CHANGES IN VILLOUS BLOOD FLOW IN RESPONSE TO INDOMETHACIN

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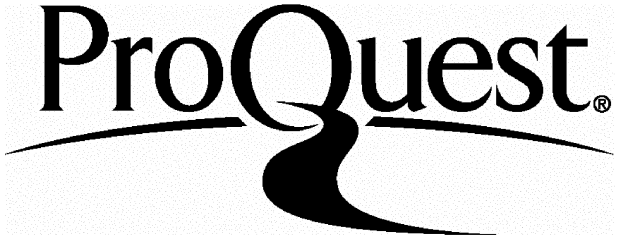

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Thesis Abstract

Introduction:Non-steroidal anti-inflammatory drugs (ie indomethacin) produce gastrointestinal ulceration. Early histology shows villous contraction, buckling of large vessels and distorted endothelium, suggesting that reduced villous blood flow might be involved at an even earlier stage. Blood flow was therefore calculated from measurements of red blood cell velocity and vessel diameters, obtained using *in-vivo* fluorescence microscopy. Villus histology was preserved at any time point by perfuse-fixation and isolation of villi displaying either stasis or reduced flow.

Aims: Firstly, to correlate histological alterations with dynamic blood flow changes, and secondly to assess the effects of the β_3-adrenoceptor agonist CL316 243 on blood flow and histology.

Results: Neither luminal or iv indomethacin altered villous blood flow or histology. However, combined luminal and iv indomethacin mimicking an oral dose, caused slowing of villous blood flow after 25 mins progressing to blood stasis within 45min. At stasis there was intense endothelial fluorescence, villous shortening, microvascular damage and distortion and epithelial damage limited to a single vessel. At slowing there was only intense endothelial fluorescence and microvascular distortion and damage. Ultrastructural analysis at the point of blood slowing showed endothelial vacuolisation and finger-like projections into the lumen of the villus microvasculature. The ultrastructural changes were more pronounced as blood stasis developed.

Activation of β_3-adrenoceptors with selective β_3-agonists such as CL316,243 prevented indomethacin-induced reduction of intestinal blood flow and prevented histological changes. CL316,243 also reversed indomethacin-induced blood stasis and histological damage.

Conclusion: Combined systemic and iv indomethacin causes

endothelial damage in villous microvasculature resulting in progressive slowing and stasis in the villus tip, which progresses to full necrosis and ulceration. These events can be prevented and reversed by activation of small intestinal β_3-adrenoceptors.

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Contents

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List of Abbreviations

ATP - Adenosine-tri-phosphate

BF - Blood flow

^{14}C - Carbon

°C - Centigrade

CCD - Charge coupled device

CNS - Central nervous system

COX - Cyclo-oxygenase

Cr - Chromium

D - Diameter

DNA-Deoxyribose nucleic acid

DTT - Dithiothreitol

EDTA - Ethylenediamine-tetra-acetic acid

EGF - Epidermal growth factor

FITC - Fluorocein Isothiocyanate

H^+ - Hydrogen ion or acid

HCO_3^- - Bicarbonate

H&E - Haematoxylin & Eosin

H_2O_2 - Hydrogen peroxide

I - Iodine

IBD - Inflammatory bowel disease

In - Inositol

L-NNA - N^G-nitro-L-arginine

mRNA - Messenger ribose nucleic acid

NA - Numerical aperture

NAC - N-acetyl-cysteine

NO - Nitric oxide

NSAID - Non-steroidal anti-inflammatory drug

O_2 - Oxygen

OH^- -Hydroxyl radical

PG - Prostaglandin

RBC - Red blood cell

RNA - Ribose nucleic acid

SIT - Silicon intensified

SMA - Superior mesenteric artery

T - Time

Tc - Technecium

TEM - Transmission electron microscopy

TGF-α - Transforming growth factor-α

TNB - Trinitrobenzene sufonic acid

V - Velocity

INTRODUCTION

The class of chemical compounds known as non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly used treatment for rheumatic conditions and gout, where they relieve joint pain and stiffness and provide analgesia. A single NSAID dose is an effective analgesic that is usually taken on demand for mild or intermittent pain (ie headaches etc). Taken regularly (ie daily) they provide long lasting analgesia and anti-inflammatory effects, making them useful in the treatment of the continuous pain normally associated with inflammatory conditions. Although there is very little difference in anti-inflammatory activity between NSAIDs, there is often considerable difference in patient response to a particular NSAID, so that several NSAIDs may have to be tried on a particular patient until an effective one is found.

NSAIDs come from a wide variety of chemical subclasses such as salicylates (aspirin), proprionic acid derivatives (naproxen), indoles (indomethacin) and oxicams (piroxicam). All have different physicochemical properties, greatly influencing where and how a particular NSAID has its effect. Lipid soluble NSAIDs such as naproxen and ibuprofen are able to penetrate the CNS and therefore have greater central effects (Netter et al, 1985; Goodwin & Regan, 1982).

The sub-classes also differ in the incidence and type of side effects they cause. The commonest side effect, usually resulting in discontinuation of drug treatment, is gastrointestinal ulceration and bleeding. Other less common side effects include hypersensitivity reactions (salicylates are especially implicated in asthma), headaches, dizziness and vertigo. Rarer side effects include blood disorders, fluid retention and reversible acute renal failure in elderly patients.

NSAIDs are the usual prescribed treatment of rheumatoid arthritis, gout, osteoarthritis and other inflammatory conditions (Bjarnason et al, 1989b). However, the well recognised gastrointestinal side effects of NSAIDs such as indomethacin form the main barrier to their use in treating inflammatory disorders. In fact NSAID use has been implicated in up to 12,000 gastric ulcer complications per annum in the UK alone, with huge financial implications in

treating these patients (Hawkey, 1996). This is supported by evidence showing that arthritis patients prescribed NSAID`s are six times more likely to develop gastrointestinal disorders, and have a 20% increased risk of developing haemorrhagic lesions than patients not on NSAID`s (Graham et al, 1988; Fries et al, 1989).

Whilst a great deal of literature and research is devoted to side effects of the upper gastrointestinal tract (ie stomach and duodenum), involvement of more distal areas has only been recognised in the last few decades. This research is still largely in its infancy, with most studies being concerned with increased intestinal permeability associated with NSAID use (Bjarnason et al, 1986; Jenkins et al, 1987; Bjarnason et al, 1989a; Aabakken et al, 1990; Bjarnason et al, 1991a, 1991b ; Davies & Rampton, 1991), whilst only a few studies attempt to define the primary pathogenic event in intestinal ulceration (Anthony et al, 1993; Anthony et al, 1995; Nygard et al, 1994). This thesis has been designed to address the early pathogenic effects of NSAIDs in the small intestine.

CHAPTER 1

REVIEW OF LITERATURE

1. Structure and Function of the Small Intestine

(a) General Structure of the Intestine:

The general structure of the small intestine is similar in both man and the rat, it can be thought of as a long tube divided into 3 segments, the proximal end joins the stomach at the pyloric sphincter and the distal end is continuous with the large bowel at the ileo-caecal junction. In man it comprises the duodenum (30-50 cm), and the jejunum and ileum (4-6m) which form the main absorptive areas of the gastrointestinal tract. The basic structure remains constant throughout the tube, consisting of serosa, smooth muscle layers, sub-mucosa, muscularis mucosae, and mucosa (Fig. 1).

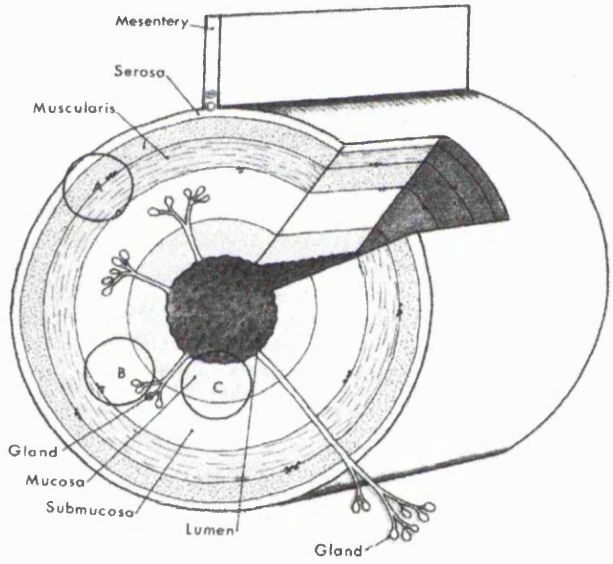


Figure 1. Transverse section of small intestine. A = muscle layers, B = sub-mucosa, C = mucosa.

The serosa is a single layer of squamous epithelial cells and connective tissue covering the smooth muscle layers. Smooth muscle is arranged in 2 layers (longitudinally and circularly). Its function is peristalsis, whereby circular muscle contracts proximally to a food bolus and relaxes distally, whilst at the same time longitudinal muscle relaxes proximal to the bolus and contracts distally. This has the effect of pulling the intestinal wall over the bolus moving it along the intestines. Peristaltic movement is co-ordinated by the enteric nervous system which is a complex organisation of ganglia, axons and interneurons arranged between the muscle layers and within the sub-mucosa.

The sub-mucosa consists of a loose irregular arrangement of connective tissue, containing lymphatic and blood vessels which supply the mucosa, muscle layers and serosa. There is also an extensive nerve plexus containing sensory fibres and parasympathetic, sympathetic and other intrinsic neurones (gut brain) involved in the control of secretions, peristalsis and blood flow. The mucosa will be described in detail later in this section.

The mucosa and sub-mucosa of the intestine form folds known as plicae circulares which encircle the lumen perpendicularly to the long axis of the tube, the number of these plicae decreases towards the ileum. Several differences occur between proximal and distal areas of the intestine; jejunum and ileum are attached to mesentery whereas the duodenum adheres to the posterior abdominal wall, towards the ileum villi gradually become smaller, lumen diameter is narrower, the intestinal wall becomes thinner and distal areas of the ileum have numerous large discrete aggregations of lymphatic tissue (Peyers patches).

(b) Mucus/bicarbonate barrier (mucosal/luminal interface):

Mucus is secreted by the surface goblet cells of the gastrointestinal tract, and comprises a continuous visco-elastic gel layer of glycoproteins adherent to the underlying epithelium. There are two dynamic phases within mucus, the upper phase in contact with the lumen, and is mostly liquid to provide lubrication; it is constantly solubilised and degraded by luminal enzymes such as pepsin. The lower phase is a thicker more viscous substance constantly renewed by hydration of mucin granules at the epithelial cell surface; it is often referred to as the unstirred layer (Allan, 1978).

The unstirred layer is essentially a layer of unmoving liquid. It has been known for a hundred years that despite the most vigorous stirring, water next to a solid surface will remain unstirred. Dietschy, (1971) was the first to demonstrate this effect within the gastrointestinal tract in the 1970`s, at which time it become a focus for intestinal absorption studies.

The mucus layer separates luminal contents from the intestinal villous

brush border. Several studies have measured the mucus layer in both man and the rat, producing values of between 81 and 830μm (Read et al, 1977; Flemstrom & Kivilaaskso, 1983; Sparso et al, 1984; Frase et al, 1985; Sarosiek et al, 1991; Allen et al, 1993; Sababi et al, 1995). However only 2 of these studies were performed in-vivo (Flemstrom & Kivilaasko, 1983; Sababi et al, 1995). The others based their results on complex mathematical calculations performed on data produced by measurement of potential difference between mucus and mucosa. They assumed in their calculations that perfect mixing was taking place, which the theory of the unstirred layer does not allow; an assumption which probably led to the very high mucus thickness values of 600μm. A mucus layer this thick would become the rate limiting factor in absorption of nutrients and would negate the advantages of increased surface area provided by villi and their brush border. Therefore the consensus was that a value of 600μm was clearly incorrect.

The two *in-vivo* studies involved insertion of micropipettes through the mucus until the tip of a villus was reached, to measure the distance between the mucus surface and villus tip. This was an error prone procedure because the authors failed to consider potential villous movement, villi are very delicate structures likely to be moved by a rigid micropipette therefore giving a false impression of mucus thickness. Mucus also obscures microscopic visualization of the villus tip, further complicating accurate placement of the pipette, accounting for the variations in results produced by both studies. Strocchi (1991), taking into account imperfect mixing, showed that the unstirred layer was around 40μm, which is probably closer to the actual value.

The mucus layer in the stomach and duodenum is alkaline to protect against harmful luminal acids secreted in the stomach. The epithelial goblet cells and Brunners glands in the duodenum constantly secrete bicarbonate ions into the mucus to provide a pH gradient within the mucus, providing the first line of defence against luminal contents and acids (Flemstrom & Kivilaasko, 1983; Sarosiek et al, 1991; Allen et al, 1993). This produces a near neutral pH micro-environment close to the mucosal surface. Microelectrodes have

demonstrated that a stable pH gradient is maintained in both the stomach and duodenum for several hours, with luminal pH at 2-3 and cell surface pH at near neutral (Scarpignato, 1995).

The release and renewal of mucus from mucosal surface goblet cells is a dynamic continuous process. Sababi (1995) demonstrated in the rat, rapid renewal of mucus within 5-15 min of its removal, confirming earlier results showing complete discharge of goblet cell intracellular mucin within minutes of physical/chemical irritation (Specian & Oliver, 1991; Allen et al, 1993). Secretion from mucosal surface goblet cells is induced by surface acting agents (Forstner, 1978). Goblet cells can refill within 60 min of the initial secretory event (Sababi et al, 1995; Specian & Oliver 1991): however while renewal continues the rate of secretion is reduced (Specian & Oliver 1991; Neutra & Forstner, 1987).

Parasympathetic nervous system activity has also been conclusively shown to depress mucus output from intestinal crypt goblet cells (Specian & Neutra, 1982), and is relatively easy to control. There is however, no way of controlling secretions from the goblet cells at the tip of the villus.

In summary the mucus layer acts by establishing a step wise gradient against harmful substances, so that the concentration of injurious agents is lower on the cell side than on the luminal side. It also provides a physical barrier preventing the penetration of proteolytic enzymes such as pepsin (Allan, 1978), and provides lubrication to facilitate passage of luminal contents, without preventing the absorption of nutrients.

***(c) Structure of the Mucosa*:**

The mucosal surface is covered by villi (around 0.5 - 1.5 mm in height), at the base of the villi are the crypts which are tube like structures opening between the bases of adjacent villi (0.3-0.5mm deep). The luminal surface of the mucosa is covered by columnar epithelium comprising (i) absorptive enterocytes which have microvilli for nutrient absorption, or (ii) goblet cells, secreting mucus. Epithelial cells are produced at the base of each villus in the

crypts of Lieberkuhn from undifferentiated columnar epithelial cells, which are shorter than the surface epithelium. These basal cells undergo mitosis to provide new enterocytes which gradually migrate to the villus tip where they are shed into the lumen after approximately 5 days (Ross & Romrell, 1990). Sub-mucosal glands are also present in the crypts and secrete mucus. Other intestinal glands at the base of the villi include Paneth cells which contain eosinophilic granules responsible for secreting lysozymic enzymes, which regulate intestinal microbial flora (Ross & Romrell, 1990).

The core of the villus (lamina propria) consists of loose connective tissue. Lymphocytes, and eosinophils are particularly prominent having migrated from blood vessels. Individual smooth muscle cells orientated length wise in the villus core, are continuous with the muscularis mucosae, and usually associated with the central lymphatic capillary. This lymphatic is involved in the absorption of fats. The lamina propria also contains the microvasculature which provides the villus blood supply (see below).

(d) Blood Supply to the Small Intestine:

The blood supply of the small intestine is derived from the superior mesenteric artery which supplies the mid gut, that is from the distal duodenum down to approximately the left colic flexure. The artery branches from the descending abdominal aorta at around the level of the pancreas, it passes in front of the duodenum towards the ileocaecal branch of the intestines where the mesentery begins. After supplying the distal half of the duodenum the superior mesenteric artery gives off around 18 smaller branches which fan out in the mesentery to supply the jejunum and ileum, these smaller arteries unite to form a series of loops and arches.

As the vessels approach the bowel they form a tier arrangement of anastomoses, eventually forming the vasa recta which pass up the sides of the intestine before entering through the muscle layers where they anastomose to form the sub-mucus plexus. From this plexus mucosal arterioles arise, pierce the muscularis mucosae and supply the mucosa, branches from this plexus also

supply the muscle layers. The number of vascular tiers supplying the intestine increases distally.

***(e) Microvasculature of Mucosa*:**

The classic study performed by Spanner 1932, quoted by Gannon (1981) using microvascular corrosion casting and direct observation microscopy suggested three blood supply patterns in the intestinal villi of the rabbit and man (Fig. 2).

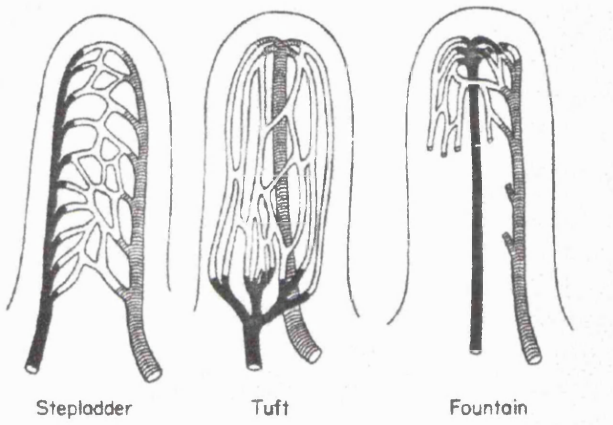


Figure 2. Diagramatic representation of villus circulation as described by Spanner (1932).

This was later modified by Gannon (1981) who showed that the villus tip received blood from a vascular fountain arrangement whereas the villus base was supplied by a tuft arrangement (Fig. 2). The same vascular arrangement was shown in rat villi by Vogt (1980), using a combination of microvascular corrosion casts, ink injection and fluorescence microscopy; these findings have since been confirmed by Anthony (1995; 1996a). The techniques are best used in combination as on their own they do not provide the full picture, ie corrosion casts and ink injection provide excellent visualization of the microvasculature but are limited in that they are artificial perfusion methods and do not always perfuse the microvessels completely. *In-vivo* fluorescence

microscopy is superior in that the animal is perfused with its own blood at the correct pressure ensuring all the arterioles, capillaries and veins are perfused; however, it can only provide a two dimensional image whereas a three dimensional image requires microvascular casting or confocal microscopy.

Comparison of the vascular architecture as described by Vogt 1980, and Anthony 1995; 1996a with that described by Gannon 1980 suggest a similar vascular pattern in human and rat villi, although no reports exist that directly compare the two. However, in the rat there is disagreement about the placement of the arteriole supplying the tip with Miller et al (1969) placing it centrally and Vogt (1980) asymmetrically. The images presented by Vogt (1980) would suggest that the central arteriole is asymmetrically orientated.

In vivo microscopy in the rat has shown the central supplying arteriole bifurcates at the villus tip into 2 vessels which run along the outer edge of the villus (arcade vessel), close to the epithelial basement membrane. Capillaries cascade away as branches from the arcade vessel, forming a fountain pattern, and converge approximately midway between the base and villus tip to form 2 venules, one each side of the villus, see figure 2a. (Vogt 1980).

The vascular fountain at the villus tip has been well described. In contrast the tuft vascular supply to the villus base is not as well described in the literature. There is a single report in the rat using microvascular corrosion casting which suggests a direct arteriolar supply (quoted in Gannon 1981). However, neither Gannon, Vogt or Miller et al state whether these two different circulations communicate with each other (not clear from the images) but Gannon demonstrated that both circulations drain into the same venules.

The microvascular anatomy has been shown to alter with age, whereby villi in older rats coalesce forming one large ridge shaped villus. The vasculature of the coalesced villus remains basically the same except that instead of one central arteriole there are two, generally arranged towards the edges of the villus (Miller et al 1969).

Placed centrally within each villus is a lymphatic capillary known as a lacteal. It begins at the villus tip as a blind capillary and drains into larger

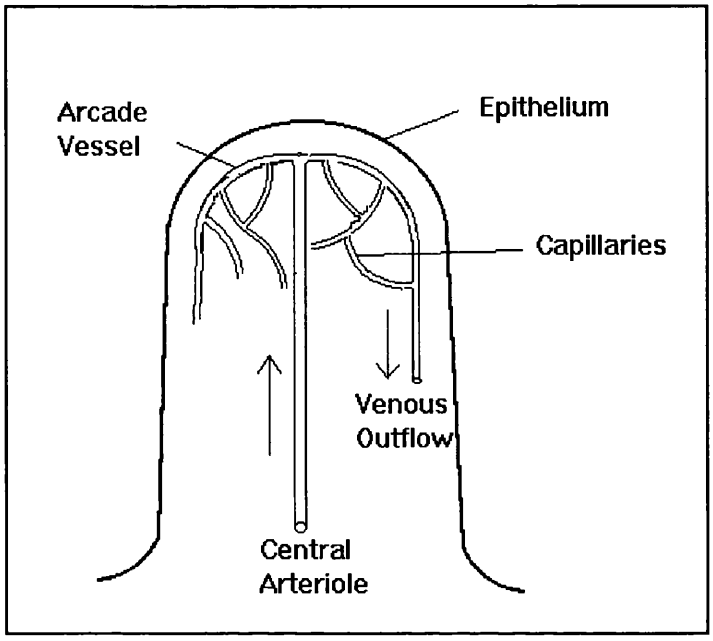


Figure 2a. Diagramatic representation of villus microcirculation as seen by *in vivo* microscopy, the arrows indicate the direction of blood flow. Arcade vessel 10-12μm, central arteriole 20-25μm.

lymphatic vessels in the sub-mucosa, its principal role is fat absorption. Consequently it becomes dilated after a meal when absorption is at its greatest, otherwise it is collapsed and not generally visible.

(f) Enteric Nerves

There are over 7 million enteric neurones controlling motility, secretion and blood flow in the gastrointestinal tract (Furness et al 1992). The enteric nervous system consists of a network of small ganglia and connecting nerve strands divided into 2 plexuses, the myenteric plexus between the circular and longitudinal muscles and the sub-mucus plexus within the submucosa.

The myenteric plexus is made up of densely packed, interlinked ganglia running from the pharyngo-oesphageal junction to the anal sphincter (Furness et al, 1990). The sub-mucus plexus is only interconnected in the small and large intestine. Within the intestinal mucosa there is a dense network of nerve fibres. These fibres are in close proximity to the epithelial cells and all the other elements of lamina propria, such as mast cells, blood vessels, lymphocytes and the muscularis mucosae.

The patterns of motility, secretion control and blood flow have been shown to be similar within most mammalian small intestine, however differences do arise in the transmitters released from these neurons in different species (Furness et al 1990).

2. NSAID-induced Pathology in Man

(a) Stomach and Duodenum:-

The general pathology of NSAID-induced gastroduodenal ulceration is outlined below. Certain features will be developed more fully in subsequent sections.

The time scale and sequence for early NSAID-induced (particularly aspirin) ulceration is now documented in the human stomach. It begins with epithelial damage comprising intracellular oedema (highlighted by lack of toluidine staining density compared with controls) and cytoplasmic

vacuolisation 10 minutes after exposure to a 1200mg aspirin solution (equivalent to 2 standard tablets) in the proximal stomach (Baskin et al, 1976). At 30 minutes endoscopic studies using a similar dose (1200-1300mg aspirin) show multiple gastric petechiae which become more pronounced at 1-2 hours (O'Laughlin et al, 1981; Graham et al, 1983; Gilbert et al, 1984). Petechiae and gastric erosions are common in patients using NSAIDs, even for only 24 hours (O'Laughlin et al 1981; Hoftiezer et al, 1982). Another study suggested that around one third of all patients using NSAIDs went on to develop gastric erosions (Larki et al, 1987). However, the presence of erosions does not predict the propensity of any given NSAID to progress to ulceration, bleeding and perforation (Graham, 1990). In fact erosions have been shown to come and go with continued NSAID use (Larki et al, 1987), which has led to confusion over choice of NSAID therapy, treatment of erosions, and their importance in pathology. The antrum is the area of the stomach most susceptible to erosions (Metzger et al, 1976; O'Laughlin et al, 1981; Hoftiezer et al, 1982), and it is also the area most prone to NSAID-induced severe mucosal haemorrhage and ulceration (O' Laughlin et al, 1981; Graham, 1983; Gilchrist et al, 1987), suggesting that erosions are not an unimportant NSAID effect.

The role of gastric acid in NSAID-induced gastric ulceration is equivocal. On the one hand the H_2-antagonist ranitidine, which reduces gastric acid output, improves gastric healing but exposure to NSAIDs reverses this healing, and on the other hand the proton pump inhibitor omeprazole, still increases the rate of ulcer healing in the presence of NSAIDs when acid production is maximally inhibited (Soll et al, 1991). It is likely that the different effects of NSAIDs with these drugs is related to their different mechanisms.

A well documented effect of continued NSAID use in man, is mucosal adaptation (Wallace, 1997), whereby the mucosa becomes resistant to NSAID-induced damage. Adaptation has been defined as "the process by which visible gastric mucosal injury lessens, and may resolve entirely despite continued administration of an injurious substance such as aspirin" (Graham et al, 1983).

It remains unknown why some individuals adapt to NSAIDs but others go on to develop ulceration and haemorrhage which further complicates our understanding of the mechanism behind gastric ulceration.

A recent animal study showed that indomethacin produced shallow erosions in the gastric corpus of fasted rats with no antral ulceration, and the reverse in fasted-refed rats (Anthony et al, 1996b). At present there are no adequate explanations to support a mechanism for this observation. However, it was hypothesised that refeeding fasted rats increased blood levels of insulin and noradrenaline. The subsequent increase in sympathetic activation would cause vasoconstriction and reduce blood flow which in conjunction with indomethacin induced reductions in mucosal blood flow causes ulceration (Kuratani et al; 1994). However, Kuratani could only postulate that reductions in blood flow were linked to systemic insulin release, as gastric mucosal blood flow in the antrum was not correlated with blood levels of insulin or noradrenaline.

No study to date has adequately addressed why NSAIDs have an effect on the gastric corpus in both man and rats (Anthony et al; 1996b; Wallace, 1997).

(b) Small Intestine:-

NSAID-induced side effects and complications in the small intestine are not as common as those in the stomach and are mostly described in case reports (reviewed by Aabakken & Osnes, 1989). Patients are often asymptomatic, and when symptoms are described they are more difficult to investigate than in the stomach or large bowel, consequently the true prevalence of NSAID enteropathy is unknown. When complications do occur they are often more serious because they are not immediately obvious so the NSAID is not immediately withdrawn as in the stomach. Complications in the small intestine differ from the stomach in that there is no luminal acid in the intestine which pre-disposes the stomach to ulceration, it is the presence of luminal bacteria and enterohepatic recirculation of NSAIDs which are strongly

implicated in the pathogenesis of intestinal lesions.

The most common NSAID-induced pathological effects in the small intestine comprise (i) increased protein loss (ii) increased intestinal permeability (iii) intestinal bleeding (iv) low grade inflammation (v) chronic inflammation ; collectively these effects are known as NSAID enteropathy (Bjarnason et al, 1993; Wallace, 1997).

(i) *Increased Protein Loss*

Increased protein loss is a controversial element of NSAID-enteropathy. It is based on the observation that 10% of all patients requiring hospitalization for rheumatoid arthritis suffer from hypoalbuminemia (Bjarnason et al, 1993). It is unclear whether increased protein loss is a function of the arthritic condition or of the NSAIDs, as only a small study of 9 NSAID using patients has been performed. Inflammation and protein loss was assessed by comparing faecal excretion of ^{51}Cr-labelled chloride with serum levels from the previous day to give a mean intestinal plasma loss (Bjarnason et al, 1987a). Six out of the 9 patients on this study had increased protein loss as well as other recognised NSAID effects, such as inflammation and increased permeability, (discussed below). However, the patients used in this study were all on NSAIDs for at least 6 months prior to the study so despite being highly suggestive no definite conclusion can be drawn. A definite conclusion about protein loss can only be made after much larger studies with better controls to rule out protein loss as an effect of the disease rather than NSAIDs.

(ii) *Increased Intestinal Permeability*

NSAID-induced intestinal permeability is widely regarded as a primary ulcerogenic mechanism (reviewed by Bjarnason et al, 1993; Aabakken & Osnes, 1989; Wallace, 1997). Increased permeability enhances the uptake of harmful macromolecules and allows the passage of luminal bacteria into the lamina propria leading to inflammation. Highly specific, noninvasive tests have been devised to assess permeability ie. its barrier function (Menzies, 1984; Cooper, 1984; Hamilton 1986). The subject is administered an oral test probe and its urinary recovery is measured to assess intestinal function (Maxton et

al, 1986). The most common probes are polyethylene glycol 400, di-monosaccharides and ^{51}CrEDTA, all of which have been used to assess intestinal permeability in rheumatoid arthritis patients (Bjarnason et al, 1984; 1986; Jenkins et al, 1987; Aabakken & Osnes, 1990). There were marked increases in intestinal permeability in all rheumatoid and osteoarthritis patients on NSAIDs, as compared to those not treated with NSAIDs and in healthy volunteers (Bjarnason et al, 1984; 1986; 1989a; 1991a; 1991b; Jenkins et al, 1987; Aabakken & Osnes, 1990; Davies & Rampton, 1991).

Only 1 study has provided a time scale for permeability increases, where 2 doses of ibuprofen (400mg), naproxen (500mg) or indomethacin (75mg) were shown to increase permeability within 12 hours in all 10 healthy volunteers (Bjarnason et al, 1986), becoming more profound if the subject did not eat prior to ingestion of NSAID (Bjarnason et al, 1992). Two reports suggest that a high luminal concentration of NSAID is responsible for increased permeability because pro-NSAIDs (which do not become active until after absorption) such as nabumetone and sulindac don't produce a high luminal concentration of active NSAID, and do not increase permeability in healthy volunteers. However, indomethacin which does produce a high luminal concentration of active drug does increase permeability (Bjarnason et al, 1991a; Davies et al, 1991).

Evidence exists to suggest that prostaglandins are important in maintaining a normal mucosal barrier, as exogenous prostaglandins reduce indomethacin-induced alterations in permeability in man (Bjarnason et al, 1989a). Prostaglandins are therefore sometimes administered together with an NSAID (Shah et al, 1993). Recent contradictory evidence suggests that although increased paracellular permeability is an early pathogenic effect it is almost certainly not the initiating factor in NSAID enteropathy. Reuter et al (1997) demonstrated that increased intestinal permeability did not necessarily progress to significant mucosal injury, based on the observation that nitrofenac (derivative of diclofenac) increased permeability in the rat without mucosal damage. Moreover, permeability changes in patients with rheumatoid arthritis

have been recognised prior to NSAID use (Tageson et al, 1983), without progressing to intestinal ulceration.

(iii) ***Intestinal Bleeding***

Intestinal bleeding is now a recognised side effect of NSAID use with a number of studies providing 3 pieces of evidence linking bleeding with NSAID-induced enteropathy. Firstly, upper endoscopic examination (stomach and duodenum) has failed to show gastroduodenal bleeding in NSAID treated patients even when blood loss has been identified in the faeces (Collins & du Toit, 1987) and has led to anaemia (Bahrt et al, 1984; Bartle et al, 1986; Collins & du Toit, 1987), suggesting blood loss from a more distal area of the gastrointestinal tract.

Secondly, in a study designed to show the localization of bleeding and inflammation, 32 NSAID patients were given ^{111}Inositol (In)-labelled neutrophils and ^{99m}Technicium (Tc)-labelled red cells and scintigraphy was performed 20 h later (Bjarnason et al, 1987a). Of the 32 patients, 19 had identical localization of labelled red cells and neutrophils in the right iliac fossa suggesting blood loss from a site of inflammation, the rest were normal. Enteroscopy of these patients would have provided much more conclusive results, but this an extremely difficult procedure and is only rarely performed, consequently only a few studies exist and are discussed below. The low grade nature of the blood loss was the reason it was not detected in the lumen of the bowel until it was collected by the natural bottleneck created by the ileo-caecal junction. However, it is also possible that inflammation was localised to the ileo-caecal region.

Finally, studies to quantitate and correlate inflammation and blood loss in NSAID patients using 4 day faecal excretion of ^{111}In-labelled neutrophils and 5 day faecal excretion of ^{99m}Tc-labelled red cells demonstrated significant quantitative correlation between inflammatory activity and intestinal blood loss (Bjarnason et al, 1988a; Teahon et al, 1990). These studies showed that faecal excretion of leucocytes was not due to simple haemorrhagic leaking from the intestine, because if 3% of the labelled leucocytes were excreted then this

should be accompanied by a 3% blood loss, which in humans would mean 150ml of blood per day. This figure was much higher than the 1-12 ml of blood per day normally lost by NSAID patient, therefore the leucocytes must be actively infiltrating the lumen of the intestine and were not a consequence of bleeding (Bjarnason et al, 1993). Further evidence for NSAID-induced small intestinal bleeding was shown in a small group of patients suffering from both rheumatoid arthritis and iron deficiency anaemia unexplained by gastroscopy or colonoscopy (Bjarnason et al, 1990). Enteroscopy of these patients with occult blood loss showed small intestinal haemorrhagic lesions, subsequently confirmed by a second study (Morris et al, 1992).

However, it is difficult to interpret whether the quantity of intestinal blood loss produced by NSAIDs is a significant factor in the anaemia associated with their use, as a normal healthy menstruating woman has an average blood loss of 5-10ml/day without becoming anaemic. This figure is similar to the 1-12ml/day of blood lost by patients with gastric and colonic malignancies who also suffer from iron deficient anaemia (Dybdahl et al, 1984). Its possible that NSAID-induced intestinal blood loss is additive to other factors such as NSAID-induced dyspepsia because of anti-acid treatments, inability to effectively reduce iron or an inability to increase iron absorption in response to low iron (Boddy et al, 1969;Weber et al, 1988), and hence lead to anaemia. Indeed iron deficiency has been shown in 50% of arthritis patients (Cartwright & Lee, 1972; Hansen et al, 1983; Vreudgenhil et al, 1990). Current studies suggest that intestinal bleeding is an effect of NSAIDs but its importance is unknown because of the difficulty in actually observing it.

(iv) ***Low Grade Inflammation***

Inflammation comprises three distinct stages; (i) an acute phase characterised by local vasodilation and increased capillary permeability (ii) a late phase characterised by infiltration of leucocytes and phagocytic cells, (iii) a chronic proliferative phase characterised by tissue degeneration and fibrosis. The late phase of inflammatory infiltration of leucocytes (in this case

neutrophils into the intestinal lumen) has been well documented in a series of excellent studies, associating NSAID use with intestinal inflammation in man (Bjarnason et al, 1984, 1986, 1987a & 1987b). The studies showed ^{111}In-labelled neutrophils being exuded from the site of inflammation into the bowel lumen where they pass down the intestines, where they were measured either in the intestines or in the faeces by scintigraphy. Large, well controlled patient groups, were used to show that 50% of rheumatoid/osteoarthritis patients on NSAIDs for at least 6 months had increased luminal neutrophil migration than comparable arthritis patients, not on NSAIDs (Bjarnason et al, 1984 & 1987b).

In NSAID-induced inflammation the inflammatory exudation of ^{111}In-labelled neutrophils into the lumen is measurable by scintigraphy 20 hours after injection of labelled neutrophils in the right iliac fossa (Segal et al, 1986; Rooney et al, 1986; Bjarnason et al, 1987b; 1993; Morris et al, 1992), the time point indicates the rate and severity of exudation. This is mild in comparison to inflammatory bowel disease which can be used as a model for severe inflammation, where labelled neutrophils are measurable after only 4 hours (Saverymuttu et al, 1983a, 1983b & 1986). However, one group suggested that inflammation was a primary result of rheumatoid arthritis and not due to NSAIDs (Segal et al, 1986). However, Segal (1986) used arthritic patients who had been sufferers for a minimum of 2 years and had been on NSAIDs for this period, with controls also exposed to NSAIDs for a few months, making it very difficult to draw a meaningful conclusion from this study.

NSAID-induced intestinal inflammation was supported by enteroscopy of the distal small intestine (Morris et al, 1992), which highlighted mucosal red spots, erosions, surrounding villus atrophy and frank ulceration, which compared well with leucocyte studies which suggested a similar inflammatory response (Bjarnason et al, 1988). The clinical importance of mucosal red spots has been questioned (Lewis, 1991), and although Morris et al (1992) suggests they are not artifact, they failed to take biopsies for histology which would have confirmed their importance.

The time scale of NSAID-induced inflammation is currently unknown,

as no prospective or sequential studies exist, although a small study by Bjarnason, (1993) did show inflammation in 4 out of 11 patients treated for less than 6 months with NSAIDs. It is known however, that once inflammation has developed it can persist for up to 16 months after cessation of NSAID therapy (Bjarnason et al, 1987b). Conclusive evidence for NSAID-induced intestinal inflammation awaits a study which combines labelled leucocytes with small bowel enteroscopy.

(v) Chronic Inflammation

Strictures and Diaphragms

Chronic inflammation is a rare event in the small intestine and difficult to document because many patients are asymptomatic. Lang (1988) in a retrospective study over 16 years found that out of 457 intestinal resection cases only 7 were caused by NSAID use. Several reports have associated NSAID use with either non specific broad based or concentric diaphragm-like intestinal strictures (Johnson, 1987; Lang et al, 1988; Bjarnason et al 1988b; Hershfield, 1992; Bjarnason et al, 1993). Histological examination of these strictures shows submucosal fibrosis with normal epithelium except for the mucosa directly overlying the stricture area which contains chronic and acute inflammatory cells. Lang (1988) postulated that strictures are formed by repeated ulceration and low grade mucosal inflammation followed by submucosal fibrosis. Unfortunately this study was performed on small bowel resection patients who had been on NSAIDs for at least 3 years, so the time scale for developing diaphragms is unknown.

NSAID-induced diaphragms are generally localised to the mid small intestine, exceptions include one patient on slow release NSAID who developed diaphragms in the ascending colon, and one rheumatoid patient with diaphragms in the terminal ileum (Teahon et al, 1991; Huber et al, 1991).

Ulceration and Perforation

In the case of intestinal ulceration and perforation, a small case-control study showed that NSAID using patients were twice as likely to develop small intestinal perforations compared to non-NSAID users (Langman et al, 1985).

Langman showed that 36 out of 161 patients who had perforated had taken NSAIDs compared to only 10 out of 161 control patients with uncomplicated lower bowel disease.

Other more anecdotal evidence has shown that indomethacin use in the treatment of patent ductus arteriosus in low birthweight babies can cause intestinal perforation (Nagaraj et al, 1981; Alpan et al, 1985; Marshall, 1985), as has the slow release indomethacin capsule Osmosin® (now withdrawn) (Day, 1983; Florence et al, 1984; Cree et al, 1985; Laidler et al, 1985). Although indicative of NSAID-induced small intestinal perforation, it is difficult to draw conclusions from case reports. Conclusive evidence for mechanisms and risk factors requires a planned retrospective study, but the rarity of this side effect precludes this approach.

(c) Colon:-

Oral NSAID doses have been shown to induce *de novo* colitis which is dependent upon patient and NSAID used. For example naproxen has been implicated in eosinophilic colitis in one patient (Bridges et al, 1990). Tanaka et al, 1991 demonstrated the general use of NSAIDs produced collagenous colitis in 19 out of 30 patients, a link suggested by other reports (Bunney, 1989; Giardello et al, 1990). Colonoscopy of patients with NSAID-induced colitis showed mild patchy erythema/edema or more serious inflammation and ulceration which often resembled ulcerative colitis (Gleeson et al, 1994). These areas had pronounced inflammatory changes shown histologically by Lee (1993), who went on to suggest that increased apoptotic bodies in the colonic crypts with intra-epithelial lymphocytes are the characteristic sign of NSAID-induced colonic inflammation.

NSAID-induced colonic strictures/diaphragms similar to those found in the small intestine, have also been shown in several studies (Huber et al, 1991; Haque et al, 1992; Whitcomb et al, 1992; Pucius et al, 1993; Halter et al, 1993). The localisation of strictures to the descending colon decreasing in frequency and severity towards the hepatic flexure with NSAID suppositories

suggests that unabsorbed drug is responsible rather than a systemic effect. If it were a systemic effect more strictures would occur in areas proximal to the hepatic flexure which do not come into contact with any topical NSAID from a suppository.

Taken as a whole NSAIDs only rarely adversely affect the large bowel. This was demonstrated by a colonoscopy study of 170 patients receiving NSAID therapy for rheumatoid and osteoarthritis none of which developed colonic inflammation (Cuvelier et al, 1987). The most dangerous effect of NSAID use is in triggering the relapse of ulcerative colitis.

3. Introduction to NSAID Mechanisms

Mucosal integrity in the gastrointestinal tract is achieved by maintaining a delicate balance between aggressive luminal and defensive mucosal factors. Aggressive factors include acid-pepsin (Soll et al, 1991) in the stomach, and bile salts (Brodie et al, 1970) and bacteria (Kent et al, 1969) in the intestines. Defensive factors fall into three categories consisting of pre-epithelial, ie. the mucus/bicarbonate barrier and surface phospholipids, epithelial which is a physical barrier to ulceration, and post-epithelial defence ie. blood flow and prostaglandin synthesis. Obviously this list is not exhaustive or an adequate description of gastro-intestinal defensive factors, but each will be discussed below in greater detail in the context of how the NSAID ulcerogenic process affects them.

Whilst the precise mechanism by which NSAIDs affect aggressive and defensive factors is unknown, the above clinical and pathological features suggest the involvement of several mechanisms. Reports suggest that inhibition of prostaglandin synthesis in the stomach is a major pathogenic factor, reducing mucus/bicarbonate secretion, blood flow, and epithelial cell turnover and repair (reviewed by Wallace, 1997). The topical irritancy of NSAIDs is also thought to be involved in gastric ulceration, particularly aspirin which becomes trapped in the gastric epithelial cells (Somasundaram et al, 1995).

However, in the intestines the pathogenesis is less well understood with

several hypotheses put forward such as increased epithelial permeability allowing absorption of luminal macromolecules and enteric bacteria leading to inflammation (Bjarnason et al 1984), reduced mucosal blood flow leading to ischaemia and necrosis (Anthony et al, 1993; Piasecki et al, 1994) and enterohepatic recirculation of NSAIDs (Hucker et al, 1966) are all implicated in ulceration. These mechanisms will be discussed in detail below.

4. Mechanisms of NSAID Ulceration.

The pathology discussed above has all been shown in man, but several animal models such as pigs, dogs, and rats have been used to elucidate the pathogenic mechanisms. The most commonly used animal model is the rat, firstly because they are inexpensive, and secondly they are a recognised model of NSAID-induced gastrointestinal ulceration. Consequently, a great deal is known about the underlying mechanisms in the rat and the majority of the mechanisms to be discussed here will have been demonstrated in the rat.

The mechanisms of NSAID-induced intestinal ulceration can be sub-divided into 2 main groups (i) non biochemical (ii) biochemical.

(a) Non Biochemical Mechanisms

(i) Bile Acids/Enterohepatic Recirculation of NSAIDs:

Almost all NSAIDs (except aspirin & nitrofenac) undergo biliary recirculation, 20-60% of an iv dose depending on the NSAID (Bjarnason et al, 1993) therefore re-exposing the small intestine to an NSAID several times over. This is important due to suggested topical irritant properties of NSAIDs which may contribute to their ulcerogenicity in the intestine (Bjarnason et al, 1993). However much of the work on this topic has been performed on the rat, which undergoes more extensive enterohepatic recirculation compared to man, particularly of indomethacin (Duggan et al, 1975; Brune et al, 1987b), therefore extrapolation to man must be done with caution.

The importance of bile in the pathogenesis of intestinal lesions in animals was first shown after ligation of the bile duct in rats prevented NSAID-induced intestinal ulceration (Hucker et al, 1966). Recirculation in bile is now recognised as a vital pre-requisite for intestinal lesions (Brodie et al, 1970; Yesair et al, 1970; Duggan et al, 1975; Fang et al, 1977; Brune et al, 1987a, 1987b; Beck et al, 1990; Reuter et al 1997). Indeed, chemical modification of a commonly used NSAID (diclofenac), so that it underwent less enterohepatic recirculation significantly reduced small intestinal damage in rats (Reuter et al, 1997). Strong supporting evidence shows that NSAIDs which undergo very little recirculation such aspirin and nitrofenac produce no intestinal ulceration(Reuter et al, 1997). It has also been shown that NSAIDs in conjunction with bile are far more cytotoxic than bile or NSAID alone (Yamada et al, 1993). Maximal ulceration would therefore be expected just beyond the bile duct, but this is not the case as other studies show maximal ulceration occurs in the mid-jejunum, some distance from the bile duct (Anthony et al, 1993; Nygard et al, 1994). The reason for this is unknown but one explanation suggests that bacterial deconjugation is necessary before the NSAID is rendered toxic (Bjarnason et al, 1993).

(ii) Enteric Bacteria:

The conclusive evidence for bacterial involvement was shown in germ free rats which were resistant to NSAID-induced intestinal ulceration (Robert & Asano, 1977). This was confirmed in studies showing that antibiotics such as metronidazole reduce the severity of experimental NSAID-enteropathy (Kent et al, 1969; Satoh et al, 1983; Davies et al, 1993; Reuter et al, 1997), and confirmed with other antibiotics. These results imply that reducing enteric bacteria prevents their mucosal infiltration through increased permeability. However, metronidazole may protect against ulceration via free radical scavenging or anti-inflammatory properties, but this has yet to be confirmed.

It has been suggested that enteric bacteria are involved in inflammation following increased intestinal permeability (review by Bjarnason et al, 1993).

This is supported by a study showing that bacterial overgrowth in the lumen occurs concomitantly with increased intestinal permeability (Reuter et al, 1997). Also, NSAIDs such as diclofenac undergo more extensive recirculation than nitrofenac and consequently produce significantly more bacterial overgrowth in the intestinal lumen (Reuter et al, 1997), supported by an earlier study (Yamada et al, 1993).

A study in man has showed that increased intestinal permeability was abolished by antibiotic treatment (Bjarnason et al, 1989a), suggesting that it is the bacteria which initiate the increased permeability. This is confusing as the literature in the rat suggests that increased permeability occurs first allowing bacterial infiltration and inflammation. A study examining the intestinal mucosa histologically for bacteria after NSAID-induced increased permeability is indicated here.

(iii) ***Activation and Adhesion of Neutrophils***:

Under normal circumstances, neutrophils roll along the vessel wall at a constant velocity with limited contact with the endothelial wall (Nazziola & House, 1992). However, during inflammation they adhere to the endothelial wall, projecting pseudopodia before migrating into the damaged tissue (Fiebig et al, 1991). This adhesion is regulated by several receptors found on both endothelium and neutrophils (Argenbright & Barton, 1992). Neutrophils then migrate out of the vessel forming an abscess which limits the infection but induces an inflammatory response.

There are conflicting reports on the importance of neutrophils in NSAID-induced ulceration in both stomach and intestine. On the one hand, Wallace and Granger (1992) first suggested that leucocyte adherence followed by infiltration were essential in NSAID-induced gastric injury. Rats treated with anti-neutrophil serum and rabbits treated with a monoclonal antibody to CD18 are resistant to NSAID-induced gastric injury (Wallace & Granger, 1992; Wallace et al, 1991). Supporting evidence comes from the fact that very low doses of indomethacin increase the number of adherent neutrophils in

mesenteric venules after only 20 minutes (Slater & House, 1993; Asako et al, 1992a). Less conclusive evidence in the intestines demonstrated increased leucocyte myeloperoxidase activity 6 hours after NSAID exposure (Miura et al, 1991), as eosinophils which are normally present in the lamina propria also contain this enzyme. On the other hand, there is convincing evidence against neutrophil involvement. Anthony et al (1996b) studied the development of the indomethacin lesion with time, after a 15mg/Kg oral dose demonstrating smooth muscle contraction and microvascular injury prior to neutrophil infiltration in the stomach after 1 hour. Similar early pathological changes comprising villous contraction, epithelial stratification, smooth muscle prominence, microvascular distortion/damage and villous tip necrosis were shown at 1 hour in the small intestine (Anthony et al, 1993). It is 3 hours before any neutrophils invade the lesion. Anthony et al (1993;1996b) also reported significant eosinophil degranulation at around 1 hour. This may account for Miura et al (1991) finding increased myeloperoxidase activity at 6 hours.

Further evidence against neutrophil involvement comes from the fact that reducing leucocyte-endothelial cell interactions in mesenteric venules with antibodies against CD11b/CD18 and E-selectin does not prevent indomethacin-induced rat jejunal injury (Arndt et al, 1995). The general consensus of opinion is that leucocyte recruitment is a consequence rather than a cause of lesions, supported by evidence showing that depletion of circulating neutrophils doesn't decrease NSAID-induced gastrointestinal ulceration (Trevethick et al, 1994; Melarange et al, 1995). This would point to neutrophils being responsible for the progression of ulceration rather than its initiation. Bjarnason et als (1990b) suggestion that infiltration of enteric bacteria is a major neutrophil chemoattractant fits in and supports the later appearance of neutrophils.

Once neutrophil recruitment begins there are several mechanisms by which they could contribute to NSAID damage. They are known to contribute to vascular damage by pressure-dependent plugging of microvessels (Slater &

House, 1993), and production/release of free radicals which are involved in ischaemia reperfusion injuries in the intestine (Parks & Granger, 1983), aspirin-induced gastric damage (Pihan et al, 1987), tissue necrosis and increased inactivation of endothelin-derived relaxing factor, therefore possibly increasing vasoconstriction (Gryglewski et al, 1986). Neutrophils also release proteases which may be involved in gastrointestinal damage (Parks et al, 1985), and alterations in mucosal permeability (Von Ritter et al, 1989).

(iv) ***Epithelial Proliferation***:

Adaptation to NSAID-induced injury is becoming a recognised event occurring in the gastric and duodenal mucosa of both animals and humans (St John et al, 1973; Graham et al, 1983; Robert, 1991; Olivero & Graham, 1992; Konturek et al, 1994a; Levi et al, 1992). Although the exact mechanism is as yet unclear, it is associated with increased epithelial proliferation and renewal of damaged epithelium, resulting in an NSAID resistant mucosa (Olivero & Graham, 1992; Konturek et al, 1994a; Levi et al, 1992).

Recent work demonstrated that adaptation of human gastric mucosa in response to repeated NSAID exposure occurred concomitantly with increased gastric mucosal blood flow (discussed later), and increased epithelial proliferation 4 days after rechallenge with aspirin (Konturek et al 1994a). Increased mitotic activity in basal mucosal cells after NSAID exposure at least once daily for several weeks compared to non-NSAID using controls (Levi S, 1990) suggests that increased proliferation occurs. This was confirmed by increased epithelial DNA and RNA synthesis 2 weeks after exposure to aspirin (Konturek et al, 1994a).

However, during the first 3 days of continued NSAID exposure a decrease in epithelial regeneration has been reported, whereby the epithelium is damaged faster than it is repaired. The basis of the sudden increase in epithelial regeneration after 3 days has yet to be explained. Increased gastric blood flow after 4 days (Konturek et al 1994a) may be involved, and the discovery that exogenous epidermal growth factor (EGF) and transforming

growth factor-α (TGF-α) prevent gastric mucosal damage suggests a role for growth factors (Konturek et al 1992a). Supporting evidence for growth factors was shown in a study where elevated tissue levels of transforming growth factor-α (TGF-α), assessed by radio-immunoassay where found in the gastric and duodenal mucosa after aspirin-induced damage in the monkey and the rat (Ramano et al, 1996).

At present there are no reports on the effects of NSAIDs on epithelial proliferation in the jejunum and ileum, probably due to NSAID-induced damage in this area of the small intestine only recently being recognised. However, adaptive increases in epithelial proliferation in the intestines have been demonstrated in other disease states such as coeliac disease (Watson et al, 1982; Savidge et al, 1995). It is therefore probable that NSAID-induced mucosal injury results in intestinal adaptation similar to that in the stomach.

(v) Morphological Changes:

Morphological changes occur very early in the pathogenesis of ulceration, typically within 1 hour of NSAID exposure in rat jejunum, and are neutrophil independent (Anthony et al, 1993).

The earliest local lesion in the small intestine has been comprehensively studied in the rat, with pre-ulcerative changes after 1-2 hours exposure to an oral dose of indomethacin (15mg/Kg). These comprised gross villus contraction, degranulation of eosinophils, distortion/stratification of epithelium, focal upper villous necrosis, damage/distortion (nuclear bulging and pinching) of endothelium, focal intravascular thrombi, buckling of large villous vessels and smooth muscle prominence highlighted by focal spasm of the contractile smooth muscle fibres (Anthony et al, 1993). The early changes gradually progress to more serious ulcerative damage, comprising neutrophil infiltration, upper villous necrosis, pink plugs of red blood cells in the upper lamina propria (3-6 hours after exposure to an oral dose of indomethacin), and eventually progressing to mucosal necrosis, large vessel thrombosis, and florid acute peritonitis(6-24 hours after exposure to oral indomethacin) in rats

(Anthony et al, 1993). Ulceration is most prominent along the mesenteric margin, an area shown to be particularly prone to NSAID-induced ulceration in the rat (Anthony et al, 1993; Nygard et al, 1994).

The above pre-ulcerative changes in mucosal endothelial cells suggests they are an early target for NSAID damage, possibly affecting the ability of the mucosa to defend itself against harmful luminal contents by reducing blood flow. Indeed, endothelial damage in the stomach has been reported in rats 15 minutes after oral aspirin (Tarnawski et al, 1990), and in pig 10 minutes after a single exposure to oral (5mg/Kg) indomethacin, resulting in extravasation of red blood cells and intracellular edema (Rainsford, 1983).

Indomethacin has been shown to cause changes comprising shortening and dilatation of the whole intestine, along with increased crypt hyperplasia and mitotic activity in apparently non-ulcerated areas in the mouse (Ettarh & Clarke, 1996) at a dose of 80mg/Kg which is far in excess of any other animal study, where doses are typically 5-15mg/Kg. However, since the small bowel ulceration in mouse was similar to that in rats the same changes probably occur in both but have not yet been reported in rats. The very high doses required in the mouse is probably due to species variation.

The early appearance and degranulation of eosinophils suggests they are involved in NSAID-induced ulceration (Anthony et al, 1993). They are known to contain collagenase (Gleich & Adolphson, 1986) and a granular protein which detaches bronchial epithelium from its lamina propria (Frigas et al, 1980), similar to NSAID-induced epithelium detachment in the jejunum (Nygard et al, 1994). Eosinophils also contain leukotriene C_4 (Weller et al, 1983), a known spasmogen which is possibly involved in pre-ulcerative villous shortening (Anthony et al, 1993). Eosinophils may also be involved in endothelial damage, as mast cell degranulation results in nuclear bulging and pinching of nuclear membranes in rat cremaster muscle endothelial cells (Majano et al, 1969), and in intestinal endothelium described by Anthony et al (1993).

(vi) ***Mucosal Blood Flow***:

Since compromising blood flow will result in cessation of mucosal defence, death and shedding of epithelium, and ulceration attempts have been made to detect changes in mucosal blood flow in ulcerative diseases. Several studies show that blood flow is decreased in NSAID induced gastric ulceration, but evidence in the small intestine is mostly circumstantial.

Gastric Blood flow

Mucosal blood flow is responsible for delivering nutrients and oxygen to allow mucosal epithelium to produce bicarbonate (HCO_3^-) which neutralizes luminal acid and to maintain mucosal integrity throughout the gastrointestinal tract (Soll et al, 1991). A delicate balance has been shown to exist between luminal H^+ and gastric mucosal blood flow, where less H^+ is able to damage the mucosa if the blood flow is reduced and vice-versa (Gyires, 1994).

All of the current research into gastric mucosal blood flow shows an initial decrease within 1 hour of exposure to NSAIDs. Clearance of ^{14}C in rats and dogs shows a significant decrease in mucosal blood flow within 30 minutes, after luminally applied indomethacin using ulcerogenic doses of 30 & 10mg/Kg (Main & Whittle, 1975; Kauffman et al, 1979). These results were confirmed using *in-vivo* fluorescence microscopy, where rats given an iv dose of indomethacin (10mg/Kg) had reduced mucosal blood flow within 30 minutes. (Kitahora & Guth, 1987; Kawano et al, 1996). Luminal aspirin in dogs also reduced mucosal blood flow, although it took around 1 hour as assessed using hydrogen gas clearance (Gana et al, 1987).

Contradictory evidence was produced by laser Doppler flowrimetry in man (Konturek et al, 1994a), where 3 days after oral aspirin (2g/day), mucosal blood flow had increased. However, blood flow was not measured in the 1 hour period after initial exposure, it is probable that the increase seen after 3 days is related to gastric adaptation as described below. It is now widely accepted that NSAIDs reduce gastric mucosal blood flow within 1 hour of administration.

The mechanism behind the initial reduction in gastric mucosal blood

flow in response to NSAIDs is controversial, several different mechanisms being proposed. There are several studies which suggest that the first site of indomethacin-induced damage is the vascular endothelium. The endothelium of the gastric mucosa is damaged within 10-15 minutes of oral indomethacin (5mg/Kg), comprising enlargement of fenestra and extravasation of red blood cells into the mucosa of pigs and rats(Rainsford, 1983; Tarnawski et al, 1990). Similar results were produced with aspirin in man after oral aspirin 150mg (McCarthy et al, 1995), and in rats and pigs after oral aspirin 250mg (Rainsford, 1983), comprising enlarged capillary fenestra and lamina proprial oedema.

Neutrophil adherence to endothelium shown histologically was associated with a 50% reduction in gastric mucosal blood flow after oral aspirin (100mg/Kg) in the rat (Konturek et al, 1994b). However, Konturek did not state the exact time at which neutrophil adherence and reduced blood flow occurred, only that it occurred on the first day of oral aspirin. This is important as other authors have shown that neutrophil recruitment does not occur until after mucosal contraction and vascular injury have begun (Anthony et al, 1996b). Unfortunately neither of these studies was able to prove conclusively whether or not reduced blood flow is associated in time with neutrophil invasion. Although Konturek did measure blood flow, it was performed with a laser Doppler flowmeter which measures general flow in an area of mucosa, neutrophils were measured histologically so their effects on blood flow were not directly demonstrated.

Vasodilatory prostaglandins (particularly PGE_2) are known to protect the gastric mucosa against NSAID damage via maintenance of mucosal blood flow (Robert, 1979), supported by studies showing that leucocyte adherence to vascular walls was prevented by prostaglandins in indomethacin (Asako et al, 1992a) and aspirin (Asako et al, 1992b) treated animals.

Other studies suggest that the initial reduction of blood flow is a result of inhibition of NO synthesis, as NSAIDs such as nitrofenac which have an attached NO moiety do not decrease gastric mucosal blood flow or produce

ulceration (Pique et al, 1989; Kubes et al, 1991; Wallace et al, 1994a; 1994b). NO has potent vasodilatory effects and prevents neutrophil adherence to vascular endothelium, therefore an attached NO group may prevent the NSAID-induced reductions in blood flow and neutrophil adherence discussed above (Pique et al, 1989; Kubes et al, 1991; Wallace et al, 1994a; 1994b).

There are studies which suggest that gastric motility may have a role in the pathogenic process of NSAID ulceration. Takeuchi (1986) demonstrated a relationship between indomethacin-induced increases in gastric motility and lesion formation, confirmed by increased smooth muscle myoelectric activity (manifest as increased electrical activity and peristaltic contractions compared to water controls) following indomethacin administration (Mersereau & Hinchey, 1988). Moreover, increased gastric peristalsis induced by field stimulation results in decreased blood flow to the antrum in rats (Livingstone et al, 1991), which is an area particularly susceptible to indomethacin-induced lesions. Natural motility of digestion generally increases mucosal blood flow, suggesting that NSAIDs/field stimulation induce a different type of motility such as prolonged muscular spasm, reducing mucosal blood flow.

However, repeated exposure to aspirin in man (Konturek et al, 1994a) and in animals (Konturek et al, 1994c) results in gastric mucosal adaptation, supported by a study with indomethacin in man (Shorrock & Rees, 1992). As well as increasing epithelial proliferation (discussed previously), there is also increased mucosal blood flow during adaptation(Konturek et al, 1994a; 1994b; 1994c). It is possible that increased blood flow results from gastric prostaglandin synthesis returning to pre-NSAID levels, as prostaglandins have been shown to increase gastric mucosal blood flow (Pihan et al, 1986). However, there is convincing evidence against this explanation, as prostaglandin synthesis is suppressed by up to 90% by the first exposure to aspirin and remains suppressed whilst adaptation still occurs (Konturek et al, 1994b). Konturek (1994c) also suggested that capsaicin sensitive nerves may be responsible for adaptive hyperaemia, based on the observations that topical capsaicin increased gastric mucosal blood flow and prevented aspirin-induced

lesions (Holtzer et al, 1989). However, ablation of these nerves increased the initial lesion but did not prevent hyperaemia and adaptation (Konturek et al, 1994c).

The evidence is equivocal as to the importance of mucosal hyperaemia in adaptation. In a study where abolishing nitric oxide (NO) mediated vasodilation in gastric mucosa with N^G-nitro-L-arginine (L-NNA) prevented adaptive hyperaemia but did not prevent mucosal adaptation to aspirin (Konturek et al, 1994b). However, the adaptive response was reduced by L-NNA compared to controls, and application of L-arginine (substrate for nitric oxide synthase) increased the adaptive response in control rats. The study by Konturek (1994b) suggests that increased blood flow (in particular NO mediated flow) has only a small part to play in gastric adaptation.

Small Intestinal Blood Flow

Only a few studies of small intestinal blood flow in relation to NSAIDs exist and they naturally fall into two main categories (a) total small intestinal blood flow, (b) local blood flow in the mucosa.

(1) Total small intestinal blood flow:

The ability of NSAIDs such as indomethacin to alter blood flow in the whole intestine is similar to that in the stomach where there is an initial decrease followed by an increase. Where a general reduction in superior mesenteric artery blood flow was observed in dogs exposed to indomethacin (Gaffney & Williamson, 1979; Feigen et al, 1981), and an increase in superior mesenteric blood flow was observed in rats exposed to indomethacin (Batterbee et al, 1996). A likely explanation for the differences, is the timing of the measurements as Batterbee et al (1996) demonstrated an increase in blood flow after 24 hours, whereas Feigen et al & Gaffney et al showed their decease within 30 minutes of administration. At 24 hours after dosing indomethacin has been shown to produce haemorrhagic ulcers throughout the distal small intestine(Anthony et al, 1993; Nygard et al, 1994), so increased superior mesenteric artery blood flow may be a part of the reparative process

involved in healing. Other explanations for the differences such as measurement artifact are unlikely as all 3 studies used similar forms of laser Doppler. The differences are also unlikely to result from different doses as the blood flow increase was produced with 7.5mg/Kg and the decrease with 10mg/Kg both of which are commonly used ulcerogenic doses.

(2) Local blood flow in the mucosa.

As in the gastric mucosa all the currently available literature has suggested a decrease in intestinal mucosal blood flow is associated with NSAIDs, but unlike in the gastric mucosa, few studies have provided conclusive evidence for this. A histological study showed early morphological damage to endothelial cells, focal contraction of smooth muscle cells around surface capillaries, plugs of red blood cells and distorted microvasculature around the villus tip, all of which were suggestive of attenuated blood flow (Anthony et al, 1993). This was supported by a study showing leucocyte rolling (ie activated just prior to adhering) and reduced blood flow in the sub-mucosal venules and villus lamina propria of the small intestine (Miura et al, 1991). These leucocytes are known to release oxygen free radicals (Del Soldato et al, 1985; Fantone et al, 1985) which then cause oxidative stress within endothelial cells and this might result in microvascular damage (Miura et al, 1991), and further vascular obstruction at the submucosal level.

More direct evidence for reduced mucosal blood flow in the aetiology of indomethacin-induced ulceration was suggested by a study showing high luminal concentrations of indomethacin (5mg/ml) produced very rapid microvascular stasis in rat jejunal villi (Piasecki et al, 1994). In fact Miura (1991)also showed sludging and blood flow stasis in villi 6 hours after sub-cutaneous dose of 20mg/Kg indomethacin.

Adrenoceptors

Adrenoceptors are receptors sensitive to catecholamines such as adrenaline (secreted from the adrenal glands) and noradrenaline (released from the adrenal glands and the sympathetic nervous system). Generally, stimulation

of the sympathetic system results in energy expenditure. The fright or flight response is the most common example comprising vasodilation in skeletal muscle, reduction of blood flow in non-vital areas such as the skin and gastrointestinal tract, lipolysis, bronchodilation and increase in heart rate and force. The effect on the gastrointestinal tract may theoretically be directly important to the mechanism of NSAID-induced reduction of blood flow, hence adrenoceptors and their role in the gastrointestinal tract are further described below.

It is postulated that reductions in superior mesenteric artery flow in response to indomethacin result from adrenergic stimulation of α-adrenoceptors leading to vasoconstriction and reduced flow in the mesenteric bed of the dog (Feigen et al, 1981). A similar α-adrenergic induced vasoconstriction has been reported in the stomach in response to feeding, which when combined with indomethacin, produced antral ulceration (Kuratani et al, 1992). An adrenergic component for indomethacin-induced enteropathy was proposed by a study showing that a β_3-adrenoceptor agonist protected against oral indomethacin (Anthony et al, 1996c). For these reasons the background pharmacology of adrenoceptors is given below.

Classification of Adrenoceptors

It was first proposed in 1948 that two distinct adrenergic sub-types existed, based on the different abilities of adrenaline and noradrenaline to regulate different physiological processes ie. α and β-adrenoceptors (Ahlquist, 1948). The α-adrenoceptors generally exert excitatory effects whereas β-adrenoceptors exert inhibitory effects, except in gut smooth muscle where α stimulation is inhibitory, and in the heart where β stimulation is excitatory. The discovery that α-adrenoceptors were part of a heterogenous family is only relatively recent, and is based on the observation that noradrenaline inhibits its own release from neurons by an action on pre-synaptic receptors (Langer, 1980). The pre-synaptic receptors were designated α_2-adrenoceptors and are pharmacologically distinct from post-synaptic excitatory α_1-adrenoceptors

(Clonidine = α_2 agonist, phenylephrine = α_1 agonist).

It was later realised that β-adrenoceptors were not homogenous and that distinct sub-populations existed. They were sub-divided into β_1 on the myocardium and β_2 on smooth muscle (Lands et al, 1967). It was not until the later development of specific β_1/β_2-adrenergic antagonists (β_1 = atenolol and β_2 = propranolol) that the classification was fully accepted.

In 1974 it was suggested that antagonism of lipolysis did not correspond to either β_1 or β_2 antagonists (Harms et al, 1974). This atypical response led to the classification of the β_3-adrenoceptor. The classification of this new receptor using novel agonists such as BRL37344 (Arch et al, 1984; Wilson et al, 1984) and its low affinity for classical β_3-adrenoceptor antagonists such as propranolol was not accepted by classical pharmacologists who preferred the use of β_3 specific agonists and anatagonists. Consequently it wasn't until the β_3-adrenoceptor was cloned in 1989 that it was finally accepted as a new receptor sub-type (Emorine et al, 1989). Acceptance of the β_3-adrenoceptor led to its identification in many diverse tissues such as adipocytes (Takahashi et al, 1992), skeletal muscle (Challiss et al, 1988), heart (Kaumann, 1989), the airways (Webber & Stock, 1992), the gastrointestinal tract (MacDonald et al, 1994; Cohen et al, 1995), and the gallbladder (Strosberg & Pietri-Rouxel, 1996).

β_3-adrenoceptors and the gastrointestinal tract

In-vitro studies have shown β_3-adrenoceptors mediate relaxation of guinea-pig ileum (Bond, 1987), small intestine (MacDonald, et al 1994), oesophageal muscularis mucosae (de Boer et al, 1995) and gastric fundus (Cohen et al, 1995). Confirmation of these effects has proved difficult due to the lack of a truly selective β_3-adrenoceptor antagonist. Recently however, one (SR 59230A) has been produced and allowed the confirmation of a β_3-adrenoceptor population in the human colon (DePonti et al, 1996) Unfortunately, mRNA expression techniques have not been able to differentiate between the layers of the gastrointestinal tract (Anthony, 1996d).

However, radioactive ligand binding studies using [^{125}I]-cyanopindolol have shown β_3-adrenoceptors in all intestinal layers ie. the mucosa, muscularis mucosae, medial smooth muscle of submucosal arteries and veins, myenteric plexus, and circular smooth muscle (Summers et al, 1995). It is however, unlikely that all the binding sites represent β_3-adrenoceptors. However, Guillaume (1995) using a polyclonal antibody against an amino acid sequence of the human β_3-adrenoceptor localised receptors to the vascular and non-vascular smooth muscle and epithelium of the human gallbladder but not in the small intestine. Unfortunately there are no antibodies with sufficient specificity to localise the receptors in the rat. Pharmacological intervention using β_3-selective agonists has demonstrated populations of β_3-adrenoceptors on microvasculature regulating vasodilation in skin (Berlan et al, 1994; Shen et al, 1994), and pancreas (Atef et al, 1996).

The relative importance of β_3-adrenoceptor activity in the layers of the gastrointestinal tract requires study, as spasm of muscle layers could restrict blood flow, since blood vessels supplying and draining the mucosa pass through both the external muscle and the muscularis mucosae. Such receptors could also directly modulate vessel tone at various levels between large vessels perforating the external muscle coat down to pre-capillary arcade vessels in villi. The recent studies discussed below suggest that the effect may be directly on both small mucosal vessels and on scattered smooth muscle fibres within the mucosa.

β_3-adrenoceptors and NSAIDS

Previous work in the stomach and intestines has suggested a protective role for both β_2& β_3 adrenergic receptors against NSAID-induced ulceration. The β_2-agonists salbutamol and salmeterol prevent NSAID-induced corpus erosions (McGeevy et al, 1981; Kuratani et al, 1994; Bahl et al, 1996), due to a reduction in corpus secretions, and the β_3-agonists CL316,243 (Fig. 3) and BRL37344 (Fig. 3) are up to 100 fold more potent inhibitors of NSAID ulceration than salmeterol in the stomach (Bahl et al, 1996).

Stimulation of gastric mucosal β_3-adrenoceptors in the rat with the β_3-agonist CL316,243 increased basal mucosal blood flow and prevented indomethacin-induced ulceration (Kuratani et al, 1994).

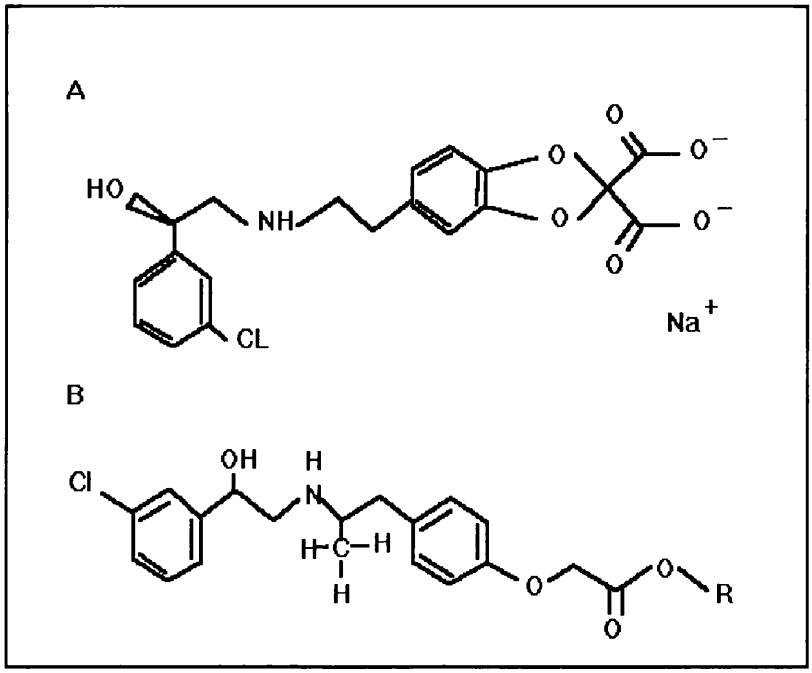


Figure 3. Molecular structure of CL316,243 (A) and BRL37344 (B), two of the most potent β_3-adrenoceptor agonists.

This result was mirrored in the intestinal mucosa, where CL316,243 prevented early indomethacin-induced focal morphological changes comprising smooth muscle shortening, microvascular damage/distortion, villous shortening and epithelial stratification (Anthony et al, 1996c). Although each study strongly suggested that protection against indomethacin was due to maintenance of mucosal blood flow, neither study attempted to measure whether indomethacin-induced blood stasis could be reversed by CL316,243, or observed the effect of concomitant administration of indomethacin and CL316,243.

There are several possible explanations for the protective effects of CL316,243, including reversal of focal vasoconstriction, decreased gastrointestinal motility, and relaxation of villus smooth muscle. Perhaps the simplest and most attractive is reversal of focal vasoconstriction within mucosal microvasculature. Unfortunately the supporting evidence is weak;

indomethacin was suggested to reduce blood flow through the superior mesenteric artery as a direct result of vasoconstriction in the superior mesenteric artery in dogs (Feigen et al, 1981). However, the vasoconstriction was neither shown or measured, and any vasoconstriction was likely to be due to vasospasm produced by vessel damage as indomethacin was injected directly into the superior mesenteric artery. Also vasoconstriction is unlikely to be the cause of the focal microvascular stasis in villus tips described by Piasecki (1994), as these vessels are capillaries with no intrinsic smooth muscle, and unlikely to be able to contract.

The observations that β_3-adrenoceptor agonists relax muscle in a variety of gastrointestinal preparations (Giudice et al, 1989; Croci et al, 1991; Arch & Kaumann, 1993), suggests relaxation of non vascular smooth muscle as the protective mechanism for this class of drugs. Indomethacin has been shown to cause non-vascular smooth muscle contraction in villi resulting in villous shortening and possibly crushing and occluding blood flow in villus tips (Anthony et al, 1993). Stimulation of β_3-adrenoceptors has been postulated to relax non vascular villus smooth muscle, promoting relaxation and reversal of indomethacin-induced villous shortening, releasing constriction around microvasculature and restoring villous blood flow (Anthony et al, 1996c).

(b) Biochemical Mechanisms

(i) Uncoupling of oxidative phosphorylation:

NSAIDs have been shown to uncouple mitochondrial oxidative phosphorylation (Whitehouse, 1964), an effect thought to be involved in the increased intestinal permeability described in NSAID pathology. NSAID absorption has been shown to uncouple enterocyte mitochondria as early as 1 hour after exposure, indicated by ultrastructural analysis of mitochondrial membranes in the rat (Hayllar et al, 1991; Somasundaram et al, 1992; 1995). The mechanism is thought to involve decreased mitochondrial adenosine tri-

phosphate (ATP) production within the enterocyte. Normal function of the intercellular junctions is regulated by an ATP-dependent process linked to the cellular cytoskeleton (Madara et al, 1987). Consequently, decreased ATP prevents normal function of junctions and results in increased paracellular permeability. Uncoupling also results in efflux of calcium and hydrogen ions from damaged mitochondria, further depleting cellular ATP and triggering the production of cytotoxic oxygen free radicals, (Somasundaram et al, 1995) and are discussed below. The increased permeability is postulated to allow the influx of enteric bacteria (Bjarnason et al, 1990b) triggering an overwhelming inflammatory response.

(ii) ***Production of Oxygen Free Radicals***:

The oxygen free radicals, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-) released as a result of uncoupling of oxidative phosphorylation, may exert their cytotoxic effect through lipid peroxidation of cellular and mitochondrial membranes (Butterfield & McGraw, 1978; Fridovich, 1978). Evidence for free radical involvement in NSAID ulceration was provided by Rainsford (1984), who reported that scavenger molecules and antioxidants prevented aspirin-induced gastric lesions in the rat. Moreover, NSAIDs themselves have been shown to scavenge free radicals, leading to the conclusion that the anti-inflammatory actions of NSAIDs are based on their ability to capture free radicals (Hiller & Wilson, 1983). However another study suggested that free radical bound indomethacin is cytotoxic, and more ulcerogenic than free indomethacin in both the stomach and the intestines (Del Soldato et al, 1985b).

As NSAIDs strongly inhibit cyclooxygenase it is likely that arachidonic acid products are then metabolised via the lipoxygenase pathway (Burka & Flower, 1979; Morris et al, 1980), resulting in free radical production. A particular effect of superoxides is degradation of hyaluronic acid which is a constituent of the basement membrane of epithelial cells, therefore providing a possible explanation for epithelial lifting and tufting described by Anthony

(1993).

(iii) Eicosanoids:

Since Vanes discovery that NSAIDs inhibit prostaglandin synthesis, and their consequential protection against ulceration, a great deal of interest has been concentrated on prostaglandins. Recently, this has lead to the development of PG analogues such as misoprostol which protect the gastrointestinal tract from NSAID damage (Bjarnason et al, 1989a), and the recognition of two isoforms of the enzyme cyclo-oxygenase (COX) known as COX1 and COX2, both of which produce prostaglandins.

COX1 is the constitutive form of the enzyme which is involved in many physiological functions such as prostacyclin production preventing platelet aggregation (Moncada et al, 1976), and PGE_2 production which is cytoprotective in the gastrointestinal tract (Whittle et al, 1980; Chan et al, 1995). There are high levels of the COX1 protein and its mRNA in most normal tissue (O'Neill & Hutchinson, 1993).

COX2 is the inducible form with a similar molecular weight and amino acid sequence to COX1 (Chan et al, 1995). COX2 is induced at sites of inflammation (Kennedy et al, 1993; Harada et al 1994; Masferrer et al, 1994; Vane et al, 1994), for example in the synovial tissue of rheumatoid arthritis patients (Crofford et al, 1994). It is induced by cytokines, endotoxins (Chan et al, 1995) and also within migratory leucocytes at inflammatory sites (Shorrock et al, 1992), but there are only negligible amounts of COX2 in normal tissue (Chan et al, 1995).

COX1 produces prostacyclin (PGI_2), thromboxane A_2 and PGE_2, whereas COX2 produces prostaglandins of both the E and F groups which are involved in inflammation. The current literature suggests that it is the site of production which dictates whether or not the prostaglandin is harmful. For example production of PGE_2 in the gastric mucosa is beneficial, but in the synovial tissue it leads to pain and arthritis.

All NSAIDs in current use are nonselective inhibitors of both COX1

and COX2 (Chan et al, 1995; Meade et al, 1993; Mitchell et al, 1993; Smith et al, 1994), therefore as well as inhibiting COX2 mediated inflammatory effects, they also inhibit COX1 mediated gastroprotection. The observation that exogenous prostaglandins have cytoprotective properties in the stomach (Robert, 1979), and the discovery of COX isoforms has lead to research into COX2 selective NSAIDs which spare the protective effects of COX1, and inhibit the inflammatory effects of COX 2. Chan et al (1995) recently described a selective COX2 inhibitor which caused no gastric ulceration and was still an effective NSAID, suggesting that COX2 selective drugs are gastrointestinal tract safe.

So far COX1 and COX 2 inhibition and prostaglandin production have only been discussed in the stomach where their protective actions are well recognised (reviewed by Wallace 1997). However, in the intestines the role of prostaglandin synthesis inhibition is equivocal. Firstly, experiments in rats have shown that aspirin, nabumetone and nitrofenac which are very potent non-selective cyclo-oxygenase inhibitors do not produce any significant intestinal damage (Reuter et al, 1997) Secondly, Nygard et al (1994) demonstrated increased PGE_2 synthesis at around 6 hours after oral indomethacin, a time point at which the mucosa is committed to deep ulceration (Anthony et al, 1993). There are several interpretations of this result, for example, increased synthesis from invading neutrophils, regeneration of cyclo-oxygenase which has a turnover time of 10 minutes (Fagan & Goldberg, 1986), or that indomethacin is not an irreversible cyclo-oxygenase inhibitor (Stanford et al, 1977). These results suggest that prostaglandins are of less importance in NSAID-induced intestinal ulceration.

However, exogenously applied PGE_2 protects the intestinal mucosa from NSAID-induced ulceration in the rat (Robert , 1975). This was supported by Whittle (1981) who demonstrated that when PGI_2 synthesis was maximally inhibited 48 hours after sub-cutaneous indomethacin, intestinal ulceration was maximal. Also, indomethacin-induced morphological changes (previously discussed) have been shown to occur concomitant with a significant decrease

in protective prostaglandins such as PGE_{1-2} (Nygard et al, 1994) both of which modulate smooth muscle tone and maintain the integrity of vascular endothelium (Wakefield et al, 1990). However, the authors showed that the initial decrease in PG levels was followed by a return to control levels, which has two possible explanations. Firstly, that decreased PG and morphological injury are independent events (Nygard et al, 1994), or secondly that it is the initial inhibition of COX which is vital to the progression of the intestinal damage (Robert, 1975).

Prostaglandins also have a controversial role in inflammatory bowel disease with both cytoprotection and pro-inflammatory roles. Levels of PGE_2 are increased in both Crohns disease (Hawkey et al, 1983) and ulcerative colitis (Sharon et al, 1978). This suggested that NSAIDs would be useful in treating IBD, but unfortunately there was no improvement and many patients deteriorated whilst on flurbiprofen and indomethacin therapy (Gilat et al, 1979; Campieri et al, 1980; Rampton & Sladen, 1981). This result suggests that inflammation in these diseases is a beneficial reaction against the disease.

In summary, there is an abundance of data, mostly in the stomach, relating to prostaglandins and their role in NSAID-induced ulceration. The literature shows that selective inhibition of inducible COX2 reduces NSAID-induced gastric ulceration (Chan et al, 1995). However, the role COX1 and 2 in NSAID-induced intestinal ulceration is more controversial, with conflicting reports. On the one hand the observations show that exogenous prostaglandins protect the intestinal mucosa, and morphological changes occur concomitantly with a significant decrease in protective PGE_{1-2}, and on the other hand not all unselective COX inhibitors produce intestinal ulceration. Consequently at present the role of prostaglandins in NSAID-enteropathy is unclear, and awaits an experiment conclusively linking decline in PGE_2 with the onset of morphological damage.

(iv) Production of Leukotrienes:

The suggestion that NSAID inhibition of COX1 and 2 diverts

arachidonic acid metabolism towards the lipoxygenase pathway (DeRubertis et al, 1984) has led to a significant amount of research into blocking the production of leukotrienes by inhibition of the 5-lipoxygenase enzyme (review by Rainsford, 1989). 5-lipoxygenase produces vasoconstrictor leukotrienes such as C_4 and D_4 and cytotoxic free radicals (Rainsford, 1987). Leukotrienes are also potent chemotactic agents (Borgeat & Samuelsson, 1979; Ford-Hutchinson et al, 1980) and are implicated as mediators of inflammation. Leukotrienes produced by 5-lipoxygenase have also been implicated in indomethacin-induced reductions in blood flow and mucosal damage in the small intestine, where they mediate leucocyte adhesion in mesenteric venules and increased free radical production after sub-cutaneous indomethacin 20mg/Kg in rats (Miura et al, 1991). These effects were abolished after administration of an oral 5-lipoxygenase inhibitor. This was supported by a study in the rat where a selective 5-lipoxygenase inhibitor accelerated ulcer healing and reduced inflammation in a trinitrobenzene sulfonic acid (TNB) model of IBD (Wallace et al, 1989). However, Wallace subsequently showed that inhibition of 5-lipoxygenase did not prevent the onset of ulceration. Increased neutrophil numbers 4 days after induction of inflammation, suggested that in this model at least, reduced leukotriene-mediated chemoattraction was not involved in the progression of ulceration. Although the progression of the lesion described by Wallace parallels the results shown in NSAID-induced ulceration the exact role of leukotrienes has yet to be conclusively proven in NSAID ulceration.

(v) ***Mucus & Surface Phospholipids***:

Gastrointestinal mucus is the first line of defence against harmful luminal substances such as acid in the stomach and pancreatic enzymes in the intestine. Therefore any reduction or alteration in mucus has the potential to increase the risk of mucosal damage. NSAID-induced inhibition of cyclo-oxygenase has been implicated in reduction of the mucus barrier as prostaglandins have been shown to stimulate duodenal bicarbonate output

(Flemstrom, 1980; Smeaton et al, 1983) and gastric mucus secretion (Bolton & Palmer, 1978; Bickel & Kauffman, 1981; Sellers et al, 1986; Davies et al, 1993; Wallace, 1993). They also affect mucus secretion in the intestines, particularly prostaglandins E_2 and I_2 whereby cyclo-oxygenase inhibition by indomethacin results in decreased mucus secretion (Sababi et al, 1995).

In addition to mucus itself, phospholipids in the mucus layer also protect the underlying epithelium by maintaining its hydrophobicity. It has recently been shown that NSAIDs interact with phospholipids in this layer to reduce hydrophobicity (Lichtenberger et al, 1995a; 1995b), an effect lasting for the duration of NSAID exposure, allowing luminal substances to penetrate the mucus layer. These observations led to the discovery that pre-association of an NSAID to a zwitterionic phospholipid attenuated the drugs gastrointestinal toxic effects (Lichtenberger et al, 1995a), and was shown to increase the NSAIDs effectiveness in reducing pain and inflammation (Lichtenberger et al, 1995a; Lichtenberger et al, 1996).

5. Summary of Mechanisms

Since gastric ulceration and haemorrhage is the main reason for discontinuation of NSAID therapy, much of the research and current literature is devoted to mechanisms and treatments of NSAID-induced gastric ulceration. However, the small bowel is now becoming a recognised site of NSAID injury, consequently current research is beginning to address this problem.

The fact that NSAIDs are extremely effective anti-inflammatory drugs, results in a contradiction, whereby their gastrointestinal side effects are a direct result of an inflammatory response. Within the intestines there are two main hypotheses for this anomaly. The first suggests that NSAIDs increase intestinal permeability, and is supported by several excellent studies. It is thought that increased permeability allows luminal contents and bacteria to infiltrate the mucosa producing an overwhelming inflammatory response (reviewed by Bjarnason et al, 1993).

The second hypothesis is that NSAIDs interfere with mucosal blood

flow in the intestines resulting in necrosis and breakdown of the intestinal mucosa, again allowing direct passage of luminal contents and bacteria causing inflammation. There have been several mechanisms suggested for cessation of mucosal blood flow, including inhibition of vasodilatory prostaglandins, inhibition of nitric oxide synthesis, vasoconstriction, non-vascular smooth muscle contraction, and damage to vascular endothelium. The aim of this thesis is to elucidate whether one or more of these mechanisms is prominent in the cessation/reduction of mucosal blood flow at the earliest stage of NSAID damage.

NSAID ulceration of the intestines shares some of the mechanisms which contribute to gastric ulceration. The involvement of neutrophils is common to both, and in the past, has been controversial. The reduction of NSAID-induced gastric ulceration by anti-neutrophil serum and monoclonal antibodies to CD18 provides compelling evidence for neutrophil involvement (Wallace et al, 1991; Wallace & Granger, 1992). The evidence for neutrophil involvement was not so convincing in the intestines, where it was based on increased myeloperoxidase activity and increased neutrophil adherence in mesenteric venules after indomethacin (Miura et al, 1991; Slater & House, 1993). However, the observations that neutrophil infiltration occurs 6 hours after the first NSAID effects in both the intestines and stomach (Anthony et al, 1993; 1996b), and that antibodies against CD11b and CD18 prevent leucocyte endothelial interactions without preventing jejunal ulceration, has lead to the general opinion that neutrophils are responsible for the progression of NSAID-induced ulceration rather than its initiation.

Inhibition of prostaglandin synthesis may be a mechanism which is common to both intestines and stomach. The available literature suggests that they play a more important role in the stomach than in the intestines. Evidence for this comes from the use of aspirin, nabumetone and nitrofenac which although strong unselective COX inhibitors, do not cause intestinal ulceration (Reuter et al 1997).

There are three differences between intestinal and gastric NSAID-

induced ulceration: (i) The presence of gastric acid pre-disposes the gastric mucosa to ulceration. This is supported by reduction of indomethacin-induced gastric lesions after administration of anti-acid secretory agents, which is not relevant in the intestines.

(ii) The presence of enteric bacteria in the small bowel, which undergo significant bacterial overgrowth concomitant with increased intestinal permeability on exposure to NSAIDs (Reuter et al, 1997), especially in the more distal areas of the intestine. However, the interaction between these effects and their role in the production of lesions is as yet unclear.

(iii) Enterohepatic recirculation is recognised as a vital pre-requisite for NSAID ulceration in the small intestine. This is confirmed by the observation that bile duct ligation prevents NSAID-induced ulceration, and NSAIDs such as aspirin and nabumetone which do not undergo recirculation are non ulcerogenic in the small intestine.

The current literature suggests β_3-adrenoceptors are potent protectants against NSAID-induced gastric and small intestinal ulceration, and are an excellent prospect for future prophylactic treatment against NSAID damage. Their postulated effect on mucosal blood flow requires direct confirmation before their worth in protecting against NSAID-induced ulceration can be measured.

Maintenance of gastric mucosal blood flow is well documented in its ability to protect against luminal acid and chemical irritants as well as NSAID-induced ulceration. It is also strongly implicated in protecting the small intestines from NSAID ulceration as compounds which increase basal gastric mucosal blood flow have also been shown to protect the intestines. Moreover, total mucosal blood flow in the intestines is reduced during the early stages following NSAIDs, and the most recent work shows that focal microvascular damage (Anthony et al, 1993) and villus stasis (Piasecki et al, 1994) precede frank ulceration.

6. Hypotheses

1. Early Ischaemic lesion

Previous results by Anthony et al (1993) demonstrated a progressive (though overlapping) series of morphological changes leading to frank small intestinal ulceration. Of the very early (occurring within 2 hour) NSAID (indomethacin) induced changes the most interesting were shortening/distortion of villi and damage/distortion of endothelium. It was suggested that these changes may occlude or severely restrict blood flow in the microvasculature at the villus tip leading to ischaemia and necrosis. Dynamic evidence for blood flow involvement was provided by Piasecki et al (1994), who showed focal blood stasis in the tip of single villi exposed to high topical doses of indomethacin. These two pieces of evidence led to the hypothesis that *focal slowing or loss of villous blood flow is an early feature in the pathogenesis of indomethacin-induced ulceration, and may be the cause of epithelial loss and alterations in villous morphology.*

2. β_3-adrenoceptor agonist mediated reversal of indomethacin-induced blood stasis.

The second part of the thesis was developed from several studies. Firstly a population of β_3-adrenoceptors was reported on smooth muscle in the arteries and veins of the gut (Summers et al, 1995). Secondly, stimulation of such receptors in other sites has been shown to increase basal blood flow in organs such as skin (Berlan et al, 1994; Shen et al 1994), pancreas (Atef et al, 1996), adipose tissue (Takahashi et al, 1992) and gastric mucosa (Kuratani et al, 1994). Thirdly, a population of β_3-adrenoceptors has been shown to exist in the gastric mucosa, stimulation of the these receptors protected against indomethacin-induced lesions (Kuratani et al, 1994). Stimulation of a separate population in the small intestine also protected against indomethacin-induced mucosal damage (Anthony et al, 1996c). However, although both of the gastrointestinal studies demonstrated β_3-adrenoceptor mediated protection, and suggested this was due to maintenance of mucosal blood flow, neither

provided conclusive evidence for the role of β_3-adrenoceptors and blood flow in protection against NSAID-induced ulceration. Therefore, the second hypothesis is that *stimulation of β_3-adrenoceptors reverses indomethacin-induced blood stasis by promoting villous blood flow.*

CHAPTER 2

METHODS

1. Introduction

(a) Selection of animal model

Rats were selected in order to relate this work to preceding studies by Anthony et al (1993), Nygard et al (1994) and Piasecki et al (1994)

(b) Selection of Study Technique

The first indomethacin-induced morphological changes are focal, occurring first in a single vessel within a villus on the mesenteric margin (Anthony et al, 1993; Piasecki et al, 1994). This thesis sought to capture histology the moment blood flow in a single vessel was altered, before any secondary changes were allowed to complicate the pathology. Therefore, this required continuous *in-vivo* observation of blood flow in a large area of mucosa overlying the mesentery (approx 40 villi). On first detection of slowing or stasis in a single vessel, the preparation could be fixed by vascular perfusion, allowing histological analysis of the epithelium, blood vessels and stroma at these points. There were 4 possible *in-vivo* techniques considered to test the thesis hypotheses:-

(i) Observe blood flow and sacrifice the preparation at a particular time point (eg. 30 minutes after exposure to indomethacin) and inject the microvessels with ink.

(ii) Continuous recording of blood flow *in-vivo* using methods such as laser Doppler flowrimetry, electromagnetic flow probe, hydrogen gas clearance, and radiolabelled microspheres.

(iii) Observe the mucosa with a microscope, then sacrifice and inject the vessels with ink.

(iv) Continuous observation and recording of mucosal blood flow with a microscope and then off-line measurement.

Continuous observation and recording with a microscope followed by off-line measurement was considered to be the most effective technique

as it allowed *in-vivo* alterations in blood flow to be simultaneously measured and observed in a large area, and was able to focus onto a particular villus vessel. The other techniques did not have this flexibility. *In-vivo* microscopy has several other advantages making it the method of choice for this thesis, firstly, as well as the ability to observe flow in large areas it can also resolve and study areas of around 600μm (ie a single villus vessel) for long periods of time. Secondly, using red blood cells (RBC) for comparison, it is possible to measure both RBC velocity and vessel diameter and hence provide a blood flow value for any microvessel. Thirdly, it can be combined with vital staining methods or intravascular markers to estimate endothelial injury or capillary leakage. However, one disadvantage is that the sample size represents a very small part of the mucosal microcirculation, but for this study it was not important as the early indomethacin-induced changes initially affect only a very small part of the mucosa (ie a single villus). Furthermore, the observer can only effectively measure surface microvasculature, but again this was not important as the initial reduction in blood flow first occurs in the surface microvasculature.

There are a large number of other techniques which could have been used to determine blood flow in a particular tissue. However, for this thesis, techniques such as electromagnetic flow probes around the superior mesenteric artery and radiolabelled microspheres could not be used as they measure whole organ perfusion rather than flow in a focal area. Hydrogen gas clearance was also ruled out because it requires around half an hour for each measurement, and is therefore insensitive to the rapid alterations in blood flow described by Piasecki et al (1994). Laser Doppler flowrimetry appeared to be an ideal choice because it is accurate, responds immediately to rapid changes in blood flow and can measure small focal areas of blood flow. Unfortunately, there are three disadvantages which disqualified the

technique. Firstly, when the project began the available probes were not small enough to measure flow within a single microvessel where the first blood flow changes begin. Secondly, because larger probes measure the average blood flow in an imprecise area of tissue they are unable to localise in which vessel a change is occurring. Thirdly, laser Doppler is also very sensitive to tissue movement producing background noise.

(c) In-vivo Microscope Set up

Since vessels in a villus are viewed through the overlying mucosa and mucus their visualization required enhancement by fluorescent labelling of the blood plasma (for vessel diameter) and RBCs (for velocity measurements).

Despite fluorescent labelling, the intensity was too low for observation by eye and insufficient for video recording, therefore an image intensified camera was used. A silicon intensified camera (SIT) was available for this purpose, which has 2 orders of magnitude greater light sensitivity than ordinary cameras. The camera has resolution of 600 lines which compared favourably with the 625 lines of the Sony Hi-8 video recorder. However, SIT cameras suffer from image lag where the live image moves faster than the camera can collect it resulting in stretched images, this can be avoided if only the leading edge of a moving image is analysed as this is not stretched.

Long working distance objectives were used to allow manipulation of the specimen whilst maintaining direct visualization. Their better depth of field was suited to observing the whole villus as required by the project. The low light gathering capabilities of long working distance objectives was offset by camera intensification.

(d) Review of measurement techniques for RBC velocity and vessel diameter in in-vivo microscopy

(i) RBC Velocity

Although *in-vivo* fluorescence microscopy is able to observe the microvasculature, it is not able to quantify blood flow without the aid of an add on measurement device/method. Most recent measurement techniques are reliant upon the fact that within the capillary microcirculation the RBCs pass as either roleauxs, or in single file with irregular spacings which remain stable over short distances. Techniques have therefore been developed to quantify the transit times for these unique patterns. For this thesis each technique was required to meet a list of stringent criteria, such as ability to clearly visualise RBC and vessel, cope with tissue movement, measure flow in fine capillaries, observe/measure flow in any direction, and make continuous measurements. The measurement techniques commonly used with *in-vivo* microscopy fall into two types (i) Non-videometric, which are generally performed on-line (ii) Videometric, which are performed on video recordings of blood flow.

Non-Videometric Analysis:-Two-slit photometry is an on or off-line method of analysis (Wayland & Johnson, 1967) it is also known as temporal correlation. Two photosensors (within a microscope eyepiece) are focused onto the vessel under study at a preset distance (D), temporal signals are picked up by both sensors with the downstream sensor signal delayed by the time (T) taken to travel D. It is therefore possible to determine RBC velocity (V) by the equation V=D/T. This technique being electronic, removes operator bias, and can calculate RBC velocity in single fine capillaries (later modifications are able to measure forward and reversed flow). The high magnifications required for observation of single microvessels mean that tissue movements are greatly magnified due to pulse and intrinsic villous movements, rendering the method ineffective.

Computerised movement correction at the time the project was started was only effective when movement was extremely slow.

Spatial correlation is a variant of the two slit method (Goodman et al, 1974), whereby the position of a RBC pattern is recorded at 2 time points (T_1 and T_2) with a constant delay between T_1 and T_2. The variable distance travelled by the RBC pattern provides the velocity.

Spatial filtering is the last correlation method and was adapted from industrial applications, where a moving object's image is projected onto a prism grating of alternating transparent and opaque lines. These lines are placed perpendicular to the direction of movement and a photosensor detects all light passing the transparent lines, the frequency at which light passes these lines is proportional to the velocity of the moving object (Slaaf et al, 1979; 1981).

Videometric Analysis:-Frame by frame analysis of RBC velocity has always been the gold standard for velocity measurements in narrow vessels where RBCs or rouleauxs move in single file (Rosenblum, 1969). The flow of RBCs is recorded and then played back frame to frame so that the distance a particular RBC moves from one frame to the next can be measured, each frame = 0.04 sec therefore providing a time. Velocity is then calculated from distance/time.

Random line flying spot is a relatively new technique developed to run on specialised software developed to measure RBC velocity (Pries, 1988). The method comprises one or more spots being superimposed on to any curved or straight vessel. The velocity of the spots is manipulated until it matches (visually) the RBC velocity in the vessel. Although operator input makes it less accurate than other electronic techniques it is nevertheless extensively used in velocity measurement on images which are difficult to observe, due in our case to mucus obscurement.

Flying spot has several advantages over other methods in that it can

measure velocity in any shape of vessel, can be used in mucus obscured preparations but most importantly it can adjust for tissue movement. However, it is very dependent upon the subjective view of the operator which is its major criticism, but this can be reduced by using only one skilled operator who will presumably make the same measurement errors each time.

A derivative of two slit photometry adapted for use with video recordings is dual window (Intaglietta et al, 1975), where two windows are set across the image of a recorded blood vessel displayed on a video monitor. Velocity is then calculated from RBCs passing beneath the windows. As its a derivative of two slit correlation it is also subject to the same problems.

Charge coupled device array (CCD) is a different technique whereby a line of image sensors are aligned along the long axis of the vessel under study (typically a few mm long). The passage of RBC is measured by the sensors which have a preset distance between each sensor (Watanabe et al, 1991). The technique is very similar to spatial correlation except it has a larger number of sensors. CCD has limited movement correction, whereby the sensor width is larger than the vessel diameter, therefore if the vessel moves left or right it is covered by the sensor and does not affect the velocity. However, there is no correction for forward or reverse movement.

Frame by frame analysis and flying spot were the two measurement techniques chosen to quantify RBC velocity because they were the least affected by tissue movement. As flying spot is dependent upon operator judgement it is prone to error, consequently, it was calibrated using frame by frame which highlighted an error of around 20-25%. Therefore random line flying spot could only be used to measure large changes in flow (for example between 100% and 75%). The degree of error renders it ineffective in quantifying subtle alterations in blood flow, for which frame by frame

analysis is used.

When measuring blood flow with any technique, it must be taken into account that flow within vessels is parabolic (Baker & Wayland, 1974). Particles at the edge of the vessel move more slowly than those in the centre, consequently, it is standard practise to measure only centre line velocity (Slaaf et al, 1990). However, this is only true in vessels with a diameter of 100μm or larger, so for this project it was not a factor. Another source of error (associated with frame by frame analysis) is the distortion of the image at the edge of a video screen. The image becomes stretched therefore giving a false impression of size so all measurements were calibrated to take this into account.

Vasomotion can occur in small vessels, where blood flow goes through alternating periods of flow and stasis, and any velocity measurements must take this into account. In any vessel which exhibited vasomotion measurements were taken over the full stasis and flow cycle and then averaged to provide the final velocity

(ii) Vessel Diameter

In order to calculate blood flow (BF)

from the equation $BF=\pi/4 \times V \times D^2$ the diameter of the vessel must be squared therefore magnifying any measurement errors. Measurement of diameter can be a problem because of out of focus specimens and the surrounding medium creating a halo of out of focus light which has been shown to alter image dimensions in fluorescence microscopy (Silver et al, 1992). The most common error is overestimation, producing errors of ± 0.6μm, therefore, significantly altering measurements of blood flow, microvascular surface area, cross sectional area and capillary resistance. Although water immersion lenses would have reduced the halo effect and improved accuracy (Gretz & Duling, 1995), they could not be used in this study because of their close working distance preventing simultaneous

manipulation and observation of the specimen. So instead extreme care was taken to ensure accurate focusing using long working distance objectives.

However, any technique requiring operator judgement, is going to be prone to error. Studies suggest that error can be reduced by using only one operator to analyse results (Silver et al, 1992), because an individual operator will consistently use the same reference point for judging the edge of a capillary, (eg. either the light or dark boundary of a capillary). Diameter can be measured from frozen still pictures with a ruler, by videometric line drawing, line scan CCD or image shearing.

Measurement of still pictures and videometric line drawing are very similar techniques in that both require the operator to superimpose a line across the diameter of the vessel, the length of the line corresponding with the vessel diameter (with the appropriate correction factor). As the accuracy of the measurement is dependent on the skill of the operator in judging the vessel edge, they are the most inaccurate of the techniques outlined here. However, they have the advantage of being very effective on motile tissue, because both use frozen images.

The line scan charge coupled device (CCD) image sensor is positioned perpendicular to the vessel, red blood cells (RBC) passing beneath the sensor cause fluctuations in sensor readings, whereas the vessel wall and surrounding tissues produce only small fluctuations. It is computer based, thereby removing the potential for operator error and bias. The accuracy of diameter measurements is increased by taking a mean value of several readings with precision accuracy of 5% for 1 diameter measurement (Rosen & Paffhausen, 1993), and is able to measure diameters of up to 80μm which would be sufficient for microvasculature. In its present form CCD requires the vessel under study to remain perfectly still as it is unable to take into account any tissue movement, and it is best suited to measurements from fairly straight vessels which seldom occur in capillaries.

Image shearing is considered the most accurate means of on and off-line diameter measurement. The image is sheared perpendicular to the direction of flow manually, across the vessel under examination, until the two opposite edges of the vessel come together; the degree of shear corresponds directly to the diameter of the vessel. Operator input is therefore unavoidable, introducing subjectivity, bias and dependence on operator skill. However, unlike manual measurement with a ruler and videometric line drawing, the operator has to judge only 1 vessel edge, reducing error by 50%, and image shearing can accommodate tissue movement and mucus obscurement.

A shearing device was under construction for this project but was never completed. However, videometric line drawing which was a module in the same software package containing the flying spot program developed by Dr Pries, (Freie Universitat Berlin, Department of Physiology) was used as an alternative.

(e) Description of Experimental Apparatus

In summary the *in-vivo* microscope structure comprised a Zeiss microscope body, modified to allow greater vertical movement for positioning of specimens, with a high-pressure mercury vapour light source (Osram HBO 100W, Burke Analytical, Glasgow, UK).

Specimens were observed using Leitz long working distance objectives, and the microscopic picture was captured with a silicone intensified camera (SIT, Panasonic Moonlight), and then recorded on a Sony EV-S9000E Hi-8 video recorder (Greenham Video, London, UK), for off-line analysis. Recorded off-line images were analysed using a standard 486 personal computer fitted with an image grabber (DataCell), with software for velocity and diameter measurement provided by Dr Pries (Freie Universitat Berlin, Department of Physiology) see figure 4 for

schematic diagram of set up. Four different microscope objectives were

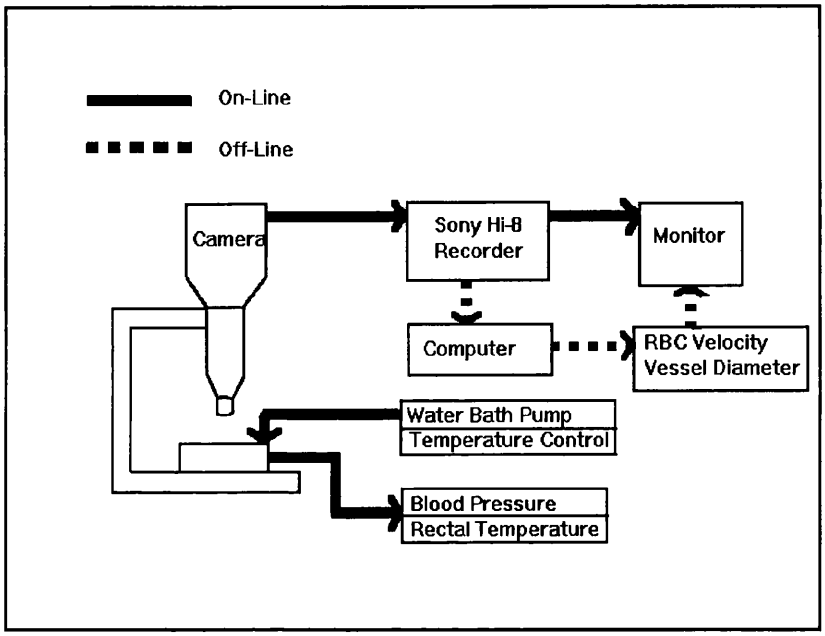


Figure 4. Schematic diagram of the hardware used in image capture and processing for *in-vivo* microscopy.

used in this project (Leitz, long working distance). The maximum resolution of each objective was calculated using Rayleighs criterion known as D_{min}, where the peak intensity of the Airy disk of the first object overlaps with the first minimum of the Airy disk of the second object. This is defined by the equation $D_{min} = 1.22\lambda/2NA$ were λ is the wavelength of light and NA is the numerical aperture. The lower the D_{min} value the better the objective resolution. The best resolutions (D_{min}) values produced by the objectives used in this study are shown in table 1.

Objective Power	Numerical Aperture	Resolution D_{Min}
× 4	0.14	2.1μm
× 10	0.22	1.4μm
× 32	0.3	1.0μm
× 40	0.65	0.5μm

Table 1. Table of objective resolutions used in this thesis.

Table 1 shows that the resolution of the ×4 objective is poor, however, this was not a major concern as it was only used to position the area of mucosa to be studied directly beneath the objectives, and not for recording velocity. Velocity measurements were carried out using the ×10 and ×32 objectives as their resolution values were more suited to the task. Measurement of capillary diameter requires a very high degree of accuracy and therefore resolution, so only the ×32 and ×40 objectives were used. Unfortunately the good resolution provided by the objectives was only fully exploited by direct observation of the preparation, because of the limitations of the camera. Even the very best cameras have inherently low resolution compared to optics (Shotton, 1993).

Blood flow was recorded at 1, 5, 10 minutes and every 5 minutes thereafter until blood flow either stopped, slowed or 90 minutes had elapsed. To avoid the deleterious effects of light (increased protein leakage) upon the microvasculature (Miller et al, 1992), preparations were exposed to fluorescent light for only 60 sec at each time point.

(f) Isolation and Histological Preparation of Single Villi

Correlation of blood flow alterations with morphology required histology from a single villus demonstrating reduced blood flow after exposure to indomethacin. The affected villus had to be marked so that it could be recognised for later microdissection, this was achieved with 2 pieces of fine suture material (1mm long) placed down into the crypts either side of the villus to prevent them being dislodged during manipulation. The pathological progression was instantly arrested in the deeply anaesthetized animals by opening the chest and inserting a needle into the heart, the animal was then cardiac perfusion-fixation. Perfusion maintained the vascular pathology, but to ensure complete fixation the section of intestine was also immersion fixed to maintain epithelial pathology. After fixation

and marking, the villus was isolated from normal surrounding villi for haematoxylin & eosin (H&E) histology or transmission electron microscopy (TEM) to ensure there was no confusion as to which villus had exhibited reduced blood flow. The preservation took into account the type of histology required, because (H&E) histology requires a different specimen fixation regimen to electron microscopy. Electron microscopy required smaller specimens to facilitate resin infiltration/impregnation of the tissue, and also because only very small specimens could be cut.

Three types of histological assessment were used in this thesis the first of which was H&E histology. H&E staining was used because all histological specimens in the study performed by Anthony (1993) were stained with H&E, consequently for direct comparison between blood flow effects and histology, specimens from this study would also have to be stained with H&E.

During preliminary experiments an intense fluorescence was observed localised within endothelial cells. Confocal microscopy was used on unstained whole mounts of these hyperfluorescent villi so that optical sections could be taken through a whole villus microvessels to assess any endothelial changes.

Evidence of endothelial damage presented by Anthony (1993) and the endothelial fluorescence seen in preliminary experiments strongly indicated transmission electron microscopy to elucidate the very earliest ultrastructural changes occurring within endothelial cells. TEM would also answer the problem of whether the villus epithelium or the villus endothelium was the primary site of indomethacin damage.

2. Description of Techniques Used in Thesis

The techniques used in this thesis were mostly standard, but specific problems such as isolating single villi and orientating them for histology

were new, requiring modifications to the standard procedures. Each new technique is outlined below.

(a) Animals

Female sprague dawley rats (150-200g) were obtained from BK Universal Ltd (Oldbrough, Hull, UK), and kept under standardized conditions for food, light and temperature. Female rats were used in preference to male rats because pilot experiments showed that they produced less mucus, enabling clearer visualisation of vessels for measuring purposes.

(b) Materials

Indomethacin, urethane, atropine sulphate, fluorocein isothiocyanate-dextran (FITC-Dextran), fluorocein isothiocyanate (FITC), N-acetyl-cysteine, dithiothreitol and sodium bicarbonate were supplied by Sigma Chemical Co. Ltd. Poole, Dorset, UK.

Glutaraldehyde, paraformaldehyde, uranyl acetate and lead citrate were supplied by Merck, Lutterworth, Leics, UK.

Osmium tetroxide was supplied by TAAB Laboratories Equipment Ltd, Aldermaston, Reading, Berks, UK.

CL316,243 (disodium-(R,R)-5-[2-[[2-(chlorophenyl)-2-hydroxyethyl]-amino]propyl]-1,3-benzodioxole-2,2-dicarboxylate) was synthesized and supplied by GlaxoWellcome PLC (Stevenage, UK.)

(c) Surgical Procedure

Rats were anaesthetized with intraperitoneal urethane 1.5g/Kg and temperature was maintained at 37°C ± 0.5°C using a homoiothermic blanket and a rectal thermometer (Harvard, Edenbridge, Kent, UK). A polyethylene cannula was inserted into the left external jugular vein. Tracheostomy was performed and an endotracheal tube inserted.

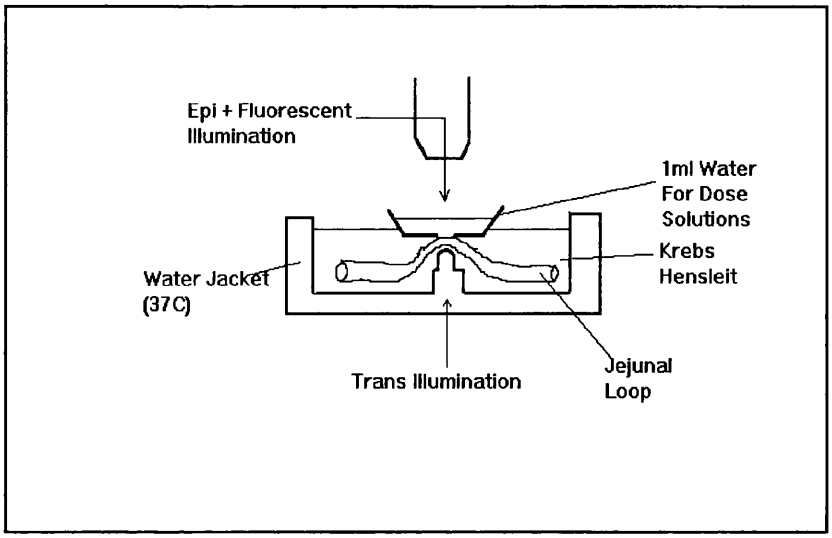


Figure 5. Water bath used to apply luminal solutions to the mucosa. It is lowered onto the mucosa using micromanipulators.

A polyethylene cannula was inserted in the left carotid artery and connected to a pressure transducer (Devices, Welwyn Garden City, Herts, UK) for monitoring mean arterial pressure. Through a right-sided laparotomy a mid-jejunal loop was exteriorised (located by removing the intestines and using only the middle section) and a 2 cm section opened along the antimesenteric border using micro-diathermy. The mesenteric border between vasa recta was observed because previous studies have shown that indomethacin-induced changes begin in this area (Anthony et al, 1996a). The loop was bathed in Krebs-Hensleit solution at 37°C, in a perspex observation chamber. A 1ml water bath was placed onto the exposed mucosa of the opened loop using micromanipulators. This prevented solutions applied to the mucosa from mixing with the Krebs-Hensleit (Fig. 5). Clingfilm was used to cover the exposed mesentery attached to the exposed loop.

Erythrocytes from an age and sex matched donor animal were labelled with Fluorocein Isothiocyanate (FITC) following the method of Sarelius (1989) and Sarelius & Duling (1982), and injected at a circulating fraction of approximately 10%. Normal rheologic behaviour of such

erythrocytes *in vivo* in the rat has been confirmed (Sarelius and Duling, 1982). Plasma was labelled with FITC-Dextran (M.Wt. 150,000) 4.4mg/Kg. Although both fluoresce green, red cells were easily distinguished from plasma as they had much greater fluorescent intensity.

(d) H&E Histology

At the earliest visible changes in villous blood flow, and selected time points thereafter, the observed villus was marked with 2 sutures placed either side of the villus into the crypt region. The animals were perfusion-fixed with 10% formol saline injected into the beating heart until the mesenteric and serosal vessels had cleared of blood. The jejunal section was removed and fixed by immersion overnight in 10% formol-saline. To isolate the marked villus from surrounding villi, the section of small intestine containing the marked villus was stretched and pinned onto a block of Silgard (Dow Corning, Germany). Surrounding villi were carefully removed and a V shape was cut into the tissue with the apex of the V 1mm below the marked villus (Fig. 6). The whole section still pinned to prevent rolling and curling of the tissue was then processed into paraffin.(Appendix A)

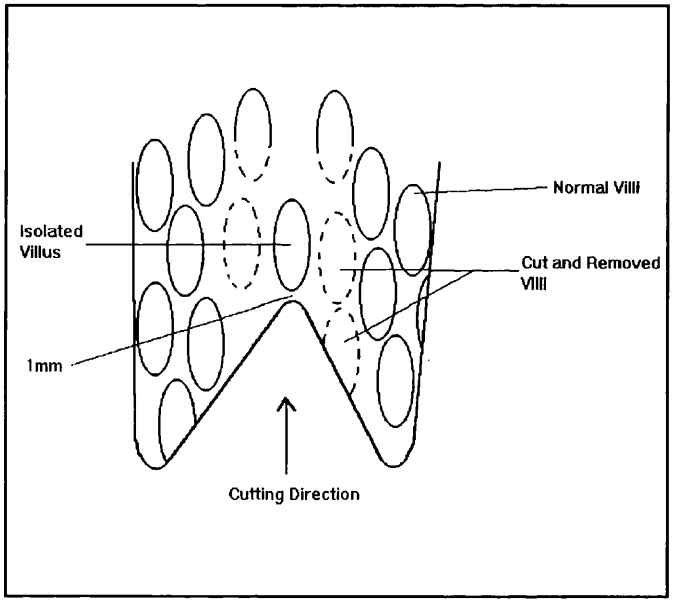


Figure 6. Schematic representation of how a villus is isolated for H & E histology

Processed tissue was wax embedded with the 2 prongs of the V facing the cutting blade, so as the paraffin block was trimmed the V would become smaller until it disappeared at which point the block was serially cut into 3μm sections. These sections were stained with haematoxylin & eosin. Sections of intestine 4 cm distal and 4 cm proximal to the marked villus were removed, fixed and cut in the same manner. Crypt depth to villous height ratios were measured in any villus exhibiting slowing or stasis using an eye-piece graticule, and compared with surrounding villi in which blood flow was normal.

(e) Transmission Electron Microscopy (TEM)

For electron microscopy, animals were vascular perfusion-fixed with 1.5% glutaraldehyde in 1% paraformaldehyde injected into the beating heart until the mesenteric and serosal vessels had cleared of blood. Marking and isolation of the affected villus was similar to H&E except that the villus was removed from the submucosa and its surrounding villi before processing (Appendix B) The fixed isolated villi were stained with 1.5% osmium tetroxide and processed into resin blocks, cut into 1μm semi-thin sections and stained with 1% toluidine blue.

Semi-thin sections were used to check the orientation of the isolated villus within the block. The plane of section was changed until good orientation was achieved of either transverse or longitudinal sections of villi was observed in toluidine sections. Thin 80 to 90nm sections were cut on an ultramicrotome (Reichert Ultracut), mounted on to copper grids (Gilder Grids, Grantham, Lincolnshire), and stained with uranyl acetate and lead citrate prior to visualization on a transmission electron microscope (Phillips Analytical, Cambridge, England).

Random groups of villi from intestines 4 cm distal and 4 cm proximal to the observed area were removed and fixed in the same manner.

(f) Confocal Microscopy

Following marking with a suture and perfusion-fixation with 10% formol saline, the marked villus was isolated by dissection, mounted in OCT (BDH, Poole, Dorset, UK) and either cut into 10μm sections on a cryostat (Bright Instruments Ltd, Huntington, UK) or examined whole after mounting in citifluor (BDH, Poole, Dorset, UK), and then imaged on a laser scanning confocal microscope MRC-600 (Bio-Rad, Hemel Hempstead, Herts, UK).

(g) Blood flow in whole small intestine

(i) Doppler Ultrasound of Superior Mesenteric Artery

Animals were prepared as in section 2c, then a mid-line laparotomy was performed and the gastrointestinal tract pushed over to the right to expose the abdominal aorta. The connective tissue around the aorta was removed to expose the superior mesenteric artery (SMA). The SMA was then carefully dissected away from its connective tissue and the adjacent lymph vessel. A 1mm Doppler ultrasound probe (Transonics system Inc, Ithca, New York, USA) was then attached to the SMA ensuring that it did not pull or kink the artery, good contact was ensured by coating the artery and probe with ultrasound gel (Henleys Medical, Brownfields, Welwyn Garden City, Herts, UK). The probe was then attached to a Transonics HT207 Doppler flowmeter.

(ii) Laser Doppler Flowimetry of Jejunal Mucosa

To measure the blood perfusion in the jejunal mucosa, a section of jejunum was diathermied and the mucosal surface exposed as described in 2c. A layer of surgical tape (3M micropore) was moistened and placed onto the mucosa and a 3mm laser Doppler probe (Moor Instruments Ltd, Millwey, Axminster, Devon, UK) attached to a Moor Instruments laser Doppler perfusion monitor was placed on to the tape. This process was

repeated with an extra layer of tape until perfusion was unmeasurable, at which point 1 layer of tape was removed. This ensured that only mucosal perfusion was being measured.

(h) Statistical Analysis

Treated groups were compared with control groups and results were analysed using the Mann-Whitney U test, except for analysis of CL316,243s effects upon basal blood flow, where the paired t-test was considered more appropriate. An associated probability of 5% or less was considered significant for all groups.

3. Experimental Groupings

(a) Attempts to improve visibility of microvessels through mucus

Three general approaches were used to remove mucosal mucus, (i) animal differences, (ii) chemical (iii) mechanical/physical mucus removal.

(i) **Animal differences**:

Hispid cotton rat:- The jejunal and ileal mucosa was visualized in 12 hispid cotton rats of either sex. Image quality and mucus secretion was then compared subjectively with 12 male sprague dawley rats.

Sex Differences:- In order to avoid the hormonal complications involved with female animals most experimental work is performed on male animals, consequently it is unknown if there whether less mucus secretion occurs in females compared to males. Therefore the mucosa was visualised in 5 female rats, and the number of experiments which had to be aborted due to mucus obscurement was compared with those in 5 male rats.

Circadian Rhythms:- The rats circadian rhythms are arranged so that in the afternoon they are asleep, and not therefore feeding or producing mucus. Mucus production in the morning was compared subjectively with

production in afternoon experiments in sprague dawley rats.

(ii) **Chemical**:

Atropine:-Since villus crypt goblet cells are known to be responsive to cholinergic stimulation (Neutra et al, 1982), attempts were made to reduce secretion with high concentrations of 100ug/ml (1.5×10^{-5}M) by 3 different routes; (i) sub-cutaneously every 30 minutes for the duration of an experiment, (ii) intravenously via the right external jugular vein every 30 minutes, and (iii) luminally together with the test solutions, where it was replaced every 15 minutes along with the test solutions. Mucus production was then compared to controls which received saline only (5 rats per group).

N-acetyl-Cysteine:-An easier, less invasive method of control was to alter the mucus after its release. N-acetyl-cysteine (NAC) is a known mucolytic agent which breaks down the structural integrity of the mucus, it is generally used to isolate mycobacterium from sputum but has also been previously used to remove gastric mucus (Kiviluoto et al, 1993). The authors applied NAC directly onto the area of study in high concentrations (50mg/ml, 0.31M) *ex-vivo*. In this *in-vivo* study it was decided to pre-dose animals with a lower dose of 1mg/ml (6.13×10^{-3}M) as described by Vogt (1980)to avoid potentially damaging high doses. NAC was dissolved in tap water to produce a concentration of 1mg/ml, together with 20.5g glucose to make it more palatable. Rats (n=5) were then given free access to this solution for 2 days prior to visualization of the mucosa. On visualization, mucus was gently removed with cotton wool, the ease of removal was compared with controls given tap water only.

Dithiothreitol:-Mucus strands are held together by disulphide bonds which can be broken by Dithiothreitol (DTT) thinning the mucus making its removal easier. Ukkonen (1987) used concentrations of 5-8 $\times 10^{-4}$M, but

this was applied to *in-vitro* nasal mucus and not *in-vivo*, for this study a lower dose was used on living tissue. DTT was applied luminally to 5 animals at doses of either 0.15mg/ml, 1×10^{-4}M or 0.31mg/ml, 2.01×10^{-4}M concomitant with any luminal bathing solutions used during the experiment. The ease with which mucus could be removed with cotton wool was compared to controls without DTT.

(iii) **Mechanical/Physical Mucus Removal**:

Micropipettes:- Sababi et al (1995) removed mucus using micropipettes, from single villi *in-vivo* via suction. As the authors only removed mucus from 1 villus it was unknown if this technique would be effective for *in-vivo* microscopy as this project required a much larger area (typically 40-50 villi) to be cleared. Microelectrodes (Borosilicate tubing 1.2mm outer diameter, 0.6mm inner diameter) were pulled on a pipette puller (Narishinge Scientific Instrument Laboratories, Tokyo, Japan) to a tip diameter of 1-2μm. Firstly, mucus was sucked through the pipette to clear an area of mucosa, and secondly, as mucus sticks to hydrophilic pipettes, they were gently stroked across the mucosal surface (5 rats for each technique).

Glass coverslips & Clingfilm:- Glass coverslips or clingfilm (5 rats each) were placed onto the mucosa and then quickly removed and mucus removal then assessed. Coverslips were also left covering the mucosa and the microvasculature was viewed through them. This was repeated using clingfilm.

Mucosal Touching with cotton wool:- This was the most simple of the physical techniques employed to remove mucus. The mucosa was gently touched once every 30 minutes with cotton wool rolled into balls of around 2mm gripped in fine forceps.

(b) Effects of Surgical Technique:

Animals were prepared according to the methods in section 2c, then a 2 cm section of jejunum was diathermied along the anti-mesenteric border, and removed for histology. Villi were not isolated but were prepared for H&E histology as described in 2d.

(c) Oral Indomethacin and Blood Flow:

The first series of experiments sought to ascertain that reduced blood flow/stasis was a feature of fully developed mucosal lesions induced by oral indomethacin. Animals received oral indomethacin (15mg/Kg) dissolved in 1.25% bicarbonate vehicle (1ml per 250g body weight), the mucosa was then observed as described in section 2c at 6 and 4 hours after dosing (n=5 per time point). Control animals were given oral bicarbonate (1.25%), 6 or 4 hours prior to mucosal observation (n=5). Animals were perfusion-fixed via the beating heart and the intestines were removed and immersion fixed, villi were not isolated but sections of the intestine were prepared for H&E histology (section 2d)

(d) Progressive Development of the Early NSAID Villous Lesion:

The model was designed, firstly, to evaluate and correlate early changes in flow with microscopic injury at early time points and, secondly, to mimic the effect of oral indomethacin in previous experiments in rats (Anthony et al, 1993). In the previous studies an oral dose of 15mg/Kg was shown to consistently produce ulceration (Anthony et al, 1993). Although this dose is high and not relevant to human therapeutic dosing, it provides a reproducible model for study. A prior pharmacokinetic study showed that this dose resulted in concentrations of approximately 100μg/ml (2.8×10^{-4}M) in plasma and 100μg/ml (2.8×10^{-4}M) in luminal contents (Anthony A, unpublished data), the dosing regimen comprised 100μg/ml luminally

(applied only on to the exposed mucosa) plus 15mg/Kg intravenously (iv) was adopted. All experimental animals were prepared as described in sections 2c. To determine the influence of administration route, 3 dosing groups were compared; luminal and iv combined, luminal alone, and iv alone.

(i) Combined Luminal and Intravenous Indomethacin:

In view of enterohepatic cycling of indomethacin, where the intestines are exposed to both luminal and systemic challenge following an oral dose of the drug, indomethacin was given both luminally and systemically. Animals were given a luminal (100μg/ml) and iv bolus dose of indomethacin (15mg/Kg) estimated to produce a plasma concentration of 100μg/ml, blood flow was then observed for 90 minutes or until blood stasis occurred (n=5). Control animals were given combined luminal bicarbonate solution (0.034%) and iv bicarbonate (1.25%), (n=5). The observed villus was isolated for H&E histology according to section 2d.

A different group of animals was given luminal (100μg/ml) and iv (bolus of 15mg/Kg) indomethacin as above and as blood stasis developed the animal was perfusion-fixed and the affected villus isolated and prepared for confocal microscopy as described in section 2f (n=5).

(ii) Luminal Indomethacin:

Luminal indomethacin 100 or 200μg/ml, dissolved in 0.034% bicarbonate was applied to the exposed mucosa, mucosal blood flow was observed for 90 minutes or until blood stasis occurred (n=5 animals per dose). Control animals were given luminal bicarbonate solution (0.034%) alone, (n=5). An end point of 90 minutes was used because in pilot experiments 90 minutes was found to be a sufficient period of time for indomethacin to exert its mucosal effect when applied luminally and

systemically. Villi were not isolated unless a blood flow effect had been observed, but histological preparation of the observed area for H&E was performed according to section 2d.

(iii) Intravenous Indomethacin:

A bolus dose of iv indomethacin 15mg/Kg or 30mg/Kg estimated to give a plasma concentration of 100μg/ml or 200μg/ml respectively was administered iv through the jugular cannula, blood flow was observed for 90 minutes or until blood stasis occurred (n=5 per dose). Control animals were given an iv bolus of bicarbonate solution (1.25%) alone, (n=5). Villi were not isolated unless a blood flow effect was observed then the observed section was removed and prepared for H&E histology as described in section 2d.

(iv) Indomethacin-induced slowing of villous blood flow:

In animals where focal vascular stasis developed it was shown to occur in a progressive and gradual manner. In order to evaluate histomorphometric changes at earlier times, when blood flow was slowing down, animals were given a combined luminal (100μg/ml) and iv bolus dose of indomethacin (15mg/Kg) and the experiment was terminated by perfusion-fixation at the moment slowing of blood flow was first observed microscopically.(n=5). The observed villus was isolated for H&E histology as in 2d. A separate set of animals were taken to the point of slowing and confocal microscopy was performed according to section 2f (n=5).

(v) Ultrastructural Pathology:

Combined luminal (100μg/ml) and iv (bolus of 15mg/Kg) indomethacin was applied to mucosa as above, and at the point of slowing the animal was perfusion-fixed with 1.5% glutaraldehyde in 1%

paraformaldehyde. The observed villus was then marked and microdissected for transmission electron microscopy (TEM) as described in section 2e (n=3). Random groups of villi were then removed proximally and distally to the observed villus and also prepared for TEM.

A separate group of 3 animals was given combined luminal and iv indomethacin and at the point blood stasis developed (visible micoscopically) the observed villus was marked and the animal perfusion-fixed with 1.5% glutaraldehyde in 1% paraformaldehyde. The observed villus was then microdissected and processed for TEM as in section 2e (n=3).

Controls comprised of animals given luminal and iv bicarbonate for 30 minutes, which is a time scale in which indomethacin affects blood flow (n=3).

(vi) Measurement of mucosal and superior mesenteric artery blood flow:

Indomethacin has been shown to reduce blood flow in the superior mesenteric artery (Feigen et al, 1981), it is therefore possible that the focal reductions in mucosal blood flow were a result of reduced flow in the whole intestine. Consequently animals were given an iv bolus dose of indomethacin (15mg/Kg) estimated to produce a plasma concentration of 100μg/ml. Blood flow in the superior mesenteric artery was measured using a Doppler ultrasound probe as described by section 2g, whilst mucosal flow was simultaneously measured using a laser Doppler probe according to section 2g for 90 minutes (n=5). Controls were given iv bicarbonate alone for 90 minutes (n=5).

(e) Pharmacological Intervention

(i) Luminal CL316,243 (β_3-adrenoceptor agonist) at Point of Stasis:

Previous studies showed that the lowest CL316,243 dose which gave maximal protection against indomethacin-induced ulceration was 1mg/Kg (Anthony et al, 1996c). It was assumed that CL316,243 was evenly distributed throughout the rat, and as each rat had an estimated blood volume of around 10ml, a 1mg/Kg dose would equate to 100ug/ml in the plasma; this concentration was then used for all subsequent experiments. Animals (n=5) were given combined luminal (100μg/ml) and iv (bolus of 15mg/Kg) indomethacin, and villous microcirculation was observed until focal blood stasis developed in a single villus. Luminal CL316,243 (100µg/ml) was then applied, and the blood flow observed for up to 90 minutes. Observed villi were isolated and prepared for H&E histology as in section 2d.

Control animals (n=5) were given indomethacin by both routes until vascular stasis developed in a single villus, followed by luminal saline.

(ii) Intravenous CL316,243 at Point of Stasis:

Animals (n=5) were given combined luminal (100μg/ml) and iv (bolus of 15mg/Kg) indomethacin, and blood flow was observed until focal blood stasis had developed in a single villus. An intravenous bolus dose of CL316,243 (1mg/Kg) estimated to produce a plasma concentration of 100µg/ml was given, and the blood flow observed for a further 90 minutes to assess the ability of an iv dose to reverse blood stasis. Control animals (n=5) were given indomethacin by both routes until stasis developed, then iv saline was given. Isolation of observed villi and H&E histology was performed as described in 2d

(iii) Luminal CL316,243 Alone:

In order to examine the effect of CL316,243 alone on basal villus blood flow luminal CL316,243 (100μg/ml) in saline was applied and blood flow was observed for 90 minutes(n=5). Control animals (n=5) received luminal saline alone. Isolation of observed villi and H&E histology was performed as described in 2d

(iv) Intravenous CL316,243 Alone:

This group examined the effect CL316,243 alone on basal villus blood flow after an iv bolus dose of CL316,243 (1mg/Kg) estimated to produce a plasma concentration of 100μg/ml, in saline. Control animals (n=5) received iv saline alone. Blood flow was then observed for 90 minutes in each group. Isolation of observed villi and H&E histology was performed as described in 2d

(v) Prophylactic Protection Against Stasis by Intravenous CL316,243:

This group was designed to test the suggestion that CL316,243 is an effective prophylactic against indomethacin-induced gastric and intestinal ulceration (Anthony et al, 1996c; Kuratani et al 1994), which may be mediated in part by preservation of mucosal blood flow. Therefore a combined luminal (100μg/ml) and iv (bolus 15mg/Kg) indomethacin was given simultaneously with an iv bolus of CL316,243 (1mg/Kg). Control animals were given indomethacin with simultaneous iv saline (n=5). Isolation of observed villi and H&E histology was performed as described in 2d.

4. Summary and Discussion of Methods

In summary, of the methods able to observe/measure RBC velocity,

only *in-vivo* fluorescence microscopy could observe villous morphology and changes in blood flow in both large and small areas of mucosa, and then focus onto a single villous vessel. Most importantly it allowed any observed blood flow changes to be correlated with villous histology. Other techniques which were able to measure/observe the mucosa could not adequately adapt to movement of the preparation, which was unavoidable due to respiration (although this was reduced by exteriorising a jejunal loop as far away from the diaphragm as possible) and the intrinsic motility of intestinal villi.

Despite fluorescent labelling, the amount of visible fluorescence was of low intensity, therefore the microscope system had to maximise the light whilst maintaining good specimen resolution. A compromise was reached where long working distance objectives (which have a poor NA compared to close working distance objectives) with the best possible NA were used. The reduction in NA was compensated for by the use of an intensified camera.

A silicon intensifier camera (SIT) was used in this thesis because it provided the best image resolution, which far outweighed its disadvantages of image lag, white noise and oversaturation. Mucus obscurement often degraded the image quality, therefore a further reduction in resolution produced by the camera was unacceptable.

I*n-vivo* microscopy alone is ideal for qualitative assessment of blood flow, but quantitative analysis of velocity and diameter requires it to be combined with a correlation or manual measurement method. However, the recent development of personal computers, and specialised software programs has lead to the development of random line flying spot which allowed quantification of RBC velocity in any vessel regardless of vessel size/shape, tissue movement, mucus or direction of flow, which none of the other described techniques was able to achieve. Its only disadvantage is its

dependence on operator input which introduces a 25% error. However, in this study it was only used to quantify large alterations in blood flow, for example between blood flow and blood stasis. In experiments where subtle velocity changes such as slowing of blood flow were quantified, frame by frame analysis of recorded video images was used. Although this technique is completely operator based the margin of error is minimal providing a RBC does not travel off the screen in 1 video frame, and measurements are only made in small capillaries, where only single RBCs pass. Otherwise it becomes difficult to identify RBC, and accurately calculate how far it travels in 1 frame length. Although more than 1 RBC was able to pass through the arcade vessel at any one time, only 10% of them were fluorescently labelled. Therefore there was only usually 1 RBC visible for measurement.

Greater accuracy was required for diameter measurements than for velocity, because diameter is squared when calculating blood flow, therefore increasing any measurement errors. The reference technique for diameter measurement is image shearing, but it was not available due to circumstances beyond control, so the next best alternative of videometric line drawing was used as it could accommodate moving preparations.

In addition to the standard techniques described here, some special procedures were developed. In particular the ability to mark and isolate a particular villus was very important to elucidate the primary indomethacin-induced pathogenic mechanisms, whereby the moment a change in RBC velocity was noticed, the preparation could be fixed, the villus in which change was observed marked and then microdissected for either H & E histology or TEM. Therefore it was possible to correlate exactly how changes in villous morphology were related to alterations in blood flow.

CHAPTER 3

RESULTS

1. Attempts to improve visibility of microvessels through mucus

A small *in-vivo* microscopy study had previously been performed on the jejunal villi of sprague dawley rats (Piasecki et al, 1994). Observation was however difficult due to mucus secretion, which obscured the villi resulting in poor images. *In-vivo* microscopy is infrequently used to observe and quantitate blood flow in the intestines consequently there are no studies to address the problem of mucus reduction/removal in sprague dawleys or any other strain of rat. Several attempts were made to solve this problem and are outlined below.

***(i) Animal differences*:**

Use of hispid cotton rats:- On laparotomy the gastrointestinal tract, although significantly shorter was otherwise the same as in sprague dawleys. Mucosal mucus was secreted in similar quantities as in sprague dawleys but was found to be almost completely translucent. Consequently the surface villous microvasculature was more easily observed in all 12 cotton rats. However, this strain of rats was found to be insensitive to indomethacin ulceration, even at 5mg/ml (50 fold higher than that required to ulcerate sprague dawleys), and could not therefore be used for this study.

Sex Differences:- To elucidate the differences in mucus secretion between male and female rats, 5 male and 5 female sprague dawley rats were prepared for microscopy and their mucus production assessed subjectively. It was found that visualisation was slightly better in female rats than in males, therefore all subsequent experiments were performed on female sprague dawley rats.

Circadian Rhythms:-Rats are nocturnal animals, and are therefore asleep, presumably not producing much mucus during the day. It was hypothesised that rats used in the afternoon would produce less mucus than rats used in the morning. On subjectively comparing 5 morning rats with 5 afternoon rats no improvements in visualisation were found.

***(ii) Chemical Removal and Reduction*:**

Atropine:-Mucus secreted from the villus crypts is under the control of cholinergic neurones and can therefore be blocked using atropine. Atropine (100μg/ml) was administered every 30 mins either sub-cutaneously or intravenously for 90 mins, it was also applied luminally with any bathing solutions (n=5 per group). However, neither sub-cutaneous, intravenous or luminal atropine produced any significant reduction in mucus secretion or improvement in mucosal observation in any animal.

N-Acetyl-Cysteine:- N-acetyl cysteine (NAC) was used because it breaks down the chemical structure of the mucus making it easier to remove and possibly more translucent. It was administered in the rats (n=5) drinking water at a concentration of 1mg/ml, the water was sweetened to disguise the taste. Animals drank the treated water more frequently than normally (assessed by the water level in the water bottle) due to the glucose in the water. Unfortunately the concentration of glucose couldn't be reduced as the rats were then able to taste the NAC and would not drink properly. Controls (n=5) were given normal drinking water.

Mucus appeared normal but it was less viscous, and when touched with small balls of cotton wool it easily came away from the mucosa, providing excellent images of the microvasculature compared to controls in all 5 test animals. However, animals developed diarrhoea and histology revealed mild to moderate erosions throughout the small intestine.

Dithiothreitol:- DTT was used because it also breaks down the chemical structure of mucus making it easier to remove, it was used in 2 groups of 5 animals at 1 or 2mM. However, neither concentration produced any change in mucus consistency or made it easier to remove in any animal.

***(iii) Mechanical and Physical Removal*:**

Micropipettes:- A study measuring the thickness of the mucus layer used suction through micropipettes to clear away mucus from single villi. The technique was subsequently attempted here in 5 sprague dawley rats, although over a much larger area of mucosa. Suction of mucus through micropipettes allowed a very small amount of mucus to be removed but was very slow taking around 5-10mins during which time alterations in blood flow may have occurred. Larger bore pipettes were ineffective as the suction required to pull the viscous mucus through the pipette was too great.

It was noticed that gentle touching of the mucosa with the pipette produced more effective and rapid mucus removal, because it adhered strongly to the hydrophilic pipette resulting in a large area of the mucosa being cleared very quickly. Unfortunately because of its thinness (2μm) it often broke causing haemorrhage.

Glass Cover Slips and Clingfilm:- The success of touching the mucus with micropipettes prompted a similar attempt using more robust glass cover slips and clingfilm (n=5 per group). However the mucus did not adhere to either coverslips or clingfilm as it appeared to adhere more strongly to the mucosa in all animals.

Mucus is extremely motile and was easily pushed aside by the coverslips and clingfilm. The mucosa when viewed through the coverslips or clingfilm presented excellent image quality, it was found that the villi were flattened with the mucus forced sideways and down into the villus crypts away from the epithelium. Unfortunately neither allowed application of luminal solutions and flattening of villi may have affected blood flow.

Mucosal Touching:-This was the most simple of the physical techniques employed to remove mucus and was adopted for this thesis. The intestinal mucosa was observed with the fluorescence microscope for 90 mins and the mucosa was gently touched every 30 mins once with cotton

wool rolled into balls of around 2mm gripped in fine forceps.

As well as being a simple technique, it was also demanding in terms of dexterity, because if pressed fractionally too hard the cotton wool damaged the villi. Nevertheless, after practise this technique was extremely effective in removing mucus because it readily adhered to dry cotton wool. Intestinal villi were generally left mucus free although occasionally poorly orientated because as the mucus was pulled away it often pulled the villi upright with it. This was the method deemed most effective in removing mucus, and was therefore used in conjunction with female sprague dawley rats in all subsequent experiments.

2. Effects of Surgical Technique

Diathermy of the anti-mesenteric margin of the jejunum severely damaged the first 5 rows of villi adjacent to each cut edge, leaving three quarters of the circumference including the mesenteric portion unaffected (Fig. 7). This and the fact that villi were almost completely removed in diathermied sections of intestine (Fig. 8) meant that indomethacin effects were easily distinguishable and did not interfere with assessment of pathology.

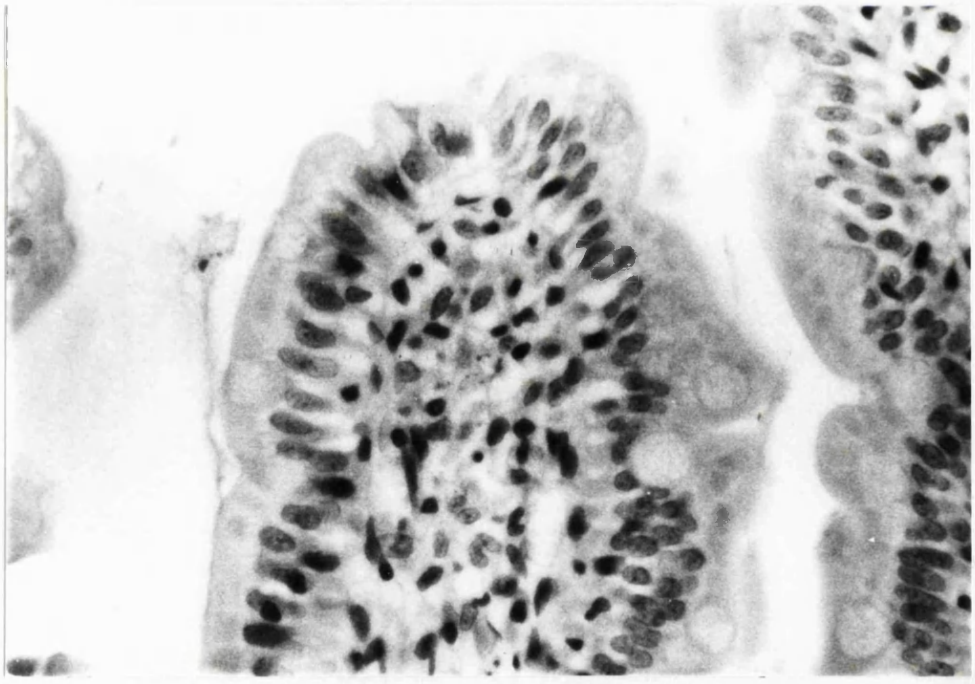


Fig. 7 A normal villus from the mesenteric portion of the jejunum after exteriorisation and diathermy of the anti-mesenteric border (Haematoxylin & Eosin × 525)

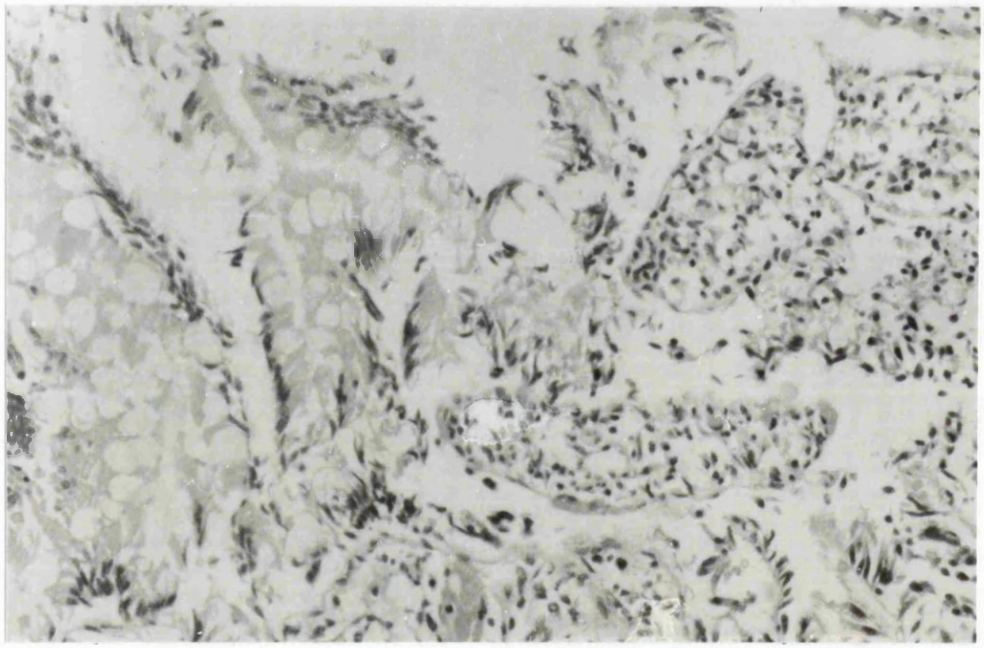


Fig. 8 Villi from the diathermied edge of the jejunum (Haematoxylin & Eosin × 525)

3. Oral Indomethacin and Blood Flow

Oral vehicle (1.25% bicarbonate) produced no macroscopically visible changes in the mucosa, and microscopic observation showed normal villous histology (Fig. 9) and blood flow, although this was not measured. In contrast, 4 hrs after oral indomethacin focal macroscopic lesions were seen from both the serosal and mucosal surfaces of the jejunum. All lesions occurred on the mesenteric aspect, between vasa recta, and appeared as translucent brown patches. Videomicroscopically, villi at the site of these lesions appeared flattened and mucosal blood flow was absent. Histological examination revealed necrosis of the upper third of affected villi (Fig. 10).

When examined at 6 hours after indomethacin, lesions were similar to those seen at 4 hours except that they had become haemorrhagic with red blood cells present in the intestinal lumen. Histology demonstrated necrosis of the upper third of the affected villi, similar to that at 4 hours. In surrounding areas of mucosa, blood flow appeared normal, and villi were histologically normal.

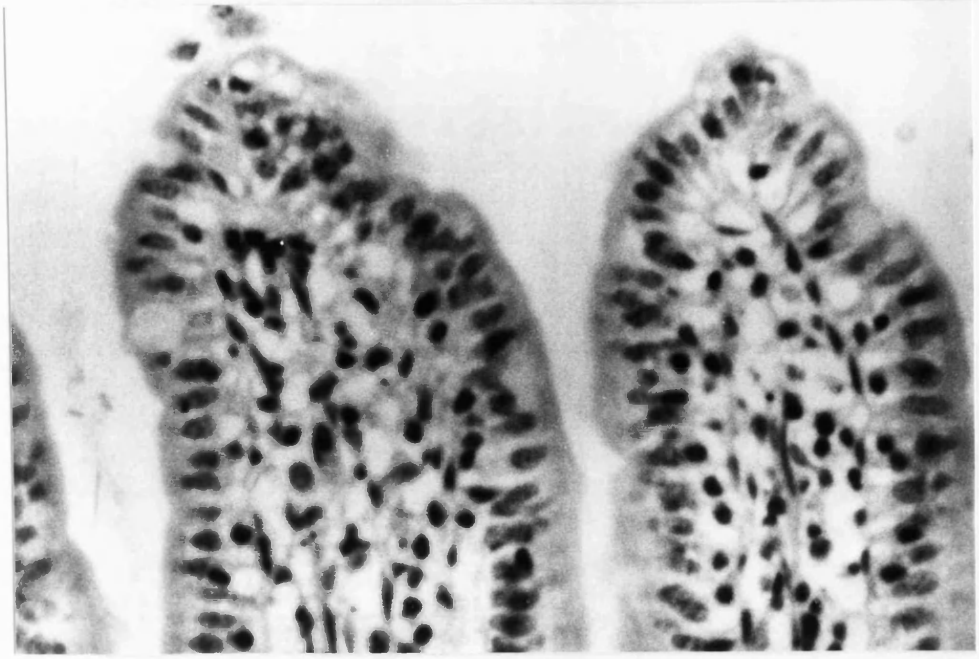


Fig. 9 Normal jejunal villi 6 hr after oral bicarbonate vehicle. (Haematoxylin & Eosin × 525)

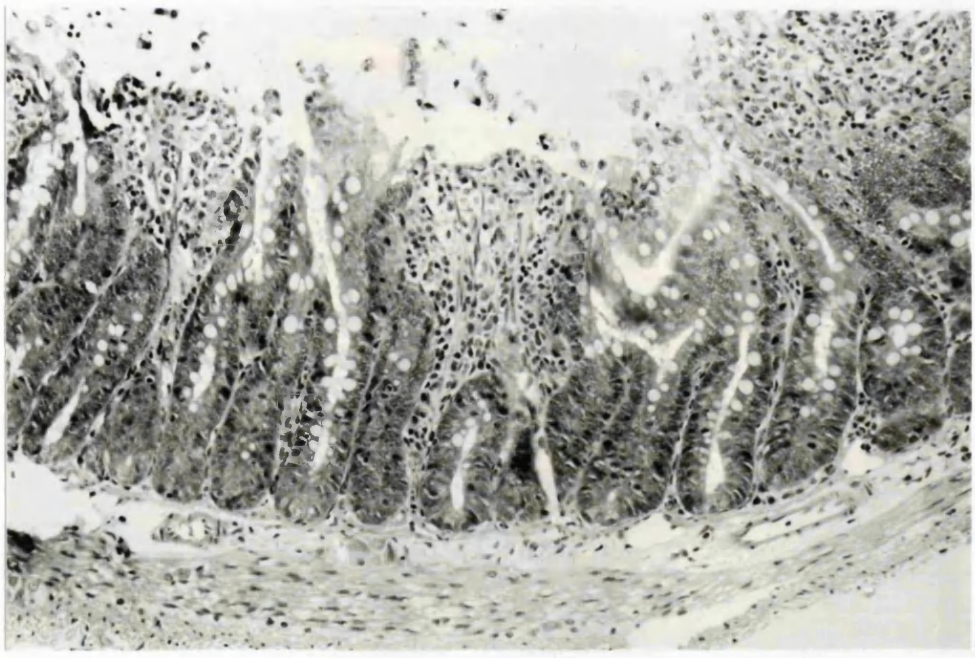


Fig. 10 Ulcerated area of jejunum overlying the mesentery 4 hr after oral indomethacin (15mg/Kg). There is necrosis of the upper portion of the villi, which has been lost into the lumen, gross villous shortening has occurred along with large vessel thrombosis. (Haematoxylin and Eosin × 40)

4. Progressive Development of the Early Indomethacin Villous Lesion

In-vivo microscopy of villous blood vessels allowed observation of the earliest changes following administration of indomethacin. Firstly, in order to mimic the effect of oral indomethacin in this animal model it was necessary to administer the drug simultaneously both luminally and systemically, in the same way that the jejunal mucosa is exposed following oral indomethacin. Secondly, the intestines were exposed to luminal and intravenous indomethacin in order to assess whether a luminal or a systemic actions was primarily responsible for indomethacin-induced effects. In preliminary studies a 15mg/Kg oral dose of indomethacin was consistently ulcerogenic to rats (Anthony et al, 1993; Nygard et al, 1994). A preliminary pharmacokinetic study (Anthony, unpublished data) showed that an oral dose of 15mg/Kg resulted in concentrations of approximately 100μg/ml in plasma and 100μg/ml in luminal contents, hence a dosing regimen was adopted comprising 100μg/ml luminally plus iv 15mg/Kg.

***Combined Luminal and Intravenous Indomethacin*:**

In order to mimic the effects of an oral dose of indomethacin, a simultaneous luminal and iv dose was applied and compared with control bicarbonate. Blood flow measurements were made from video recordings taken every 5 mins, each lasting 1 min, from each rat. Control luminal and iv bicarbonate had no effect on blood flow for up to 90 min, (n=5, Fig. 11) or on the histology (Fig. 12). Using combined iv and luminal indomethacin administration at a dose of 100μg/ml, blood slowing occurred first in the arcade artery of single villi within 15 min and progressed to permanent and irreversible stasis within 40 min (Fig. 11, $n = 5$ $P < 0.02$). The time scale for the development of stasis was also variable for each rat, blood flow data are shown in table 2. Around this focus of vascular stasis in one villus,

adjacent villi exhibited only slowing of blood flow (not measured). Stasis consistently developed only in the arcade vessel along the villus tip, three quarters of the way between the centre and edge of the leaf-shaped villus (Fig. 13a & b).

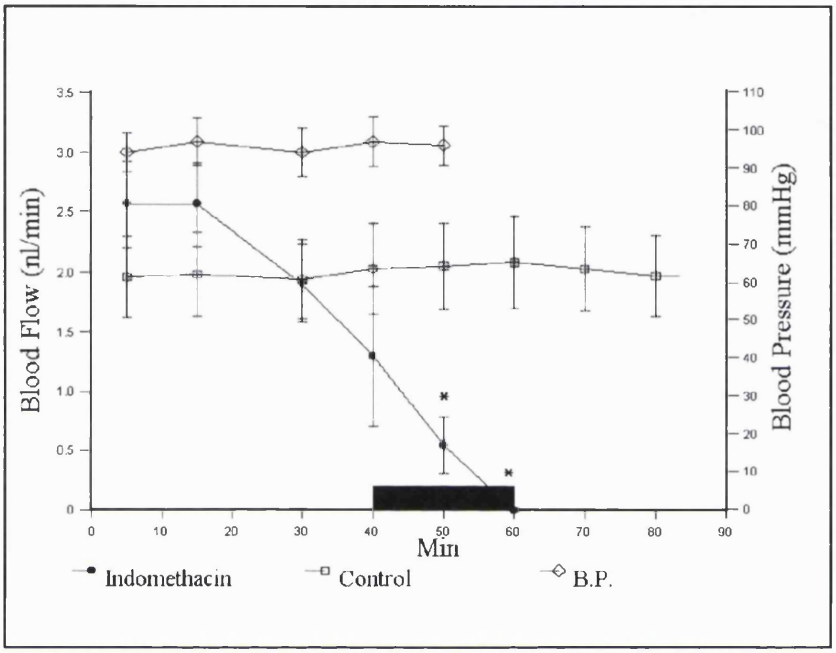


Figure 11 Blood flow in single jejunal villi after combined luminal (100μg/ml) and iv (bolus of 15mg/Kg) indomethacin and bicarbonate control given at 15 mins. The shaded area represents the 20 min period during which all 5 animals developed blood stasis in a single villus. Each point represents the mean of 5 animals ± SEM. (* $p<0.02$, Mann Whitney U)

In addition, intense endothelial fluorescence occurred in this segment of the vessel as slowing and stasis began to develop. Confocal microscopy demonstrated that the fluorescence was localised exclusively to the endothelium, and only affected the vessel in which stasis had developed. The part of the arcade vessel on the other side of the T-junction (ie the other end of the villus) showed no signs of increased fluorescence (Fig. 14a & b) nor slowing or stasis. Histology of single villi in which blood stasis occurred, showed corrugation of epithelial cells (tufting), smooth muscle prominence, endothelial damage/distortion and villous shortening (Fig. 15),

highlighted by crypt depth/villous height ratios of 0.92±0.2 compared with 0.42±0.06 in surrounding villi without vascular stasis (Table 3, Mann-Whitney U test $P < 0.05$).

Mins	RAT 1	RAT 2	RAT 3	RAT 4	RAT 5
5	2.95 nl/min	3.1 nl/min	2.04 nl/min	2.04 nl/min	1.86 nl/min
15	3 nl/min	2.97 nl/min	1.98 nl/min	1.98 nl/min	2.04 nl/min
30	0.9 nl/min	3 nl/min	2.04 nl/min	2.1 nl/min	1.68 nl/min
40	1.8 nl/min	2.94 nl/min	0 nl/min	0 nl/min	2.1 nl/min
50	0.9 nl/min	1.26 nl/min			0.57 nl/min
60	0 nl/min	0 nl/min			0 nl/min

Table 2 Blood flow values for the single villi which developed blood stasis on exposure to combined luminal (100µg/ml) and iv bolus estimated to give a plasma concentration of 100µg/ml in all 5 rats. Velocities and diameters used to calculate these values can be found in appendix C.

Control Villus	Ratio	Test Villus	Ratio
Crypt=20.3µm Villus=46.9µm	0.47	Crypt=17.3µm Villus=24.7µm	0.7
Crypt=17.3µm Villus=74.1µm	0.23	Crypt=19.8µm Villus=49.4µm	0.47
Crypt=14.8µm Villus=32.2µm	0.46	Crypt=19.7µm Villus=14.8µm	1.3
Crypt=27.8µm Villus=55.1µm	0.5	Crypt=55.1µm Villus=45.6µm	1.2

Table 3 Villus height and crypt depths in the villi in which stasis occurred compared to surrounding control villi which maintained normal flow. Crypt depth to villus height ratios indicate the degree of shortening, (high numbers indicating greater shortening).

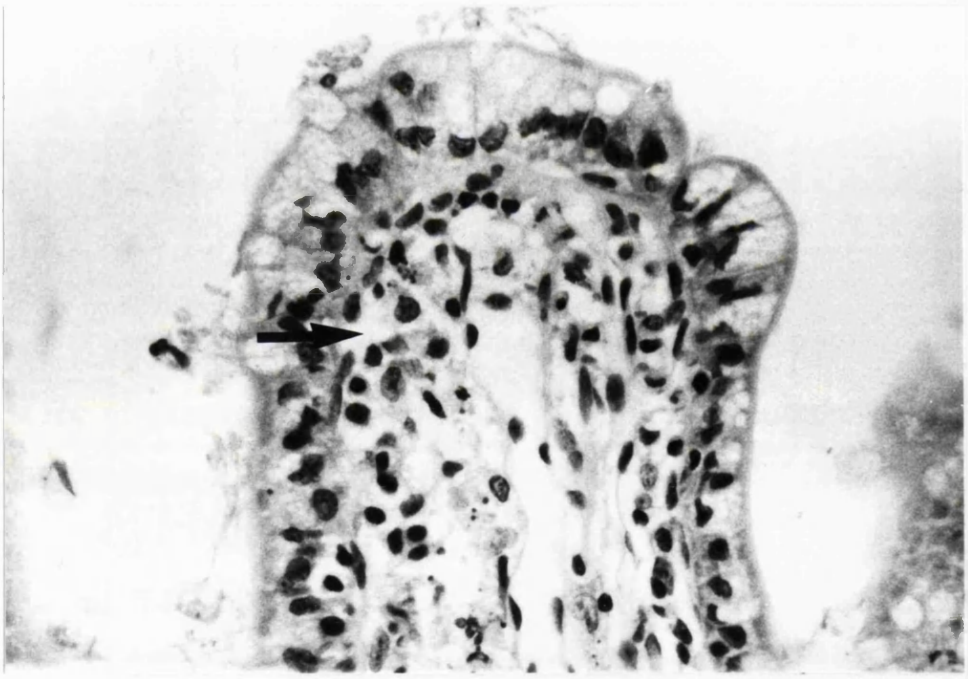
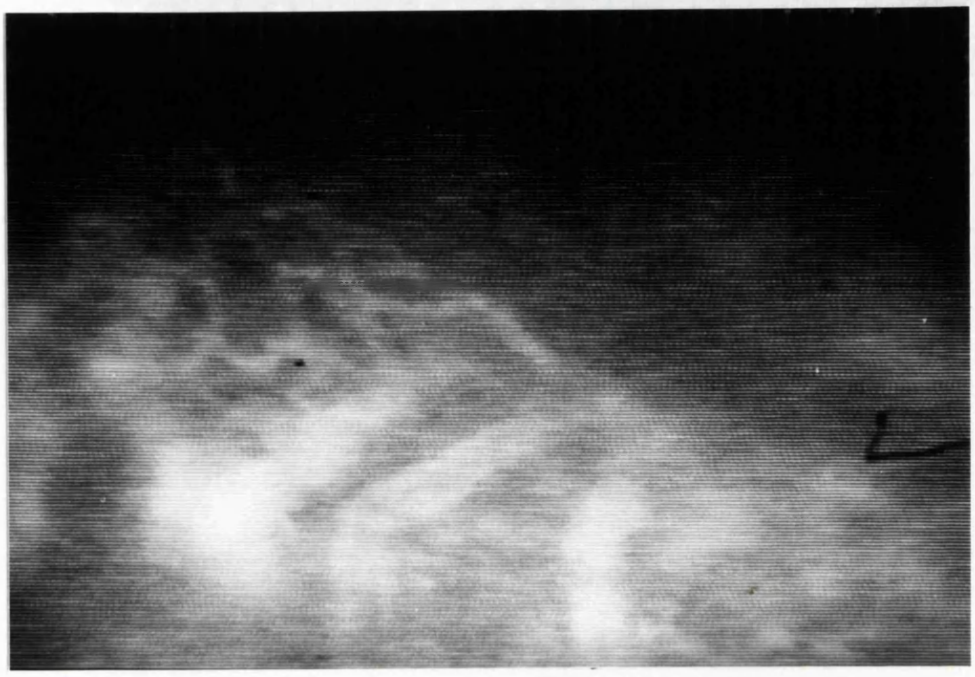


Fig. 12 Normal jejunal villous tip from a rat which received combined luminal & iv bicarbonate and perfusion fixed 90 min after dosing. Subepithelial capillaries are cleared of blood (arrow) indicating no vascular occlusion/blockage, with good preservation of villous architecture. (Haematoxylin and eosin, × 525).



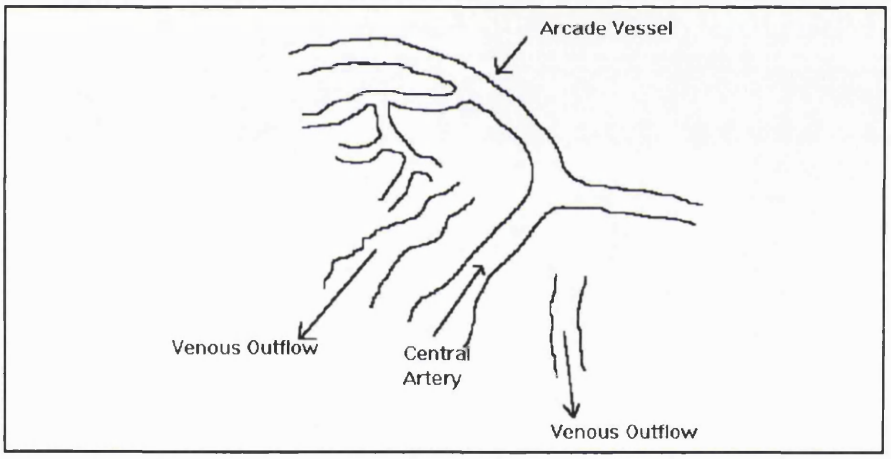
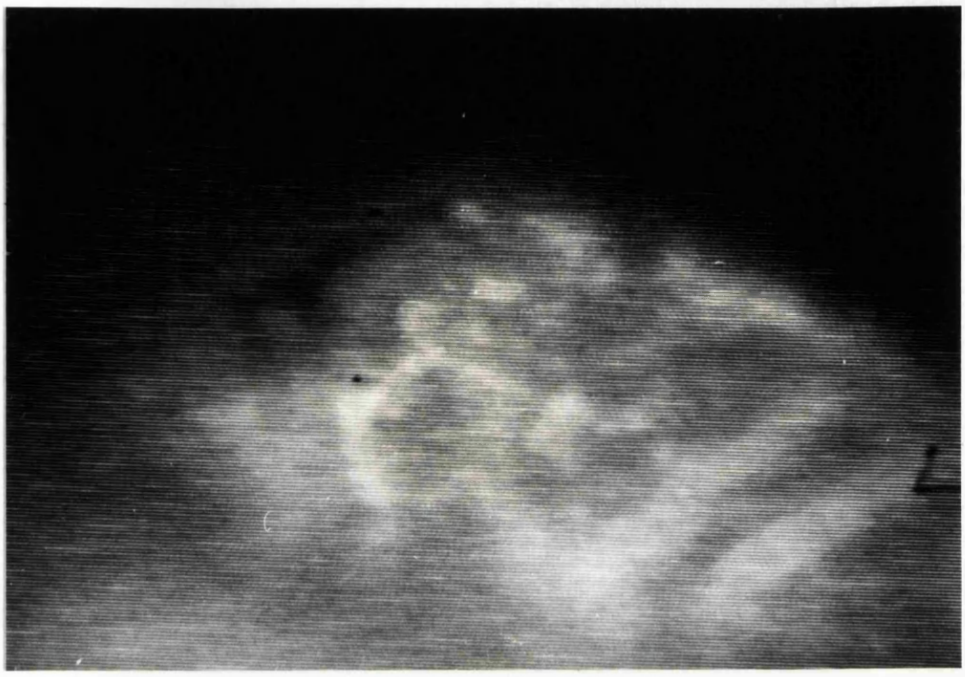


Fig. 13(a) The upper photograph is a video image obtained from a normal in-vivo villus prior to application of indomethacin, the vasculature is highlighted with FITC-Dextran in the plasma. The lower line diagram demonstrates the vascular anatomy of this villus. Notice central artery ascending the centre of the villus and dividing at the tip as a T-junction to form the arcade vessel. This runs along to the left along the tip to supply the left half of the villus, and along the right tip for the right half of the villus (Magnification × 330).



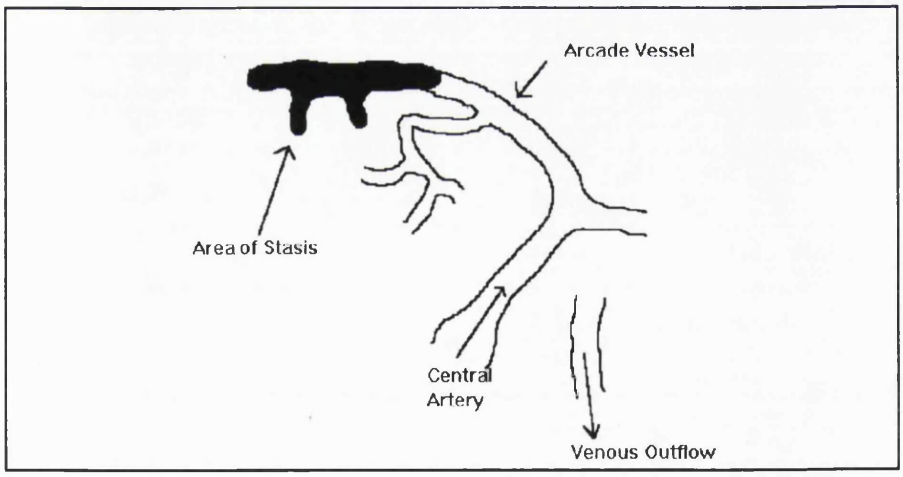
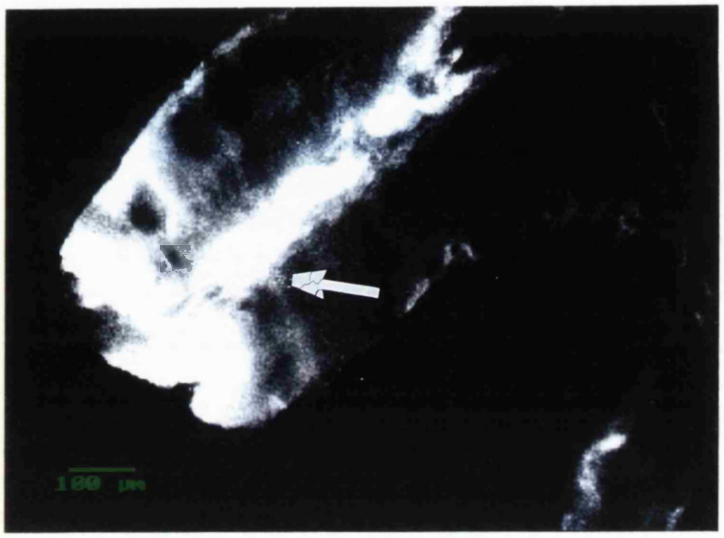


Fig. 13(b) This is the same villus as 13(a) after application of indomethacin, again the upper picture is a photograph of a video image where the darkened area in the upper left half of the picture is an area of blood stasis. This is demonstrated more clearly on the lower line diagram (Magnification × 330).



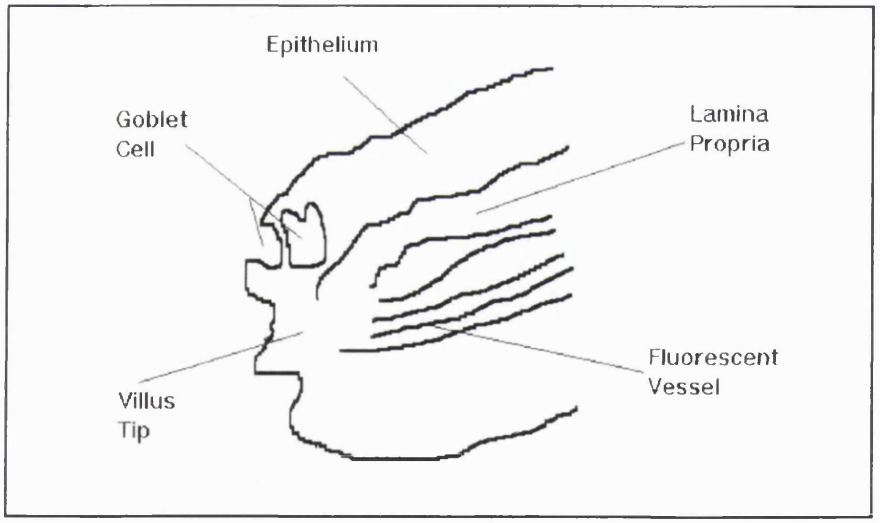
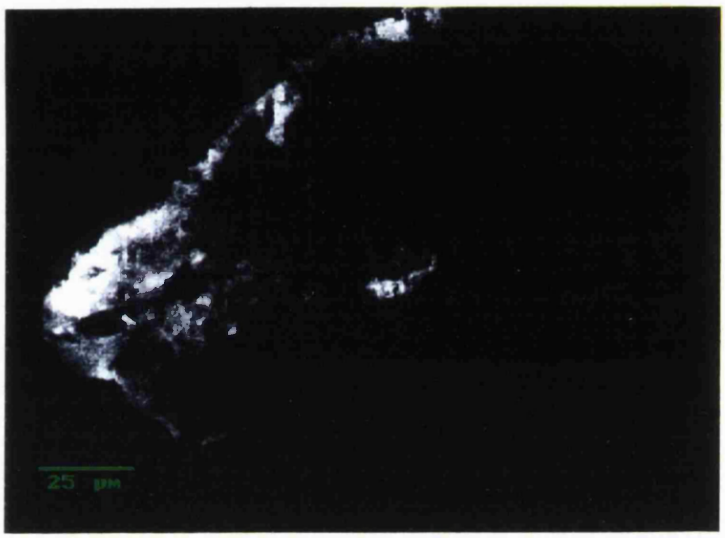


Fig. 14(a) The upper image was taken from a confocal microscope and shows an isolated villus from rat jejunum after combined indomethacin (100μg/ml), taken from the left half of the villus which showed stasis and increased endothelial fluorescence (arrow), at which point the villus was perfuse fixed, the area of fluorescence to the left of this vessel is epithelium. This is more clearly shown in the lower line diagram.



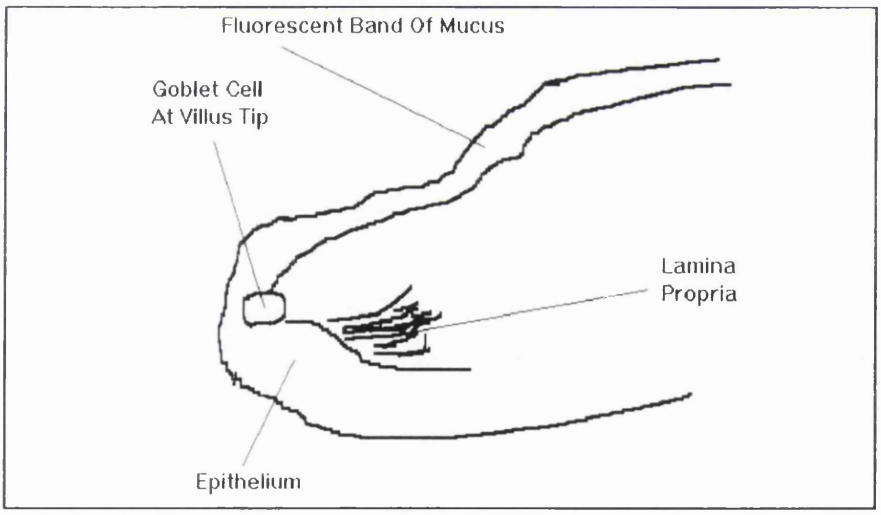


Fig 14(b) Image from other right side of villus (a) which had normal flow and no fluorescence except for surface mucus, shown on the lower line diagram.

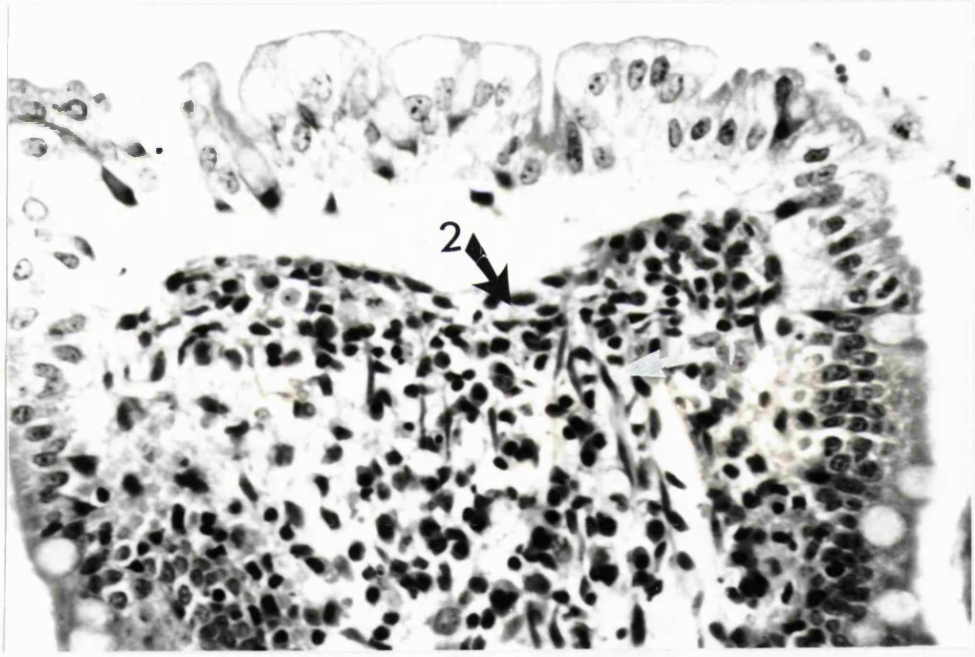


Fig. 15 Villus tip from rat jejunum showing blood stasis after combined luminal (100μg/ml) and iv (15mg/Kg) indomethacin, surface epithelium beginning to lift and fall away from lamina propria. There is contraction of surface smooth muscle cells manifest by prominent contractile elements (arrow 1) causing occlusion of surface vessels (arrow 2), there is also degeneration of the lamina propria. (Haematoxylin and Eosin × 410).

***Luminal Indomethacin*:**

In order to assess the topical effect, indomethacin was applied luminally at doses of 100 and 200µg/ml, and the blood flow and histology compared to controls given luminal bicarbonate alone. A 90 min exposure to control bicarbonate did not alter either blood flow (Fig. 16) or histology (Fig. 17).

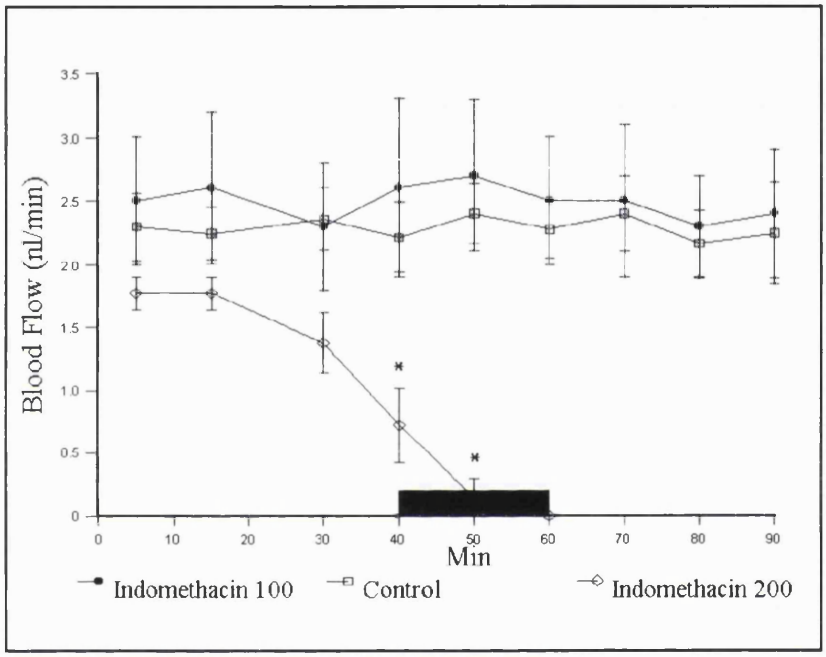


Figure 16 Jejunal villus blood flow after luminal indomethacin (100μg/ml & 200μg/ml) or bicarbonate control at 15 mins. The shaded box represents the 20 min period during which all 5 rats developed blood stasis. (* p< 0.02, Mann Whitney U)

Luminal indomethacin given at the lower dose of 100µg/ml also did not alter villous blood flow for up to 90 mins (Fig. 16). Histology at this time point showed that villi were normal (Fig. 18) in all 5 rats. However, when given at a concentration of 200µg/ml it resulted in a discontinuous step-wise slowing of blood flow, visible by eye. Whereby flow stopped briefly in the arcade vessel, and then restarted at a lower velocity followed by another brief period of stasis and restart. This process was repeated with

progressively longer periods of stasis and greater reductions in RBC velocity until complete and permanent stasis occurred. The flow data for each rat is shown in table 4, which shows that the time point at which each rat developed permanent stasis varied between 40 and 60 mins (25 and 45 mins after exposure to indomethacin, n=5, $P<0.02$). Around the focus of vascular stasis, adjacent villi exhibited only slowing of blood flow, assessed subjectively. Histological examination at the point of permanent stasis, from the single villus in which stasis first developed, showed the typical pre-ulcerative changes of shortened/distorted villi, smooth muscle prominence, focal upper villous necrosis endothelial damage and buckling of large vessels in all 5 rats (Fig. 19).

Mins	RAT 1	RAT 2	RAT 3	RAT 4	RAT 5
5	1.5 nl/min	1.9 nl/min	1.62 nl/min	2.15 nl/min	1.90 nl/min
15	1.46 nl/min	1.86 nl/min	1.60 nl/min	2.2 nl/min	1.92 nl/min
30	1.08 nl/min	1.26 nl/min	1.08 nl/min	2.1 nl/min	0.98 nl/min
40	0.42 nl/min	1.3 nl/min	0 nl/min	1.2nl/min	0 nl/min
50	0 nl/min	0 nl/min		0.58 nl/min	
60				0 nl/min	

Table 4 Blood flow values for the single villi in each rat which developed blood stasis on exposure to luminal indomethacin 200µg/ml. The velocity and diameter values used to calculate blood flow can be found in appendix D.

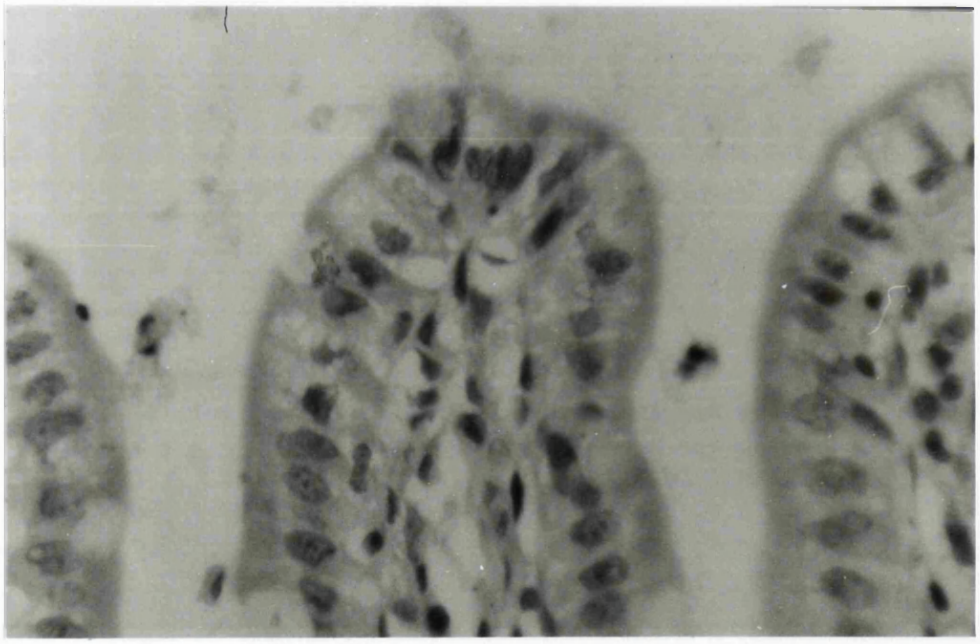


Fig. 17 Normal jejunal villous tip from a rat which received luminal bicarbonate control and perfusion fixed 90 min after dosing. Subepithelial capillaries are cleared of blood indicating no vascular occlusion/blockage, with good preservation of villous architecture. (Haematoxylin and eosin, × 525).

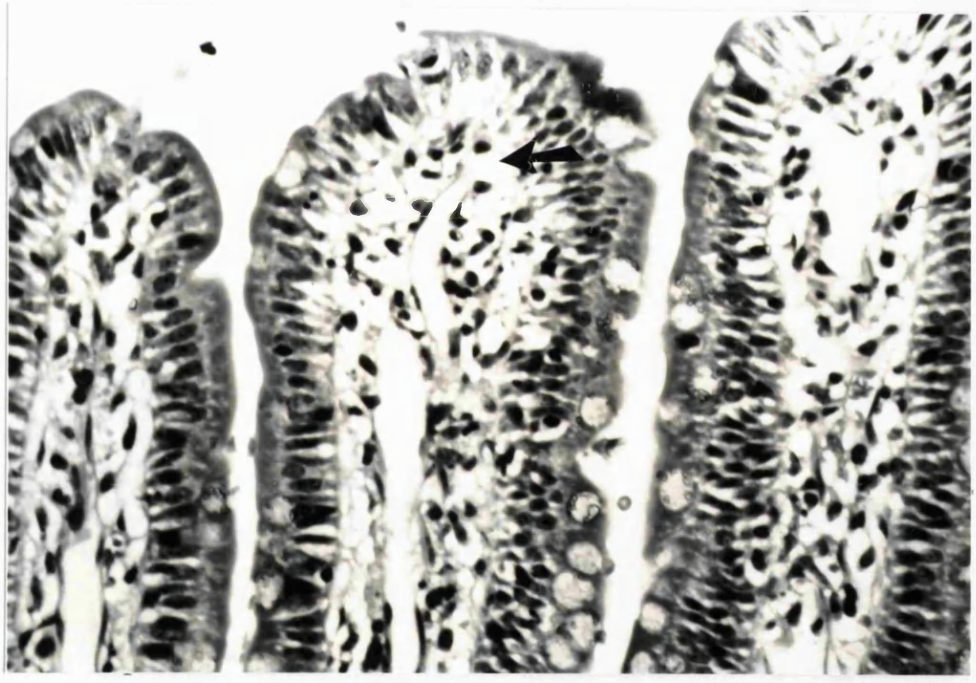


Fig. 18 Normal jejunal villous tip from a rat which received luminal indomethacin (100μg/ml) and perfusion fixed 90 min after dosing. Subepithelial capillaries are cleared of blood (arrow) indicating no vascular occlusion/blockage, with good preservation of villous architecture. (Haematoxylin and eosin, × 410).

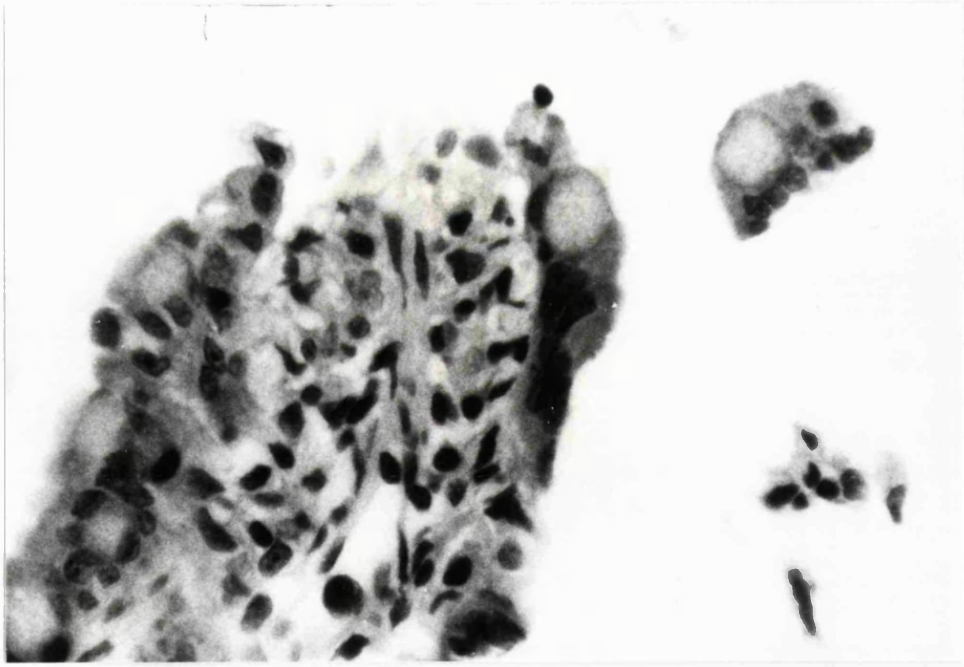


Fig. 19 A shortened jejunal villus which developed blood stasis after 50 mins exposure to luminal indomethacin (200μg/ml), and then perfusion fixed. Epithelium at the tip has been shed into the lumen, underlying smooth muscle cells are prominent and contracted. (Haematoxylin & Eosin, ×760)

Intravenous Indomethacin

The systemic effect of indomethacin on mucosal blood flow and histology was compared to control iv bicarbonate. Bicarbonate control given over 90 mins had no effect on blood flow (n=5, Fig. 20), and histologically the villi were as shown in figure 17.

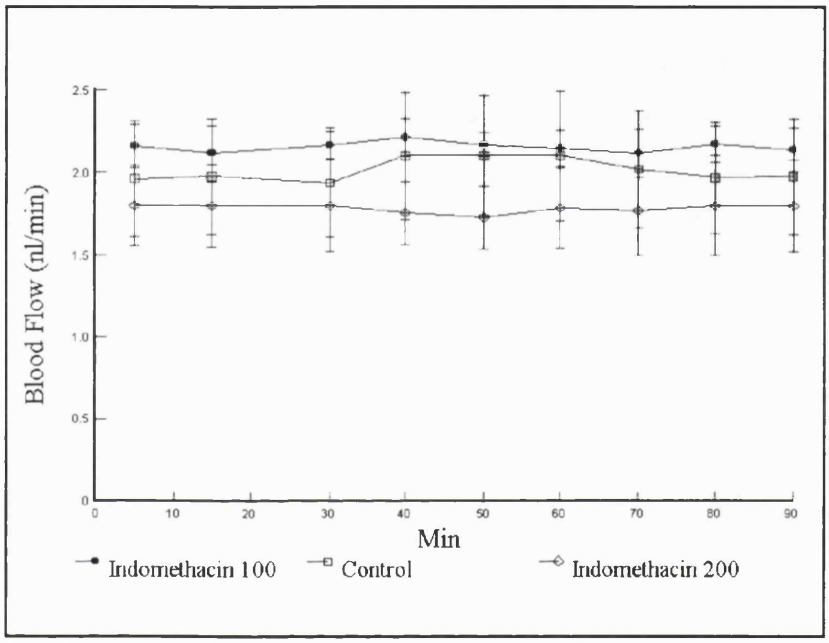


Figure 20 Jejunal villous blood flow after an iv bolus dose of indomethacin estimated to produce a plasma concentration of 100 & 200μg/ml or bicarbonate control, applied at 15 mins. Each point represents the mean of 5 animals ± SEM. (p = NS, Mann Whitney U)

Intravenous bolus doses of indomethacin (15 & 30 mg/Kg) estimated to give plasma concentrations of 100μg/ml and 200μg/ml respectively produced no alterations in blood flow over 90 mins (n=5 per dose, Fig. 20), and histologically the villi were also as shown in figure 17 in all animals.

***Indomethacin-induced slowing of villous blood flow*:**

In animals receiving both intravenous and luminal indomethacin, slowing of blood flow was shown to progress to focal vascular stasis. Accordingly, in order to evaluate histomorphometric changes at earlier times, when blood flow was slowing down, a separate group of 5 animals received combined luminal and iv indomethacin, and the experiment was terminated by perfusion fixation at the moment slowing of blood flow was first observed microscopically, later confirmed by off line measurements.

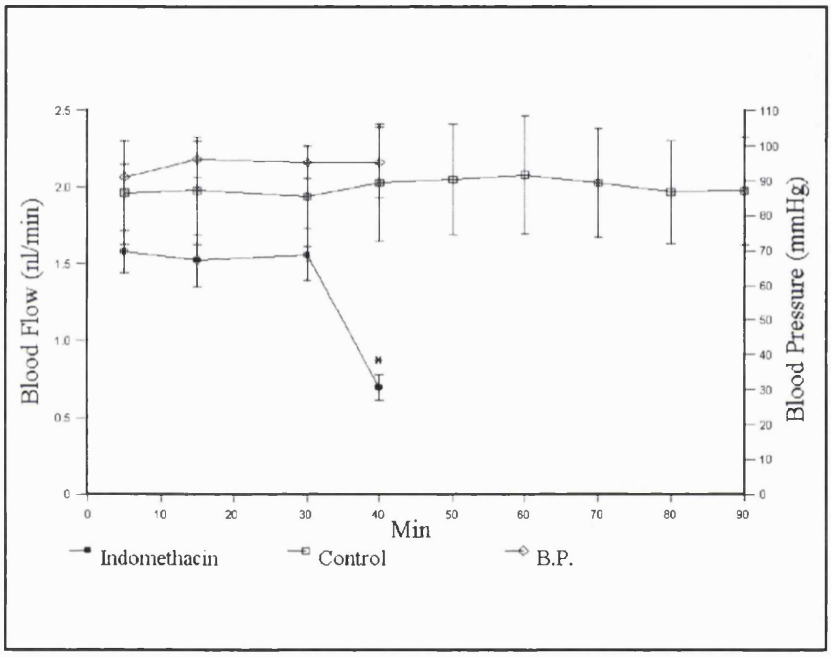


Figure 21 Jejunal villous blood flow after combined luminal (100μg/ml) and iv (bolus of 15mg/Kg) indomethacin until blood flow began to slow. Indomethacin and bicarbonate control were administered at 15 mins. Each point represents the mean of 5 animals ± SEM. (* $p<0.03$, Mann Whitney U)

Slowing was first noticed at 20-30 min after indomethacin in a small focus within a single villus (Fig. 21) originating consistently in the arcade vessel along the villus tip, three quarters of the way between the centre and edge of the leaf-shaped villus. In addition endothelial fluorescence occurred in the segment of the vessel as blood flow began to slow (Fig. 22a & b). The

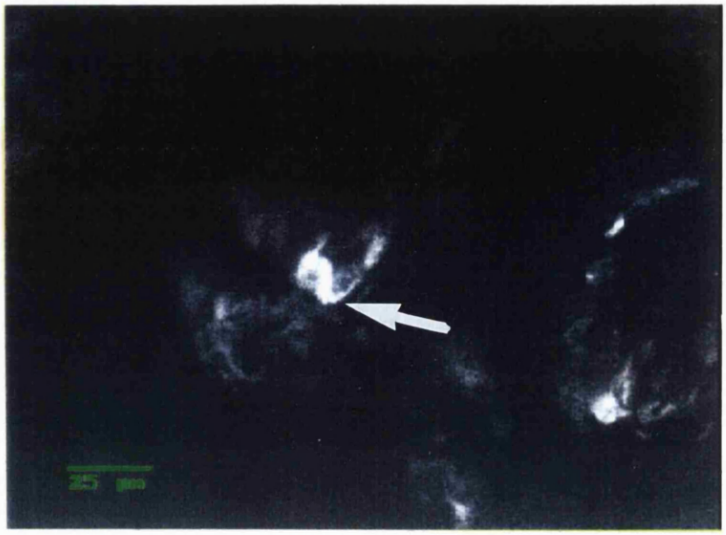
moment slowing was first evident microscopically, and confirmed by velocity measurements performed later, off-line, the gut was fixed by vascular perfusion. The time scale for the slowing to develop, like stasis, varied between rats (Table 5). Histological examination of single villi in which slowing occurred showed slight microvascular distortion and epithelial stratification, but no villous shortening at this stage (Fig. 23); mean crypt depth to villous height ratios in these villi were 0.356±0.02 and 0.386±0.01 in surrounding villi with normal circulation, (Table 6, NS Mann Whitney U test P=<0.2, n=5).

Mins	RAT 1	RAT 2	RAT 3	RAT 4	RAT 5
5	1.92 nl/min	1.2 nl/min	1.44 nl/min	1.44 nl/min	1.9 nl/min
15	2 nl/min	1.02 nl/min	1.5 nl/min	1.32 nl/min	1.74 nl/min
30	2.04 nl/min	1.14 nl/min	1.4 nl/min	1.3 nl/min	1.92 nl/min
35	1.98 nl/min	0.58 nl/min	1.5 nl/min	1.26 nl/min	1.93 nl/min
40	0.54 nl/min		1.38 nl/min	1.14 nl/min	0.62 nl/min
45			0.9 nl.min	0.78 nl/min	

Table 5 Blood flow values obtained from the single villi which developed slowing of blood flow on exposure to combined luminal (100µg/ml) and an iv bolus of indomethacin estimated to produce a plasma concentration of 100µg/ml. The velocity and diameter values used to calculate blood flow can be found in appendix E.

Control Villus	Ratio	Test Villus	Ratio
Crypt=24.7μm Villus=57μm	0.42	Crypt=20.9μm Villus=55.1μm	0.38
Crypt=22.8μm Villus=57μm	0.4	Crypt=19μm Villus=51.3μm	0.37
Crypt=20.9μm Villus=51.3μm	0.41	Crypt=19μm Villus=47.5μm	0.4
Crypt=17.1μm Villus=47.5μm	0.36	Crypt=17.1μm Villus=57μm	0.3
Crypt=20.9μm Villus=60.8μm	0.34	Crypt=19μm Villus=57μm	0.33

Table 6 Villus height and crypt depths in villi which developed slowing of blood flow. Crypt depth to villous height ratios indicate that no shortening took place as blood flow slowed.



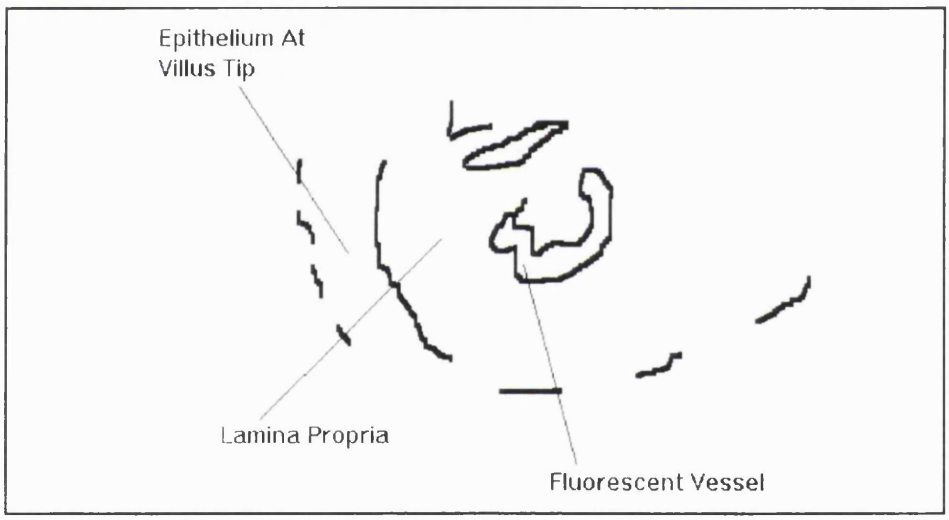


Fig. 22(a) Transverse confocal image of an isolated villus from rat jejunum after a combined luminal (100μg/ml) and iv (15mg/Kg) indomethacin, and perfusion fixed as blood flow slowed at 30 min. Image is taken from the right side of the villus which exhibited slowing of flow and endothelial fluorescence of the arcade vessel (arrow), which is surrounded by epithelium showing slight fluorescence due to surface mucus, demonstrated on lower line diagram.

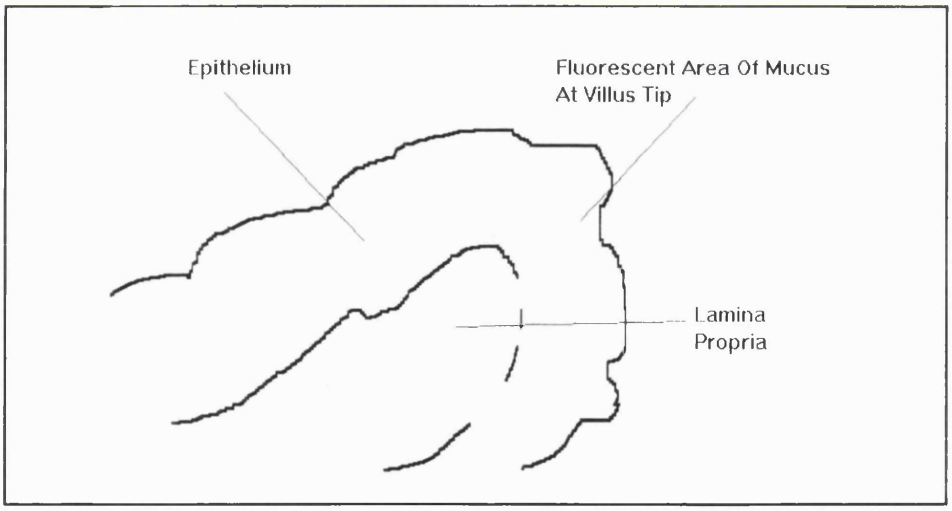


Fig. 22(b) The left side of the villus which maintained normal flow. The only fluorescence again comes from surface mucus on the epithelium, which is more clearly observed on the lower line diagram.

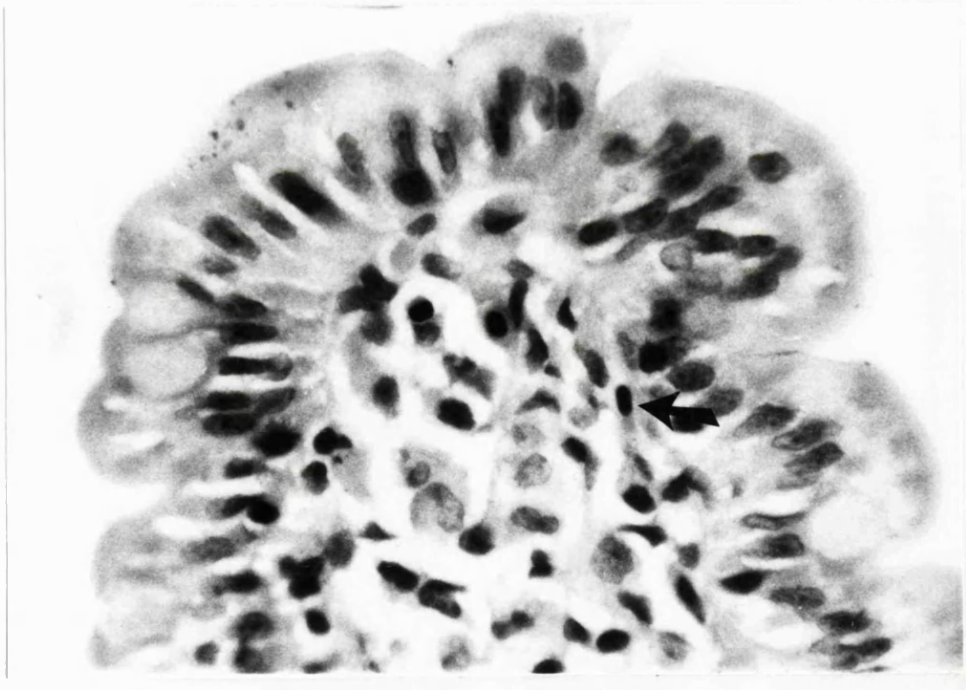


Fig. 23 Villus tip from rat jejunum showing blood slowing after combined luminal (100μg/ml) and iv (15mg/Kg) indomethacin. Surface epithelium is intact, but there is distortion of surface microvasculature, and contraction of capillary endothelial nuclei (arrow). (Haematoxylin & Eosin × 760)

***Ultrastructural Pathology*:**

As blood flow began to slow and progress to stasis in the arcade vessel, intense fluorescence was observed localised to the endothelial cells. This suggested that they were a primary site for indomethacin effects, therefore 2 separate groups of 3 animals were perfuse-fixed at the point blood slowing and then blood stasis. The villi in which a blood flow change was observed were isolated for ultrastructural analysis. Controls comprised of luminal and iv bicarbonate applied for 45 mins which the previous groupings demonstrated as the maximum amount of time required for indomethacin-induced blood stasis to occur.

Controls:- In control animals (n=3) given luminal and systemic bicarbonate, the columnar epithelium at the villus tip (involving 7-8 cells right at the tip) was degenerative, in that there was a decrease in organelle integrity (Fig. 24) and mitochondrial swelling (Fig. 25). However the surface brush border appeared intact as were the tight junctions. The epithelium from the side of the villus was normal showing none of the changes observed at the tip (Fig. 26). Endothelial cells of the arcade vessel at the villus tip and other capillaries within the villus lamina propria were all normal (Fig. 27). Groups of villi taken from proximal and distal areas of the small intestine all had the same ultrastructural appearance as the observed villus.

Indomethacin-induced blood slowing:- In isolated villi from 3 rats which had exhibited slowing of blood flow the epithelium at the tip had the same degenerative characteristics described in the controls (Fig. 28). Side epithelial cells were again normal. Endothelial cells from the arcade vessel demonstrated finger-like projections into the vessel lumen, vacuolisation of the endothelial wall, and irregular thickening of the cytoplasm (Fig. 29). Vessels deeper in the villus with normal blood flow showed no signs of indomethacin-induced damage, their appearance was the same as in the control capillaries. A significant amount of plasma was still present at the

villus tip, suggesting that slowing of blood flow restricted vascular perfusion with fixative. Some surrounding capillaries also contained plasma, but did not have any luminal projections or vacuolisation. Villi removed proximally and distally which had been exposed only to systemic indomethacin showed no signs of ultrastructural damage, and were as described in the controls.

Indomethacin-induced blood stasis:- In a separate group of animals where blood stasis developed in the arcade vessel the endothelial finger like projections and vacuolisation had become more pronounced (Fig. 30). Epithelium was either degenerate or had begun to lift away from the lamina propria (Fig. 31). Perfusion with fixative at the villus tip was again poor with a significant amount of plasma present in the capillary lumen. The surrounding capillaries had no projections or endothelial cell vacuolisation. Villi removed distally and proximally were exposed only to systemic indomethacin, their ultrastructural appearance was the same as for control bicarbonate.

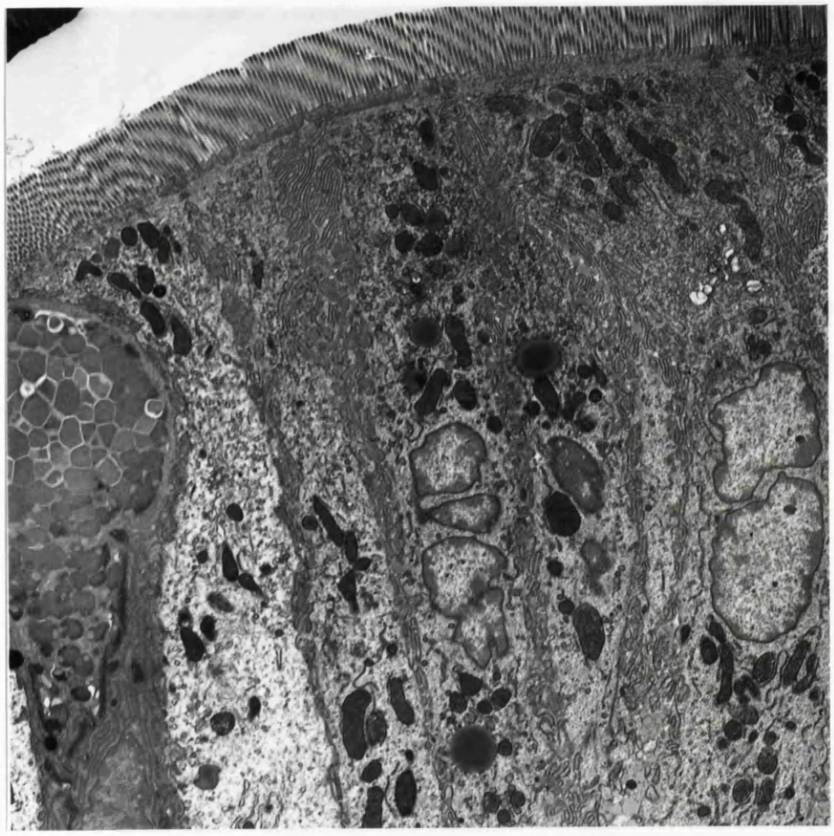


Fig. 24 Normal jejunal epithelium from the villus tip after combined luminal and iv vehicle for 45 min, intestines were perfusion-fixed and then immersion fixed to maintain vascular and epithelial integrity. Brush border and tight junctions are intact but intracellular organelles appear to be breaking down, particularly the mitochondria. Large numbers of lipid globules can be seen within the cell. (Magnification × 3,852).

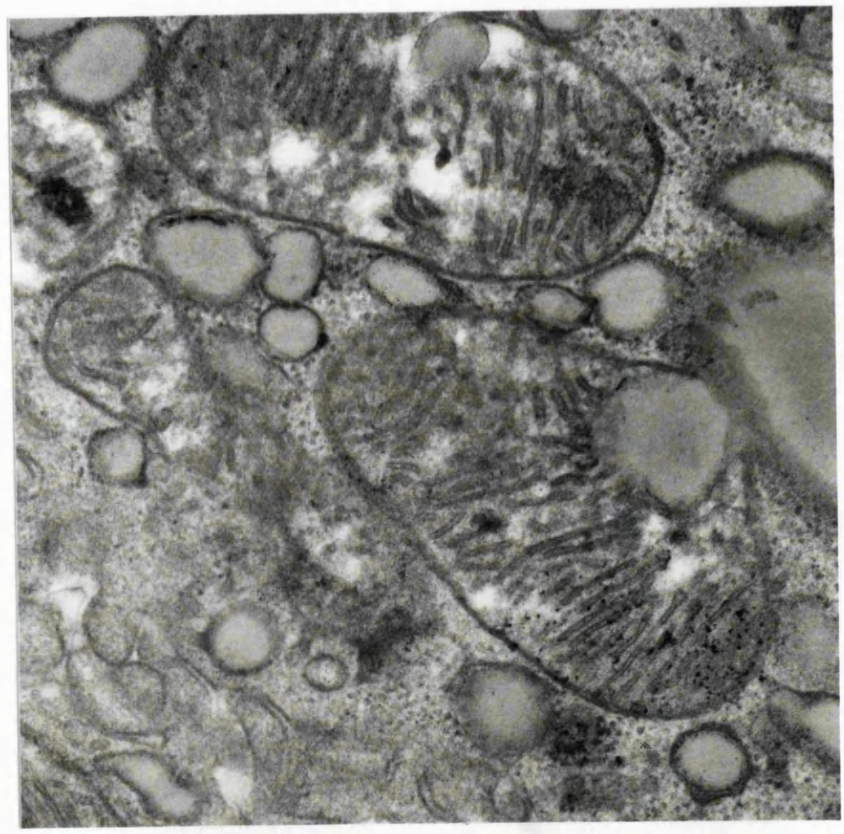


Fig. 25 High power view of the epithelial mitochondria. Mitochondria are swollen and degenerate, denoted by fragmentation of the cristae. (Magnification × 48,125).

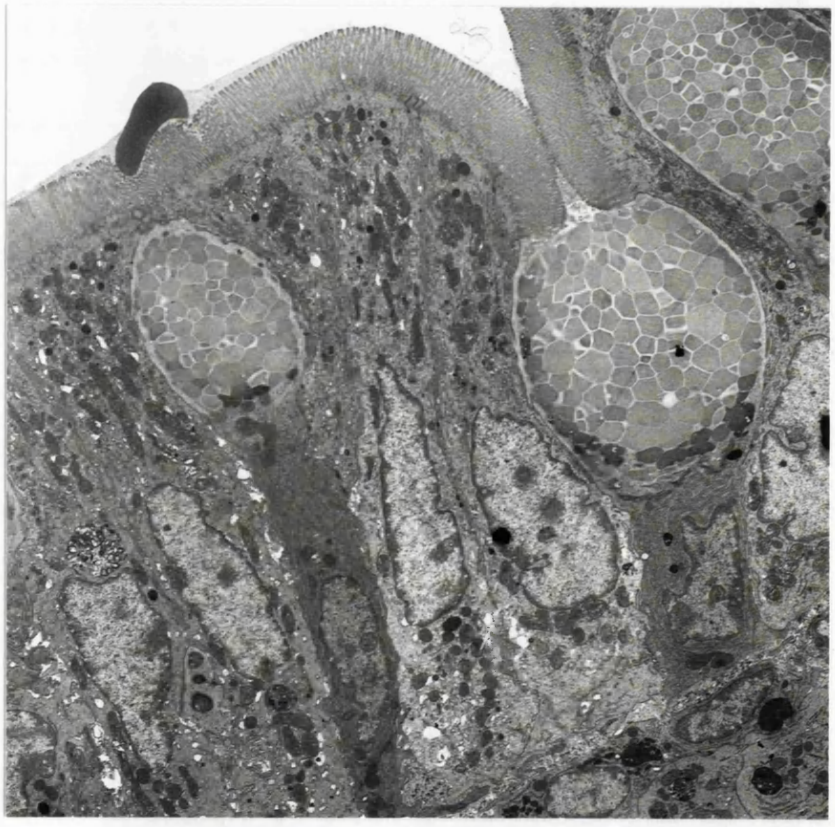


Fig. 26 Normal jejunal epithelium taken from the side of the villus. Brush border and tight junctions are intact, the intracellular organelles are in excellent condition with very few lipid globules within the cell. Three undischarged mucus granules can also be seen. (Magnification × 3,220)

Fig. 27 Endothelial cell from the arcade vessel of a villus exposed to control vehicle only, for 45 min. There is a red blood cell present in the lumen of the vessel but no plasma is present. All cellular membranes appear intact with no signs of degeneration, and the nucleus appears normal. (Magnification × 13,293).

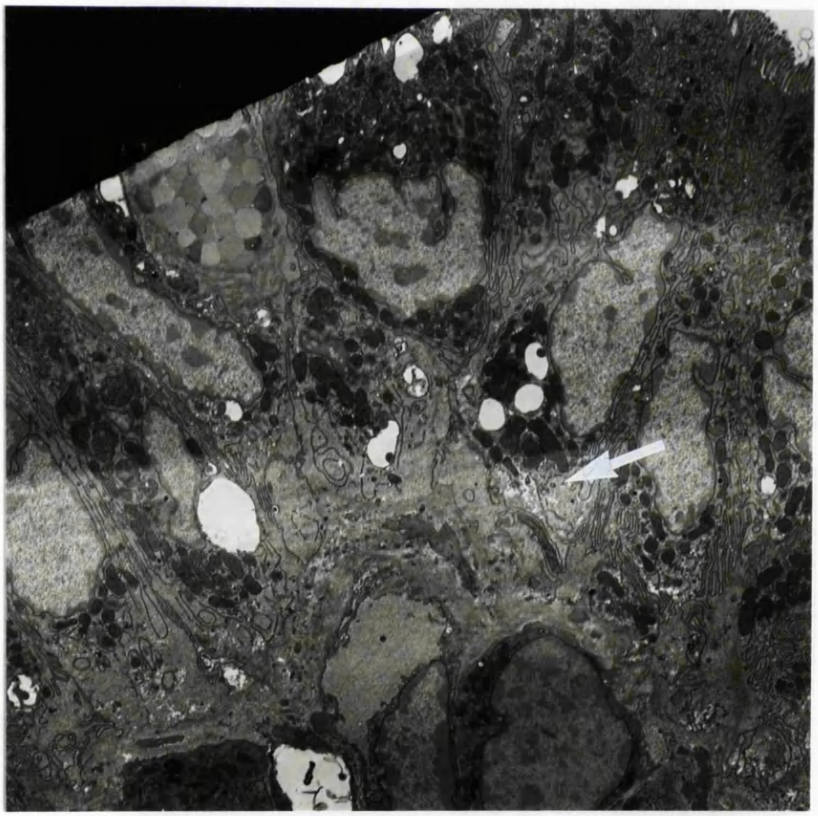
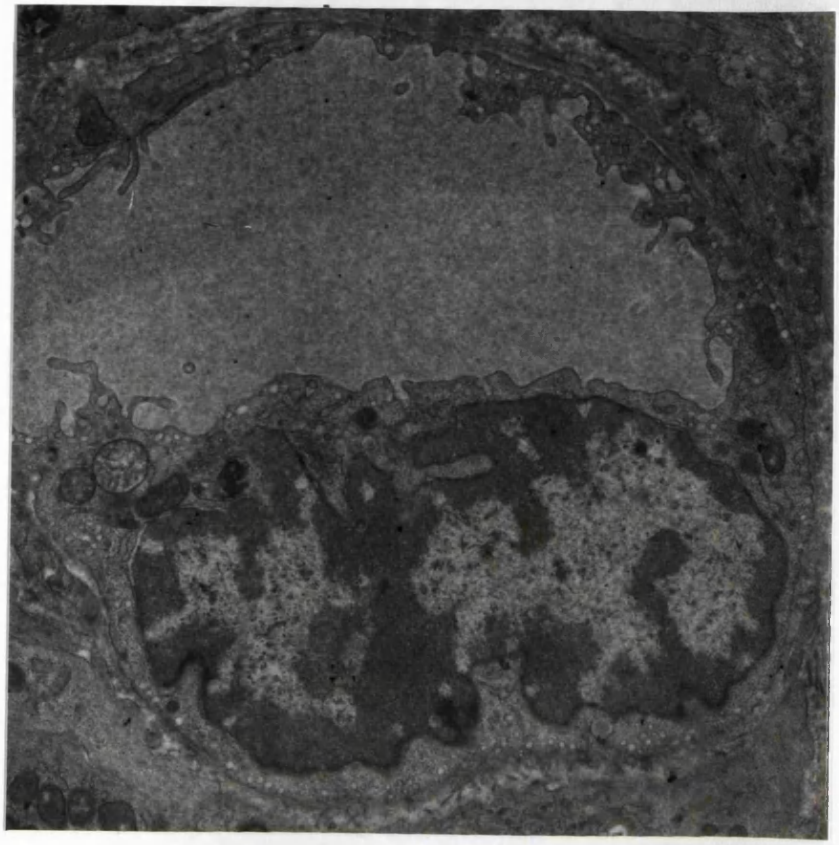
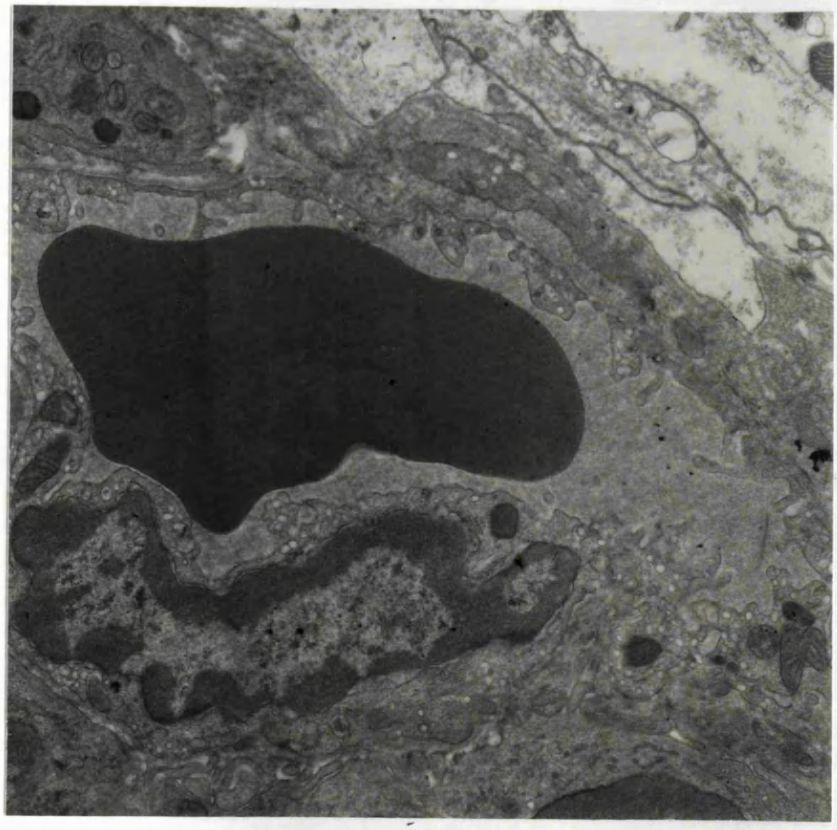


Fig. 28 Normal epithelium taken from the tip of a villus which had developed slowing of blood flow. Note cell vacuoles and degeneration around the base of the cell (arrow) (Magnification × 3,852)





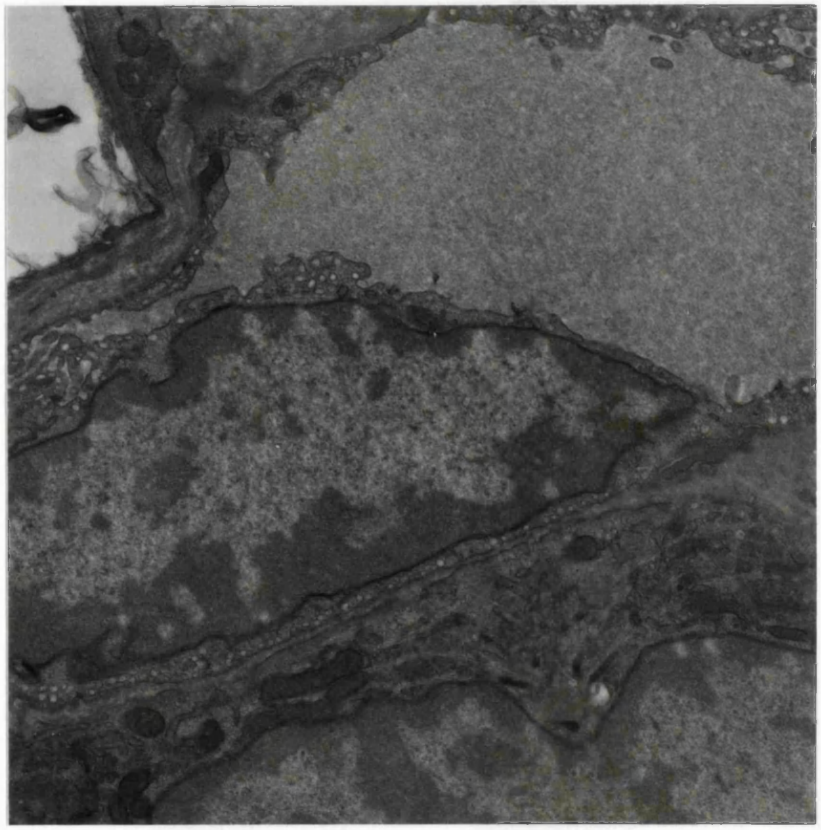
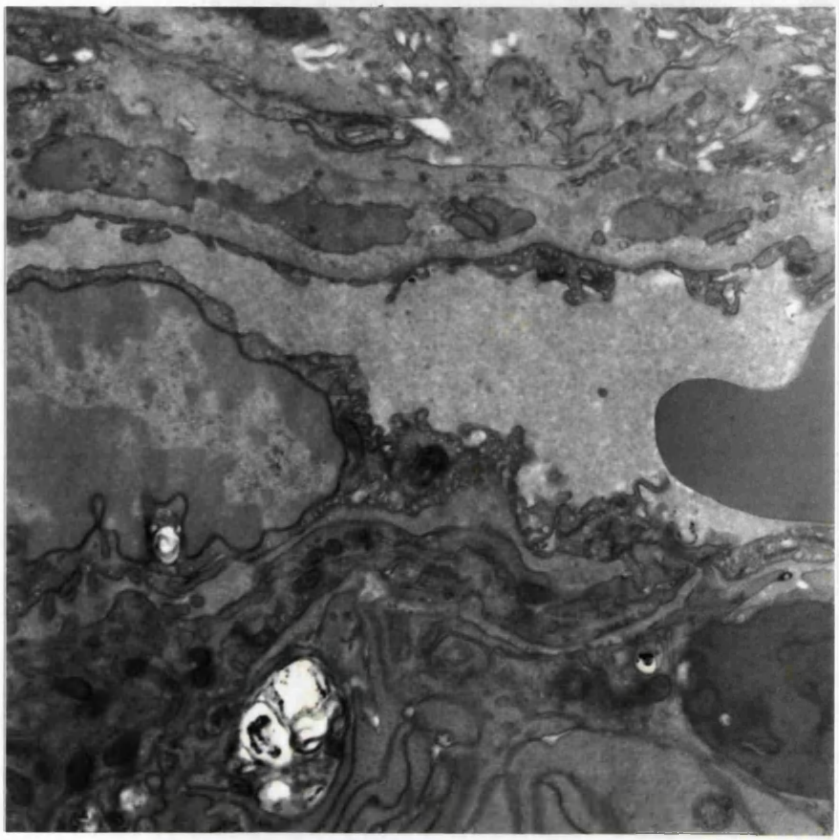
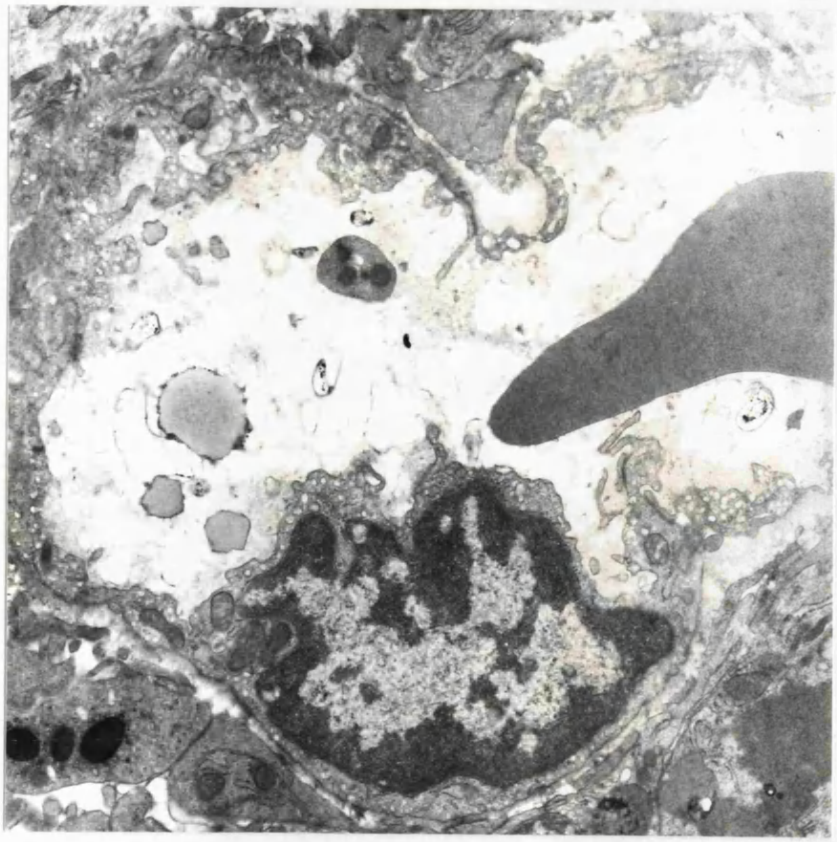


Fig. 29 Three endothelial cells taken from the arcade vessel in separate animals exposed to combined luminal (100μg/ml) and iv (15mg/Kg bolus) indomethacin until slowing occurred in the arcade vessel. All the cell membranes are intact but there is severe vacuolisation of the cell wall and finger like projections into the vessel lumen. The dark granular material within the vessel lumen is unperfused plasma. (Magnification × 13,293).





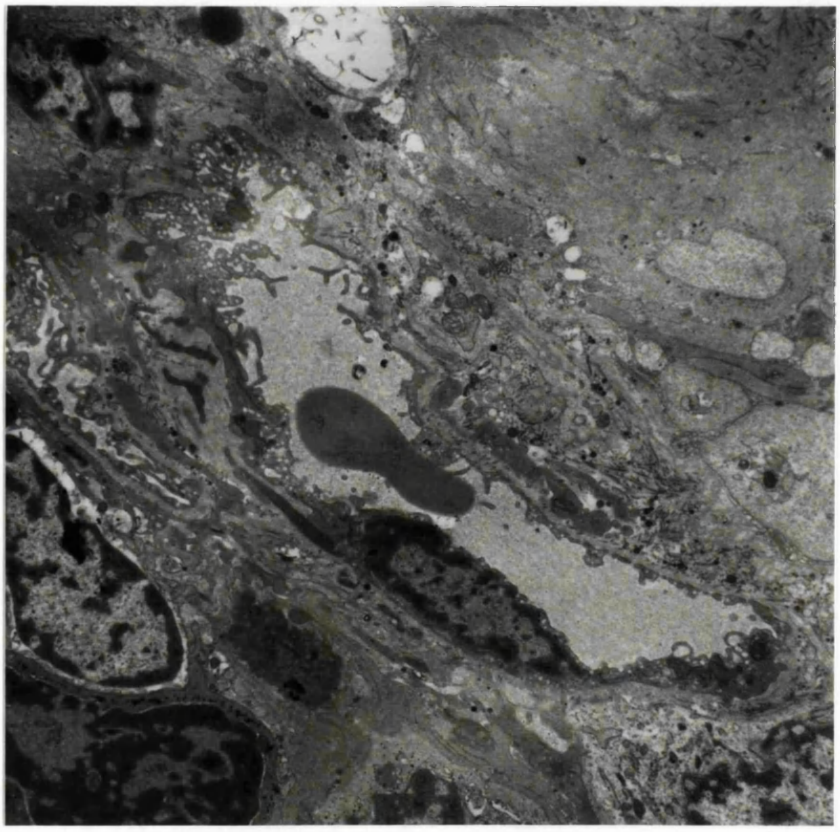


Fig. 30 Three endothelial cells from the arcade vessel in separate animals exposed to luminal (100μg/ml) and iv (15mg/Kg bolus) indomethacin until stasis occurred in the arcade vessel. The cell membranes are intact but the vacuolisation and finger like projections into the lumen have become more pronounced. As well as unperfused plasma there is cellular debris in the lumen of one vessel. (Magnification × 13,293 & 8,382)

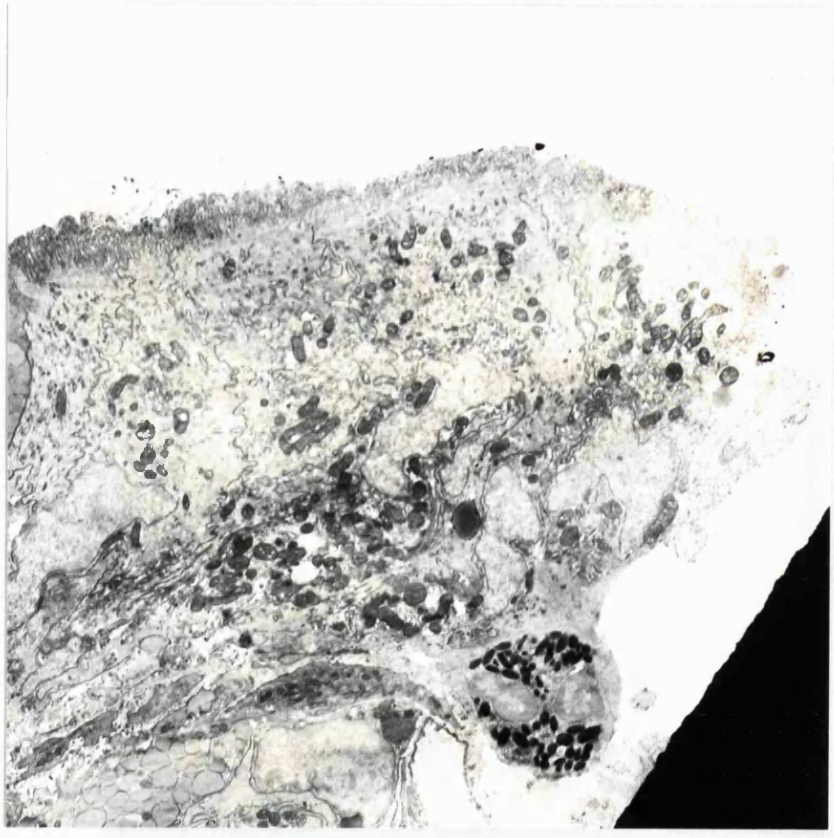


Fig. 31 Severely degenerate epithelium from a villus in which blood stasis developed. The brush border is beginning to shed into the lumen and the tight junctions are open. The mitochondria are clearly fragmented, and the other intracellular organelles have disintegrated. (Magnification × 3,852).

***Measurement of Mucosal and Superior Mesenteric Artery Blood Flow*:**

Previous authors demonstrated a reduction in the superior mesenteric artery blood flow after systemic indomethacin, this may have contributed to the blood flow effects observed in the previous experiments.

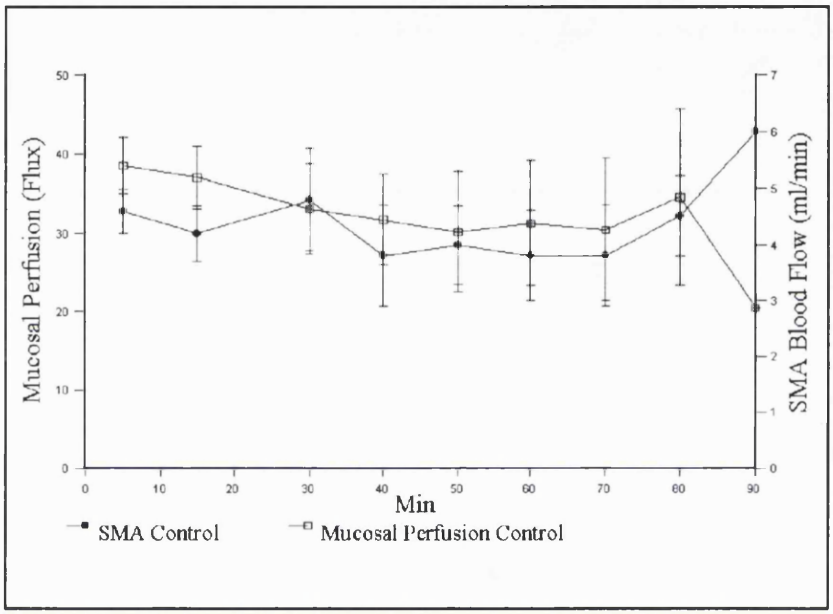


Figure 32 Blood flow in the superior mesenteric artery and mucosal perfusion after an iv bolus dose of bicarbonate control administered after 15 mins. Each point represents the mean of 5 animals ± SEM. (p = NS, Mann Whitney U)

It was repeated in this thesis whilst simultaneously measuring mucosal flow so that any reduction in SMA flow could be correlated with mucosal reductions. Animals were given 15 min for intestinal blood flow to reach equilibrium after surgery before the iv bolus doses were given. No significant reduction in either superior mesenteric artery (SMA) blood flow or mucosal perfusion was observed after iv saline, n=5 (Fig. 32). An iv bolus dose of indomethacin also produced no significant reduction in either SMA blood flow or mucosal perfusion. (Fig. 33).

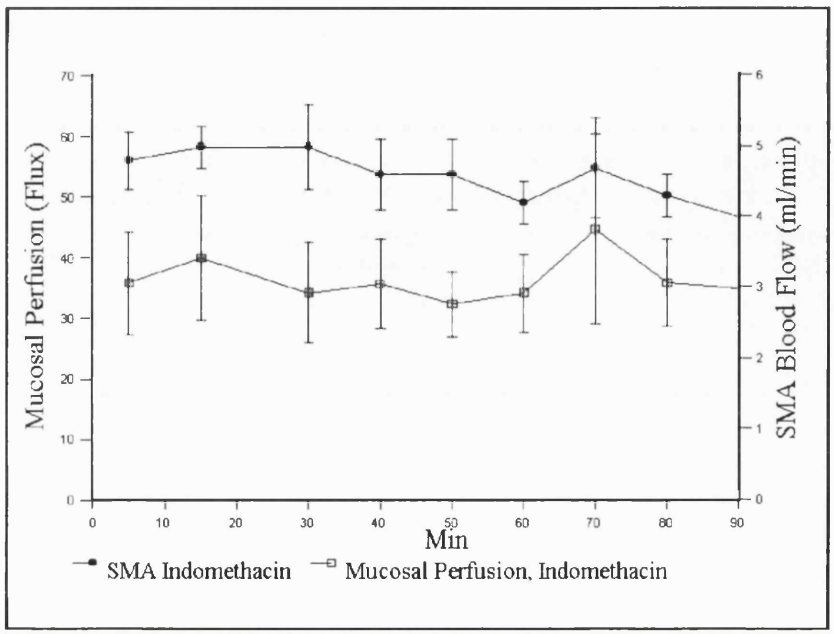


Figure 33 Blood flow in the superior mesenteric artery and mucosal perfusion after an iv bolus dose of indomethacin estimated to produce a plasma concentration of 100μg/ml, administered after 15 min. Each point represents the mean of 5 animals ± SEM. (p = NS, Mann Whitney U)

5. Pharmacological Intervention with β_3-Adrenoceptor Agonist CL316,243

***Luminal CL316,243 at Point of Stasis*:**

Previous studies showed that small intestinal ulceration induced by oral indomethacin could be abolished using the β_3-adrenoceptor agonist CL316,243 (Anthony et al, 1996c), suggested to result from maintainence of blood flow. CL316,243 was therefore given luminally at the point of indomethacin-induced stasis to assess its ability to return blood flow to control levels.

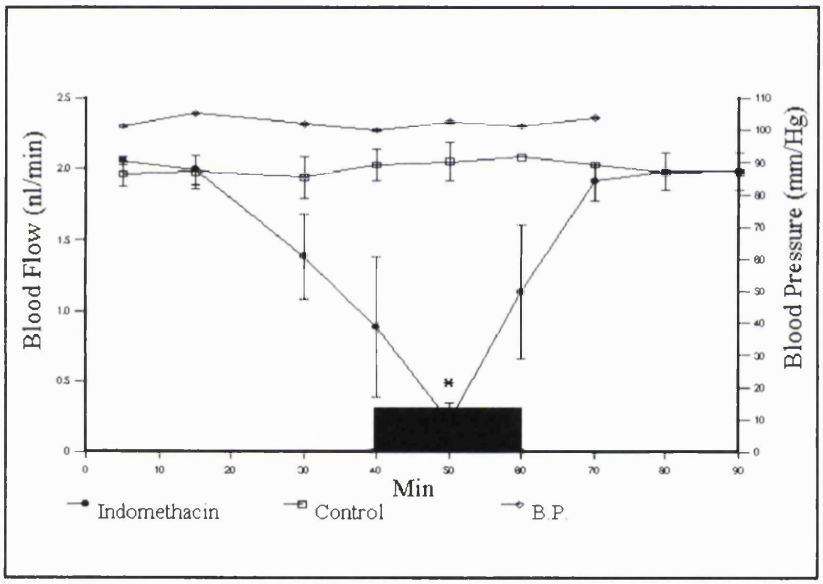


Figure 34 Jejunal blood flow after combined luminal (100μg/ml) and iv (bolus of 15mg/Kg) indomethacin at 15 mins. Shaded area represents the 20 min period in which blood stasis developed in each animal. At stasis luminal CL316,243 (100μg/ml) was applied. Each point represents the mean of 5 animals ± SEM. (*p< 0.005, Mann Whitney U)

CL316,243 (100µg/ml) applied luminally at the point of blood stasis returned blood flow to normal control levels within 10-20 min of application

(Fig. 34) and the endothelial fluorescence faded. As with the development of stasis, the return to normal flow was also variable between rats, the blood flow data for each rat is shown in table 7. When terminated at 90 mins, histology of single villi in which blood stasis had been reversed was normal, with mean crypt depth to villous height ratios of 0.45±0.06 in test and 0.5±0.01 in surrounding villi which had not developed stasis (Table 8, Mann Whitney U NS, Fig. 35). In contrast with the mode of onset of vascular stasis, return of blood flow was a smooth process with none of the step-wise changes which were a feature the progression to stasis. In 5 separate control animals saline applied luminally at the point of blood stasis did not reverse blood stasis, which spread to the rest of the villus, with surrounding villi also developing slowing and stasis. Histology of the affected villus showed the typical pre-ulcerative of villus shortening, distortion and stratification of the epithelium, microvascular damage/distortion, and smooth muscle prominence.

Mins	RAT 1	RAT 2	RAT 3	RAT 4	RAT 5
5	1.98 nl/min	2.04 nl/min	2.16 nl/min	2.04 nl/min	2.1 nl/min
15	2.04 nl/min	2.4 nl/min	1.92 nl/min	1.74 nl/min	1.86 nl/min
30	2.16 nl/min	0.84 nl/min	0.48 nl/min	1.26 nl/min	2.16 nl/min
40	2.16 nl/min	0.3 nl/min	0.12 nl/min	0 nl/min	1.8 nl/min
50	0.57 nl/min	0 nl/min	0 nl/min	0 nl/min	0.48 nl/min
60	0 nl/min	2.28 nl/min	1.56 nl/min	1.8 nl/min	0 nl/min
70	1.7 nl/min	2.4 nl/min	1.98 nl/min	1.56 nl/min	1.86 nl/min
80	1.74 nl/min	2 nl/min	2 nl/min	1.7 nl/min	2.04 nl/min
90	1.8 nl/min	2.5 nl/min	2.04 nl/min	1.68 nl/min	2 nl/min

Table 7 Blood flow values from all 5 rats for the reversal of indomethacin-induced blood stasis in single villi by luminally applied CL316,243 (1mg/Kg). The velocity and diameter values used to calculate blood flow can be found in appendix F.

Control Villus	Ratio	Test Villus	Ratio
Crypt=49.4μm Villus=104.5μm	0.47	Crypt=57μm Villus=100.7μm	0.57
Crypt=24.7μm Villus=57μm	0.42	Crypt=20.9μm Villus=55.1μm	0.38
Crypt=47.5μm Villus=89.3μm	0.53	Crypt=36.1μm Villus=60.8μm	0.6
Crypt=49.4μm Villus=102.6μm	0.48	Crypt=28.5μm Villus=79.8μm	0.36
Crypt=38μm Villus=76μm	0.49	Crypt=36.1μm Villus=87.4μm	0.41

Table 8 Crypt depths and villus heights in villi where indomethacin-induced blood stasis was reversed by luminal CL316,243 (100μg/ml. Crypt depth to villus height ratios show that as well as reversing stasis, CL316,243 reversed the concomitant shortening.

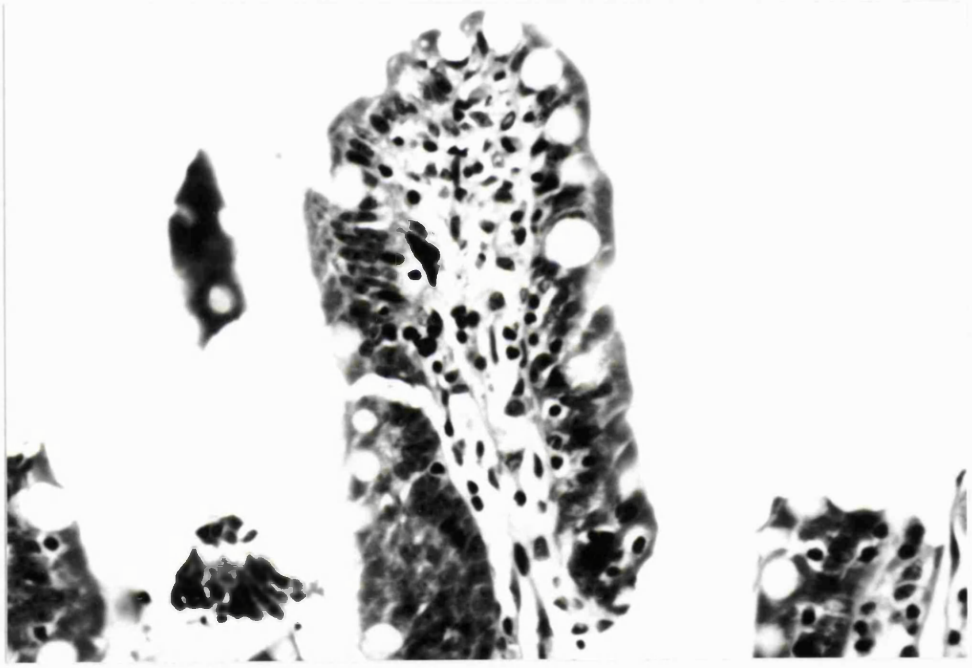


Fig. 35 A normal jejunal villus after combined luminal (100μg/ml) and iv indomethacin (15mg/Kg) until blood stasis developed at 40 min. Then luminally applied CL316,243 (100μg/ml) was given, which returned the blood flow. The intestines were then perfusion-fixed and the villus removed for histology after 90 min. (Haematoxylin and Eosin × 410)

***Intravenous CL316,243 at blood stasis*:**

On exposure to luminal and iv indomethacin (100μg/ml) blood stasis developed as described previously, an iv bolus of CL316,243 (1mg/Kg) estimated to produce a plasma concentration of 100μg/ml was administered at the point of stasis in this group to assess the ability of systemic CL316,243 to reverse stasis. Systemic CL316,243 did not reverse stasis or the endothelial fluorescence in the observed villus which gradually spread to the rest of the villus, affecting the whole villus by 90min. Blood flow in surrounding villi which had not developed stasis returned to normal although this was not quantified. Histologically, the villus which had developed blood stasis showed stratified and distorted epithelium, endothelial damage, smooth muscle prominence, and villous shortening, with mean crypt depth to villous height ratios of 1.44±0.22 in test against 0.38±0.01 in controls (Table 9, Mann Whitney U $p < 0.02$) in controls.

Control Villus	Ratio	Test Villus	Ratio
Crypt=43.7μm Villus=114μm	0.38	Crypt=41.8μm Villus=39.9μm	1.05
Crypt=38μm Villus=95μm	0.4	Crypt=41.8μm Villus=34.2μm	1.22
Crypt=39.9μm Villus=108.3μm	0.36	Crypt=38μm Villus=22.8μm	1.67
Crypt=55.1μm Villus=127.3μm	0.37	Crypt=36.1μm Villus=58.9μm	0.61

Table 9 Villus heights and crypt depths from villi which developed indomethacin-induced blood stasis not reversed by iv CL316,243 (1mg/Kg). Crypt depth to villus height ratios show that shortening had occurred.

There were also more severe histological changes comprising of upper villous necrosis and buckling of large villous vessels (Fig. 36). Surrounding villi which

appeared to maintain blood flow had mild histological changes such as epithelial stratification in 2 animals. In 5 separate control animals given iv saline at the point of stasis showed the same sequence of events where stasis in 1 part of a villus spread to the rest of the villus and eventually to the surrounding villi.

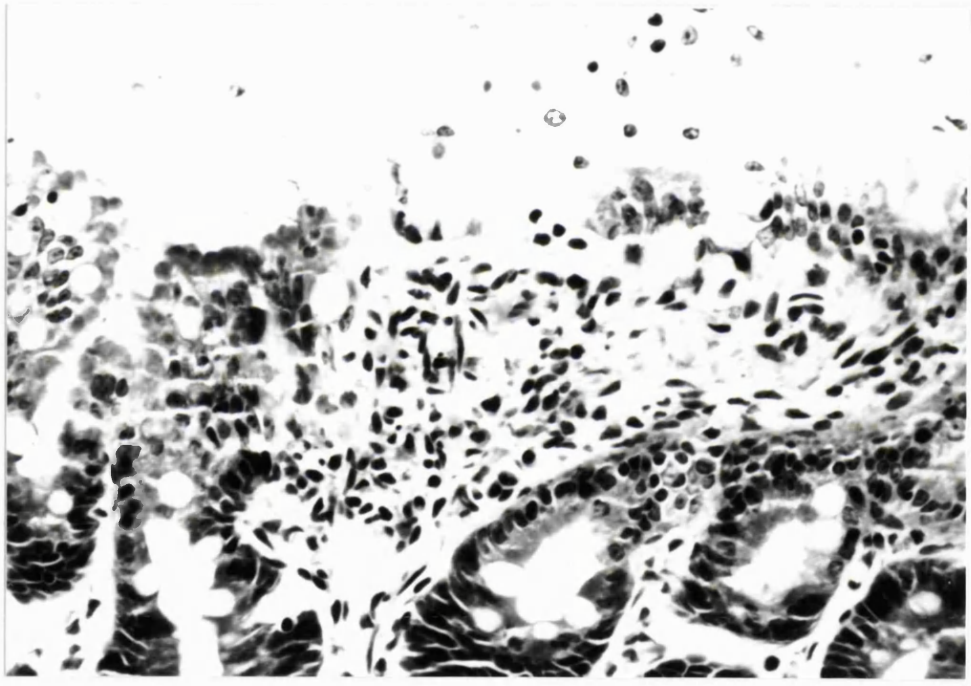


Fig. 36 A jejunal villus given combined luminal (100μg/ml) and iv indomethacin (15mg/Kg) until blood stasis developed at 45 min, then iv CL316,243 (1mg/Kg) was given. The intestines were perfusion-fixed and removed after 90 min. Villus shows epithelial shedding, shortening and clumping of lamina proprial cells. (Haematoxylin and Eosin × 410)

***Luminal and intravenous CL316,243 alone*:**

CL316243 has been shown to significantly raise basal blood flow in the gastric mucosa, it was therefore administered by both the luminal and iv route to repeat the experiments in the intestines and to elucidate which route had the greatest effect on blood flow. Luminal CL316,243 (100μg/ml) alone significantly raised villous basal blood flow ($P < 0.003$) with no effect on systemic blood pressure, although the degree of increase was not as large as that described by Kuratani (1994) in the gastric mucosa (Fig. 37).

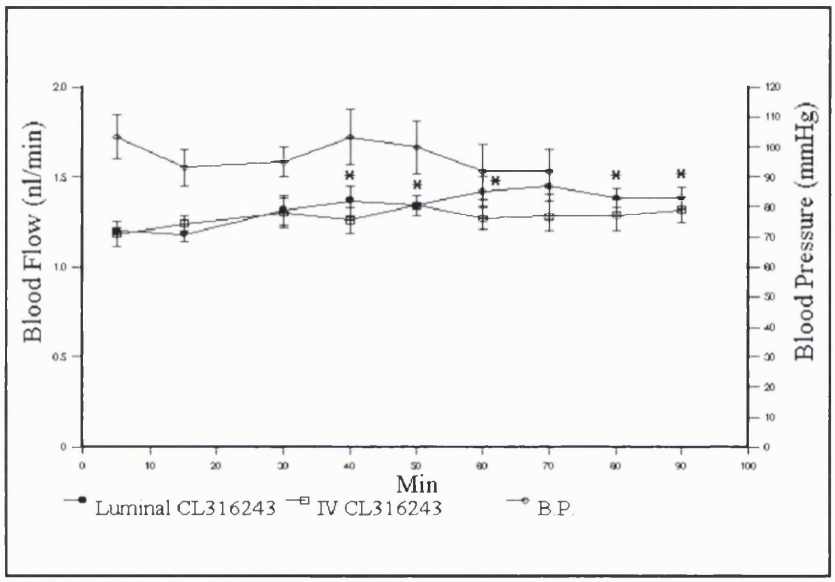


Figure 37 Jejunal villous blood flow after an iv bolus dose of CL316,243 (1mg/Kg) and luminal CL316,243 (100μg/ml) given at 15 mins. Each point represents the mean of 5 animals ± SEM. (*$p<0.03$, Paired t test)

In contrast, iv CL did not raise villous basal blood flow (Fig. 37). The blood flow data from each of the 5 rats is shown in tables 10 & 11. Histology of the observed villi showed no pathological changes after either luminal or iv CL316,243 (Fig. 38).

Mins	RAT 1	RAT 2	RAT 3	RAT 4	RAT 5
5	1.2 nl/min	1.2 nl/min	1.08 nl/min	1.08 nl/min	1.1 nl/min
15	1.32 nl/min	1.25 nl/min	1.09 nl/min	1.15 nl/min	1.2 nl/min
30	1.56 nl/min	1.38 nl/min	1.2 nl/min	1.1 nl/min	1.38 nl/min
40	1.5 nl/min	1.5 nl/min	1.26 nl/min	1.08 nl/min	1.5 nl/min
50	1.4 nl/min	1.44 nl/min	1.3 nl/min	1.14 nl/min	1.44 nl/min
60	1.68 nl/min	1.4 nl/min	1.38 nl/min	1.14 nl/min	1.5 nl/min
70	1.74 nl/min	1.38 nl/min	1.5 nl/min	1.2 nl/min	1.44 nl/min
80	1.6 nl/min	1.35 nl/min	1.4 nl/min	1.1 nl/min	1.38 nl/min
90	1.54 nl/min	1.37 nl/min	1.38 nl/min	1.2 nl/min	1.4 nl/min

Table 10 Blood flow values from all 5 rats given luminal CL316,243, agonist was applied after 15 mins. The velocity and diameter values used to calculate blood flow can be found in appendix G.

Mins	RAT 1	RAT 2	RAT 3	RAT 4	RAT 5
5	1.38 nl/min	1.2 nl/min	1.02 nl/min	1.02 nl/min	1.32 nl/min
15	1.4 nl/min	1.14 nl/min	1.26 nl/min	1.14 nl/min	1.26 nl/min
30	1.08 nl/min	1.26 nl/min	1.5 nl/min	1.27 nl/min	1.44 nl/min
40	1.14 nl/min	1.14 nl/min	1.32 nl/min	1.2 nl/min	1.5 nl/min
50	1.2 nl/min	1.3 nl/min	1.5 nl/min	1.26 nl/min	1.4 nl/min
60	1.08 nl/min	1.32 nl/min	1.3 nl/min	1.2 nl/min	1.44 nl/min
70	1.14 nl/min	1.26 nl/min	-	1.24 nl/min	1.52 nl/min
80	1.4 nl/min	1.1 nl/min	-	1.14 nl/min	1.5 nl/min
90	1.38 nl/min	1.14 nl/min	-	1.26 nl/min	1.56 nl/min

Table 11 Blood flow values from all 5 rats given an iv bolus dose of CL316,243. The agonist was given after 15 mins. The final 3 readings were not possible in rat 3 because of obscurement by mucus. The velocity and diameter values used to calculate blood flow can be found in appendix H

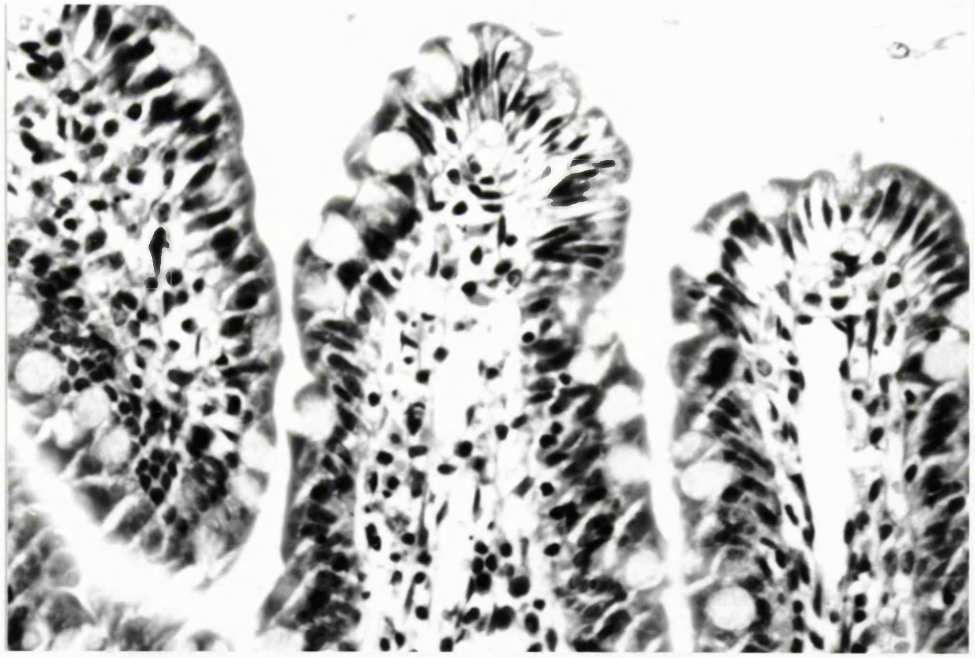


Fig. 38 A normal jejunal villus 90 min after luminal CL316,243 (100μg/ml) (Haematoxylin & Eosin × 410).

***Prophylactic Protection Against Stasis by Intravenous CL316,243*:**

Combined luminal (100μg/ml) and iv (bolus of 15mg/Kg) indomethacin applied with concomitant iv CL316,243 (1mg/Kg) did not result in any visible change in mucosal blood flow. Slowing and stasis did not occur in any of the 30-40 villi observed in 5 test animals within 90mins, and no significant increase in blood flow was observed after iv CL316,243 (paired t test NS, Fig. 39).

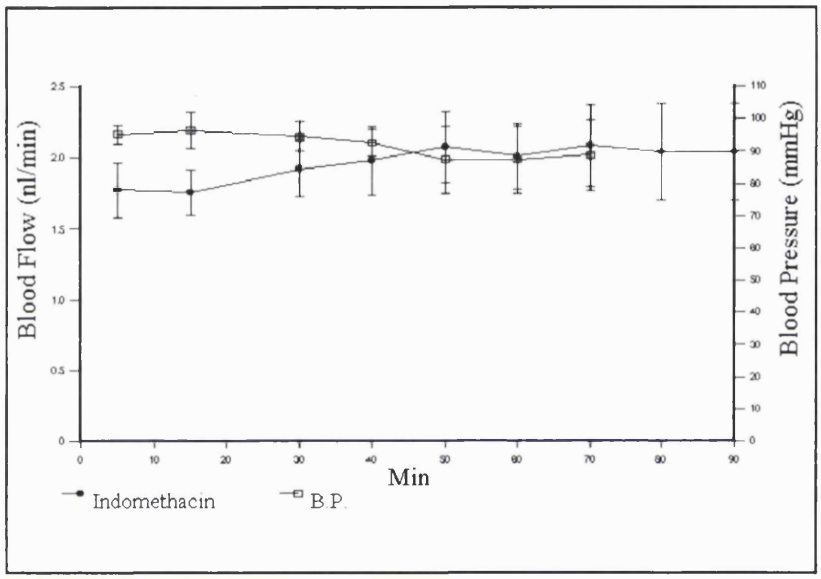


Figure 39 Jejunal villous blood flow after combined luminal (100μg/ml) and iv (bolus of 15mg/Kg) indomethacin concomitant with an iv bolus of CL316,243 (1mg/ml) given at 15 mins. Each point is the mean of 5 animals ± SEM.

Histology of the isolated villi showed no pathological changes (Fig. 40). Surrounding villi also had normal histological profiles. In the 5 control animals given iv saline and indomethacin developed non-reversible slowing and stasis as previously described.

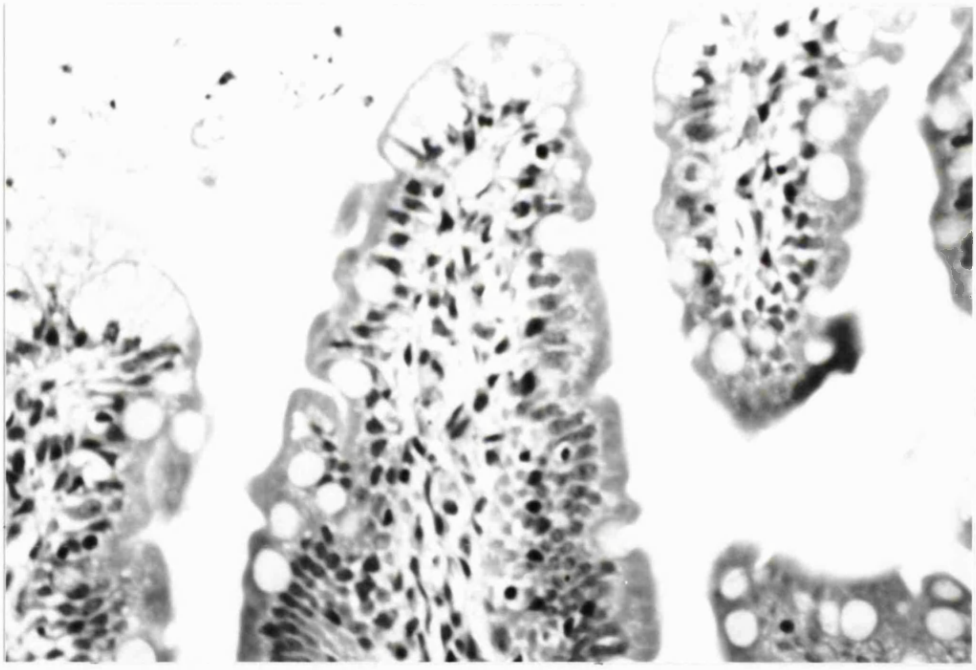


Fig. 40 Normal villus 90 min after combined luminal and ie. indomethacin (100μg/ml) and concomitant iv CL316,243 (1mg/Kg). (Haematoxylin and Eosin × 410).

6 Discussion

Attempts to increase visibility through mucus:

The presence of mucus blurring the vessel image is reported to result in measurement errors (Gretz & Duling, 1995), consequently it had to be removed. Attempts to remove and reduce mucus formed a significant part of this thesis, as it was a major barrier to visualisation of the mucosa. With practise and under direct observation intestinal mucus was best removed by touching the mucosa with small cotton wool balls revealing the microvasculature beneath. This technique was particularly good in that any artifact caused by touching resulted in almost instant blood stasis whereas indomethacin effects were more gradual, therefore "cotton wool" damage was immediately obvious. This allowed rejection of experiments with "touch damage", and so complete avoidance of this artefact in the presented experiments.

Chemical Interference:

Attempts to control the release of mucus chemically proved to be difficult as surface goblet cells secrete in response to luminal irritants whilst crypt goblet cells are controlled by parasympathetic neurotransmitters such as acetylcholine (Neutra et al, 1982). Consequently only crypt mucus could be effectively targeted, using cholinergic antagonists such as atropine. In practise this gave disappointing results. Increasing the dose would have caused suppression of respiration, tachycardia due to blocking of vagal tone to the heart, and inhibition of gastrointestinal motility (Neal, 1990). In any case, it is more likely that mucus from surface goblet cells was responsible for obscuring the microcirculation of the arcade vessel, and these cells cannot be inhibited as they are not under nervous or hormonal control.

Mucus itself was targeted after release from goblet cells with mucolytics such as Dithiothreitol (DTT) and N-acetyl cysteine (NAC)

(Kiviluoto et al, 1993; Ukkonen and Julkunen, 1987). DTT proved to be a very poor mucolytic in the intestines with little or no discernable effect upon visibility through the mucus. NAC on the other hand was extremely effective in reducing mucus viscosity, facilitating easy removal. Unfortunately it caused intestinal erosions which were similar to those produced by indomethacin, therefore interpretation of histology would have been almost impossible, ruling out the use of NAC.

Physical Removal:

Physical removal of mucus produced excellent results. Cling-film and glass cover slips significantly reduced visual impairment by pushing mucus away from the surface epithelium down into the villus crypts or away into the surrounding water bath. Both techniques produced excellent villus orientation, flattening the villi to present a perfect side view of the microvasculature. However, it is doubtful whether they allow unrestricted blood flow within the microvasculature as they apply weight to flatten the mucosa. Most importantly however, neither technique allowed solutions to be applied luminally.

Micropipettes were ineffective when used to suck the mucus away from the surface because the cohesion between mucus strands was too great to allow passage through a micropipette. Gently touching the mucosa with the micropipette was more effective due to mucus strongly adhering to the hydrophilic pipettes. Micropipettes were not used because they were extremely easy to snap with the resultant glass shards puncturing villi, leading to haemorrhage. Surprisingly, larger diameter pipettes weren't as effective because they were not able to slip through the mucus surface, which was important as the pipette had to get beneath the mucus layer to lift it away from the mucosa.

Female Sprague Dawley Rats

Female sprague dawleys produced slightly less mucus than their

male counterparts. Although the difference was not statistically significant it was considered large enough to warrant their use instead of males.

Cotton Rats

Despite the success of physically removing mucus with cotton wool, the ideal situation was not to have to interfere with mucus at all. This was found to be the case in hispid cotton rats (available for another study) which presented excellent visualisation of mucosal microcirculation without the need for any interference. The quantity of mucus produced was similar to sprague dawley rats, but it was more translucent and posed no real barrier to observation of the mucosa. Unfortunately they were not susceptible to NSAID-induced ulceration, despite oral concentrations five-fold higher than those required to produce ulceration in sprague dawley rats.

A small pharmacokinetic study performed on 5 hispid cotton rats showed that even after an iv bolus dose of indomethacin (15mg/Kg), plasma levels were almost negligible 15 mins after injection. A number of possible mechanisms may be postulated to account for this insensitivity to indomethacin. It may be due to either high plasma protein binding or rapid metabolism/excretion. However, high luminal doses (50 fold higher than required in sprague dawley), bypassed metabolism as they exposed the mucosa to high concentrations of free indomethacin but failed to ulcerate the intestine. One explanation might be that cotton rats may have a more extensive intestinal blood supply with none of the watershed areas described in sprague dawley (Anthony et al 1995), whereby indomethacin-induced local reductions in blood flow are compensated for by extensive anastomoses in the cotton rat. Although cotton rats were of no use in this project they may provide an excellent model for intravital microscopy in other studies as the jejunal mucosa is not obscured by mucus.

In summary, despite the excellent mucosal visualisation possible in hispid cotton rats, non-sensitivity to indomethacin prevented their use in

this particular project. However, for other applications they may provide an excellent study model for mucosal microcirculation. The best solution for removing mucus was found to be frequent removal with cotton wool in female sprague dawley rats.

Localisation and development of indomethacin-induced lesions

The localisation of indomethacin-induced lesions to the mesenteric border is a source of interest (Anthony et al, 1996a). At 24 hours rats exposed to oral indomethacin exhibited full thickness lesions localised exclusively to the mesenteric margin of the jejunum. At earlier time points (Anthony et al, 1993), there was a further localisation within the mesenteric border, with lesions lying only between vasa recta, avoiding the areas overlying these vessels, confirmed in this study. Indeed during the 20-35 minutes of progression from the first visible slowing to stasis, no other similar lesions were detected in surrounding areas. There are three possible explanations for this observation. Firstly, these areas may be specially susceptible to the vascular effects of indomethacin; secondly, anatomical studies of jejunal vascular anatomy show a paucity of blood vessels between vasa-recta, and lesions begin in these watershed areas (Anthony et al, 1996a); and thirdly, indomethacin has been shown to reduce mesenteric blood flow (Feigen et al, 1981). Therefore a reduction in mesenteric blood flow will affect the sensitive watershed areas described by Anthony (1996a).

However, the results presented here do not support a general reduction in intestinal blood flow, because if reduced intestinal flow was responsible then slowing/stasis should have occurred in several villi rather than in a single vessel in a small part of a single villus. It is also possible that any general reduction in mesenteric flow would be compensated for by shunting of intestinal blood flow away from the muscle layers towards the mucosa, in order to maintain the integrity of the mucosa as a whole.

However, the best evidence against reduction of intestinal blood flow is that following indomethacin, there was no decrease in superior mesenteric artery flow or mucosal flow for 90 minutes. Although this is in direct conflict with previous findings (Feigen et al, 1981), the discrepancy is probably due to a species difference as Feigen performed it in the dog.

Another question is why does the lesion start in a single villus, and in a single vessel at the tip? At present there are no clear explanations for this observation; the intense endothelial fluorescence suggests damage to the luminal surface of endothelial cells. At the site of slowing, confocal microscopy highlighted damaged endothelial cells which were fluorescent, suggesting a cause for the slowing but not for the predisposition for the tip of the villus. It can be speculated that as mucus is continually secreted from the base of the villus and is removed from the tip by passing contents, the base of the villus has a lower contact with luminal indomethacin compared with the tip, where the lesion first occurs. Another consideration is that older epithelial cells at the tip may be more permeable to indomethacin, leading to exposure of the underlying vessel to the drug. Indeed, ultrastructural analysis of villi from control animals showed that villus tip epithelial cells were degenerate and that those further down the villus were in good condition, therefore it is probable that tip epithelium is more permeable to indomethacin.

The intense endothelial fluorescence observed at slowing and stasis may be due to the high molecular weight FITC-Dextran (M.Wt = 150,000) entering the endothelial cell and becoming trapped because it was too large to leave the undamaged non-luminal side of the cell. Although a smaller molecular weight dextran would have demonstrated this effect more clearly, the resultant increase in background fluorescence would have prevented observation of the early blood flow effects.

Reports in the literature state that a high local, rather than systemic,

concentration of an NSAID is the most important factor in lesion pathogenesis (Bjarnason et al, 1991a). However, this thesis provides equivocal results; on the one hand luminal doses of 200μg/ml caused stasis in villous surface microvasculature, but an iv bolus dose estimated to produce a systemic concentration of 200μg/ml did not. This is supported by ultrastructural analysis of proximal and distal villi (exposed only to systemic indomethacin) which showed no abnormal pathology compared with observed villi (which were exposed to combined luminal and systemic indomethacin) . On the other hand lower doses of indomethacin (100μg/ml) caused no blood flow effects when given either luminally or systemically alone, whereas a combined dose did produce slowing/stasis. This is a difficult problem to resolve as the systemic dose is subject to metabolism and plasma protein binding, whereas the luminal dose is not. A pharmacokinetic study allied to increasing intravenous doses is indicated here to test whether or not systemic indomethacin is ulcerogenic.

Individual Variability of Animals

The interpretation of results is complicated by variability between animals. Each animal developed indomethacin-induced blood stasis/slowing over different time scales. The flow within the villi themselves also varied between animals, but this was probably due to variations in superior mesenteric artery flow which correlated with villous flow (in each animal). Superior mesenteric artery blood flow and mucosal perfusion showed a variation of between 3-7ml/min and 20-60 flux units respectively in controls. One reason for these changes may have been varying amounts of circulating sympathetic amines released in response to the fight or flight reflex, induced during handling prior to the experimental procedure. Release of amines such as adrenaline would cause vasoconstriction in the gastrointestinal tract therefore reducing the flow within the intestines and hence producing lower flow values (Neal, 1990). It is, however, unknown

if such effects would be maintained during 1 hr of anaesthesia, and surgery (surgery was the same in all animals).

Measurement Variations

Variations were also present in the measurement of velocities and diameters. Calibration of flying spot analysis against frame by frame analysis (velocity measurement) demonstrated a 25% error in the flying spot method. However, as only one operator performed the velocity measurements making the same errors each time, the error was steady and consistent, and did not therefore greatly affect results, accounting for the relatively small fluctuations seen in the blood flow graphs. However, a 25% error was not significant when compared to indomethacin-induced changes, where velocity was measured at full, half and zero flow.

The small differences in villous blood flow shown within individual control animals was probably due to vasomotion. Unfortunately the very subtle alterations in vessel diameter which cause vasomotion would not be detected using the videometric line drawing technique employed in this thesis. However, such subtle diameter changes had no bearing upon the recognition of indomethacin effects which were more gross.

Calibration of videometric line drawing used in diameter measurements highlighted a possible error of ± 20% therefore a 40% error is possible. The equation used to calculate blood flow from velocities and diameters ($BF=\pi/4\times V\times D^2$) squares the diameter therefore greatly magnifying any errors. Fortunately only one operator measured diameters using the same reference point within the vessel (light or dark border) each time, reducing the possible error from 40 to 20%.

The Transonic flow probe used to measure SMA blood flow highlighted large variations between animals but showed only very small fluctuations within individual animals. This suggests that the flow probe was correctly placed on each animal with good contact between probe and

vessel. This was also found with the laser Doppler flow probe used to measure mucosal perfusion.

Endothelial cell damage was assessed *in vivo* by an increase in fluorescent intensity, although it should be noted that this was unlikely to be an increase in FITC fluorescent properties and more likely to be an increase in the concentration of FITC-Dextran within the endothelial cell. It is possible that the increase in fluorescence could result in diameter measurement errors whereby the intense fluorescence observed in damaged endothelial cells would have masked any endothelial swelling and narrowing of the vessel lumen. Therefore luminal narrowing would not have been observed, as diameter was measured using the fluorescent border of the vessel under observation. However, as far as could be discerned there were no changes in the diameter of the arcade vessel either before or during blood stasis.

CHAPTER 4

GENERAL DISCUSSION

General Discussion and Summary

Hypotheses:-

1. *Focal slowing or loss of villous blood flow is an early feature in the pathogenesis of indomethacin-induced ulceration, and may be the cause of epithelial loss and alterations in villous morphology.*

2. *Stimulation of β_3-adrenoceptors reverses indomethacin-induced blood stasis by promoting villous blood flow.*

The previous studies in the rat had shown the early histological/dynamic events (within 1 hour) occurred randomly along the mesenteric border where the mesenteric vessels attach to the jejunum, but not overlying the point of attachment (Anthony et al, 1993). These primary events were further localised to areas between vasa recta and in a single capillary within a villus (Anthony et al, 1995; Piasecki et al, 1994). The technique best suited to observe and record these early changes was *in-vivo* microscopy where blood flow could be observed in single villus vessels and the moment slowing or stasis of blood flow was observed, the preparation could be perfusion and immersion-fixed to preserve vascular and epithelial histology at this time point, and the affected villus isolated. This allowed histological changes to be directly correlated with blood flow at a very early stage, uncomplicated by later progressive changes.

The histological pathogenesis of early indomethacin-induced intestinal ulceration has been studied by Anthony et al (1993) and Nygard et al (1994) who showed that villous contraction, epithelial stratification, endothelial cell damage/distortion and smooth muscle prominence were all early events, occurring within 1 hour of oral indomethacin (15mg/Kg).

Piasecki et al (1994) then showed that luminally applied indomethacin induced blood flow slowing/stasis in the tips of villi. However, up till now there have been no studies in which blood flow was observed and then directly correlated with histology.

The histological study performed by Anthony et al (1993) suggested that decreased blood flow was secondary following villus shortening, where contraction of villus smooth muscle elements might crush the fine capillary network at the villus tip, occluding villus blood flow. However, isolation and histological examination of villi which had developed blood stasis confirmed villus shortening but the microvasculature at the tip, although occluded (RBC plugs), was not crushed. This suggests that the primary ulcerogenic effect is vascular rather than due to smooth muscle.

A vascular aetiology for indomethacin-induced ulceration has been suggested by studies showing increased leucocyte rolling and reduced blood flow in sub-mucosal venules of the small intestine 6 hours after sub-cutaneous indomethacin (Miura et al, 1991). Much earlier microvascular damage was reported by Anthony et al (1993), who showed endothelial cell damage within 1-2 hours of oral indomethacin, and Piasecki et al (1994) demonstrated rapid focal microvascular stasis in villus tips within 5 minutes after high doses of indomethacin (5mg/Kg) applied to the jejunal mucosa. Dynamic *in-vivo* results from this thesis also support a vascular aetiology, because at the point when villus blood flow begins to slow, endothelial changes are already present comprising intense endothelial fluorescence localised to the vessel wall, vacuolisation and finger like projections into the lumen. Indeed, there are several ultrastructural studies in the stomach which have highlighted the mucosal vascular endothelium as a very early site of NSAID damage within 15 minutes of exposure to oral aspirin and indomethacin (Rainsford et al, 1983; Tarnawski et al, 1990; McCarthy et al 1995; Gyomber et al, 1996). However, the type of endothelial damage

described in this thesis differs from that observed in the gastric mucosa, where endothelial cell changes comprised increased capillary permeability (Rainsford, 1983; Gyomber et al, 1996), or increases in the size of capillary fenestra leading to permeability (McCarthy et al, 1995). Increased endothelial permeability was not a feature of the endothelial changes in the jejunum as tissue oedema would have been obvious on electron microscopy. The studies in the stomach and the results from this thesis show different forms of endothelial damage but all strongly suggest that the endothelium is a very early site for indomethacin-induced lesions.

The ultrastructural study performed in this thesis is the first study to relate epithelial and endothelial changes at the same time point, in the same jejunal villus in response to NSAIDs, and also that these endothelial changes are concomitant with reduced mucosal blood flow. Ultrastructural analysis of normal control villi with normal mucosal blood flow and endothelium (as described by Vogt, 1980) showed that tip epithelial cells were already degenerate, (specifically the mitochondria in these cells were uncoupled). Other authors however, have cited epithelial degeneration as a primary NSAID effect for several years in both rats (Rainsford, 1988; Sigthorsson et al, 1997) and in man (Bjarnason et al, 1984; 1986; 1987b; 1991a). These conflicting results are difficult to explain as on the one hand this thesis would suggest that degeneration of tip epithelium is a normal function of epithelial shedding into the lumen. On the other hand epithelial degeneration has been reported by several authors to be directly caused by indomethacin.

Degeneration of villus epithelium has been postulated to increase intestinal permeability in rats (Rainsford, 1988; Sigthorsson et al, 1997) and in man (Jenkins et al, 1987; Bjarnason et al, 1984; 1986; 1991b). The mechanism is thought to involve NSAID-induced uncoupling of mitochondrial oxidative phosphorylation, indeed aspirin has been known to

uncouple mitochondria for several years (Whitehouse, 1964; Haas et al, 1985;). Uncoupling has been shown in rat intestinal epithelium 1 hour after exposure to indomethacin (Hayllar et al, 1991; Somasundaram et al, 1992; 1995). It results in a decrease in the cellular adenosine triphosphate (ATP) vital to normal control of intercellular junctions, which are regulated by an ATP-dependent cellular cytoskeleton (Madara et al, 1987), and ultimately leads to increased paracellular permeability.

Although permeability probably has a role to play in the pathogenesis of NSAID ulceration, the results presented here suggest that it may not be the initiating factor. This is supported by the observation that in villi where villus blood flow is slowing the epithelium is still relatively normal, showing the same degenerative changes observed in the tips of control villi. However, the endothelial cells from the arcade vessel in villi which developed slowing 25 minutes after indomethacin, were vacuolated with finger like projections into the lumen. Therefore it seems that the primary morphological change involves endothelial damage, leading to reduced blood flow. This is in direct conflict with the results of Hayllar et al (1991) and Somasundaram et al (1992, 1995) who reported no epithelial degeneration in controls, but observed epithelial degeneration almost identical to that observed in the controls of this thesis, within 1 hour of oral indomethacin. The conflict cannot be totally resolved by the available results as Hayllar et al and Somasundaram et al both used indomethacin in the rat at similar doses to those used in this thesis. However, Hayllar et al and Somasundaram et al took random villi from the intestine and are likely to have missed the first few villi with developing lesions, and they were not able to provide any data on the blood flow in the selected villi. It is therefore possible that they observed villi with normal blood flow but degenerate tip epithelium just prior to shedding.

The type of endothelial damage seen here may provide an

explanation for the step wise slowing of blood flow (as opposed to a uniform and gradual slowing of flow) seen after indomethacin. Normally, when endothelium is damaged a thrombus forms, firstly by platelet activation then adherence, together with leucocyte activation, rolling and adherence. Later more leucocytes and platelets adhere to the damaged endothelium. This was unlikely to have occurred here, since adherent leucocytes and platelets would have been apparent in both light and electron microscopy. However, electron microscopy does reveal finger like projections into the vessel lumen, which increase in severity from slowing to stasis, suggesting they may have a role in the progression towards blood stasis, apparently by a non-thrombotic mechanism. The step wise change in flow may be due to red and white blood cells becoming trapped by the endothelial projections forming the period of stasis, the subsequent back pressure then forces the red/white cell past the projections, restarting flow. This process is repeated until the projections are large enough to stop the red and white blood cells for sufficiently long periods to form adhesions and so permanent stasis. The step wise slowing is unlikely to be a feature of transient vasoconstriction, since any vessel narrowing would have been evident during videomicroscopy.

The projections themselves have never been described before in NSAID-induced damage but they appear to be very similar to those seen in ischaemia (Ward, 1997). However, the projections appear to be the cause of the ischaemia rather than a result of it, as they occur as the blood flow slows rather than after it has stopped. They may also be a result of endothelial contraction demonstrated by Anthony et al (1993) whereby folding of the endothelial membrane could resemble projections. However, the endothelial contraction described, was based on the H&E histological appearance of the endothelial nucleus, and the resolution was not great enough to show an actual contracted endothelial cell. If endothelial

contraction was involved there would also have been folding of the non-luminal surface of the cell, which was not the case. It could also be argued that these changes were due to inadequate fixative perfusion, as plasma was present in the vessels at the villus tip. However, plasma was also present in several surrounding capillaries which did not show any of the changes, therefore reducing the likelihood of inadequate fixation as the cause of the changes.

Endothelial vacuolisation may provide an explanation for the intense endothelial fluorescence of the vessel wall (as seen best in confocal microscopy) observed as blood flow began to slow. The endothelium may take up the fluorescent marker in the plasma, and concentrate it in the vessel wall, therefore producing fluorescence. It may also be a type of endothelial permeability, whereby labelled plasma leaks into endothelial cells, but does not pass through them and out into the surrounding lamina propria. Whatever the mechanism, the ultrastructure described here provides very convincing evidence that endothelium may be a primary site of action for indomethacin.

The results are limited however, in that only morphological rather than biochemical events were studied. Significant prostaglandin reductions concomitant with microvascular damage have been reported 1 hour after oral indomethacin (Nygard et al, 1994), but it can only be speculated whether or not slowing of blood flow at the much earlier time point of 25 minutes occurred concomitantly with a reduction in prostaglandins. This is a difficult problem to resolve in view of the highly focal nature of the vascular changes, since focal COX changes may not be detectable in tissue homogenates which include a large proportion of normal tissue. However, immunohistochemical labelling of prostaglandins on histological sections, may clarify their role in intestinal inflammation.

Pharmacological Intervention with CL316,243

Two prior studies have shown reversal and prevention of indomethacin-induced ulceration in the stomach (Kuratani et al, 1994) and intestine demonstrated histologically (Anthony et al, 1996c). It has been hypothesised that in the stomach, β_3-adrenoceptor agonists such as CL316,243 mediate their protective effects by activation of a population of β_3-adrenoceptors on the antral microvasculature (Kuratani et al, 1994). CL316,243 was shown to increase gastric mucosal blood flow, and protect against indomethacin-induced ulceration, supporting the hypothesis of an ischaemic pathogenesis for this injury. In the intestine, stimulation of the β_3-adrenoceptors with CL316,243 was shown histologically to reverse indomethacin-induced villus shortening and microvascular damage (Anthony et al, 1996c). Both of these studies were highly indicative of an effect on blood flow effect, but neither conclusively demonstrated such a role for blood flow, as there was no direct visualisation of villous blood flow after indomethacin.

The results from this study in rat jejunal villi demonstrated β_3-adrenoceptor mediated (CL316,243) reversal of indomethacin-induced blood stasis and prevention of pre-ulcerative histology. There are several possible mechanisms for reversal of indomethacin-induced blood stasis, including reversal of vasoconstriction, endothelial cell effects or relaxation of smooth muscle.

This thesis suggests that since no vasoconstriction was seen with indomethacin, vasodilation is an unlikely mechanism, any gross vasoconstriction would have been visible during *in-vivo* observations. When focal slowing of blood flow was observed in a short segment of the surface villous vessel, flow and vessel diameter in the rest of the villous microvasculature appeared unchanged.

It is possible that β_3-adrenoceptors might mediate a subtle form of

blood flow control in the arcade vessel by affecting endothelial cells, indicated by the observation that increased fluorescence and swelling of the arcade vessel wall resolved after luminal CL316,243. Endothelial cells have been reported to contract and/or swell obstructing the lumen (Anthony et al, 1993), only a small degree of swelling would be required to reduce blood flow severely, since flow varies proportionately with the square of the diameter. It is possible that restoration of blood flow is due to activation of β_3-adrenoceptors on endothelium, resulting in reversal of swelling or contraction. If β_3-adrenoceptor mediated reversal of endothelial swelling/contraction did take place, narrowing of the lumen would escape detection because diameter measurements were taken from the width of the fluorescence. Therefore if a swollen endothelial cell fluoresces it would be taken on video appearance as part of the overall vessel diameter.

The above features imply that β_3-adrenoceptors may control the permeability of the luminal surface on endothelium, preventing the ingress of further FITC-Dextran into the cell in reversal experiments and preventing it in the first place in protection experiments. The β_3-adrenoceptors may prevent NSAID-induced uncoupling of oxidative phosphorylation (Whitehouse, 1964; Haas et al, 1985), which may be reversible in its early stages, but at later stages it becomes irreversible.

Another explanation for CL316,243 effects would be reversal of the endothelial projections, thus releasing trapped red and white blood cells and reversing the stasis, this would also provide an explanation for why iv CL316,243 given at the point of stasis is ineffective as the agonist would not come into contact with the projections because of the stasis. Luminal CL316,243 would however, come into contact with the projections as it is absorbed from the intestinal lumen.

An intravenous dose of CL316,243 also failed to increase basal villous blood flow, it is therefore possible that the concentration within the

villous vasculature as a result of metabolism or plasma protein binding is not high enough after intravenous administration to increase basal blood flow. Unfortunately there was insufficient CL316,243 for further dose response studies (since it had to be specially manufactured for these studies).

Supporting evidence for CL316,243 mediated activation of β_3-adrenoceptors comes from radioactive ligand binding and immunohistochemical studies which have identified β_3-adrenoceptor binding sites on the smooth muscle of human colon sub-mucosa (Summers et al, 1995), vascular smooth muscle of human gallbladder (Guillaume et al, 1995), and in human intestinal smooth muscle layers (Anthony et al, 1997). However these studies have not had sufficient resolution to localise the receptors to specific cell types, such as endothelium.

It has been proposed that a population of β_3-adrenoceptors exists on villus smooth muscle, which upon stimulation promote relaxation and hence reverse indomethacin-induced villous shortening, and microvascular injury (Anthony et al, 1996c). However, this study shows that endothelial fluorescence, projections and slowing of villous blood flow occur prior to villous shortening. Villous shortening occurs after the endothelial cells are damaged, suggesting that shortening is likely to be a protective mechanism which might distort and constrict microvessels (Anthony et al, 1993) to prevent haemorrhage after an initial focal injury.

However, this does not totally rule out a role for smooth muscle relaxation in reversing blood stasis, although the lack of any discernable changes in vessel diameter rules out focal smooth muscle contraction around vessels. Indeed, villus contraction was actually observed *in-vivo* in two animals after blood flow was slowing and endothelial fluorescence had begun to develop in the arcade vessel. It is possible that contraction acts in conjunction with endothelial projections to cause blood stasis. Initially the

endothelial projections produce slowing of flow leading to hypoxia, which may then trigger a protective contraction of the whole villus. The shortening may then interfere in some way with blood flow within microvasculature which is already partly occluded by the projections further reducing blood flow until stasis develops in the affected vessel at the villus tip. β_3-adrenoceptor agonist mediated reversal of villus shortening releases stasis, restarting villous blood flow and maintaining H&E histology. It is also possible that reversal of villus shortening allows small foci of stasis to move downstream allowing blood flow to restart. Supporting evidence for reversal of villus shortening was provided by Bianchetti (1990), MacDonald (1994), and Cohen (1995) who demonstrated the spasmolytic properties of CL316,243 in the gastrointestinal tract. In order to conclusively prove this theory it would require further continuous *in-vivo* observation of stages after the initial onset of the first focus of blood slowing, together with histology and ultrastructure, and calculation of crypt depth to villus height ratios to assess villus contraction. An ultrastructural study is also strongly indicated here to elucidate whether or not CL316,243 reverses the endothelial projections and vacuolisation.

β_3-adrenoceptor agonists such as CL316,243 may have clinical applications in other intestinal diseases such as Crohns disease, which arises from impaired vascular perfusion (Wakefield et al, 1989). Spasm of colonic muscularis mucosae has been reported in the pathogenesis of ulcerative colitis (Goulston and McGovern, 1969) which may now be treatable using anti-spasmodics such as CL316,243 (Anthony et al, 1996d).

The results presented in this thesis allow speculation as to the possible initial pathogenic mechanism of indomethacin-induced ulceration. Firstly, the degenerate epithelial cells seen in controls may be more permeable than cells at the side of the villus, this is supported by the presence of large numbers of lipid droplets within tip enterocytes compared

to those at the side of the villus. This suggests that increased permeability at the tip is a normal intestinal process and not a result of any NSAID effect as reported in the literature (Jenkins et al, 1987; Bjarnason et al, 1984; 1986; 1991a; Sigthorsson et al, 1997). In animal experiments the extensive surgery necessary for *in-vivo* microscopy may lead to shock and a reduction in blood flow to the whole gastrointestinal tract. This reduction in general flow may be responsible for increasing the degeneration of tip epithelial cells reported here throughout the intestines. However, oral indomethacin in rats has been shown to ulcerate the intestines in exactly the same areas of the intestine as described here, and the histological profile was also identical. Most importantly, the epithelial damage described in controls did not increase when indomethacin-induced blood slowing developed. This provides evidence that the indomethacin effects seen in this thesis are not a result of surgical shock. The results presented here strongly suggest that increased epithelial permeability is not caused by indomethacin at the earliest stages, but it may allow indomethacin to enter the stroma. This would allow indomethacin to act directly upon the microvasculature at the villus tip, specifically the arcade vessel which runs just below the epithelial basement membrane. The first morphological change occurs within the endothelial cells which form projections into the vessel lumen which may interfere with normal blood flow causing slowing. As the projections gradually become more severe they may trap red and white blood cells leading to stasis. At the point of stasis many of the pre-ulcerative changes have developed such as epithelial stratification and villus contraction. Endothelial vacuolisation may lead to thickening of the endothelium due to uptake of fluorescent probe further restricting blood flow at the tip. However, Whittle et al (1995) suggested that damage to the intestinal microvasculature was mediated by iNOS, which was induced by increased intestinal bacteria

Conclusions

In conclusion, this thesis has demonstrated that focal slowing of blood flow concomitant with finger like projections into the vessel lumen, are primary morphological and probably primary pathogenic events in indomethacin-induced intestinal ulceration. These changes then progress to blood stasis, villous shortening and other associated pre-ulcerative changes such as degranulation of eosinophils, distortion/stratification of epithelium, focal upper villous necrosis, and buckling of large villous vessels.

Whilst it is unlikely that indomethacin-induced ulceration is the product of a single factor, this study indicates that focal vascular events at the villus tip may be more significant than have hitherto been recognised. The data demonstrate that the initial morphological lesion which occurs in only a portion of the villus arcade vessel, results in ultrastructural damage to the endothelial cells.

The data also show that the β_3-adrenoceptor CL316,243 is a potent inhibitor of indomethacin-induced focal blood stasis and histological damage in the rat jejunum when given either concomitantly with indomethacin or luminally as stasis begins. The mechanism is unclear but it is likely to involve either reversal of villous smooth muscle contraction or an effect on endothelium to reverse the endothelial damage.

Future Work

A. Ultrastructural analysis of villi in which β_3-adrenoceptor mediated reversal of blood stasis has occurred is strongly indicated by these results.

B. β_3-adrenoceptor specific binding studies to elucidate the exact localisation to a specific cell type will give an important clue as to the mechanism of action. Unfortunately the location of these receptors within rat intestine cannot be demonstrated at present due to a lack of sensitivity of currently available antibodies.

C. The possibility exists that CL316,243 is acting at a site remote from

β_3-adrenoceptors to elicit its protective effects, possibly at β_2-adrenoceptors which also relax gut smooth muscle (Lefkowitz et al, 1989). The recent development of a highly specific β_3-adrenoceptor antagonist (Manara et al, 1996) will help to unravel this problem.

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Appendix A

Processing to paraffin

Following fixation in 10% formol-saline the specimen was:-

1. Dehydrated with graded alcohols from 50 to 70, 90 and three changes of 100% alcohol.

2.Cleared in xylene to make specimen miscible with paraffin wax.

3. Impregnated with paraffin wax at 56°C under vacuum.

4. Specimen was correctly orientated and embedded in paraffin wax blocks, then cut into 3μm sections on a microtome.

H&E Staining

1. Sections were de-waxed by two washes in xylene.

2. Sections were rehydrated by passing through graded alcohols from 100% to 70% and then into water.

3. Stained with Harris haematoxylin for 5 minutes, then rinsed in water.

4. Washed briefly in 1 % acid alcohol until adequate differentiation of staining was acquired.

5. Washed in alkalinised water to produce blue haematoxylin staining..

6. Stained with 1 % eosin for five minutes, then rinsed in water. to remove excess.

7. Sections were dehydrated through graded alcohols, from 70, 90 and two changes of 100%.

8. Sections were cleared in two changes of xylene, then coverslipped using DPX mountant.

Appendix B

Following fixation in 1.5% glutaraldehyde the specimen:-

1. was placed into a screw cap tube and washed with phosphate buffered saline (PBS) for 10 mins.
2. Washed in a further 2 changes of PBS for 10 mins.
3. Post-fixed in osmium tetroxide solution (in fume cupboard) for 1 hr 30 mins.
4. Washed in water 3 × 15 mins.
5. Dehydrated through graded alcohols 30%, 50%, 70% and 90%, 2 × 15 mins each.
6. Washed in absolute alcohol 3 × 20 mins.
7. Lemix resin was warmed to room temperature and made up in a 50:50 mixture of resin and absolute alcohol. Tissue was placed into mixture for 4hrs-overnight to allow resin to infiltrate.
8. Specimen then placed into 100% resin for 5-6hrs to finish infiltration.
9. Specimen embedded in 100% fresh resin in labelled moulds then polymerized at 70°C overnight.

Appendix C

	RAT 1	RAT 2	RAT 3	RAT 4	RAT 5
Diameters	13.7μm	11.4μm	12.9μm	13.0μm	11.47μm
RBC velocities in μm/sec					
5 Mins	544	513	466	359	332
15 Mins	578	528	466	359	317
30 Mins	113	255	112	332	326
40 Mins	0	0	228	391	0
50 Mins			114	141	
60 Mins			0	0	

Vessel diameter and RBC velocities from the observed single villi which developed blood stasis on exposure to combined luminal (100μg/ml) and iv bolus estimated to give a plasma concentration of 100μg/ml in all 5 rats.

Appendix D

	RAT 1	RAT 2	RAT 3	RAT 4	RAT 5
Diameters	9.8μm	11.8μm	10.9μm	11.6μm	11.9μm
RBC Velocities in μm/sec					
5 Mins	327	282	327	320	366
15 Mins	331	282	287	327	308
30 Mins	233	188	180	331	369
40 Mins	93	175	0	188	290
50 Mins	0	0		91	168
60 Mins				0	0

Vessel diameter and RBC velocities from the observed single villi in each rat which developed blood stasis on exposure to luminal indomethacin 200μg/ml.

Appendix E

	RAT 1	RAT 2	RAT 3	RAT 4	RAT 5
Diameters	11.6μm	11.1μm	10.4μm	11.1μm	10.94μm
RBC Velocities in μm/sec					
5 Mins	395	354	353	330	255
15 Mins	447	343	353	298	230
30 Mins	298	286	227	326	212
35 Mins	109	286		339	227
40 Mins				284	212
45 Mins					137

Vessel diameter and RBC velocities from the observed single villi which developed slowing of blood flow on exposure to combined luminal (100μg/ml) and an iv bolus of indomethacin estimated to produce a plasma concentration of 100μg/ml.

Appendix F

	RAT 1	RAT 2	RAT 3	RAT 4	RAT 5
Diameters	12.4μm	12.4μm	11.9μm	10.7μm	11.74μm
RBC Velocities in μm/sec					
5 Mins	255	282	328	376	327
15 Mins	284	327	291	327	282
30 Mins	301	152	188	237	336
40 Mins	297	0	21	0	282
50 Mins	78	308	0	0	78
60 Mins	0	312	297	330	0
70 Mins	240	326	297	288	284
80 Mins	227	331	298	273	315
90 Mins	242	326	298	315	315

Blood flow values from all 5 rats for the reversal of indomethacin-induced blood stasis in single villi by luminally applied CL316,243 (1mg/Kg).

Appendix G

	RAT 1	RAT 2	RAT 3	RAT 4	RAT 5
Diameters	12.19μm	11.5μm	11.3μm	10.7μm	12.03μm
RBC Velocities in μm/sec					
5 Mins	175	192	187	170	175
15 Mins	187	188	178	178	175
30 Mins	220	225	200	203	200
40 Mins	212	237	207	203	217
50 Mins	200	231	210	212	212
60 Mins	237	225	229	203	220
70 Mins	243	225	250	218	208
80 Mins	218	218	233	200	200
90 Mins	218	220	230		210

Velocity and diameter values obtained from all 5 rats given luminal CL316,243, agonist was applied after 15 mins.

Appendix H

	RAT 1	RAT 2	RAT 3	RAT 4	RAT 5
Diameters	10.7μm	11.0μm	10.6μm	10.8μm	11.7μm
RBC Velocities in μm/sec					
5 Mins	200	218	233	200	175
15 Mins	250	200	250	207	192
30 Mins	200	225	275	217	225
40 Mins	212	200	250	217	230
50 Mins	216	200	277	225	220
60 Mins	200	233	250	217	225
70 Mins	212	217	-	220	233
80 Mins	250	204	-	207	233
90 Mins	215	200	-	225	230

Velocity and diameter values obtained from all 5 rats given an iv bolus dose of CL316,243. The agonist was given after 15 mins. The final 3 readings were not possible in rat 3 because of obscurement by mucus.