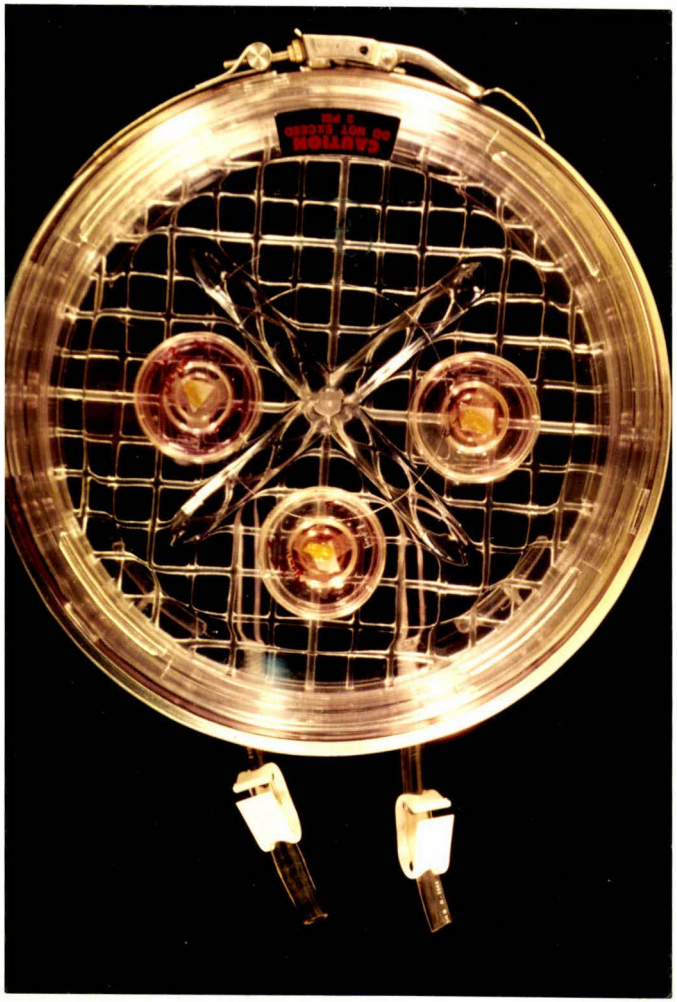
2.4 SELECTION OF CULTURE CONDITIONS AND MEDIA

Pieces of foetal lamb intestine (approximately 5 mm long) were slit lengthwise using sterilised Wolf iris scissors and fine forceps, and the mucosal side was gently washed with phosphate buffered saline (pH 7.4) (PBS), and then with T199 medium, to remove meconium and other debris. Small pieces of intestine were examined under a dissecting microscope to ensure that the mucosal surfaces were facing up. Each piece was then placed on a small piece of triangular filter paper covering a fan-shaped stainless steel grid, lying over the central well of an organ culture dish (60x15 mm)* as shown in Figure 2.2.

Six groups of foetal lamb intestine were prepared,(2 pieces per group) and put into six separate culture dishes. The six culture media to be tested (T199, T199 + 10% foetal calf serum , Trowell's medium , Trowell's medium + 10% foetal calf serum, RPM1, and RPM1 + 10% foetal calf serum) were added to the central well's of the culture dishes (one medium per dish) until a thin layer of the medium was drawn over the villous surface by capillary action. The central well was surrounded by an outer well containing a cotton pad saturated with 0.9% NaCl solution.The dishes were covered, placed in a modular incubator chamber (Figure 2.3) and gassed with 5% CO2 and 95% O2 for two minutes.They were then maintained at 37 C to determine optimal culture conditions.The culture media were renewed every day and cultures were regassed. The viability of the cultures was also checked daily under a dissecting microscope, being verified by observing the rhythmical contraction of the smooth muscle. Cultures maintained in serumless media were removed from the dishes after 3 days and the

Figure 2.3

Modular incubator chamber showing organ culture dish containing pieces of foetal lamb intestine in T199 medium + 10% foetal calf serum.


CAUTION
DO NOT EXCEED
3 PSI

cultures maintained in media with serum were removed from the dishes after 6 days. The reason for removing the groups at different times was that it had been observed in preliminary experiments that the organ cultures could be maintained for 3 days in serumless maintenance media and for a maximum of 6 days in maintenance media with serum. On removal they were immersed for two hours in Bouin's solution (Appendix II) and then transferred to 70% ethanol for 48 hours. Transverse sections of foetal lamb intestine were embedded in paraffin wax and sections of 5um were cut and stained with haematoxylin and eosin, for light microscopic examination.

2.5 PREPARATION OF THE TEST ORGANISM CAMPYLOBACTER JEJUNI

An ovine intestinal isolate of Campylobacter jejuni was obtained from the Microbiology Laboratory,Department of Veterinary Pathology and Public Health, Massey University, and cultured on Vibrio Selective Agar(VSA)(Appendix 1). After 48 h primary culture, individual colonies were subcultured onto blood agar plates (Difco Colombia-blood agar base with 7% sheep blood and 1 ml of 0.05% solution of ferrous sulphate,sodium metabisulphite and sodium pyruvate supplement per 50 ml of agar). They were incubated at 42 C for 48 hours. Organisms were subcultured three times to ensure purity.One to two colonies from a pure culture were suspended in heart infusion broth in a sterile bijou bottle. After overnight growth the bacterial suspension was serially diluted 1 in 10 with T199 medium. Bacterial counts were made for each of the dilutions using the Miles and Misra (1938) technique. The dilution used for infecting the cultures was found to contain 1.15×10^6 bacteria in 1.25 ml, the amount to be inoculated into each infected culture medium.

2.6 INTERACTION OF C.JEJUNI WITH FOETAL LAMB INTESTINAL ORGAN CULTURE

The intestines of two foetuses were prepared for organ culture as described in Sections 2.1, 2.2 and 2.4. The tissues were allocated randomly to the Control and Infected treatment groups. Six experimental groups were established, 3 groups infected with C.jejuni (Infected treatment) and 3 groups containing uninfected tissues, acting as the Control. Each of the Control and Infected groups consisted of 3 pieces of foetal intestine (measuring 4-5 mm) in an organ tissue culture dish (60 X 15 mm). The dishes were placed in a modular incubator chamber, as shown in Figure 2.3. 1.25 ml of the diluted bacterial culture, containing approximately 1.15 X 10^6 microorganisms, was used to infect the organ cultures.

One of the Control and one of the Infected culture dishes were removed from the modular incubator chamber at 6, 13 and 15 hours post-infection and prepared for microscopic examination as described below.The medium in the remaining two culture dishes in each of the Control and Infected groups was aspirated and replaced by freshly prepared medium as described in section 2.4, and the cultures returned to the incubator and gassed as before. The tissues collected were treated as follows: tissues for transmission and scanning electron microscopy were immersion fixed in modified Karnovsky's solution (Appendix III) and tissues for Light microscopy were immersion fixed with Bouin's solution for two hours.

2.6.1 Light Microscopy (LM)

In addition to routine H & E staining of tissue sections, selected sections were stained by the Warthin Starry technique (Young, 1969) in order to demonstrate the microorganisms. Seven villi from each section were chosen at random and the height and width of the epithelial cells of the villi were measured. The heights of ten randomly selected villi were measured. Results were analysed statistically by application on the Reg.Program (Gilmour,1985) and Duncan's Multiple Range Test (Steel and Torrie, 1981).

2.6.2 Scanning Electron Microscopy (SEM)

Specimens cut into 4x4 mm pieces were fixed with modified Karnovsky's solution overnight.After several washings in two changes of 0.1M PBS (pH 7.2) at 4C for one hour,the specimens were dehydrated by consecutive immersion in graded ethanol solutions of 25%, 50%, 70%, 90% and twice in 100% ethanol at room temperature, for 20 minutes each.The dehydrated tissues were dried in a critical drying apparatus*. All the tissue samples were mounted on aluminium stubs with silver conducting paint, sputter coated with 200A gold by routine methods and examined under a Cwikscan/100 field emission scanning electron microscope.

2.6.3 Transmission Electron Microscopy (TEM)

Specimens for Transmission Electron Microscopy were washed and fixed in a similar manner as for Scanning Electron Microscopy ,then were cut into 1 mm cubes and post fixed in 1% osmium tetroxide(Appendix IV) in 0.1M PBS (pH 7.2) for one hour at 4 C. After washing and dehydrating in alcohol in the same way as for SEM, the tissues were immersed in propylene oxide for 20 minutes and placed overnight in propylene oxide with 25% epoxy resin**, on a stirrer. The next day the tissues were embedded in freshly prepared 100% epoxy resin and left for 48-72h, at 60 C to polymerise. Sections of one to two micron thickness were cut from each block and picked dry off the glass knife with an eyelash. They were transferred onto a drop of water on a glass slide and then heated on a hot plate at 80 C until the water evaporated. The thick sections were immediately covered with a drop of 1% toluidine blue and heated on a hot plate at 80 C until the drop of stain evaporated at the edges. The samples were then washed thoroughly with distilled water, dried, and mounted in DPX

* Polaron E3000/series 2.

** Durcupan Fluka

(Distrene, Dibutyl phthalate and Xylene). After the heating,washing,and drying the thick sections were examined under a light microscope, both to evaluate the histological features of normal structures and to determine areas of interest for electron microscopy. Following this, the block face was trimmed down to an area of interest for ultra-thin sectioning. Thin sections were cut on an LKB 111 ultramicrotome, stained with saturated uranylacetate (Appendix V) in 50% ethanol and lead citrate (Appendix VI) for 6 minutes each,and examined on a Philips EM201C transmission electron microscope.

2.7 STATISTICAL METHODS

The data was analysed using a Generalized Linear Models Computing Package (REG), Gilmour (1985). The model assumed to describe the data was:

$$Y_{ijk} = u + A_i + B_j + e_{ijk}$$

where

Y_{ijk} is the kth observation in the ith time and jth treatment.

u = the general mean

A_i = the effect of the ith time

Levels of significance are denoted throughout by the letters assigned to means on the basis of Duncan's multiple range test; means with letters not in common are significantly different and the level of significance is given in the text.

The following abbreviations are used to denote the levels of statistical significance used in the analysis of variance tables:

NS = Not significant or $P > 0.05$

* = $P < 0.05$

** = $P < 0.01$

B_j = the effect of the jth treatment

e_{ijk} = the effect of random residual assumed to be normally and independently distributed with mean zero and variance.

CHAPTER III

RESULTS

3.1 SELECTION OF SUITABLE MEDIUM FOR OVINE INTESTINAL ORGAN CULTURE

The experiment was designed to evaluate the effects of different media, with or without serum, on the survival and growth of foetal lamb intestine in organ culture. The aim of the experiment was to select a suitable medium for the maintenance of viable ovine foetal intestinal organ culture, to study the pathogenicity of Campylobacter jejuni.

The media tested were T199 medium, T199 with 10% foetal calf serum, Trowell's medium (T8), Trowell's medium with 10% foetal calf serum, RPMI medium and RPMI with 10% foetal calf serum.

3.1.1 The Effects of Different Media on the Morphology of Primary Intestinal Organ Culture.

3.1.1.1 T199

Organ culture was maintained in this medium for three days. The mucosal cells were viable but changes were seen in the morphology of the epithelial lining cells. The absorptive cells were cuboidal and their nuclei were round to ovoid in shape and were situated either centrally or basally (Figure 3.1). However, some of the epithelial cells were squamous and had elongated nuclei occupying most of the cell (Figure 3.2).

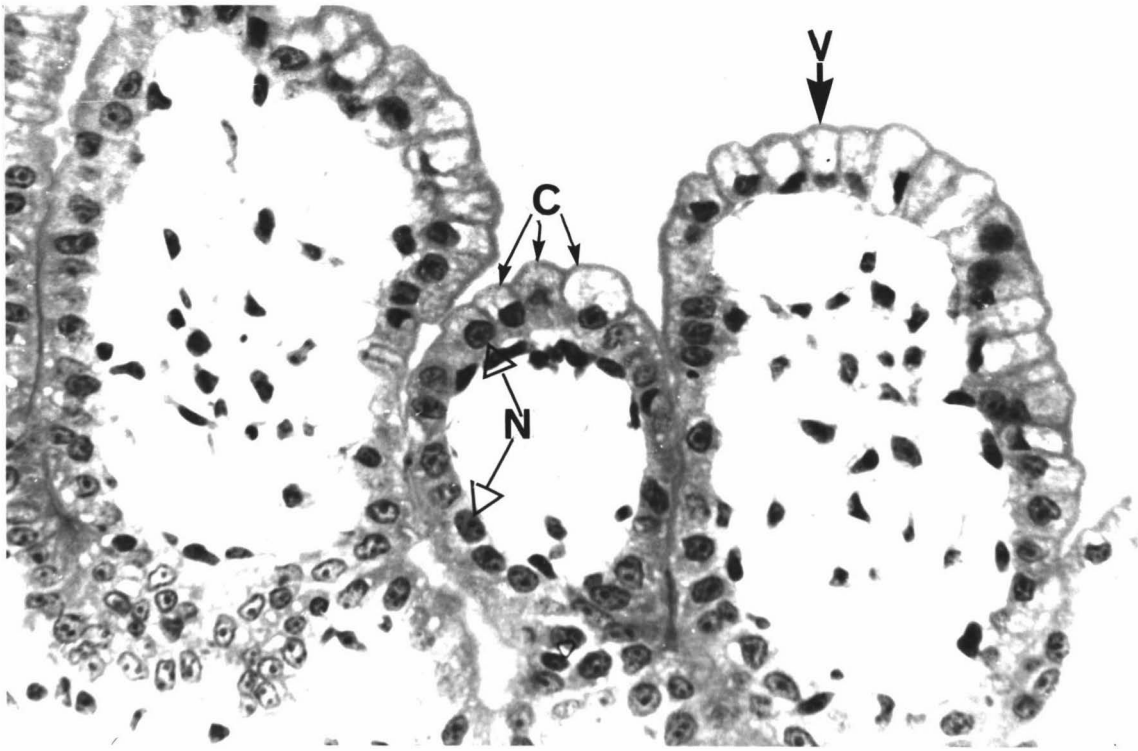
The morphology of the villi changed from normal elongated to dome-shaped (Figure 3.1). The crypts were shorter and wider than normal (Figure 3.2).

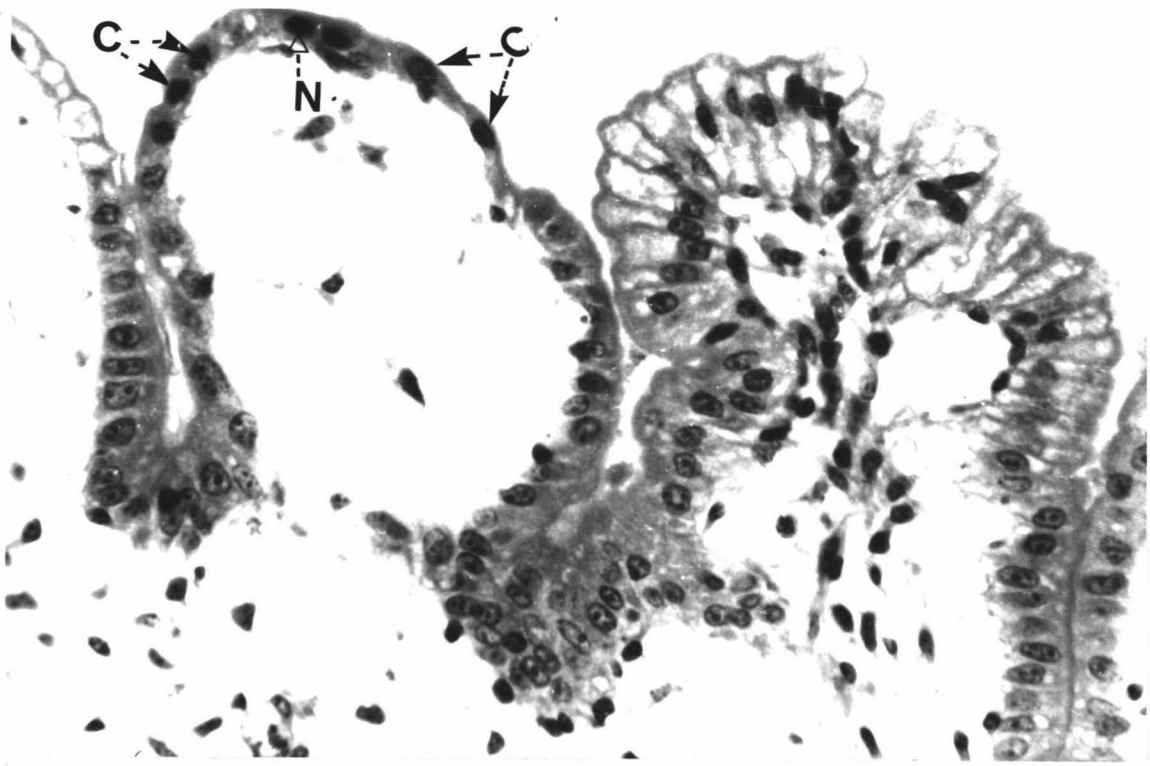
Figure 3.1

Foetal lamb intestine after 3 days culture in T199 medium . The villi (v) are dome-shaped and are lined by cuboidal epithelial cells (C). Their nuclei (N) are oval to round and occupy the base of the cells. (H&E X200).

Figure 3.2

Foetal lamb intestine after 3 days culture in T199 medium . The epithelial lining cells are squamous in appearance (C). Their nuclei are elongated to ovoid in shape and occupy most of the cytoplasm . (H & E X200).


V
C
N


C
N
C

3.1.1.2 T199 with 10% Foetal Calf Serum

In this medium, the architecture and the cellular composition of the mucosa were consistently well preserved during the six days of culture (Figure 3.3). Most of the epithelial cells were columnar with oval nuclei which occupied the base of the cells. The intestinal crypts were well developed. No goblet cells were seen (Figure 3.3).

3.1.1.3 Trowell's Medium (T8)

The culture was maintained in this medium for three days . The villi were shorter and wider than normal and had a dome-shaped appearance. The enterocytes were more cuboidal towards the apical part of the villi (Figure 3.4). The nuclei were oval in shape and large in relation to the amount of cytoplasm (Figure 3.4).Goblet cells could not be defined. The intestinal crypts were smaller when compared with uncultured tissue.

3.1.1.4 Trowell's Medium with 10% Foetal Calf Serum

The culture was maintained for six days in Trowell's medium enriched with 10% foetal calf serum (Figure 3.5). The villi on the epithelial surface showed an uneven appearance and some of the villi were severely reduced in height. Crypts of Lieberkuhn were not well developed. The enterocytes were cuboidal to round in shape, with rounded to oval nuclei (Figure 3.5). Most of the nuclei occupied the central part of the cells and few of them occupied the basal portion. Some of the absorbtive cells showed vacuolation of the apical cytoplasm. Goblet cells were scarce.

3.1.1.5 RPMI

Cultures were maintained for three days in RPMI. The villi were thin and tall with an irregular mucosal surface (Figure 3.6). The epithelial lining cells of the villi were squamous. The nuclei were either round or elongate and occupied most of the

Figure 3.3

Foetal lamb intestine after 6 days culture in T199 medium + 10% foetal calf serum. The epithelial lining cells (C) are well preserved. The absorptive cells (C) are columnar with oval-shaped nuclei occupying the base. The intestinal crypts (Cr) seen here are well developed. (H & E X200).

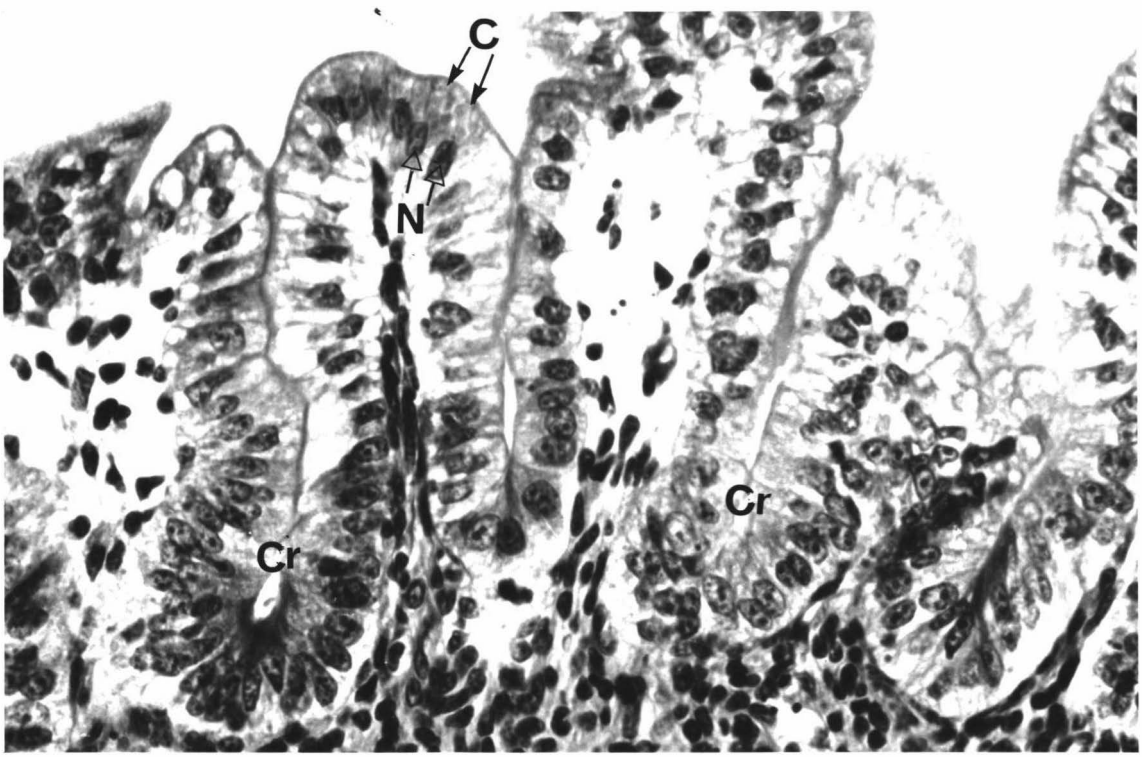
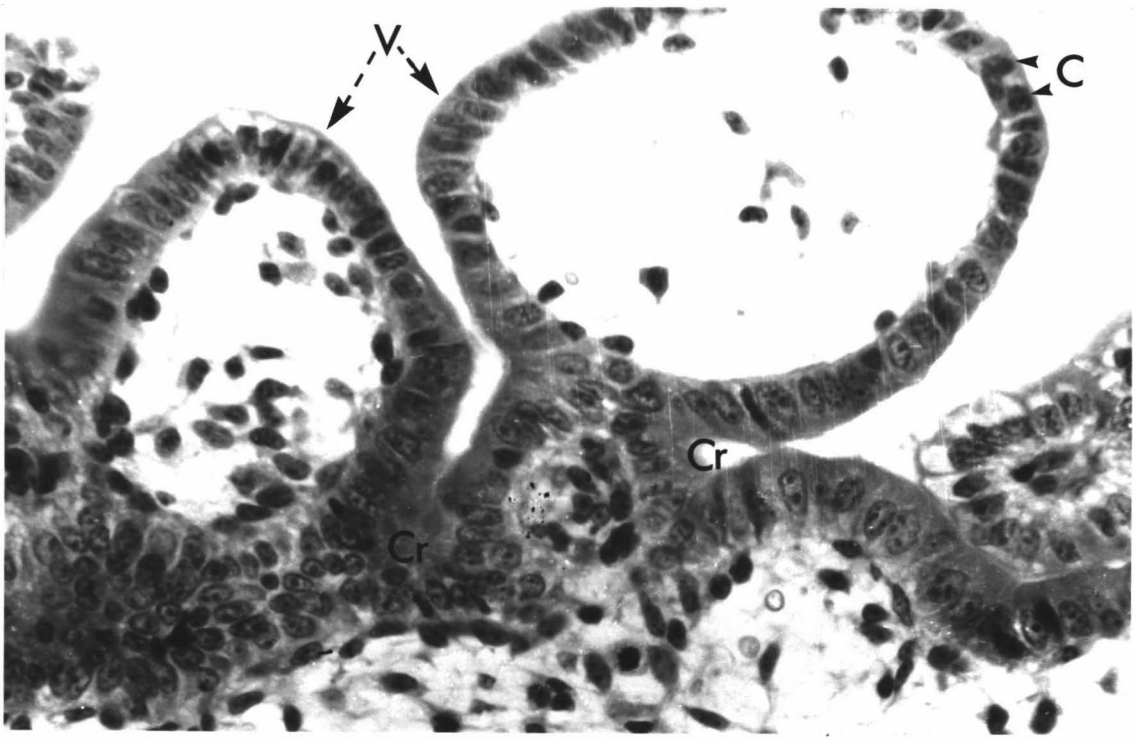

C
N
Cr
Cr

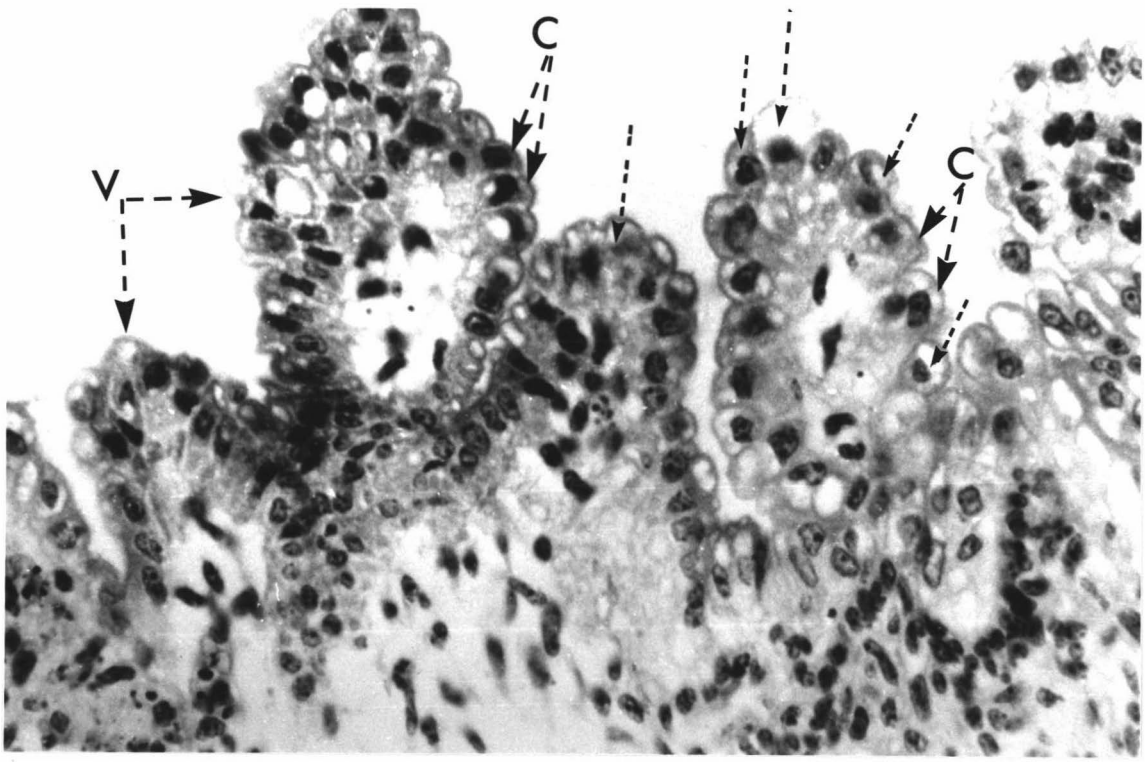
Figure 3.4

Foetal lamb intestine after 3 days culture in Trowell's medium (T8). The villi (V) are shorter and wider than normal and have a dome-shaped appearance. The enterocytes (C) are more cuboidal towards the apical part of the villi. The intestinal crypts (Cr) are smaller than in uncultured intestinal tissue. (H & E X200).

Figure 3.5

Foetal lamb intestine after 6 days culture in Trowell's medium (T8) + 10% foetal calf serum. The villi (V) have an uneven surface and are reduced in height. The enterocytes (C) are cuboidal to round in shape and show a number of vacuoles (small arrow) in the apical part of the cytoplasm. (H & E X200).


V
C
Cr
Cr


C
V
C

cell's cytoplasm. Goblet cells were not observed. The intestinal crypts were shallow ,narrow and not well developed.

3.1.1.6 RPMI with 10% Foetal Calf Serum

In this medium foetal intestinal organ culture was maintained for six days. The villi were severely reduced in height and showed a dome-shaped appearance (Figure 3.7). The epithelial lining cells were round and irregularly arranged. The cytoplasm bulged towards the luminal surface (Figure 3.7). The nuclei were round in shape and occupied most of the cytoplasm of the epithelial lining cells. Most of the absorbing cells showed supranuclear vacuolation (Figure 3.7).

On the basis of the results obtained, T199 medium enriched with 10% foetal calf serum was chosen, because it gave more consistent results in maintaining foetal intestinal explants for a period of six days in culture. This medium was subsequently used for ovine foetal intestinal organ culture for the study of the pathogenicity of Campylobacter jejuni .

3.2 STUDIES OF SOME ASPECTS OF THE PATHOGENESIS AND PATHOGENICITY OF C.JEJUNI USING OVINE FOETAL INTESTINAL ORGAN CULTURE AS A MODEL

Three microscopic techniques (Light Microscopy,Transmission and Scanning Electron Microscopy) were used to study the pathogenesis and pathogenicity of Campylobacter jejuni on foetal lamb intestinal organ cultures maintained in T199 medium enriched with 10% foetal calf serum.The tissues were experimentally infected and the morphological changes studied at 6,13 and 15 hours post-infection. The changes observed were compared with control uninfected culture tissues at 6 , 13 and 15 hours.

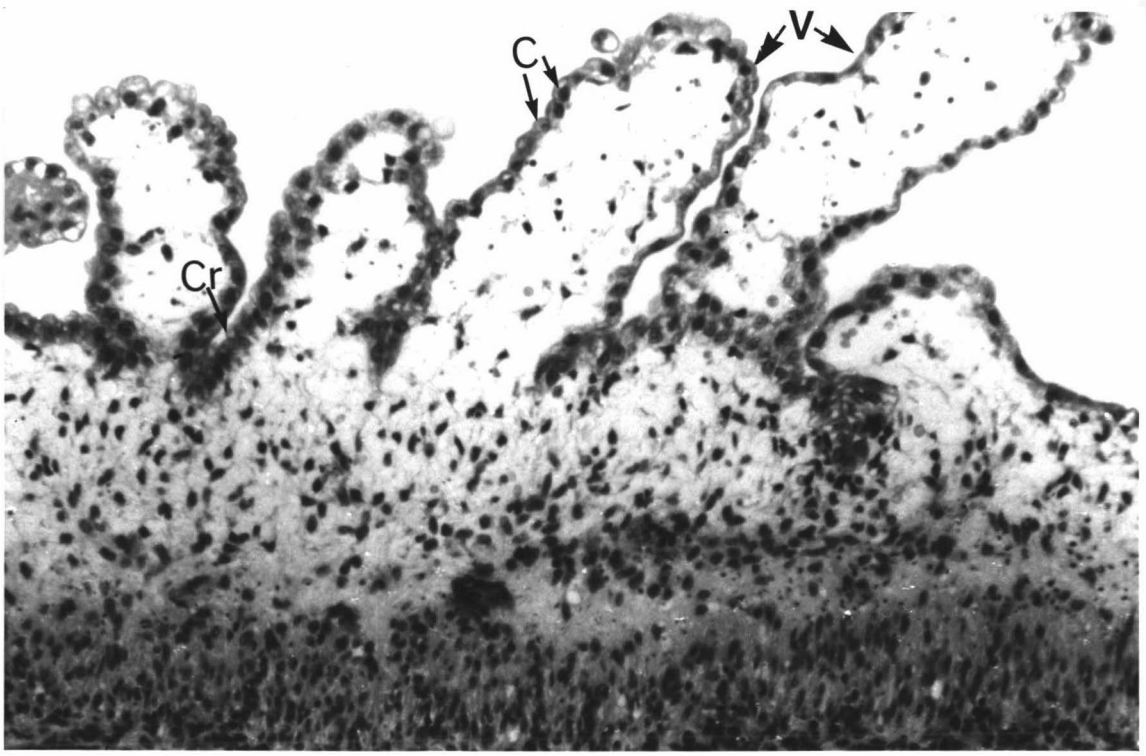
3.2.1 Light Microscopy

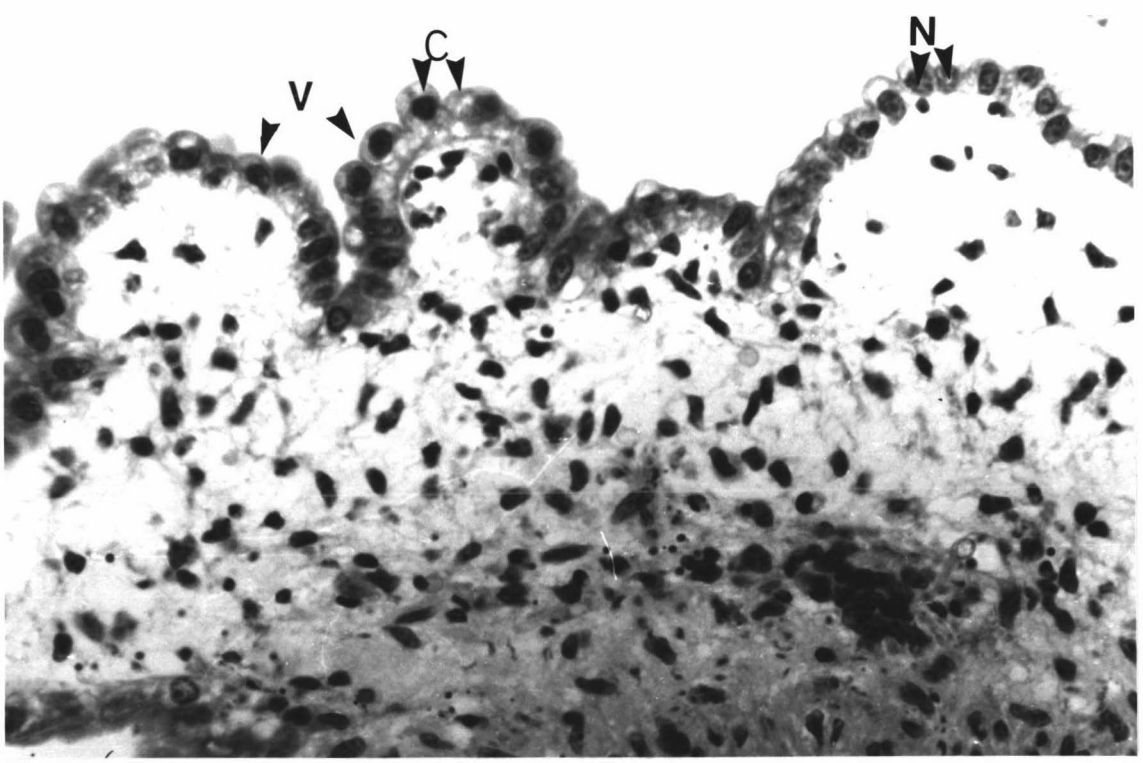
Figure 3.6

Foetal lamb intestine after 3 days culture in RPM1. The villi (V) are tall and thin with an irregular surface (arrow). The epithelial cells (C) are squamous with small round nuclei occupying most of the cytoplasm. The intestinal crypts (Cr) are shallower and narrower than normal. (H & E X200).

Figure 3.7

Foetal lamb intestine after 6 days culture in RPM1 + 10% foetal calf serum. The villi (V) are severely stunted and have a dome-shaped appearance. The absorptive cells (C) are round with uneven surfaces. Their nuclei (N) are round and occupy the entire cytoplasm. (H & E X200).


C
V
Cr


V
C
N

3.2.1.1 Control

3.2.1.1.1 : Zero Time

This tissue provided the normal appearance of the intestinal epithelium from which comparative assessments were made(Figure 3.8).

The lining epithelial cells of the uncultured foetal intestine consisted of simple columnar cells. The nuclei of these cells were ovoid to elongate in shape and occupied the basal part of the cells. Chromatin content of the nuclei varied from dense to light. The epithelial cells showed no vacuolation. The cytoplasm of the epithelial cells was homogeneous and basophilic in character.

The microvilli of the epithelial lining cells were prominent. The epithelial cells had an even striated border. The villi were tall and prominent, with tapering ends. The bases of the villi appeared densely cellular (Figure 3.8).

The intestinal glands were prominent and lined by simple columnar cells (Figure 3.9). The nuclei of the lining cells were ovoid in shape and located at the basal part of the epithelial cells of the apical portion of the gland. A few goblet cells were present at the mid and distal parts of the villi. The epithelial cells of both the villi and the intestinal crypts were not vacuolated. The intestinal crypts were lined with simple columnar epithelial cells (Figure 3.8).

3.2.1.1.2 : Six hours

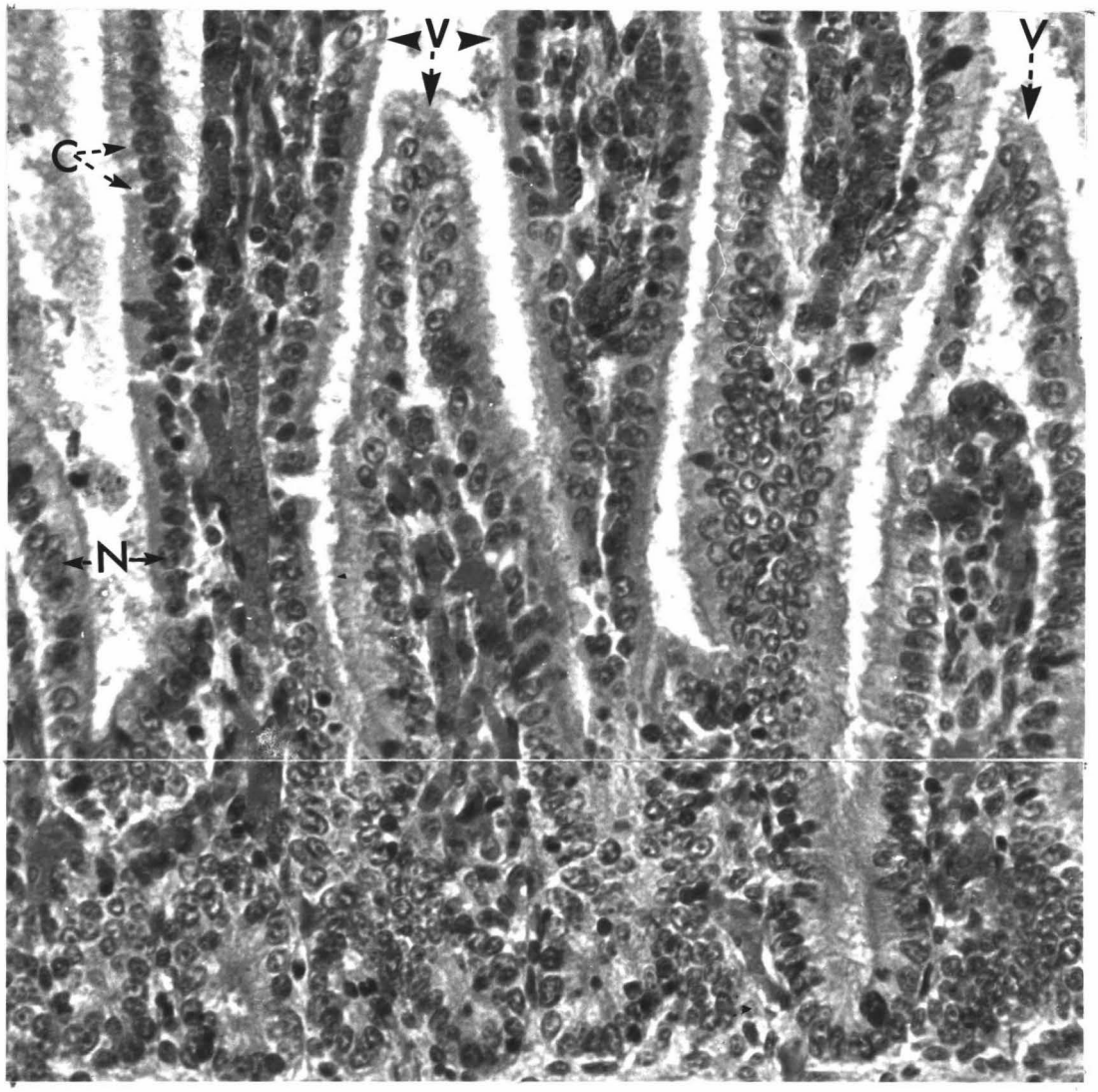
The cellular morphology of the epithelium of uninoculated, control foetal intestinal organ cultures was essentially similar to that of the uncultured intestine (Figure 3.10). The intestinal crypts, although well developed,were shorter and wider than the controls at zero time (Figure 3.10 and 3.11). The intestinal

Figure 3.8

Uncultured foetal lamb intestine from a 98 day old foetus. The villi (V) are tall and pointed with prominent microvilli (MV). The epithelial lining cells (C) are columnar with an even striated border, and oval elongated nuclei (N) occupying their bases. (H & E X400).

Figure 3.9

Morphology of an intestinal gland from uncultured foetal lamb tissue. The intestinal glands (Gl) are healthy and lined by simple columnar cells (C). (H & E X400).


V
V
C
N

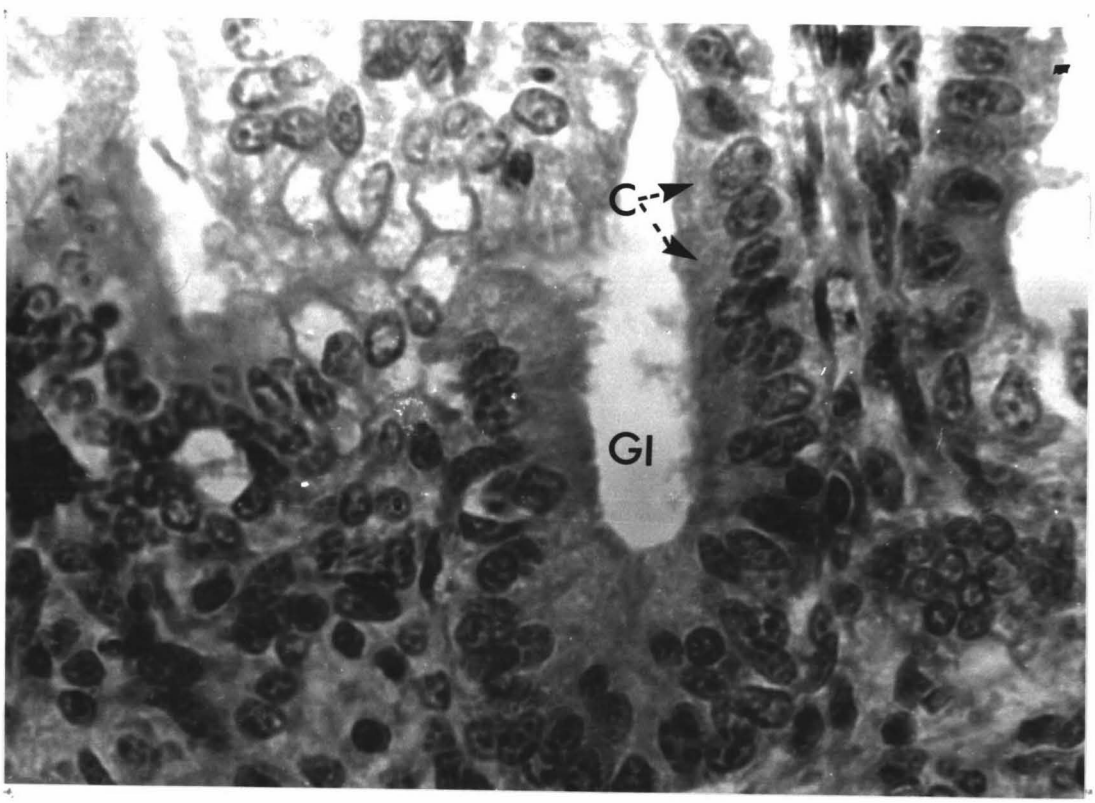
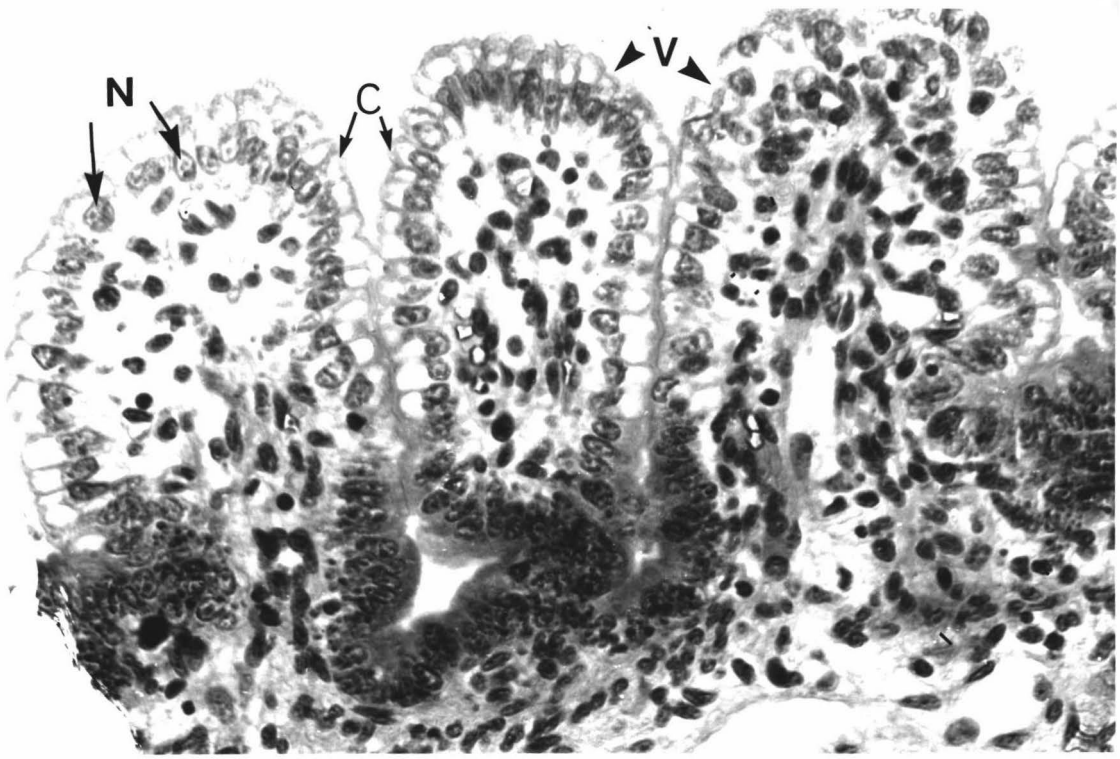

C
GI

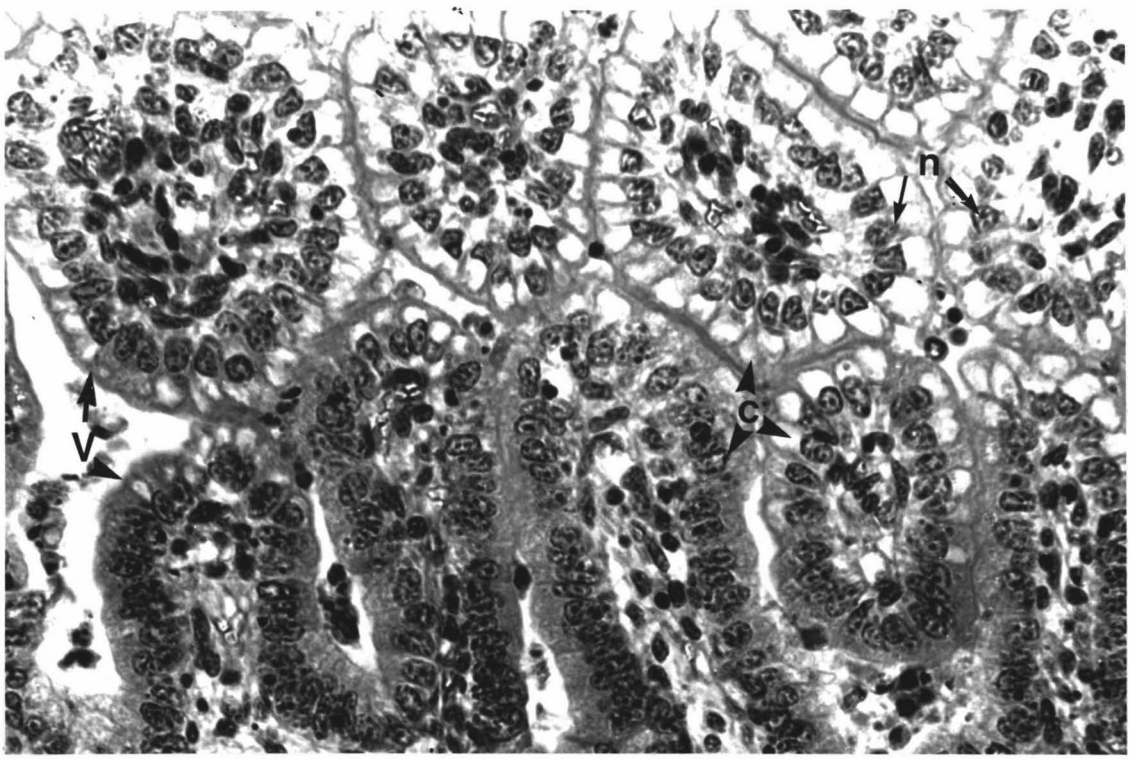
Figure 3.10

Control foetal lamb intestine after 6 hours culture in T199 medium + 10% foetal calf serum. The morphology of the intestinal villi (V) is well preserved. The epithelial lining cells (C) are columnar with round to elongate nuclei (N). The intestinal crypts (Cr) are well maintained and lined with simple columnar cells. (H & E X200).

Figure 3.11

Cross section of control foetal lamb intestine after 6 hours culture in T199 medium + 10% foetal calf serum. The villous epithelium (V) is well preserved. The absorptive lining cells (C) are simple columnar in shape, with round to elongate nuclei (N). (H & E X400).


N
C
V


n
V
C

glands were preserved, although the shapes of the epithelial lining cells ranged from low columnar to cuboidal in appearance, with rounded nuclei (Figure 3.10 and 3.12). No goblet cells were seen.

3.2.1.1.3 : Thirteen hours

Although the epithelium of the mucosa remained intact, the cells were columnar to cuboidal in appearance (Figure 3.13). Some of the epithelial lining cells were vacuolated (Figure 3.13).

The intestinal crypts were normal. Some of the epithelial cells had degenerated and had sloughed . No goblet cells were seen.

3.2.1.1.4 : Fifteen hours

The mucosal morphology of the uninoculated control after 15 hours culture was histologically similar to the 13 hours control culture, except that the villi were thinner (Figure 3.14).

3.2.1.2 Infected

3.2.1.2.1 : Six hours

Foetal intestinal organ culture, maintained in T199 medium enriched with 10% foetal calf serum, was infected with approximately 10^6 colony forming units/ml (CFU/ml) of Campylobacter jejuni .

The morphological changes appeared to be limited to the extreme tips of the villi (Figures 3.15,3.16 and 3.17). The changes included slight vacuolation of the cytoplasm of the cell, between the nucleus and the striated border (Figures 3.16 and 3.17). The nuclei of affected cells were relatively normal in appearance (Figure 3.16). There was a slight shedding of epithelial cells from the villous tip and the desquamated cells

Figure 3.12

Control foetal lamb intestine after 6 hours culture in T199 medium + 10% foetal calf serum. The intestinal glands (Gl) are well maintained and lined by simple columnar epithelium (C). (H & E X400).

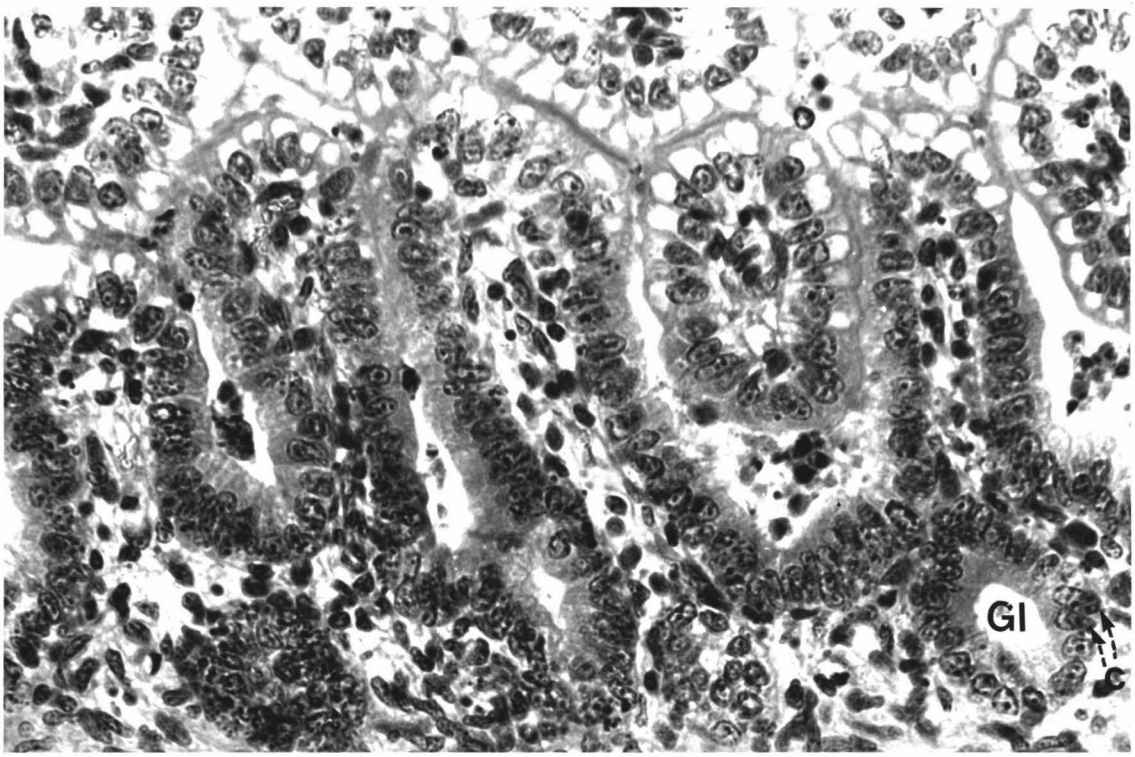

GI

Figure 3.13

Control foetal lamb intestine after 13 hours culture in T199 medium + 10% foetal calf serum. The villous epithelial cells (C) are intact and well preserved. The epithelial lining cells (C) are columnar to cuboidal in shape with round to elongate nuclei (N). Some of the absorptive cells (C) have cytoplasmic vacuoles (arrow). (H & E X400).

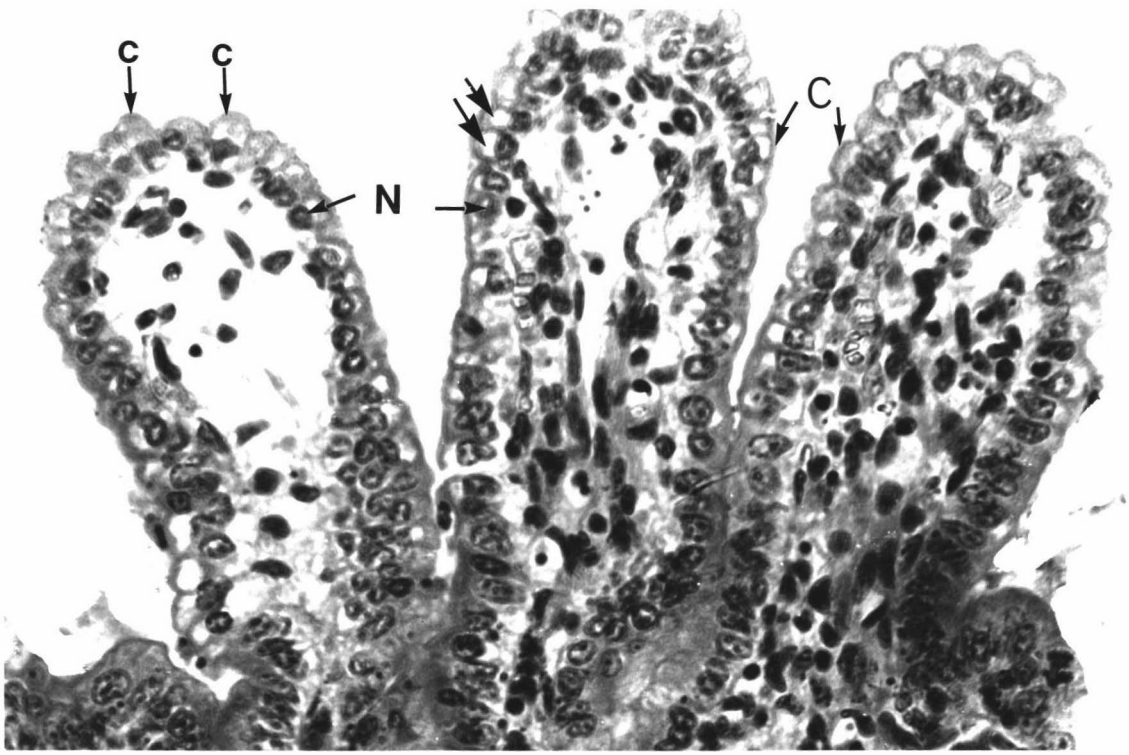

C
C
N
C

Figure 3.14

Control foetal lamb intestine after 15 hours culture in T199 medium + 10% foetal calf serum. The mucosal epithelium is well preserved. (H & E X200).

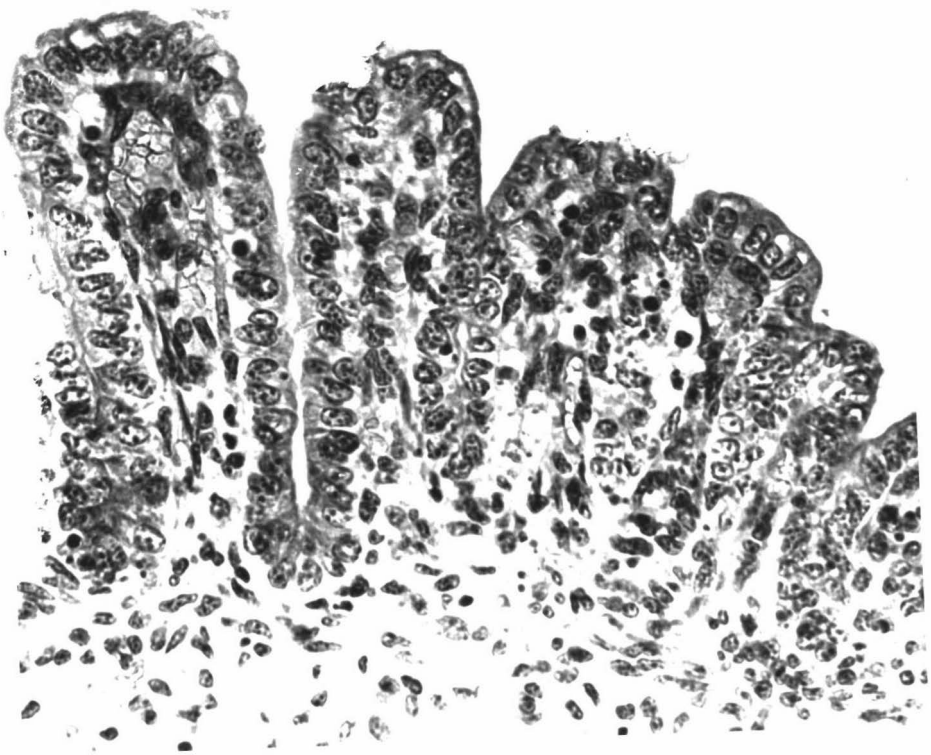
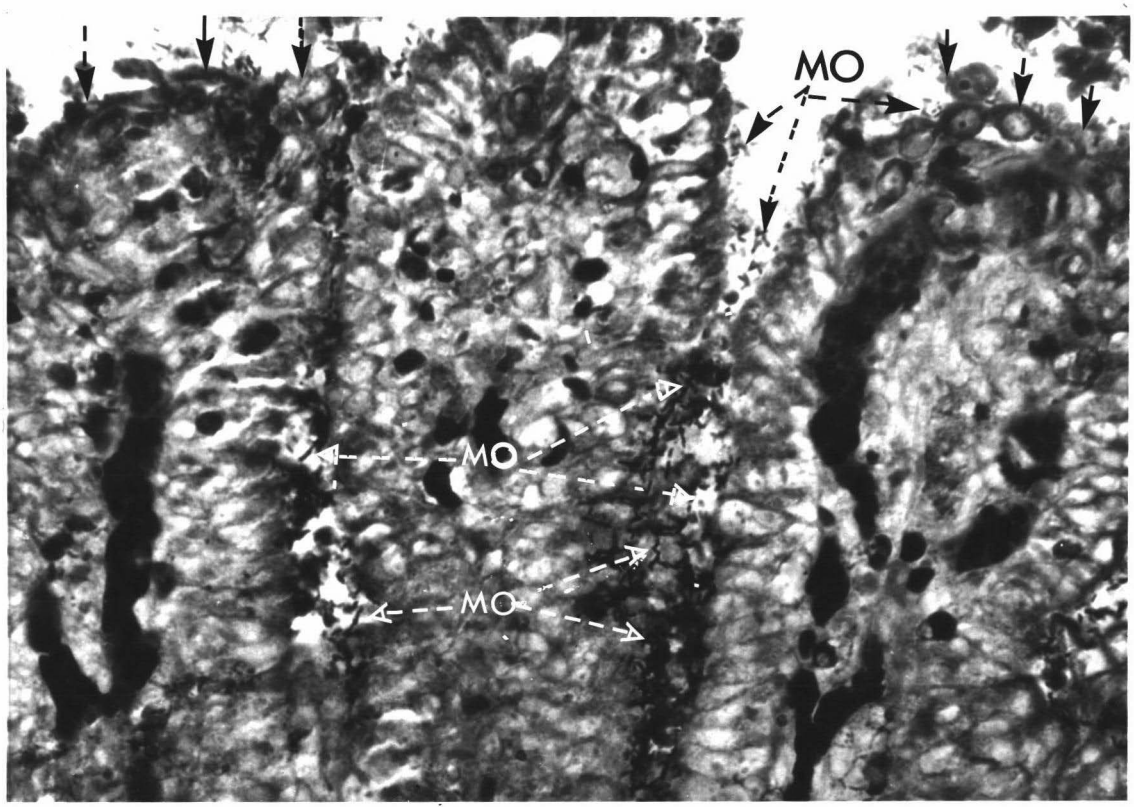


Figure 3.15

Foetal lamb intestine cultured in T199 medium + 10% foetal calf serum, infected with approximately 10^6 CFU/ml of Campylobacter jejuni, showing changes that have occurred 6 hours postinfection . Mild necrosis (arrow) can be seen at the tips of the villi, with a slight shedding of epithelial cells. The microorganisms (MO) are attached to the surface of the villous and crypt epithelia (long arrow). (Warthin Starry Stain X200).

Figure 3.16

Foetal lamb intestine cultured in T199 medium + 10% foetal calf serum, infected with approximately 10^6 CFU/ml of C.jejuni,showing changes that have occurred 6 hours postinfection .The epithelial cells at the tips of the villi are disorganised (arrow) and show exfoliation. Some also show cytoplasmic vacuolation (small arrow). (H & E + Warthin Starry Stain X200).


MO
MO
MO

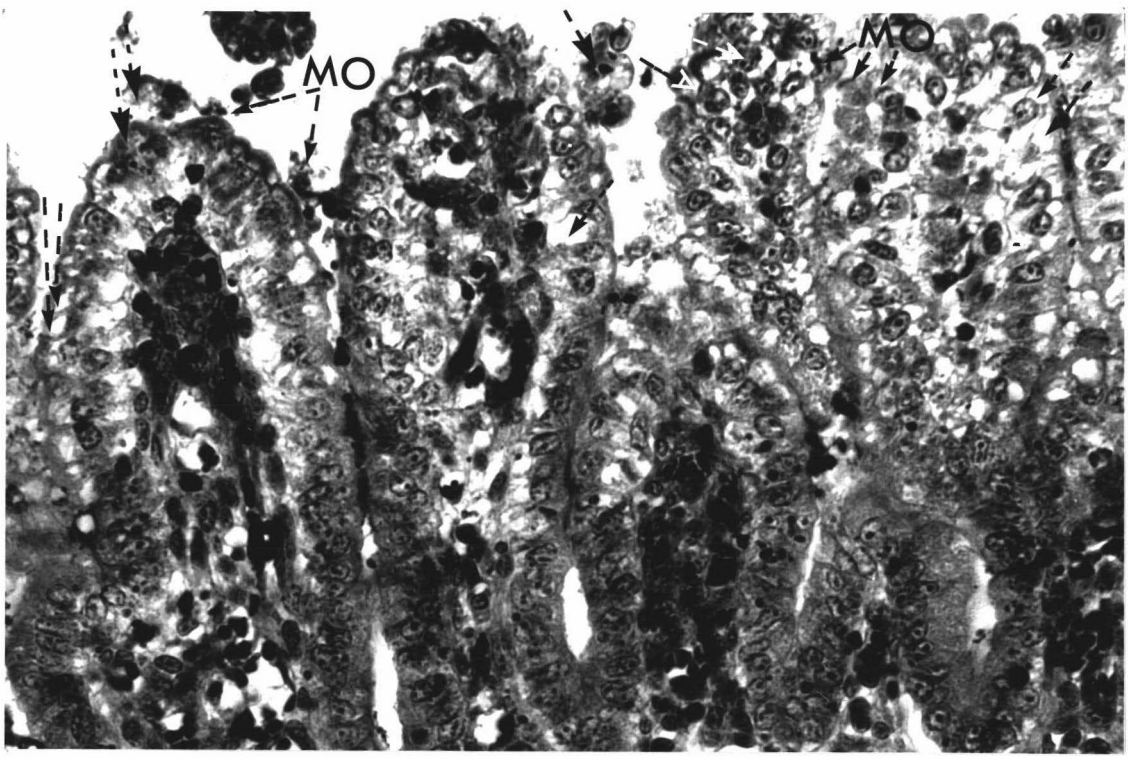
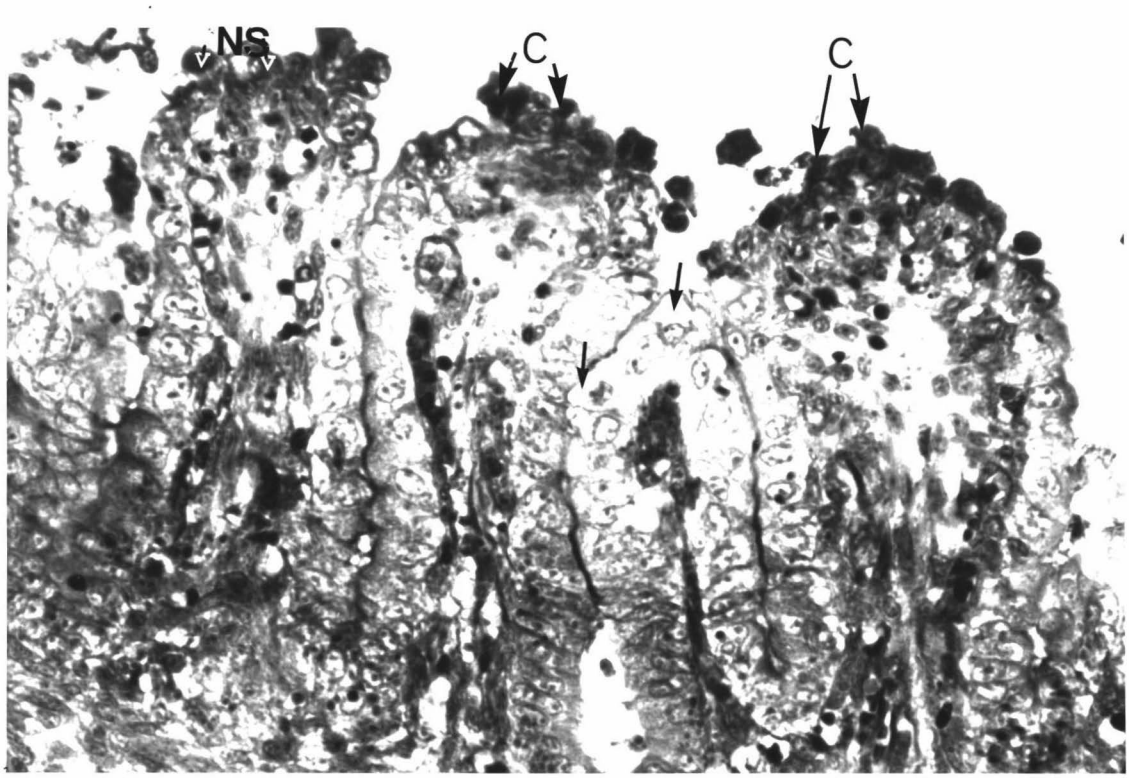

MO
MO

Figure 3.17

Foetal lamb intestine cultured in T199 medium + 10% foetal calf serum, infected with approximately 10^6 CFU/ml of C.jejuni, showing changes that have occurred 6 hours postinfection. The epithelial cells (C) at the tips of the villi are slightly damaged. A few of the epithelial cells (C) show cytoplasmic vacuolation (arrow), nuclear swelling (NS), and chromatin margination (CM). (H & E + Warthin Starry Stain X200).


NS
C
C

were round in shape (Figures 3.15, 3.16 and 3.17). There was an increased eosinophilia of the cytoplasm (Figure 3.17). Microorganisms were demonstrated attached to the surface of the villous and crypt epithelial cells in silver stained sections (Figure 3.15). Most organisms were attached in the region of the tips of the villi. Some microorganisms had invaded the epithelial cells.

3.2.1.2.2 : Thirteen hours

Thirteen hours after inoculation the intestinal mucosa showed increased necrosis at the tips of the villi (Figures 3.18 and 3.19). This was characterised by a loss of epithelium (Figures 3.18 and 3.19).

The desquamated cells were round in shape and the nuclei showed prominent peripheral lumping of chromatin (Figure 3.19). The infected villous epithelial cells showed nuclear swelling with cytoplasmic eosinophilia and marked vacuolation (Figures 3.20 and 3.21).

The epithelial cells of the crypts, from the base to the mid-portion of the villi, were relatively normal in appearance (Figure 3.19). Stained silver sections revealed rod- and spiral-shaped microorganisms attached to the surface and within the epithelial cells (Figures 3.18, 3.20 and 3.21). The microorganisms varied in length between 2.5-5 microns . The intestinal crypts were also invaded by the microorganisms (Figure 3.18).

3.2.1.2.3 : Fifteen hours

There was severe damage to the epithelial cells at the tips of the villi (Figures 3.22 and 3.23) in intestinal tissue 15 hours postinfection. Epithelial cells were commonly exfoliated and had prominent vacuolation of their cytoplasm (Figures 3.22 and 3.23).

Figure 3.18

Foetal lamb intestine cultured in T199 medium + 10% foetal calf serum, infected with approximately 10^6 CFU/ml of C.jejuni, showing changes that have occurred 13 hours postinfection. The cells at the tips of the villi show a moderate necrosis. Spiral to rod-shaped organisms (MO) can be seen attached to the villous epithelial surface. A few organisms can also be seen within the epithelial cells (thin arrow). (Warthin Starry Stain X200).

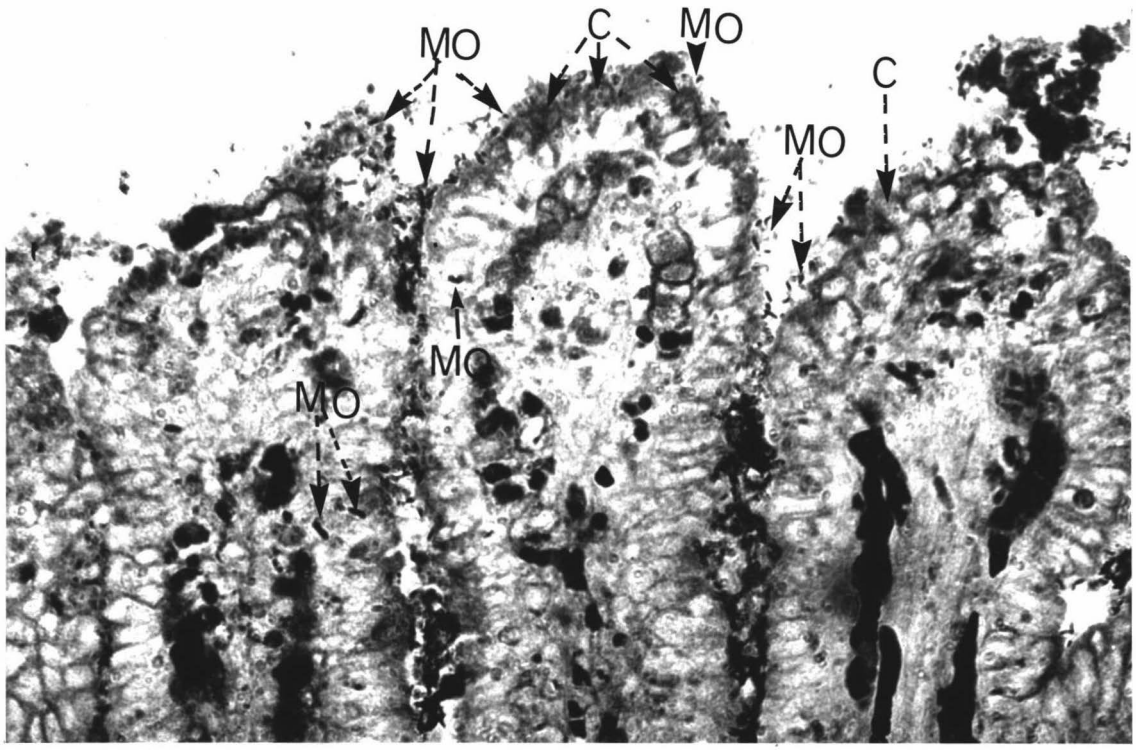

MO
C
MO
C
MO
MO
MO

Figure 3.19

Foetal lamb intestine cultured in T199 medium + 10% foetal calf serum, infected with approximately 10^6 CFU/ml of C.jejuni, showing changes that have occurred 13 hours postinfection. The necrotic epithelial cells (C) are being shed from the tips of the villi. The infected cells are round with swollen nuclei (N). The uninfected epithelial lining cells (small arrow) appear unchanged. (H & E + Warthin Starry Stain X200).

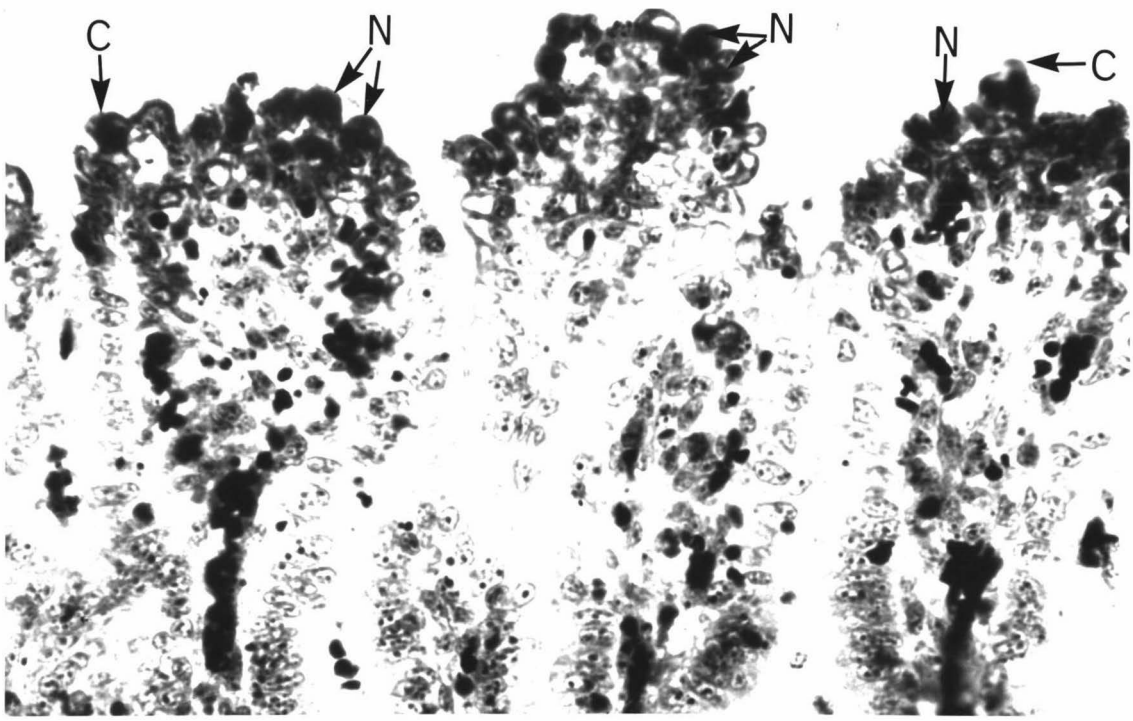

C
N
N
N
C

Figure 3.20

Foetal lamb intestine cultured in T199 medium + 10% foetal calf serum, infected with approximately 10^6 CFU/ml of C.jejuni, showing changes that have occurred 13 hours postinfection. The infected epithelial cells show moderate vacuolation (arrow) and nuclear swelling (NS). The microorganisms (MO) are attached to the external surface of the villi and the crypt epithelium . (Warthin Starry Stain X200).

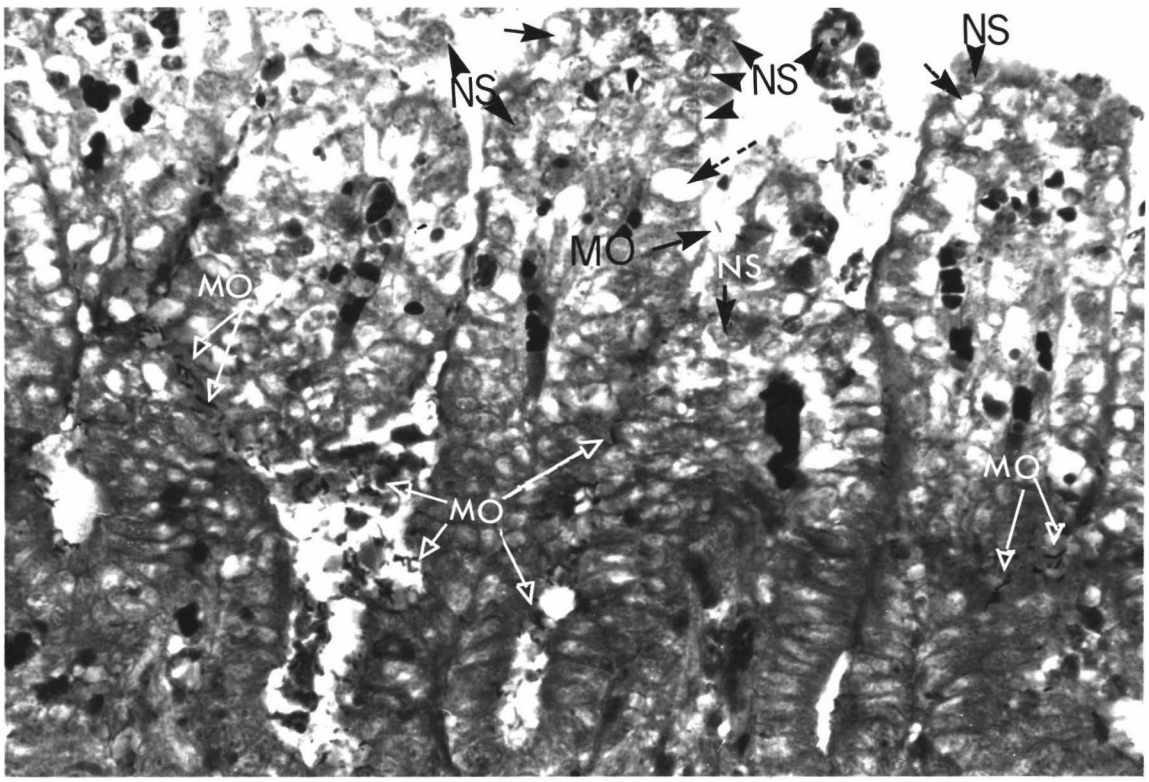
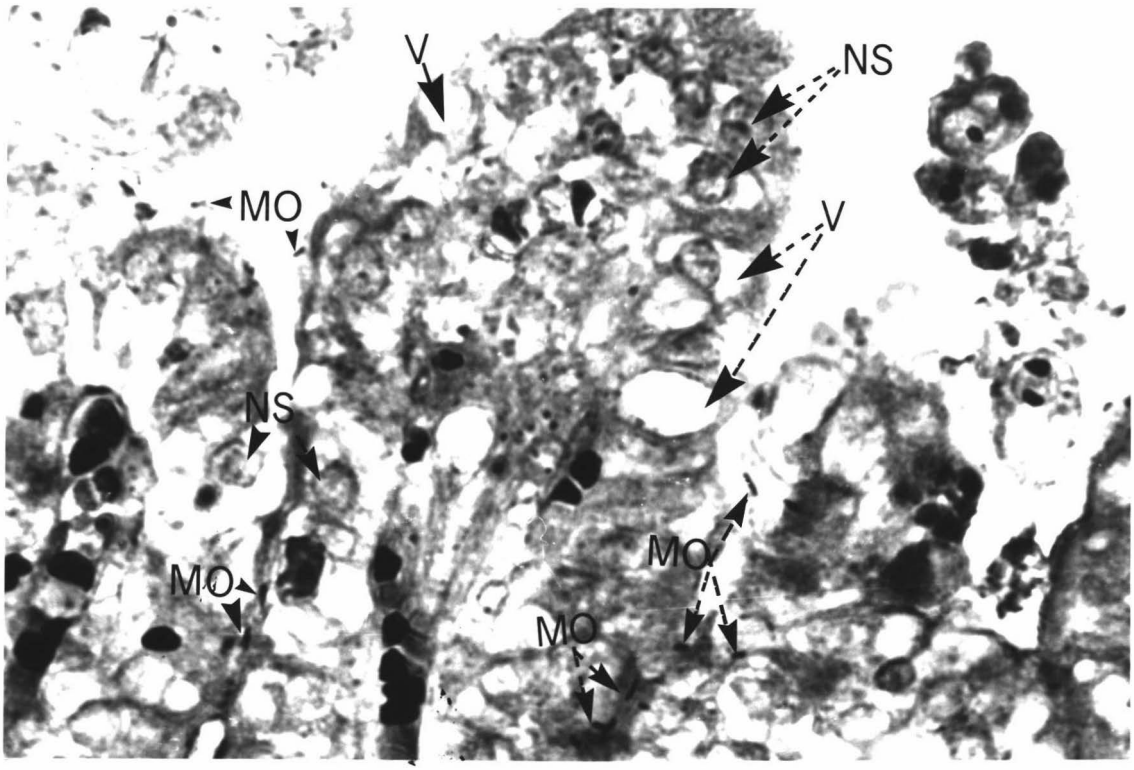

NS
NS
NS
NS
MO
MO
NS
MO
MO

Figure 3.21

Higher magnification of Figure 3.20, showing the morphology of the epithelial cells 13 hours postinfection. The absorptive cells (C) show cytoplasmic vacuolation (V) and necrosis at the tips of the villi. Microorganisms (MO) can be observed attached to the epithelial cells. (Warthin Starry Stain X400).


V
NS
MO
V
NS
MO
MO
MO

Sections stained with Warthin starry showed that some spiral-shaped microorganisms were attached to the external surface of the villous epithelial cells. Other microorganisms were found adhering to the sloughed necrotic cells (Figures 3.22 and 3.23).

3.2.2 Transmission Electron Microscopy

3.2.2.1 Control

3.2.2.1.1 : Six hours

The absorptive cells had well preserved regular microvilli and were covered by a well developed glycocalyx (Figure 3.24). They had maintained their normal morphological shape and orientation and had oval-shaped basal nuclei (Figure 3.25). The cytoplasm was compact and contained free ribosomes. The cytoplasmic organelles, including mitochondria, elements of endoplasmic reticulum and golgi apparatus, were normal in appearance and distribution (Figure 3.25). Occasional microvesicles were observed at the apical portion of the cytoplasm, just under the terminal web (Figure 3.26). A large digestive vacuole was observed in the cytoplasm of a few absorptive cells.

3.2.2.1.2 : Thirteen hours

The intestinal epithelial cells showed well preserved brush borders with microvilli of uniform length. The apical cytoplasm of the absorptive cells contained prominent lysosomal organelles containing multifocal aggregations of glycogen granules (Figure 3.27). The mitochondria, golgi apparatus and endoplasmic reticulum, were normal in morphology and distribution. The ultrastructural features of the cells were essentially similar to those seen after six hours culture, however there was an increase in the number of apical vesicles. (Figures 3.27 and 3.28).

Figure 3.22

Foetal lamb intestine cultured in T199 medium + 10% foetal calf serum, infected with approximately 10^6 CFU/ml of C.jejuni, showing changes that have occurred 15 hours postinfection. Severe necrosis and exfoliation of the epithelial cells (C) can be seen at the tips of the villi. Spiral microorganisms (MO) appear to be colonising the external surface of the cells. (Warthin Starry Stain X200).

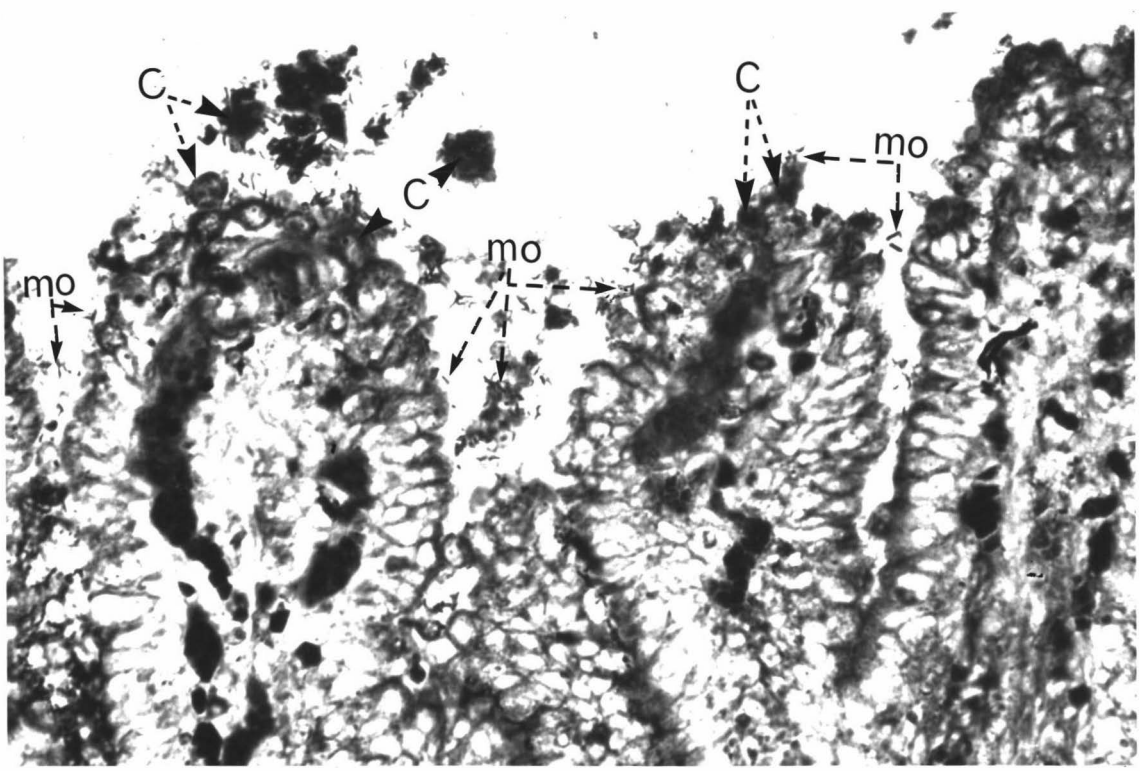

C
C
C
mo
mo
mo

Figure 3.23

Foetal lamb intestine cultured in T199 medium + 10% foetal calf serum, infected with approximately 10^6 CFU/ml of C.jejuni, showing changes that have occurred 15 hours postinfection.The infected cells (C) have marked cytoplasmic vacuolation (arrow). Severe exfoliation of the necrotic cells (NC) can be seen. (Warthin Starry Stain X200).

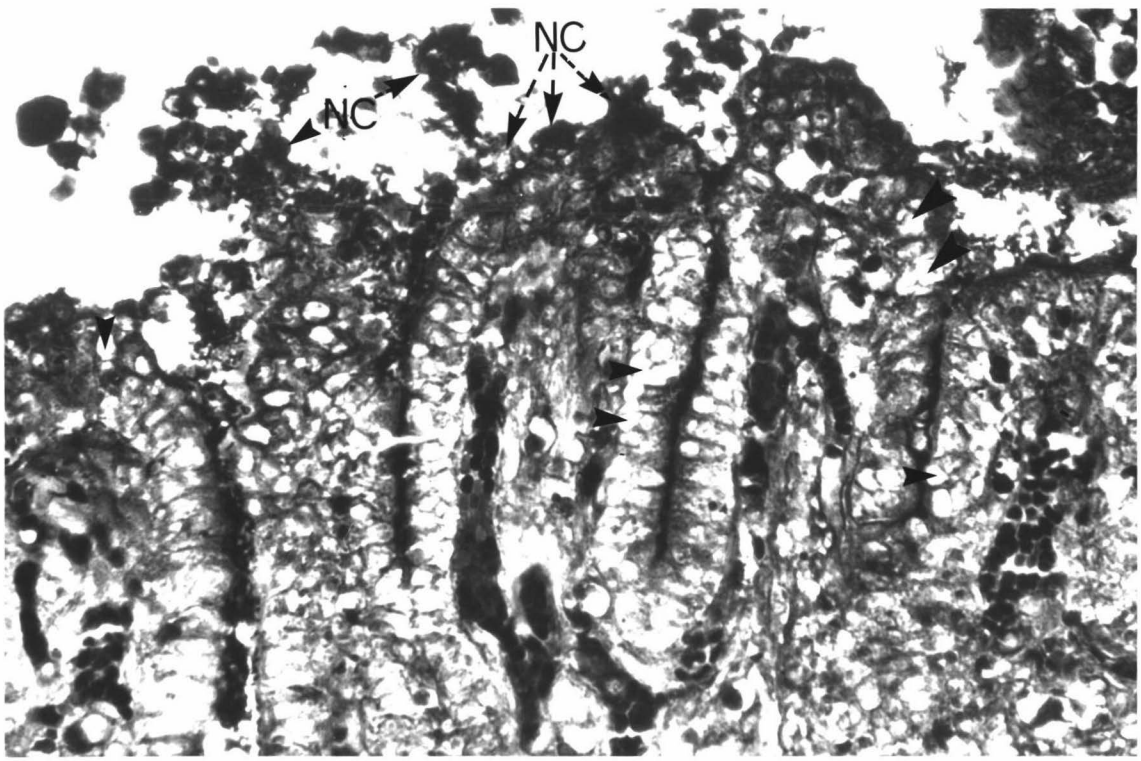
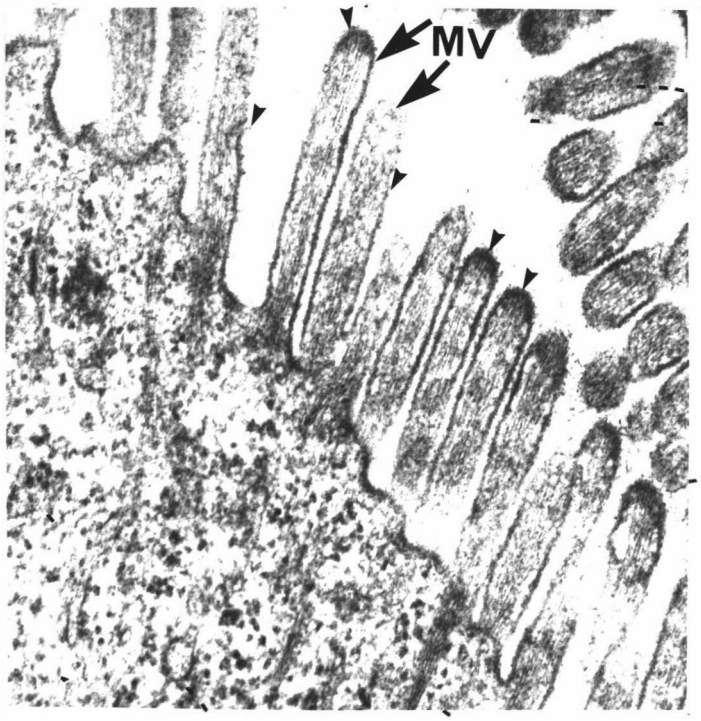

NC
NC

Figure 3.24

Control foetal lamb intestine after 6 hours culture in T199 medium + 10% foetal calf serum, showing the brush border of the intestinal epithelial cells. The normal structure of the microvilli (MV) can be observed and the fuzzy coat of the glycocalyx is clear (arrow). (TEM X48,600).

Figure 3.25

Control foetal lamb intestine after 6 hours culture in T199 medium + 10% foetal calf serum, showing well preserved epithelial cells (C), nuclei (N), microvilli (MV), terminal web (TW), junctional complex (JC), dense supranuclear mitochondria (M), rough endoplasmic reticulum (ER) and occasional golgi apparatus (G). (TEM X11,200).


MV

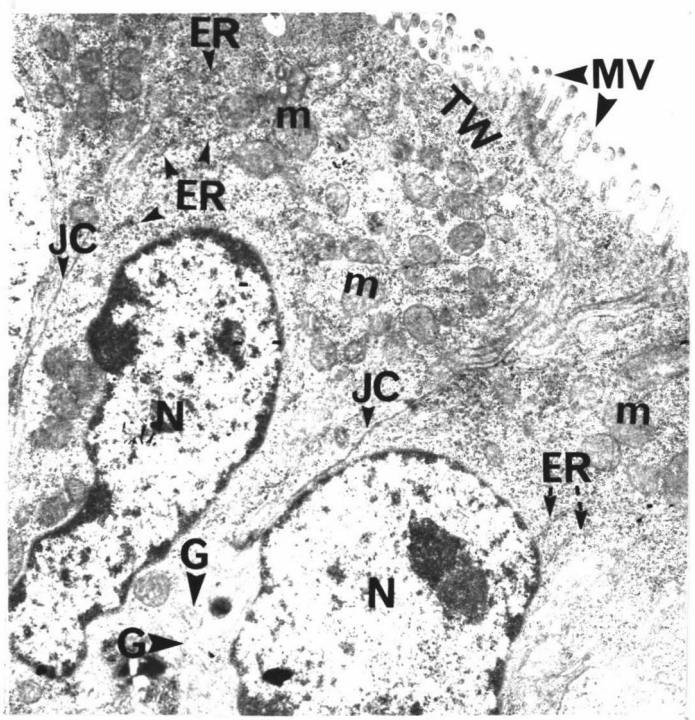

ER
MV
m
TW
ER
JC
m
JC
N
m
ER
G
N
G

Figure 3.26

Ultrastructure micrograph of control foetal lamb intestine cultured for 6 hours in T199 medium + 10% foetal calf serum, showing an occasional microvesicle (MV) under the terminal web and large digestive vacuoles (DV). A Few dense lysosomes (L) are present. (TEM X11,200).

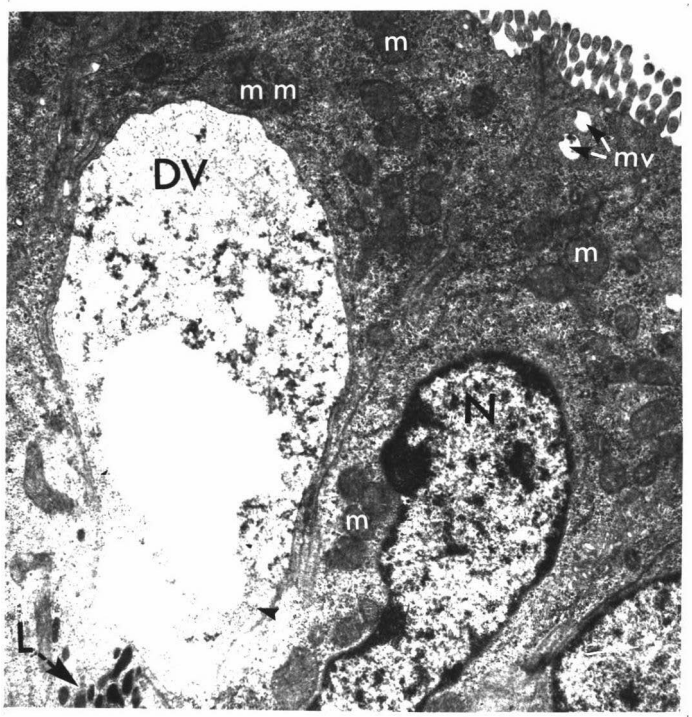
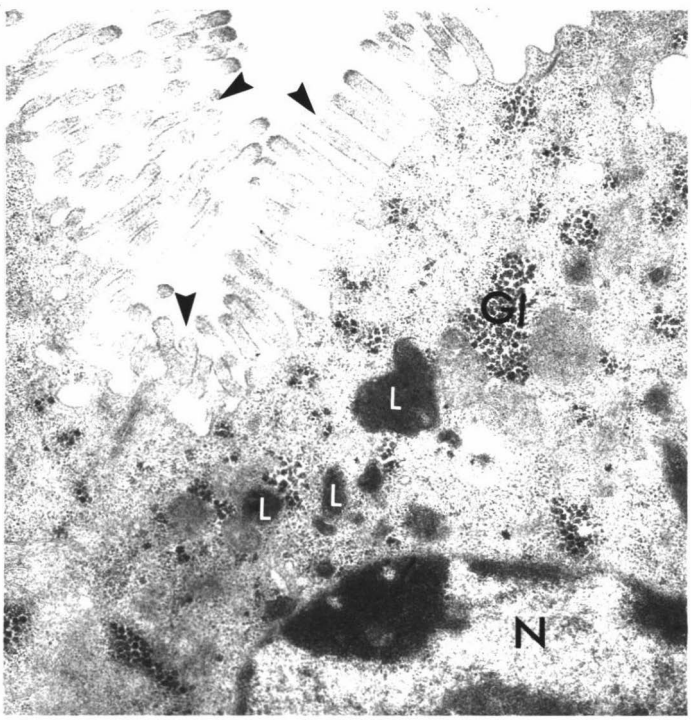

m
m m
DV
mv
m
N
m
L

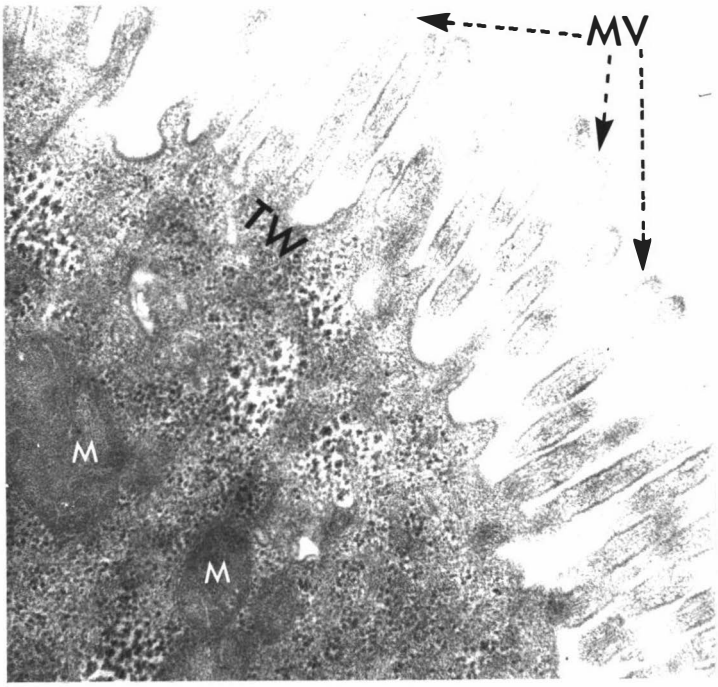
Figure 3.27

Control foetal lamb intestine after 13 hours culture in T199 medium + 10% foetal calf serum. The epithelial cells show a well preserved brush border (arrow), prominent lysosomal organelles (L) in the apical cytoplasm and multifocal aggregations of glycogen granules (Gl). (TEM X13,500).

Figure 3.28

Ultrastructure micrograph of control foetal lamb intestine after 13 hours culture in T199 medium + 10% foetal calf serum. The microvilli (MV) are uniform in length and well preserved. The terminal webs (TW) and mitochondria (M) can be observed. (TEM X31,800).


GI
L
L
L
N


MV
TW
M
M

3.2.2.1.3 : Fifteen hours

The ultrastructure of the cells was similar to the control culture at six hours. However, small changes in the microvilli, mitochondria and ribosomes were seen. The microvilli on the luminal surface were more numerous and closely packed. The mitochondria were heavily packed,larger in size and located predominantly in the supranuclear cytoplasm (Figure 3.30). Occasional lysosomal-like structures were present in the apical and supranuclear cytoplasm. The number of unattached ribosomes appeared to be higher (Figure 3.30). The presence of a large number of small vesicles was noted in the apical cytoplasm (Figure 3.29).

3.2.2.2 Infected

3.2.2.2.1: Six hours

Microorganisms were present in close proximity to the brush border. The brush border was intact (Figure 3.31). The epithelial cell brush borders were irregular in shape, shortened in length and thickened, when compared with the uninoculated six hours control. The microvilli appeared to be directed towards the microorganisms (Figure 3.31).

Higher magnification revealed that one microorganism was attached to the microvillus,by a dense, blurred, pilus-like structure. The microorganism was surrounded by a fuzzy coat (Figure 3.32). An occasional microvillus showed degeneration. The filamentous cores, the fuzzy coat (glycocalyx) and the terminal webs of the microvilli close to the microorganism were obscured (Figure 3.32).

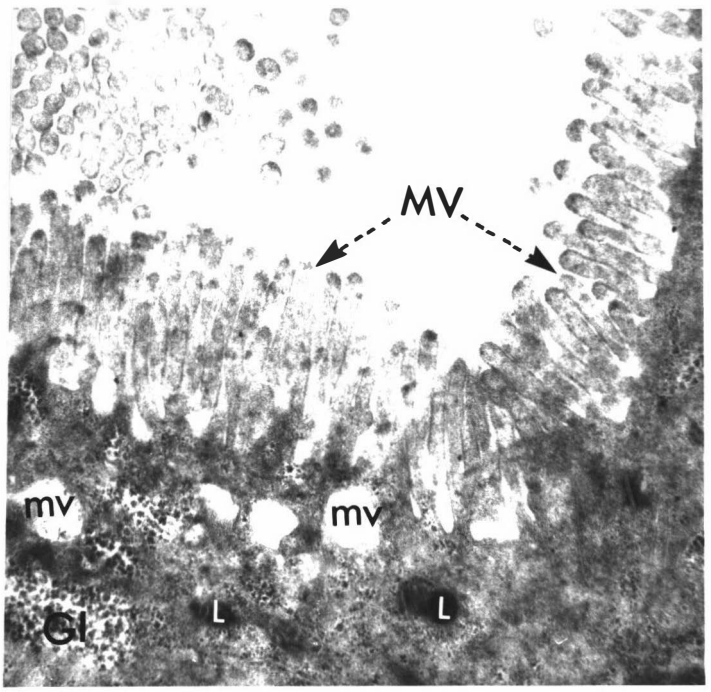
The morphological changes at six hours post-infection appeared to be limited to the extreme tips of the villi. The mitochondria and other cytoplasmic organelles of affected cells were unchanged, and only occasional focal aggregations of glycogen

Figure 3.29

Control foetal lamb intestine after 15 hours culture in T199 medium + 10% foetal calf serum. The microvilli (MV) are more numerous and closely packed than after 6 hours and 13 hours culture. Occasional lysosomal-like structures (L) are present in the apical and supranuclear cytoplasm. Moderate numbers of microvesicles (Mv) are present. Many glycogen granule aggregations (Gl) can be observed. (TEM X21,200).

Figure 3.30

Higher magnification of Figure 3.29 showing the microvillous coat (glycocalyx) (Gx). Free ribosomes (R) are present in the apical portion of the cytoplasm. The mitochondria (M) are large, well preserved and located in the supranuclear region. (TEM X72,100).


MV
mv
mv
Gl
L
L

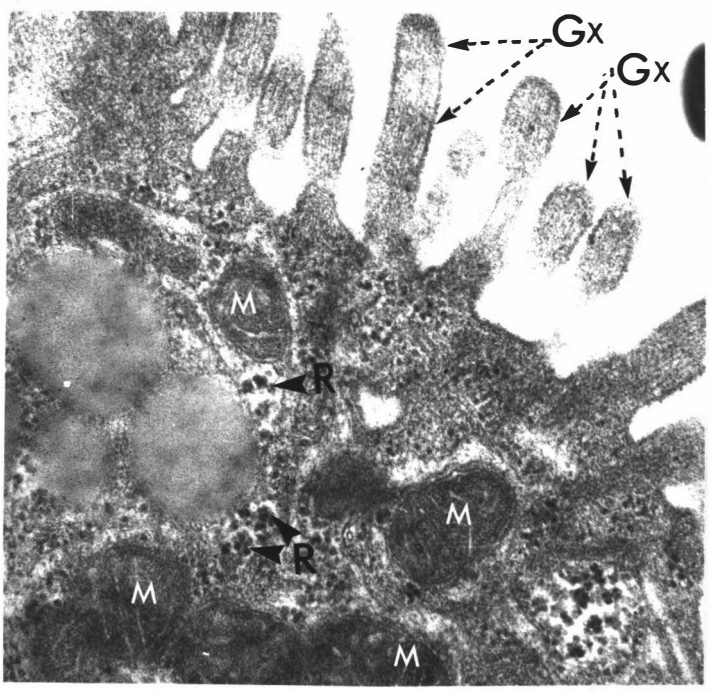
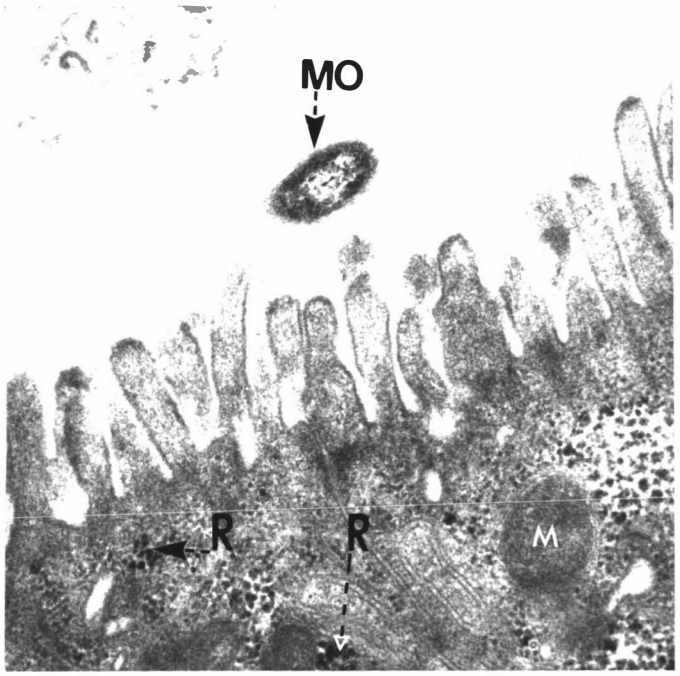

Gx
Gx
M
R
M
R
M
M

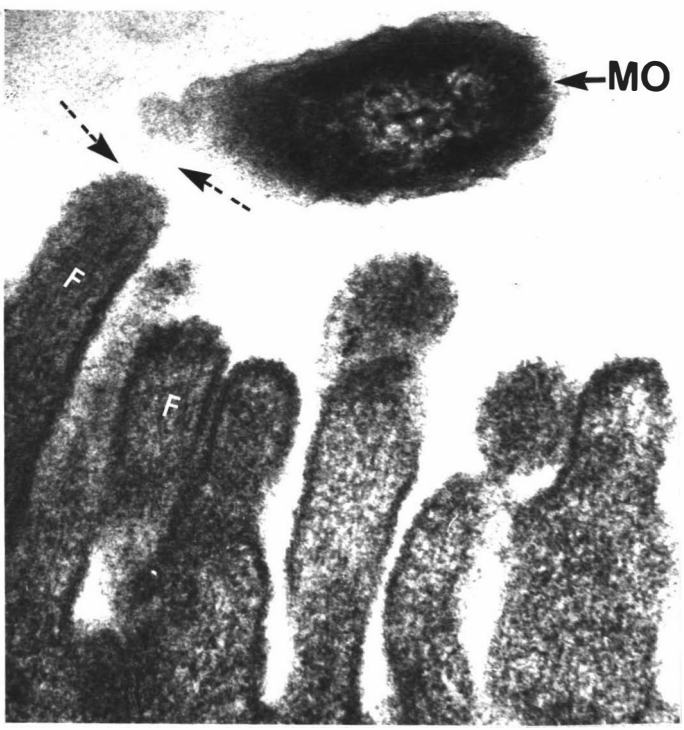
Figure 3.31

Organ culture of foetal lamb intestine in T199 medium + 10% foetal calf serum, infected with approximately 10^6 CFU/ml of C.jejuni, 6 hours postinfection, showing one microorganism (MO) in close proximity to the brush border. The brush border is intact and irregular in shape. Free ribosomes (R) are present in the apical cytoplasm of the absorptive cells. (TEM X31,800).

Figure 3.32

Higher magnification of Figure 3.31 showing the attachment of the microorganisms (MO) to the tip of the microvillus (MV) by a blurred pilus-like structure (arrow). The microvillus is directed towards the microorganism. The filamentous core (F) and the fuzzy coat are obscured. (TEM X72,100).


MO
R
R
M


MO
F
F

were seen (Figure 3.31).

A large macrophage was found in the lamina propria.Two microorganisms (oval and spiral-degenerated in shape) were observed in the cytoplasm of the macrophage (Figure 3.33).

3.2.2.2.2 : Thirteen hours

The villous epithelial cells were irregularly arranged when compared to the control culture (Figures 3.40 and 3.41). The microvilli had degenerated, and were shortened (Figure 3.34), elongated (Figures 3.34 and 3.35) and vesiculated (Figure 3.36). Occasional villous epithelial cell showed extrusion of cytoplasm. A large intracytoplasmic autophagic vacuole was observed and contained some of the disrupted organelles and debris (Figures 3.40 and 3.41). Figure 3.37 shows the presence of curved microorganisms in close association with the microvilli.

Degenerative changes were also noted in the ultrastructure of the cells. The nuclei varied in shape and showed margination of the nuclear chromatin (Figures 3.37 and 3.40). Some of the mitochondria, located in the apical part of the cytoplasm, were swollen (Figure 3.38). The cristae were also observed to have degenerated. Ocassional dilation of the endoplasmic reticulum was seen (Figure 3.35), together with an increase in the number of cytoplasmic vacuoles (Figures 3.34 and 3.35). Free ribosomes were seen (Figure 3.39). Few lysosomes were present (Figure 3.40).

3.2.2.2.3 : Fifteen hours

The degenerative changes noted at the ultrastructural level at 13 hours post-culture were more severe at 15 hours. A moderate to severe loss of microvilli could be seen . The microvilli were generally shorter, denuded and fragmented (Figures 3.42 and 3.43). Curved microorganisms were observed in close association with the brush border (Figure 3.42). A microoganism was seen attached to the tip of one microvillus by a plug-like structure (Figure 3.44)

Figure 3.33

Ultrastructure micrograph of foetal lamb intestine cultured in T199 medium + 10% foetal calf serum, infected with approximately 10^6 CFU/ml of C.jejuni, 6 hours postinfection showing a macrophage in the lamina propria. The macrophage has two phagolysosomal vacuoles containing degenerated oval and spiral-shaped microorganisms (MO). (TEM X21,300).

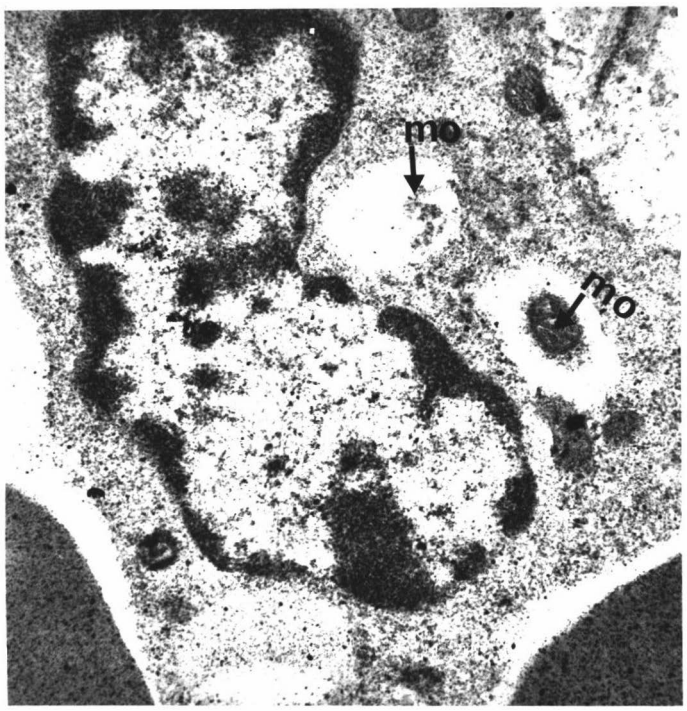
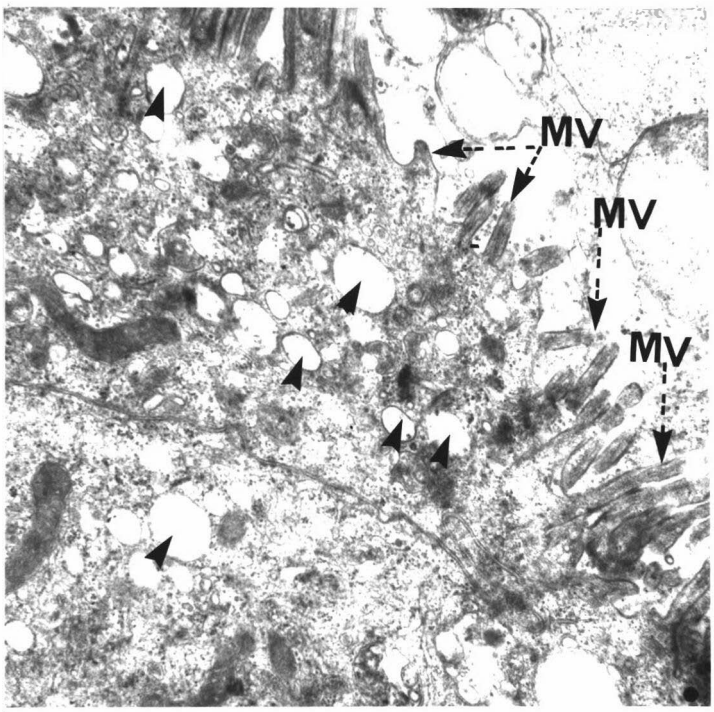

mo
mo

Figure 3.34

Organ culture of foetal lamb intestine in T199 medium + 10% foetal calf serum, infected with approximately 10^6 CFU/ml of C.jejuni, showing the changes that have occurred 13 hours postinfection. The microvilli (MV) are irregularly distributed and are both shortened and elongated. Numerous vacuoles (arrow) are present in the apical cytoplasm. (TEM X15,300).

Figure 3.35

Higher magnification of Figure 3.34 showing an elongation of the microvilli (MV). An occasional dilation of the endoplasmic reticulum (ER) can be observed. Free ribosomes (R) are distributed in the apical cytoplasm. (TEM X31,800).


MV
MV
MV

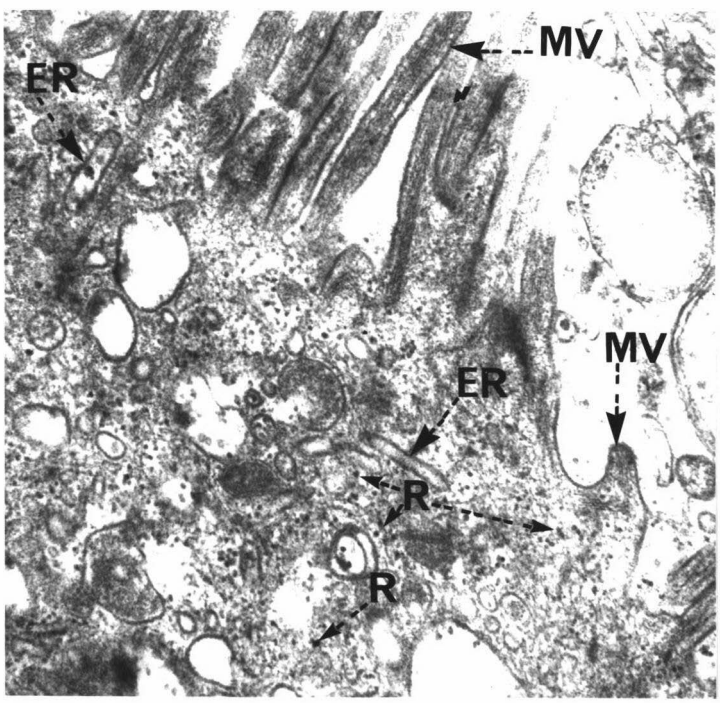
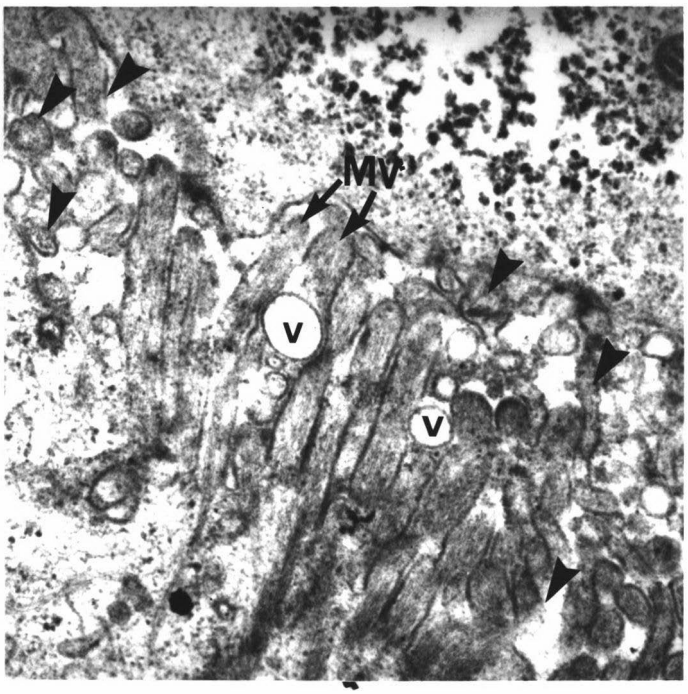

MV
ER
MV
ER
R
R

Figure 3.36

Foetal lamb intestine cultured in T199 medium + 10% foetal calf serum, infected with approximately 10^6 CFU/ml of C.jejuni, showing the changes that have occurred 13 hours postinfection. The apices of the absorptive cells can be observed with vesiculation (V) of the microvilli. Some of the microvilli have degenerated and sloughed (arrow). (TEM X15,300)

Figure 3.37

Organ culture of foetal lamb intestine in T199 medium + 10% foetal calf serum, infected with approximately 10^6 CFU/ml of C.jejuni, showing the changes that have occurred 13 hours postinfection. Curved microorganisms (MO) can be seen in close association with the microvilli. The nuclei (N) of the epithelial cells show margination of the nuclear chromatin (arrow). (TEM 21,200).


Mv
v
v

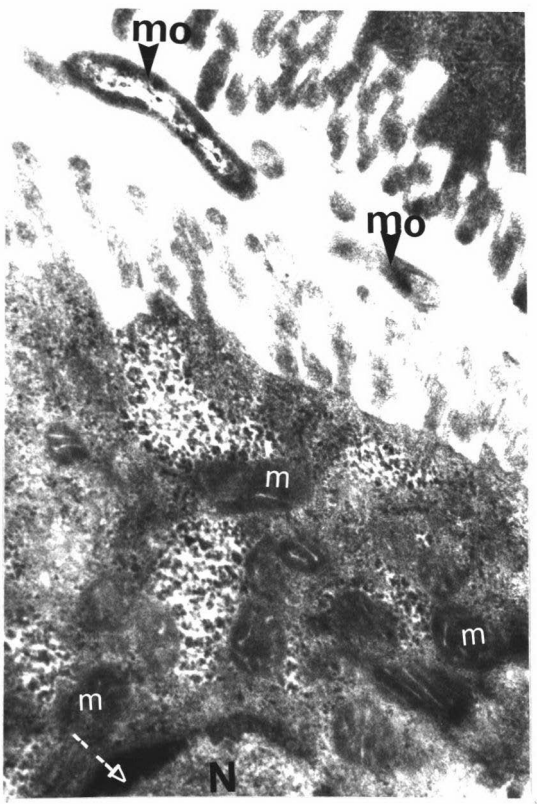
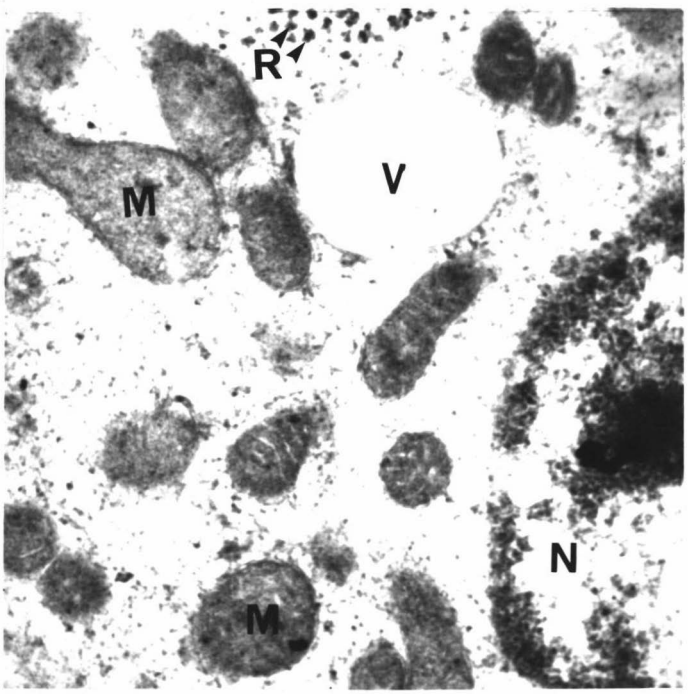

mo
mo
m
m
m
N

Figure 3.38

Foetal lamb intestine cultured in T199 medium + 10% foetal calf serum, infected with approximately 10^6 of C.jejuni, showing changes that have occurred 13 hours postinfection. Swollen mitochondria (M) are seen with a loss of cristae in the mid portion of a villous epithelial cell . Free ribosomal granules (R) and medium sized phagocytic vacuoles (V) can be observed. (TEM X15,300)

Figure 3.39

Apical portion of villous epithelial cell cytoplasm of foetal lamb intestine cultured in T199 medium + 10% foetal calf serum, infected with approximately 10^6 CFU/ml of C.jejuni, 13 hours post-infection, showing free ribosomes (R) with a moderate number of endoplasmic reticuli (ER) and an occasional golgi apparatus (G). Variable-sized cytoplasmic phagocytic vacuoles (V) are present. (TEM X7,800).


R
V
M
N
M

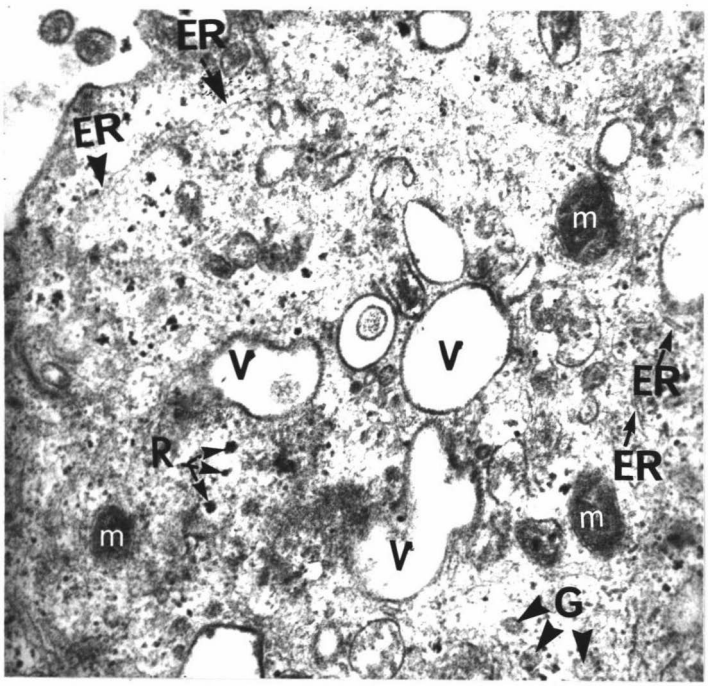
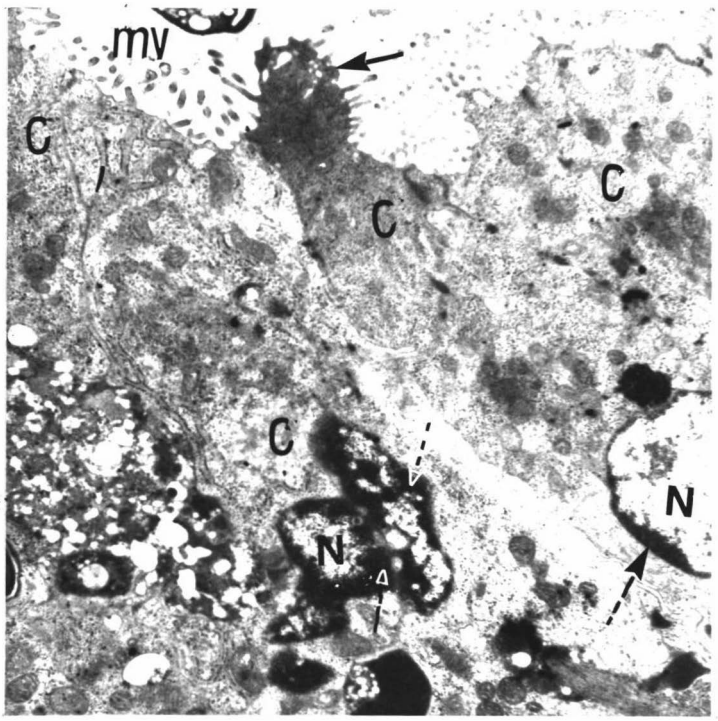

ER
ER
m
V
V
ER
R
ER
m
m
V
G

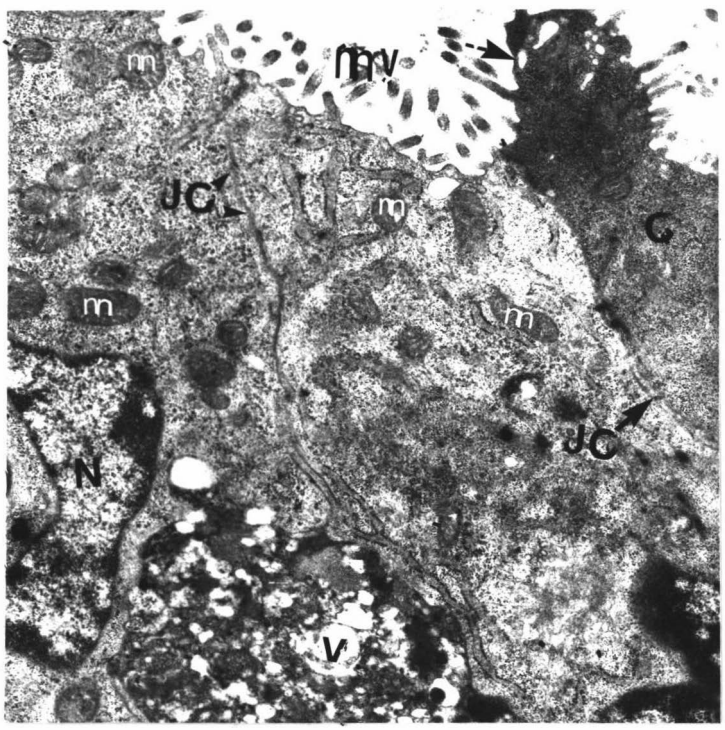
Figure 3.40

Organ culture of foetal lamb intestine in T199 medium + 10% foetal calf serum, infected with approximately 10^6 CFU/ml of C.jejuni, showing changes that have occurred 13 hours postinfection. The villous epithelial cells (C) are disorganised. The microvilli (MV) are degenerated and an occasional epithelial cell shows an extrusion of cytoplasm, (arrow). Margination of the nuclear chromatin (long arrow) and a variation in nuclear shape (N) can be observed. (TEM X7,800).

Figure 3.41

Organ culture of foetal lamb intestine in T199 medium + 10% foetal calf serum, infected with approximately 10^6 CFU/ml of C.jejuni, showing changes that have occurred 13 hours postinfection. The absorptive cells (C) show cytoplasmic budding (arrow) and degeneration of the microvilli (MV). A large intracytoplasmic autophageal vacuole (V) can be seen, containing disrupted organelles and undifferentiated debris. Most of the mitochondria (M) have accumulated near the apex. (TEM X11,200).


mv
C
C
C
C
C
N
N


m
mv
JC
m
C
m
m
JC
N
V

and it appeared that the other microvilli were directed towards it. The filamentous core, the terminal web, and the glycocalyx were indistinct (Figures 3.42 and 3.44).

Many cytoplasmic vacuoles were observed in the apical part of the cytoplasm (Figure 3.42), with an abnormal aggregation of mitochondria. Occasional lysosomal structures were observed (Figures 3.42 and 3.43). Neither the endoplasmic reticulum nor the golgi apparatus could be seen.

After 15 hours post infection, some of the epithelial cells were degenerated and exfoliated (Figure 3.45). The desquamated epithelial cells contained many cytoplasmic vacuoles (Figure 3.45). One oval-shaped microorganism was present lying in the interstitium of the submucosal layer (Figure 3.46).

3.2.3 Scanning Electron Microscopy

3.2.3.1 Control

3.2.3.1.1 : Six hours

An examination of uninfected foetal intestine cultures, maintained in T199 medium enriched with 10% foetal calf serum for 6 hours, showed that the epithelial surface was regularly arranged in geometrical patterns, consisting of numerous densely packed, polygonal epithelial cells. The outline of each cell was well defined and represented by a shallow furrow or depression of the luminal surface (Figure 3..47).

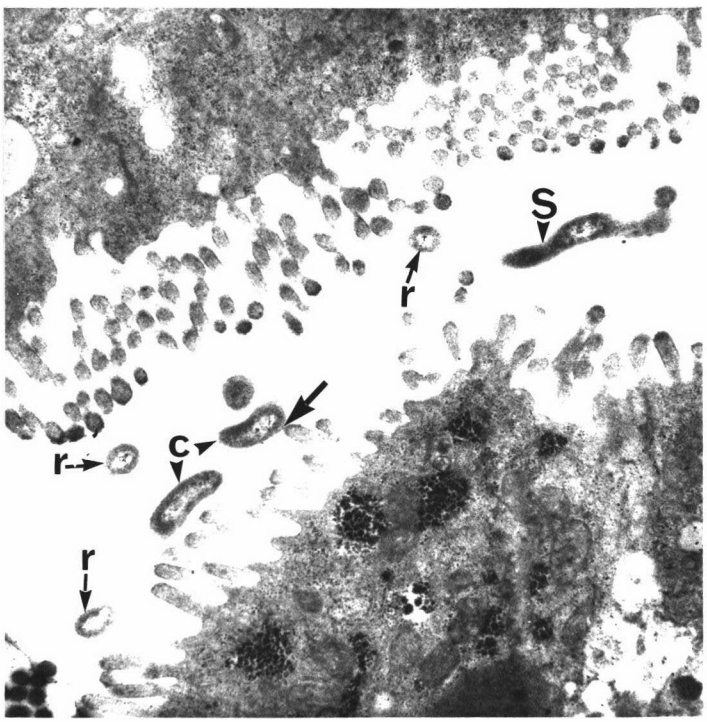
Both flat and dome-shaped projections were clearly visible at the apices of the cells (Figure 3.47). The polygonal epithelial cells were densely covered by small, nodular, rod-shaped microvilli (Figure 3.47). At higher magnification the polygonal absorptive cells showed microvilli, rod-shaped in structure, separated from each other by spaces of approximately 0.01 to 0.02 microns (Figure 3.48). The diameter of the microvilli was between 0.1 and 0.2

Figure 3.42

Organ culture of foetal lamb intestine in T199 medium + 10% foetal calf serum, infected with approximately 10^6 CFU/ml of C.jejuni, showing changes that have occurred 15 hours postinfection. Numerous microorganisms varying in shape, (curved (c), spiral (s), and round (r)) are present on the luminal surface. Some of the microorganisms are in close contact with the microvilli (arrow). The microvilli are shorter than those in the control cultures. (TEM X21,200)

Figure 3.43

Organ culture of foetal lamb intestine in T199 medium + 10% foetal calf serum, infected with approximately 10^6 CFU/ml of C.jejuni, showing changes that have occurred 15 hours postinfection. A marked shortening of the microvilli (MV) can be seen.Occasional lysosome-like structures (L) are present in the mid portion of the epithelial cytoplasm. (TEM X15,300).


S
r
r
c
r

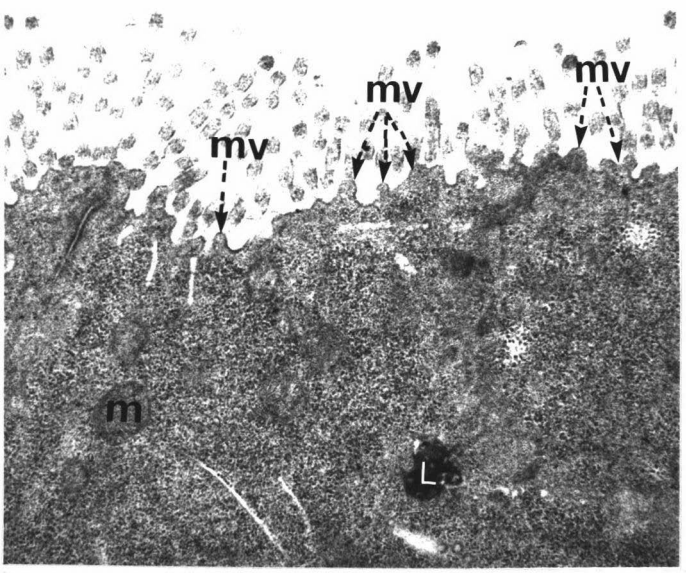

mv
mv
mv
m
L

Figure 3.44

Higher magnificatioin of a portion of Figure 3.42 showing attachment of the microorganisms (MO) to the tip of a microvillus by a plaque-like structure (arrow). The microvilli are directed towards the microorganism. The filamentous core (F) and terminal web (TW) are obscured. (TEM X48,600).

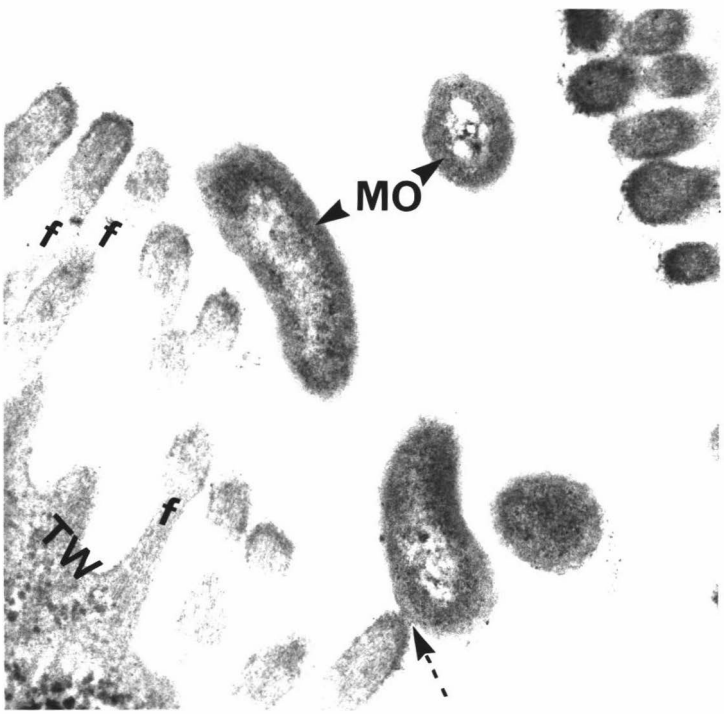

MO
f
f
TW
f

Figure 3.45

Organ culture of foetal lamb intestine in T199 medium + 10% foetal calf serum, infected with approximately 10^6 CFU/ml of C.jejuni, showing changes that have occurred 15 hours postinfection. The exfoliation of a villous epithelial cell (C) can be observed, with disruption and loss of the surface coat. The cell at the lower left margin (arrowed a) is in the process of being extruded. It has lost microvilli (MV) and contains multiple aggregations of glycogen particles (Gl). The epithelial cell in the upper part of the micrograph (arrowed b) appears to be completely detached from the epithelium. The cytoplasm contains dark amorphous material (arrow) and a limited number of cytoplasmic vacuoles (V). (TEM X7,800).

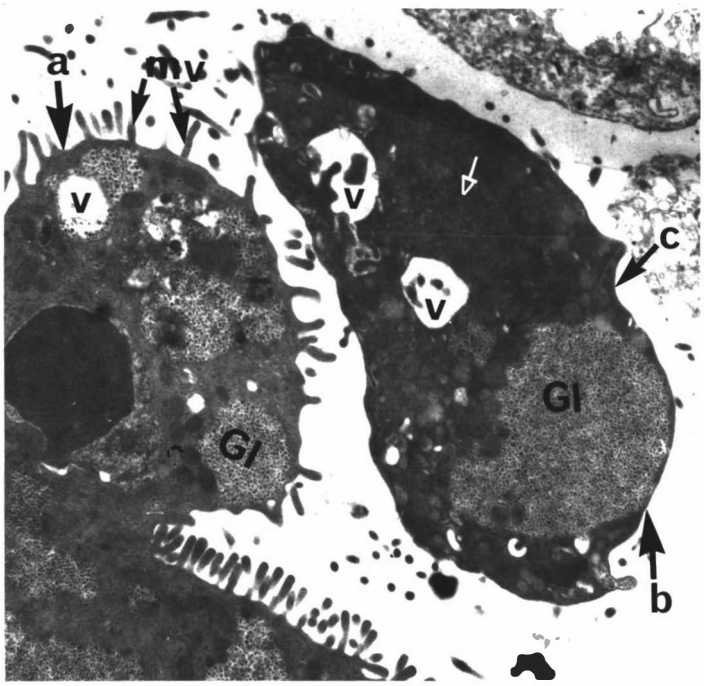
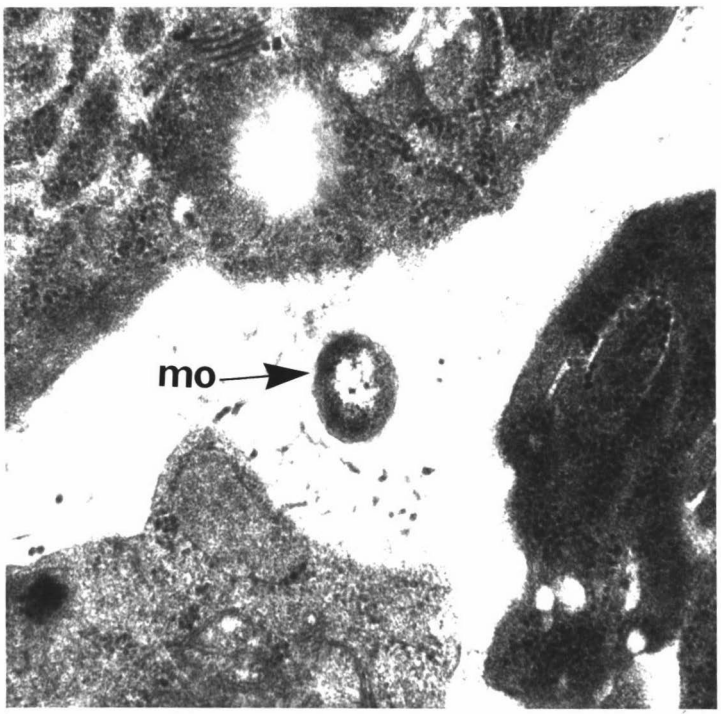

a
v
v
v
v
c
Gl
Gl
b

Figure 3.46

Organ culture of foetal lamb intestine in T199 medium + 10% foetal calf serum, infected with approximately 10^6 CFU/ml of C.jejuni, showing changes that have occurred 15 hours postinfection. An oval-shaped microorganism (MO) can be seen lying free in the interstitium of the submucosal layer. (TEM X21,200).


mo

microns. No mucus blanket was seen on the surface of the epithelial cells (Figures 3.47 and 3.48).

3.2.3.1.2 : Thirteen hours

The cells were similar to those seen at 6 hours post-culture, except for a greater variation in cell size (Figure 3.49).

A circular to oval hole was seen opening directly into the external side of the villous surface, representing an empty goblet cell. No mucus shreds were seen on the surface of the epithelial cells (Figure 3.49). The cell apices showed a surface granularity caused by a regular, densely packed pattern of tiny nodules representing the tips of the microvilli (Figure 3.49).

3.2.3.1.3 : Fifteen hours

The topographical morphology of the cells at 15 hours post-culture was essentially similar to that seen at 6 hours and 13 hours. However, it was noted that the absorptive cells varied in size (Figure 3.50). One goblet cell was intercalated among the absorptive cells (Figure 3.50).

The epithelial cells covering the villous surface were separated from the adjacent villous surface by a deep cleft which contained flecks of mucus debris (Figures 3.50 and 3.51).

3.2.3.2 Infected

3.2.3.2.1 : Six hours

The epithelial cells had a coarse surface appearance due to irregular distribution of the microvilli (Figure 3.52). The absorptive cells appeared disorganised (Figure 3.52) when compared to the 6 hours control. Most of the absorptive cells remained intact and were covered by unevenly distributed microvilli (Figure 3.53).

Figure 3.47

Scanning electron micrograph (SEM) of the mucosal surface of control foetal lamb intestine after 6 hours culture in T199 medium + 10% foetal calf serum. The epithelial cells (C) are polygonal in shape and are regularly arranged. The outline of each cell is defined by either a shallow furrow (x) or a deep depression (d). (SEM X4620).

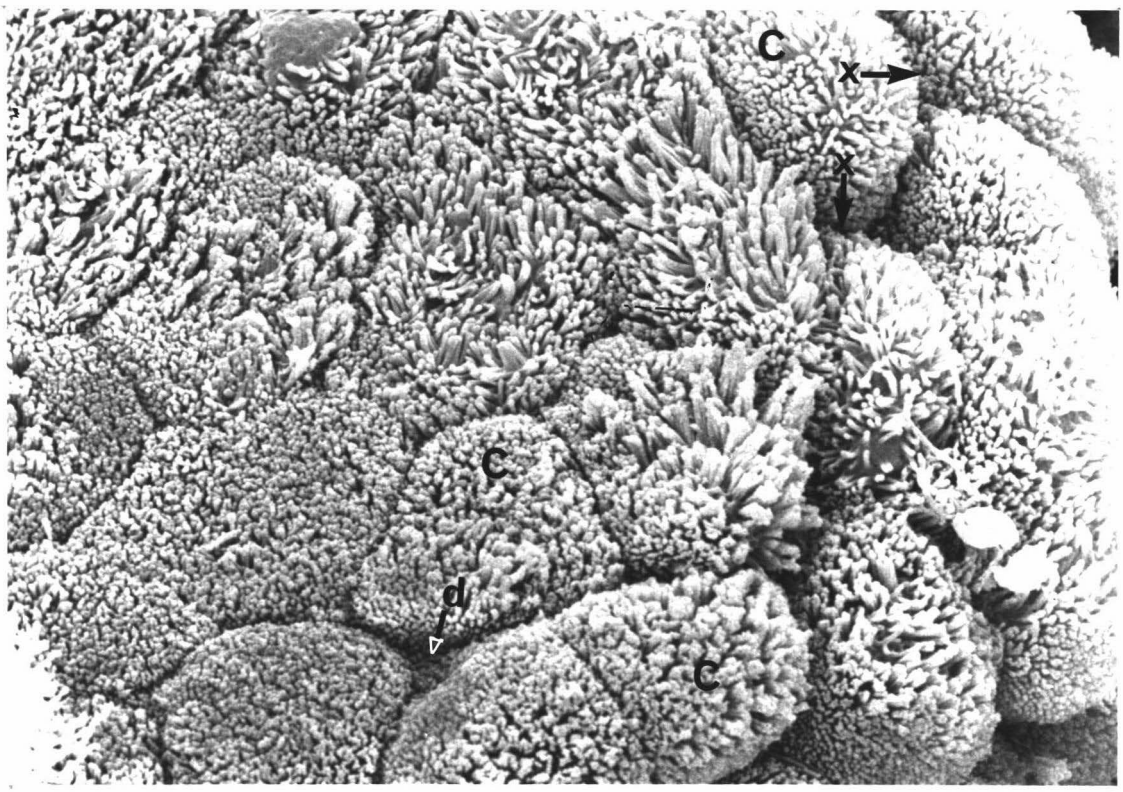

C
X
X
C
d
C

Figure 3.48

Higher magnification of the epithelial cells of Figure 3.47 .Heavily packed, rod-shaped microvilli (MV) can be seen covering the polygonal-shaped epithelial cells. The average distance between each microvillus is 0.1-0.2 um. Mucus blankets are not apparent. (SEM X14000).

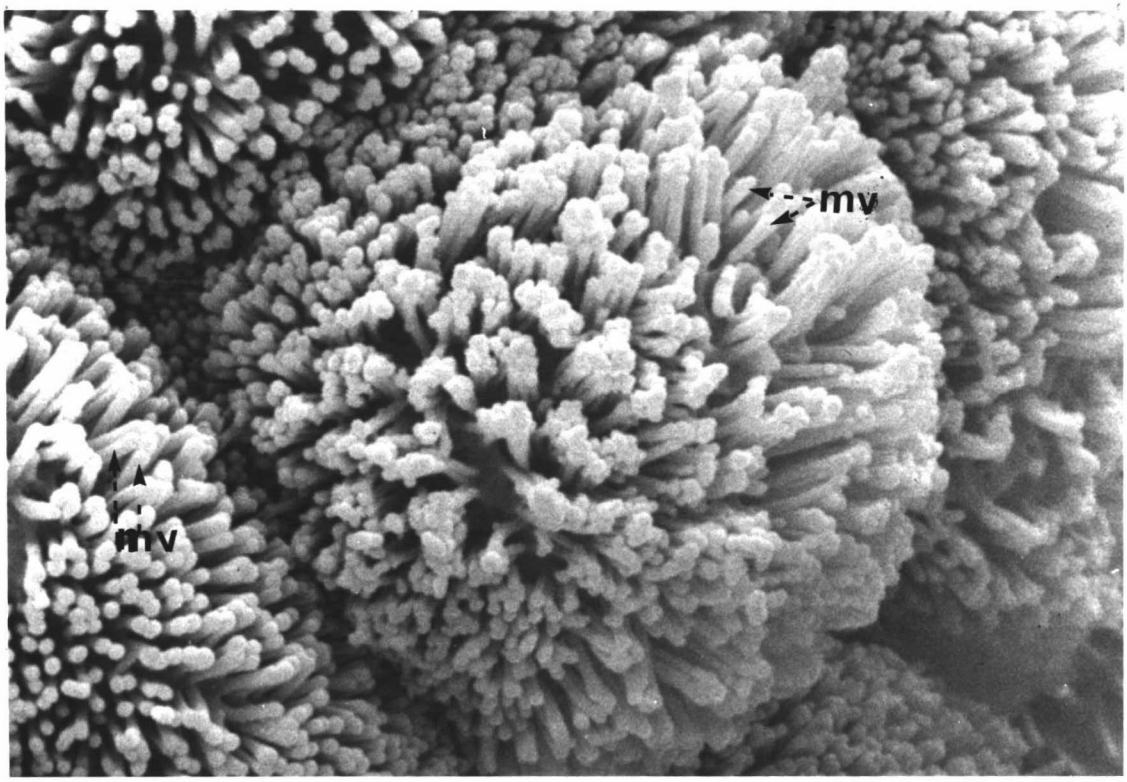

mv
mv

Figure 3.49

SEM of the tips of the villi of control foetal lamb intestine after 13 hours culture in T199 medium + 10% foetal calf serum. The surface is divided into polygonal units (epithelial cells) and is well defined by furrows. A goblet cell pit (G) can be observed as an oval hole, surrounded by absorptive cells. The apices of the epithelial cells are covered by small, densely packed nodules representing the tips of the microvilli (MV). (SEM X3080).

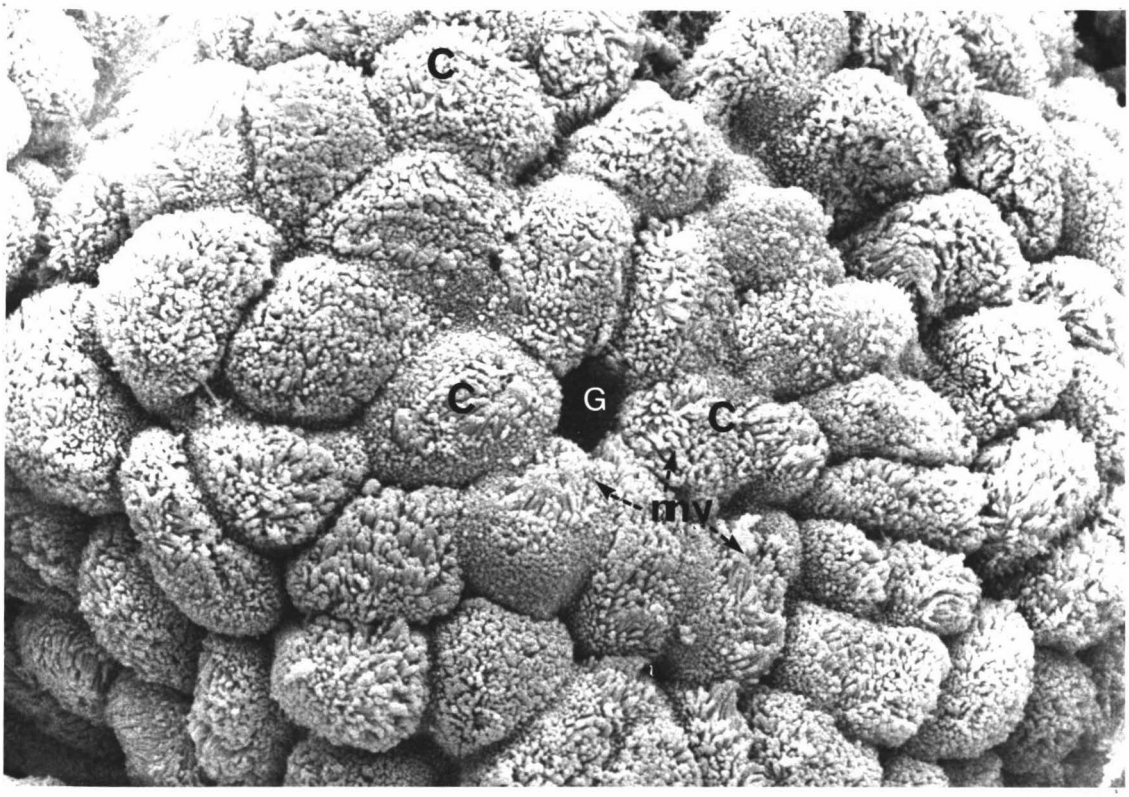
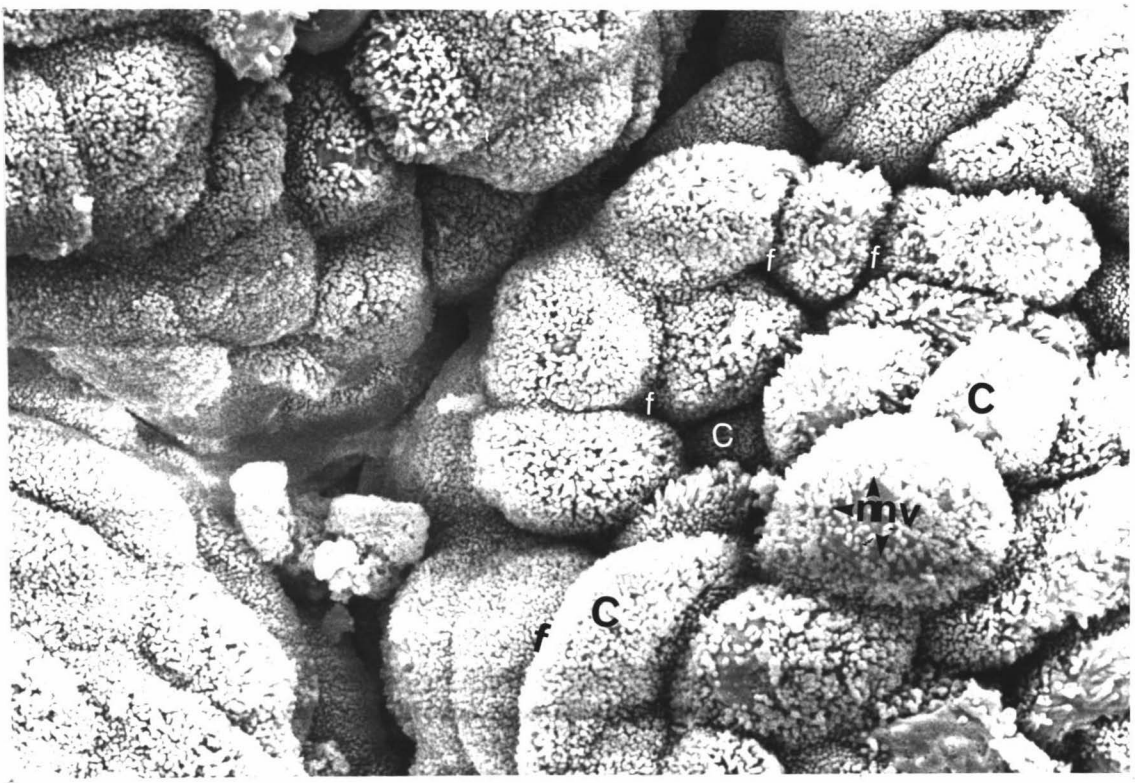

C
C
G
C
mv

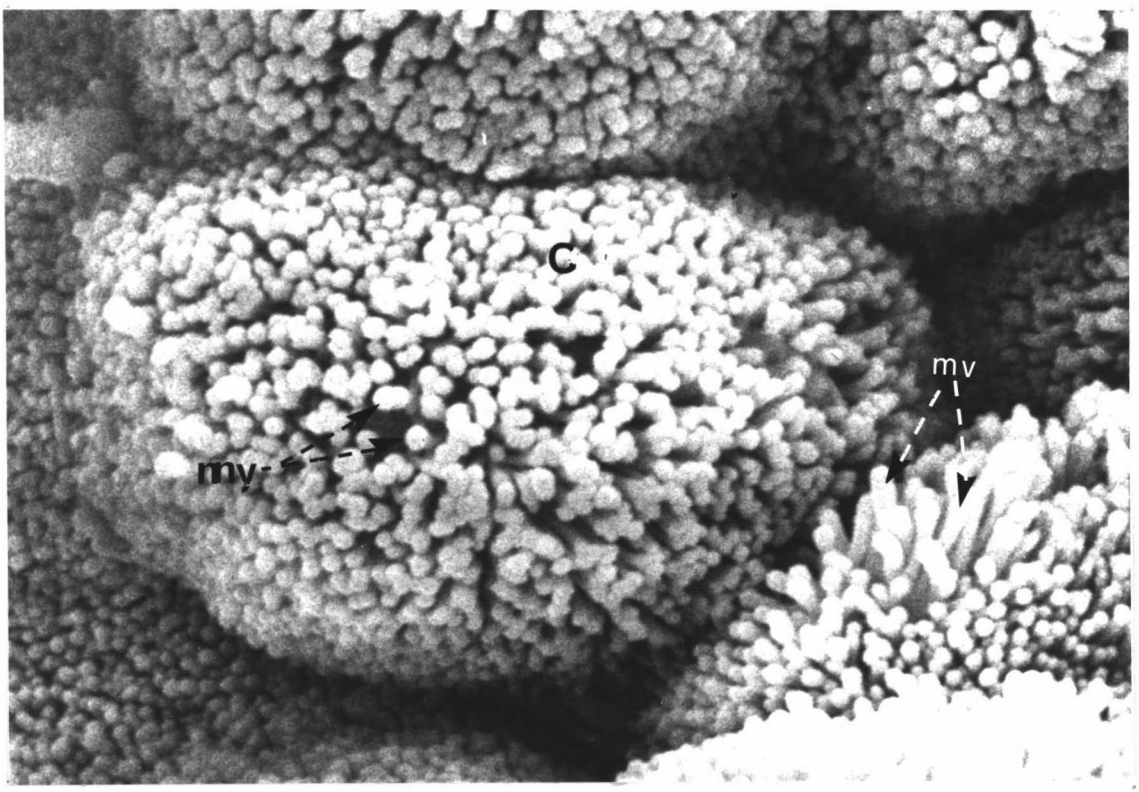
Figure 3.50

SEM showing the surface of the intestinal villi of control foetal lamb intestine after 15 hours culture in T199 medium + 10% foetal calf serum. The surface is divided into polygonal units, separated by furrows (f). Nodular-shaped microvilli (MV) cover the tips of the villi. A goblet cell (G) can be seen surrounded by absorptive cells. (SEM X3300).

Figure 3.51

Higher magnification of an absorptive cell of control foetal lamb intestine after 15 hours culture in T199 medium + 10% foetal calf serum. The absorptive cell (C) is covered with densely packed, rod shaped microvilli (MV). (SEM X16,500).


f
f
f
C
C
C
mv
f
C


C
mv
mv

Some of the absorptive cells showed a moderate loss of microvilli (Figure 3.53), others exhibited a severe denudation. (Figures 3.52 and 3.54). Occasional strands of mucus were observed on the surface of the absorptive cells (Figures 3.52 and 3.53). At higher magnification long filamentous Campylobacter jejuni were observed, (Figure 3.54), attached to the apices of epithelial cells which showed a complete loss of microvilli.

3.2.3.2.2 : Thirteen hours

The absorptive epithelial cells were disorganized, and varied in size and shape. The cells had lost their normal polygonal pattern (Figure 3.55).

The infected epithelial cells were swollen and protruded into the luminal surface due to a disruption of the cell boundaries (Figures 3.56 and 3.58).

The surface of the villous epithelial cells was roughened and irregular due to an abundant coating of thick white mucus secretion containing microorganisms. This had accumulated on and between the infected epithelial cells (Figures 3.56, 3.57 and 3.58). Many holes and pits were observed on the surface of the villous mucosa which may have indicated the presence of goblet cells (Figure 3.56). The infected epithelial cells showed a severe loss of microvilli when compared with the uninfected control (Figure 3.55). A large number of organisms had colonised the surface of the epithelial cells. The microorganisms observed were spiral, filamentous and ring-shaped in appearance (Figure 3.57).

3.2.3.2.3 : Fifteen hours

The epithelial cells were various in shape, from round to elongate, and had a disorganised pattern (Figure 3.59). The prominence of individual villous epithelial cells caused the villous surface to have an irregular and roughened appearance

Figure 3.52

SEM of the epithelial cells of an organ culture of foetal lamb intestine in T199 medium + 10% foetal calf serum infected with approximately 10^6 CFU/ml of C.jejuni, showing changes that have occurred 6 hours postinfection. The epithelial cell surfaces (C), have a coarse appearance and appear disorganised when compared with the six hour control (Figure 3.38). Some of the absorptive cells show either a moderate loss of microvilli (small arrow) or a severe denudation (large arrow) and some of them are exfoliated (Ex). (SEM X3080).

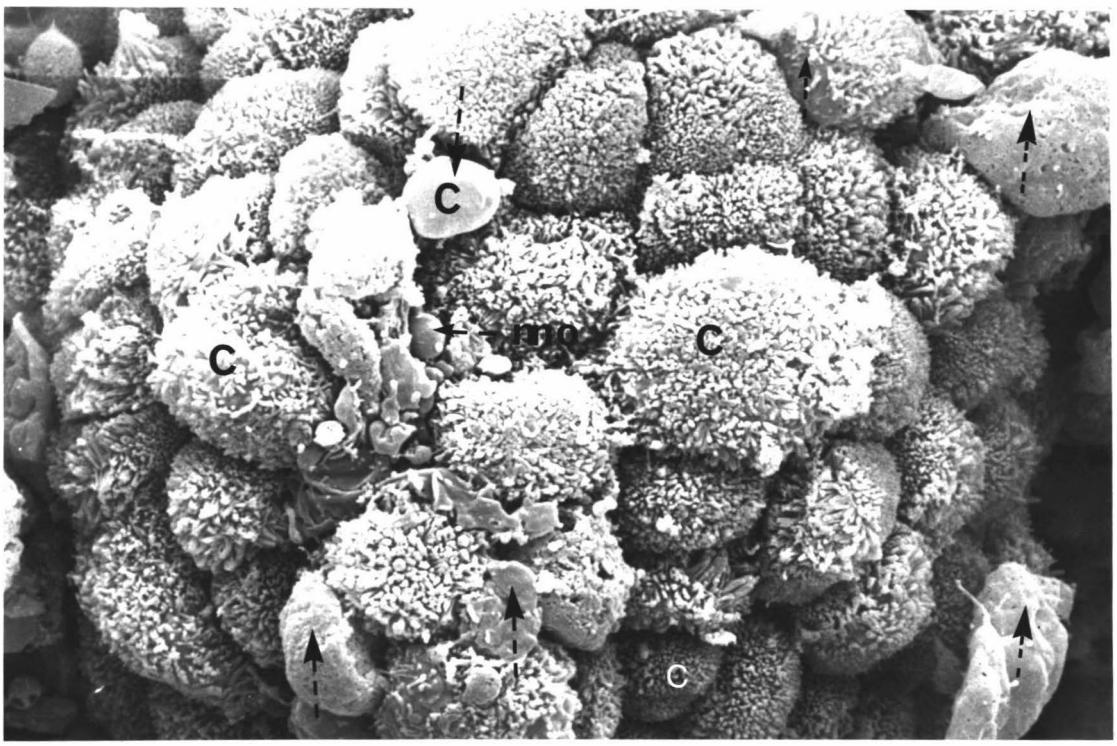

C
C
C
C
C

Figure 3.53

Higher magnification of the epithelial cells of Figure 3.52. The epithelial cells (C) are covered unevenly by microvilli (MV). Some of the epithelial cells show a moderate loss of microvilli (arrow). (SEM X4840).

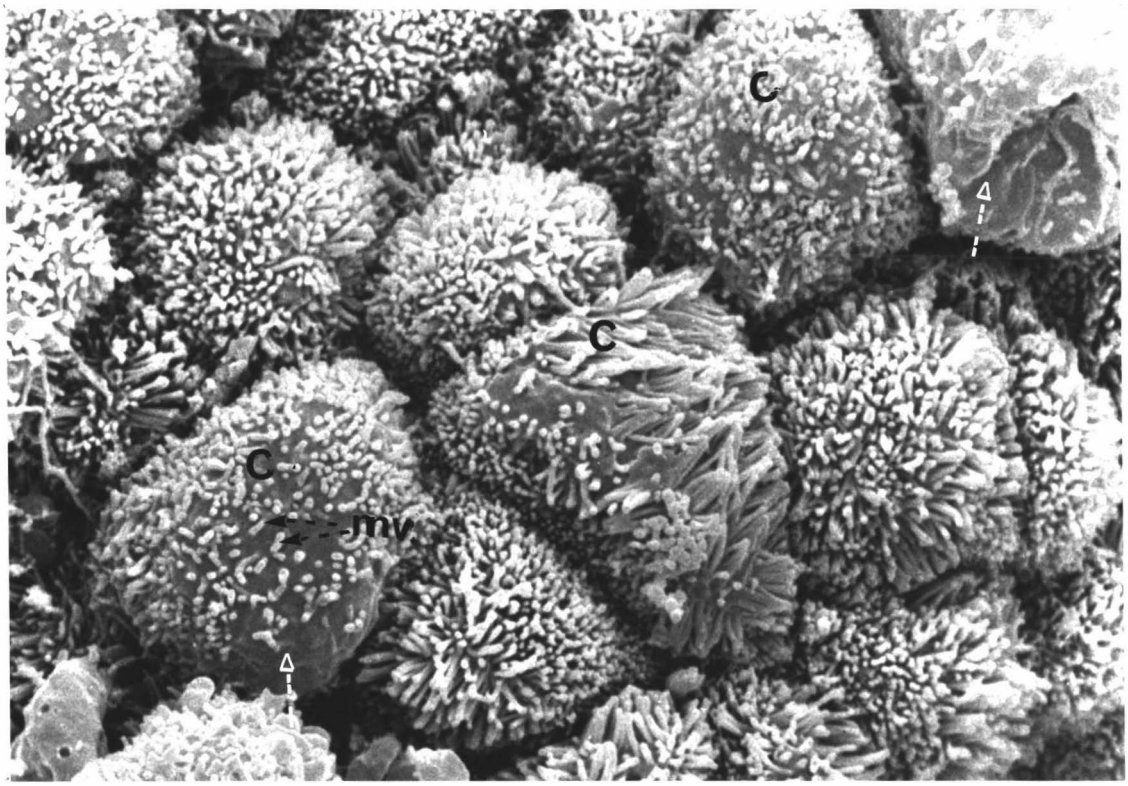

C
C
C
inv.

Figure 3.54

Organ culture of foetal lamb intestine in T199 medium + 10% foetal calf serum, infected with approximately 10^6 CFU/ml of C.jejuni, showing changes that have occurred 6 hours post infection. A filament-shaped microorganism can be observed attached to the tips of the epithelial cells. The infected epithelial cells (C) show a severe loss of microvilli. (SEM X8,250).

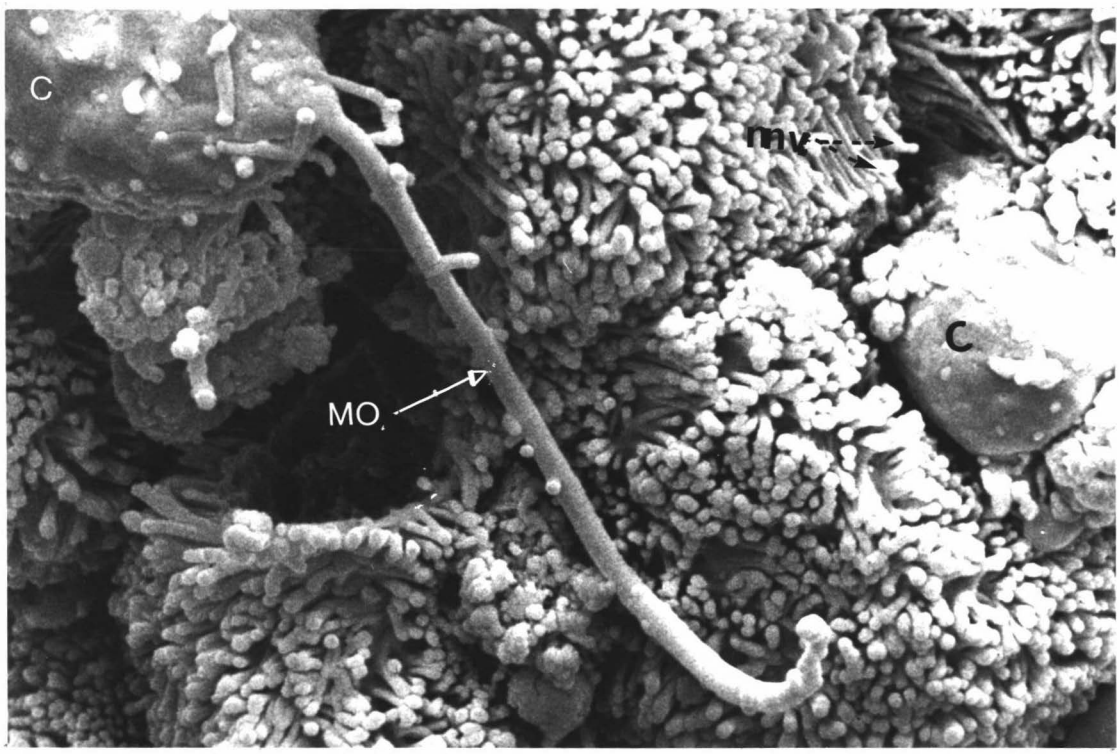
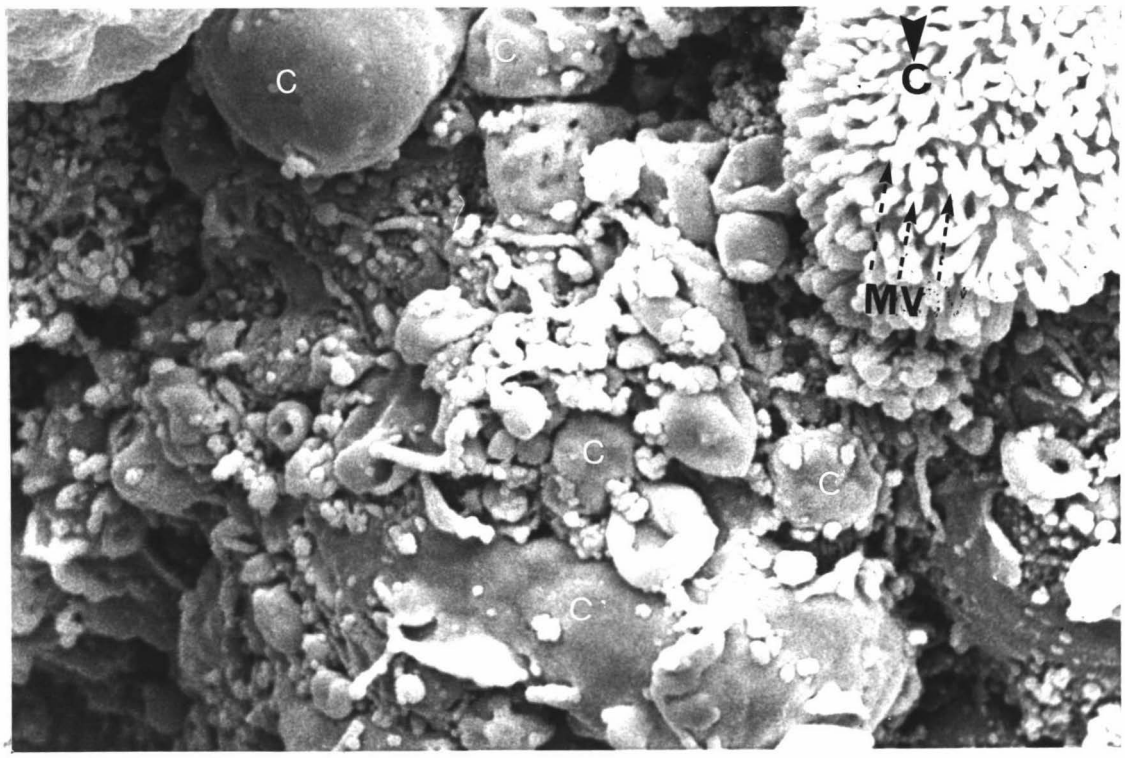

C
mv
C
MO

Figure 3.55

Organ culture of foetal lamb intestine in T199 medium + 10% foetal calf serum, infected with approximately 10^6 CFU/ml of C.jejuni showing changes that have occurred 13 hours postinfection. The infected epithelial cells (C) are disorganised and have lost their normal polygonal pattern and their microvilli when compared with the 13 hour control culture (Figure 3.49). One normal epithelial cell (arrow) remains covered with densely packed rod-shaped microvilli (MV). (SEM X7,700).

Figure 3.56

Organ culture of foetal lamb intestine in T199 medium + 10% foetal calf serum, infected with approximately 10^6 CFU/ml of C.jejuni, showing changes that have occurred 13 hours postinfection. The infected epithelial cells (C) are swollen and protrude towards the luminal surface. A layer of thick white mucus (mu) can be observed covering the surface of the infected cells. Pits and holes are present (arrow) and represent goblet cells (G). Occasional spiral-shaped (s) to rod-shaped (r) microorganisms can be seen within the mucus. (SEM X12,100).


C
C
C
C
MV
C
C
C

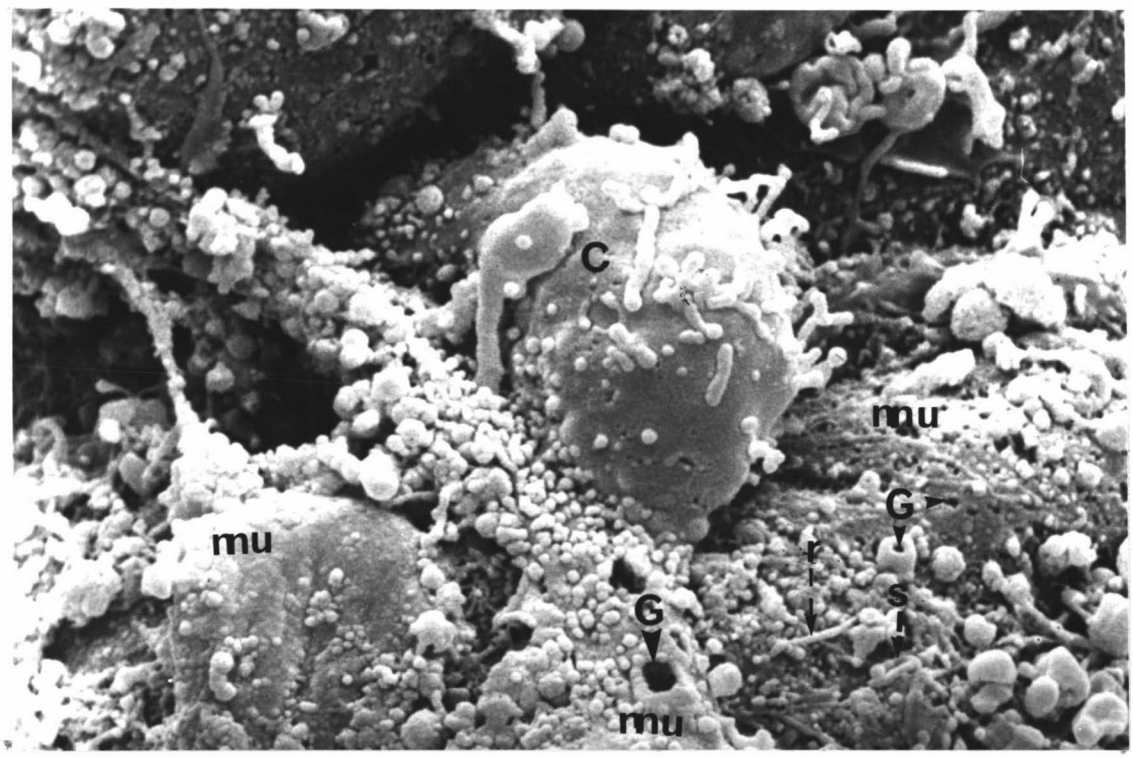
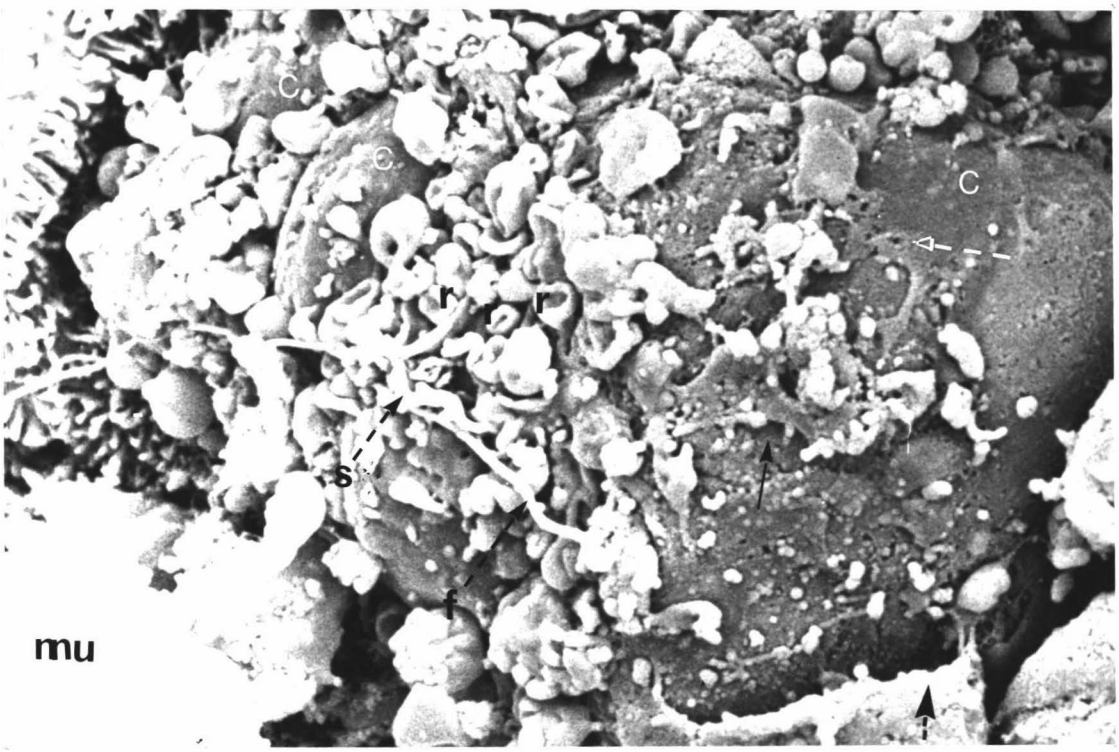

C
mu
G
mu
mu
G
s

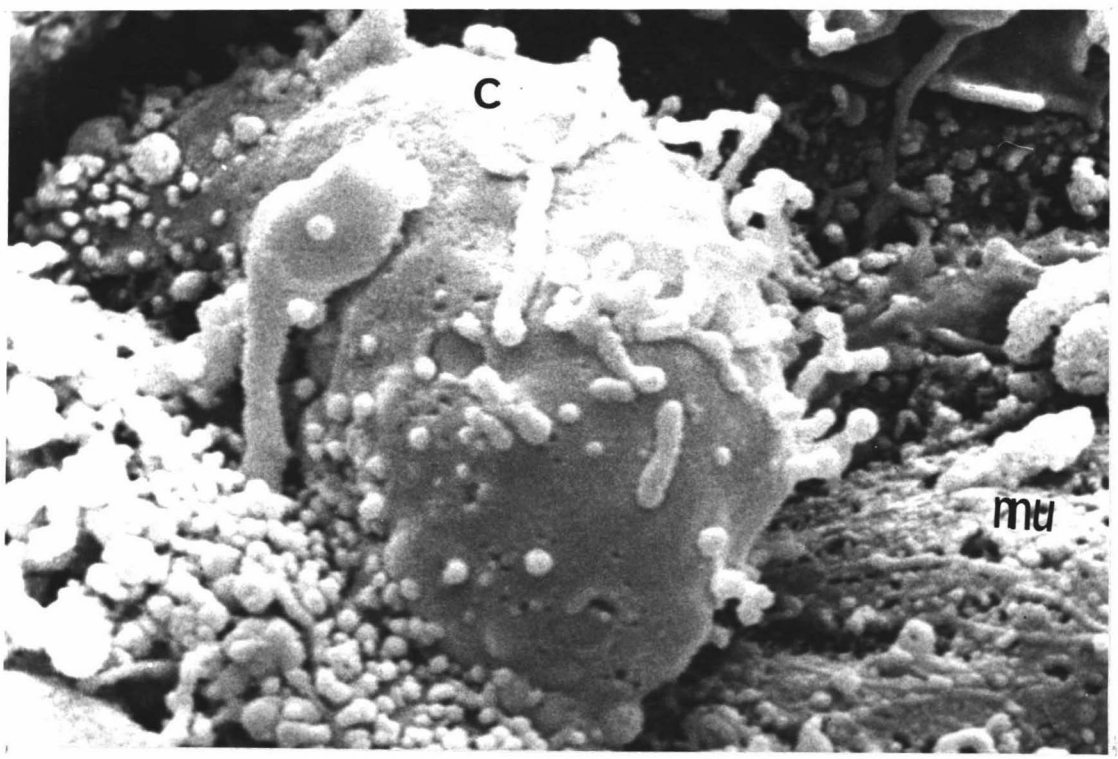
Figure 3.57

Organ culture of foetal lamb intestine in T199 medium + 10% foetal calf serum, infected with approximately 10^6 CFU/ml of C.jejuni, showing changes that have occurred 13 hours postinfection. The surface of the villous epithelium is roughened and irregular and covered with thick mucus (arrow). The epithelial cells (C) show a severe loss of microvilli. Spiral (s), filamentous (f) and ring-shaped (r) microorganisms can be observed attached to the surface of the infected epithelial cells. (SEM X6600)

Figure 3.58

Higher magnification of Figure 3.57. The infected epithelial cells (C) are swollen and protrude into the luminal surface. They show a severe loss of microvilli and are covered with thick, white strands of mucus. (SEM X12,100).


C
C
C
r
r
s
f
mu


C
mu

(Figure 3.60). Some of the absorptive cells had desquamated from the villous surface (Figure 3.59).

The infected absorptive cells showed a moderate to severe loss of microvilli (Figures 3.60 and 3.61). Some of the villi exhibited necrosis and exfoliation of the epithelial cells from the tips, exposing the lamina propria (Figure 3.61) In other villi the intact epithelial cells were disorganised (Figure 3.61).

3.3 STATISTICAL RESULTS

Figure 3.62 and Table 3.1 show the effects of Campylobacter jejuni on the height of the villi at 6 hours, 13 hours and 15 hours post-infection.

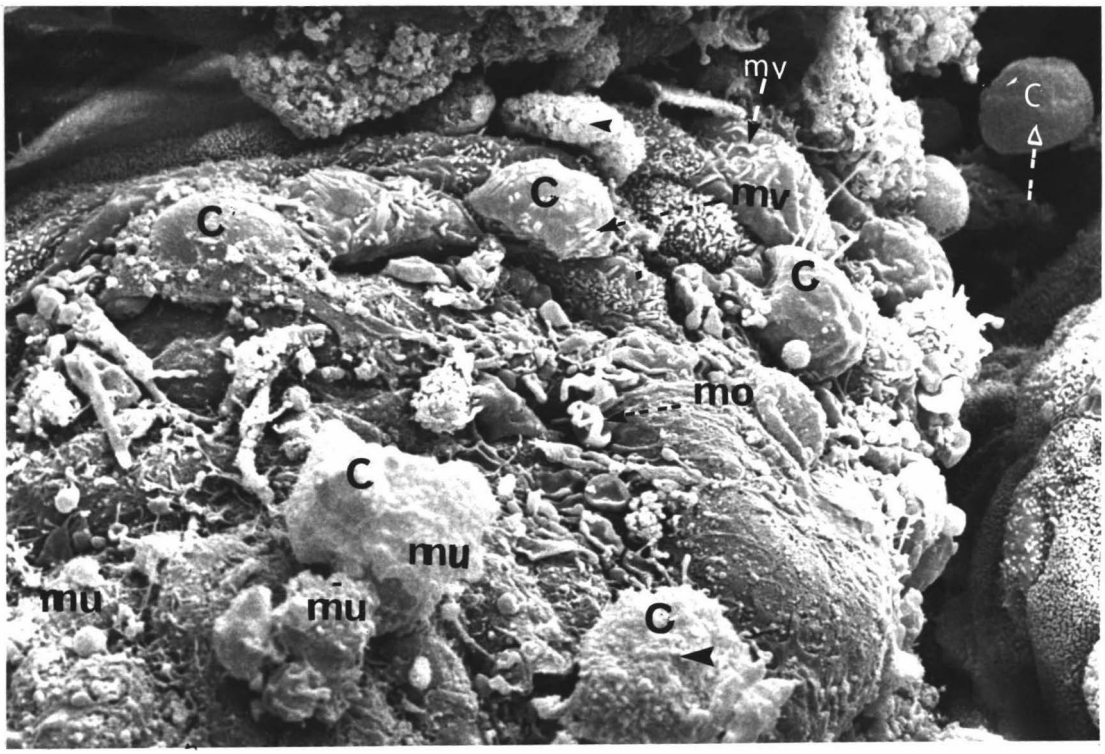
The effect of the medium on the control and infected cultures was significant ($P<0.01$) at 6 hours, 13 hours and 15 hours postinfection. However there was no significant difference ($P>0.05$) in the height of the villi, between the control and infected cultures at 6 hours, 13 hours and 15 hours postinfection.

Figure 3.59

Organ culture of foetal lamb intestine in T199 medium + 10% foetal calf serum, infected with approximately 10^6 CFU/ml of C.jejuni, showing changes that have occurred 15 hours postinfection. The epithelial cells (C) are severely disorganised, round to elongate in shape (arrow) and some show a severe loss of microvilli (MV). A few are exfoliated (Ex). The external surface of the epithelial cells is roughened and covered with thick strands and plugs of white mucus (M). Occasional-spiral shaped microorganisms (MO) are attached to the surface of the epithelial cells. (SEM X2640).

Figure 3.60

Higher magnification of Figure 3.59 showing the irregular, ridged surface of the absorptive epithelial cells (arrow). A severe loss of microvilli can also be observed. (SEM X9,900).


mv
C
C
mv
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mo
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mu
C

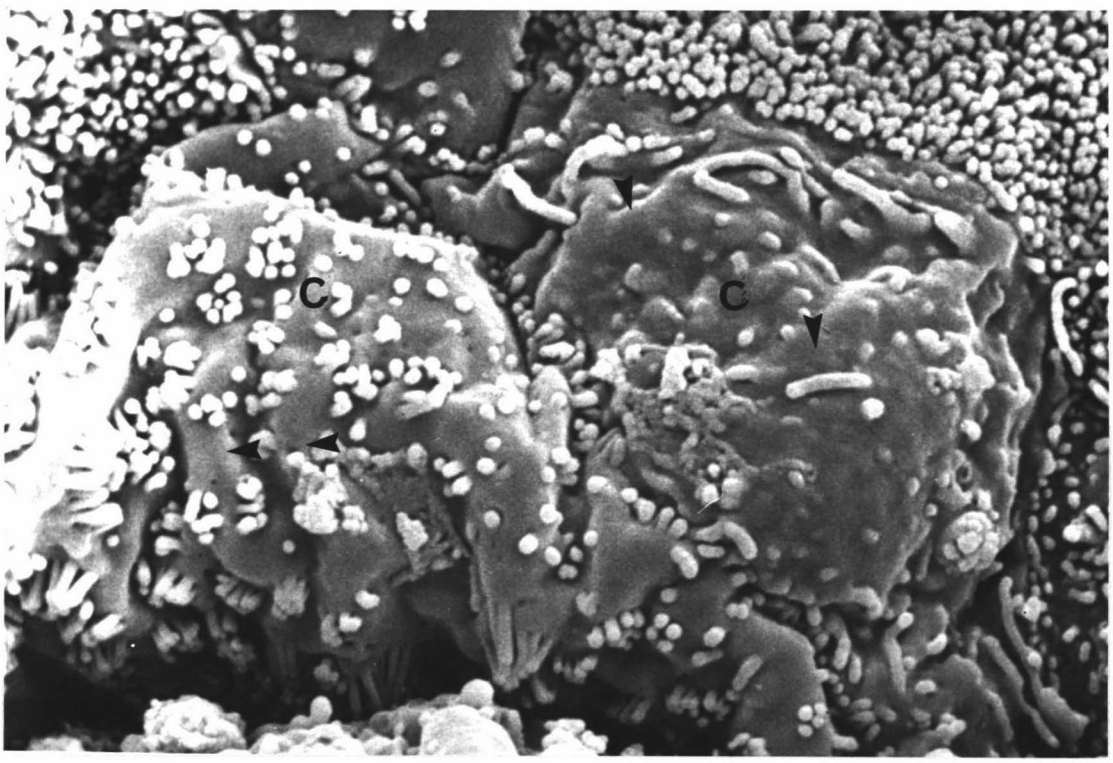
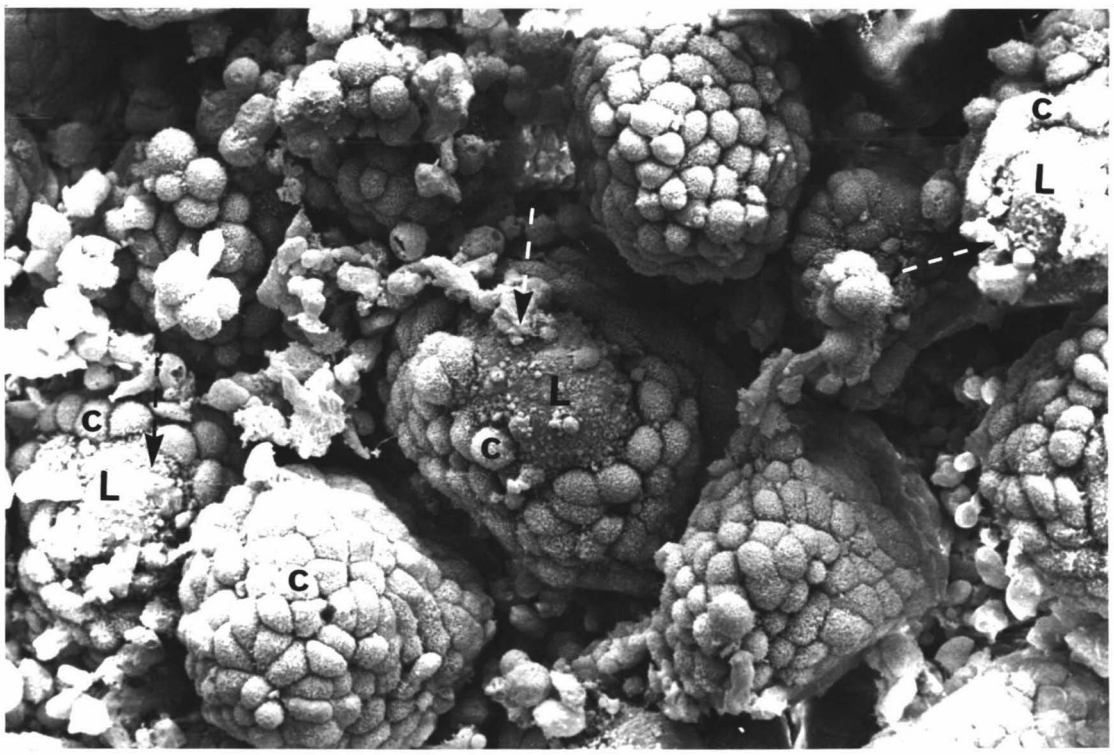

C
C

Figure 3.61

Organ culture of foetal lamb intestine in T199 medium + 10% foetal calf serum, infected with approximately 10^6 CFU/ml of C.jejuni, showing changes that have occurred 15 hours postinfection. Necrosis and exfoliation of some of the epithelial cells can be observed at the tips of the villi (arrow). The lamina propria (L) is exposed and protrudes above the remaining epithelial cells (C). Some of the intact epithelial cells show a disorganised pattern (long arrow) when compared to the 15 hour control culture (Figure 3.50). Occasional shreds and plugs of mucus (MU) cover the desquamated cells (small arrow). (SEM X6050).


C
L
L
C
C
L
C

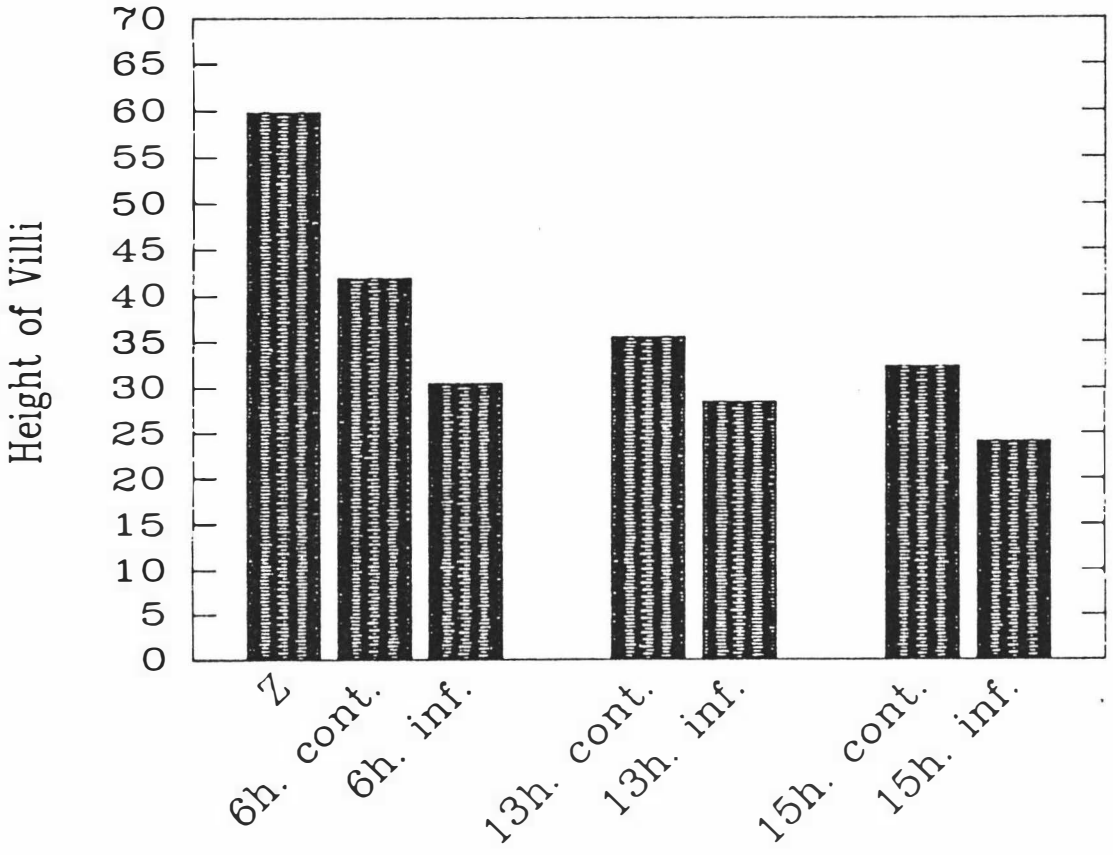


Fig.3.62. Histogram comparing the means of epithelial villus heigh in foetal lamb intestinal organ cultures infected with C.jeju and controls, at 6, 13 and 15 hours post-infection.

Table 3.1: Effect of C.jejuni on the height of villi (um) ($\bar{X}$ ± SEM) at 6 hours,13 hours and 15 hours post- infection.

Time Hours Post-infection	Height of villi(um) Zero #	Control	infected	Significance Z.Vs.C	Z.Vs.I	C.Vs.I
06	149 ±14.61	105 ±5.55	076 ±4.63	* *	* *	NS
13	149 ±14.61	089 ±4.09	071 ±4.44	* *	* *	NS
15	149 ±14.61	081 ±9.61	060 ±5.00	* *	* *	NS

: Zero (Z) : Preculture of foetal lamb intestine organ culture.

* : Significant difference (P<0.05)

**: Significant difference (P<0.01)

NS: Non significant.

C : Control.

I : Infection.

$\bar{X}$: Mean

SEM: Standard error of mean

Statistical analysis of the data using Duncan's Multiple-Range Test failed to show any significant difference (P>0.05) between the control and infected groups at 6 hours, 13 hours and 15 hours postinfection although the infected cultures showed a consistent decrease in the height of the villi by 20% , 26% and 28% at 6 hours, 13 hours and 15 hours postinfection respectively.

Figures 3.63, 3.64 and Table 3.2 show the width and height of epithelial cells of the villi of control and infected tissues at zero hours, 6 hours, 13 hours and 15 hours postinfection.

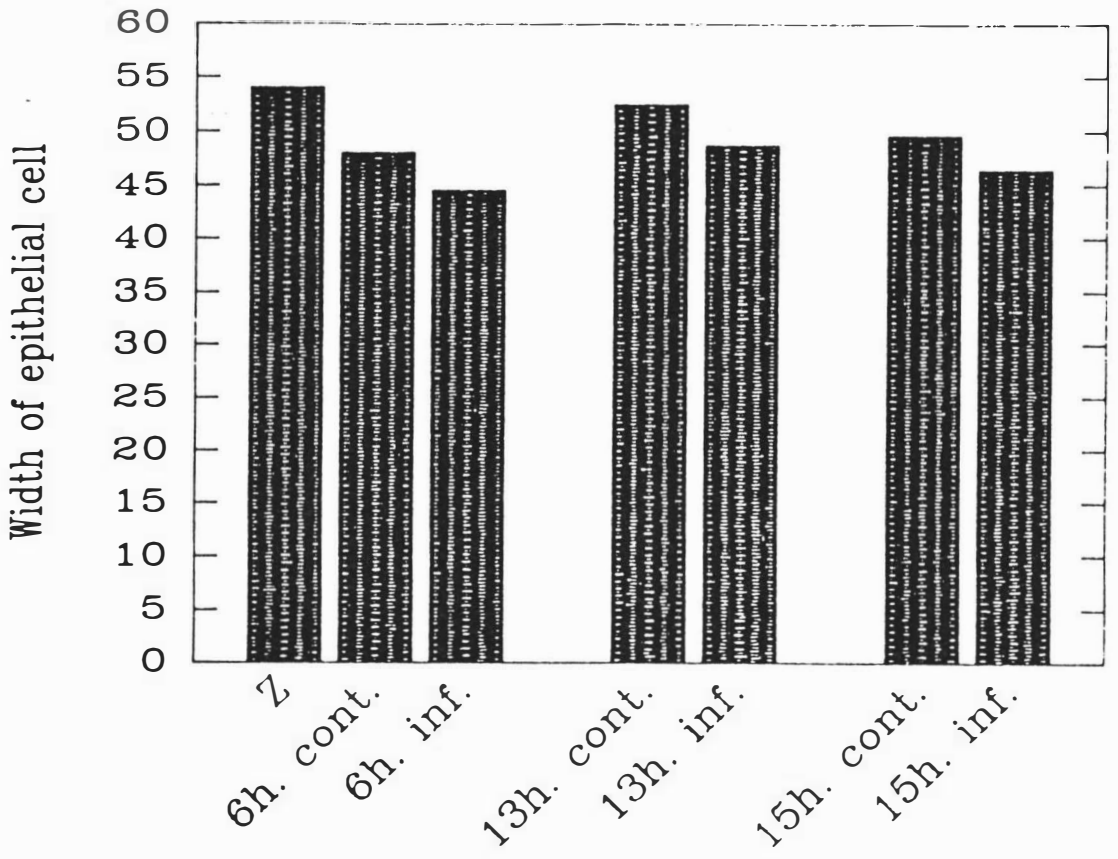


Fig.3.63. Histogram comparing the means of epithelial cell widths in foetal lamb intestinal organ cultures infected with C.jejuni, and controls, at 6, 13 and 15 hours post-infection.

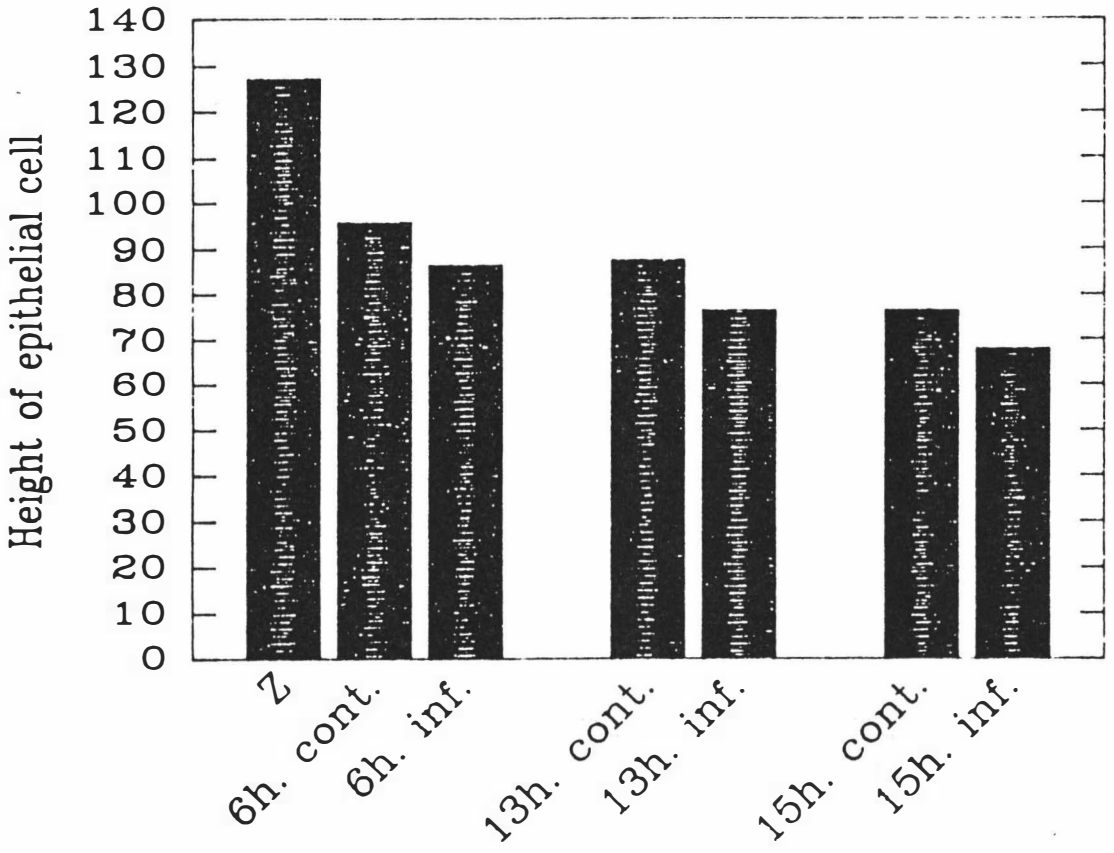


Fig.3.64. Histogram comparing the means of epithelial cell heights in foetal lamb intestinal organ cultures infected with C.jejuni, and controls, at 6, 13 and 15 hours post-infection.

Table 3.2 : Effect of C.jejuni on the width and height of epithelial cells of villi (um) ($\bar{X}$ ± SEM).

Time	Width(um)	Height(um)
Zero hours	7.71 ±0.37 S.E.M	18.16 ±0.44 S.E.M
6 hr. control	6.86 ±0.38 S.E.M	13.60 ±0.47 S.E.M
6 hr. infected	6.36 ±0.25 S.E.M	12.35 ±0.49 S.E.M
13 hr. control	7.08 ±0.28 S.E.M	12.51 ±0.50 S.E.M
13 hr. infected	6.50 ±0.23 S.E.M	11.07 ±0.28 S.E.M
15 hr. control	7.48 ±0.28 S.E.M	10.91 ±0.50 S.E.M
15 hr. infected	6.52 ±0.19 S.E.M	9.71 ±0.39 S.E.M

$\bar{X}$ = Mean
SEM= Standard Error of Mean

The width and the height of the epithelial cells of the villi in control and infected cultures were measured after 6 hours, 13 hours and 15 hours culture. The same measurements were also made on the villi at zero hours (ie on the pre-culture tissues), to determine the effect of the medium on the width and the height of the epithelial cells.

It was found that the medium did not significantly affect the width of the epithelial cells of either the control or the infected cultures after 6 hours,13 hours and 15 hours culture ($P>0.01$). There was also no significant difference ($P> 0.01$) between the width of the cells of the control and infected cultures, although the width of the cells of the infected cultures was always smaller than the control: (at 6 hours: 7.3% reduction in width, at 13 hours: 8.2% reduction and at 15 hours 12.8% reduction).

The medium seemed to significantly affect ($P<0.01$) the height of the epithelial cells of the villi of both the control and the infected tissues after 6 hours, 13 hours and 15 hours post-culture.

There was no significant difference in height ($P>0.01$) between the control and the infected epithelial cells of the villi after 6 hours , 13 hours and 15 hours culture. However, it was observed that the infected villi showed epithelial cells reduced in height at all three times (at 6 hours : 9.2% reduction in height, at 13 hours: 11.5% reduction in height , at 15 hours: 11.0% reduction in height).

CHAPTER IV

DISCUSSION AND CONCLUSION

It appears that there is no published data on the use of intestinal organ culture in the study of the pathogenicity of Campylobacter jejuni in sheep.In addition, there is a lot of controversy in the literature at present on the role of C.jejuni as a cause of enteritis in sheep (Russell,1955, Smibert,1965, Jopp and Orr,1980, Vandenberghe et al., 1980, Stephens,1983).

The present study was carried out to determine the pathogenicity of C.jejuni on foetal lamb intestine. Organ culture was used , as it maintains the architecture of the tissues and thus offers a unique opportunity to simulate "in vivo" conditions.

At the beginning of the experiment it was essential to choose the correct medium for the maintenance of the foetal lamb intestines.Several media were tested for their ability to maintain viable foetal intestinal organ cultures.T199 with 10% foetal calf serum was selected and used for reasons described previously.

The interaction of C.jejuni with the foetal lamb intestines in organ culture and the pathogenicity, were then determined at the cellular level using three microscopic methods, namely Light Microscopy ,Transmission and Scanning Electron Microscopy, at six, thirteen and fifteen hours post-culture.

A zero-time treatment was also used to assess the effects of the medium on the culture of both the infected and control tissues. This was essential to isolate the effects of the medium "per se" from the activity of the microorganisms on the foetal lamb intestines.. Only Light Microscopy was used to determine these effects at the cellular level.

4.1 SELECTION OF MEDIUM

Of the six media tested, T 199 + 10% foetal calf serum was found to be the most appropriate, with the architecture of the cells being well maintained for six days. T199 alone did not appear to maintain the organ culture as efficiently as T199 + 10% foetal calf serum as the organ culture was maintained for only three days. Changes occured in the epithelial lining cells and the morphology of the villi .These changes were probably due to physical factors such as temperature, pH of the culture medium and oxygen tension, or to nutritional levels.The addition of calf serum to the media semed to improve these conditions.

RPM 1 with 10% foetal calf serum maintained intestinal organ culture for six days,but severe morphological changes occurred in the tissues. RPM 1 alone maintained the intestinal organ culture for only three days. Similar results were obtained with Trowell's medium.

The results showed that the addition of foetal calf serum aided explant survival. This agreed with the findings of Ferland and Hugon(1979), who reported that serumless media caused severe morphological changes in adult mouse intestinal culture after 24 hours. They achieved the best results with DMEM-HEPES and NCTC-135 enriched with 10% foetal bovine serum. Alkassi (1986), successfully used T199 medium enriched with 10% calf serum to maintain tracheal organ culture and used this to study the effects of different microorganisms associated with pneumonia. T199 + serum appears to maintain both foetal and adult organ tissues in culture.

Changes in the culture conditions were not measured in the present study. These changes are more likely to occur at the physical level (lowering of the pH due to lactic acid production, and a shift in the oxygen tension) and at the nutrient level (depletion of glucose and amino acids).It would be interesting in a further study to determine changes in the culture medium with

time as this would enable improvements to be made in the lifespan of the explant tissues.

Calvert and Micheletti (1981), evaluated the capacity of six culture media to maintain foetal mouse duodenal culture. They found that T199, Trowell's (T8) and Mc Coys' 5A (all with added serum) maintained duodenal mucosa in culture for 72 hours. Johansen (1970), also showed that T199 and Trowell's (T8) with 10% calf serum satisfactorily maintained human rectal mucosa in culture. Medium T199 with 10% foetal calf serum has been found to sustain human foetal trachea, monkey adult trachea, rabbit adult trachea, ferret adult trachea and pig trachea in culture (Hoorn and Tyrrell, 1969).

In a comprehensive study of the explant culture of human colon, Autrup et al., (1978), and Autrup (1980),used different media and types of sera. The most consistent results were obtained using CMRL 1066 with foetal bovine serum (5%) or foetal bovine albumin (5%). Autrup et.al., (1978), and Autrup (1980), stressed the importance of sera in extending the lifespan of organ cultures. This agrees with the results obtained in the present study.

Trowell's (T8) with 10% foetal calf serum has been used successfully to culture adult guinea-pig duodenum, maintaining the culture for 24 hours (Kedinger et al., 1974). In the present study, signs of degeneration and necrosis appeared after three days of culture in Trowell's (T8) medium alone. The addition of 10% calf serum significantly improved the lifespan of the explant allowing the culture to be maintained for six days. Trowell's (T8) with 10% calf serum has also been used in the culture of human rectal mucosa (Eastwood and Trier, 1973),human colonic mucosa (Mak et al., 1979) and human intestinal mucosa (Howdle,1983). The explants were maintained for 24 hours without any change in the architecture of the tissues .

It appears that after 48 hours severe degenerative changes take place in adult tissues cultured in Trowell's (T8) medium. Kedinger et al.,(1974) suggested that adult tissues may be more difficult to maintain in culture. This may explain the difference in the lifespan of the culture obtained by the above authors and the present study. Mitus et al.,(1970) reported that foetal human intestine was maintained in culture for 14-21 days in Eagles' medium with 10% calf serum. Similarly, Wyatt et al.,(1974), cultured foetal human intestine for 12 days in L-15 (Leibovitz) medium.

Falchuk et al., (1974), used RPM1, enriched with 10% foetal calf serum, glutamine, glucose and insulin to study the effect of Gliadin on intestinal human epithelial cells. Various antibiotics (Penicillin, Streptomycin and Neomycin) were added to the medium which was also sterilised using Millipore filtration (0.45 um filter). The epithelial cells maintained their integrity and orientation for 48 hours after which degeneration was seen. RPM1 with 10% foetal calf serum maintained human colon mucosa in organ culture for 24 hours (Mak et al., 1979). In the present study , RPM1 with 10% foetal calf serum sustained the growth of the foetal lamb intestine for six days.

CONCLUSION

In the present study, T199 enriched with 10% foetal calf serum satisfactorily maintained explant tissues of foetal lamb intestine in culture for six days. The architecture of the tissues was well preserved. It appears that the addition of serum to the medium significantly increases the lifespan of the culture. Further studies are necessary to improve the lifespan of tissues in organ culture and nutritional and physical changes in the medium over time are probably important factors.

4.2 PATHOGENICITY OF C.JEJUNI IN SHEEP

This study clearly demonstrates that C.jejuni causes morphological changes at the cellular level in the inoculated foetal intestinal organ culture.

The three microscopic methods used to study the effects (LM,TEM,SEM) confirmed these morphological changes. An attempt was made to assess the effects of C.jejuni on the height and width of the epithelial cells of the villi. The microscopic methods used revealed the attachment of C.jejuni to the surface of the villous epithelial cells. The microorganisms were also found within the epithelial cells and the luminal crypts.

4.2.1 Morphological Changes

The severity of the infection with time was studied after six, thirteen and fifteen hours. In a preliminary trial, it was observed that the viability of the tissues was maintained until fifteen hours culture. After 18,21 and 24 hours culture the infected tissues showed a high rate of autolysis making it impossible to study the interaction of microorganisms and tissues. Changes occured from six hours post-infection , with an increase in the severity of the infection with time.

A number of workers have infected intestinal organ cultures from other animal species with C.jejuni and observed the following results. Humphrey et al., (1986) working with adult hamster intestinal tract, observed cellular abnormalities after 48 hours post-inoculation. Welkos (1984), observed changes in the lower gastrointestinal tract of chicks 48-72 hours after inoculation. Necrosis of the gizzard epithelium was detected by electron microscopy in infected chickens studied six hours after inoculation. Experimentally infected mink showed a severe necrosis of the ileo-colonic junction 96 hours post-infection (Hunter et al., 1986).

From the above results it can be seen that the model used to study the pathogenicity of C.jejuni has to be well defined as experimental animals may show a variable resistance to inoculation of the pathogen [Robert et al., (1980); Ruiz-Palacios et al., (1981); Humphrey et al., (1986)].

Organ culture systems therefore provide special advantages when studying the effects of microorganisms in vitro. They eliminate biological variations between individuals as both the control and test tissues are taken from a single animal. Organ culture models do not possess a blood supply and hence there is no interaction of host immunity, hormonal factors or nutritional factors. It can also be argued that foetal organ cultures present a better model for pathogenicity studies by virtue of a complete absence of competing microorganisms.

It appears that most authors agree on the morphological changes in the cellular architecture of the tissues affected by C.jejuni. Various animals have been studied: hamster (Humphrey et al., 1986); mink (Hunter et al., 1986); wister rats (Vandenberghe and Verheyen 1985);chicks (Welkos, 1984); humans (Moon et al., 1983); cats and dogs (Bruce et al., 1980 and Collins and Libal,1983); pigs (Roland and Lawson 1974 and Lomax et al., 1982); rabbits (Moon et al., 1974); and adult sheep (Tucker and Robertstad,1965).

The present study revealed that infected tissues showed a mild necrosis of the epithelial cells at six hours post-infection, as observed by Light Microscopy. The severity of the necrosis increased with time as indicated by the changes seen at 13 hours and 15 hours post-infection. Changes were limited to the extreme tips of the villi. In addition there was a marked vacuolation of the cytoplasm of the affected absorptive cells. Other authors, using Light Microscopy have reported similar lesions in epithelial cells of the intestinal tract following infection by Campylobacter species: Humphrey et al., (1986), on hamster; Vandenberghe and Hoorns (1980), Stephens (1983), on infected lambs; Duffy et

al.,(1980) on humans and Moon et al., (1974) on rabbits. There have been other cases reported in the literature where the lesions were restricted to the tips of the villi,as in the present study. Kurtz et al., (1979) showed that the ilea of pigs naturally infected with C.mucosalis had villous tip erosions. Hampton and Rosario (1965) reported that lesions were restricted to the villous tip in the infected ileum of the mouse.

Further observations of the infected tissues by Transmission and Scanning Electron Microscopy showed that the microvilli were irregular in shape, distorted ,shortened in length and thickened in appearance . The observations made by Light Microscopy correlated well with the findings of Transmission and Scanning Electron microscopy . Some of the infected epithelial cells showed a complete loss of microvilli . These results tend to suggest that Campylobacter species have a direct action on the microvilli of epithelial cells. The findings of other authors support the results of the present study [Moon et al. (1974); Kurtz et al.,(1979); Vandenberghe and Hoorens (1980); Field et al., (1981); Ruiz-Palacios et al., (1981); Lomax et al., (1982); Pearson et al.,(1982); Stephens (1983), Stephens et al., (1984); Vandenberghe et al., (1985); Humphrey et al., (1986); and Hunter et al., (1986)].

Similar changes have also been noted in shigellosis and following intestinal invasion by Escherichia coli and Clostridium difficile [Takeuchi et al.,(1968); Staley et al., (1969); Takeuchi (1971); Newman et al., (1977) ; Humphrey et al., (1979)].

Scanning Electron Microscopic studies of the infected tissues showed that the villous surface was roughened and irregular. The infected epithelial cells were swollen and protruded into the lumen of the intestine. These results agree with the findings of Olson et al., (1973).

It is not known how Campylobacter species cause alterations to the shape and size of the microvilli although it appears that this may be due to production of toxic substances in the intestinal lumen. Frederick et al., (1984), and Johnson and Lior (1984) provided some evidence that C.jejuni and C.coli produce cytotonic or cytotoxic toxin. Ruiz-Palacios et al., (1983) showed that C.jejuni produced heat-labile enterotoxin.The enterotoxin was found to raise intracellular cyclic AMP levels and induced intra-luminal fluid secretion in rat ileal loops.

The abnormalities noted in the microvilli may also be due to an abnormal development of enterocytes , although there is no evidence to support this. Electron microscopic studies also revealed morphological changes in the nuclei , mitochondria, golgi apparatus and endoplasmic reticulum of the infected epithelial cells . These findings are supported by similar observations made by Humphrey et al., (1986).

Quantitative measurements made by Light Microscopy on the height and width of the epithelial cells showed that C.jejuni did not significantly ($P>0.01$) affect either parameter. The height of the villi was also not significantly affected by the microorganisms .Taylor and Olubunmi (1981) reported that C.fetus caused a reduction in the height of the villi in pigs. Unfortunately there is not much published data in the literature on the effects of the microorganisms on the length and width of the epithelial cells . The changes in the morphology might be a direct effect of the attachment of the bacteria to the cells. This will be discussed later.

The microvilli forming the brush border of the intestinal epithelial cells function actively in absorption and transport of nutrients and increase the total available absorptive surface. Any lesions caused by the microorganisms on the villous surface would seriously affect absorption in infected animals and such findings appear to characterise the pathogenesis of Campylobacter jejuni. Further research is required to investigate the effects

of Campylobacter species on the surface area and number of microvilli per unit area on infected intestinal epithelial cells. Both Transmission and Scanning Electron microscopy can be used to investigate these parameters.

CONCLUSION

The combined use of Light, Transmission and Scanning Electron microscopy techniques gave a more accurate description of the intestinal lesions than any single technique . The lesions and morphological changes observed by the three methods correlated well and provided a more complete picture. The changes in the villous epithelial cell morphology were determined more accurately by Light and Electron Microscopy. The three dimensional visualisation of the villi by Scanning Electron Microscopy made it possible to study the surface of the mucosa. Severe lesions and modifications occurred in the infected cells and these were clearly demonstrated by all three microscopic methods. These observations were supported by a number of other workers.

4.2.2 Attachment and Penetration of C.jejuni into the Epithelial Cells of Foetal Lamb Intestine

Attachment and invasiveness are requisite virulence factors for the pathogenicity of some microorganisms. Epithelial cell penetration by Campylobacter species is now recognised as an essential step in the pathogenesis of associated enteritis.

The three microscopic techniques used in the present study clearly demonstrated that microorganisms resembling C.jejuni attached to the surface of the villous epithelial cells. The microorganisms showed an intense colonisation of the brush border of the villous epithelial cells, especially at the tips of the villi.

Light Microscopy revealed an invasion of the villous and crypt epithelial cells at 6 ,13 and 15 hours postinfection. Microorganisms also invaded the luminal crypts. Warthin starry

staining clearly showed the attachment to, and invasion of, Campylobacter jejuni into the intestinal epithelial cells. Transmission Microscopy failed to show the presence of microorganisms within the cytoplasm of the infected cells. This might have been due to the weak staining affinity of the microorganisms with lead citrate and uranyl acetate. Likewise the sections taken might also have been inappropriate. However the technique did show that two microorganisms lying within phagolysosomes, were engulfed by macrophages in the lamina propria. These observations are supported by the findings of Vandenberghe and Hoorens (1980), Ruiz-Palacios et al.,(1981), Jubb et al ., (1985), Vandenberghe et al., (1985).

Epithelial cell attachment and penetration by Campylobacter species have been demonstrated in a number of animal species: humans: (Butzler and Skirrow (1979); Duffy et al., (1980); Manninen et al.,(1982)); rats and mice:(Hampton and Rosario (1965); Reimann (1965), Field et al.,(1981); Newell and Pearson (1984); Vandenberghe et al.,(1985)); lambs:(Hoorens et al., (1977); Vandenberghe and Hoorens (1980); Firehammer and Myers (1981) and Stephens et al., (1984)); mink: (Hunter et al 1986); chickens:(Ruiz-Palacios 1981); pigs: (Staley et al., (1969); Love and Love (1979); Lomax et al., (1982)); cattle:(Firehammer and Myers (1981) ; Taylor (1982)) and rabbits: (Moon et al., 1974).

On the other hand, Prescott et al., (1981) failed to observe any invasion of the mucosa of puppies by C.jejuni. Fox et al., (1985) reported that Campylobacter attached to the epithelial cells of adult beagles but did not invade them.

It has long been recognised that a number of pathogenic bacteria are capable of penetrating intestinal epithelial cells. Shigella ,E.Coli and Salmonella organisms have all been reported to attach and to penetrate the epithelial cells of infected tissues [Laberec et al., (1964); Takeuchi et al., (1965); Takeuchi,(1966); Staley et al., (1969); Vogelweid and Elmore

(1983); and Sherwood et al., (1985)].

The results of the study showed that Campylobacter jejuni has a strong staining affinity with warthin starry . This agrees with the reports of Collins and Cibal (1983); and Hunter et al., (1986). This finding made it possible to study the shape of the microorganisms and, more importantly,to detect a possible mechanism of penetration by the organism. Warthin starry staining sections showed that the microorganisms varied in shape, with rods and spirals being seen, and Scanning Electron Microscopy showed that the microogranisms were also filamentous in shape. Higher magnifications using Transmission Electron Microscopy revealed that the microorganisms were approximately 0.776 um long and 0.31 um.wide. IFA staining (Lomax et al., 1982) and crystal violet staining (Stephens et al., 1984) have also been used to identify the microoganisms. Studies on the morphology of Campylobacter species suggest that the microorganisms exist in different shapes : curved, spiral, comma-shaped, S-shaped, ring shaped (Donuts), coccoid, dimple shaped, gull shaped and filamentous (Kurtz et al., 1979 ; Lomax et al., 1982; Pearson et al., 1982 ; Collins and Libal,1983; Stephens, 1983; Stephens et al., 1984; Ng et al., 1985; and Humphrey et al., 1986) .

Humphrey et al., (1986), suggested the possibility that Campylobacter changed its structure in different environments or during developmental stages. Ng et al., (1985), showed that the cells of a single colony of Campylobacter are heterogeneous depending on age and physiological state. A variation in the morphology of Campylobacter is also supported by the findings of Field et al., (1981).

The results of the present study on the length and width of Campylobacter jejuni are in agreement with the studies of Lomax et al., (1982) and Stephens et al., (1984) .

There appears to be no published data on the mechanism of penetration of Campylobacter into infected cells.The Transmission Electron Microscopy technique used in this study showed that the microorganisms were surrounded by a dense,blurred,pilus-like structure (or filament).These structures seemed to mediate the attachment of the bacteria to the microvilli. Although there is no conclusive evidence to support the observation it appears that the filament might be involved in the invasion of the cells. Transmission Electron Microscopy displayed the filaments of the microorganisms to better advantage. Erlandsen and Chase (1974) reported a similar specialised attachment segment ("holdfast" or filament) in Giardia species. Merrell et al., (1981) failed to observe any pili on C.fetus subspecies jejuni but found a fibroid meshwork surrounding the microorganisms and suggested that this might represent an alternative attachment and penetration mechanism.

The exact portal of entry of the microorganism has not yet been determined .

Conclusion

Campylobacter species attach to the surface of infected cells. Colonisation of the brush border of the epithelial cells causes it to be destroyed. There is ample evidence from the literature and from the present study to suggest that the microorganisms invade the infected cells . Information on the mechanism of entry of Campylobacter species into the infected cells is not available. This field deserves more research with a view to a better understanding of the pathogenesis of Campylobacter species. More detailed study of the sequential morphological events surrounding attachment and invasion of the microorganism is also required. The three microscopy methods used in the present study have shown that they complement each other and correlate well.

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APPENDICES

APPENDIX 1

Vibrio Selective Agar

(Preparation of the Modified Preston's Medium, VSA)

The modified Preston's medium was prepared in a similar manner to the modified selective medium of Skirrow, but with the addition of antibiotics recommended by Bolton and Robertson (1982) rather than Skirrow's antibiotic supplement.

A solution was prepared containing 3.125 mg of polymyxin B* dissolved in 10 ml of distilled water (3.125 mg/ml). The solution was kept in a universal bottle at 2-3 C, and 1 ml was added to 500 ml of medium (51 ug/ml).

Fifty (50) mg of rifampicin* was dissolved in 10 ml of distilled water (5 mg/ml). The solution was kept in a universal bottle at 2-3 C and 1 ml was added to 500 ml of medium (10 ug/ml).

Fifty (50) mg of trimethoprin* was dissolved in 10 ml of distilled water (5 mg/ml). The solution was kept in a universal bottle at 2-3 C and 1 ml was added to 500 ml of medium (10 ug/ml).

Five hundred (500) mg of actidione* was dissolved in 10 ml of distilled water (50 mg/ml). The solution was kept in a universal bottle at 2-3 C and 1 ml was added to 500 ml of medium (100 ug/ml).

* Sigma Chemicals Co., Products, Nos P1004, No. R-3501, T-7883 and C-6255 respectively, St.louis, Mo 63178, USA.

APPENDIX II

Bouin's Solution

Picric acid, saturated solution in 95% alcohol 80 ml

Formalin (40%) formaldehyde) 25 ml

Glacial acetic acid 5 ml

APPENDIX III

Modified Karnovsky's Fixative

Paraformaldehyde 2.0 gm

Gluteraldehyde 12 ml

$Na_2HPO_4.12H_2O$ 2.51 gm

KH_2PO_4 0.41 gm

1.0N NaOH 0.1 ml

To make 100 ml:

A) Heat 2.0 gm paraformaldehyde in 80 ml distilled water to 60-70 C.

B) 1.0N NaOH was slowly added, dropwise until the solution cleared.

C) Add buffer salts 2.51 gm $Na_2 HPO_4.12H_2O$, 0.41 gm KH_2PO_4.

Add 12 ml of 25% gluteraldehyde.

D) The solution was made up to 100 ml and stored at 4 C.

APPENDIX IV

Osmium Tetroxide

Osmium tetroxide was made up as a 1% aqueous solution in 0.1M phosphate buffer and kept at 4 C in a dark bottle.

APPENDIX V

Uranyl Acetate Stain

Add uranyl acetate to 50% ethanol until it will no longer dissolve, centrifuge and store supernatant in a brown glass bottle.

APPENDIX VI

Lead Citrate Stain

Lead citrate 0.25 gm

Distilled water 10 ml

1.0N NaOH 0.1 ml

Shake vigorously until dissolved