2nd.

AN INVESTIGATION INTO INTESTINAL UPTAKE OF MICROPARTICLES AND RELEVANCE TO DISEASE

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A thesis submitted to the University of Edinburgh for the Degree of Doctor of Medicine, October 2001

FOR MY PARENTS

DECLARATION

I declare that this thesis has been composed by me and that the work contained within it was performed by me, except where clearly stated otherwise. The entire work was performed while I held a post at the Gastrointestinal unit, Western General Hospital, Edinburgh. The thesis or part of it has not been submitted for any other degree or professional qualification.

Ramendra Nath Mazumder, MBBS

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ABSTRACT

Intestinal uptake and translocation of polystyrene microparticles across epithelium have been studied in animals, but similar studies in human subjects are scarce. Inorganic microparticles have been reported in the intestinal mucosa of patients with inflammatory bowel disease, and inorganic microparticles conjugated with lipopolysaccharide have been shown to stimulate IL-1β secretion from lamina propria macrophages. Accordingly, it is possible that dietary non-degradable microparticles are involved in the pathogenesis of IBD.

The aim of this thesis was to investigate the uptake of inert microparticles by human intestinal mucosa *in vitro* and to consider a possible relationship with disease pathogenicity. Another aim of this project was to investigate the extent of microparticle-laden macrophages in mucosa affected by inflammatory bowel disease.

A model to study uptake of inert microparticles across human colon mucosa has been developed. Uptake of inert microparticles was examined by confocal and electron microscopic studies in cultured human colon mucosa. Also, the effect of lipopolysaccharide on the uptake of inert microparticles by inflamed colon mucosa was studied. The survival of cultured biopsies was assessed by bromodeoxyuridine labelling of crypt proliferating cells and the morphology of the

tissues was assessed by light and electron microscopy. In resected human intestine, inorganic microparticles were examined by X-ray microanalysis and microparticle-laden cells were quantitated by image analysis in immunostained cells.

In vitro labelling of cultured colon biopsies by bromodeoxyuridine was comparable to *in vivo* bromodeoxyuridine labelled cells in mice. Lipopolysaccharide had no effect on the morphology of human colon epithelium as examined by light and electron microscopy. In uptake studies with organ culture, the numbers of inert microparticles in inflamed intestinal mucosa were greater than in normal intestine. The numbers of microparticles were increased significantly in lipopolysaccharide-treated inflamed tissue. The increased formation of macropinosomes indirectly in response to LPS may explain the observed increased uptake of inert microparticles in IBD. An image analysis technique was applied to quantify microparticle-laden macrophages and revealed a significant increase in microparticle-laden macrophages in Crohn's disease. By X-ray microanalysis, inorganic microparticles were confirmed as compounds of titanium, silicon, aluminium and chromium. Detection of chromium in resected human intestine has not been reported before. Chromium has been reported as granulogenic in other tissues, but not in the intestine.

This study has shown for the first time that human colonic cells can take up microparticles *in vitro* and that this uptake is enhanced in the presence of

inflammation. It is further enhanced in the presence of LPS. Microparticles of aluminium, silicon, titanium and chromium may be found within the macrophages in inflamed intestinal mucosa from IBD patients. Chromium is granulogenic in certain tissues. The routes of uptake of microparticles are discussed in detail in this thesis.

LIST OF PUBLICATIONS BASED ON THIS THESIS

Mazumder RN, Bode J, Campbell S, Church NI, McIntyre MM, Ghosh S. Lipopolysaccharide increases microparticle uptake in inflamed human colon in vitro. [Abstract] Gastroenterol 2001; 120 (Suppl. 1): A697.

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The above scientific study received the "President's nomination" as one of the outstanding top ten posters and was subsequently invited for oral presentation in plenary session on 22 March, 2001, at the Scientific meeting of British Society of Gastroenterology.

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ABBREVIATIONS

IBD	Inflammatory bowel disease
UC	Ulcerative colitis
IgG	Immunoglobulin
IL	Interleukin
TNF	Tumour necrosis factor
CLSM	Confocal laser scanning microscopy
TEM	Transmission electron microscopy
SEM	Scanning electron microscopy
EDAX	Energy dispersive analysis of X-ray
CD	Cluster of differentiation
TBS	Tris buffered saline
NRS/TBS	Normal rabbit serum in TBS
'M' cell	Microfold cell
PP	Peyer's patch
DNA	Deoxyribonucleic acid
BrdU	Bromodeoxyuridine

CHAPTER 1: INTRODUCTION AND AIMS

Transepithelial transport of bacteria and viruses across intestinal epithelium has been reported in both animal experiments and in cultured cell lines. Experiments with animal tissues, both *in vivo* and *in vitro*, have demonstrated uptake and translocation of microparticles across intestinal mucosa. However, the pathways and mechanisms of uptake of microparticles are not totally clear. It was believed that small microparticles may be transported through the epithelial cells of the intestine but uptake and translocation of 2-μm-sized microparticles across intestinal epithelium have been demonstrated in animal experiments.

Microparticle uptake studies with colonic tissues are very few, and it is generally thought that the colon is relatively impermeable to microparticles.

Most of the microparticle uptake studies have been conducted with a view to the microencapsulated delivery of a drug or vaccine. It is widely believed that the uptake of polystyrene microparticles occurs across the intestinal mucosa in experimental animals. A corollary to the results of these experiments is whether the intestinal uptake of non-degradable microparticles is harmful in man.

There is no experimental evidence that non-degradable microparticles are taken up by the human intestinal mucosa. Also, the effect of any luminal factors such as lipopolysaccharide on this uptake is not available.

Non-degradable microparticles have been reported in inflamed human intestinal mucosa. The possible benefit of a microparticle-free diet in Crohn's disease is now being cautiously considered in the knowledge that environmental microparticles ingested in our diet as food additives and in pharmaceutical products as adjuvants may be related to the perpetuation of Crohn's disease.

Therefore in this thesis, the following research questions have been addressed:-

1. Does intestinal uptake of non-degradable microparticles occur in man?
2. Does any pre-existing pathology affect this uptake process, or do any luminal factors such as lipopolysaccharide influence it?

3. What is the extent of microparticle uptake by the gut in health and disease in human?
4. What are the types of microparticles and extent of microparticle-laden cells in the human intestine?

CHAPTER 2: REVIEW OF LITERATURE

Barrier function of the gastrointestinal tract and microparticle uptake

Background

The gastrointestinal tract is the largest organ in the human body exposed to noxious agents present in the external environment (1), and a major function of this organ is to protect the host from such noxious agents as microbes, microparticles and macromolecules present in the gastrointestinal lumen. This is, of course, secondary to the absorption of nutrients but it is an important function of the gastrointestinal tract. It is achieved by the development of a barrier of epithelium, i.e., cells joined together by tight junctions in the intestinal tract (2,3) and by covering the apical surfaces of these intestinal cells with secretory IgA antibodies and mucus to prevent the entry of harmful agents into the interior milieu of the intestine (4,5).

It is widely accepted that secretory IgA provides a first line of defence against bacterial invasion of mucosal surfaces. Secretory IgA consists of a secretory component (SC) and immunoglobulin A (IgA). Secretory component is a transmembrane polypeptide (MW ~ 80,000), preferentially expressed on the basolateral aspect of the intestinal epithelial cells. It has five extracellular domains, thus placing SC in the immunoglobulin super family (6). SC functions as the cellular receptor of polymeric IgA and IgM secreted by lamina propria

plasma cells. The J-chain on polymeric IgA or IgM probably binds to secretory component. Following binding the receptor complex is internalised and transported through the cells into the lumen (7). The process by which secretory IgA prevents invasion of bacteria is not totally clear (8,9).

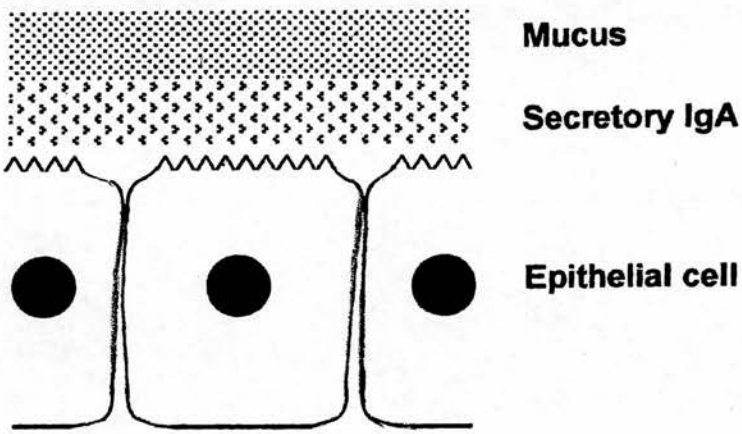

Figure 2. 1: Schematic diagram of intestinal barriers to the luminal contents

The intestinal mucus (also called glycocalyx) creates a diffusion barrier as well as a highly degradative microenvironment within the gastrointestinal lumen (10,11). Mucus secreted by goblet cells in the intestinal epithelium contains specific receptors to bind endogenous enteric bacteria (12), and this binding prevents potential pathogens from binding to (and colonising) the mucus layer (13). The negative ionic charges of mucus glycoprotein and the ability of mucus to create a hydrophobic luminal barrier enhance the protective ability of the gastrointestinal

mucus (14). The degradative function of the glycocalyx is achieved by entrapping pancreatic and hydrolytic enzymes that impede the access of macromolecules and microorganisms to the apical cell membrane of the intestinal epithelial cells (10).

Even with this highly developed barrier function in the gastrointestinal tract, there is both clinical and experimental evidence that luminal contents traverse the epithelium (15,16). Mechanisms of entry of microbial pathogens i.e., bacterial translocation or passage of macromolecules such as antigens across the intestinal mucosa have been well studied. However, studies related to the entry of other luminal contents such as microparticles and nutritionally insignificant intact protein (non-nutritive protein) in the human intestine are very few. Most of the microparticle uptake studies in animals have been done with an aim to observe encapsulated drug or vaccine delivery through the intestine. It is now emerging that the entry of microparticles may be of significance in the process of inflammation, e.g., in Crohn's disease (17, 18). Similarly, entry of intact proteins across the intestinal mucosa may result in a completely new type of infection such as the transmissible spongiform encephalopathies (TSEs) (19). Most studies to assess the barrier function of the intestinal epithelium have been conducted to investigate the translocation of bacteria and bacterial products. Studies on the uptake of microparticles and TSE agents (prions) across the human intestinal epithelium, with reference to pathogenesis, are almost non-existent. For example, entry of microparticles in this way may perpetuate

inflammation and entry of prions may initiate variant Creutzfeldt-Jacob disease (vCJD). A better understanding of the limitations of the protective functions of the gastrointestinal tract which has a surface area of approximately 200-300 m^2 should enable us to improve defences against exogenous noxious agents, and should also help to develop further preventive and therapeutic applications.

In the following chapters, maintenance of the barrier function of the gastrointestinal tract will be considered with special reference to the ways in which environmental agents and possibly prions may challenge the defence mechanisms of the human gut.

Structure of the gastrointestinal tract

The gastrointestinal tract extends from the mouth to the anus and its basic tubular structure varies with its appendices and evolutionary modifications in different species. Research work in this thesis will focus only on the large and small intestine, and discussion therefore, in this thesis will be limited to these parts of the intestine. Although animal models must be considered the principal interest will be the human gut. The gastrointestinal mucosa is the interface between nutrients and extraneous substances and the internal milieu and therefore plays a crucial role in protecting the host from harmful substances (20).

Organisation of the gastrointestinal mucosa

The gastrointestinal mucosa consists of (1) epithelium; (2) lamina propria of loose connective tissue; and (3) the muscularis mucosa. The intestinal epithelium consists of columnar cells, ~ 20 μm high x 8-10 μm wide, each with an oval nucleus in the basal half of the cell. Columnar epithelial cells are interspersed with mucus-secreting goblet cells and intraepithelial lymphocytes. The epithelial cells are held together by junctional complexes (tight junctions) that prevent passage of unwanted substances via the intercellular spaces. Beneath the junctional complex lies the gap junction that allows small molecules and ions to diffuse between adjacent cells. In the small intestine mucosa there are finger- like projections (villi) that again have microscopic projections (microvilli). The purpose of these two special developmental adaptations is to increase the surface area for absorption. The large intestine is devoid of villi.

Other than epithelial cells, a special type of cell called microfold or 'M' cell overlies the lymphoid follicles in the intestine. Lymphoid follicles are organised lymphoid tissues in the gut or "gut associated lymphoid tissues"(GALT).

Organised lymphoid tissues of the gastrointestinal tract

Organised lymphoid tissues are situated from the proximal intestine to the rectum (21,22). The basic difference between organised lymphoid tissues in the gut (GALT) and secondary lymphoid structures such as spleen, is the absence of a capsule and lack of a draining system as afferent and efferent lymphatics. Also, the epithelial lining of the GALT is different from that of the secondary lymphoid tissues both functionally and structurally.

In the small intestine, lymphoid tissues are in aggregated forms called Peyer's patches, whereas in the appendix and colon they are called lymphoid follicles.

Peyer's patches of the small intestine

In 1667 De Peyer first described the organised lymphoid tissue of the small intestine. These organised areas of lymphoid tissue in the mucosa appear as small round blebs on the mucosa.

The numbers of Peyer's patches in the small intestine vary according to age, highest in adolescence (~ 250) and lower at the extremes of age (~ 100) (23). It is unknown why there is an increase in the number of mucosal lymphoid tissues from birth followed by a decline in old age. However, the general view is that the

presence of luminal antigen stimulates the increase in the number of mucosal lymphoid tissues (24,25). The number of Peyer's patches increases from the duodenum to the terminal ileum in mammalian intestine.

Other than Peyer's patches, solitary lymphoid nodules and lymphocyte-filled villi have been reported in human small intestine (26,27). However, the functional significance and structural details of these structures has not been as well studied as Peyer's patches.

Lymphoid follicles of the colon

In 1926, organised lymphoid tissues (lymphoid follicles) in human colon were first described by Dukes and Bussey (21). Small, white nodules 1 to 4 mm in size are studded in the colonic mucosa. There are two types of lymphoid follicles: non-dome or flat type and dome type; the proportion of these two types of lymphoid follicles in the colon is 10:42 (26). In flat type follicles 'M' cells have not been reported in the follicle-associated epithelium. As with Peyer's patches, the average numbers of follicles per cm^2 in old age decrease to 3 from 8 in childhood. Anatomically, lymphoid follicles are distributed all over the colon, but the number gradually increases from the caecum distally to the rectum (21,28). Similar structures have been reported as lymphoglandular complexes in animals (29).

Differences between lymphoid follicles of the colon and Peyer's patches in the small intestine

The lymphoid follicles in Peyer's patches of the small intestine occur in aggregated forms whereas follicles in the colon appear in isolation. Also, lymphoid follicles in Peyer's patches lie above the muscularis mucosa, but the lymphoid follicles of the colon may appear below the muscularis mucosa or disrupt the muscularis mucosa and become very superficial close to the lumen.

Follicle associated epithelium (FAE)

In general, structurally organised lymphoid tissues in the human gut consist of a follicle or germinal centre. The follicle centre is surrounded by a zone of small lymphocytes known as the mantle zone. The mantle zone merges into a mixed cell zone. Towards the luminal side the mixed cell zone forms a dome like structure and is covered by a specialised epithelium (follicle associated epithelium, FAE) in both forms of organised lymphoid tissue.

Follicle-associated epithelium is cuboidal in shape and contains fewer goblet cells but has specialised types of microfold cells or 'M' cells described by Owen RL *et al.* (30). Morphological features such as poorly organised brush border, deficient apical glycocalyx and alkaline phosphatase, expression of Sialyl Lewis

A antigen and presence of lymphocytes within the pockets of these cells are most often used to differentiate 'M' cells from absorptive enterocytes [Figure 2.2], although the absolute specificity of these is debated. Functionally these cells are believed to be specialised to sample antigens from the gut lumen. However, the developmental origin of these cells is yet unknown (31).

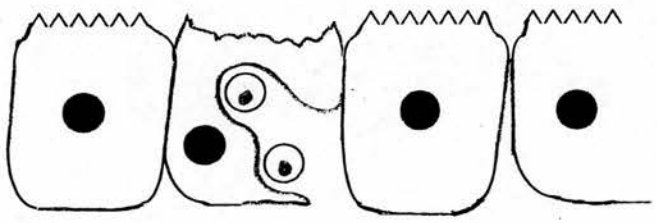

Figure 2. 2: Schematic diagram of follicle associated epithelium showing M cell with lymphocytes in its pocket

There are basically two schools of thought on the development of 'M' cells: [a] studies indicate that M cells are developed through a completely separate process of differentiation from crypt cells (32); [b] alternatively, 'M' cells are derived from the phenotypic conversion of mature intestinal epithelial cells with induction from the surrounding lymphocytes (33). After extensive research over

the last three decades since their description in 1974 there is disagreement on the criteria for identification of M cells. In a recently published elaborate study on 'M' cells, Onori and his colleagues concluded, "the concept that distinct cell types (enterocytes and M cells) can be identified in the rat dome epithelium does not appear to be valid". Similarly, other researchers have questioned whether 'M' cells are a separate entity or a dynamic morphofunctional shape of normal intestinal epithelial cell (34-36). There are differences in the organisation of gut-associated lymphoid tissues between animals and man (37) and this makes it difficult to relate work with experimental animals directly to human models.

Microfold or 'M' cells

Less than 1% of mucosal epithelial cells are M cell (38). These are deficient in cytoplasmic acid phosphatase and microvillus-associated alkaline phosphatase (39) and are probably HLA-DR-ve (40). It is unlikely, therefore, that these play a role in antigen presentation, but are more likely a portal for entry of luminal contents into the dome area of the follicle. This has been clearly demonstrated by entry of reovirus and horseradish peroxidase, which adhere and are transported through 'M' cells in mice (41,42). Similarly, uptake of poliovirus by humans was demonstrated by Scinski *et al*, [1990] (43). It has been postulated that soluble proteins, microparticles, and live microorganisms may traverse through the M cells by transcytosis and are delivered to the basolateral side of the cell packed

with T and B lymphocytes. It has also been demonstrated that HLA-DR+ epithelial cells of the dome epithelium have a capacity to process and present antigen (44).

Beneath the epithelium, the lamina propria consists of loose connective tissue containing immune cells such as plasma cells, macrophages, and lymphocytes.

Absorptive functions of the gastrointestinal tract

The major functions of the gastrointestinal tract include the secretion of fluids and digestive juices; absorption of nutrients; mixing and propulsion of foods and digestive secretions; and the neurohumoral co-ordination of these processes.

The small intestinal epithelium absorbs nutrients. A significant amount of research has been done to elucidate the mechanisms of absorption and different pathways by which a variety of dietary nutrients are absorbed every day in mammals. In brief, carbohydrate, protein and fat in the intestinal lumen are degraded from polymers to simpler forms by gut secretions and processing, prior to their absorption. Nutrient molecules enter the enterocytes at the apical cell membrane and exit at the basolateral side. The first step in this process is the attachment of a nutrient molecule to its receptors in the apical cell domain.

Macromolecules with the capacity to evoke an immune response can also cross the intestinal epithelial cells and this is a physiological phenomenon. From published work, it is now clear that there are two major pathways by which macromolecules can cross the epithelium. They can bind to receptors and shuttle through the absorptive cells or they can pass through specialised 'M' cells. A third possibility is that antigenic fragments may cross the epithelial cells for presentation by class II MHC molecules at the basolateral membrane. Antigenic fragments could be generated during epithelial transport or could be processed from whole macromolecules that have traversed the epithelium. The definitive evidence for this mode of transport is yet to be confirmed.

Since the description of 'M' cells by Owen *et al.* [1974], other than macromolecules, 'M' cells in the Peyer's patches have been widely accepted as a route for the entry of microparticles, bacteria and viruses (38). However, transport of microparticles and bacteria through intestinal epithelial cells (transcellular transport) has not been ruled out. In animal studies it has been demonstrated that microparticles can traverse through the epithelial cells (45) and bacteria have developed strategies to traverse through the intestinal epithelial cells other than by passing through the 'M' cells (46-48).

It therefore appears that a mechanism to exclude harmful gut luminal contents that can cross the barrier of the gastrointestinal mucosa is as important as

absorption of nutrients to protect the host from the damaging effects of the noxious agents.

So far, little attention has been paid to this important aspect of intestinal function. If this exclusion mechanism is not absolutely efficient, it is important to determine and define substances that can get through the epithelial barrier of the host and to identify any consequence of such entry. As mentioned earlier, of the three major noxious agents in the gastrointestinal lumen, entry of bacteria and their products including antigens has been well studied but entry of microparticles from the potentially pathological point of view has been virtually ignored. The pathological role (if any) of ingested microparticles and their subsequent location in the intestine requires further study.

In the following sections, microparticles and their routes of entry across the gastrointestinal mucosa will be considered. In the published literature, microscopic particles have been described as particulates, ultrafine particles and microparticles. There is no general consensus in terminology. In this thesis, the term microparticles will be used for all of these.

Entry of non-nutritive proteins across the gastrointestinal mucosa

Although highly efficient hydrolytic systems exist in the gastrointestinal mucosa that form a chemical barrier, studies have shown that in the physiological state a fraction of intact macromolecules (e.g., proteins) bypasses the gastrointestinal

barrier and these are absorbed through the gastrointestinal tract (49,50). This finding is established particularly in neonate and young animals as well as in young human infants. Studies have also demonstrated absorption and transport of intact macromolecules in adult mammals, although to a lesser extent. It is well established that penetration of epithelial barriers by the antigens is the first step in the development of protective mucosal and systemic immune responses (24), but the significance of the transport of intact macromolecules across the intestinal epithelium in adults is not fully understood (51).

Gastrointestinal uptake of microparticles

The wall of the mammalian gastrointestinal tract was for long assumed to be an impermeable barrier to the passage of microparticles (52,53). However, the passage of large non-dissolved food microparticles from the intestinal lumen into the circulation was reported as early as 1844 by Herbst (54). At the beginning of the twentieth century, intact starch microparticles in blood and urine were observed after per oral administration by Hirsh R [1906], and by Verzár F [1911](54). In the early 1960's, Payne *et al.* (55), and others have demonstrated the intestinal uptake of microparticles in animals (56).

The transmucosal transport of microparticles in the gastrointestinal tract was most extensively studied by Volkheimer (57). He demonstrated microparticles of

corn (3-10 μm), potato (5-10μm), rice (3-10μm), pollen (10-12 μm), diatoms (5-12 μm) and cellulose in the blood and urine after peroral administration.

A significant number of studies were conducted to demonstrate uptake and translocation of microparticles by the gastrointestinal mucosa and to understand the mechanisms of this process (Table 2.1).

Table 2.1 1: Summary of studies investigating uptake and translocation of inert, non-motile microparticles by the intestinal epithelium.

Species Studied	Route of inoculation	Microparticles	Site of particle location	Reference
Rat	Oral	Starch	Epithelial cells	58
Human	Oral	Starch	Venous blood	54
Human	Oral	Mineral ≤ 10 μm	Urine	59
Mouse	Oral	Carbon (20-50 nm)	Macrophage in PP*; Lamina propria	60
Chicken	Oral or Intracloacal	Carbon	Bursal epithelial cells; Macrophages	61
Hamster	Ileal loops	Ferritin (50-60 Å)	Epithelial cells, macrophages	62
Chicken Rabbit Mouse	Bursa Appendix Ileal loop	Ferritin, india ink	Epithelial cells	63
Chicken	Intracloacal	Latex micro-particles (1 μm)	Bursal epithelium; Macrophages	61
Mouse	Oral	Latex micro-particles (2 μm)	Macrophages; Dome of Peyer's patches	64
Mouse	Oral	Latex micro-particles (1 μm)	Mesenteric lymph nodes; Phagocytes	65

*PP = Peyer's patch;

Three possible routes of entry of microparticles across the gastrointestinal epithelium have been suggested: (1) uptake by a paracellular pathway; (2) uptake by the M-cells in the Peyer's patches; and (3) transcellular uptake i.e., transport via the epithelial cells lining the intestinal mucosa.

1. Uptake by a paracellular pathway

Volkheimer (57) and Alper *et al.* (66) have detected a significant quantity of microparticles in the blood 10 minutes after peroral administration in experimental animals. This seems to be extremely rapid and can only be explained by a paracellular pathway.

It has been reported that paracellular passage of microparticles between the enterocytes was the major mechanism of intestinal uptake of larger (> 1μm) particles. Scanning electron microscopic studies had revealed a loosening of the tight junctions of the intestinal mucosa in the immediate neighbourhood of the goblet cells enabling the passage of the microparticles.

Microparticles in the nanometre range have been reported in the intercellular spaces by Aprahamian *et al.* (67). Microparticles of ferritin and virus microparticles were reported in the intercellular spaces of absorptive cells in rats and this was attributed to the disruption of junctional complexes as a result of a protein-deficient diet (68).

In a number of animal studies, macromolecular tracers were not observed in the paracellular spaces surrounding undamaged epithelial or goblet cells (69).

Therefore when the normal barrier of epithelial tight junction is considered, the passage of microparticles (>1μm) between absorptive cells (paracellular route) seems rather less likely than passage through the cells (intracellular or via 'M' cells). In normal circumstances, the zona occludens (or tight junctions) between the epithelial cells is thought to act as an effective barrier against the absorption of all but the smallest molecules. However, in *vitro* studies it has been demonstrated that if this barrier is damaged by various treatments e.g., aspirin (70), alcohol (71,72) and hypertonic solutions, there is an enhanced uptake of macromolecules (73).

Aprahamian *et al.* (67) also observed microparticles (< 1μm, nanoparticles) in defects of the mucosa between 10 and 15 min after intraluminal injection into the intestine of Beagle dogs and described this phenomenon as 'persorption'.

2. Uptake via the M-cells

LeFevre *et al.* (64) clearly demonstrated involvement of Peyer's patches in the uptake of 2 μm latex microparticles in mice. Their results indicated accumulation

of latex microparticles in Peyer's patches during chronic feeding of microparticle suspensions to mice.

Peyer's patches are located in the antemesenteric wall of the small bowel and similar structures are also found in the colon as lymphoid follicles. Follicle-associated epithelium (FAE) overlying Peyer's patches contains 'M' cells. FAE contains a decreased number of goblet cells and the resulting decrease in mucus secretion has been assumed to make the M-cell's surface environment more accessible to microparticles or bacteria (74).

Membranous epithelial cells (M-cells) are believed to be specialised epithelial cells overlying lymphoid follicles of the gastrointestinal and respiratory tracts (75). These cells transport antigens from the lumen to the extracellular space, allowing subsequent access to lymphocytes, macrophages, and plasma cells. In suckling and adult animals they have been shown to be involved in the transport of macromolecules (50).

Jani *et al.* (52) have observed uptake of fluorescent polystyrol nanoparticles of sizes between 100 nm and 1 μm in the Peyer's patches after administration by gavage to mice for 10 days. The particles were not distributed randomly in the tissues, but were concentrated at the serosal side of the Peyer's patches. Histologic investigations have revealed a translocation of the microparticles of 1 μm in size and smaller from the Peyer's patches to the mesenteric lymph nodes.

The microparticles were easily visualised in ducts connecting the patches and lymph nodes. A comparison between ^{125}I-labelled 100 nm and 1 μm microparticles demonstrated a higher uptake of the smaller microparticles. Especially the smaller particles were also observed in the liver and spleen.

Studies involving polylactide, polyhydroxybutyric acid, polymethyl methacrylate, ethylcellulose, cellulose acetate hydrogen-phthalate, cellulose triacetate, and polystyrene microparticles (76) have shown that microparticles of diameter 1-10 μm are taken up by the Peyer's patches, whereas microparticles larger than 10 μm are excluded. Microparticles of a size below 5 μm traverse the Peyer's patches, enter the mesenteric lymph nodes, and eventually enter the circulation via the thoracic duct. Microparticles of a size between 5 and 10 μm do not migrate out of the Peyer's' patches and can stay there for months.

3. Transcellular uptake (i.e., transport through the epithelial cells)

It is generally considered that small macromolecular complexes in the luminal contents, e.g., microparticles and bacteria, may traverse the gastrointestinal epithelium, but the exact mode for their transport varies with the structure of the intruding substances.

Polystyrene microparticles of a size of 220 nm were observed within jejunal epithelial cells 1 h after intragastric administration in the rat (56). The

microparticles were occasionally observed between the microvilli or in the terminal web. However, they were very numerous in the cytoplasm of the epithelial cells, where they were enclosed in vesicles indicative of transcellular uptake. At 2-4 h after their administration, the particles were observed in the interstices of the lamina propria and in the lymphatics of the mucosa.

The observation of microparticle uptake by cells lining the intestinal mucosa has been supported by electron-microscopic autoradiographic investigations (77). Kreuter *et al.* [1989] observed radioactivity in the interior of epithelial as well as of goblet cells between 90 min and 6 days after peroral administration of polyhexylcyanoacrylate microparticles labelled with ^{14}C. Earlier studies indicated that smaller microparticles could be transported through the mucosal epithelial cells. In 1995, Hodges *et al.* confirmed uptake and translocation of 2 μm fluorescent polystyrene microparticles in rat enterocytes (48). They have clearly demonstrated microparticles within the enterocytes and goblet cells of the epithelial layer, in the lamina propria, and within crypt epithelium. In a number of studies this group have demonstrated uptake and translocation of polystyrene microparticles >1 μm within the epithelial cells of the animal intestine (48). Similarly, Cartwright-Shamon J *et al.* have demonstrated 5 μm size yeast microparticles within the apical enterocytes in mice and recommended that microparticles move through the mucosal epithelial cells (45). It is now emerging that M cells are not the only cells involved in uptake and transport of

microparticles or bacteria; intestinal epithelial cells also take up and transport microparticles and bacteria transcellularly (46,78).

However no study has yet demonstrated uptake and transport of polystyrene microparticles in human intestinal mucosa.

In summary, evidence exists for the transport of microparticles in the nano- to micrometre range by all three described routes. In a number of studies (52,56,67,79,79) the simultaneous occurrence of more than one pathway has been reported. It is possible that the major uptake pathway may vary in different parts of the intestine.

The surface properties of microparticles may have an influence on the uptake mechanism. However, in some studies there were no differences in uptake of different materials (52,80). It is also possible that the gastrointestinal mucosa in different species handles microparticles with different physicochemical surface properties differently.

Regarding size of the microparticles, it appears that the uptake of smaller microparticles is significantly greater than that of larger microparticles (52,55,57,76,81). Also, smaller microparticles often accumulate in clusters after uptake (56,64,67,77,82).

Cellular pathways of microparticle uptake

The cellular pathway by which microparticles are translocated is not totally clear. Sanders *et al.* [1961] observed polystyrene microparticles (220 nm) within the cytoplasm of the jejunal epithelial cells in rats, where they were enclosed in vesicles (56). This finding was supported by electron microscopic investigation (77). The presence of microparticles enclosed in vesicles within the cytoplasm of the cells suggests an endocytic uptake mechanism. This mode of uptake and transport was suggested as a second pathway for translocation of luminal microparticles (52).

Endocytosis

Endocytosis refers to the process by which cells take up macromolecules, microparticles and even other cells in special cases without passing through the membrane (83,84). Endocytosis is an active process that requires metabolic energy. The term encompasses both pinocytosis and phagocytosis. Phagocytosis occurs in specialised cells e.g., macrophages.

Of all of the endocytic mechanisms, receptor-mediated endocytosis has been most extensively studied.

Four morphologically distinct endocytic pathways have been characterised and are illustrated in Figure 2.3. They differ in the composition of the coat (if any) and in the size of the detached vesicle.

1. Receptor-mediated endocytosis

invaginations of the cytoplasmic membranes and small transport vesicles decorated by an electron-dense 'bristle' coat were first detected in insect oocytes by conventional electron microscopy. Yolk proteins were clearly concentrated within these 'coated' pits and vesicles, leading to the conclusion that they were the vehicles of receptor-mediated endocytosis (85). Isolation of the coated vesicles led to identification of the coat constituents as clathrin, which forms a polygonal lattice on the surface of the membrane, and adaptor (or assembly) protein (AP) complexes, which mediate the assembly of the clathrin lattice on the membrane (86). Macromolecules to be taken up are recognised and bound by the specific membrane receptors in the coated pits. The binding often leads to aggregation of receptor -ligand complexes, and the aggregation triggers endocytosis. Coated vesicles range in size from ~100-150 nm in diameter (Figure 2.3a).

2. Caveolae

The discovery of non-coated invaginations of the plasma membrane, 50-80 nm in diameter (Figure 2.3b) preceeded the discovery of coated pits. Caveolae are highly abundant in epithelial cells and it has been suggested that these vesicles participate in transcytotic transport.

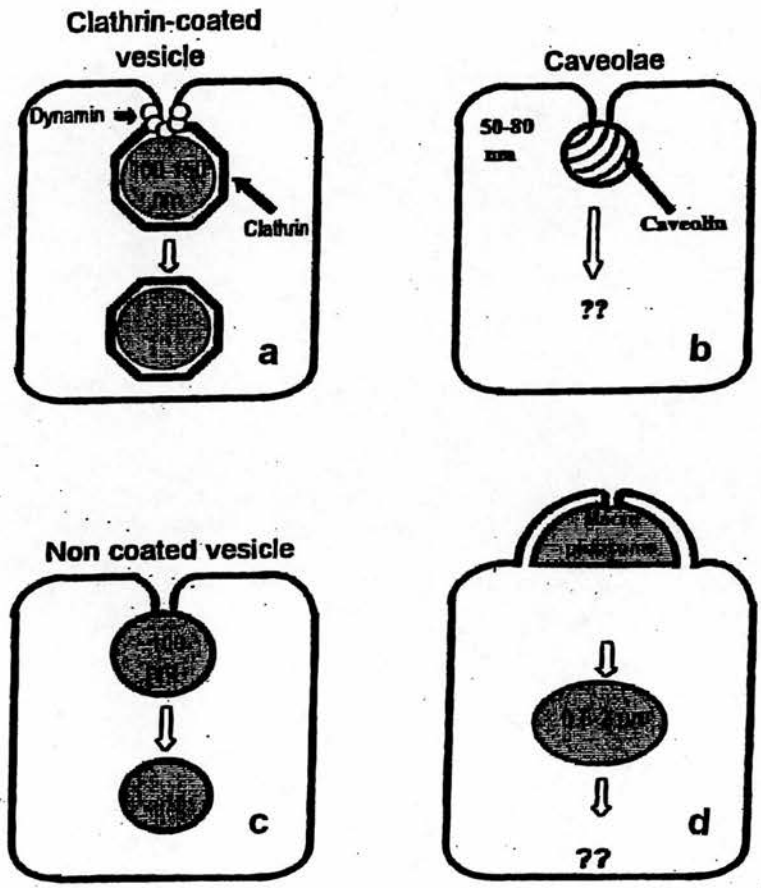

Figure 2. 3: Multiple endocytic pathways in mammalian cells (a) clathrindependent pinocytosis (b) caveolae (c) non-coated vesicle (d) macropinocytosis.

However, failure to detect detached vesicles unambiguously derived from the plasma membrane invaginations or to identify ligands transported by them had led to uncertainty as to whether caveolae mediate classical endocytic events (87,88). Quick-freeze images of caveolae revealed a granular, spiralling coat structure not detectable by conventional electron microscopy (89). It has been proposed that caveolin/VIP21, a 21kDa integral phosphoprotein can immunolocalize caveolae and is a major structural constituent of this coat. In a recent study, however, treatment of cells with cholesterol oxidase caused the quantitative redistribution of caveolin to the Golgi complex without the appearance of caveolae at the cell surface (90). A structural role for caveolin remains to be firmly established.

3. Non-coated vesicles

It is apparent that not all 'non-coated' invaginations observed on the plasma membrane by conventional microscopy are caveolae (88). Non-coated invaginations that pinch off from smooth vesicle carrying the fluid-phase marker horseradish peroxidase (HRP) into cells have been extensively characterised in a number of cell types (91,92). These non-coated vesicles are on average larger than individual caveolae and slightly smaller than coated vesicles (Figure 2.3c).

The presence or absence of the caveolar marker caveolin will help to distinguish unambiguously these two classes of non-coated vesicles.

4. Macropinocytosis

Macropinosomes are non-clathrin-coated vesicles, mostly between 0.5-2.5 μm in diameter, formed preferentially at the leading edge of the cell and upregulated in response to growth factors and phorbol esters (93). It has been reported that a significant number of macropinosomes are several microns in diameter (94). The formation of macropinosomes is accompanied by, but not necessarily dependent on, membrane ruffling (Figure 2.3d). Whether their formation occurs constitutively or transiently is not totally clear. The uptake of polystyrene microparticles has been suggested as a process of macropinocytosis and in a recent published study it has been proposed that macropinocytosis and phagocytosis are functionally identical processes (95).

The uptake of microparticles across the gastrointestinal tract mucosa is an important phenomenon that may have pathological and immunological implications. We ingest thousands of microparticles as part of our diet or as a result of environmental contamination. The potential problems that can occur as a result of uptake of a small percentage of these ingested microparticles are not known. The presence of non-degradable microparticles e.g., titanium in the intestinal milieu has attracted scientific interest and debate about a possible

relationship between ingested inorganic microparticles and perpetuation of inflammation in inflammatory bowel disease (17,96).

Gut luminal bacteria and lipopolysaccharide

The human intestine contains a complex and dynamic microbial ecosystem and at any given time, a specific region of the large intestine may contain > 400 bacterial species (97). Various bacterial products may be harmful to the host and bacterial endotoxin has attracted much attention by the scientific community for its role in the intestine. Bacterial endotoxins are distinguishable from exotoxins by the following characteristics: (i) endotoxins are an integral part of the outer layer of the bacterial cell wall; (ii) they are heat stable; (iii) they are toxic but much less potent and less specific in their cytotoxic effect than bacterial exotoxin; (iv) they are not convertible to toxoids; and (v) they are not rendered non-toxic when combined with homologous antibody.

Pfeiffer [1892] first suggested that part of the bacterial structure could be harmful to the host when released by disintegration of the cell and he called it endotoxin (98). It was subsequently evident that endotoxins can be released from intact bacteria without noticeable structural damage to the cell membrane. Chemically, endotoxins consist of lipopolysaccharides (LPS) which are long-chain phosphate containing heteropolymers with three distinct regions [Figure 2.4].

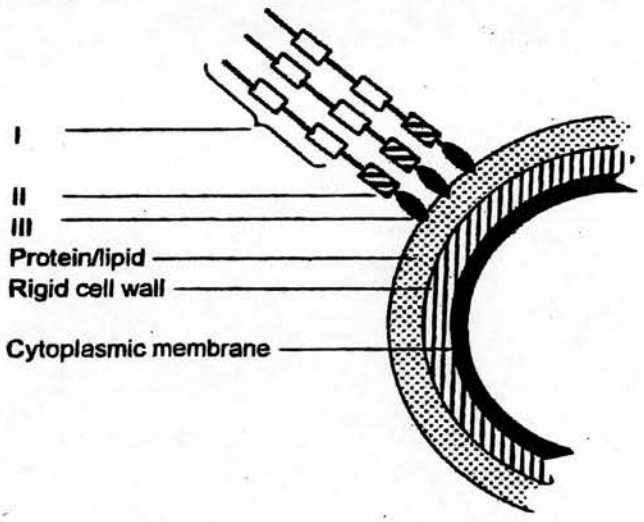

Figure 2. 4: Schematic diagram of a gram-negative bacterial cell wall. Region I - O- specific units of oligosaccharides. Region II - core structure common to a single genus. Region III - lipid component closely associated with the cell bound protein.

Region 1 consists of repeating units of oligosaccharides; particular sugar units, especially the terminal ones, confer immunological specificity on the respective O-antigens.

Region 2 is the core structure that is specific to a gram-negative bacterium but varies among different genera.

Region 3 is the component that links the lipopolysaccharide macromolecule to cell-bound lipoprotein. The endotoxic properties of the lipopolysaccharide are associated with this region which consists of two components: A and B. These lipid components are separable and lipid B can be easily detached and is biologically inactive. Lipid component A consists of chains of glucosamine disaccharide units connected by pyrophosphate bridges attached to a number of long-chains fatty acids. β-hydroxymyristic acid (a C_{14} fatty acid) is always present and is unique to this lipid structure. There is variation in the other fatty acid constituents in different bacterial species.

The lipopolysaccharide macromolecule can be broken down by phenol extraction that separates the lipopolysaccharide moiety from the protein and most of the phospholipid fraction.

Macrophages in the human intestinal wall

The gastrointestinal tract contains the highest number of resident macrophages compared to any other organ in the human body (99). These macrophages are predominantly located beneath the epithelial layer within the lamina propria in the

human intestine. Human intestinal macrophages express a range of markers including CD68 (100-102). Granules present within the lamina propria macrophages express epithelial cell-associated antigens such as cytokeratin and carcinoembryonic antigen; this suggests that they may play a role in the removal of apoptotic epithelial cells and mucosal epithelial renewal.

Regarding the developmental origin of these phagocytic cells, it is now established that tissue macrophages are haemopoietic in origin i.e., they are derived from pluripotent stem cell precursors formed in the bone marrow. Under the influence of growth factors, pluripotent stem cells divide and differentiate into committed progenitor cells, which then further differentiate along restricted cell lines [Figure 2.5]. The monoblast is the first cell that may be recognised as a monocyte precursor because of its non-specific esterase content and the presence of lysoszyme. These cells have IgG receptors and the capacity to phagocytose. The monoblast develops into the promonocyte, which is the direct precursor of the monocyte. Newly formed monocytes remain within the bone marrow for a brief period (~24h) before entering into the circulation. In the circulation, the half-life of monocytes is 1-3 days depending on the host animal species.

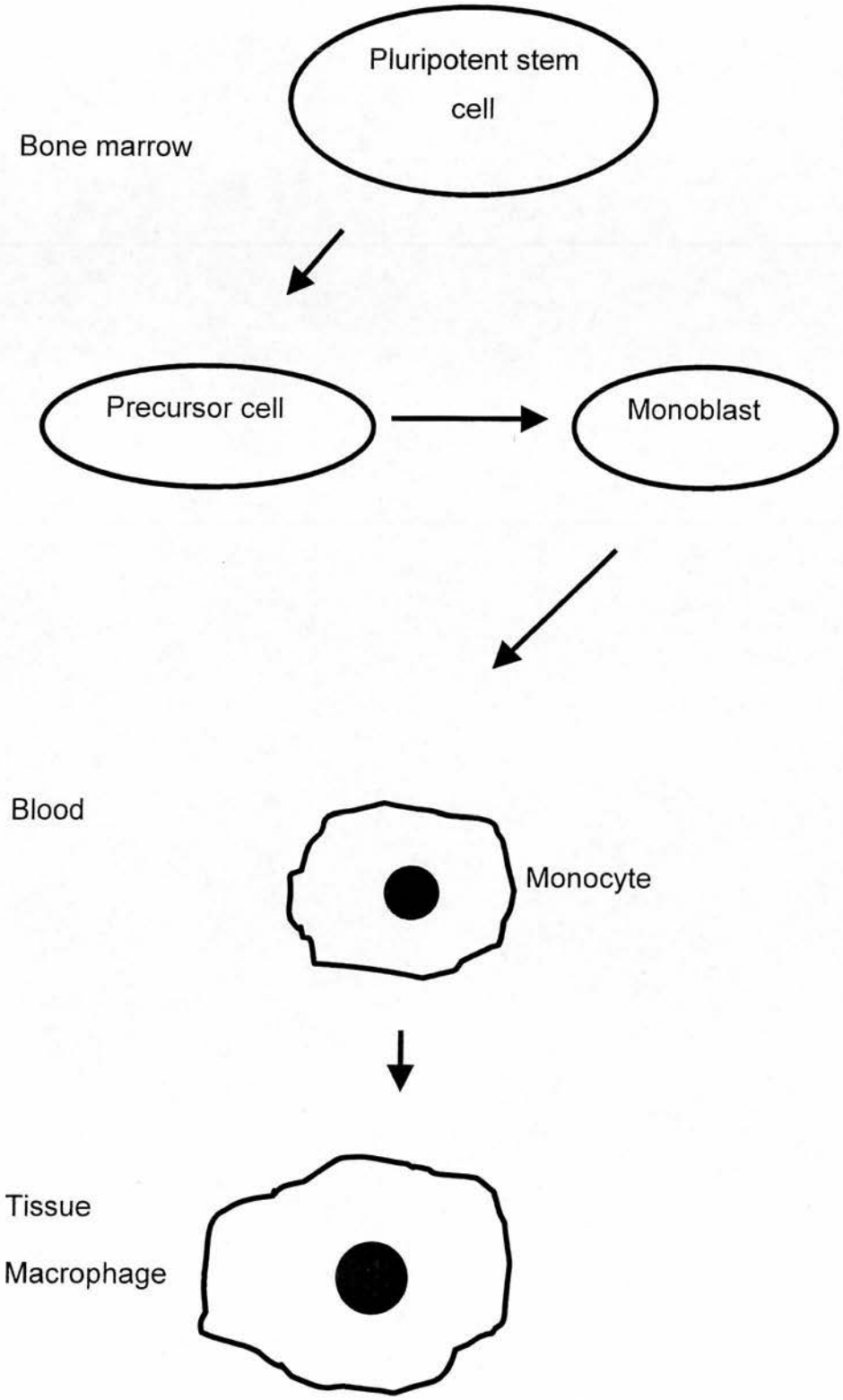

Figure 2. 5: Origin and differentiation of macrophages at different tissue levels

When monocytes migrate into the tissues, they differentiate into resident macrophages and may be within the tissues for days or months. The process involved from differentiation to maturation and becoming "resident macrophages" is not totally clear. Once within the tissue, the exact turnover time of tissue macrophages is also unknown; it probably depends on the location of the tissues, the microenvironment, and local inflammation or inflammatory process. Under the influence of cytokines or lipopolysaccharides, localised macrophages may be activated or stimulated so that they are morphologically, functionally and metabolically changed to their resident state. The morphological change probably increases the capacity to enter tissues, phagocytose and produce cytokines including TNF α, IL-1, IL-6, and IL-8.

The half-life of peritoneal macrophages has been estimated to be approximately 2 weeks. Macrophages may persist in non-inflamed skin for many months, but the ultimate fate of resident and activated macrophages is totally unknown. It has been observed that after scavenging or phagocytosis, macrophages do not die locally; instead they migrate from tissues to regional lymph nodes in an irregular fashion (103). The fate of macrophages when they have reached the regional lymph nodes is also uncertain.

In the lamina propria of the large and small intestine macrophages are able to present antigens (44).

Fox *et al* (1989) have detected viral RNA in macrophages in the lamina propria but not in the epithelial cells in human rectum (104). Subsequently Fiocchi *et al.* [1990] infected isolated human mononuclear cells with HIV *in vitro* but failed to infect epithelial cells (105). It seems logical to accept that entry of any luminal agent into intestinal epithelial cells, which are constantly shedding, may not produce a significant impact on the mucosal pathogenesis of any disease. On the other hand, luminal bacteria may persist in the macrophages of the human intestine for unknown periods. Similarly, fluorescent microparticles (>1 μm) that were detected in macrophages 7 days after peroral administration indicate retention properties of these phagocytes (65).

Gastrointestinal mucosa and cytokines

Intestinal epithelial cells are in constant equilibrium between proliferation and loss under physiological conditions. Stem cells in the basal crypt possess a high mitotic capacity and their daughter cells differentiate into mature cell types such as enterocytes, goblet or M cells over lymphoid follicles along the crypt-villus axis. Once at the villus tip, these highly differentiated post-mitotic cells die via apoptosis and are extruded into the lumen (106-108). Thus, maintenance of the physiological function of intestinal cells depends on a delicate balance with appropriate proliferation as well as loss. The physiological factors that regulate crypt cell proliferation, differentiation and finally apoptosis are unknown.

However, it is widely believed that a variety of growth factors, cytokines, hormones, luminal nutrients and mesenchymal structures are involved in this regulatory process. It has been demonstrated in vitro that immature crypt cell proliferation is stimulated by factors such as TGF-α (109-111) and TNF-α (112), whereas TGF-β (113,114) and IFN- γ (115,116) inhibit mitotic activity.

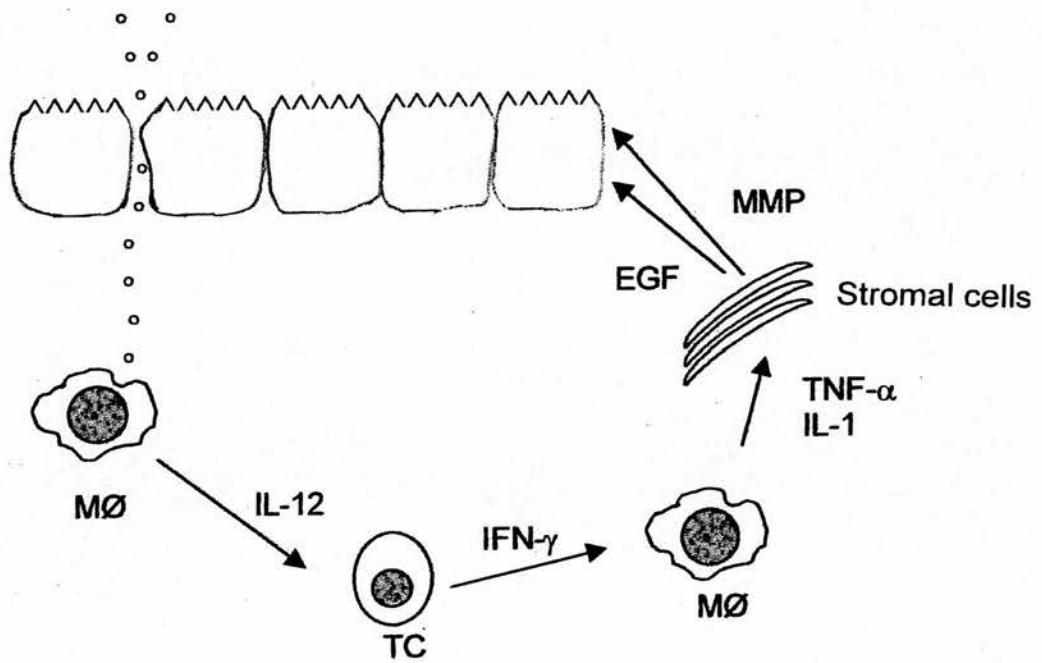

Figure 2. 6: A schematic model of immunopathogenesis of mucosal inflammation and involvement of cytokines. ° = antigen; MØ = macrophage;TC = T cell; IFN = interferon; IL= interleukin; KGF = keratinocyte growth factor; MMP = matrix metalloproteinase.

Studies have shown that intestinal epithelial cells produce and secrete several cytokines such as IL-6, IL-8, TNF-γ and TGF- β indicating the potential for autocrine and paracrine responses (117,118). In a variety of immune-mediated pathological conditions such as Crohn's disease and ulcerative colitis there is a dysregulation in the production of cytokines and this is believed to induce morphological changes in the intestinal mucosa and functional derangement (119,120). It is established that in the inflamed intestine of Crohn's disease and ulcerative colitis there is an increase in the number of inflammatory cells. It is also now becoming increasingly evident that because of increased epithelial cell turnover and a high rate of apoptosis, cytokines released locally by infiltrating inflammatory cells are playing a significant role in the pathogenesis of inflammation in IBD [Figure 2.6].

Factors that could trigger the release of cytokines in the intestinal mucosa are unknown. The human gastrointestinal tract is host to many millions of microbes including many hundreds of bacterial species and it also contains microparticles that might be involved in the liberation of proinflammatory cytokines (121).

From a substantial number of studies it appears that among the inflammatory cells the number of macrophages are increased in Crohn's disease (122-126). It has been suggested that bacterial endotoxin may activate macrophages and

other reticuloendothelial cells to elaborate proinflammatory cytokines, which, in turn, mediate the host responses (127).

Mucosal inflammation and microparticles

Background

The earliest histological features of mucosal inflammation are hyperaemia, oedema, and infiltration of inflammatory cells such as polymorphonuclear cells (PMNs), macrophages, and lymphocytes. These common pathologic manifestations in inflammatory bowel diseases (IBD) are accompanied by an imbalance in mucosal cytokine homeostasis. There is an up-regulation of proinflammatory cytokines and down regulation of antiinflammatory cytokines. Mucosal cytokines are in a delicate balance to maintain the epithelial cell homeostasis. The triggering factor that imbalances pro- and antiinflammatory cytokines in the intestinal mucosa is not totally clear. However, it is widely believed that this imbalance in cytokine homeostasis is probably a secondary event in mucosal inflammation.

Inflammatory bowel disease

Inflammatory bowel disease is a chronic relapsing and remitting inflammation of the intestinal tract. Although there has been progress in defining the pathogenesis of these diseases, until now the aetiology of IBD remains obscure (128).

Crohn's disease was first documented by Morgagni in 1761 and was followed by reports early in the 19th century by Combe and Saunders. In 1913, a Scottish surgeon Dalziel described a number of cases. Crohn, Ginzburgh and Oppenheimer from Mount Sinai Hospital in New York in their classic paper emphasised this inflammatory disorder as a distinct clinical and pathologic entity (18). Similarly, ulcerative colitis was recognised as an entity distinct from bacillary dysentery by Samuel Wilks of London in 1859 (129).

Crohn's disease and ulcerative colitis have similarities and some differences in their presentation. Some features of these two diseases are so similar that in ~ 10% of cases it is difficult to distinguish them and to make a diagnosis (130). These similarities raise the possibility of a different expression of one disease.

Epidemiological studies to identify risk factors relating to IBD have observed an association with early age, sex, smoking, ethnic population and geographical

location, social class, life style, food and dietary habits. It is clear that environmental and genetic factors are involved in developing IBD.

In this section I shall focus on the environmental and genetic factors that have attracted most attention.

Environmental factors and IBD

Mycobacteria, measles virus and Crohn's disease

Crohn's disease can involve any part of the gastrointestinal tract but most commonly involves the terminal ileum. Considering granuloma as a pathognomonic feature for Crohn's disease, a bacterial aetiology was considered most probable in the pathogenesis of Crohn's disease. Caseating granuloma is a typical feature of tuberculous infection, but the typically non-caseating granuloma of Crohn's disease raised the possibility of an atypical form of tuberculous infection produced perhaps by *Mycobacterium paratuberculosis*(131-134). The recovery of an unclassified acid-fast microorganism related to *M. paratuberculosis* from Crohn's tissue was an exciting finding (131,135). Neonatal goats fed such an organism developed granulomatous inflammation in the terminal ileum and there was evidence of both cellular and humoral immunity against the microorganism (136). However, subsequent results of similar

experiments were inconsistent. Also, there was insignificant elevation of antibody titres to mycobacterial antigens (132,133). Similarly, equivocal results of anti-tubercular therapy in controls made it difficult to accept beyond doubt the role of Mycobacterium species in the aetiology of Crohn's disease (137). In addition, the results of sophisticated technology such as the polymerase chain reaction (PCR) have been inconsistent in detecting mycobacterial DNA in intestinal tissue affected by Crohn's disease (134,138,139). However, the homology between an immunodominant mycobacterial antigen and human heat-shock protein (HSP60) present in Crohn's disease tissue warrants further scientific work (140).

Similarly, the possible aetiological role of infection by measles virus has drawn great attention in the last decade. A Swedish study suggested that perinatal or in utero exposure to measles might be related to the increased incidence of Crohn's disease (141). In a longitudinal study it has also been shown that measles vaccinated children are more likely to develop Crohn's disease (142). Detection of paramyxovirus-like particles by transmission electron microscopy suggested the presence of measles virus in Crohn's tissues (143). However, no viral genome has yet been extracted from any Crohn's disease tissue (144).

Bacterial aetiology of ulcerative colitis

Since the description of ulcerative colitis in the early nineteenth century, debate continues on the aetiology of ulcerative colitis (145). The introduction of tinned foods and the use of preservatives have not been ruled out as a possible cause of the disease. In the early nineteenth century, ulcerative colitis was believed by some investigators to be an atypical form of dysentery and some investigators also considered the disease to be psychosomatic. No claim for the presence of any specific microorganism in the affected tissue was substantiated. As the normal colon harbours so many bacteria, it has been postulated that a commonly occurring bacterium in the intestinal lumen is probably playing a key role in the initiation of the disease. Of these microorganisms, Escherichia coli is considered to be a prime candidate because of its continued mutations and probably releasing some products that might damage the mucosa (146). Strains of E.coli were isolated from ulcerative colitis patients which was different from those isolated from patients with normal colon but this was not universally accepted as significant because of the isolation from established cases after relapse which can not explain the primary event of disease (147). However, it has been suggested that any mutant strain may damage the gastrointestinal mucosa by liberating certain enzymes or products yet to be identified. The increased expression of adhesins by colonic mucosa in ulcerative colitis patients strengthens the view that E.coli in colitis patients probably may have a tendency

to adhere more to the colonic epithelial cells compared to normal and that may initiate the mucosal damage (148). The results of a clinical trial of tobramycin, an effective drug against E.coli, support this view (149). Our clinical knowledge has advanced on ulcerative colitis since Sir Arthur Hurst first made a complete description of the disease differentiating it from bacillary dysentery (129). However, the chronic nature of the disease remains unexplained. An infective aetiology responsible for the initiation of inflammatory bowel disease has not been excluded and the search continues (150).

Genetics of Inflammatory Bowel Disease

It is widely believed that genetic factors are involved in the pathogenesis of inflammatory bowel disease. In the following paragraphs, studies related to genetic predisposition in Crohn's disease and ulcerative colitis are discussed.

Genetics of Crohn's disease

Several lines of investigation indicate that genetic factors are involved in the pathogenesis of IBD. A higher prevalence of Crohn's disease among Ashkenazi Jews of middle European origin compared to other parts of the world supports this view (151). Family studies have documented a higher incidence of Crohn's disease among relatives of patients with inflammatory bowel disease (152,153). With one exception (154) most studies have ruled out Crohn's disease as X-

linked (155). A Swedish twin study has found that ~40% unselected monozygotic twins were concordant for Crohn's disease compared with ~6% monozygotic twins in ulcerative colitis (156). A recent Scottish study has shown a 50% concordance for Crohn's disease between monozygotic twins, 3% for dizygotic twins and 2% for other siblings (157). It therefore appears that Crohn's disease has a greater genetic element than ulcerative colitis, but the concordance rate even among monozygotic twins is less than 100% which cannot be consistent with a Mendelian autosomal recessive mode of inheritance.

Inherited chromosomal fragility found in Crohn's disease patients compared to normal also indicates a genetic involvement of the disease. Chromosome 6 and the major histocompatibility complex have been considered as the locus of a susceptibility gene by segregation analysis (158). In studies based on segregation analysis and familial aggregation of Crohn's disease, Monsen and co-investigators concluded that Crohn's disease is an oligogenically inherited disease (152).

Experimental models of inflammatory bowel disease point to an unregulated immune response to unidentified luminal antigens in inducing chronic inflammation in genetically susceptible subjects. Microbial antigens are likely to trigger the unregulated immune response and there could be a critical link between environmental factors and the genetic make-up of an individual in the pathogenesis of Crohn's disease. Therefore it has been suggested that luminal

factors such as bacteria or their products must play a role in the clinical expression of Crohn's disease.

Genetics of Ulcerative colitis

For many years ulcerative colitis was believed to be a familial disease but there is no absolute evidence in support of this theory. However, there is general agreement that a familial association exists within first-degree relatives. It has also been found that affected family members may have either ulcerative colitis or Crohn's disease (159,160).

Genetic anticipation: Children in affected families develop disease at an earlier age compared to their affected parents and this has been observed in IBD. This age onset supports a high degree of concordance among affected siblings, and this is also true for the site of inflammation and the type of clinical presentation (161).

Genetic susceptibility is partly understood in ulcerative colitis (156). Early European studies documented increased frequency of HLA-A11 and HLA-A7 in UC and a decreased frequency of HLA-A9 in CD (162). Studies in Japanese and Jewish patients found an association between ulcerative colitis and the 1502 allele of human leukocyte antigen HLA - DR2 (163). This allele is almost non-existent among northern Europeans. Genotype studies can predict extent or

severity of disease in a defined population. This finding indicates a genetic diversity in IBD (164).

Recent studies have identified linkages to specific chromosomes. A French study observed a susceptibility locus on chromosome 16 (165), and linkage studies in Britain have produced evidence for susceptibility for both CD and UC on chromosomes 3, 7, and 12, suggesting that these are distinct disorders sharing some susceptibility genes (166). In a recently published article Hugot *et* al. [2001] and Ogura *et* al. [2001] have reported that mutations of the gene encoding NOD2 are strongly associated with the development of Crohn's disease (167,168). These findings will provide new insight into potential IBD genes that may be involved in pathogenesis of IBD.

However, the rapid increase in incidence of Crohn's disease during the last few decades cannot be explained by changes in genetic makeup of stable populations during the same period (169). Therefore, non-genetic i.e., environmental factors are presumably also involved in the pathogenesis of IBD.

Luminal factors and Inflammatory Bowel Disease

Investigations on the bacteria and bacterial products within the gastrointestinal tract indicate that these microorganisms can breach the intestinal mucosal barrier

and enter the intestinal epithelium (170). In animal studies it has been observed that microparticles can cross the epithelial barrier too (48). In the human intestine, non-digested microparticles have been observed (17). No single factor has yet been confirmed for the initiation process of inflammation in IBD. However, persistence of inflammation might be due to any of several factors either alone or in combination.

Lipopolysaccharide and mucosal inflammation

It is widely accepted that genetic susceptibility and environmental factors are involved in the pathogenesis of inflammatory bowel disease. Genetic knockout animal models do not develop overt manifestations of Crohn's disease and ulcerative colitis in a pathogen-free environment (171,172). It is therefore likely that bacteria and bacterial products such as LPS are playing a part (involved) in the pathogenesis of inflammatory bowel disease.

Studies have shown lipopolysaccharide (LPS) found within the intestinal lumen can recruit neutrophils as well as initiate an inflammatory process (173,174). In animal experiments it has been demonstrated that injection of bacterial cell wall products e.g., LPS into the wall of the intestine sets up a chronic inflammatory condition that resembles Crohn's disease. This of course, bypasses the barrier function of the intestinal epithelium, but it is interesting that luminal contents of

the intestine can both initiate and sustain inflammatory responses within the intestinal wall.

It has been reported that mammalian cells respond to LPS ((175)), a major component of the outer wall of the bacterial cell wall by activating Toll-like receptors (TLRs) ((176,177). In humans, a family of proteins homologous to Toll, TLRs have recently been described. So far nine human TLRs have been cloned (TLR1-TLR9) (178). TLR 4, a member of the family of TLRs, is a type I integral membrane protein that contains leucine-rich motifs on their extracellular portions and an intracellular signalling domain homologous to that of the IL-1 receptor (179). In response to LPS, TLR 4 initiates a cascade of serine/threonine kinase activity that eventually leads to the activation of NF-κB in macrophages/monocytes. NF-κB activates a variety of inflammatory mediators and cytokines including tumour necrosis factor-α (TNF-α) and interleukin12 (IL-12) which are involved in the inflammatory process of inflammatory bowel disease (180).

Intestinal microparticles- any role in intestinal inflammation?

The finding of inorganic microparticles such as titanium in the human intestine suggests an unwanted breach of the intestinal barrier that could initiate an unregulated inflammatory process in IBD (18,181). Shepherd (1987) first reported the presence of cells containing microparticles in sections of small

intestinal tissue (181). Similar cells were subsequently reported in sections of both small and large intestine (181,182). Detailed examination of the inorganic microparticles by confocal microscopy demonstrated their intracellular localisation and X-ray microanalysis confirmed that the microparticles consisted of compounds of titanium, silicon, and aluminium of <1μm in size. It is likely that these have been ingested as a contaminant or as a result of dietary contamination (183). Aluminium and silicon occur as silicates/allumino-silicates in soil throughout the world. Aluminosilicate as kaolinate and titanium oxide in its polymeric form as anatase are used in the food industry as preservatives. These inorganic microparticles are highly resistant to degradation. A report from the UK indicates that the average daily intake of anatase is 5.4 mg/person equivalent to 10^{12} microparticles/person/day (184). Most studies on microparticle-related injury have been done in lung tissues and in prosthetic implants and found to be granulogenic (185,186). Similar studies in intestinal tissues are scanty. It is important to know the effects that microparticles may produce in the human intestine. Inorganic microparticles may be inert in isolation but during passage through the intestinal tract may adsorb luminal contents such as lipopolysaccharide and this may alter the properties of these microparticles. At present we lack data on the number of microparticle-laden cells in the human intestinal wall and information on whether microparticles may have a role in intestinal inflammation is inadequate.

Summary

Most of the microparticle uptake studies have been done with the aim of delivery of a pharmaceutical agent across the gastrointestinal mucosa. No experimental study in humans on uptake and translocation of non-degradable microparticles from a possible pathologic point of view has been done. It may be of relevance, if non-degradable microparticles are taken up by human intestinal mucosa, to examine the cells involved in this process.

Non-degradable microparticles have been reported in the human intestine and macrophages retain these microparticles. Macrophages are definitely involved in the inflammatory process in IBD. No quantitative data on the involvement of microparticle-laden macrophages in inflamed intestine is available. It is therefore also important to study the extent of involvement of microparticle-laden macrophages in human intestine.

It is now established that other than providing nutritional benefit, a small fraction of intact protein crosses the intestinal barrier but the purpose of this transport is not known. Any protein or incomplete protein such as prions may be of significance in contracting the human form of 'mad cow disease'. It is now widely believed that per oral route of entry of prions is most likely in the pathogenesis of variant Creutzfeldt-Jacob disease (vCJD). Many studies on the pathogenesis of vCJD have been conducted in animals by injecting infectious materials either into

the brain or peritoneum. A large study has been completed in the UK with orally dosed calves. Similar oral challenge experiments have been done by other workers, but little research so far been focussed on the cellular site of entry of prions in the gut or the mechanisms of passage from the gut. It is necessary to produce evidence that uptake of prions occurs by human intestinal epithelium. Then it will be necessary to determine the subsequent handling of prions by the mucosal cells in the intestine.

CHAPTER 3: MATERIALS AND METHODS

Sections from surgically resected intestine were lightly stained with H & E and reviewed. Microparticles within the tissues were determined by confocal laser scanning microscopy, and microparticles in the subcellular structures were assessed by transmission electron microscopy. The microparticle-laden cells in the tissues were identified by CD68 antibody specific for tissue macrophages. Automated image analysis allowed microparticle-laden CD68+ cell areas to be measured, quantitated and compared in tissues from patients with Crohn's disease or ulcerative colitis and from control subjects.

Subjects

Biopsies were collected from adult human subjects for culture and experimentation with fluorescent polystyrene microparticles with or without lipopolysaccharide of *Escherichia coli* O55:B5.

Consent and Ethical approval

Before colonoscopy, either for diagnosis or cancer screening, informed consent was obtained from subjects to collect biopsies for this research. The Medicine and Oncology Subcommittee of the Lothian Ethics in Research Committee approved this study.

Endoscopic biopsies were collected, as stated, from subjects undergoing colonoscopy for the diagnosis of disease or cancer screening. For IBD biopsies, areas of the intestine had been assessed for the presence or absence of disease by an experienced colonoscopist on a scale of 0-3. The scale, 0 = normal, 1 = mild inflammation with loss of vascular pattern with granularity, 2 = severe inflammation with contact bleeding, 3 = severe disease with friability, ulcers or spontaneous bleeding (187). In this study biopsies from IBD patients were collected from subjects in grade 0 and 1.

The subjects in this study were control subjects and IBD patients. Control subjects were disease controls and comprised individuals in whom investigation had been undertaken for gastrointestinal symptoms and with normal intestinal histology. They comprised patients with irritable bowel syndrome, or patients who had colonoscopy for cancer screening. Diagnosis of Crohn's disease and ulcerative colitis was made according to the criteria set out by Binder in 1982

(188) and Lennard-Jones in 1989 (189). The histories of the patients were obtained prior to collection of biopsies and subjects were excluded if necessary.

Microparticle uptake study

The aim of this thesis was to examine any uptake of inert microparticles such as polystyrene latex microspheres by human intestinal tissues. This experimentation is not ethically permissible *in vivo* in humans. Therefore organ culture of human intestinal biopsies was chosen as a feasible way to conduct such an experimental work. Organ culture of gastrointestinal mucosa has been successfully applied to study the toxic effect of substances such as gliadin, deoxycholate or indole, which could not be done in vivo. Similarly organ culture of respiratory mucosa has been most widely used to study the uptake of environmental microparticles such as asbestos, polystyrene, diesel particles etc. which could not be done in vivo, but it is important to understand any pathogenic effect of these substances.

Organ culture - Culture of human colon biopsies

Organ culture has been defined as a "Tissue culture technique in which the structure and integrity of an organ can be maintained *in vitro* with a minimum outgrowth whilst preserving normal histology". Initially, researchers used flat

plasma clots as a solid base for growth of embryonic tissues. Thereafter, mature tissues were cultured using liquid medium in high oxygen concentration (190). Trowell (1959) pioneered the organ culture technique that could be used to culture adult animal tissue and his method formed the basis of all subsequent methods of organ culture (191). Browning and Trier (1969) successfully cultured adult human intestinal mucosal biopsies for 24 hours (192). Thereafter, almost all research work on the culture of gastrointestinal mucosa followed their method with minor modifications.

The basic principle of organ culture is to maintain an explant tissue at the interface between a fluid culture medium and a gas phase. Explant tissues are maintained on an iron mesh or filter membrane in a specially designed culture dish (organ culture dish, Becton Dickinson, UK) in an oxygen-rich environment at body temperature (37°C). The central well of the culture dish is filled with a culture medium so as to touch the under-surface of the explant tissue and a thin layer of the medium is drawn over the apical surface by capillary suction. The whole system is maintained at 37°C in an atmosphere of 95% O_2 and 5% CO_2. The most important point for a successful culture of explant tissues is the gas-fluid interface and optimum oxygen concentration. Hodges *et al* (1976) observed that supplementation of Weymouth's MB 752/1 medium with small amounts of ascorbic acid, ferrous sulphate and hydrocortisone enhanced the preservation, growth and development of foetal mouse tissue (193). By adding foetal calf serum, Defries *et al* (1977) were able to maintain adult mouse colon in organ

culture for several months (194). Following the same method, it was possible to maintain adult human colon mucosa for up to 14 days (195).

The technique of Senior *et al.* (195) was adapted for organ culture of colonoscopic biopsies. The basic medium used in the present study was as described by Hodges *et al* (1976). This comprised Weymouth's MB 752/1 medium (ICN, UK) supplemented with 10% foetal calf serum (ICN, UK), ascorbic acid 300 μg/ml (Sigma, UK), ferrous sulphate 0.45 μg/ml (Sigma, UK), L-glutamine 200 mM (Sigma, UK), penicillin 5000 i.u/ml (Sigma, UK), streptomycin 5000 μg/ml (Sigma, UK) and gentamicin 50 μg/ml (Sigma, UK) (196).

A second modification to the technique of Senior *et al* was the use of a sealed modular incubator chamber (Bilrups-Rothenburg Corporation, California, USA). This is a Plexiglas chamber fitted with trays that can hold up to 30 organ-culture dishes. Humidity was maintained by placing sterile water in a petri dish at the bottom of the tray and the entire chamber was gassed with a mixture of 95% O_2 and 5% CO_2 and sealed at a pressure of up to 2 atmospheres. It is specifically designed to allow rapid exchange of gases and the gas phase reaches an equilibrium within 20 seconds when flushed at 5 L/min. All biopsies were blinded after culture for 24 hours and sections were coded.

No organ culture model has been described for the study of uptake of polystyrene microparticles in human intestinal tissues. Therefore, initially it was

necessary to be acquainted with the methods of culturing human intestinal tissues, and then to adapt these for the present study. The first difficulty was that tissues grown on iron grids were not suitable to study the uptake of particles. The pore size of these iron grids ~1mm allowed the particles (2 μm) to fall through. Therefore, the first modification needed was the use of cellulose filter membranes in place of iron grids. Immediately after collection biopsies tend to curl-up. It was observed that this curling could be prevented if biopsies were placed on a flat sterile petri dish with a drop of medium. The surface tension of the medium flattened the biopsies on the petri dish and facilitated spreading on the filter membranes. After trying filters with different pore size it was observed that filter membranes of 0.45-μm pore size were most suitable to study the uptake of polystyrene microparticles. The tissues grew well on these and their viability was assessed by studying the morphology and uptake of the thymidine analogue bromodeoxyuridine by the cultured tissues. For particle uptake studies it was also necessary to study the integrity of the tissues by electron microscopy. In this part of the study Dr. Margaret McIntyre was consulted. The development of this original organ culture model for the study of the uptake of polystyrene particles in cultured human intestinal tissues took about 3 months to set up in a reproducible form.

Survival of tissues in organ culture

Various methods have been applied to assess the survival of explant tissues in organ culture. Good morphological preservation, steady-state protein synthesis and enzyme activities indicate survival of tissues. Morphology of the tissues is usually examined by light microscopy but examination of cultured tissues by transmission electron microscopy provides the opportunity to evaluate the integrity of the epithelium, activity of the goblet cells, and organisation of the subcellular structures. Enzymatic activities such as alkaline phosphatase and sucrase of the brush border are also used to assess the metabolic functions of the cells in organ culture. Incorporation of tritiated thymidine or thymidine analogue bromodeoxyuridine by the dividing cells is the most frequently used method to assess the viability of the tissues in organ culture and indicates the synthetic ability of the undifferentiated dividing cells .

Bromodeoxyuridine

Bromodeoxyuridine is a thymidine analogue and is incorporated as tritiated thymidine [^{3}H] in deoxyribonucleic acid (DNA) of the cells in the S-phase of cell division. Monoclonal antibody specific to bromodeoxyuridine enables the detection of cells that have been dividing.

Polystyrene microparticles

Fluorescent, plain (non-ionic) mono-disperse polystyrene microparticles (Polysciences, Warrington, USA), 2 μm in diameter were used in experiments on uptake. The microparticles have 2.5% solid content with a yellow-green excitation with emission minimum and maximum of 545 and 610 nm. The stock suspension of microparticles was diluted in sterile de-ionised water to obtain a concentration of 5.68×10^6 per 0.1 ml.

Quantitative analysis of microparticle uptake

Numerous methods have been applied to evaluate microparticle uptake and translocation in tissues. Of the various methods in detecting microparticles after oral or intestinal administration, two methods have been widely used in studies using polystyrene microparticles. Each method has its advantages and limitations.

1. **Quantitation of microparticles in macerated tissues:** the major strength of this technique is that data are gathered from a large mass of tissue. From the view of microencapsulated delivery of a drug or vaccine to a tissue, this may be more informative. A major weakness of this method is lack of information at the cellular level, which would be more relevant in studying the potential for pathological consequences. A particular problem in studies

of the uptake by gastrointestinal tissue is that it is impossible to ascertain, even after thorough washing, whether counted microparticles were located within the tissue or were associated with the mucosal exudate attached to the apical surface of the intestinal epithelial cells.

2. ***In situ* visualisation of microparticles and quantitation**: this method can be applied to various forms of microscopy to localise microparticles within a tissue. Of the various microscopical techniques, confocal laser scanning microscopy provides the facility of optical sectioning (≤1 μm), a convenient means of locating microparticles within frozen tissue. Confocal imaging of cryosections minimises the possibility of artefactual movement of microparticles during tissue preparation and provides unequivocal evidence of the location of microparticles within a thick (15 μm) tissue section. In addition, merging confocal images allows the relationship between microparticles and tissue site to be determined. Furthermore, associated epifluorescence microscopy provides a sensitive method for quantitating fluorescent microparticle uptake by optical counting of microparticles *in situ*.

3. **Electron microscopy (EM):** EM is used to determine cellular and subcellular location of microparticles. Ruska and Knoll first constructed the transmission electron microscope (TEM) in 1931 (197) and since then many modifications and improvements have been made to study subcellular structures in detail. The basic principle of an electron microscope is that a

high velocity homogeneous electron beam passed through an ultrathin tissue section producing an emergent beam of transmitted electrons that is then refracted by a system of lenses to form a magnified, two dimensional image of the specimen. The magnification, which can be increased to ~ 500 000 X, allows the examination of minute details of a structure in biology and reveals subcellular structures in tissues.

Most microparticle uptake studies have used fluorescent polystyrene microparticles with detection at tissue level by confocal microscopy and at cellular and subcellular level by electron microscopy (48,52).

Microparticle-laden macrophages

Paraffin-embedded sections from surgically resected intestine from patients with Crohn's disease or ulcerative colitis or from control subjects with distal bowel cancer were collected. An unaffected part of the cancerous intestine was used for the controls. Selected specimens were from ileum, ileocaecal valve and colon, and were macroscopically normal. Microscopically, tissues were mild to moderately inflamed in Crohn's disease and ulcerative colitis. Controls were free from polymorph infiltrates, significant lymphoid or plasma cell infiltrates, fibrosis, regenerative epithelium or crypt destruction.

Immunostaining of macrophages

Streptavidin/biotin-peroxidase method

Sections were deparaffinized and antigen was unmasked at high temperature by exposure to microwave at 800 W for 20 min. Tissue endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Then sections were incubated with 20% filtered normal rabbit serum in 0.05 M Tris buffered saline (TBS), pH 7.6 followed by incubation with a range of mouse monoclonal anti-human CD68 antibody (PG-MI, Dako, UK). Sections were then incubated in rabbit anti-mouse biotinylated horseradish peroxidase (HRP) antibody (IgG) at a dilution of 1:300. Then sections were incubated with streptavidin/biotin-peroxidase. Sections were rinsed and washed in TBS after each step. Highly chromogenic brown reaction products were produced by incubation with 3,3' - diaminobenzidine (0.05%) in 0.01% hydrogen peroxide in TBS. Sections were counterstained with 0.01% methyl green for detection of brown stained CD 68+ cells (tissue macrophages) for image analysis. Serial sections from surgically obtained tonsils were prepared as positive controls and sections stained without primary antibody were used as negative controls. In CD68 positive sections, cytoplasm appeared as dark brown and in negative sections cytoplasm was not stained.

Serial dilutions of CD68 antibody were tested from 1:10 to 1:320. The optimal antibody dilution was assessed by two observers who agreed that 1:40 gives uniform staining without background staining.

Streptavidin/biotin-alkaline phosphatase method

Serial sections were also immunostained by alkaline phosphatase method to enumerate CD68+ cells with inorganic microparticles by image analysis. In brief, after incubation with primary antibody (mouse monoclonal antihuman CD68, PG-MI, Dako, UK) at dilution 1:40, sections were then incubated with rabbit anti-mouse biotinylated alkaline phosphatase at a dilution of 1:300. This step was followed by incubation with streptavidin/biotin-alkaline phosphatase. After each incubation sections were rinsed and washed in TBS. Highly chromogenic bright red reaction products were produced by incubation with New substrate system (Dako, UK). Sections were counterstained with Harris haematoxylin which helps to detect blue stained nuclei and black microparticles on a pink-red background, and facilitates enumeration of CD68+ cells with or without microparticles by automated image analysis. Positive controls were surgically resected tonsils and negative controls were sections without primary antibody as before.

Image analysis of tissue macrophages

Qualitative descriptions of tissues routinely made by histologists have limitations for quantitative analysis. The measurement of microscopic images of tissues (histometry) facilitates the quantitative analysis of tissues and can be done in various ways:

1. Measurements could be made by reflecting an image from a microscope slide projector on a screen or on a paper and measurements can be made onto a measuring grid drawn on cardboard or on a transparent material.
2. The use of eyepiece graticules allows quantitative measurement of tissues. This method was most widely used, before the development of computerised image analysis.
3. ***Computerised image analysis:*** In recent years, this is increasingly used in research and is now the most convenient and accurate method for quantitative analysis of tissues. A specially designed television camera scans microscopic images and sends signals to a detector unit. The detector unit is a densitometer which classifies each image according to grey level and allows densitometric data to be determined as well as morphometric parameters. By pattern recognition it is possible to measure two parameters simultaneously. This pattern recognition capability allows the exclusion of background extraneous matter that would otherwise be a

source of error. The electrical profiles of the features are fed to a computer and analysed (198).

In this thesis, computerised image analysis (Leica, Germany) was used for the quantitative analysis of microparticle-laden macrophages in resected human intestine.

X-ray microanalysis

To determine the chemical composition of the microparticles in tissues, energy-dispersive analysis of X-rays (EDAX) was done in association with transmission electron microscopy.

EDAX is a modification of an electron microscopy technique and can be used to undertake a chemical analysis of the specimen. Basically, when a high-energy electron in the probe strikes a thin section interactions between the primary electrons of the probe and atoms in the specimen occur. Transmitted electrons may pass through the specimen unchanged, or they can be scattered through different angles. These transmitted and scattered electrons are used to produce images of the ultrastructural characteristic of the specimen.

Secondary electrons are also produced when electrons within the specimen are ejected from their atoms by collisions with a high-energy probe electron. These

secondary electrons have a low energy and can provide information on the composition of the surface of the specimen. Secondary electrons are utilised in scanning electron microscopy.

Atoms consist of the nucleus, which is surrounded by electrons circulating in orbits. Each orbit corresponds to an energy level of the electrons. The number of these orbits and their energy levels depend on the size of the atom. They are grouped in units called shells, each shell has a number of energy levels. Elements are characterised by their nuclear charge and the energy distribution of their electrons. The shells are noted by the letters K, L, M, etc. and heavier elements have large atoms with large numbers of electron orbits and shell units.

In the analytical mode, it is the characteristic X-rays, that are produced when an electron from the electron probe collides with, and removes from its orbit, an electron previously bound to an atom. The removed electrons are replaced by other electrons from higher shells, and in doing so a precise amount of energy in the form of a spectrum of X-ray is liberated. The spectrum is very characteristic for an element and provides information on the element involved in the interaction. The X-rays are collected and analysed in an energy dispersive X-ray analysis system. Different types of X-ray analysis can be performed by this technique. The most commonly used techniques are: (a) point analysis, a complete system to analyse all the elements present at one particular point which can be as small as 1 nm; (b) X-ray distribution analysis or mapping, for

determining distribution of up to sixteen elements; (c) X-ray line scans to determine variations in the amount of a single element in relation to their location over a specimen; and (d) quantitative analysis to determine the concentration of a particular element.

EDAX in combination with transmission electron microscope enables directly to image ultrastructural detail, elemental composition of selected particles. The spectrum of an element is compared against standard spectra. Spectra for every element in the periodic table are stored in a data base within the software used for the analysis. The test spectrum is compared with this data. Confirmation of the presence of specific elements is done by taking a background reading from a resin not containing metallic particles.

Analysis of inorganic microparticles in this thesis has been done in a transmission electron microscope (TEM Philips CM120, Eindhoven, Holland) and EDAX (EDAX DX4 Eindhoven, Holland) attached to it. Software that has been used to analyse the samples is EDAX bDX Biological TEM, version 1.53, Eindhoven, Holland).

Statistical analysis

The data were entered in a personal computer and analysed by SPSS PC+ program (SPSS PC+ Inc. Chicago, IL, USA). Graphs and bar charts were prepared by Microsoft Excel program (Microsoft Corporation, USA).

The mean of a series of observations from a single case was taken to represent the value for that observation and was included in the final analysis. Descriptive statistics included mean and standard error of the mean of the parameters in the analysis. Values from specific subgroups of patients were then compared using t-test. A probability (p) value of ≤ 0.05 was considered as significant.

Data relating to labelling indices of cells as assessed by uptake of bromodeoxyuridine (BrdU) was presented as mean and standard error of the mean, and was compared using t-test.

The data regarding immunostained macrophages, microparticle laden-macrophages and macrophage areas were analysed by t-test. The mean of observations from a case was included in the final analysis. Descriptive statistics included mean and standard error of the mean of the parameters in the analysis. A probability (p) value of ≤ 0.05 was considered as significant.

The value for uptake of fluorescent polystyrene microparticles (microparticle count/mm^2) were presented as mean and standard error of the mean and the comparison between groups was assessed by t-test. A probability (p) value of $\leq$ 0.05 was considered as significant. The mean of observations from a case was included in the final analysis.

CHAPTER 4-METHODS: ASSESSMENT OF SURVIVAL OF ORGAN CULTURE OF HUMAN COLON

Introduction

Organ culture of human intestinal mucosa was introduced by Browning and Trier in 1969 (192), and since then this technique has been widely applied to investigate structure and function of human intestinal mucosa in vitro. It has been observed that in this technique intestinal mucosa remains viable for 24 hours with good morphological preservation and steady-state protein synthesis in man and animals (199-202). The continuing synthesis of deoxyribonucleic acid (DNA) by the epithelial cells indicates their physiological state.

DNA is synthesised during the S - phase of cell division when doubling of the genome takes place. In 1951, Howard and Pelc studied cell division (203) and they observed that the S-phase is followed by a G2 or second gap phase during which biochemical events take place in preparation for cell division. G2 phase is followed by mitosis after which cells enter G1 or first gap phase during which cells proceed to further division or quit cell division. Cells that are not in the process of division are termed as G0 cells (204). G0 cells may proceed for differentiation or may re-enter the process of cell division.

Synthesis of DNA in a tissue can be studied using a variety of techniques, namely scoring of the mitotic index, or measuring the uptake of thymidine analogues such as tritiated thymidine (^{3}H-tdr) by autoradiography or scintillation counting, or by determining the DNA content of the cells by microdensitometry or flow cytometry. All of these methods have limitations and advantages. (205).

In early cell cycle studies, tritiated thymidine [^{3}H] was the most commonly used technique and was largely restricted to studies in animals. The thymidine analogue bromodeoxyuridine (5-Bromo-2-deoxyuridine, BrdU) is also incorporated as thymidine in the DNA of the dividing cells and is free of radioactivity. Development of a monoclonal antibody to bromodeoxyuridine by Gratzner has opened up a much simpler immunohistochemical technique to study cell kinetics (206). BrdU labelling has been found to be comparable to tritiated thymidine labelling both in vivo and in vitro (207). BrdU is highly specific, without false background positivity, and can be detected at very low levels. In the past, most of its use was in flow cytometry. *In vivo* labelling of dividing cells in man was reported by Khan *et al* (208) and *in vitro* labelling of dividing cells in human biopsies was reported by Risio *et al* (209).

Detection of the S-phase of cell division by bromodeoxyuridine has become a widely applied method for determining the viability of tissues in culture and cell proliferation studies. However, application of this method has been mostly in flow cytometry studies, though initial studies with bromodeoxyuridine were mainly

concerned with frozen tissues and used immunofluorescence microscopy (210). The BrdU technique of detecting cells in S-phase was advanced by Morstyn *et al* (1986) who worked with ethanol-fixed tissues (211). Suguhara *et al* developed an enzymatic pre-digestion method to expose the BrdU binding site in formalin-fixed tissues (212). It has been found that BrdU labelling, i.e., in the S-phase of cell division, is best achieved by fixing the tissues in Carnoy's fixative that preserves the nuclear structure as well as allowing paraffin wax processing (213).

Aims

The aims of the present study was to assess survival of the human colonic biopsies in short-term organ culture by evaluating incorporation of bromodeoxyuridine in the dividing crypt cells and to compare results with *in vivo* bromodeoxyuridine labelled cells in murine colon.

Materials and methods

Development of positive control - Mice

In developing the immunohistochemical method, CBF mice were used as a positive control. CBF mice housed in standard laboratory conditions were labelled by intraperitoneal injection of 50 μg/kg of bromodeoxyuridine (Becton-Dickinson Antibody Data Sheet) 12 hours before sacrifice. The large intestines

were removed and fixed in Carnoy's fixative for 12-16 hours followed by in 99% IMS for another 12 hours and processed in paraffin wax. After embedding, 5 μm sections were cut in a microtome (Leica, Germany) and plated on slides coated with poly-L-lysine (Sigma, UK).

Immunohistochemical detection of bromodeoxyuridine labelled cells in mice

For immunohistochemical staining, paraffin embedded sections were dehydrated by serial immersion in Histoclear, 2 changes for 5 minutes, followed by industrial methylated spirit for a further 10 minutes. Sections were then placed in 1 % hydrogen peroxide in methanol for 20 minutes to block tissue endogenous peroxidase activity. After washing in running tap water, sections were placed in 1N HCL acid at a temperature of 60°C for 8 minutes. Treatment with acid releases purines (adenine and guanidine) from the double helix DNA and exposes the aldehyde group of deoxyribose sugars which includes bound BrdU making it accessible to the BrdU antibody (Becton Dickinson). After rinsing in running tap water for 5 minutes, sections were blocked with a 1:4 dilution of normal rabbit serum in Tris buffered saline (pH 7.6) for 10 minutes. Thereafter the slides were drained off and a range of dilutions of anti-BrdU antibody (monoclonal) was applied for 60 minutes. The slides were then rinsed in a stream of TBS and washed in a rocker for 5 minutes. The sections were then incubated with biotinylated rabbit anti-mouse immunoglobulin in a dilution of 1:300 for 40

min. After washing as before, the sections were incubated in avidin/biotin-peroxidase complex (ABC, Dako, UK) in TBS for 30 minutes. Finally the sections were incubated in diaminobenzidine (DAB) solution at concentration of 0.5 mg/ml for 10 minutes. Sections were again rinsed in running tap water and counterstained in Harris haematoxylin for 5 minutes, followed by acid alcohol and lithium carbonate. After dehydrating through graded alcohol, clearing in Histoclear and xylene, sections were mounted in synthetic resin (DPX). In BrdU-positive sections, nuclei appear as dark brown and in negative sections appear as pale blue. Serial dilutions of anti BrdU antibody were tested from 1:25 to 1:800. Optimal antibody dilution was assessed subjectively by two observers who agreed that 1:200 gave optimal uniform staining without background staining.

Subjects

Biopsies from the sigmoid colon were taken from each subject with informed consent who were undergoing colonoscopy either for diagnosis or cancer screening in the fasting state. All patients were fasted from midnight as required for the purpose of colonoscopy. The Medicine and Oncology Subcommittee of the Lothian Ethics in Research Committee had approved this study.

Culture medium

The culture medium comprised Weymouth's MB752/1 medium (ICN, UK. Catalogue No. 252254), supplemented with 10 % foetal calf serum (ICN, UK. Catalogue No. 2910154), L-glutamine 200 mM (Sigma, UK. Catalogue No. G-7513), ascorbic acid 300 μg/ml (Sigma, UK. Catalogue No. A-4544), ferrous sulphate 0.45 μg/ml (Sigma, UK. Catalogue No.F-8633), penicillin 5000 i.u. /ml, streptomycin 5000 μg/ml (Sigma, UK. Catalogue No.P-4458), and gentamicin 50μg/ml (Sigma, UK, Catalogue No. G-1272).

Organ culture of human colon biopsies - protocol

Colonoscopic biopsies were taken between 9 and 11 am to exclude any theoretical effects of diurnal variation. Immediately after removal, biopsies were transported to the laboratory, which is close to the endoscopy suite. The transport was done in complete medium, pre-gassed with 95% O_2 and 5% CO_2 at 37 °C and the tissues were established in organ culture within 10 minutes of biopsy. In brief, after washing in warm medium, biopsies were oriented on Millipore filters (pore size 0.45 μm, diameter 25 μm, Millipore Corporation, Bedford, MA, USA. Catalogue No. HAWP 02500) with mucosal surface up under a dissecting microscope (Watson, Barnet, England) and the biopsy was completely flattened; (it usually tends to curl up after excision). The filter membrane was then placed on the rim of organ culture dishes (Falcon, Becton

Dickinson UK Ltd. Cat. # 3707) 5.5 cm in diameter with a central 2 cm well in which 1 ml of complete medium with BrdU was added. In our laboratory, it has been demonstrated by a series of experiments by Wilson *et al* (1990) that bromodeoxyuridine at a concentration of 100 μM gives optimal labelling of the dividing cells at 10 μl /ml of complete medium (196). The central well of the organ culture dishes is surrounded by an outer well in which 1 ml of 0.9% NaCl solution was added and covered. The dishes were then placed on a plastic tray in a Plexiglas modular chamber [Bilups-Rothenburg™, California, USA] and gassed with a mixture of 95% O_2 and 5% CO_2 for 5 minutes prior to sealing the outlet of the chamber until the pressure inside the chamber rose to 2 atmospheres. Then the chamber was placed in an incubator at 37°C for periods of up to 24 hours.

After 24 h, explant tissues were fixed in Carnoy's fixative for 12-16 hours followed by immersion in 99% IMS for another 12 hours and processed in paraffin wax.

To measure the labelling index and the position of the proliferative cells within the crypt, each biopsy was embedded on the edge so that subsequent tissue sections are cut at 90° to the horizontal plane of the crypts. All sections were cut on a standard staged microtome (Leica, Germany) at a thickness of 5 μm and placed on slides coated with poly-L-lysine (Sigma UK). The slides were coded and examined blinded.

Immunohistochemical staining of human explant tissues

Five-micron sections of the explant human colon tissues were stained by avidin/biotin-peroxidase (ABC kit, Dako, UK) technique as described above. In brief, sections were de-waxed, passed through Histoclear (2 x) and graded alcohol 99% IMS, 70% alcohol for 5 minutes in each step. Next, sections were incubated for 20 minutes in 1% hydrogen peroxide (H_2O_2) in methanol to block tissue peroxidase activity. After washing in Tris buffered saline (TBS) at pH 7.6, DNA (deoxyribonucleic acid) was denatured by immersing sections in 1N HCL at 60°C for eight minutes to expose bound bromodeoxyuridine for immunohistochemical staining. Tissue sections were successively incubated at room temperature in 20% normal rabbit serum in TBS (NRS: TBS) at a dilution 1:4 for 10 minutes, and in 1:200 mouse monoclonal anti-BrdU (Becton-Dickinson) for 60 minutes, then in 1:300 biotinylated rabbit anti-mouse immunoglobulin for 40 minutes, and finally in streptavidin/biotin-peroxidase complex for 30 minutes. After each step, sections were rinsed in TBS and finally sections were incubated in 0.05 M TBS containing 0.5 mg/ml diaminobenzidine for 10 minutes. Sections were counterstained with Harris haematoxylin, dehydrated through graded alcohol and cleared in Histoclear and were mounted in synthetic resin (DPX).

Immunohistochemically, BrdU-stained sections were systematically reviewed for crypt architecture, epithelial integrity, nuclear orientation, oedema and cell debris

in the lamina propria, any crypt necrosis and amorphous material surrounding the crypts.

Scoring of BrdU labelled cells

Four well-oriented tissue sections were examined from each biopsy and four longitudinally sectioned crypts from each section were scored for BrdU labelling under a light microscope at a magnification of x 400. Total numbers of cells, and the numbers and positions of BrdU positive cells of each hemicrypt were directly keyed to a microcomputer for further analysis. The labelling index (LI) for each crypt was determined by estimating the percentage of BrdU positive cells out of the total number of cells within that column. The total number of positive cells obtained for each four crypts was used to calculate the mean % LI for each individual biopsy. The control Mouse BrdU-labelled sections [Figure 4.1] were scored in a similar way.

Statistics

Mean labelling indexes were compared by Students' t-test using SPSSPC+ (SPSSPC+ Inc. Chicago, IL) and a minimum probability of significance value of $p \leq 0.05$.

Results

Biopsy specimens were obtained from four subjects with diagnosis of irritable bowel syndrome and one normal for cancer screening. The subjects were 1 male and 4 females with a median (range) age of 59 (35-74).

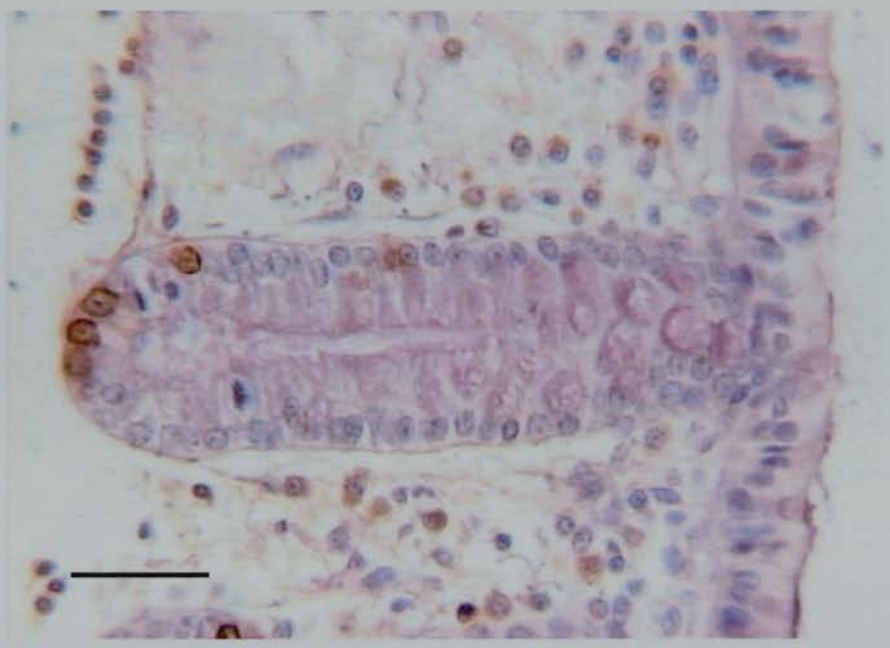

Figure 4.1. In vivo bromodeoxyuridine labelled (brown) murine colonic crypt cells. Scale bar = 20 μm

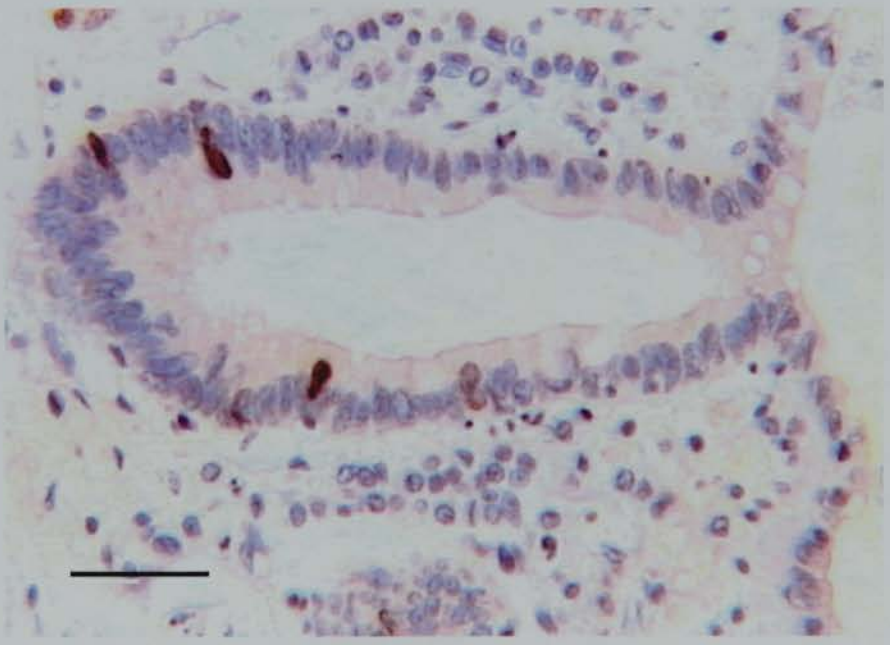

Figure 4.2. In vitro bromodeoxyuridine labelled (brown) human colonic crypt cells. Scale bar = 20 μm

Survival of the biopsy specimen was found in all specimens of cultured tissue examined for up to 24 h and was determined by retention of normal morphology of the tissues and the continued appearance of mitotic cells [Figure 4.2]. Crypt architecture was maintained well in human colon biopsies subjected to in vitro organ culture. Variable amounts of heterogeneous material, presumably consisting of mucus and cellular debris, were noted on the surface of the epithelium. Cells in the lamina propria appeared normal. Many mitoses were present in the crypts, even after culture for 24 hours and mostly were present within the lower two-thirds of the crypts.

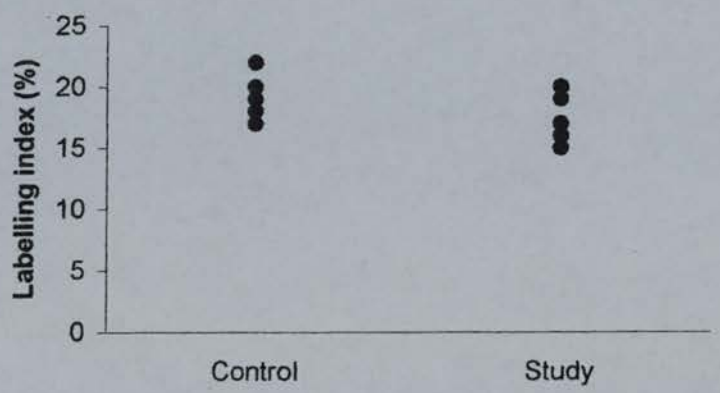

Figure 4. 3: Mean labelling indices (% LI) of colonic crypt cells.

Control = mouse; study = human.

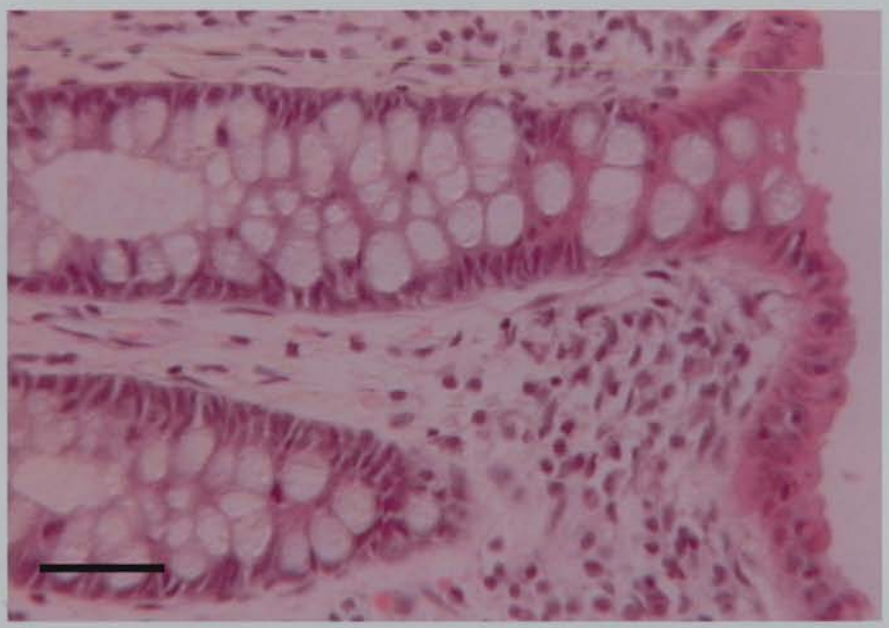

Figure 4.4. Light micrograph of human colonic mucosa. Counter stained with H & E. Scale bar = 20 μm

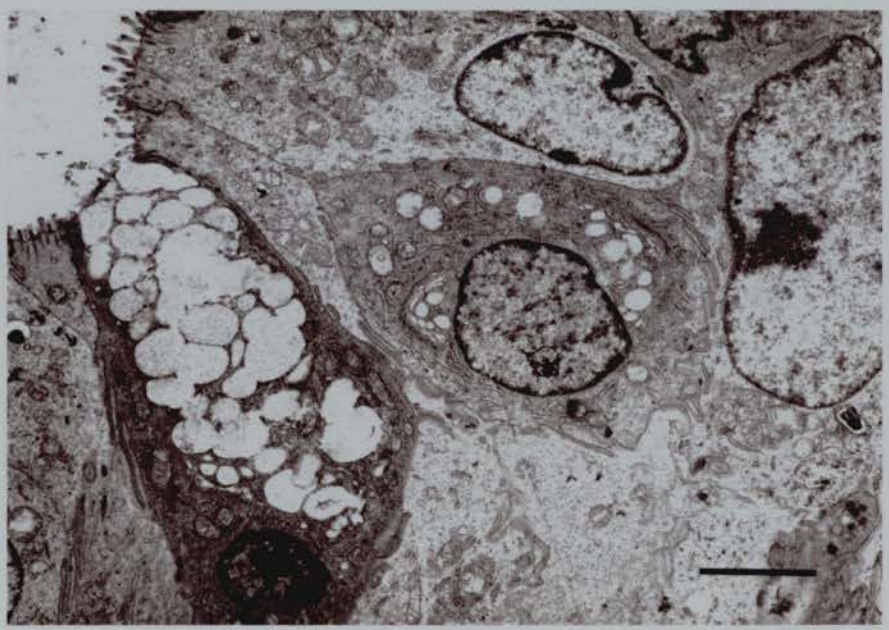

Figure 4.5. Electron micrograph of human colonic epithelium showing goblet cell with a nuclei and filled with mucus. Scale bar = 5 μm

The total number of BrdU-labelled cells in the subjects studied was 60 and the total number of cells was 316. In the mouse studies, BrdU-labelled cells were 78 and the total number of cells was 418. The percentage of labelled cells ranged from 2-5 per hemicrypt at 24 hours in cultured human mucosa and 3-5 in murine colon. The mean (SEM) labelling index (LI) in human biopsies was not significantly different from that found for the mice: 17 (0.8) vs. 18 (0.8), $p = 0.1$ [Figure 4.3].

Light micrograph (H & E stained) and electron micrograph of human colonic mucosa have been presented in figure 4.4 and figure 4.5.

Discussion

In this study, it appears that *in vitro* BrdU labelling of human colon crypt cells is analogous to *in vivo* labelling of mouse colon cells. In animal studies it has been documented that the lower two thirds of the colonic crypt is the principal site of cell division (214). In the upper third of the crypt, fully differentiated cells lose their capacity to proliferate and do not synthesize their DNA in normal conditions. This study confirms that observation. The values obtained for human colonic mucosa are similar to those reported using ^{3}H-Thymidine (215).

Most organ culture studies of human colon have been conducted to determine the proliferation of cells in colon cancer and few studies have been done to study the toxic effect of gliadin *in vitro*. Cole *et al* in their work to develop *in vivo* flash labelling methods in advanced colon cancer subjects have demonstrated uptake of 3H-thymidine by the cells at the base of the crypt and gradual progression of labelled cells towards the lumen (216).

Incorporation of BrdU into the nuclei of undifferentiated crypt cells provides evidence that epithelial cells were actively synthesising deoxyribonucleic acid. The presence of mitoses in crypts of biopsies continued *in vitro* for 24 h and migration of BrdU-labelled cells from crypt base to the lumen indicate epithelial cell proliferation and migration in this organ culture technique. The rate of migration of BrdU-labelled epithelial cells in vivo in mice and in cultured human colon epithelium *in vitro* was not significantly different. In both situations, cells have migrated from the crypt base.

The viability of tissue in organ culture for a prolonged period is dependent on detailed technique. In organ culture, the tissue sample is not immersed (as in tissue culture) but rather it is placed on the surface of the medium and the medium seeps over the specimen by capillary action. This allows better oxygenation of the tissue and survival is prolonged.

In the present study, human colon biopsies were successfully cultured for up to 24 h and the culture methods have been validated for subsequent studies in modified conditions.

CHAPTER 5: EFFECTS OF LIPOPOLYSACCHARIDE ON ORGAN CULTURE OF HUMAN COLON

Introduction

Lipopolysaccharides (LPS), also called endotoxin, are elements of the cell wall of gram-negative bacteria. Under physiological conditions, the gastrointestinal epithelium is considered to be a barrier to these toxins(217). The epithelial barrier is maintained by a delicate balance between proliferation of progenitor cells in the crypts and shedding of fully differentiated functional epithelial cells at the top of the villi or crypts (218). The functional cells of the epithelium are located at the top of the crypts and once they have fulfilled their differentiated function, the cells senesce and are shed off into the lumen as a programmed cell death or apoptosis (106). There is a continuous loss of epithelial cells, and in order to maintain epithelial integrity and sustain the epithelial barrier, the lost cells must be replaced at an equal rate. The epithelium of the gastrointestinal tract is one of the most rapidly renewing tissues and has a very large number of cycling cells. The proliferating cells are located in the bottom two-thirds of the crypts referred to as the proliferative zone. In the healthy state, the rate of cell production and loss is balanced under stringent conditions. Studies have shown that in physiological condition, the undifferentiated cells in the basal crypts have the capacity to proliferate but fully differentiated mature cells in the upper parts of the

crypts lose their capacity to proliferate (196,215,219). The physiological factors regulating proliferation of undifferentiated cells in the crypts are largely unknown. It is believed that a variety of growth factors, cytokines, hormones, and luminal nutrients are regulating factors (220,221).

The large intestine hosts a huge number of indigenous bacteria and is exposed to potentially pathogenic bacteria. Extensive studies have suggested that gram-negative bacteria may interact with the intestinal epithelium by attachment to membrane glycoproteins or glycolipid receptors and through this mechanism induce signal transduction to modify effector responses in epithelial cell structure and /or function for their own purposes (222).

Although endotoxin is a large macromolecule, it has been demonstrated that bacterial lipopolysaccharide can cross the intestinal epithelium and is capable of altering the function of the epithelium. *In vitro* studies have demonstrated that endotoxin alters the secretory properties of the intestinal epithelium. LPS is also a potent stimulator of mucosal cells and liberates proinflammatory cytokines and growth factors. It has been observed that LPS increases secretion of cytokines by activating NF- κB (223). Intestinal epithelial cell homeostasis depends on a delicate balance between pro- and anti-inflammatory cytokines produced in the mucosa. Endotoxin may not have an impact on terminally differentiated intestinal epithelial cells but it may have an effect on the progenitor cells residing in the

crypt. LPS has also been reported to increase proliferation of nasal epithelial cells in the rat (224).

Organ culture provides a good model to study proliferation of crypt cells and has been widely used to study tumourogenesis in the human colon. However, studies on the effect of endotoxin on human crypt cell proliferation are lacking. The aim of the present study was to determine whether endotoxin may have any effect on epithelial cell proliferation and morphology in organ culture of human colon.

Subjects and methods

Biopsies were obtained from 5 patients, M: F = 1: 4, with a median age of 58 (range 35-74) years. Patients had normal colonic mucosa at colonoscopy for non-inflammatory conditions such as irritable bowel syndrome or during colorectal cancer screening.

Biopsies, ~2 mm in size were collected from sigmoid colon with informed consent from each subject who underwent colonoscopy for diagnosis of abdominal complaints or for cancer screening. All patients were fasted from midnight as required for colonoscopy on the following morning.

Culture medium

The culture medium (100 ml) was Weymouth's MB752/1, 77 ml (ICN, UK. Catalogue No. 252254), supplemented with 10 ml of 10 % foetal calf serum (ICN, UK. Catalogue No. 2910154), L-glutamine 200 mM, 1.5 ml (Sigma, UK. Catalogue No. G-7513), ascorbic acid 300 μg/ml, 10 ml (Sigma, UK. Catalogue No. A-4544), ferrous sulphate 0.45 μg/ml, 20 μl (Sigma, UK. Catalogue No.F-8633), penicillin 5000 i.u /ml and streptomycin 5000 μg/ml, 1 ml (Sigma, UK. Catalogue No.P-4458), and gentamicin 50μg/ml, 0.5 ml (Sigma, UK. Catalogue No. G- 1272). To each ml of complete medium 10 μl of bromodeoxyuridine (conc. 200 μM) was added for labelling proliferating crypt cells (Sigma, UK. Catalogue No. B-5002).

Lipopolysaccharide-containing medium was constituted by the inclusion in complete medium of 50-μg/ml lipopolysaccharide of *Escherichia coli* 055:B5 (Sigma, UK. Catalogue No. L- 2880). The biopsies were cultured in parallel and under identical conditions (200) in the presence or absence of LPS.

Culture procedure

Immediately after removal, biopsies were transported to the laboratory which is close to the endoscopy suite. The transport was done in complete medium, pre-

gassed with 95% O_2 and 5% CO_2 at 37 °C and the tissues were established in organ culture within 10 minutes after biopsy. Biopsies were washed twice in warm media. Under the dissecting microscope, biopsies were oriented, luminal surface up, on Millipore filters (pore size 0.45 μm, diameter 25 μm, Millipore Corporation, Bedford, MA, USA. Catalogue No. HAWP 02500). Filters were placed on the rim of the organ culture dishes 5.5 cm in diameter with a central 2 cm well containing 1 ml of complete medium (Becton Dickinson UK Ltd.). The central well is surrounded by an outer well into which 1 ml of 0.9% NaCl solution was added. The dishes were covered, placed on a plastic tray and sealed in a Plexiglas modular chamber [Bilups-Rothenburg™, California, USA]. The modular chamber was gassed with a mixture of 95% oxygen and 5% carbon-dioxide for 5 min at 5L/min, then sealed and maintained at 37°C in an incubator.

Histology technique

After 24 h of culture, explant tissues were rinsed and fixed by submerging in Carnoy's fixative for 12-16 h followed by 99% industrial methylated sprit (IMS) for another 12-16 h. Explant tissues were then processed to paraffin wax. Sections of the entire width of the biopsy were then cut 5 μm thick with a microtome (LEICA 2045 Multicut, Nussloch, Germany) and placed onto 0.1% poly-L-lysine coated slides. The slides were coded.

Electron microscopy of explant colon biopsies

Biopsies were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (Sigma) overnight at 4°C. Tissues were then washed in cacodylate buffer for 30 min and post-fixed in 1% osmium tetroxide for 1 h in cacodylate buffer. Then tissues were dehydrated through grades of alcohol 10% alcohol (3 changes, 10 min per change), 50% alcohol (1 change 15 min), and 100% alcohol (3 changes 30 min per change). Tissues were then dehydrated through propylene oxide (2 changes 20 min per change) and embedded in Araldite resin and polymerised in an incubator at 56°C for 2-3 days. Thin sections (0.5-1 μm) were cut with a glass knife and dried on a hot plate. The dried sections were stained with toluidine blue in 1% borax for approximately 30 sec. Tissues processed for transmission electron microscopy were chosen after observation of the thin sections by light microscopy. Ultrathin sections were cut using a diamond knife, interference of colours determined the section thickness (60-80 nm). The sections were placed onto 300-mesh copper grids and allowed to dry for 30 min. and grids were stained with uranyl acetate and lead citrate. The grids were examined in a JEOL 100 CX II transmission electron microscope (Tokyo, Japan) and photographs were taken at 60 kV.

Immunohistochemical staining for BrdU

Sections were de-waxed and placed in 1% hydrogen peroxide (H_2O_2) in methanol for 20 min to block endogenous peroxidase. After washing in TRIS buffered saline (TBS), pH 7.6, DNA was denatured by immersion of the slides in 1 N HCL for 8 min at 60°C to expose bound BrdU for immunostaining with a streptavidin-biotin peroxidase technique. Tissue sections were incubated successively in 20% rabbit serum in TBS for 10 min, 1:200 mouse anti-BrdU (Becton-Dickinson, UK) for 60 min at room temperature, then 1:300 biotinylated rabbit anti-mouse for 40 minutes, and finally in streptavidin/biotin- peroxidase complex (ABC) for 30 min. After each step, sections were rinsed in TBS. Then sections were incubated in 0.05 M TRIS/HCL buffer (pH 7.6) containing 0.05% diaminobenzidine and 0.1% H_2O_2 for 10 min. Sections were counterstained with haematoxylin, dehydrated through graded alcohol, cleared in Histoclear, and mounted in synthetic resin. Sections were examined by light microscopy for morphology of the tissue including epithelial cell shape and nuclear orientation, oedema and crypt necrosis. The slides were coded and examined blinded.

Scoring of BrdU labelled cells

Four well-oriented tissue sections were examined from each biopsy and four longitudinally sectioned crypts from each section were scored for BrdU labelling under a light microscope at a magnification of x 400. Total numbers of cells, and

the numbers and positions of BrdU-positive cells of each crypt were directly keyed to a microcomputer for further analysis. The labelling index (LI) for each crypt was determined from the percentage of BrdU positive cells out of the total number of cells in that column. The total number of positive cells obtained for each four crypts was used to calculate mean % LI values for each individual biopsy.

Statistical analysis

Data were entered in a microcomputer and analysed in SPSSPC+ (SPSS PC+ Inc. Chicago, IL, USA). Continuous data were analysed by Mann-Whitney U - test using a minimum probability of significance value of $p \leq 0.05$. Discontinuous data were evaluated by Chi square analysis or Fisher's exact test.

Results

The histologic pictures of the colonic mucosa are shown after culture for 24 h in control media, figure 5.1 or media containing LPS, figure 5.2. After culture in control media, the morphology of the biopsy tissue was well preserved. The epithelium was intact, cells were columnar in shape with basically oriented nuclei. The crypts were regular with well -preserved cells, and in the upper part cells were numerous and compact with slender nuclei. At the lateral margins of

the cultured biopsy, areas of partial necrosis were noted. In these areas the epithelium was lower and had cut surfaces. These areas were omitted on histological judgement and morphometric analysis of biopsies. The biopsy surface was covered by translucent mucus. Many mitoses were present in the crypts, even after 24 h, as demonstrated by the incorporation of BrdU in the dividing cells. All biopsies were examined in pairs from the same subject.

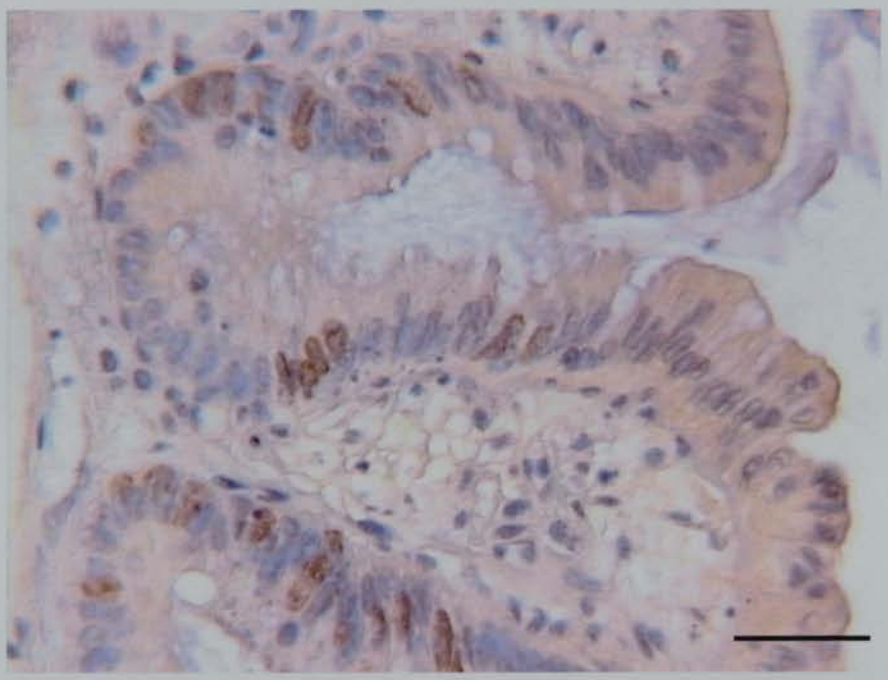

Figure 5.1. Micrograph of bromodeoxyuridine labelled human colonic crypt cells cultured in control media. Scale bar = 20 μm.

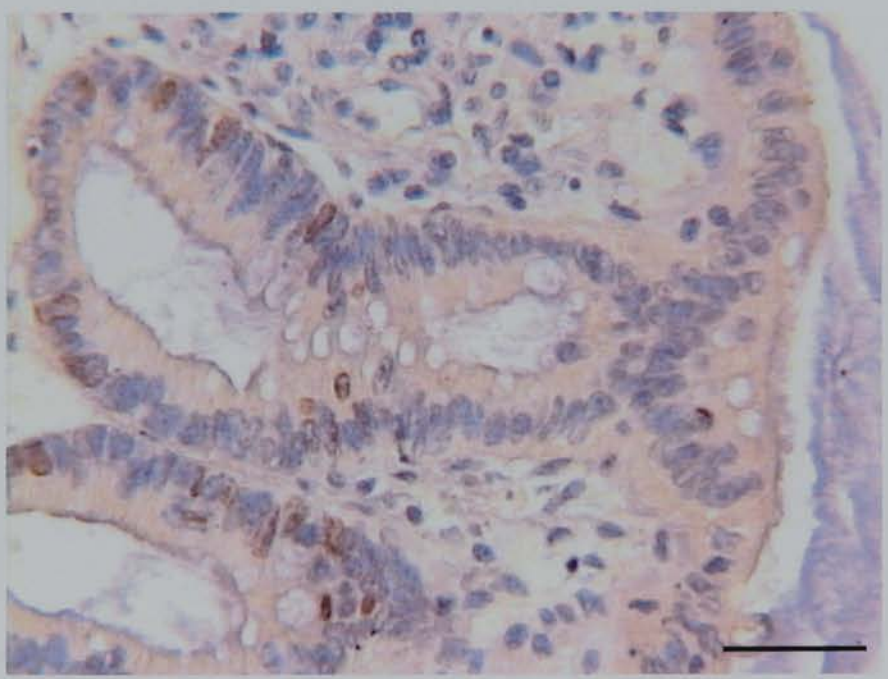

Figure 5.2. Micrograph of bromodeoxyuridine labelled human colonic crypt cells cultured in LPS containing media. Scale bar = 20 μm.

Histologic evaluation of the explant human colon biopsies from healthy subjects illustrated that the cultured colon epithelium was intact after 24 h of culture in complete medium (not incubated with LPS), with no apparent area of ulceration or breach of epithelium. Parallel biopsies incubated with 50 μg of LPS per ml in complete medium appeared indistinguishable from non-LPS treated cultured biopsies.

The numbers of BrdU labelled cells were 2-5 per hemicrypt and a total of 75 labelled cells in control biopsies. In LPS-treated tissues, BrdU-labelled cells ranged from 2-6 per hemicrypt and the total number of labelled cells was 73. Mean (SEM) labelling indices (LI) in biopsies were not significantly different between groups; 17(0.7) vs. 18(18), $p = 0.3$.

Transmission electron microscopy confirmed the impression gained by light microscopy. The morphology of the epithelium was well maintained during in vitro culture. Epithelial cells appeared to maintain integrity with neighbouring cells.

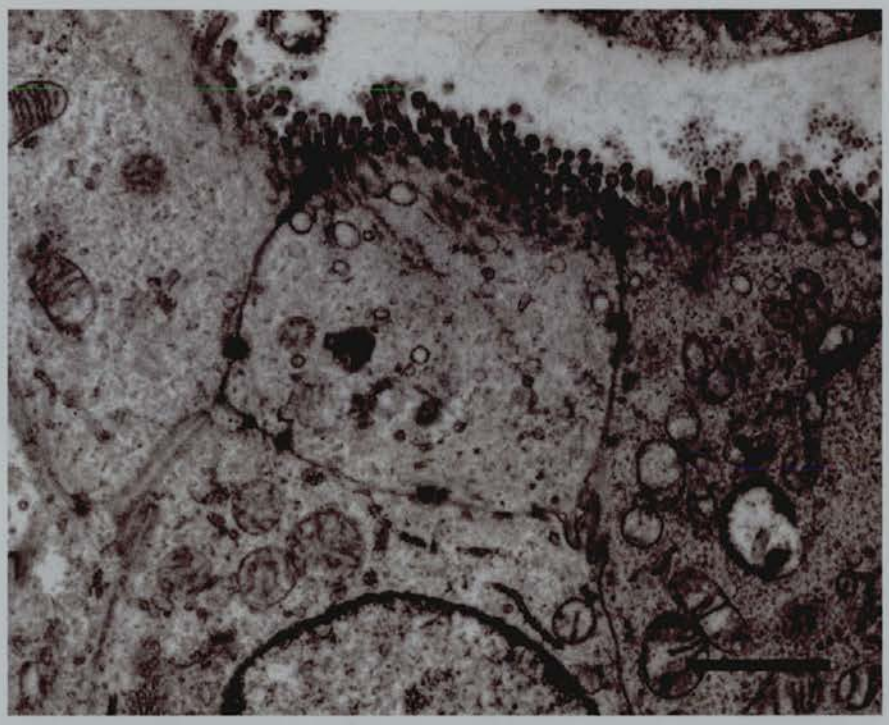

Figure 5.3. Electron micrograph showing human colon epithelium with intact tight junctions (cultured in LPS containing media). Scale bar = 2.25 μm

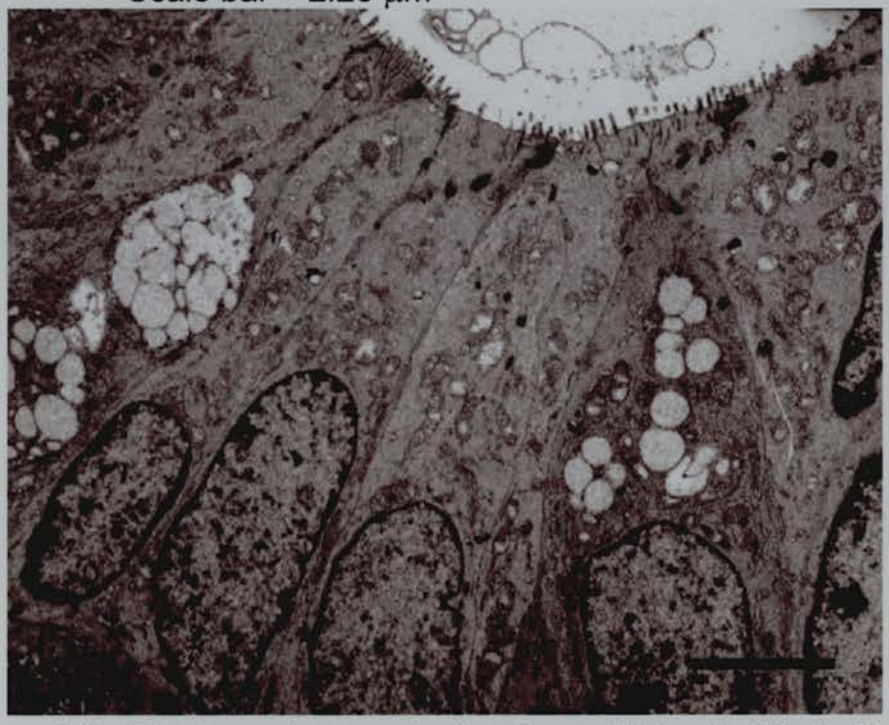

Figure 5.4. Electron micrograph showing goblet cells filled with mucus and organised with surrounding epithelial cells (cultured in LPS containing media). Scale bar = 7.5 μm

Colonic epithelium in LPS-treated biopsies appeared indistinguishable from control biopsies. Their cytoplasm was compact, and the cytoplasmic organelles, including mitochondria, elements of endoplasmic reticulum, Golgi material and lysosomes were normal in appearance and distribution. The apical surfaces and basal lamina were structurally intact as evident by intact tight junctions [Figure 5.3].

Goblet cells in the crypts were filled with large mucous granules and appeared normal morphologically when viewed with electron microscope [Figure 5.4].

Discussion

Good morphological preservation of the tissue with intact epithelium after 24 h of culture was observed in the explant human colon tissues in the present study. Similar findings have been reported in organ culture of intestinal biopsies in man and other species (199,201,202). It has been reported that after culture for 48 hours, tissue morphology is not so well preserved as cell proliferation and synthetic function deteriorates after 24 hours of culture.

In the present study, human colon biopsies were cultured for 24 h and morphology of the explant tissues has been well maintained as evident by light and electron microscopy. No morphological changes were observed in endotoxin-treated organ culture of human colon mucosa. This finding

corroborates the findings of other investigators on the effects of LPS on morphology of the intestinal epithelium.

There are very few reported studies on the effects of lipopolysaccharide on cultured human intestinal tissues. In organ cultures of rabbit jejunal biopsies, it has been observed that lipopolysaccharide at a concentration of 100 μg/ml affects the morphology of the epithelial cells in the intestine (237). Walker *et al.* reported that LPS at a higher concentration damages the integrity of the tissues and disrupts tight junctions of the epithelial cells causing paracellular leakage (238).

The stimulatory effect of lipopolysaccharide has been well established in animal models, particularly to study the secretory activity in relation to the transport of solutes and ions. LPS of *E.coli* at a concentration of 50μg/ml has been shown to induce secretion of cytokines e.g., IL-1 at detectable levels in cultured human intestinal tissues (225). At this dose the morphology of the tissues was not affected during treatment of the tissues with 50μg/ml of LPS. The dose of LPS in the present study was adopted from the above-mentioned experimental study. In the present study, it has also been observed that at a dose of 50 μg/ml of LPS of *E.coli* the morphology of the cultured human intestinal tissues was unaffected i.e., intercellular tight junctions remained intact, activity of goblet cells was preserved as evident by presence of mucus within the cells, and subcellular organelles remained intact.

CHAPTER 6: QUANTITATION OF INORGANIC MICROPARTICLE-LADEN MACROPHAGES IN THE HUMAN INTESTINE

Introduction

Non-degradable microparticles have been reported in the human intestinal wall and implicated as a probable cause of chronic inflammation in inflammatory bowel disease (17,182). The possible source of microparticles is considered to be dietary in food preservatives and in preparations used as adjuvant in pharmaceutical products. In a recent clinical trial, a microparticle-free diet has been found to improve the symptoms in Crohn's disease (96). It is now considered that environmental microparticles present in the intestine may have a role in the pathogenesis of IBD (18).

The aetiology of inflammatory bowel disease (IBD) typed as Crohn's disease and ulcerative colitis is unknown. Both environmental factors and genetic susceptibility have been implicated in the pathogenesis of IBD (226) but environmental factors that may initiate the development of IBD are unknown. Genetic knock out animal models deficient in IL-10 and IL-2 develop forms of enterocolitis, but not in germ-free environments (171,227). Accordingly, studies to find a possible microbial cause of IBD have been intensified over the last two decades. Mycobacteria have received special attention (131-135). The possible

role of measles virus has been vigorously postulated (141-143). To date, the evidence for the role of any specific pathogen in IBD is inconclusive. Accordingly, the search has been widened to discover any other agent that may initiate and /or perpetuate inflammation in IBD. In the early days of the description of IBD, it was considered that dietary non-degradable components might be responsible for inflammatory bowel disease (145), and it is widely held that environmental factors probably contribute to the process of inflammation in IBD, particularly in Crohn's disease (18).

In the inflamed intestine of Crohn's disease and ulcerative colitis there is an increase in the number of inflammatory cells, and the increased numbers of macrophages in Crohn's disease are well documented (122-126). An increase in the recruitment of peripheral blood monocytes in the intestinal mucosa has been reported in IBD (228,229) and it has been recently noted that newly recruited macrophages can be located in the gut mucosa even before Crohn's disease has fully developed (230). In chronic inflammatory bowel disease the macrophages are located sub-epithelially and are considered as resident macrophages (100,228,229,231).

Shepherd (1987) first described inorganic microparticles in the human intestine (181). Although analytical studies on the composition of the microparticles were extensive, the cells that contain the microparticles were relatively ignored (182). Inorganic microparticles have been reported in non-inflamed intestinal tissue but

without quantitative data it is impossible to assess the role of microparticle-laden macrophages in the inflamed intestine. It therefore seems important to study more extensively macrophages that contain microparticles in the inflamed intestine, with special reference to Crohn's disease and ulcerative colitis. The aims of the present study were (1) to quantify microparticle-laden macrophages in the human intestine by image analysis, (2) to compare microparticle-laden macrophages from subjects with Crohn's disease, ulcerative colitis and normal controls, (3) to determine the proportion of the area occupied by microparticle-laden macrophages in Crohn's disease, ulcerative colitis and control subjects, and (4) to identify the types of microparticles present in the human intestinal wall.

Materials and Methods

Subjects

Paraffin-embedded sections from 15 surgically resected intestines were examined in this study. The patients consisted of three groups, Crohn's disease (n=5), ulcerative colitis (n=5) and controls (n=5). Control subjects had surgical resections from operations undertaken for colon cancer. Unaffected intestine was selected to act as control tissue. Clinical and demographic data of the cases have been presented in tables 6.1a, 6.1b and 6.1c.

Table 6.1a: Clinical and demographic data of patients with Crohn's disease

Patient	Age	Sex	Clinical diagnosis	Length of disease	Involved bowel	Indication for surgery	Disease state	Treatment	Particles X-ray analysis	Dietary information
A	48	M	Colonic Crohn's	4 years	Colon	A Colonic Peroration	Active	Prednisolone	Al, Si,	-
B	28	F	Crohn's disease	4 years	T. Ileum	Stricture & fistula	Active	-	Ti, Al, Si,	-
C	31	M	Crohn's disease	5 years	T. Ileum	SBO & Perforation	Active	Prednisolone	Cr,	-
D	65	F	Crohn's disease	6 years	Ileum	Stricture	Active	-	Ti, Si,	-
E	42	F	Crohn's disease	3 years	Ileo-caecal	Fistula with abscess	Inactive	-	Cr, Si,	Elemental diet

SBO=small bowel obstruction; Al=aluminium; Si=silicon; Cr=chromium; Ti=titanium;

Table 6.1b: Clinical and demographic data of patients with ulcerative colitis

Patient	Age	Sex	Clinical diagnosis	Length of disease	Involved bowel	Indication for surgery	Disease state	Treatment	Particles X-ray analysis	Dietary information
F	35	M	UC	4 years	Left colon	Refractory to medical Rx	Active	Prednisolone	Ti, Al,	-
G	25	M	UC	6 years	Transverse + Left colon	Fulminant colitis	Active	-	Si, Ti,	-
H	44	F	UC	5 years	Left colon	Refractory to medical Rx	Active	-	Al, Si,	-
J	38	M	UC	11 years	Left colon	Recurrent severe attacks	Inactive	Prednisolone Colifoam	Si, Ti	-
K	43	M	UC	20 years	Transverse +Left colon	Low grade Dysplasia	Inactive	-	Ti, Al,	-

Al=aluminium; Si=silicon; Ti=titanium;

Table 6.1c: Clinical and demographic data of control patients

Patient	Age (y)	Sex	Diagnosis	Particles (X-ray analysis)	Involved bowel
L	70	F	Cancer	Si,	Caecum
M	70	F	Cancer	-	Sigmoid colon
N	59	F	Cancer	-	Left colon
O	60	M	Cancer	-	Sigmoid colon
P	50	F	Cancer	-	Rectum

Si=silicon.

In Crohn's disease and ulcerative colitis cases, macroscopically evident areas of small bowel/colon involved by perforation, fistula, thickening , or with any polypoid lesion were excluded from sampling. Macroscopically normal areas near the proximal or distal ends of the specimens were sampled. Tissue histology was studied in H&E stained sections from the samples which showed infiltration of inflammatory cells and indicated activity of the disease.

Control sections were free of polymorph infiltrates, significant lymphoid or plasma cell infiltrates, oedema, fibrosis, regenerative epithelium or crypt destruction.

Three 5-μm sections were cut from each block. One was lightly stained with haematoxylin and eosin (H & E) and the other two were prepared for immunohistochemistry. All slides were coded and examined blinded.

Immunostaining for CD68 (Tissue macrophage)

Paraffin-embedded sections were de-paraffinized in Histoclear and graded alcohols. Antigens were unmasked at high temperature (microwave at 800W for 20 min in citrate buffer). Tissue endogenous peroxidase activity was blocked with 1% hydrogen peroxide (H_2O_2) in methanol, washed and rehydrated in 0.05 M Tris-buffered saline (TBS), pH 7.6. Then sections were incubated with 20% filtered rabbit serum (Sappu) in TBS for 10 min prior to incubation with primary

antibody, mouse monoclonal anti-human CD68, (1:40) (PG-M1, Dako, UK, Catalogue No. M0876) for 60 min. Then sections were rinsed and washed in TBS and incubated in rabbit anti-mouse biotinylated immunoglobulin (1:300, Dako, UK, Catalogue No. E0413) for 60 min. Sections were re-washed, incubated in streptavidin/biotin-peroxidase (ABC, Dako, UK, Catalogue No. K377-B) for 50 min. After washing in TBS, sections were incubated in 0.05%, 3,3' - diaminobenzidine and 0.01% H_2O_2 in 0.05 M TBS (pH 7.6). Sections were counterstained with 0.01% methyl green for easy detection of light brown stained CD68+ cells (tissue macrophages) for image analysis. Serial sections from surgically obtained tonsils were prepared as positive controls and sections stained without the primary antibody were used as a negative control (232).

Serial sections were also immunostained by streptavidin/biotin-alkaline phosphatase method. In brief, in the third step of immunostaining streptavidin/biotin-alkaline phosphatase (ABC, Dako, Catalogue No. K391) was used instead of streptavidin/biotin-peroxidase at a dilution of 1:300. After washing in TBS, sections were incubated with New fuchsin substrate system (Dako, UK, Catalogue No. K698) and sections were counterstained with Harris hematoxylin. Highly chromogenic bright reaction products help to detect blue stained nuclei and black microparticles on a pink-red background, and facilitates enumeration of CD68+ cells with or without microparticles in the tissues (233).

Image analysis of macrophages (CD 68+ cells)

Measurement of CD68+ve area

Immunostained sections were initialised at magnification 400 and calibrated for measurements in square microns (μm^2). After initialisation, a dark brown colour as indicator of CD68 positive cells was detected and confirmed with the colour intensity throughout the field; then areas of positive cells in consecutive 25 frames (13014 μm^2) per tissue were measured by an in-built automatic colour detection system. Results were expressed as total tissue area, area occupied by CD68 positive cells in the tissues in square microns, and also as the proportion of CD68 positive area to the total tissue area.

Count of CD68+ve cells

Macrophages were counted by image analysis (LEICA, Germany). In brief, immunostained sections were initialised at magnification 400 and calibrated in square microns as before and in 25 consecutive frames CD 68+ cells were analysed. First, all CD68+ve cells in a field were marked and confirmed with colour detection, and subsequently marked cells were counted automatically by a *Leica* in-built program. In the second step, immunostained microparticle-laden cells (that appear as black dots on a pink-red background) were marked and

automatically counted as before. CD68 positive cells not loaded with microparticles were derived from the two counts. The number of CD68+ve cells, with or without particles were expressed as total number per mm^2 of tissue and as a proportion of the total cells.

Confocal microscopy

The locations of inorganic microparticle-laden in the tissues were noted and were investigated by confocal laser scanning microscopy (CLSM, Carl-Zeiss, Germany). The cells were initially identified under CLSM with transmitted light using differential interference contrast optics, x 63 objective and x 40 to 160 zoom. Later an argon ion laser with helium neon of 633 nm wavelength was used to obtain confocal images of a cell at an interval of 1 μm and photographed. The inorganic microparticles, unlike tissue, reflect the laser light and become visible in the surrounding non-reflecting area of the nucleus.

X-ray microanalysis

An area of tissue was selected using H & E section and a piece of tissue was removed from this area in the paraffin blocks of resected intestine using a single edged razor blade. The tissue was placed in chloroform for 2 x 2 hours to remove the wax. The chloroform was removed by washing the specimen in 740P sprit 3 x

1 h. Whilst in alcohol the specimen was trimmed or sliced into smaller pieces to aid penetration of the resin. The specimen pieces were then infiltrated with 50:50 LEMIX epoxy resin (TAAB, Birkshire, UK), 740P spirit, overnight. The following morning the specimen was placed in 100 LEMIX resin for 8 hours. Individual pieces of tissue were then embeded in fresh resin and polymerised at 70°C overnight. One micron semi-thin sections were cut using a glass knife on a Reichert Ultracut ultramicrotome (Leica, UK) and stained, on a 90°C hot plate, aniline blue/azure II for 10 mins followed by 0.01% basic fuchsin for 40 seconds and reviewed under a light microscope. The microparticles were clearly visible within the cytoplasm of the macrophages in an area of section subsequently subjected to X-ray microanalysis. Then 100 μm ultrathin sections were cut using a diamond knife and mounted on Pioform (Agar Scientific, UK) coated grids. One section was stained with saturated uranyl acetate in 50% ethanol, followed by Reynold's lead citrate and rest were left unstained for EDAX.

Then unstained sections were viewed under a transmission electron microscope (Philips CM 120, Holland). Microparticles were identified within vesicles in the cytoplasm of the cells and microanalysis was performed using an EDAX (Energy dispersive analysis of X-rays) DX4 attached to the microscope with a transmitted electron detector at magnifications of 10,000 and above. This technique yields information on all elements present within a tissue with an atomic weight greater than that of sodium (atomic weight 22). The analysis conditions were 80kV, 60nm spot size. A detailed description of the technique has been made in the Methods

section (Chapter 3). Briefly, the spectrum of an element was analysed by DX4 that matches the standard spectrum of the element in the database.

Statistics

The mean of a series of observations from a case was taken to represent the value for that observation and was included in the final analysis. Descriptive statistics included mean and standard error of the mean. Values from specific subgroups of patients were then compared using t-test. A probability of value of ≤ 0.05 was considered as significant.

The number of macrophages is the mean of observations for a case in this analysis. Similarly, microparticle-laden macrophages, proportion of microparticle-laden macrophages, macrophage area, and proportion of macrophage area to the tissue area measured (as a percentage) is the mean of the observations from a single case. The values have been included in the final analysis for comparison between groups. The mean and standard error of the mean of the parameters have been presented and comparison between groups was done using t-test. A probability of value of ≤ 0.05 was considered as significant.

Data were entered in a personal computer and analysed in SPSS PC+. The results have been presented in table 6.2a and table 6.2b.

Results

Light microscopy

In lightly stained (H & E) sections, inorganic microparticles and cells that contained microparticles were noted. By conventional light microscopy at magnification 400, inorganic microparticles appear as dark black dots but are difficult to detect below that magnification.

Immunostaining with CD68 antibody specific for tissue macrophages confirmed that positively stained microparticle-laden cells in the resected tissues are macrophages. The macrophages were located in the mucosa and submucosa of the intestinal tissues. Microparticle-laden macrophages were identified immediately beneath the epithelium in the lamina propria of IBD tissues [Figure 6.1]. Macrophages were also located in and around the Peyer's patches and colonic lymphoid follicles.

Table 6.2a: Data on macrophage number, microparticle-laden macrophages and proportion of microparticle-laden macrophages of patients with Crohn's disease, ulcerative colitis and controls.

Crohn's				UC				Control			
Patient	**MØ**	**MØpt**	**Proport**	**Patient**	**MØ**	**MØpt**	**Proport**	**Patient**	**MØ**	**MØpt**	**Proport**
A	8.6	6.0	69.7	F	10.1	7.0	69.3	L	5.8	1.0	1.7
B	14.6	14.2	97.2	G	11.3	10.3	91.1	M	5.7	0	0
C	13.3	10.8	81.2	H	9.5	2.3	24.2	N	8.0	0	0
D	7.3	1.3	17.8	J	11.8	6.7	56.7	O	2.3	0	0
E	16.5	13.8	83.6	K	5.1	0.3	5.8	P	9.8	0	0
$\underline{X}$ **± SEM**	12±1.7[a]	9.2±2.4[d]	69.9±13.7[g]	$\underline{X}$ **± SEM**	9.5±1.2[b]	5.3±1.9[e]	49.4±15.3[h]	$\underline{X}$ **± SEM**	6.3±1.2[c]	.02±.02[f]	.3±0.3[j]

MØ =macrophage number, MØpt = particle-laden macrophages, proport=percent of microparticle-laden macrophages; values are mean of twenty-five frames from a case. a vs. b, p =ns; a vs c, p =0.02; b vs c, p=0.09; d vs e, p = ns; d vs f, p=0.006; e vs f, p=0.01; g vs h, p=ns; g vs j, p=0.001; h vs j, p=0.01; *t-test.*

Table 6.2b: Data on macrophage area and proportion of macrophage area of patients with Crohn's disease, ulcerative colitis and controls.

Crohn's				UC				Control			
Patient	**Mtiss**	**MØar**	**Proport**	**Patient**	**Mtiss**	**MØar**	**Proport**	**Patient**	**Mtiss**	**MØar**	**Proport**
A	13014	525	4.03	F	13014	470	3.61	L	13014	310	2.38
B	13014	1088	8.36	G	13014	608	4.68	M	13014	307	2.35
C	13014	690	5.3	H	13014	479	3.68	N	13014	245	1.88
D	13014	355	2.72	J	13014	773	5.94	O	13014	331	2.54
E	13014	1466	11.27	K	13014	406	3.12	P	13014	369	2.84
$\bar{X}\pm$**SE**	13014	824.8 ± 201^{a}	6.3 ± 1.5^{d}		13014	547.2 ± 65^{b}	4.2 ± 0.5^{e}		13014	312.4 ± 20^{c}	2.4 ± 0.16^{f}

Mtiss=measured tissue area (μm^2); MØar=macrophage area; proport=percentage of macrophage area; Values are mean of 25 frames from a single case. a vs b, p=ns; a vs c, p=0.03; b vs c, p=0.009; d vs e, p=ns; d vs f, p=0.03; e vs f, p=0.009. t-test.

Image analysis

By image analysis the numbers of macrophages, and microparticle-laden macrophages were determined. The average of observations from each case was presented in table 6.2a. The numbers [Mean ± SEM] of macrophages were: 12 ± 1.7, 9.5 ±1.2 and 6.3 ± 1.2 in Crohn's disease, UC and control tissues respectively. Similarly, the number inorganic microparticle-laden macrophages were: 9.2 ± 2.4, 5.3 ±1.9 and <1 ±0.02 in Crohn's disease, UC and control tissues respectively. The percentage [mean ± SEM] of microparticle-laden macrophages was 69.9 ± 13.7 vs. 49.4 ± 15.3 vs. <1 ± 0.3 in Crohn's disease vs. ulcerative colitis vs. controls ($p < 0.01$) [Table 6.2a]. In one control tissue, microparticles were located within a CD68 positive cell.

By image analysis macrophage area (μm^2) was measured optically by detecting area of the brown stained CD 68+ cells on a light green background [Figure 6.2]. The macrophage area (mean± SEM) was: 825± 201, 547± 65 and 312± 20 in Crohn's disease, ulcerative colitis and control tissues respectively (p=<0.01)[Table 6.2b]. The percentage of macrophage area was [mean ± SE]: 6.3 ± 1.5, 4.2 ± 0.5 and 2.4 ± 0.16 in Crohn's disease, ulcerative colitis and controls respectively [Table 6.2b]. The measured area was the same (25 x 13014 μm^2) for all groups.

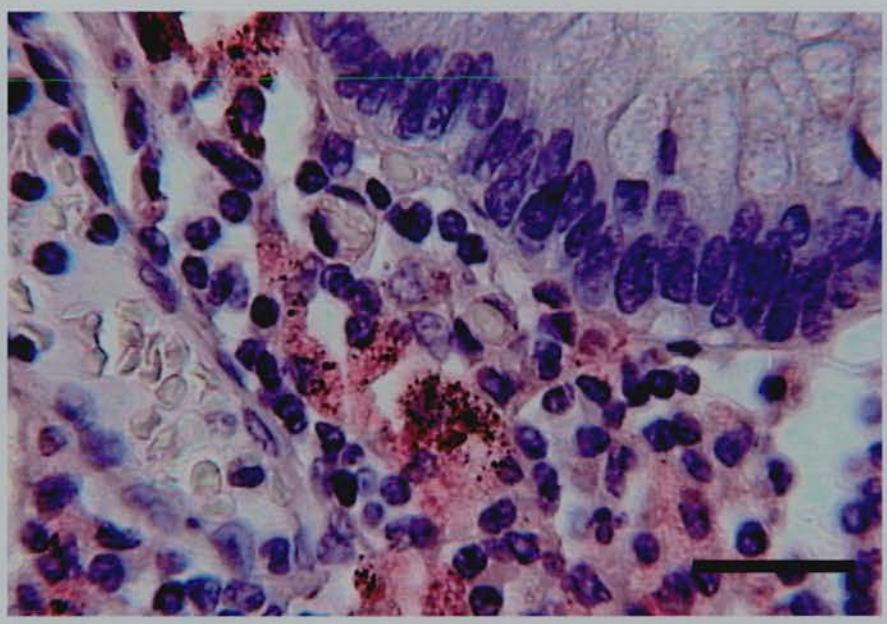

Figure 6.1. Immunolabelled microparticle-laden macrophages beneath the epithelium in a Crohn's tissue. Counter stained with hematoxylin. Scale bar = 15 μm

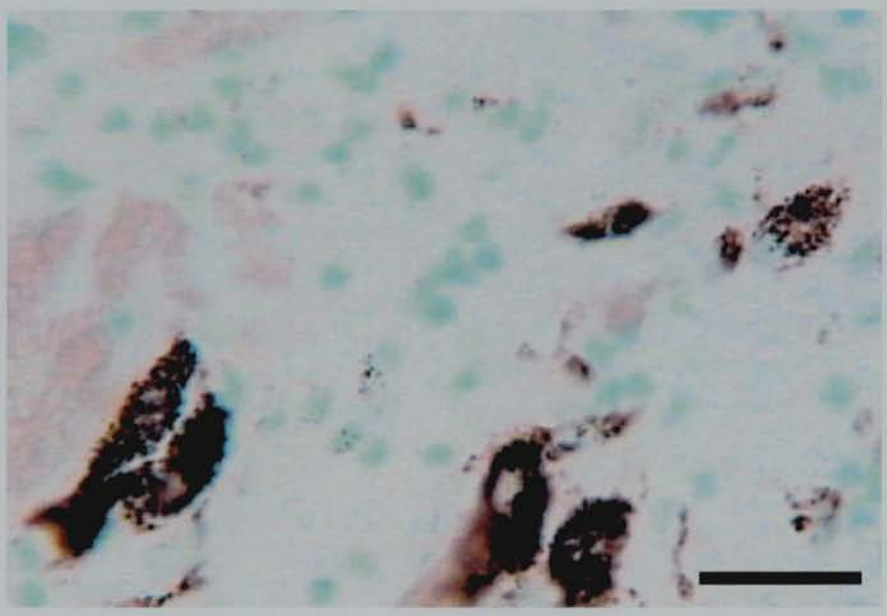

Figure 6.2. Immunostained (CD68+) microparticle-laden macrophage in a Crohn's section. Counterstained with 0.01% methyl green. Scale bar = 15 μm

Both microparticle-laden macrophages [Figure 6.3] and microparticle-laden macrophage area [Table 6.2b] were significantly increased in Crohn's disease compared to ulcerative colitis and controls [$p<0.01$].

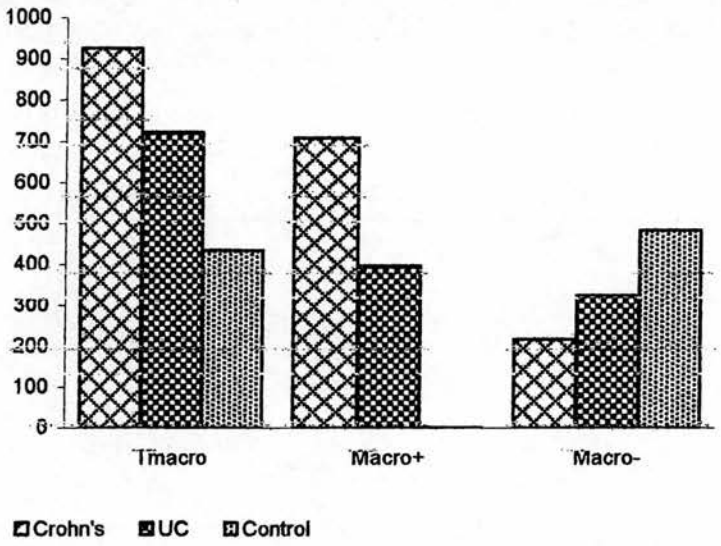

Figure 6. 3 Macrophage (CD68+) count/mm^2 in resected intestine of patients with Crohn's disease, UC and control. Tmacro = Total number of macrophages; Macro+ = microparticle-laden macrophages; Macro- = macrophages not containing microparticles.

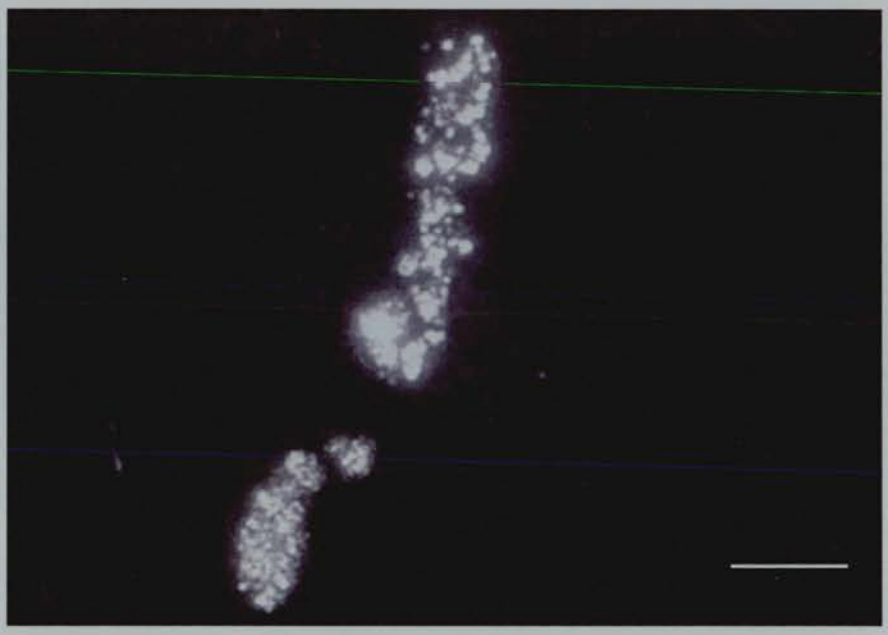

Figure 6.4. Confocal micrograph showing inorganic microparticles reflecting laser light and visible in the surrounding non-reflecting area including nucleus. Scale bar = 25 μm

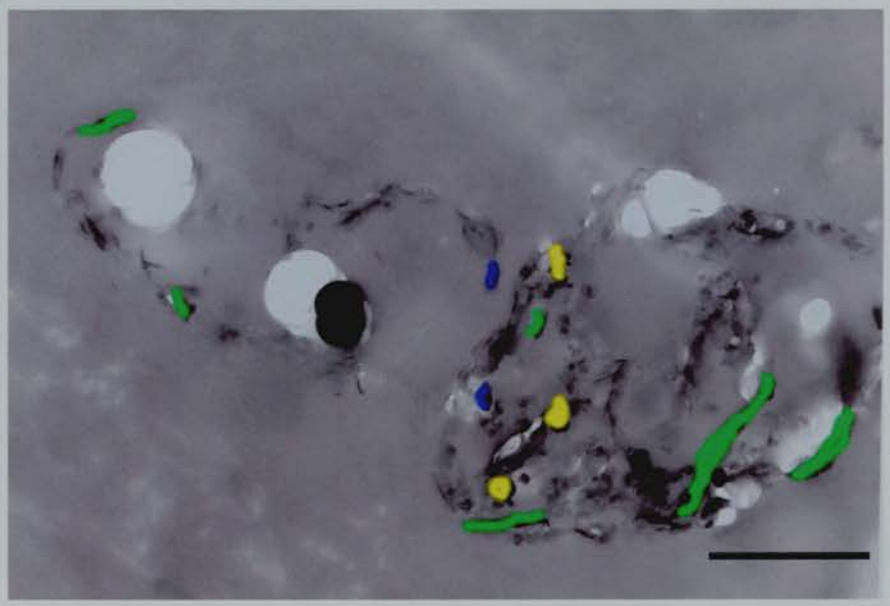

Figure 6.5. X-ray micrograph of microparticles in Crohn's intestine. Yellow = titanium; Green = aluminium; Blue = silicon Scale bar = 1 μm

Confocal and electron microscopy

Using Confocal laser scanning microscopy microparticles were located in clusters within the macrophages in the mucosa [Figure 6.4]. Thin sections stained with aniline blue/azure II and basic fuchsin confirmed this finding. By transmission electron microscopy, sub-micron size particles were observed packed within membrane vesicles that had the appearance of phagolysosomes.

X-ray microanalysis

By X-ray microanalysis in association with transmission electron microscopy sub-micron size particles were determined as compounds of silicon, titanium, aluminium and chromium [Figure 6.5]. The compounds of chromium have not been reported before in human intestinal tissues. Figure 6.6 shows the X-ray spectrum in an area with electron dense microparticles of chromium (the copper and nickel peaks are from the grids). One of the double peaks is an "escape peak" generated by the program.

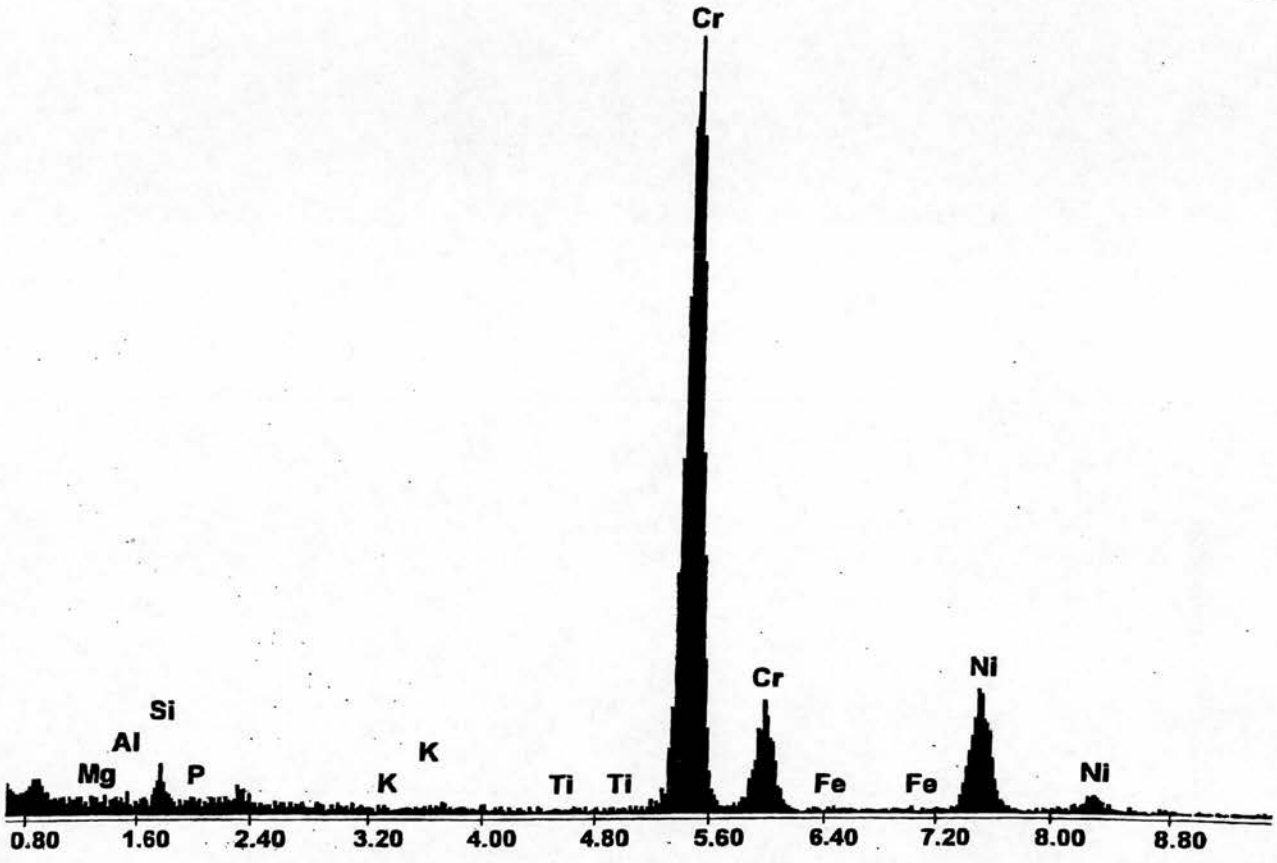

Figure 6. 6 X-ray spectrum of a chromium microparticle (nickel peaks are from grids and background).

Discussion

In the present study, chromium has been detected for the first time in human intestinal mucosa. Chromium is a hard, steel-grey brittle metal with a molecular weight of 52. It is found as chromite, a mixed chromium-iron oxide, which occurs as a dark-brown or black mineral. Local exposure to chromium particles has been reported to cause ulceration of the skin and mucus membrane (234). Experimental rats exposed to chromium particles have been shown to develop chronic interstitial inflammation in the alveolar septa, characterised by infiltration of inflammatory cells including macrophages (235). There are no reports of inflammatory reactions to chromium in the intestinal tissues of experimental animals. Although in two cases chromium was found in human intestinal macrophages in the present study, the finding cannot be correlated to with any clinical features or complications, as in other cases with similar complications chromium was not identified. However, it is possible that chromium may cause damage to the intestinal tissues as in lung tissues and this needs to be explored in a larger number of cases.

To identify the possible source of chromium, Ministry of Agriculture, Food and Fisheries (MAFF) was contacted: it appears that vitamin preparations and mineral supplements contain chromium. On checking (in various food stores) it was advised that vitamins and mineral supplements for human consumption e.g.

Centrum, Multibionta, contain chromium. This is a possible source of the chromium particles that have been identified in resected specimens in this study.

So far there is only one published report (182) on X-ray microanalysis of human intestinal tissues with inorganic particles. In this thesis a second report on X-ray microanalysis of human intestinal tissues has been presented, and chromium has been identified for the first time in Crohn's disease affected intestine.

In the present study inorganic microparticles were clearly observed within the macrophages in the lamina propria of the intestine in Crohn's disease and ulcerative colitis. This finding confirms a previous report of inorganic microparticles in inflamed human intestine (182). It had been reported that inorganic microparticles were present only in the basal part of the Peyer's patches and colonic lymphoid follicles of the human intestine but, in the present study, inorganic microparticles were observed immediately beneath the epithelium in the mucosa and also in the submucosa, in addition to their presence around the gut associated lymphoid tissues. Studies on inorganic microparticles in human intestine have previously focussed on the elemental analysis of the microparticles. The present study of inorganic microparticle-laden macrophages in human intestinal cells has considered the extent of their involvement in IBD. In the present study, a specific marker (CD68) was used that identifies tissue macrophages and by image analysis it has been conclusively shown that inorganic microparticle-laden macrophages are significantly

increased in Crohn's disease compared to UC and controls. Specifically, the proportion of the area occupied by microparticle-laden cells was found to be significantly increased in IBD compared to non-inflamed control tissues. A quantitative analytical study of inorganic microparticle-laden macrophages in inflamed human intestine has not been reported to date.

Inorganic microparticles as compounds of titanium, silicon and aluminosilicate were documented in phagosomes of the macrophages in the present study. These findings support previous reports on the intracellular location of inorganic microparticles (17,128). The source of these microparticles is presently unknown. Many pharmaceutical preparations contain titanium, and compounds of aluminium and silicate are widely used in the food industry in preservative preparations. It is likely that the microparticles that have been found in the present study and by others in resected human intestine were ingested and subsequently translocated into intestinal mucosa.

An increase in the number of macrophages in the mucosa has been reported in the early stages of inflammation of Crohn's disease (230) and these cells have been shown to be newly recruited peripheral monocytes because of their expression of CD 14+L1, which are not expressed by resident macrophages (99,230,236). The stimulus for the recruitment of macrophages into tissues of the intestine is not known. However, luminal factors such as lipopolysaccharide of the resident gram-negative bacteria or any inflammatory pathology already

present in the intestine may have some role in the uptake of inorganic microparticles by the intestinal mucosa. It is possible that the presence of inorganic microparticles conjugated with LPS may stimulate the recruitment of macrophages from the peripheral blood.

The fate of macrophages that have phagocytosed inorganic microparticles is unknown. Inorganic microparticles conjugated with LPS have been shown to increase secretion of proinflammatory cytokines e.g., IL-1β in human intestinal macrophages (121). Macrophages play a significant role in removing apoptotic T cells, which are increased in inflammatory bowel disease. Studies have shown a decreased phagocytic capacity of macrophages to engulf apoptotic cells is other diseases (237). It is possible that engulfed inorganic microparticles may cause dysfunction of macrophages in efficient removal of the apoptotic cells that may be involved in the perpetuation of the inflammatory process in IBD. The observed benefit of a low inorganic microparticle-containing diet in Crohn's disease supports this view (96).

CHAPTER 7: LIPOPOLYSCCAHRIDE INCREASES UPTAKE OF MICROPARTICLES IN INFLAMED HUMAN COLON MUCOSA IN VITRO

Introduction

In animal studies, uptake and translocation of 2-μm-size polystyrene microparticles have been demonstrated in the small intestine *in vivo* (48). In the earlier studies, it had been observed that microparticles in the nanometre to micron range were taken up by the gastrointestinal mucosa (238,239) and it was believed that smaller (< 1μm) microparticles could only be transported across the intestinal mucosa. Most studies related to the uptake of microparticles have been done from the point of view of a drug or vaccine delivery (240). However, transcytosis of such microparticles may have pathological implications (241). The exact mechanisms and pathways of microparticle transport are not totally clear (242). Three possible pathways of microparticle uptake across gut mucosa are: (i) transcellular(48); (ii) through specialised M-cells (243); and (iii) paracellular (67,79). Most of the microparticle uptake studies have been done in animals and are limited to the small intestine (244). Studies on the uptake of polystyrene (or similar) microparticles with human tissues are non-existent, and for ethical reasons will not be possible in the foreseeable future. It is generally believed that the colon is impermeable to microparticles, and there is a lack of data on the uptake of microparticles by human colon mucosa (241).

The colon hosts a number of bacteria and one of their products, lipopolysaccharide (LPS) has been shown to alter epithelial cell metabolism and/or damage the mucosal barrier (245-247). In animal studies, it has been observed that if bacterial cell wall products such as LPS traverse the intestinal epithelial barrier, a chronic granulomatous inflammatory condition resembling Crohn's disease may develop (248). Increased transmucosal transport of macromolecules has been observed in inflamed human colonic mucosa in vitro (249) and this has been suggested as secondary to the action of bacterial toxin (250). However, there is no experimental evidence of the effect of LPS on the uptake of microparticles in healthy or inflamed human colonic mucosa.

The aim of this study was to (i) demonstrate whether transport of micron-size particles occurs across colon mucosa *in vitro* and (ii) determine the effect of LPS on the uptake of microparticles, in non-inflamed colonic mucosa from normal subjects and inflamed mucosa from patients suffering from IBD.

Materials and Methods

Subjects

Four biopsies from the sigmoid colon were taken, with informed consent, from ten fasting patients undergoing colonoscopy either for diagnosis or for cancer

screening. All patients were fasted from midnight as required for colonscopy on the following morning. The Medicine and Oncology Subcommittee of the Lothian Ethics in Research Committee approved this study.

Culture medium

The culture medium was Weymouth's MB752/1 (ICN, UK. Catalogue No. 252254), supplemented with 10 % foetal calf serum (ICN, UK. Catalogue No. 2910154), L-glutamine 200 mM 1.5 ml (Sigma, UK. Catalogue No. G-7513), ascorbic acid 300 μg/ml (Sigma, UK. Catalogue No. A-4544), ferrous sulphate 0.45 μg/ml (Sigma, UK. Catalogue No.F-8633), penicillin 5000 i.u. /ml, streptomycin 5000 μg/ml (Sigma, UK. Catalogue No.P-4458), and gentamicin 50μg/ml (Sigma, UK. Catalogue No. G- 1272).

Culture procedure

Colonoscopic biopsies were obtained between 9 and 11 am to exclude any theoretical effects of diurnal variation. Immediately after removal, biopsies were transported promptly to the laboratory, which is close to the endoscopy suite. The transport was done in complete medium, pre-gassed with 95% O_2 and 5% CO_2 at 37 °C and the tissues were established in organ culture within 10 minutes after biopsy. Biopsies were washed twice in warm media. Under the dissecting microscope (Watson, Barnet, England) biopsies were oriented, luminal surface

up, on Millipore filters (pore size 0.45 μm, diameter 25 μm, Millipore Corporation, Bedford, MA, USA. Catalogue No. HAWP 02500). The filters were then placed on the rims of organ culture dishes. Sterile plastic organ culture dishes (Falcon, Becton Dickinson UK Ltd. Cat. # 3037) 5.5 cm in diameter were used, with a central 2 cm well into which 1 ml medium was placed. The central well is surrounded by an outer well in which 1 ml of 0.9% NaCl solution was added. The dishes were covered, placed on a plastic tray and sealed in a Plexiglas modular chamber [Bilups-Rothenburg™, California, USA]. The modular chamber was gassed with a mixture of 95% O_2 and 5% CO_2 for 4 min at 5L/min, then sealed and maintained at 37°C in an incubator.

Biopsies were cultured in (i) complete medium with bromodeoxyuridine (BrdU); (ii) complete medium with microparticles; (iii) complete medium with LPS and microparticles; and (iv) complete medium with BrdU and LPS. The biopsies were cultured in parallel and under identical conditions (200).

The organ culture technique that has been used in this study is essentially that described by Prichett *et al* (251). Monoclonal antibody against the thymidine analogue BrdU permits identification of cells in S-phase of mitosis by an immunohistochemical technique (206). Retention of normal morphology of the tissue and incorporation of BrdU in DNA of dividing cells were considered as indices of the survival of explant tissues (196). In these experiments 10 μl of

BrdU (200 mM, Sigma, UK. Catalogue No. B-5002) was added to each ml of complete medium.

*Lipopolysachari*de

Lipopolysaccharide (LPS) of *Escherichia coli* O55:B5 (Sigma, UK. Catalogue No. L- 2880) 50 μg / ml was added to the test culture media at 0 hour to study the effect of endotoxin on microparticle transport. *In vitro* studies have demonstrated that this concentration of the LPS of *E.coli* liberated cytokines and growth factors without altering morphology of the intestinal mucosa (225,252).

Microparticles

To study microparticle uptake and translocation, plain (non-ionic), mono-disperse, yellow-green (Y-G) fluorescent polystyrene latex microparticles (2.5 % solid content, 2.0 μm in diameter) with Y-G dye excitation (Polysciences Inc., Warrington, USA. Catalogue No. 18338) and with emission minimum and maximum of 545 and 610 nm, respectively, were used. The stock suspension was diluted in sterile de-ionised water to obtain a microparticle concentration of 5.68×10^6 per 0.1 ml. After 3 hours of incubation, 50 μl of microparticle suspension was added to the explant tissues. Explant tissues were cultured at 37°C for a further 21 h.

To test viability after 24 h of culture, explant tissues were rinsed and fixed by submerging in Carnoy's fixative for 12-16 h followed by 99% industrial methylated sprit (IMS) for another 24 h. Explant tissues were then processed to paraffin wax. Sections of the entire width of the biopsy were then cut at 5 μm with a microtome (LEICA 2045 Multicut, Nussloch, Germany) and placed onto 0.1% poly-L-lysine coated slides for subsequent immunostaining. The slides were coded.

Confocal laser scanning microscopy

Of the numerous methods for evaluating uptake and translocation of microparticles, two techniques are well established: quantification of particulates in bulk macerated specimens and *in situ* visualisation (253). Each of these methods has advantages and limitations. The major weakness of the maceration technique is lack of information provided at cellular level and particularly so in gastrointestinal tissues where even after thorough washing, maceration makes it impossible to decide whether microparticles were originally within the mucosa or were merely overlying the mucosal surface. In this study therefore, the second method was adopted.

Explant tissues (incubated with polystyrene microparticles) were coded and mounted in Cryo-M-Bed embedding compound (OCT) and frozen at -52°C (Bright Cryospray 134) and stored at -70°C until cryosections were made. Thirty 15-μm-

thick cryosections (Bright's Hacker cryostat, UK) from each explant tissue were taken on poly-L-lysine-coated glass slides and dried at room temperature (22° C).

The uptake of microparticles into the mucosa was assessed in propidium iodide (Sigma, UK. Catalogue No. P- 4170) stained cryosections. Briefly, cryosections were incubated in 0.001 mg/ml of propidium iodide in 0.1 M sodium cacodylate buffer for 30 min and mounted without dehydration, in fluorescence mounting medium (DAKO, UK. Catalogue No. S-3023). Particle distribution across mucosal sites was analysed by epifluorescence microscopy. The first 10 good quality i.e., not fragmented, 15 μm sections were used from a pool of 30, giving a total thickness of 150 μm of tissue sampled from each biopsy.

The localisation of the fluorescent microparticles within tissue compartments was determined using a confocal laser scanning microscope (LSM; LEICA TCS NT, Leica Microsystems, Mannheim, Germany) with an argon ion laser. At first, the LSM with filters for FITC and TRITC excitation was adjusted. The lateral and basal one-fourths of the sections were excluded from the analysis because of the cut during biopsy. After targeting the area to be counted, optical sectioning (1 μm) by argon laser was employed to locate microparticles within the mucosa. Fluorescent microparticles were marked and counted automatically by the UHTHA Image tool program and measurement of tissue area were done by marking the area using the same software (The University of Texas Health

Science Centre, San Antonio, USA). Counts and measurements were done by two observers with 83% agreement ($\kappa = 1$).

Immunostaining for BrdU

Sections were de-waxed and placed in 1% hydrogen peroxide (H_2O_2) in methanol for 20 min to block endogenous peroxidase. After washing in 0.05 M TRIS buffered saline (TBS), pH 7.6, DNA was denatured by immersion of the slides in 1 N HCL for 8 min at 60°C to expose bound BrdU for immunostaining with a streptavidin-biotin peroxidase technique. Sections were incubated successively in filtered rabbit serum in TBS (1:4, NRS/TBS) for 10 min, in mouse monoclonal anti-BrdU antibody (Becton-Dickinson, UK) in NRS/TBS (1:200) for 60 min, in biotinylated rabbit anti-mouse immunoglobulin in NRS/TBS (1:300) for 40 minutes, and finally in streptavidin/biotin-peroxidase (ABS, Dako, UK) for 30 min. at room temperature. After each step, sections were rinsed in TBS. Then sections were incubated in 0.05 M TRIS/HCL buffer (pH 7.6) containing 0.05% diaminobenzidine and 0.01% H_2O_2 for 10 min. Sections were counterstained with Harris hematoxylin, dehydrated through graded alcohol, cleared in histoclear, and mounted in synthetic resin.

BrdU-stained sections in each experiment were systematically reviewed for crypt architecture, epithelial cell shape, and nuclear orientation, oedema and cell

debris in the lamina propria, crypt necrosis, and amorphous material surrounding the crypts.

Electron microscopy

To confirm the location of microparticles within the cytoplasm of cells, 1 mm cubed pieces of tissue were fixed overnight at 4°C in 3% glutaraldehyde in 0.1M sodium cacodylate buffer. Tissues were then washed in cacodylate buffer for 30 min and post-fixed in 1% osmium tetroxide (1h) in cacodylate buffer; dehydrated through various grades of alcohol, 10% (3 changes 10 min per change), 50% alcohol (1 change 15 min), 100% alcohol (3 changes 30 min per change). Then dehydrated through propylene oxide (2 changes 20 min per change) and embedded in Araldite resin and polymerised in a 56°C incubator for 2-3 days. Thin sections (0.5 - 1 μm) were cut with a glass knife and dried on a hot plate. The dried sections were stained with toluidine blue in 1% borax for approximately 30 sec. The sections were examined under light microscope and areas with microparticles were selected. Ultra thin sections were cut using a diamond knife; interference colours determined section thickness (60-80 nm). The sections were placed on copper grids (mesh size 300) and allowed to dry for 30 min. The grids were stained with uranyl acetate and lead citrate. The grids were viewed in a JEOL 100CXII-transmission electron microscope (Tokyo, Japan) and photographs were taken at 60 kV.

Statistical analysis

Data were entered in a personal computer and analysed in SPSSPC+ (SPSS PC+ Inc. Chicago, IL, USA).

The mean of a series of observations from a case was taken to represent the value for that observation and was included in the final analysis. Values from specific subgroups of patients were then compared using t-test. Descriptive statistics included mean and standard error of the mean (SEM).

The value for uptake of fluorescent polystyrene microparticles (microparticle count/mm^2) were compared by using t-test. A probability (p) value of ≤ 0.05 was considered as significant. The mean of observations from a case was included in the final analysis.

Results

Biopsies (~2mm) were obtained from ten patients, four men and six women with a median age of 47 years (range 30 - 66 years). Clinical and demographic data of patients with Crohn's disease and ulcerative colitis has been presented in

Table 7.1.Three patients had established colonic Crohn's Disease and two had ulcerative colitis.

For Crohn's and ulcerative colitis biopsies, areas of intestine were assessed for the disease states by an experienced colonoscopist. Biopsies were collected from areas appearing endoscopically normal or mildly inflamed (loss of vascular pattern). Areas of severe inflammation with contact bleeding or severe disease with friability, ulcers or spontaneous bleeding were excluded for biopsy (Gomes *et al.*). H & E stained sections of the biopsies were examined for the tissue histology.

Table 7.1: Clinical and demographic data of patients with inflammatory bowel disease

Patient	Age	Sex	Clinical diagnosis	Length of disease	Involved bowel	Disease state	Treatment	Dietary information
A	54	F	Colonic Crohn's	6 years	Caecum, ascending , transverse	Active	Asacol	-
B	51	F	UC	20 years	Pancolitis	Inactive	Sulphasalazine	
C	66	M	Colonic Crohn's	30 years	Pancolitis	Active	Prednisolone Azathioprine	-
D	30	F	Colonic Crohn's	4 years	Sigmoid, rectum	Active	Prednisolone	-
E	45	F	UC	3 years	Rectum, sigmoid, descending	Inactive	Sulphasalazine	-

Morphology and BrdU labelling

Morphology of the cultured colonic mucosa was well maintained in culture for 24 h. Many mitoses were present in the crypts, even after 24 h, as demonstrated by the incorporation of BrdU in the dividing cells. Epithelium was intact and crypt architecture was well maintained. Biopsies from normal controls cultured in medium containing LPS were morphologically indistinguishable from the biopsies cultured in control medium. Similarly, biopsies from IBD patients cultured in medium containing LPS were indistinguishable from the biopsies cultured in control medium. Figure 7.1 is light photomicrograph of a section from a IBD patient cultured in control media, and 7.2, represents a similar section from an inflamed tissue cultured in media containing LPS.

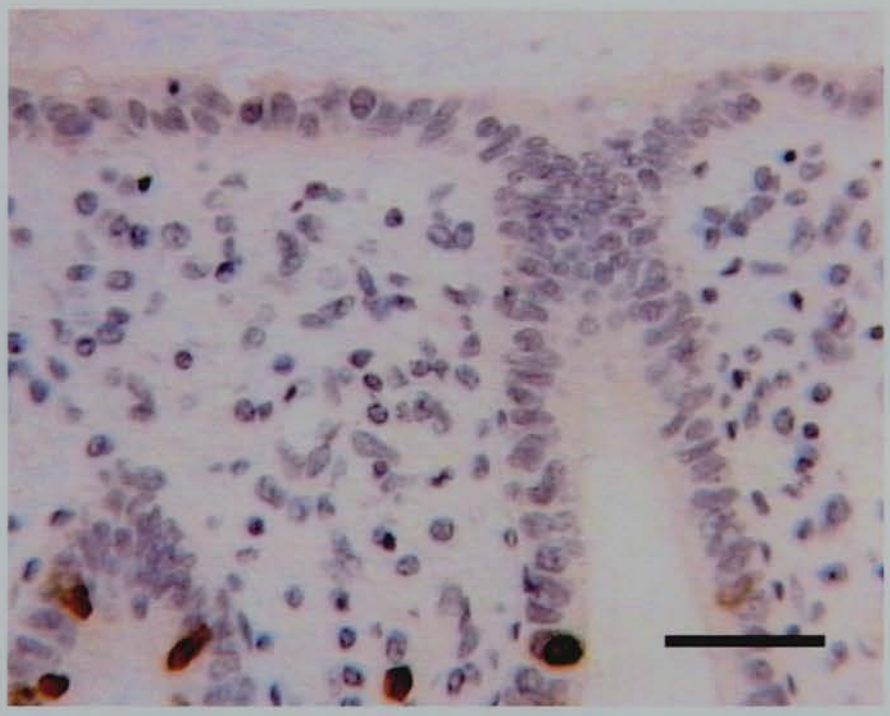

Figure 7.1. Micrograph of IBD mucosa cultured in control media. BrdU labelled cells appear as brown. Scale bar = 20 μm

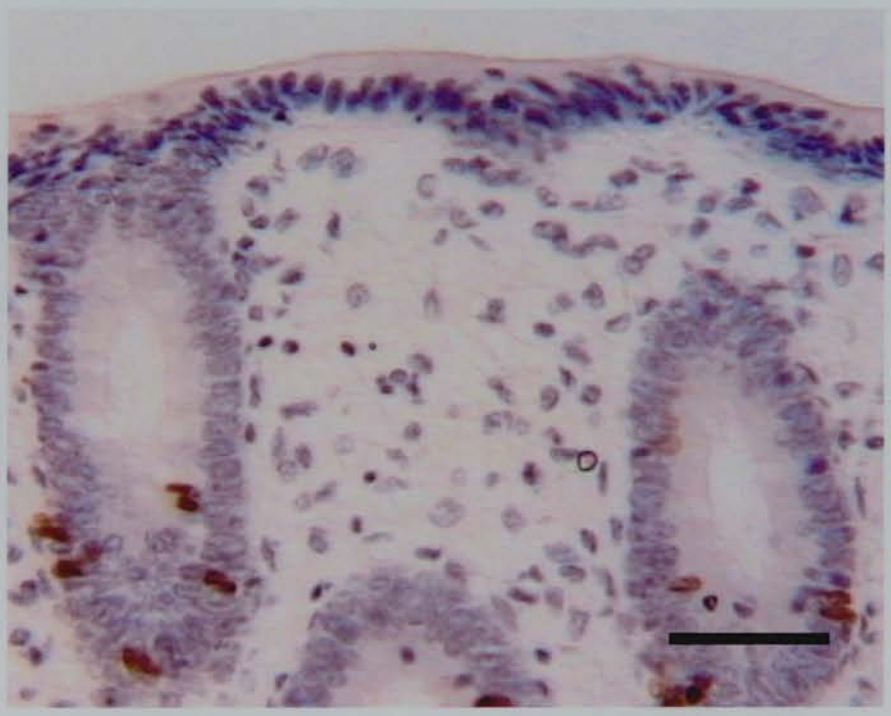

Figure 7.2. Micrograph of IBD mucosa cultured in LPS containing media. BrdU labelled cells appear as brown. Scale bar = 20 μm

Localisation and quantitation of polystyrene microparticles

Microparticles were located beneath the epithelium and in the lamina propria in propidium iodide stained cryosections of cultured human colon mucosa examined by the confocal laser-scanning microscopy (LSM). Fluorescent microparticles were observed in the associated mucus along the mucosal surface within colonocytes and goblet cells of the epithelium and lamina propria. Microparticles were also located with cryptal epithelium and pericryptal stroma. Serial optical sectioning (1 μM) by confocal microscopy clearly identified the position of the fluorescent microparticles within the tissues and established that their presence was not artefactual [Figure 7.3 and 7.4].

The mean numbers of fluorescent microparticles found within the cultured human colonic biopsies differed between normal and IBD groups (Table 7.2). In normal tissues, the numbers of fluorescent microparticles were similar irrespective of the incubation with or without LPS. The numbers of microparticles were markedly greater in IBD mucosa compared to normal mucosa. The numbers of fluorescent microparticles were further increased in IBD mucosa when incubated with LPS [Figure 7.5].

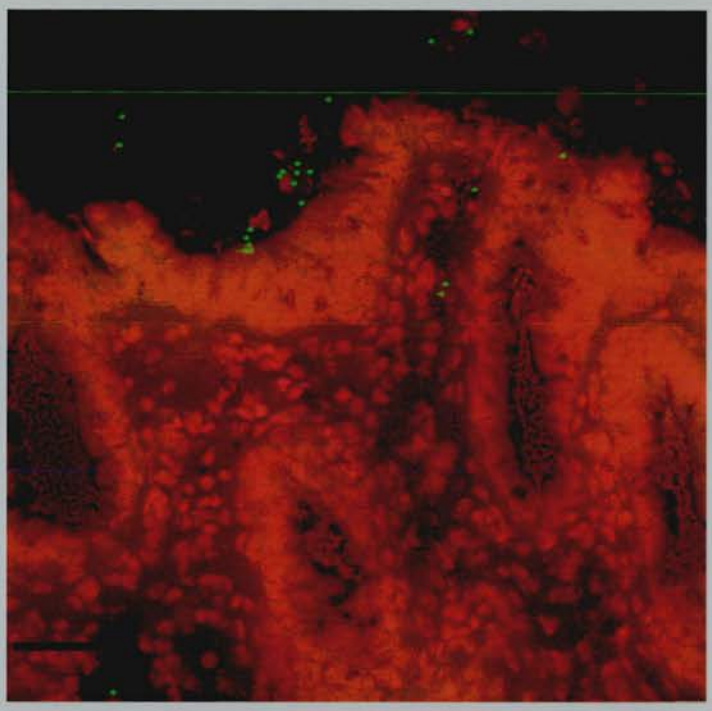

Figure 7.3. Confocal micrograph showing 2 μm fluorescent microparticles in IBD mucosa cultured in control media. Cryosections stained with propidium iodide. Scale bar = 20 μm.

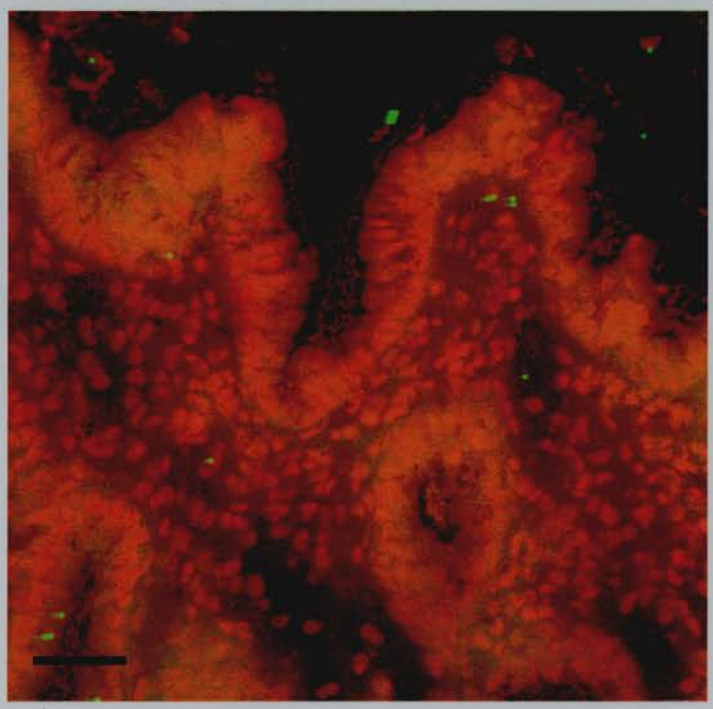

Figure 7.4. Confocal micrograph showing 2 μm fluorescent microparticles in IBD mucosa cultured in LPS containing media. Cryosections stained with propidium iodide. Scale bar = 20 μm.

Table 7. 2: Effect of lipopolysaccharide on transcytosis of fluorescent polystyrene microparticles in explant human colon mucosa

	Control (N=5)	Control +LPS (N=5)	IBD (N=5)	IBD+LPS (N=5)
Tissue area (mm^2)	2.83	2.78	2.78	2.82
Total count	81^a	98^b	211^c	686^d
†Count/mm^2	28 ± 2.5^e	36 ± 3.6^f	77 ± 21.7^g	245 ± 31.4^h

†Values are mean ±SEM; a vs. b, p = NS; a vs c and d, $p<0.05$; b vs c and d $p<0.05$; c vs d $p<0.01$; e vs f, p=ns; e vs g, $p=0.05$; e vs h, $p<0.01$; f vs g, $p=0.09$; f vs h, $p<0.01$; g vs h, $p<0.01$; t- test.

When IBD cases were dissected out to evaluate the effect of LPS, it appears that in four cases (2 Crohn's and 2 UC) LPS increased the uptake of microparticles (Figure 7.5 and Table 7.3). In one Crohn's cases there was minor increase compared to others. When the cases were evaluated the uptake of microparticles did not correlate to any clinical parameters examined.

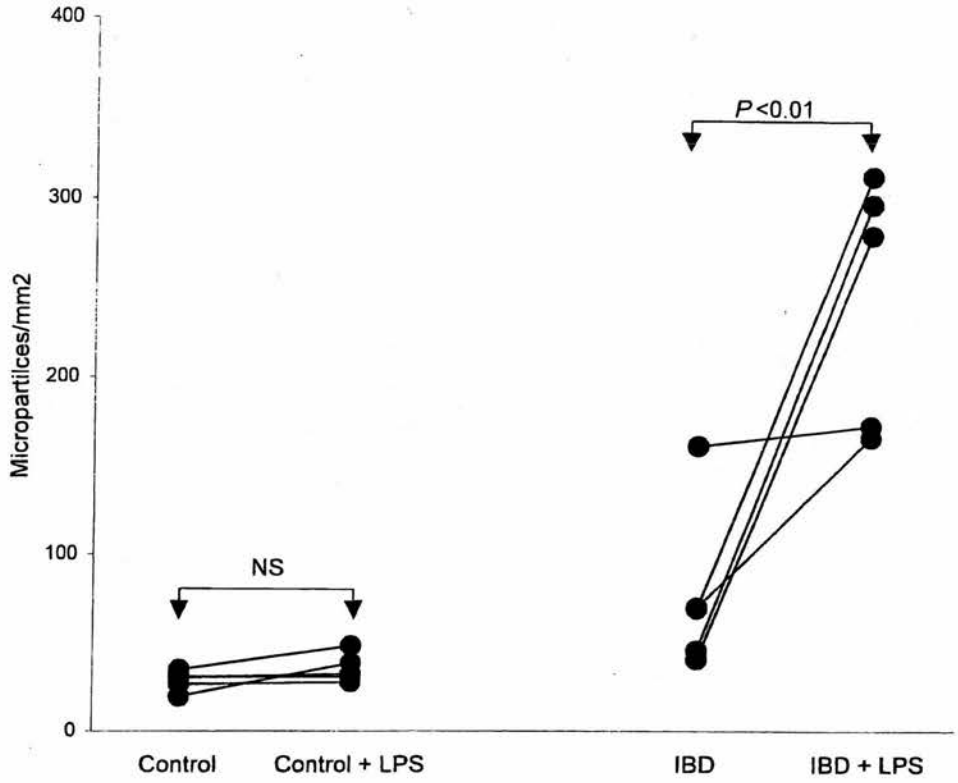

Figure 7. 5 Numbers of polystyrene microparticles/mm^2 in control and IBD subjects in presence of LPS. The values are mean of ten observations from a case. N=5.

Table 7.3: Data on number of microparticles/mm^2 in IBD patients

Patients	**Clinical diagnosis**	**IBD**	**IBD+LPS**
A	Crohn's	40.5	278.7
B	UC	69.1	311.7
C	Crohn's	160.8	172.2
D	Crohn's	70.0	165.6
E	UC	45.7	296.0
Mean±SEM		77.2±21.7[a]	244.8±31.4[b]

Values are mean of ten observations from a case. a vs b, $p<0.01$; t-test.

Electron microscopy

In transmission electron microscopic (TEM) studies, polystyrene microparticles were observed within the epithelial cells of the human colon in vesicles [Figure 7.6]. In the lamina propria, microparticles were present within macrophages [Figure 7.7]. The TEM studies confirmed the impression gained with the light microscopy that the epithelial morphology was well maintained during in vitro culture of human colon biopsies for 24h. Colonocytes appeared to maintain integrity with neighbouring cells as evident by intact tight junctions when the biopsies were cultured in medium containing LPS. The cytoplasm was compact, and the cytoplasmic organelles, including mitochondria, elements of endoplasmic reticulum, golgi complex and lysosomes were normal in appearance and distribution. The apical surfaces and basal lamina were structurally intact. The goblet cells in the crypts were filled with large mucus granules and appeared normal morphologically when viewed with the electron microscope. No polystyrene microparticles were seen in intercellular junctions.

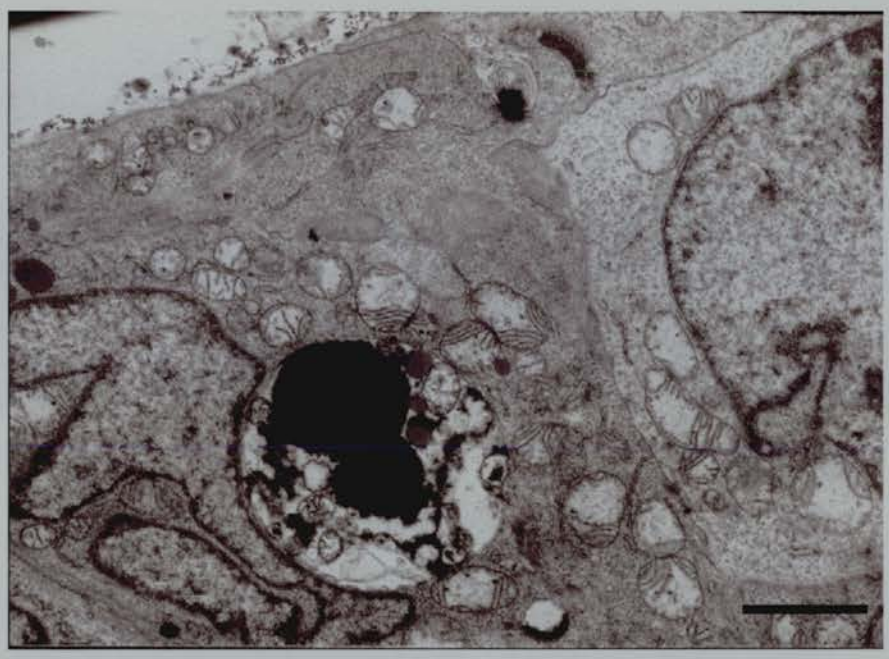

Figure 7.6. Electron micrograph showing 2 μm polystyrene microparticle within a colonocyte in LPS treated IBD tissue. Scale bar = 2.25 μm

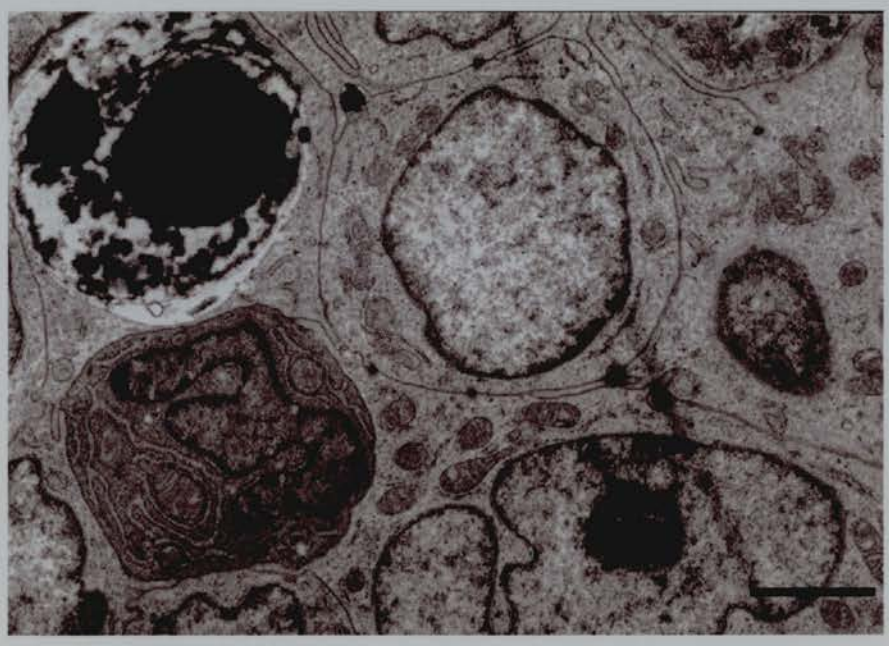

Figure 7.7. Electron micrograph showing 2 μm polystyrene microparticle within a macrophage in LPS treated IBD tissue. Scale bar = 2.25 μm

Discussion

Uptake and translocation of microparticles have been observed within the intestinal mucosa in previous studies after intragastric administration of polystyrene microparticle to rodents (20,48,253,254). In the present *in vitro* model, for the first time, uptake and translocation of polystyrene microparticles by human colonic epithelial cells have been demonstrated. Beneath the epithelium, fluorescent microparticles were observed in the lamina propria. Serial optical sections viewed by confocal laser scanning microscopy permit identification of fluorescent microparticles within a tissue (255), but the intracellular location of microparticles cannot be determined by this technique. By transmission electron microscopy, microparticles were located within the epithelial cells and in the macrophages in the present study. Uptake studies in animals by other investigators also indicated that microparticles were located within the cytoplasm of the epithelial cells and macrophages in vesicles (64,256). In this study, microparticles were present in the epithelium and beneath the epithelium in the lamina propria. The microparticles were attached to or in close proximity to the apical surface of the colonocytes, and the observation of microparticles within the cytoplasm of the colonocytes suggests that microparticles had entered through the epithelium of the human colonic mucosa in this experimental model. The observed presence of microparticles in vesicles suggests an endocytic uptake mechanism (56,63,77,242).

Microparticles were transcytosed in very small numbers by control colonic mucosa and LPS did not increase the numbers.

It is established that there is increased infiltration of newly recruited macrophages in the lamina propria of the inflamed intestine e.g., Crohn's disease (228-230). This has also been observed in this thesis (Chapter - 6). Lipopolysaccharide has been shown to liberate epidermal growth factors from mucosal cells such as macrophage (265). Addition of epidermal growth factors has been shown to increase uptake of microparticles (94). In the inflamed intestine examined in the present study, this effect is more pronounced due to the presence of increased number of macrophages and possibly would be less marked in the non-inflamed intestine due to the smaller number of macrophages. This is most likely the explanation for the lower uptake of microparticles by the control (non-inflamed) tissues observed in this thesis.

The significance of the transport of small numbers of microparticles in health is unknown. At least *in vitro*, healthy human colonic mucosa cannot completely exclude the uptake of microparticles as large as 2-μm in diameter. In IBD biopsies, greater numbers of microparticles were taken up compared to controls and this may be due to mucosal inflammation and altered histology. However, the significantly increased uptake of microparticles in the presence of LPS by the inflamed mucosa is an important observation and appears to indicate a possible effect of LPS on inflamed mucosa in IBD. LPS has been shown to liberate

epidermal growth factors (EGF) from the mucosal cells such as macrophages, fibroblasts and epithelial cells (252). EGF acts on EGF receptors (EGF-R) of the epithelial cells and has been shown to stimulate increased macropinocytosis in human epidermal cell culture studies (94). The molecular mechanism of increased macropinocytosis is not totally clear but growth factor induces membrane ruffling of the "edge end" of the cells and this appears to be regulated by members of the ras family and particularly the rho sub-family of ras-related GTPases. Increased macropinocytosis is the most likely explanation for the increased uptake of microparticles in IBD biopsies in the present study.

Transport of microparticles as well as bacteria and viruses through M cells has been documented in animal models (42,43,257). However, transport by transcellular (256) and paracellular routes has not been excluded in these models. In this *in vitro* experiment with human colon biopsies, microparticles were identified as inclusions within the cytoplasm of the colonocytes; the entry appears to be transcellular and this is likely to be by macropinocytosis (94,258). It has been suggested that microparticles may move through the epithelial barrier in a paracellular way (54), but in our study we have not found any evidence for this. The uptake and transcytosis of bacteria, 2-μm-size microparticles and yeast particles by intestinal epithelial cells has been demonstrated. The route seems to involve a transcellular step and not paracellular pathway (45,48,170).

The human colon is host to many commensal bacteria including *E.coli* and their cell wall products (LPS) and from this study it appears that LPS may increase the uptake of luminal microparticles. The significance of this uptake in relation to possible pathological states requires further studies.

CHAPTER 9: GENERAL DISCUSSION

For the first time, particles of chromium have been reported in Crohn's intestine. In this study, chromium particles were detected by energy dispersive analysis of X-ray (EDAX) in Crohn's intestine. Chromium is found most commonly as chromite. Chromite, chromium-iron oxide, occurs naturally as a dark brown or black mineral. The molecular weight of chromium is 52. Chromium as its compound may be present in various states: (i) Divalent chromium ((Cr (II)): chromous chloride and chromous sulphate; (ii) Trivalent chromium (Cr(III)): chromic oxide, chromic sulphate and chromic chloride; (III) Hexavalent chromium (Cr(VI): chromium trioxide, chromates, dichromates and polychromates. Chromium is widely used as metallic chromium in preparing special steels and alloys. Also, it is widely used in the glass and ceramic industries, in preparing paints and as a preservative in the wood industry. The adverse effect of chromium on health is associated with the valency state and water solubility. Hexavalent chromium is of most concern. The half-lives of chromium after a single exposure to rats is 84 days (259). The principal adverse effect of chromium on exposure is ulceration of the skin and mucous membrane. In experimental rats exposed to chromium, the metal has been shown to cause infiltration of alveolar macrophages and interstitial inflammation in lungs and is considered as immunogenic. There is no similar study in relation to the intestinal tissues. Thus the effect of chromium in human intestinal tissues is unknown and studies are required to evaluate the possible role of chromium in the

pathogenesis of chronic intestinal inflammation. Other than chromium, particles of aluminium, titanium, and silicon have been identified in IBD tissues. These particles are non-degradable and exist in their compound forms. Particles of aluminium, silicon, and titanium have been reported in a previous study. In this thesis, a second report on inorganic particles has been presented.

The second important observation in this thesis is the experimental evidence of luminal uptake of polystyrene microparticles by the human intestinal mucosa, and the increased uptake of microparticles by inflamed human intestinal mucosa in the presence of lipopolysaccharide. In other work with animal models, it has been observed that inert microparticles are taken up by intestinal epithelial cells. However, we did not have any information on whether such inert microparticles are taken up by human intestinal mucosa. In the present thesis this question has been investigated in an organ culture model of human intestinal tissues as it is not permissible to conduct such a study *in vivo* in humans. Organ culture has been widely used in respiratory medicine to study the uptake of environmental microparticles and to understand the pathogenesis of various diseases. However, studies with organ cultures of gastrointestinal tissues have been limited to investigating the toxic effects of substances such as gliadin, etc.

In this thesis, for the first time, the uptake of polystyrene latex particles has been clearly demonstrated in human intestinal tissues in an organ culture model. In this experimental model, fluorescent polystyrene microparticles were shown to be

taken into human intestinal tissues in organ culture. The uptake of polystyrene fluorescent microparticles by intestinal cells *in vivo* has been demonstrated by other workers. In the present thesis, fluorescent polystyrene microparticles were shown to be present in the human colonic epithelium and beneath the epithelium in the lamina propria by confocal laser scanning microscopy. This finding was further substantiated by electron microscopy showing polystyrene microparticles present on the apical side of the human colonic epithelial cells in vesicles. Polystyrene microparticles were not observed between the epithelial cells [paracellular]. This finding supports the view that the monodisperse latex microparticles were endocytosed by the human colonic epithelial cells. Regarding the mechanism of uptake of microparticles, no coated pits were visible in the vesicles, and this excludes the possibility of clathrin coated receptor-mediated endocytosis. Other than receptor-mediated endocytosis, the possibility of caveolin-mediated and non-coated vesicular uptake can be excluded by the size of the microparticles (2 μm) used in these experiments. Macropinosomes are mostly 0.5 - 2 μm but can be up to several microns in size. Therefore the uptake of the polystyrene microparticles observed in these experiments appears to be by macropinocytosis. This mode of uptake of microparticles by the intestinal epithelium has been proposed by other investigators in animal studies *in vivo*.

In this thesis an organ culture model has been developed to study the uptake of inert particles by human intestinal mucosa. The observation of the experimental studies presented demonstrates the endocytic mechanism of luminal uptake of

particulates by the epithelial cells. This also explains the possibility of uptake of inorganic luminal particulates such as chromium. However the possible pathogenic role of chromium in human intestinal tissues needs to be explored.

In the experiments presented in this thesis, human colonic biopsies were shown to be viable in short-term organ culture for up to 24 hours. Viability of the cultured tissue was assessed by good morphological preservation of the tissues as judged by light and electron microscopy. Incorporation of the ^{3}H-thymidine analogue bromodeoxyuridine in the DNA of the human colonic crypt cells indicates the synthetic capacity of the tissues in the present organ culture system. Morphology of the cultured tissues in the present study was well maintained as examined by light microscopy. This finding has been strengthened by ultrastructural examination of the cultured tissues by electron microscopy. Transmission electron microscopic examination of the cultured intestinal tissues has clearly demonstrated the integrity of the epithelium as indicated by intact tight junctions, detailed cell structure and organised subcellular structures such as active mitochondria, endoplasmic reticulum and secondary lysosomes. These findings indicate the stability of the present organ culture system and its suitability for studies related to luminal uptake of particulates.

Organ culture of human intestinal tissues offers several advantages. In this model it is possible to study the uptake of substances such as polystyrene microparticles or chromium that cannot be conducted *in vivo*. It provides a model

in which both epithelial and interstitial tissues are present in their anatomical arrangement. This is clearly an advantage over cell culture studies. The microparticles have been taken up by the epithelial cells and subsequently into the interstitium over the time of the study, mimicking the process in *vivo*.

However, this model has some limitations for uptake studies and these limitations must be kept in mind. *In vitro*, the process of uptake may be slower compared to the *in vivo* situation as only small amount of tissue is available for the experimentation. Regarding dosing of microparticles and size, it is difficult to predict what dose and size of microparticles really reflects the *in vivo* situation. This *in vitro* experimentation is in a static condition whereas *in vivo* the uptake of microparticles occurs in a dynamic state. However, in the present experiments biopsies were collected from the colon, where the dynamic state is less marked compared to that of the proximal intestine. Therefore, any environmental particles present in the lumen will remain for a longer period in this segment of the intestine. From this point of view, this model for uptake studies is probably not very much different from the real situation. The work presented in this thesis might be the beginning of further studies on uptake of microparticle in cultured intestinal mucosa. This model can be extended to study the effect of dose of lipopolysaccharide on uptake of the particles in tissues from different segments of the intestine. It is possible that uptake of polystyrene microparticles would be different if a higher dose of LPS had been used in the experiments presented in this thesis (chapter 7).

The direct observation of microparticles within the epithelium and in the lamina propria is one of the major strengths of this model in interpreting the results. Even then, the results may not be reflective of events that occur in the intestinal lumen where different factors are operating their role.

Most of the published literature suggests that M cells in follicle-associated epithelium are the portals of entry for bacteria, viruses and microparticles. It has now emerged that bacteria and microparticles (2-μm) are transcytosed by the intestinal epithelial cells. Results of *in vitro* experiments described in this thesis confirm this observation for human colonic epithelial cells, particularly in IBD.

Beneath the epithelium, polystyrene microparticles were observed in macrophages. This finding correlates well with the identification of immunostained macrophages containing inorganic microparticles in the lamina propria in the resected intestine of IBD patients presented in this thesis.

In this thesis, macrophages containing inorganic particles have been demonstrated in human intestinal mucosa. Macrophages in the intestine have been reported to be increased in inflammatory bowel disease. However, there are no data on inorganic microparticle-laden macrophages in human intestine. For the first time, data on inorganic-microparticle-laden macrophages have been presented in this thesis. The results of the quantitative image analysis of inorganic microparticle-laden macrophages in the human intestinal mucosa

indicate that numbers of inorganic microparticle-laden macrophages were significantly greater in intestinal tissues of Crohn's patients compared to controls. Also, it has been demonstrated that area occupied by macrophages in the inflamed intestine is significantly increased in inflammatory bowel disease. This is also the first reporting of the application of an image analysis method to study macrophages in human intestine.

Macrophages in the inflamed intestine are believed to be secreting proinflammatory cytokines e.g., TNF-α which is probably the key event in perpetuation /activation of inflammatory bowel disease. Several studies have demonstrated an increase in the macrophage population in IBD and the increase in microparticle-laden macrophages observed in the present study suggests that microparticles present in the lumen alone or in combination with other factors such as LPS may play a role in the activation of intestinal macrophages. It is therefore necessary to investigate the role of microparticles such as chromium in activating macrophages, which can be studied by *in vitro* experiments either by harvesting intestinal macrophages or by studies with a macrophage cell line. We do not have any information on the fate of macrophages that have phagocytosed inorganic microparticles whether ingested microparticles cause any damage or dysfunction of these cells. This also merits investigation to understand the duration of the functional capacity of the macrophages involved.

In conclusion, attempts have been made in this thesis to answer a few key questions on the possibility of luminal uptake of microparticles by human intestinal epithelium. The apparently important findings are:

1. Uptake of non-degradable microparticles does occur in human intestine. Any pre-existing pathology such as inflammation increases this process of uptake.
2. Uptake of microparticles is an endocytic process, more specifically macropinocytosis.
3. Lipopolysaccharide further augments the uptake of microparticles in IBD.
4. Inorganic microparticle-laden macrophages are significantly increased in Crohn's intestine.

Prospective studies on the possible role of non-degradable microparticles in the pathogenesis of IBD are merited and may yield useful information. It is possible to record the numbers of microparticles in biopsy specimens taken from newly diagnosed Crohn's patients and to correlate these findings with the course of the disease.

In parallel, similar studies are required in a population not exposed to diets with food additives, which are likely the main sources of non-degradable microparticles. A country with an agriculture-based economy is probably the right place to conduct such a study.

In this thesis, an organ culture model has been successfully applied to study uptake of microparticles by human intestinal tissues. This model may be applied to study uptake of similar other particles across human intestinal epithelium which is not feasible *in vivo*. Any study on definitive uptake of prion particles across human intestinal epithelium is lacking. This experimental model may be applied to study uptake of prion particles across human intestinal epithelium and may be extended to study the internalisation process of infectious prion proteins.

The technical work involved in these studies is time consuming. Organ culture, processing of tissues, confocal and electron microscopy, X-ray microanalysis and other related work such as image analysis are labour-intensive, and demanding processes. If time permitted, it might be rewarding to study uptake of microparticles in organ culture of human intestinal tissues at different concentrations of lipopolysaccharide and to evaluate whether an increased dose of LPS has any effect on increased uptake of microparticles by human intestinal tissues.

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