THE PERIVASCULAR INNERVATION OF HUMAN MESENTERIC BLOOD VESSELS IN HEALTH AND INFLAMMATORY BOWEL DISEASE

A thesis submitted to the University of London for the degree of Doctor of Medicine

by

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Abstract

Perivascular innervation of human mesenteric blood vessels in health and inflammatory bowel disease (IBD)

Vascular abnormalities and ischaemia have been implicated in the pathophysiology of Crohn's disease and ulcerative colitis. Perivascular nerves and the endothelium form the dual control mechanism for local blood flow. This study investigates the structure and function of the perivascular nerve plexus to identify changes in IBD which may contribute to the disease process.

Immunohistochemical techniques using computerised quantitative image analysis were used to assess the nerve density and structure of the nerve plexus in resected colonic specimens. Noradrenaline (NA), neuropeptide Y (NPY), substance P (SP), calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP) and 5-hydroxytryptamine (5-HT) were identified in the perivascular nerves. The neuronal marker, protein gene product 9-5 (PGP), was used to determine the total number of nerves. In the control arteries, the number of nerves containing NPY, NA and VIP, in proportion to those with PGP-like immunoreactivity (LI), was 100%, 60% and 30% respectively, compared to 100%, 85% and 30% in the veins. 5-HT nerves were sparse and SP and CGRP were identified in less than 5% of the nerves of the control vessels: CGRP-LI was absent in the control veins.

In IBD there was an increase in the number of nerve fibres in both the arteries and veins (control vs IBD arteries: 6.94 ± 1.22 vs 19.83 ± 1.52, p=0.005; veins: 9.31 ± 1.06 vs 14.39 ± 0.59, p=0.01; mean PGP total fluorescent area ± 95% CI; Student's t-test).
This increase was mainly of NA and NPY-containing sympathetic nerves and was accompanied by the appearance of a less organised nerve plexus. The relative proportion of nerve types was similar to the control vessels, although 5-HT-LI was identified more consistently and CGRP-LI was found in the veins.

The response of the vessels to vasoactive substances and to transmural electrical field stimulation (EFS) was examined. NA and adenosine triphosphate (ATP), cotransmitters with NPY in sympathetic nerves, produced concentration-dependent contractions of the arteries and the veins. The strength of the contraction to NA was greater in the arteries than the veins (control arteries vs veins; 124.3 ± 19.3 vs 64.1 ±19.0, p=0.0008; % maximal contraction to KCl (E_max); mean ± 95% CI; Student’s t-test). NPY produced dose-dependent contraction in the veins, but not in the arteries. In control veins, but not arteries, the presence of a sub-threshold dose of NPY increased the sensitivity to NA, and increased the force of contraction.

There was no difference in the maximal contraction to NA of the IBD vessels compared with controls, but there was a right-shift of the concentration-response curves of the IBD arteries to ATP, indicating decreased sensitivity (3.22 ± 0.15 vs 2.59 ± 0.45, p=0.021; [-log] producing 25% of the maximal contraction of the vessel to KCl 120mM (p[A]_25); mean ± 95% CI; Student’s t-test). The modulatory effects of NPY on the contractions of the veins to NA were not seen.

Transmural EFS of perivascular nerves produced contractions of the veins but not of the arteries which was abolished by tetrodotoxin (1μM). The neurogenic contractions were partially blocked by the α₁-adrenoceptor antagonist prazosin (1μM), P₂-purinoceptor antagonism with suramin (10μM) or desensitisation of P₂X-purinoceptors with α, β-methylene adenosine triphosphate (1μM). In the presence
of both $\alpha_1$-adrenoceptor and purinoceptor blockade any small residual contraction was abolished by the addition of the $\alpha_2$-adrenoceptor antagonist, yohimbine. In IBD, there was a significant enhancement of the contractile response (control vs IBD veins; $10.2 \pm 4.2$ vs $33.0 \pm 14.35$, $p=0.038$; % maximal contraction to 120mM KCl; mean $\pm$ 95% CI; Student’s $t$-test). The proportion of this response to $\alpha_1$-adrenoceptor stimulation was less than in the control vessels. After P$_2$-purinoceptor antagonism, there was often a large residual contraction which was abolished by yohimbine, but in some, it was abolished only by tetrodotoxin.

Electron microscope studies on control vessels identified differences between the arteries and veins, including wide separation of nerves and vascular smooth muscle with interposed collagen and fibroblasts, which may explain the lack of responsiveness to nerve stimulation. The increase in nerve density in IBD arteries was confirmed.

This study defines changes in the structure and function of human mesenteric perivascular nerves in IBD. These changes in the mechanism of blood flow control may contribute to the ischaemia hypothesised as a factor in the pathogenesis of IBD.
Statement of originality

The studies described and presented in this thesis are the original work of the author. All studies were performed by the author with the following exceptions:

All electron microscopy was performed in conjunction with Mr M Turmaine in the Department of Anatomy, University College London.

Specimens of bowel mesentery were obtained at surgery by Professor PB Boulos at the Middlesex Hospital and Mr P Hawley at St Mark’s Hospital.

No part of this work has been submitted to any other university for consideration for a higher degree.

All the studies in this thesis were performed in accordance with protocols approved by the Ethical Committee and with patients’ informed consent.
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Finally, I would like to thank my parents, Julia and Winnie for their unending and cheerful support during my research.
List of abbreviations

2D.................................two-dimensional
3D.................................three-dimensional
5-HT...............................5-hydroxytryptamine; serotonin
AECA..............................anti-endothelial cell antibodies
ANOVA............................analysis of variance
ANS...............................autonomic nervous system
ATP...............................adenosine triphosphate
CD.................................Crohn’s disease
CGRP.............................calcitonin gene-related peptide
ChAT..............................choline acetyltransferase
CI.................................confidence interval
CLSM.............................confocal laser-scanning microscopy
CNS...............................central nervous system
CRC...............................concentration-response curve
EDHF..............................endothelium-derived hyperpolarizing factor
EDRF..............................endothelium-derived relaxing factor
EEL...............................external elastic lamina
EFS...............................electrical field stimulation
ENS...............................enteric nervous system
ET.................................endothelin
FITC..............................fluorescein isothiocyanate
GALT.............................gut-associated lymphoid tissue
HMA...............................human mesenteric artery
HMV...............................human mesenteric vein
IBD...............................inflammatory bowel disease
ID.................................intercept density
IEL...............................internal elastic lamina
Ig.................................immunoglobulin
IMA...............................inferior mesenteric artery
-LI.................................-like immunoreactivity
LID...............................longitudinal intercept density
NA...............................noradrenaline
NDS...............................normal donkey serum
NGF...............................nerve growth factor
NO...............................nitric oxide
NOS...............................nitric oxide synthase
NPY...............................neuropeptide Y
NSAID............................non-steroidal anti-inflammatory
PACAP.........................pituitary adenylate cyclase-activating peptide
PAF...............................platelet activating factor
PBS...............................phosphate-buffered saline
PGP...............................protein gene product 9-5
PHI...............................peptide histidine-isoleucine
PSB...............................pontamine sky blue
SMA...............................superior mesenteric artery
SP...............................substance P
SSRI.............................selective serotonin-reuptake inhibitor
TFA...............................total fluorescent area
TH...............................tyrosine hydroxylase
TID...............................transverse intercept density
UC...............................ulcerative colitis
VACht............................vesicular acetylcholine transporter
VIP...............................vasoactive intestinal peptide
List of equipment and reagents

α, β me-ATP.... Sigma, St. Louis, USA
Adenosine triphosphate .......... Sigma, St. Louis, USA
Biotinylated donkey anti-rabbit.... Amersham Ltd, Amersham, UK
Cacodylate buffer ....... Agar Scientific, Luton, UK
Force-displacement transducer.... Grass Instrument Co, Quincy, MA, USA
Glutaraldehyde ..... Agar Scientific, UK
JEM 1010 electron microscope... JEOL, Japan
Magneto optical disc ........ Sony Corp, Japan
Neuropeptide Y ....... Sigma, St. Louis, USA
NIH image ............ National Institute of Health, USA
Noradrenaline .. Sigma, St. Louis, USA
Normal donkey serum .......... Gibco, Paisley, UK
Osmium tetroxide ...... Agar Scientific, Luton, UK
Polygraph ............. Grass Instrument Co, Quincy, MA, USA
Pontamine sky blue ... BDH Chemicals, Poole, UK
Prazosin ............ Sigma, St. Louis, USA
Rabbit anti-5-HT .......... Incstar, USA
Rabbit anti-CGRP .......... Affiniti, UK
Rabbit anti-ChAT ...... Biogenesis, UK
Rabbit anti-NOS ......... Ultraclone, UK
Rabbit anti-NPY ........ Biogenesis, UK
Rabbit anti-PGP ........ Ultraclone, UK
Rabbit anti-SP ............ Genosys, UK
Rabbit anti-TH............ Affiniti, UK
Rabbit anti-VIP .......... Incstar, USA
SD9 stimulator.... Grass Instrument Co, Quincy, MA, USA
Streptavidin-fluorescein .... Amersham Ltd, Amersham, UK
Suramin ............ Sigma, St. Louis, USA
Sylgard ......... Dow Corning, Poole, UK
TCS 4D confocal microscope.... Leica, Wetzlar, Germany
Tetrodotoxin..... Sigma, St. Louis, USA
Triton X... Fisons Corp, Bedford, Mass
Uranyl acetate....... Agar Scientific, UK
Yohimbine ...... Sigma, St. Louis, USA
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Preface

The aetiology of IBD is multifactorial. Bowel ischaemia and vascular abnormalities have been proposed as contributing factors. The endothelium and the perivascular nerves form the 'dual control' mechanism for local blood flow and vascular tone. Extensive work to investigate these mechanisms has been carried out on blood vessels from a number of vascular beds in a variety of animal species. There have been relatively few studies on human blood vessels. In this study, the structure and function of perivascular nerves in normal human mesenteric arteries and veins is investigated and compared to IBD vessels to identify any changes which may contribute to the pathogenesis of the condition.

In Chapter 1, the subject is introduced. This chapter is divided into three sections. The first is a description of the mesenteric vasculature, with reference to the anatomy of the vascular bed and mechanisms of blood flow control. The complexity of the innervation of the vessels is highlighted, and the short and long term (trophic) effects of neurotransmitters on both vascular smooth muscle and endothelium, are discussed. The second section describes the inflammatory bowel disease, with reference to current concepts on its aetiology and the role of vascular abnormalities. The third section describes the aims and experimental plan of the study.

Chapter 2 describes the experiments to identify the pattern of perivascular innervation and the chemical coding of neurotransmitters, using immunohistochemistry, confocal laser-scanning microscopy and computerised quantitative image analysis. Characteristics of control vessels are described and
compared with the findings in IBD vessels. Differences identified between the groups are discussed.

In Chapter 3, organ-bath pharmacology experiments are described. Section 1 outlines the effects of exogenous neurotransmitters on the vessels, and compares the response of control vessels to those in IBD. In Section 2, the contractile responses of vessels to electrical nerve stimulation are described and the two groups compared.

Chapter 4 outlines ultrastructural studies on mesenteric arteries and veins. The findings of this part of the study are discussed with reference to the findings in the previous chapters.

In Chapter 5, the General Discussion, the interrelationships of the findings in earlier chapters and difficulties encountered during the study are considered. The relevance of the results to the pathophysiology of IBD is discussed, and suggestions are made for further work arising from this study.
Chapter 1    Introduction and historical review
1.1 The Mesenteric Vasculature

1.1.1 Macroscopic Anatomy

The gut and associated organs are divided embryologically into three: the foregut, midgut and hindgut. The foregut comprises the lower oesophagus, the stomach and most of the duodenum. The midgut is the largest of these three areas. It is the part if the alimentary tract which emerges into the extra-embryonic coelom during early fetal life, and consists of the remainder of the duodenum, the jejunum and the ileum, and the proximal two-thirds of the colon; the remainder of the colon and the rectum comprise the hindgut.

Each of these regions is supplied by one of the three ventral branches of the abdominal aorta. The coeliac axis emerges just caudal to the diaphragm, and is the artery of the foregut. It is a short vessel, and divides into the hepatic, splenic and left gastric arteries. It thus supplies the liver, spleen and other upper abdominal organs, as well as the intestine. The superior mesenteric artery (SMA) is the largest of the three vessels. It arises 1 cm below the coeliac axis and supplies the midgut, as well as sending some branches to the pancreas. The inferior mesenteric artery (IMA) arises three or 4 cm above the bifurcation of the aorta, and supplies the distal colon, the rectum and the upper anal canal.

This is the general pattern, but there are wide variations in the blood supply to the gut and in particular the degree of anastomosis between the vessels. This can have important implications in the clinical effect of embolism or thrombosis of the large vessels.
The major vessels supplying the gut divide into intermediate arteries within the mesentery. Branches of the SMA divide and re-anastomose, forming the jejunal and ileal arcades. From the terminal arcades, vasa recta are given off, to supply alternate sides of the bowel. These arcades increase in complexity along the length of the small bowel, until the terminal ileum is reached. Here, the terminal branch anastomoses with the ileal branch of the ileocolic artery.

The intermediate vessels of the colon have a rather different arrangement. The large, right-sided branches of the SMA and the upward running left colic branch of the IMA form the marginal artery of the colon. This vessel travels parallel to the colon, linking the supplying vessels, and giving off vasa recta and vasa brevia to supply the colon. There is a degree of variability in the marginal vessel in the region of the splenic flexure, at the site of the anastomosis between the left colic and the middle colic arteries. If these vessels are not well developed, there exists an area of critical blood supply in this area. Any impairment of the flow in either the SMA or IMA may result in ischaemic damage.

The more distal branches of the IMA are the sigmoid arteries. These are two or three in number, and anastomose with the marginal artery. This vessel is continuous and anastomoses with branches of the superior rectal artery. This region was thought to represent another 'critical point' of the circulation, but anatomical studies have refuted this (Williams 1995).

Blood draining from the splanchnic organs passes to the liver via a portal system before rejoining the systemic circulation. The midgut is drained via the superior mesenteric vein. This is a large vein, which is joined behind the pancreas by the splenic vein, which drains the spleen, some of the stomach and much of the
pancreas. The hepatic portal vein is formed by the junction of these tributaries, carrying blood to the liver. The rest of the foregut is drained by small vessels into the portal vein. The vein of the hindgut is the inferior mesenteric vein which joins the splenic vein prior to its joining the superior mesenteric. The blood of the portal system thus passes through two sets of exchange vessels, the capillaries of the intestine and the sinusoids of the liver. The importance of this arrangement is that the blood containing absorbed nutrients is processed by the liver before it rejoins the systemic circulation.

1.1.2 Vessel structure

1.1.2.1 Basic structure

The cross-sectional structure of blood vessels varies considerably with vessel size and type, but classically consists of three distinct layers.

The outermost layer is the *tunica adventitia*. This layer consists mainly of connective tissue, which blends with the surrounding fibro-areolar tissue. The lymphatics and the nutrient blood vessels of the vessel, the vasa vasorum, lie in this layer. In the outer adventitia lie large nerve bundles. These 'paravascular' nerves travel with the vessels to and from the organs, but play no part in the innervation of the vascular smooth muscle.

Deep to the adventitia is the *tunica media*, which is separated from the adventitia by the external elastic lamina (EEL). The media is a fibro-muscular layer, with differing proportions of elastic tissue in different vessel types. At the adventitial/medial border lie the perivascular nerves, forming a plexus that innervates the vessel. Between the media and the innermost layer is the internal
elastic lamina (IEL), another condensation of elastic tissue, separating it from the
*tunica intima.*

The intima consists of a monolayer of flattened cells, the endothelium, which is in
contact with the blood. These cells lie on a basal lamina, which is supported by a
sub-endothelial layer of connective tissue. This basic structure varies considerably
throughout the vascular tree.

### 1.1.2.2 Arteries

The thickness of the vessel wall is largely dependent upon the pressure within the
vessel. Arteries therefore have thicker walls than their corresponding veins. In large
arteries, the tunica media consists largely of elastic tissue. These vessels are known
as 'conduit arteries' and are concerned with converting the pulsatile flow from the
heart into a more continuous stream throughout the arterial tree. Smaller arteries
contain an ever-increasing amount of smooth muscle within their walls. These
distributing arteries are responsible for controlling overall blood flow to organs and
large tissue masses.

The smallest muscular arteries branch into muscular arterioles. These vessels are of
between 50 - 100μm diameter and contain four or five layers of smooth muscle cells
within their walls. These branch into terminal arterioles, which have one or two
layers of muscle. These small arterioles open into the capillary bed, their terminal
portions being surrounded by pre-capillary sphincters. These small vessels comprise
the resistance vessels, which provide precise control of blood flow through the
microcirculatory units of the organs.
1.1.2.3 The capillary bed

The capillaries consist of a tube of endothelial cells surrounded by the basal lamina with occasional supporting pericytes. The thin wall of the capillaries allows efficient exchange of nutrients, gases, metabolites and water between the blood and surrounding tissues. Capillary bed density varies considerably, and is dependent upon the metabolic requirements of the tissues supplied. The endothelial lining may be continuous or, more commonly, fenestrated. Continuous capillaries occur in areas, such as the brain, which require a physiological barrier to large molecule diffusion. Fenestrated capillaries are found in areas where there is ready exchange of large molecules, for example in the intestinal villi. The endothelium in these vessels is perforated by pores of between 30-100nm. In the intestine, these endothelial cells also possess numerous endocytic vesicles, which further enhance the passage of nutrients.

1.1.2.4 Veins

Venules are formed by the confluence of a number of capillaries. These are of between 20-30μm in diameter. The immediate post capillary venules consist of a layer of endothelium surrounded by some fibrous tissue, fibroblasts and pericytes. The larger venules are also partially enclosed by a media of a few smooth muscle cells. The immediate post-capillary venules are important sites of fluid exchange and leukocyte migration; they are sensitive to inflammatory agents that promote the permeability of these vessels to fluids and inflammatory cells.

The basic structure of the veins is similar to that of the arteries. The endothelium is supported by a basal lamina overlying a fine network of elastic tissue, which replaces the definite IEL of the arteries. In most veins, the endothelium is
specialised in places into valves, which direct the flow of blood to the heart. The media contains relatively more connective and elastic tissue than the arteries, along with smooth muscle fibres. There is no definite EEL. The adventitia consists of areolar and elastic connective tissue, containing nerves as well as vasa vasorum in the larger veins. The venules and small veins comprise the postcapillary resistance vessels. These vessels do not control blood flow as such, but their tone is one of the main determinants of hydrostatic capillary pressure and the rate of hydrodynamic fluid exchange across the capillary wall (Lundgren 1989).

The venous compartment as a whole forms the capacitance section of the vascular bed. Changes in the tone of these vessels alter regional blood volume without significant changes in the total regional vascular resistance (Lundgren 1989). The capacitance vessels therefore form a 'pool' of mobilizable blood. In some species, the spleen can actively contract and acts as a blood reservoir. In man, however, the contractile function of the spleen is limited, so constriction of the capacitance vessels fulfils this role.

1.1.3 Control of blood flow

1.1.3.1 General considerations

Poiseuille’s law governs steady flow in a cylindrical tube: \( Q = \frac{P r^4}{8\eta L} \), where \( Q \) = volume flow (i.e. volume per unit time); \( P \) = pressure drop; \( r \) = radius of tube; \( L \) = length of tube; \( \eta \) = viscosity. This equation holds under conditions of laminar, undisturbed flow and disregards factors such as the effect of pulsatility of flow and the elasticity of the vessel walls. However, it can be seen that small changes in the vessel diameter have a great influence in the blood flow, with a reduction in the
vessel diameter of 20% reducing flow by about 60%. Details vary in different vascular beds, but in general, blood flow to organs is largely controlled by the relatively large, muscular distributing arteries and the smaller resistance vessels, responsible for about 15% and 40% of total peripheral resistance respectively. Control of the diameter of these vessels is of the greatest importance in controlling blood flow. The capillaries are responsible for about 30% of peripheral resistance, but are incapable of altering their diameter (Ledingham and MacKay 1988). Blood flow to each tissue or organ is governed by local and general chemical and neural mechanisms, which integrate the needs of the organ with those of the organism as a whole.

1.1.3.2 Control mechanisms

The complexity of neurohumoral control mechanisms of blood flow has become apparent over the last two decades. There is wide variability in these mechanisms between species, and in different vascular beds within species, which makes the extrapolation of results difficult. For many years, the study of these control mechanisms concentrated on the role of adrenergic and cholinergic autonomic nerves, and catecholamines released by the adrenal medulla. The true picture is much more complex, and it is now known that, as in the central nervous system (CNS), there are a wide variety of neurotransmitters in the autonomic nervous system (ANS) which are involved in neurotransmission and neuromodulatory mechanisms. The endothelium plays a major role in the control of vascular tone, responding to circulating or locally produced vasoactive substances. It also produces transmitter substances in response to changes in haemodynamic forces and shear stress in the vessel wall, which mediate constriction or dilatation of the vessel.
The balance between the actions of the endothelium and the perivascular nerves comprises the concept of the dual control of vascular tone (Burnstock 1988).

**Endothelium**

The endothelium is a continuous monolayer of cells, which line the lumen of all blood vessels. This layer was previously thought to act only as a non-wettable surface on which the blood cannot clot. The importance of the endothelium in the control of vascular tone was first discovered in 1980 (Furchgott and Zawadzki 1980). They showed that the endothelial cells have a crucial role in the vasodilator response to ACh via the release of a diffusible mediator, which was termed 'endothelium-derived relaxing factor' (EDRF). This substance was proposed to be nitric oxide (NO) in 1986 (Ignarro et al. 1987). There is a continuous basal release of NO from blood vessels, which contributes to resting vascular tone. Inhibition of this release or endothelial damage will thus lead to an increase in vessel tone (Burnstock and Ralevic 1994).

The endothelium also produces a number of other substances, including constricting factors, hyperpolarizing factors and prostaglandins. These are released following the stimulation of endothelial cells by a number of vasoactive substances, or by other physiological stimuli such as changes in blood flow or oxygenation. The endothelium therefore acts to integrate these signals to play a major role in the local control of blood flow (Burnstock and Ralevic 1994).

**Endothelium-dependent vasodilatation**

Nitric oxide synthase (NOS) has been localised in endothelial cells at both light and electron microscope levels (Dikranian et al. 1994; Nichols et al. 1994). In addition
to NO, other substances stored within endothelial cells may be released to give rise to endothelium-dependent vasodilatation. Prostaglandins, released from endothelial cells in response to shear stress and hypoxia, cause smooth muscle relaxation. Endothelium-derived hyperpolarizing factor (EDHF) is distinct from NO and also contributes to smooth muscle relaxation in blood vessels. It is released from endothelial cells on stimulation by SP, ACh and ATP (Furchgott and Vanhoutte 1989). It has been suggested that EDHF may be hydrogen peroxide (Matoba et al. 2000).

A variety of vasoactive substances acts upon endothelial cells, stimulating NO production. The NO diffuses from the endothelium to act upon vascular smooth muscle, causing vasodilatation. The receptors to these substances are of different subtypes to those occurring on vascular smooth muscle, and often have the opposite action. This is exemplified by the response to ATP, which produces vasodilatation via endothelial P_{2Y} receptors, whilst ATP released from perivascular nerves produces constriction of vascular smooth muscle via P_{2X} receptors (Burnstock and Kennedy 1985).

Choline acetyltransferase (ChAT) was localised in endothelial cells of capillaries and small vessels in the rat cerebral cortex in 1985 (Parnavelas et al. 1985). Further ultrastructural studies using immunohistochemical methods have identified endothelin-1, arginine-vasopressin and SP in a high proportion of endothelial cells, co-localised with NO (Dikranian et al. 1994). Other studies have also identified ChAT, SP, 5-HT, vasopressin, and angiotensin II in the endothelium of a variety of blood vessels (Loesch and Burnstock 1988; Milner et al. 1989; Lincoln et al. 1990; Loesch et al. 1991a, b; Cai et al. 1993). The role of these transmitters appears to be
that they are released from the endothelium in response to local stimuli, and act on adjacent endothelial cells, which produce EDRF, which in turn acts on the smooth muscle to produce vasodilatation.

One of the main stimuli for the release of these agents appears to be shear stress in the wall of the vessel (Burnstock 1999). An increase in blood flow increases the shear stress in the endothelial cells. The response of arteries to this stimulus is an endothelium-dependent vasodilatation (Holtz et al. 1984; Smiesko et al. 1985; Hull et al. 1986; Rubanyi et al. 1986; Bevan,JA and Joyce 1988). There is evidence that the mediators of this increased release of NO are the transmitters stored within the endothelium. In the perfused rat hindlimb, an increase in flow stimulates an increased level of SP in the effluent (Ralevic et al. 1990), which is no longer evoked after removal of the endothelium by air bubbles (Ralevic et al. 1989). The source of the SP has been shown not to be the perivascular nerves, as it is resistant to treatment with capsaicin (Ralevic et al. 1990). Flow-induced release of SP, ATP and ACh has also been identified from cultured endothelial cells (Milner et al. 1990), and of ATP from the rat perfused mesenteric arterial bed (Ralevic et al. 1992).

Hypoxic vasodilatation is also known to be endothelially-mediated. Coronary endothelial cells have been shown to release ACh, ATP, SP and 5-HT in response to hypoxic perfusion of the isolated rat heart (Paddle and Burnstock 1974; Burnstock et al. 1988; Milner et al. 1989). The vasodilatation may be to protect the organ from the harmful effects of ischaemia, as it has been shown that the endothelially-induced dilatation can override the vasoconstrictive effect of perivascular adrenergic nerves (Ishibashi et al. 1997).
Local mediators may also elicit endothelium-dependent vasodilatation. Substances such as arachidonic acid, bradykinin and histamine, are produced during the inflammatory response by immune cells. These substances may produce their vasodilatory effects via an endothelially-mediated mechanism (Furchgott and Vanhoutte 1989).

Endothelial cells carry both $\alpha_2$ and $\beta$ adrenergic receptors (Miller, VM and Vanhoutte 1985; Howell et al. 1988; Langer and Schoemaker 1989; Vanhoutte and Miller 1989; Aikawa and Akatsuka 1990; Durieu-Trautmann et al. 1991; Bockman et al. 1996). These are likely to respond to circulating catecholamines, and may contribute to the vasodilator effect of catecholamines in certain vascular beds (e.g. coronary, splanchnic). They also serve to blunt the vasoconstrictor response to sympathetic nerve-mediated vasoconstriction (Vanhoutte and Miller 1989). Other circulating hormones, such as vasopressin, cause variable effects, depending on the particular vascular bed involved. It has been shown that vasopressin dilates cerebral vessels, but not other systemic vessels (Vanhoutte et al. 1984). This is the opposite action to the response in most vessels, which raises the possibility that vasopressin is responsible for the redistribution of blood flow under certain conditions (Furchgott and Vanhoutte 1989).

There are important interactions between the endothelium and blood constituents, particularly platelets. Aggregating platelets release adenine nucleotides and 5-HT, both of which can evoke endothelium-dependent relaxation and endothelium-independent constriction (Vanhoutte 1988; Golino et al. 1990; Shepherd et al. 1991). The endothelium thus plays a protective role against intra-luminal platelet aggregation and thrombus formation. Damage to the endothelium, on the other
hand, promotes vasoconstriction in response to platelet products. In the case of haemorrhage, this is an appropriate response, but in disease states such as atherosclerosis and hypertension, the inappropriate activation of these mechanisms may give rise to pathological effects.

Endothelium-dependent vasoconstriction

As well as vasodilatation, the endothelium also has a mechanism for inducing vasoconstriction. It has been found that there are a number of endothelium-derived constricting factors, which are released by different parts of the vascular system in response to differing stimuli. Endothelially-dependent constriction occurs in response to arachidonic acid (De Mey and Vanhoutte 1982), 5-HT (Auch-Schwelk and Vanhoutte 1991), hypoxia (De Mey and Vanhoutte 1983) and stretch (Katusic et al. 1987), and to platelet-derived substances such as ATP and ADP (Shirahase et al. 1990). Caffeine (Jino et al. 1994) and nicotine (Shirahase et al. 1988) have also been found to cause endothelium-dependent constriction. These agents appear to act via a number of pathways.

A number of the responses can be inhibited by cyclo-oxygenase inhibitors, indicating that a product of arachidonic acid metabolism is involved. This appears to be thromboxane A2, as thromboxane synthetase inhibitors antagonise the response (Shirahase et al. 1987; Katusic et al. 1988; Auch-Schwelk et al. 1990). ATP (via P2X receptors) (Shirahase et al. 1991), angiotensin II (Manabe et al. 1989), nicotine (Shirahase et al. 1988) and acetylcholine (Shirahase et al. 1987) all cause endothelially-mediated vasoconstriction via this mechanism.

Other mediators of endothelium-dependent constriction include the superoxide anion (Katusic and Vanhoutte 1989) and a yet unidentified contracting factor released
from hypoxic endothelial cells (Rubanyi and Vanhoutte 1985). The nature of this factor is unknown. It is not metabolised from arachidonic acid or other autacoids, and the rapid time course of its action makes it unlikely to be a peptide.

In 1988 a vasoconstrictor peptide, endothelin, was isolated from endothelial cells (Yanagisawa et al. 1988). A family of endothelins, with a wide variety of actions, has now been identified. Endothelin-1 is a potent vasoconstrictor in resistance and capacitance vessels in man, and produces a sustained vasoconstriction (Haynes 1995). Hypoxia and ischaemia augment ET secretion and in turn stimulates the secretion of NO, arginine vasopressin, atrial natrurietic peptide (Levin 1996) and vasodilator prostaglandins (Haynes 1995). The precise role of ET has yet to be established, but one of its actions appears to be the long-term maintenance of vascular tone (Haynes and Webb 1994; Webb 1995).

Autonomic nervous system

The classical concept of the ANS as an antagonistic parasympathetic cholinergic and sympathetic adrenergic system dominated thinking for over 50 years at the beginning of the century. Over the past thirty years, however, there have been great advances in our understanding of the ANS, which have completely changed our understanding of its structure and function.

Since the recognition of non-adrenergic, non-cholinergic nerves in the 1960’s, there has been the identification of a large number of substances as neurotransmitters, including purines, amino acids, peptides, monoamines and NO. Co-transmission has been recognised, in which more than one transmitter is synthesised, stored and released by nerves (Burnstock 1976). This is in contrast to the previous idea of ‘one nerve, one transmitter’, known as ‘Dale’s Principle’, proposed by a pioneering
investigator of the ANS (Dale 1935). Co-transmission is the control of a single

target cell by two or more substances released from one neurone in response to a
single neuronal event (Campbell 1987). The demonstration of more than one
putative transmitter substance within a nerve is not enough to establish that they are
cotransmitters. It is also necessary to show post-junctional actions of each via their
own specific receptors (Burnstock 1987). Where cotransmission occurs, the actions
of the cotransmitters are often synergistic. The interaction can be more complex,
with the cotransmitters having opposite actions on different target cells, or different
post-junctional actions depending on the tone of the effector muscle (Burnstock
1987). This occurs in some situations (Lundberg et al. 1985a), but in others the
transmission appears to be mediated by a single transmitter. In these cases, the co-
released substances may have a role in neuromodulation.

Neuromodulation describes the modification of the process of neurotransmission by
another substance. Neuromodulators may be circulating hormones, endothelially-
derived substances, local agents such as prostanoids, bradykinin or histamine, or
neurotransmitters released from the same or adjacent varicosities on the same nerve
fibre. The neuromodulator can either alter the amount of a transmitter released by a
pre-junctional mechanism, or have a post-junctional effect on the time course or
intensity of action of that transmitter.

It appears that most, if not all nerves release more than one transmitter. Patterns are
emerging within the ANS, whereby different types of nerves contain different
patterns of neurotransmitters, with well-defined central connections and peripheral
projections. This principle is well recognised in the enteric nervous system (ENS)
(Costa et al. 1986; McConalogue and Furness 1994) and the intrinsic nerves of other
organs (Masuko et al. 1991), and the CNS (Shimazu 1981; Bieger 1993). It also applies to perivascular nerves.

Another important advance has been the recognition of the importance of the intrinsic nervous systems of many organs. The best developed of these is the ENS, but other organs (such as the heart, airways and bladder) also have intrinsic ganglia. These ganglia, previously thought to be simple parasympathetic relay stations are now known to be capable of sustaining and modulating complex local activity independent of the CNS.

The sensory-motor nerves are another class of nerve, the importance of which is now being appreciated. The axon reflex has been recognised in the skin for many years (Lewis 1927), but the importance of similar reflexes in other organ systems was not recognised. It is now known that antidromic impulses from sensory nerves are important in the regulation of activity in many organs, including the gut, lungs, heart and bladder, as well as blood vessels.

The autonomic neuroeffector junction

In perivascular nerves, the autonomic neuroeffector junction consists of varicose nerve fibres forming a branching plexus at the adventitial-medial border of most blood vessels. The small terminal axons are devoid of Schwann cells and comprise a series of varicosities (1-2 μm diameter) separated by intervaricose regions (0.1-0.3 μm diameter) (Burnstock 1986; Smolen 1988). The varicosities are the site of storage and release of neurotransmitters. The transmitters are released ‘en passage’ by depolarising nerve impulses, and diffuse across a junctional cleft to act on vascular smooth muscle cells. The distance between the nerve fibres and smooth muscle cells varies considerably, from about 80 nm to 2 μm in larger vessels.
(Burnstock 1987). The myocytes themselves have no post-junctional specialisation, unlike striated muscle cells that possess the motor end plate. The smooth muscle cells are in electrical continuity via gap junctions (Fujiwara et al. 1983; Moore and Burt 1995), so that nervous impulses initiated at the adventitial-medial border are propagated throughout the media via these channels.

**Sympathetic nerves**

**Noradrenaline**

The main transmitter in most sympathetic perivascular nerves is NA. There are two main sub-classes of adrenoceptor, \( \alpha \) and \( \beta \). The effect of stimulation of these receptors is usually antagonistic: \( \alpha \)-receptors are excitatory and \( \beta \)-receptors are inhibitory. Each of these groups has been further sub-classified.

Whereas earlier attempts at subclassification of receptors were based on anatomical location, current classifications are based on the pharmacological characteristics of the receptors. There are two main subdivisions of \( \alpha \)-receptor, \( \alpha_1 \) and \( \alpha_2 \), each of which have been further subdivided into four subgroups (Docherty 1998). \( \beta \)-receptors have also been subdivided into three subgroups, \( \beta_1 \), \( \beta_2 \) and \( \beta_3 \). Initially, post-junctional receptors were classified as \( \alpha_1 \) and pre-junctional as \( \alpha_2 \) receptors, but it is now known that post-junctional \( \alpha_2 \) receptors have an important role in vasoconstriction in some vessels (McGrath 1982).

Pre-junctional \( \alpha_2 \) and \( \beta_2 \) receptors exist on perivascular sympathetic nerves. The pre-junctional \( \alpha_2 \) receptors are inhibitory, and reduce the amount of transmitter released by the nerve. Blockade of these receptors by yohimbine has been shown to
increase the amount of circulating NA in rats (Remie and Zaagsma 1986). The inhibitory function of these receptors extends to other transmitters released from sympathetic nerves. In the rabbit mesenteric circulation, inhibition of the pre-junctional $\alpha_2$-adrenoceptors increased the motor response in blood vessels, the dominant component of which is purinergic (Bulloch and Starke 1990).

The role of the facilitatory $\beta_2$ receptors is hard to define, as demonstration of this effect requires prior blockade of the $\alpha_2$ auto-inhibitory effect, and $\beta$-adrenoceptor antagonists do not reduce transmitter output from in vitro vascular preparations (Wilson and Dunn 1996). It has been shown that adrenaline enhanced neurogenic responses in the dog mesenteric artery, without affecting the response to exogenous NA. This response was sensitive to $\beta_2$-adrenoceptor blockade. It may be therefore that the pre-junctional $\beta_2$-receptors are activated by circulating adrenaline (Borkowski et al. 1989).

The main post-junctional effect of NA released from perivascular nerves is vasoconstriction, mediated by $\alpha_1$, and often $\alpha_2$, receptors. The two types of receptor are often present on the same post-junctional membrane, and may act synergistically (McGrath et al. 1989). Post-junctional interactions between the two receptor types can give confusing results during receptor blockade experiments, as can the absence of humoral agents normally present in vivo. In the rabbit saphenous artery, it was found that NA mediated a contractile response via post-junctional $\alpha_1$ receptors. These receptors also facilitated a contractile response by post-junctional $\alpha_2$ receptors, but both were sensitive to $\alpha_1$ adrenoceptor antagonists, such as prazosin. On removal of the influence of $\alpha_1$ receptors, the presence of tachyphylactic concentrations of angiotensin II provided the positive influence necessary for the
expression of the contractile response of the $\alpha_2$ receptor, leading to a prazosin-resistant contraction (Dunn et al. 1989).

Receptors of the $\alpha_2$ sub-type have been found more consistently in veins than in arteries (De Mey and Vanhoutte 1981), although there are wide intra-species variations. Unequivocal demonstration of $\alpha_2$-adrenoceptor was first shown in the canine saphenous vein (Constantine et al. 1982). In most cases $\alpha_2$ receptors have been shown to coexist with $\alpha_1$ receptors, and demonstration of the $\alpha_2$ receptor has required blockade of the $\alpha_1$ with an antagonist such as prazosin. Some venous preparations have been shown not to possess functional $\alpha_2$ receptors (Dunn et al. 1991).

In arterial preparations, $\alpha_2$ receptors have been demonstrated most reliably in smaller vessels (Parkinson et al. 1992). Again, there are species differences. Human and porcine mesenteric resistance vessels have been found to possess post-junctional $\alpha_2$ receptors, whereas those of the rat and the rabbit do not (Nielsen et al. 1991). The location of these receptors on resistance vessels indicates that they have an important role in the control of blood flow and blood pressure.

As well as the $\alpha$-adrenoceptors, $\beta$-adrenoceptors are found post-junctionally in some vascular beds. Sympathetic adrenergic stimulation of these receptors leads to vasodilatation in some vessels, such as rabbit facial veins (Pegram et al. 1976), equine digital veins (Elliott and Soydan 1995) and feline pulmonary vessels (Hyman et al. 1981). However, some of these responses only occur after $\alpha$-adrenoceptor blockade, which raises doubts over their physiological role. The role of the endothelium in these cases was variable. In some vascular beds the vasodilator
response was endothelium-dependent (Hempelmann and Ziegler 1993). However, a study of the dog coronary artery found that coronary artery vasodilatation was due to direct stimulation of the \( \beta_1 \) receptors on smooth muscle, and not due to endothelial \( \beta_2 \) receptors (Macdonald, PS et al. 1987).

Both \( \beta_1 \) (Najafipour and Ferrell 1993) and \( \beta_2 \) (Kazanietz et al. 1989) receptors have been identified in these neurogenic relaxations. There is evidence that the \( \beta_2 \) receptors are stimulated by adrenaline, which is taken up from the circulation by sympathetic nerves and released on depolarisation of the nerves (Berecek and Brody 1982; Diana et al. 1990). The adrenaline in this case is acting as a 'false neurotransmitter'. It has been suggested that there is a functional interaction between the vasoconstrictor response of the vessels to \( \alpha_2 \) adrenoceptors and the vasodilatory response to \( \beta_2 \) receptors to control vascular tone in situations where the circulating level of adrenaline is elevated (Kazanietz et al. 1989).

Adenosine 5'-triphosphate (ATP)

ATP is a co-transmitter in many sympathetic nerves (Burnstock and Sneddon 1985). It is synthesised in the mitochondria and incorporated into small and large dense-cored vesicles along with NA (Franco-Cereceda 1996). ATP is also present in the cytosol and used for energy metabolism, but the transmitter released from the nerve on stimulation is only secreted from storage vesicles. There is a great deal of inter-species variability in the contribution made by ATP to sympathetic transmission, and within species there is variability between blood vessels in different vascular beds (Burnstock and Ralevic 1994).
Purinergic receptors were initially divided into two types, P₁ and P₂. Adenosine is the most potent P₁ agonist of the receptors, and ATP of the P₂ receptors. The P₁ receptors have since been subdivided into A₁, A₂ and A₃ subtypes (Tucker and Linden 1993; Ohana et al. 2001). Numerous sub-types of P₂ receptors have been identified (Kennedy 2000), the most important of which, in blood vessels, are the P₂ₓ and P₂ᵧ receptors. P₂ₓ stimulation results in contraction of vascular smooth muscle, P₂ᵧ and A₂ receptors cause vasodilatation, and pre-junctional A₁ receptors attenuate transmitter release (Franco-Cereceda 1996). As previously mentioned, P₂ᵧ receptors are present on endothelial cells and cause smooth muscle relaxation by release of NO.

The observed contractile effects of ATP and NA vary according to the stimulation parameters applied. The release of ATP is often favoured by low stimulation frequencies of short duration (Kennedy et al. 1986), whereas prolonged stimulation favours NA release. The released ATP results in a brief, rapid response, whilst the noradrenergic component of the contraction is slower and more sustained (Sneddon and Burnstock 1984; Sjöblom-Widfeldt 1990). The significance of cotransmission in these nerves may be that a much wider range of responses of the blood vessels is possible, allowing greater functional complexity and ‘fine tuning’ of responses.

Neuropeptide Y

NPY is the most frequent peptide transmitter in the sympathetic nervous system. It is synthesised in the nerve cell body, stored in large dense-cored vesicles and transported to the peripheral nerve terminal by axonal transport. NPY-positive neurones have been localised in sympathetic ganglia, and there are high concentrations of NPY in the cardiovascular system (Franco-Cereceda 1996). Many
blood vessels are densely innervated by NPY-positive fibres, where it is generally co-stored with NA (Ekblad et al. 1984). Five subtypes of NPY receptor have been recognised to date (Jackerott and Larsson 1997). The Y1 receptor is located post-junctionally and causes vasoconstriction, whilst the Y2 receptor is located pre-synaptically and inhibits NA release.

NPY has both direct stimulatory actions and indirect neuromodulatory actions on vascular smooth muscle. In some preparations it has profound vasoconstrictor effects, but there is wide variability between species, between different vascular beds within the same species, and between the arteries and veins in the same vascular bed (Edvinsson et al. 1984; Ekblad et al. 1984; Lundberg et al. 1985b). The contraction evoked is characteristic of peptides, namely a slowly developing and long-lasting response. In many preparations NPY elicits a contraction only at high concentrations, but enhances responses to NA at much lower levels (Lundberg et al. 1985b). This neuromodulatory effect has also been shown in post-junctional constrictor responses to ATP (Saville et al. 1990). It appears that the main function of NPY is neuromodulation, reducing the release of NA and ATP pre-junctionally, and post-junctionally enhancing their actions (Burnstock and Ralevic 1994). In vivo experiments show profound pressor effects of NPY. Experiments on human forearm vessels showed direct vasoconstrictor effects of NPY which were independent of noradrenergic α1 receptors (Pernow, J et al. 1987; Clarke et al. 1991). NPY has also been found to inhibit the vasodilator response to acetylcholine and SP, and may have a role in opposing vasodilatation (Fallgren et al. 1989).
Serotonin

Serotonin (5-HT) has been identified in sympathetic nerves in a number of sites (Gale and Cowen 1988; Chang et al. 1989). It appears that 5-HT in some sympathetic nerves is a 'false neurotransmitter', as it is taken up and stored by the nerves, and released upon stimulation, but not manufactured by the nerves (Saito and Lee 1987; Jackowski et al. 1989). Some sympathetic neurones are able to synthesise 5-HT in tissue culture, so this feature remains unresolved (Sah and Matsumoto 1987). 5-HT can have both vasoconstrictor or vasodilator actions (Vanhoutte 1987). The direct stimulation of S₂ serotinergic receptors leads to constriction of vascular smooth muscle (Arneklo-Nobin et al. 1985). 5-HT can also act as a neuromodulator, enhancing the actions of NA and acting pre-junctionally on S₁ receptors to reduce the release of NA (Shepherd and Vanhoutte 1985). The endothelium also has an important role in the actions of 5-HT. Acting on S₁ receptors, 5-HT causes the release of NO (Vanhoutte 1987). The net action of 5-HT on the vessel wall thus depends upon the pre-existing vascular tone and the integrity of the endothelium, and probably the level of circulating 5-HT.

Parasympathetic nervous system

Acetylcholine

Acetylcholine is the classical transmitter of post-ganglionic parasympathetic nervous system. Previously, the parasympathetic nervous system was thought to innervate some vascular beds via cholinergic fibres, which acted on muscarinic receptors on smooth muscle, causing vasodilatation. This system was envisaged as being in antagonism to the vasoconstrictor sympathetic nervous system. The situation is now
known to be more complex, and the role of acetylcholine itself is being questioned in many situations (Bell 1996). Many of the vasodilatory mechanisms previously ascribed to acetylcholine are now known to be due to other mechanisms.

Post-ganglionic cholinergic nerves release acetylcholine, which act on muscarinic receptors, all of which are antagonised by atropine. There are five (Eglen and Watson 1996; Reever et al. 1997) sub-types of these receptors, of which three have been found to be involved in control of peripheral blood flow. M1 and M2 receptors have been identified on endothelial cells, and M3 blockade inhibited the endothelium-dependent vasodilatation of the rat mesenteric bed (Rubanyi 1991; Hendriks et al. 1992). Muscarinic receptors are found on vascular smooth muscle. In spite of this, a definite role for cholinergic parasympathetic nerves in blood vessels is difficult to identify. There is evidence of cholinergic innervation of blood vessels in skeletal muscle, especially in cats and dogs, which may allow vasodilatation of these vessels in anticipation of exercise, before metabolite build-up leads to vasodilatation. Some of this response is, however due to stimulation of β-adrenergic receptors by adrenaline. Cerebral and pial vessels of many species are supplied with cholinergic neurones that originate from the cranial parasympathetic ganglia, but their role in vasodilatation is unclear. In the salivary glands, there is good evidence of cholinergic parasympathetic innervation, but these nerves appear to control protein output from the gland, rather than control vasodilatation (Lundberg et al. 1984).

Other parasympathetic transmitters

Many of the effects of parasympathetic nerve stimulation are atropine-resistant, indicating the presence of other neurotransmitters. Some parasympathetic nerves
contain VIP as a co-transmitter. This was first shown in the salivary gland of the cat (Lundberg 1981). At low stimulation frequencies, ACh is released from parasympathetic nerves to increase salivary secretion and elicit some vasodilatation. At higher stimulation frequencies, co-released VIP produces marked atropine-resistant vasodilatation. It also acts as a neuromodulator to enhance the post-junctional effect of ACh on acinar cells, and pre-junctionally increases the release of ACh.

VIP is present in many neurones in the cranial parasympathetic ganglia, and in cerebral perivascular nerves. This has led to the extrapolation that VIP can be used as a marker for parasympathetic nerves in general, as the histological and ultrastructural methods of localising cholinergic nerves are somewhat unreliable (Bell 1996). However, many other cotransmitters have been identified within parasympathetic perivascular nerves, either with or without VIP. These include peptide histidine-isoleucine (PHI), NPY, NO, SP and CGRP. VIP also exists in nerve populations other than the parasympathetic system, either on its own or with other transmitters. For example, many nerves in the ENS contain VIP along with a variety of other transmitters. VIP has been identified in 'intestinofugal' nerves projecting from the gut to autonomic ganglia (Luckensmeyer and Keast 1996). It is clear that the presence of VIP alone is not necessarily indicative of innervation by the parasympathetic nervous system.

In general, in the areas where a parasympathetic perivascular innervation has been identified, its actions are to vasodilate the vessels, usually in antagonism to the sympathetic innervation. However, this mechanism is neither so widespread as previously thought, nor as straightforward. The sympathetic nervous system has
vasodilator mechanisms of its own, and the overall actions of the innervation on the vascular bed is dependent upon many other variables, such as the integrity of the endothelium and the presence of vasoactive substances in the circulation.

**Sensorimotor nerves**

Primary afferent nerves relay sensory information from the peripheral organs to the CNS. These nerves have their cell bodies in the dorsal root or cranial sensory ganglia and send fibres in both central and peripheral directions. The peripheral nerve endings may be receptors themselves or be connected to specialised sensory structures. In addition to their sensory function, it has been known for many years that stimulation of the peripheral stump of transected dorsal roots resulted in vasodilatation of the supplied skin areas. Only more recently has the ubiquity of this peripheral effect of 'antidromic vasodilatation' become apparent. The peripheral terminals of sensory nerves not only serve a sensory role but release neurotransmitters to take part in local effector mechanisms, including vasodilatation and immune responses (Holzer 1988). The classical 'triple response' of skin in response to mechanical or other stimulation is dependent upon an intact sensory nerve supply. It is unclear whether these responses occur via an 'axon reflex' mechanism, or whether the stimulated nerve endings release neurotransmitters themselves, independently of an axon potential. Whatever the process, this system, termed the 'sensorimotor' or 'sensory-effector' mechanism, is widely involved in the control of blood flow and other responses in peripheral tissues.
Substance P

Sensorimotor nerves have been found to contain a number of neuropeptides. The most frequent of the neuropeptides identified within these nerves are the tachykinins. These form a family of peptides, the most frequently identified of which is SP (Pernow, B 1985). This peptide is present in all peripheral nerves containing cutaneous or visceral afferents, but appears to be absent from primary afferent nerves of special sensory organs. In particular, SP occurs in the varicose terminals found in the walls of blood vessels, as well as the free nerve endings of epithelial surfaces and the smooth muscle of visceral organs (Holzer 1988).

Calcitonin gene-related peptide

CGRP is often identified as a co-transmitter with SP in primary afferent nerves (Lee et al. 1985). In some species, for example the guinea pig, all capsaicin-sensitive SP-containing nerves also contained CGRP (Wharton et al. 1986). There is also evidence for ATP as a co-transmitter in sensory nerves, and many other peptides have been identified as probable sensory co-transmitters (Holzer 1988).

The discovery that capsaicin, the ingredient that provides the heat in fruits of the genus Capsicum, is a selective sensory neurotoxin, was a major advance in the study of these nerves. At low doses, capsaicin stimulates the release of transmitters from these nerves, which is followed by desensitisation of the nerves (Holzer 1988). At high doses, much higher than those used for spicing food, capsaicin is neurotoxic to a proportion of sensory nerves. Its effect varies with the dose, the route of administration and the species and age of the animals. For example, systemic treatment of newborn rats leads to the destruction of the majority of SP
immunoreactive neurones in the dorsal horn of the spinal cord and the trigeminal ganglion, but not in any of the other central or peripheral ganglia containing SP, nor in the SP-containing nerves of the ENS (Buck and Burks 1986). A similar, but less dramatic effect is seen in adult rats treated with a similar dose. Studies using capsaicin to destroy sensory nerves selectively, have given valuable insights into the role of these nerves.

Effects of sensorimotor nerves

Cutaneous vasodilatation is the most obvious manifestation of the action of sensorimotor nerves. It is now known that similar vasodilator fibres are widespread throughout the body. The vasodilatation is often associated with an increase in vascular permeability, particularly in venules. Only a minor component of the vascular effects of irritants such as mustard oil and capsaicin are due to their direct actions on the tissues: most are due the effects of sensory neurones. This may be a direct effect of the neurotransmitters released from the nerves, but there is evidence that mast cell degranulation is responsible. Mast cells are closely apposed to many sensory nerve fibres (Stead et al. 1989), and SP can lead to their degranulation (Suzuki et al. 1995). Antihistamines can reduce the wheal produced in the triple response, although this is not always observed (Holzer 1988). Other agents can also result in the activation of sensorimotor nerves. Protons have been found to stimulate capsaicin-sensitive nerves via the same mechanism as capsaicin itself (the activation of ion channels) (Bevan, S and Geppetti 1994). This may result in a protective mechanism, whereby the build-up of metabolites actively dilates the vascular bed.

In the gut, capsaicin-sensitive nerves are involved in controlling mucosal blood flow (Gustaw et al. 1995). It has been suggested that an action of these nerves is to
protect the mucosa from ulcerogenic factors by increasing blood flow, thus 'flushing away' the back-diffusing hydrogen ions (Raybould and Mayer 1991; Guth 1992).

Sensorimotor nerves also have effects on non-vascular smooth muscle. The response may be contraction or relaxation, depending on the organ. In the bladder, tachykinins have been found to cause contraction (Maggi et al. 1991), and are probably involved in the micturition reflex. In the gut, capsaicin-sensitive neurons affect motility, and have been found to cause both contraction and relaxation (Mayer et al. 1990).

Transmitters released from sensorimotor nerves have immunologic effects. SP has been found to enhance the phagocytic effects of both polymorphonuclear leukocytes and macrophages, and is chemotactic for these cells. SP also stimulates the synthesis of immunoglobulins in lymphocytes. Other neuropeptides released from these nerves, such as VIP, bombesin and somatostatin, also have immunologic effects (Holzer 1988). The immunological and other effects of transmitters released from sensory nerves will be considered more fully below.

**Intrinsic nerves**

The intrinsic nerves of most organs were considered mainly post-ganglionic parasympathetic fibres, which had limited integration in small intramural ganglia, and were largely under the central control of the ANS. The ENS was known to have a much more complex structure, and was termed the 'third arm of the ANS' by Langley as early as 1927. It was then regarded as an exception. The ENS is largely autonomous, and most gut functions can continue with minimal apparent disruption after extrinsic denervation. This is demonstrated by small bowel transplantation, in which the extrinsic nerves are severed, and take months to partially re-innervate the
organ. The intrinsic nerves are maintained, and most of the functions of the gut are preserved with little disturbance (Sugitani et al. 1994). More recently, the importance of intrinsic nerves has been appreciated in many other organs, such as the bladder (Brading and Mostwin 1989), the airways (Dey et al. 1988; Masuko et al. 1991; Watson et al. 1993), the eye (Flugel et al. 1994) and the heart (Corr et al. 1990). The intrinsic nerves contain a wide variety of neurotransmitters and display a different chemical coding from the other classes of nerves. For example, CGRP has not been found in the intrinsic nerves of the ENS of the rat and guinea-pig although it is co-localised with SP in the sensorimotor nerves (Gibbins et al. 1985; Hirose et al. 1993). It is thus possible to identify intrinsic SP nerves by excluding those containing CGRP in colocalisation studies. It has been found that all intrinsic nerves in the guinea pig ENS contain SP or VIP, but not both (Llewellyn-Smith et al. 1988). On the other hand, SP and VIP are co-localised in the intrinsic nerves of the cat airways (Dey et al. 1988). NPY, nitric oxide synthase (NOS; a marker for neuronal nitric oxide) and 5-HT have been found in the intrinsic nerves of the heart (Hassall et al. 1992), whilst in the tongue, VIP, NOS and ACh are co-localised in intrinsic ganglia (Yoshida and Toda 1997).

Some of these neurones project to blood vessels, forming part of the perivascular plexus. In the gut, there are projections of the ENS to submucosal vessels, but the central extent of these nerves has not been identified. The intrinsic nerves of the heart and tongue have projections to their blood vessels (Corr et al. 1990; Yoshida and Toda 1997). Organs can thus exert some control over their own blood supply by active neural mechanisms, and not merely by the build-up of metabolites.
This variety of transmitters indicates a degree of complexity in the intrinsic innervation of many organs that has yet to be widely investigated. It appears that the intrinsic nerves of many, if not most, organs are capable of complex interactions and control many aspects of the function of these organs independently of the CNS.

Summary

Perivascular nerves derive not only from central projections of the ANS, but also from sensorimotor nerves and from intrinsic nerves. The combined influence of these nerves contributes to vascular tone and blood flow. The endothelium also plays a major role, both by responding to signals from the nerves and circulating agents, and to self-produced vasoactive substances. The integration of these actions is complex, involving afferent signals from the sensory nerves to the CNS. The relative contribution of each of these systems to the control of blood flow at any particular time must depend on a variety of factors, including the metabolic needs of the organ supplied, the overall activity of the organism and the presence of toxic metabolites or ischaemia.

1.1.4 Blood flow to the gut

1.1.4.1 Microcirculation of the gut

The microcirculation of the gut exhibits many specialised features. The architecture is similar in the small bowel and the colon, although the mucosal pattern differs. There are parallel circuits of capillaries supplying the muscle, the submucosa and the mucosa, with the control to each controlled by a different set of resistance vessels. After giving off a small vessel, which contributes to the serosal plexus of vessels, the vasa rectae pass obliquely through the muscularis. They occasionally give off a
small lateral branch to the muscularis before passing to the submucosa and forming a circumferential plexus of large vessels. From here, smaller branches pass to the mucosa and recurrently to the muscularis. The recurrent vessels form an inner muscle plexus from which vessels pass to supply the muscle. These anastomose with vessels from the serosal plexus, giving the muscle a dual supply. This may explain the relative resistance to ischaemia of the muscularis.

The vessels passing to the mucosa form a different pattern in the colon and the small bowel. In the small bowel, the vessels pass to the base of the mucosa, forming a basal mucosal plexus. From here, a single vessel passes up through the centre of the villus to reach its tip, before branching into several descending capillaries that form a network on the surface of the villus.

In the colon, the submucosal plexus sends vessels towards the mucosa, where they too form a basal mucosal plexus. Straight, parallel vessels pass up to the mucosa where the basal mucosal plexus forms a honeycomb network in the mucosa, surrounding the glands.

1.1.4.2 Special considerations

The gut is an immensely complex organ system with a number of functions, which require a highly specialised microcirculation. The three major functions of the gut are motility, secretion and absorption.

The contribution of changes in blood flow to secretory processes is unclear. It is thought that secretion is an active process involving the transportation of solutes. Shedding of epithelial cells may also play a part. In active transport, the rate of blood flow per se would not affect the rate of secretion. An increase in flow would
however be necessary to supply the metabolic needs of the mucosa, as well as
supplying the necessary electrolytes.

In the case of absorption, blood flow fulfils two requirements. The blood supplies
the nutrients to the absorptive cells to enable them to transport absorbed material
actively across the epithelium. The blood is also one of two transport mechanisms
for the absorbed materials (the other being the lymphatic system). The exact effect
of changes in blood flow is therefore difficult to ascertain, as a variation in flow may
alter absorption directly or as an indirect effect on cell metabolism.

The gastrointestinal tract in man receives 15 - 20% of resting cardiac output. This is
about half that of the brain and heart, but considerably greater than that of the
skeletal muscle. The small bowel has a higher requirement than the large bowel,
which receive 40 and 20 ml min\(^{-1}\) 100g\(^{-1}\) respectively. This can, however increase
greatly. In the cat, it has been shown that the blood flow can increase to 200-250 ml
min\(^{-1}\) 100g\(^{-1}\) after a vasodilator has been given, whilst blood flows in the human gut
have been estimated at 150-200 ml min\(^{-1}\) 100g\(^{-1}\) in fulminating ulcerative colitis.

Blood flow is not homogeneous throughout the gut wall. The metabolic
requirements of the smooth muscle of the gut wall are lower than those of the
mucosa. The mucosa therefore receives 2 - 4 times the flow to the muscle, per unit
weight of tissue. There are a number of reasons for this. The specialised functions
of the mucosa mean that the blood flow required is not merely a reflection of the
metabolic requirements of the tissue. Whilst secretion and absorption are
metabolically demanding, the blood also supplies fluid for secretion and as a vehicle
for absorption. The mucosa therefore receives more blood than its metabolic
requirements demand, as is the case in the renal cortex.
One feature of the splanchnic circulation is that the veins are valveless. Blood can theoretically flow in either direction, and changes in portal pressure are transferred directly to the gut. Tissue pressure in the gut can thus be influenced by events in the liver as well as the systemic circulation. Postcapillary resistance vessels may have a role in buffering these pressure changes.

The mucosal capillaries represent a huge surface area for exchange. It has been estimated that the total surface area of the villus capillaries is around $2\text{m}^2/100\text{g}^{-1}$ with a length of approximately $100\text{km}/100\text{g}^{-1}$. These compare with corresponding values for skeletal muscle of $0.7\text{m}^2$ and $28\text{km}/100\text{g}^{-1}$.

This vast area has the potential to pose problems if the capillary hydrostatic pressure is not kept within very narrow limits, as a small rise could result in the translocation of a large amount of fluid into the extravascular space. There are therefore mechanisms to keep the mean capillary pressure constant, despite variations in arterial blood pressure.

Autoregulation of blood flow is of great importance in the mesenteric circulation. Flow is maintained within perfusion pressures of 50 - 160mm Hg. This ability is less pronounced than in the brain or kidney, but greater than in skeletal muscle. It is unclear whether the stimulus for autoregulation is to maintain the blood flow or to maintain a constant capillary hydrostatic pressure.

The main autoregulatory vessels are the precapillary arterioles. Flow to the villi is better regulated than that to the intestine as a whole, indicating that the muscularis has less well-developed autoregulation.

Blood flow in the mucosa remains relatively constant despite a reduction in perfusion pressure to 30 - 40mm Hg. This is achieved by increasing the perfused
blood volume by increasing the number of perfused villi. The mean transit time through the hairpin vascular loops in the villi is also increased.

There are two theories as to the mechanism of autoregulation. The myogenic theory ascribes the constancy of blood flow to the properties of the vascular smooth muscle. An increase in the transmural pressure induces contraction of the vascular smooth muscle and vice versa. According to the other hypothesis, the accumulation of vasodilating metabolites leads to relaxation of the vascular smooth muscle. This appears to be the case in the cerebral and skeletal muscle circulations, but has not been shown in the mesenteric circulation. This may be because the resistance vessels are at some distance from the area of metabolism, unlike other vascular beds. A build-up of metabolites in the villi would not be able to influence the smooth muscle of the resistance vessels directly as they would have to diffuse too far to be of direct influence. There may be other signalling mechanisms, such as local nervous reflexes, that influence these vessels.

1.1.4.3 Capacitance function

The venules and veins beyond the postcapillary resistance vessels constitute the capacitance section of the circulation. The control of capacitance vessels is of importance to whole body and cardiac filling, and has little functional effect on the region drained by the veins. It was recognised in 1964 that the venous system was at least as reactive and well controlled as other elements in the circulation and had an important part to play in cardiovascular integration (Folkow and Mellander 1964). Changes in vascular capacitance form the basis of a rapidly mobilizable, centrally controlled intravascular fluid reserve, which is necessary for good pump performance in a closed flow circuit. The intestinal blood volume is 2-4 times
greater than that of skeletal muscle, i.e. between 5-10ml 100g\(^{-1}\), or 20% of total blood volume. It has been shown that the blood vessels in the muscle and subcutaneous tissues make very little contribution to the body's capacitance control (Hainsworth 1986). This leaves the blood within the capacitance vessels of the splanchnic circulation. These are much more distensible than veins elsewhere in the body, with a compliance of 10ml kg\(^{-1}\) kPa\(^{-1}\).

In some animals, it can be shown that a very fast reduction in the volume of the splanchnic vascular bed can occur, which is not dependent on changes in blood flow. In the dog, 40% of this can be attributed to the contraction of the spleen (Hainsworth 1986). The spleen in man has little, if any, contractility, but the intestinal veins are able to contract. It appears that changes in the tone of the splanchnic veins are responsible for changes in vascular compliance. These vessels are sensitive to low frequencies of sympathetic nerve activity and to stimulation of the carotid sinus.

The main function of the control of vascular capacitance is the regulation of cardiac output. Cardiac output is dependent upon the mean circulatory filling pressure. This is dependent upon the volume of blood in the circulation and the state of constriction of the capacitance vessels, as most of the blood is in the veins. The pressure of blood in the veins is not only dependent upon active constriction of the vessels.

Changes in blood flow (controlled by the resistance vessels) and external pressures can alter mean circulatory filling pressure and hence cardiac output.

It has been estimated that the total mobilizable blood pool available to the circulation from the contraction of the splanchnic capacitance vessels is of the order of 300 ml. This amount is probably of great significance in man. Humans regularly make changes in posture which result in the elevation of the heart to a level above
the feet greater than that of any other animal except the giraffe (Williams 1995). This results in a reduction of cardiac output of about one-third. Without a readily mobilizable pool of blood, postural hypotension would be a major problem.

1.1.4.4 Effects of ischaemia

Major vessel occlusion

Ischaemia due to occlusion of the large vessels supplying the gut is a clinical catastrophe with a high mortality. As described above, the main superior and inferior mesenteric arteries are effectively end arteries, with only minor potential anastomotic sites. In chronic ischaemia, these vessels to open to supply the requirements of the ischaemic gut, but acute occlusion of these vessels leads to death of the gut as the anastomoses are inadequate to supply the metabolic requirements of the gut.

The anatomy of the blood supply to the small bowel is such that ischaemia due to occlusion of the intermediate arteries is unlikely to lead to clinically apparent gut ischaemia. Division or occlusion of mesenteric vessels close to the gut leads to complete focal ischaemia of the segment of gut supplied, but occlusion more proximally has little effect because of the numerous anastomoses. However, partial occlusion of the vessels may lead to reduced perfusion pressure. The autoregulatory mechanisms described above provide a means to ensure constant flow in the face of varying perfusion pressures. However, these mechanisms may contribute to mucosal ischaemia at very low pressures.
**Mucosal ischaemia**

The anatomical arrangement of the blood vessels within the villus, described above, means that the direction of blood flow in the central artery of the villus is in the opposite direction to that of the subepithelial capillaries. This arrangement gives rise to a countercurrent exchange mechanism, which causes an osmotic gradient along the villus, with an osmolality of 700 - 800mOsm kg\(^{-1}\) H\(_2\)O at the tip. This creates a force for villous water absorption. However, since oxygen is a small lipophilic molecule, it diffuses easily from the central artery and is washed out by the subepithelial capillaries. This creates an oxygen gradient with a low \(PO_2\) at the tip of the villus.

In haemorrhagic shock, it has been shown that the intestinal mucosa of experimental animals exhibits mucosal ulceration. The reason for this appears to be a side effect of the autoregulatory mechanisms, which maintain a constant villous flow in the face of a fall in blood pressure.

Partial occlusion of the SMA to reduce perfusion pressure of the gut from 100mmHg to 40mmHg decreases total blood flow to between one half to one third of resting flow, whilst villous blood flow remains almost unchanged. In normal circumstances, mucosal blood flow is not homogeneous: some villi are well perfused whilst others are under-perfused. A decrease in perfusion pressure leads to relaxation of the precapillary sphincters, resulting in perfusion of most of the villi. Perfusion of a single villus thus oscillates at rest, whilst in hypotension it has a constant blood flow of low velocity (Lundgren 1989).

This leads to an increase in villous blood volume and mean transit time. At rest, mean plasma transit time in the mucosa is between 4-8 seconds. In hypotension,
mean transit time increases to 20-30s. The transit time in the central arteriole is about one-tenth the total mucosal transit time, about 2-3s. This allows much greater oxygen transfer between the ascending and descending limbs of the countercurrent exchange mechanism, leading to severe ischaemia at the tip of the villus. Prolonged hypotension can thus lead to mucosal ulceration.

There is evidence to support this mechanism for the ulceration observed in hypotension. Perfusing the gut lumen with isotonic saline equilibrated with oxygen prevented the appearance of mucosal ulcers, whilst saline equilibrated with nitrogen had no protective effect (Lundgren 1989).

This indicates that ischaemic damage can occur in the absence of vessel occlusion. In addition, breakdown of the mucosal barrier results in inflammation. The nature of gut luminal contents also modifies the pathological processes so a range of responses in the bowel wall may result.

1.1.5 Other roles for neurotransmitters

The most important effector tissue for neurotransmitters released by the perivascular nerves is the vascular smooth muscle. Many transmitters, especially the neuropeptides, also have effects on other tissues. Many substances previously recognised as endocrine or paracrine hormones have also been identified as neurotransmitters. These neurotransmitters often display actions other than their vasoactive ones, and this may indicate complex interactions with the vessel wall.

1.1.5.1 Trophic effects

The condition of 'denervation atrophy' in skeletal muscle is well recognised. Efferent denervation leads to atrophy of the muscle, associated with abnormal
excitability and increased sensitivity to circulating acetylcholine. The regeneration of the motor nerve results in recovery, indicating a trophic effect on the muscle.

Disorders of the ANS suggest that it too has trophic functions. The condition of 'reflex sympathetic dystrophy', or Sudeck's atrophy, is a painful condition, often precipitated by minor trauma or electrical injury, which is thought to be due to autonomic nervous dysfunction (Gordon 1996). The condition results in trophic changes to the skin, vasomotor instability in the affected limb and osteoporosis. This aspect of the ANS has only recently begun to be investigated, and the role of the various neurotransmitters has yet to be defined. Sensorimotor nerves may be involved (Berthelot et al. 1997).

The ANS is known to be involved in the normal development of the embryo via trophic substances released from the nerves. In the developing rat embryo, VIP or a related peptide regulates the proliferation of neuroblasts and glial cells (Waschek 1995), and the peptide promotes survival of sympathetic nerves prior to them reaching their target tissues, when target tissue interactions become more important (Pincus et al. 1994). ET is produced by post-ganglionic neurons and cells adjacent to the neurones. ET also promotes survival and differentiation of neurones in culture, and enhances the activity of nerve growth factor (NGF) (Damon 1999). There is two-way communication between the target organ and the nerves, which are also dependent upon substances, such as NGF, released by the target tissue for their correct growth.

It has been proposed that the term neurotropism should be restricted to long-term maintenance regulations that are not mediated by nerve impulses (Gutmann 1976). However, in noradrenergic, and probably other, nerves random quantal release of
NA goes on independently of nerve impulses. This release would be difficult to differentiate from release due to nerve impulses. It is also difficult to differentiate between trophic factors due to innervation, and the effect of stimulation itself causing growth in the target tissue (Folkow 1983). However, it is clear that some mature tissues are under trophic control of their nerves.

In the heart, the normal development of cardiac muscle is dependent upon an intact sympathetic nerve supply (Renick et al. 1997). It appears that ATP, not NA, is the transmitter responsible for this effect (Osswald 1991). Hypertrophy of the heart in spontaneously hypertensive rats has been proposed to be due to the hyperinnervation observed in these animals (Mangiarua and Lee 1990).

The ENS appears to control the growth of the intestinal mucosa. Destruction of the myenteric nerves leads to hypertrophy of the mucosa (Hadzijahic et al. 1993). The mechanism for this is not known.

It has been speculated that perivascular nerves may have trophic effects on the vessels themselves. The relative density of noradrenergic and peptide-containing nerves varies in the early development of the guinea pig. In the carotid and mesenteric arteries of the guinea-pig, the peptide containing nerves are at their most numerous at birth and decline thereafter, whilst the noradrenergic nerves reach their maximum density later in development (Dhall et al. 1986). This may indicate a trophic role for the peptide-containing nerves. There is evidence that the sensory innervation of blood vessels affects the expression of vasoactive substances by the endothelium (Milner and Burnstock 1996). Sensory denervation increases the sympathetic vasoconstriction in rat mesenteric arteries, an effect which is thought to be due to long-term trophic changes in the vessels (Ralevic et al. 1995). Much has
yet to be learned about the possible trophic effects of neurotransmitters, but these effects may explain the presence of nerves containing substances that appear to have little direct effect on the vascular smooth muscle.

1.1.5.2 Secretory and absorptive effects

One of the major advances in the study of the ANS has been the identification of known endocrine and paracrine hormones as neurotransmitters. Gastrin, secretin, cholecystokinin and somatostatin, for example, have all been identified as neurotransmitters. The concept of the neuroendocrine system has thus been developed whereby hormones affecting many metabolic functions are released by nerves under local or central nervous control. In many exocrine organs, such as the salivary glands and the pancreas, and in the gut, the secretion of fluid is under the control of the ANS. Somatostatin is found in endocrine D-cells in the pancreas and mucosa of the stomach, where it performs an endocrine role, whereas it is mainly localised to enteric nerves in the lower GIT. It is concerned with the control of secretion of other hormones in the GIT and of acid secretion in the stomach (McIntosh 1985). Tachykinins have been identified as secretagogues in nerves of the pancreas and salivary glands, usually as cotransmitters with acetylcholine. They act both by their vasodilator effects and by direct stimulation of protein secretion (Gallacher 1983; Pawlik et al. 1992). Projections of nitrergic nerves from the ENS to the pancreas have been identified which are involved in the secretion of amylase (Kirchgessner et al. 1994). Elsewhere in the gut, it has been found that the ENS controls the secretion of fluid by enterocytes. It is thought that the enterochromaffin cells of the mucosa are sensory cells, which are stimulated by certain luminal stimuli such as rubbing and distension, and act by releasing 5-HT. This then acts on enteric
nerves, which stimulate the enterocytes via a neural reflex (Hubel 1985).

Cholinergic tone increases the secretion of fluid, and there are numerous VIP receptors on the mucosa of dogs, which may indicate a role for this peptide in the control of secretion (Zimmerman et al. 1988). Neuroendocrine tumours secreting VIP cause severe diarrhoea, presumably due to this action. Secretomotor neurones containing NPY have been identified in the ENS of the guinea pig (Uemura et al. 1995); NPY has been found to inhibit ion secretion in vitro (Nguyen et al. 1990).

Intestinal absorption is also under nervous control. Extrinsic noradrenergic nerves increase absorption by acting both directly on enterocytes and on intrinsic nerves (Hubel et al. 1989), whereas cholinergic nerves decrease absorption of electrolytes in dogs, cats and man (Hubel 1985). Cholinergic nerves govern the absorption of macromolecules such as albumin in the rat jejunum (Kimm et al. 1994), and it appears that psychological and physical stresses can affect absorption via neural pathways (Barclay and Turnberg 1987, 1988). A wide spectrum of gastro-intestinal tract function is thus under control of the ANS. Damage to or abnormalities of these systems could have a range of clinical effects.

1.1.5.3 Neurally-mediated inflammation and immune responses

Interactions between the CNS and the immune systems have been postulated for many years. There is an obvious link between psychological and emotional stress and the susceptibility to disease, which has attracted a variety of explanations. The nervous control of secretion of glucocorticoids, with their well-known immunoinhibitory actions, provided one explanation of this phenomenon. However, this explanation is rather simplistic, and the evidence for a much more complex, bidirectional communication between the nervous and immune systems has been
accumulating over recent years. Recent developments include the recognition of the widespread distribution of neuropeptides within the ANS, and the presence of receptors for some of these peptides on cells of the immune system.

The non-specific pro-inflammatory actions of sensorimotor nerves have already been mentioned. The 'triple response' mediated by sensory nerves is an example of such a response. The noxious stimulation of sensory nerves results in reflex vasodilatation and an increase in vascular permeability, leading to increased blood flow and fluid extravasation (Pernow, B 1985; Holzer 1988). SP released by sensory nerves is also chemotactic to polymorphonuclear cells and macrophages, and enhances phagocytosis in these cells. SP is most commonly associated with neurogenic inflammation, but other transmitters may also be involved. CGRP, VIP, somatostatin and acetylcholine have all been implicated in these processes (Mayer et al. 1988). The responses are often mediated by capsaicin-sensitive, that is sensorimotor, nerves. In the gut, SP and VIP are found in large numbers of nerves in the ENS. This raises the possibility of the nerves being involved both in the inflammatory response and in the motility changes observed in inflammatory processes (Mayer et al. 1988).

Purines have a role in the control of inflammation. ATP is a co-transmitter in many nerves, and adenosine and ATP can be released by endothelial cells and neutrophils (Fredholm 1997). Neutrophils possess receptors for ATP and adenosine. The effect of these mediators is dependent upon the receptor types stimulated. ATP causes degranulation, leukocyte adhesion to the endothelium and potentiates the oxidative burst via P2 receptors, whereas adenosine antagonises these effects via A2A receptors. The effect of these substances is clearly defined by the receptors present
in the tissues, and it is of interest that there are reports of changes in the receptors present in chronically inflamed tissues. ATP has been shown to promote inflammatory events in vivo (Ziganshina et al. 1996), whilst adenosine has been identified as a potent endogenous anti-inflammatory agent (Le Moine et al. 1996).

NO is also involved in increased vascular permeability and leukocyte adherence and emigration, but it is likely that this NO is derived from inducible sources in the endothelium, and not from nerves (Kurose et al. 1994).

The pro-inflammatory responses proposed above are all non-specific processes. There is a large body of evidence of specific, two-way interactions between nerves and the immune system. Nerves have been identified associated with lymphoid tissue, and it has been found that mast cells are closely apposed to nerves in the gastrointestinal mucosa (Stead et al. 1989). A number of peptides have been identified as having immunomodulatory effects. The most widely studied mediators include SP, VIP and somatostatin. CGRP, NPY, cholecystokinin and the opioid peptides (leu- and met-enkephalin) may also regulate the immune response (Sirinek and O'Dorisio 1991). These interactions have been identified in many organs including skin, joints, the eye and the urogenital and respiratory tracts, as well as the gastrointestinal tract (Holzer 1988).

The gut-associated lymphoreticular tissues (GALT) constitute the largest single component of the mucosal immune system. They are separate from the peripheral immune system and regulated differently. There is extensive innervation of GALT both by nerves of the ENS and extrinsic nerves. Of the ‘peptidergic’ nerves, those containing VIP and SP are the most frequent. Both SP NK-1R and VIP receptors have been identified on lymphocytes (Pascual et al. 1994). Adrenergic and
peptidergic nerves have been identified in T-cell dependent areas of GALT, but are absent from B-cell follicular areas. It is thought that the failure to innervate areas populated by undifferentiated B-cells, although these cells bear SP receptors, is to prevent premature stimulation of these B-cells by SP (Pascual et al. 1994). The final differentiation of B-cells into IgA-producing plasma cells occurs in mucosal tissues that are heavily innervated by SP-containing nerves, and it is thought that SP plays a part in this process. SP-containing nerve fibres are seen in contact with perifollicular T-cells and antigen presenting cells, suggesting that SP may be directly involved in T-cell activation. SP treatment of CD4+ T cells releases IL-5, which induces IgA synthesis in B-cells. Other immune cells can be activated by SP. Macrophages can be induced by SP to produce increased levels of the inflammatory cytokines, TNF-α, IL-1 and IL-6, which may also promote IgA synthesis.

Peyer's patches and other areas of GALT are also innervated by nerve fibres containing VIP. VIP has been found to have negative regulatory functions in PP. It inhibits mitogen-induced proliferation of T-lymphocytes in mice, and decreases IgA production in PP cell cultures. These contrasting effects suggest opposing regulatory mechanisms for mucosal immune responses (Sirinek and O'Dorisio 1991; Pascual et al. 1994).

VIP containing nerves are the dominant type in the lamina propria of the bowel wall, and many of these nerve fibres follow blood vessels. Macrophages are also found in the lamina propria and may be involved in antigen presentation and processing at this site (Pascual et al. 1994). It has been suggested that the VIP nerves are involved in the suppression of the continual stimulation of the GI lymphoid system.
Somatostatin is a common transmitter in the ENS, occurring in the myenteric and submucosal plexuses, as well as in short mucosal neurones. Receptors to this peptide have been found in T- and B-lymphocytes, as well as on macrophages. Its effects are largely inhibitory, suppressing the stimulated proliferation of lymphocytes, and inhibiting the production of immunoglobulin (Ottaway 1991; Sirinek and O'Dorisio 1991). The immunomodulatory actions of other neuropeptides are only beginning to be investigated, and results to date are rather contradictory. Nerve fibres containing CGRP, NPY, the enkephalins and CCK have all been identified in immune tissues, and there have been reports of variable effects of the peptides on immune cells (Sirinek and O'Dorisio 1991).

Reports of the production of neuropeptides by lymphoid cells raise the possibility of two-way communication between the two systems. VIP and somatostatin have been isolated from mast cells, human eosinophils and human mononuclear cells (Ottaway 1991; Leceta et al. 1996). In the rat, all lymphoid tissue contains a novel VIP-related neuropeptide, pituitary adenylate cyclase-activating peptide (PACAP). Granulomas in murine schistosomiasis have been shown to generate SP, VIP and somatostatin (Weinstock and Blum 1990; Weinstock et al. 1990). These findings, coupled to the presence of receptors for the peptides on the cells and the proximity of nerves to the lymphoid cells, indicates an interaction between nerves and lymphoid tissue.

Interleukin-1, a cytokine released during the inflammatory response, stimulates the synthesis of neurotrophic factors including NGF (Creange et al. 1997). NGF has many physiological actions. It is vital for the normal growth and development of sympathetic and small fibre sensory neurones (Apfel 2000). In the mature animal,
target tissues produce NGF to maintain their nerve supply (Di Mola et al. 2000). NGF also has effects on tissues of the immune system, including spleen, thymus, T- and B-lymphocytes, eosinophils and mast cells (Aloe et al. 1997). Immature mast cells have been shown to mature in vitro in the presence of NGF (Welker et al. 1998). Interactions have also been identified with sensory neurones. In skin inflammation induced by exogenous SP or mustard oil, an irritant that produces neurogenic inflammation, caused an increase in the level of NGF in the tissues. This effect was antagonised by treatment of the rats with the tachykinin NK-1 receptor antagonist SR140333 (Amann et al. 2000). This indicates a two-way interaction between neurones and their target tissues via NGF. Chronic inflammation in skeletal muscle caused by injection of Freund’s adjuvant gave rise to an increase in the number of nerves displaying SP-LI and NGF-LI (Reinert et al. 1998). Nerves with GAP-43-LI also increased in number, indicating axonal sprouting, regeneration and synaptic reorganisation.

The role of higher neural centres in these interactions is an interesting area of research. Circulating levels of NGF are elevated in animals and humans under stress, and appears to be part of the neurogenic response to a number of environmental and psychological stressors (Aloe et al. 1997). In a study to investigate the neuroimmune axis, sensitised rats were exposed repeatedly to an enteritis-producing antigen paired to a pattern of audio-visual signals to provide a conditioning stimulus. The release of a specific protease produced by mucosal mast cells was measured to assess mucosal activation. After conditioning, exposure to the audio-visual stimulus alone, in the absence of the antigen, resulted in mast-cell activation. This indicates the possibility of environmental stressors having an effect
on mucosal immune responses via the neurohumoral-immune axis (MacQueen et al. 1989), and has interesting implications for a number of pathological conditions.
1.2 *Inflammatory bowel disease*

Inflammatory bowel disease (IBD) is the term given to a group of diseases of unknown origin, characterised by inflammation of the gut. There are three recognisable groups, ulcerative colitis (UC), Crohn's disease (CD) and the group known as indeterminate colitis, which includes those cases that do not fit into the other two categories. This classification excludes other inflammatory diseases of the bowel for which the cause is known, such as ischaemic and infective colitis.

1.2.1 *Ulcerative colitis*

Ulcerative colitis was first described by Wilks and Moxon in 1875 (de Dombal et al. 1993), although case descriptions may even go back to Roman times. They described a condition that was idiopathic and involved the rectum and the colon. The mucosa was predominantly affected, and the inflammation was continuous. They recognised that there was a distinction between this condition and other ulcerating colonic conditions common at that time, such as tuberculosis and dysentery.

Ulcerative proctocolitis is defined as an inflammatory disease of unknown origin, characterised by recurrent attacks of bloody diarrhoea, and pathologically by diffuse inflammation of the wall of the large bowel. The inflammatory changes spread proximally from the rectum; and are confined to (or are most severe) in the colonic and rectal mucosa (Myren 1986). Some cases are restricted to the rectum; these cases of proctitis are regarded as the same disease as proctocolitis.
1.2.1.1 Clinical features

UC mainly affects young adults with an equal sex distribution. The annual incidence is around 10 per 100,000 population and has changed little over the last 30 years (Nicholls 1998).

The disease is characterised by episodes of diarrhoea with the passage of blood per rectum. In proctitis, bleeding and mucus secretion are the most frequent features, usually accompanied by an increased frequency of defaecation. There is usually also some degree of urgency. Systemic features, such as malaise, anorexia and weight loss vary greatly in degree, but are usually mild in proctitis.

In more extensive disease, there is a worsening of the symptoms of frequency and urgency. Severe attacks are characterised by debilitating urgency; sometimes urge incontinence can occur. Urgency is often the dominant symptom in these cases. Severe colitis also leads to significant fluid, blood and protein losses. This results in malnutrition and anaemia, and may cause growth retardation in children. Acute fluid and electrolyte losses can lead to hypovolaemia and collapse.

The course of the disease is usually chronic and relapsing. Acute episodes are usually interspersed by periods of complete resolution. Some cases are probably mild and self-limiting, but a few run a severe, continuous course. Fulminating and sometimes fatal forms are not rare.

1.2.1.2 Macroscopic appearance

Anatomically, UC is always restricted to the large bowel. The rectum is always affected, although local treatment can lead to apparent rectal sparing. The disease is diffuse, without intervening segments of unaffected mucosa. Rectal sparing in the
absence of local treatment should raise the suspicion of CD. At presentation, approximately 50% of cases have disease confined to the rectum (proctitis). In 30%, the disease extends to the left colon (proctosigmoiditis); in the remainder, the disease extends beyond the splenic flexure (extensive colitis). There is a sharp demarcation at the ileum, although in some cases of total colitis there is sometimes some ileal inflammation - so-called ‘backwash ileitis’.

Early UC gives a velvety, congested appearance to the mucosa. There is loss of the normal vascular pattern, petechial haemorrhages and bleeding. This is followed by punctate erosions, which coalesce into ulcers in more severe disease. This stage is associated with extensive muco-purulent discharge and haemorrhage. The remaining mucosa between the ulcers becomes swollen and hyperplastic, giving rise to pseudo-polyposis. Between exacerbations, there is healing of the mucosa, and fibrous stenosis of the bowel is rare.

1.2.1.3 Microscopic appearance

The disease is confined to the mucosa except in fulminant colitis when the inflammatory process may extend into the muscularis propria. During active inflammation, the mucosa is congested and infiltrated with leukocytes, mainly plasma cells. There is goblet cell depletion. Crypt abscesses, characterised by the accumulation of neutrophils, eosinophils, erythrocytes and mucus within the crypt lumina, are conspicuous, but not specific for UC. The crypt lining breaks down, leading to the formation of frank ulcers. Ulceration seldom extends more deeply than the submucosa. The pseudopolyps consist of islands of surviving hyperplastic mucosa.
During remission, surface re-epithelialisation takes place, but there are few glands within the healed mucosa. In long-standing cases, dysplasia of the mucosa is almost invariable, and carcinoma of the colon is common. This complication is most common in those with total colitis, especially when the duration of the disease is greater than ten years. It is usually difficult to identify the areas of dysplasia visually, but they may be recognisable endoscopically as raised plaques.

1.2.1.4 Local Complications

The most urgent of these is perforation of the colon, which is usually associated with pronounced toxic dilatation of the colon. About 5-10% of patients present with acute severe colitis, in which severe local symptoms are associated with fluid and electrolyte disturbance. Aggressive medical management usually leads to remission, but 30-40% of these patients will require urgent surgery. In some cases severe colitis progresses to toxic dilatation, confirmed by colonic diameter of greater than 8cm on a plain abdominal radiograph. Abdominal distension associated with localised tenderness raises the suspicion of perforation, the signs of which can be masked by treatment with high-dose steroids. This complication carries a mortality of over 40%.

Perianal disease is less common than in CD, occurring in 10% of patients with severe disease requiring proctocolectomy. The lesions are usually minor, such as low fistulae or fissures.

Bleeding requiring emergency surgery is a rare complication of UC. The source of bleeding is often rectal ulceration and emergency proctocolectomy may be required, leaving the anus and a short cuff of rectum for future reconstruction.
Carcinoma of the colon is the most serious long-term complication. Duration and extent of disease are recognised as independent risk factors. There is a 20-fold higher incidence in severe, extensive UC, with an average age of onset 20 years younger than in the general population (Harpaz and Talbot 1996). In contrast to sporadic cancer, its precursor is flat mucosal dysplasia; endoscopic surveillance for dysplasia is used as a means to reduce cancer mortality. However, dysplastic epithelium is often not colonoscopically distinguishable from non-dysplastic epithelium, and despite surveillance, there is still a significant incidence of invasive carcinoma in these patients. Techniques such as dye spraying are being tested to identify early mucosal changes. Prophylactic colectomy with restorative proctocolectomy is sometimes advised in those with extensive disease. Unequivocal dysplasia on biopsy is associated with a high risk of coexistent or future colorectal cancer, and surgery is usually recommended in these cases (Willenbucher 1996).

1.2.1.5 Extra-alimentary manifestations

Arthopathy is the commonest extra-alimentary manifestation. Activity-related polyarthropathy occurs in up to 20% of patients and is more likely in those with extensive disease. It affects mainly the large joints of the limbs and is rheumatoid factor negative. It disappears when a remission is induced with medical treatment or by proctocolectomy.

Ankylosing spondylitis occurs in up to 5% of patients, the majority of whom are HLA B27 positive. In addition, isolated sacroileitis can occur in HLA B27 negative subjects. These conditions are unaffected by treatment of the underlying colitis.
1.2.2 Crohn’s disease

Dr Burril B Crohn is credited with the first description of the eponymous disease in 1932. However, descriptions of a disease similar to tuberculosis of the bowel, but in which acid-fast bacilli could not be isolated, had been made by several authors decades before. Dalziel, in 1913, described several patients with chronic intestinal enteritis that, although very similar to intestinal tuberculosis, he believed to be a new disorder.

Crohn’s disease is an idiopathic, chronic disease that affects any part of the alimentary canal. It most commonly affects the gut and may be ileal, ileocolic or colonic. ‘Skip lesions’ are common, with areas of normal mucosa between inflamed areas. Perianal disease may coexist with any of these patterns. There may be associated with extra-intestinal manifestations. Lesions are focal and transmural, and characterised histopathologically by granulomata or giant cells. Long remissions may occur. There is a recurrence rate of about 50% within 20 years of operation.

1.2.2.1 Clinical features

The condition affects young adults of both sexes, with an annual incidence of 5-6 per 100,000 in northern Europe, Scandinavia and North America. There are wide regional differences, with a very low rate in Japan. Studies of migrant populations indicate an environmental effect, with British Asian and West Indian populations having a higher rate of the disease than their home populations.

The symptoms are usually ill defined at first, with mild diarrhoea and vague abdominal pain the usual complaints. Unlike UC, an acute first presentation is
unusual, although ileal disease can present like acute appendicitis, and colonic disease as a fulminating colitis. Diarrhoea may result from mucosal inflammation, entero-enteric fistulas, short bowel due to previous resections, bacterial overgrowth in obstructed bowel and bile salt malabsorption from terminal ileal disease. Chronic intestinal obstruction may develop due to fibrotic narrowing of a segment of small bowel. Rectal bleeding is uncommon in small bowel disease, but is a feature of colonic disease. Proctitis leading to decreased rectal compliance causes tenesmus and increased bowel frequency. Perianal disease is relatively common, often presenting with fissures or complex fistulas, which may extend into neighbouring organs.

1.2.2.2 Macroscopic appearance

Crohn’s disease is segmental and can affect any part of the gastro-intestinal tract. There is a predilection for the ileocaecal region. There is no demarcation between the small and large intestine, and the colon may be affected primarily. Segmental lesions are discontinuous - so-called ‘skip lesions’.

The earliest macroscopic lesions in CD are multiple aphthous ulcers that develop on the surface of submucosal lymphoid nodules. In more advanced disease, there is intense oedematous thickening of the bowel wall, with extension of the mesenteric fat onto the bowel, known as fat wrapping. Often, there is marked narrowing of the lumen. This may be sufficient to cause obstruction, with dilatation of the proximal bowel. Several strictures may be present, with multiple dilated segments in between. Linear ulcers of the oedematous mucosa are characteristic, producing the typical ‘cobblestone’ appearance. Fissures penetrate the entire bowel wall and may
extend into adjacent structures, producing fistulas. The mesentery is thickened and oedematous, and the regional lymph nodes are usually enlarged.

1.2.2.3 Microscopic appearance

The initial lesion in CD is in the lymphoid follicles and Peyer’s patches, in distinction from UC. Hyperplasia of these is followed by the aphthoid ulceration typical of early Crohn’s. In more advanced cases, inflammation extends throughout the entire thickness of the bowel wall. The most constant microscopic change is a focal lymphocytic infiltrate, usually most prominent in the submucosa. In about 60% of cases epithelioid-cell granulomas occur. These are similar to the granulomas seen in sarcoidosis and are of great diagnostic value. Granulomas are usually a non-specific reaction to an insoluble antigen. Patients with high numbers of granulomas have a better prognosis, which has led to speculation that the causative agent is being removed in these patients (de Dombal et al. 1993). The observation has also led to speculation that there may be two forms of CD, a granulomatous and a non-granulomatous form. The identification of the initial lesion in the lymphoid tissue has led to the theory that CD is a primary disorder of lymphoid tissue, which would account for its predilection for the terminal ileum and anus.

There is always marked oedema in the initial stages of the disease, most conspicuous in the submucosa. Fibrosis becomes increasingly important in later stages of the disease. In addition to the ulceration, the mucosa displays pyloric metaplasia, which may be due to secondary infection of the lesions. The fissures arise from the ulcer bases and are lined by granulation tissue and foci of suppuration caused by secondary infection.
1.2.2.4 Local Complications

Stenosis and obstruction are common complications. If due to active disease, medical treatment with steroids can be effective in relieving the obstruction. As the disease progresses, the strictures become fibrotic. Medical therapy will not affect this type of stricture, and surgery is necessary.

Fistula formation may occur into adjacent loops of bowel, other hollow viscera or the skin. Perianal sepsis and fistulae are quite common, and may be the presenting feature. This is commoner in colonic disease, but not exclusive to it.

Carcinoma is also a problem in CD, especially in extensive colonic involvement (Harpaz and Talbot 1996; Willenbucher 1996). As in UC, patients with extensive colonic disease should undergo regular surveillance colonoscopy.

1.2.2.5 Systemic Complications

Weight loss is a common symptom. There are several reasons for this, including anorexia, food-fear, and diarrhoea. Malabsorption is a less common cause. Malabsorption is sometimes due to the inflammatory disease itself, but more often bacterial overgrowth in blind loops, areas of chronically obstructed bowel or from coloenteric fistulae. Iron-deficiency anaemia is common, and macrocytic anaemia may occur due to interference with vitamin B₁₂ absorption. Other features of malabsorption syndrome may occur, especially in diffuse ileo-jejunal disease.

1.2.2.6 Extra-alimentary Complications

As in UC, some of the extra-intestinal manifestations can be related to disease activity whilst others are not. In general, they are more common in Crohn’s colitis than in small bowel disease, and are similar to those seen in UC (Thompson-Fawcett
and Mortensen 1998). They can precede or herald active inflammatory disease. Acute arthritis, uveitis and skin lesions such as erythema nodosum are the most frequently seen manifestations that are related to disease activity. A condition known as metastatic CD occurs rarely. This presents as nodular ulcerating skin lesions at sites including the vulva, limbs and sub mammary areas. Non-caseating granulomas are present in biopsies. Treatment of the disease results in the resolution of these features.

Sacroileitis and ankylosing spondylitis occur in 10-15% of patients. These conditions are unrelated to disease activity. Liver disease, such as primary sclerosing cholangitis, is rare, although minor abnormalities of liver function are common in acute attacks. Gallstones are common (15-30%) due to malabsorption of bile salts in the terminal ileum.

Amyloid is a rare but serious condition. It is associated with long-standing low-grade inflammation and causes organ damage due to amyloid deposition in the kidneys and other organs. It is reported in 25% of patients at post-mortem, but only about 1% have clinical manifestations (Thompson-Fawcett and Mortensen 1998). Treatment of the inflammation prevents progression, but organ damage is usually permanent.

1.2.3 Indeterminate colitis

The differentiation between ulcerative and Crohn’s colitis is based on histological criteria. In some cases of idiopathic colitis, insufficient of the diagnostic attributes are present to allow a firm diagnosis to be made. Sometimes macroscopic or microscopic features of the disease indicate the possibility of CD or UC, and this is indicated by the pathologist, as further evidence one way or the other may be
recognised in due course. A number of cases remain unclassifiable, are termed indeterminate colitis. The proportion of cases with this condition varies from series to series, but is estimated to be between 3-15% (de Dombal et al. 1993). It is not clear whether indeterminate colitis represents a distinct disease entity, or whether the label merely represents an inability to make a firm diagnosis. In general the condition tends to behave more like CD than UC (Nicholls 1998).

1.2.4 Aetiology of IBD

The aetiology of IBD is, by definition, unknown. Both UC and CD are multifactorial diseases. The available data suggest that UC and CD are heterogeneous disorders of multifactorial aetiology in which hereditary and environmental factors interact to produce the disease. The gut may be in a constant state of controlled inflammation, and it is probable that the mechanisms controlling this are disturbed in IBD. There are likely to be a number of environmental factors, which, acting on genetically predisposed individuals, give rise to the diseases (Jarnerot 1996). A wide variety of initial injuries or perturbations of immunoregulatory pathways can lead to similar phenotypes of intestinal injury (Sartor 1995).

Environmental factors exerting a primary effect at an early age may cause the disease to manifest itself in teenagers or young adults. Secondary environmental factors may alter disease expression, severity or influence relapse (Whelan 1990). The inflammation which sometimes occurs as a complication in continent ileo-anal pouches, pouchitis, can be thought of as a human model of IBD, and unravelling the aetiology of this condition may improve our understanding of IBDs in general (Mignon et al. 1995).
The epidemiology of IBD is interesting, as although there is a great deal of difference in the epidemiology of IBD among populations, there is little difference within these populations between CD and UC. There are notable exceptions, such as cigarette smoking, which is commoner in patients with CD, but appears to be protective for UC. However, with these exceptions, it is possible that CD and UC are different expressions of the same disease (Lashner 1995).

1.2.4.1 Genetic factors

There is a familial component in the aetiology of both CD and UC, which is more marked for CD. Between 10 and 20% of affected individuals have a first-degree relative with IBD, their relative risk being 10 to 20-fold (Satsangi et al. 1994). The characteristic genotypes for CD and UC are different: that of CD is more complex (de Dombal et al. 1993). In UC there is a higher incidence among family members, particularly in first-degree relatives (Griffel and Das 1996). Identical twin studies provide the best evidence for the role of genetic factors in the aetiology of IBD. In CD, there is disease concordance in monozygotic twins of nearly 50 (Tysk et al. 1988). It is likely that the chronicity of the disease depends on continued exposure to toxic luminal components and genetically determined host susceptibility. The mechanisms of this are unclear, but defective down-regulation of inflammation may be one factor (Sartor 1995).

Ethnic differences in incidence are seen, but it is difficult to separate genetic from environmental factors. There is a higher rate of IBD in whites than in black or Arab populations, but IBD is less common in underdeveloped countries in general, with evidence of an increasing incidence with increasing wealth. This points to
environmental factors being responsible for at least some of the observed differences (Tysk et al. 1988).

1.2.4.2 Environmental factors

The role of the environment is difficult to assess. Epidemiological clues mentioned above indicate that there is interplay between environmental and genetic factors. Studies of migrant populations provide evidence of an environmental influence, with West Indian and Asian immigrants in the UK having a higher incidence than in their home country. Of the external, environmental factors, cigarette smoking is the most intriguing. There is an association between non-smoking and UC; and smoking and CD (Cope et al. 1987; Osborne and Stansby 1992; Koutroubakis et al. 1996). Other factors for which associations have been found include oral contraceptives, refined sugar, perinatal events, childhood infections, microbial agents and domestic hygiene. Further evaluation of these is necessary to define the strengths of the associations. Measles virus has also been implicated, as it persists in intestinal tissue; early exposure to the virus may be a risk factor in the development of CD (Koutroubakis et al. 1996). The role of xenobiotic substances producing reactive metabolites in the liver, and damaging both the liver and colonic mucosa in UC has recently been questioned, and needs to be investigated further (Crotty 1994).

1.2.4.3 Psychological Factors

There are many anecdotal reports that emotional and other stress can lead to occurrences of IBD (Shanahan 1999). The psychoneuroendocrine system regulates the inflammatory and immune responses, and this is likely to play a part in IBD (Shanahan 1994; Ottaway 1996). There is relatively little research on this aspect of
the aetiology of the diseases. Stress-related increases in circulating levels of NGF, which leads to the activation of cells in the immune system have been identified in IBD (Aloe et al. 1997). A conditioned response resulting in mast cell activation has been developed in rats after pairing exposure to an enteritis-producing antigen to a pattern of audio-visual signals (MacQueen et al. 1989). This indicates that in a sensitised bowel, an inflammatory response may be possible in the absence of the original stimulus, as a result of emotional stress. Psychological factors may be important contributing or eliciting factors, but are not the primary aetiology of the condition.

1.2.4.4 Infection

Infection as an aetiologically factor has been extensively investigated, but there are yet no conclusive results. There are changes in the intestinal flora, which may be secondary to changes in intestinal motility or intestinal contents. This makes the identification of an infectious agent difficult. The altered flora may have a role in causing some of the symptoms in IBD by secondary mechanisms (de Dombal et al. 1993). Atypical agents such as mycoplasma have been investigated (Baseman and Tully 1997). Infective agents which elicit a granulomatous response, such as mycobacteria, are an obvious possible cause, but none has yet been definitively identified in CD (Gitnick 1996; McFadden and Fidler 1996). Mycobacterium paratuberculosis causes a disease in cattle (Johnes' disease) with many of the features of CD. It has been suggested that CD is the human form of this disease. However, immunological reactivity to M. paratuberculosis cannot be demonstrated in CD, and antituberculous therapy is not effective, so the role of this infective agent is not clear.
As mentioned previously, measles virus has been found to persist in intestinal tissue, particularly in that affected by CD (Smith and Wakefield 1993). An association between measles vaccination and CD and UC has been described, leading to the conclusion that the virus may be implicated in the development of IBD (Thompson et al. 1995; Wakefield et al. 1995). It has been suggested that CD results from a granulomatous vasculitis secondary to measles virus infection. Other workers have found no evidence of persistent measles virus, however, and it may be that the damage to vascular endothelium is secondary to the inflammation rather than its cause.

It may be that further advances in diagnostic techniques will lead to the recognition of an infective agent (Zumla and James 1996).

1.2.4.5 Immunity

There are profound disturbances in immunity in IBD. Major alterations of immunoglobulin (Ig) synthesis have been identified in vitro - especially of Ig A. There is also an altered humoral and cellular immunological response. These changes are not confined to IBD, however, and similar changes have been identified in coeliac disease and colonic cancer (de Dombal et al. 1993).

Since the humoral and cellular responses to gut-related antigens occurs in IBD and a wide variety of other intestinal diseases, it could be concluded that they are secondary to an increased antigenic challenge resulting from antigen absorption across the damaged, inflamed mucosa. These responses may therefore represent a secondary phenomenon grafted onto the primary inflammatory process.
Even if these responses are secondary, they may play a major role in the outcome and course of the disease. Anti-immune therapy often has a dramatic effect on the outcome of the disease, which points to the importance of these responses. There are, however, differences in the immune responses in CD and UC.

CD appears to be primarily a condition of chronic T-lymphocyte activation with tissue damage induced by secondary macrophage activation. This persistence of T-cell and macrophage activation may be related to an inability of the immune system to eradicate one of a number of luminal antigens (Pavli and Gibson 1992). In UC, there is no strong evidence for T-cell activation, and humoral mechanisms predominate (Pavli and Gibson 1992; MacDonald,TT and Murch 1994; Collins 1996).

Autoimmune mechanisms have also been investigated. In UC, antibodies are produced against colonic epithelial proteins (Griffel and Das 1996). An animal model has been produced in which interleukin-2 knockout mice develop a colonic lesion similar to that in human UC, which is initiated by the normal colonic flora. These mice possess anti-colon antibodies (MacDonald,TT 1994). However, it is not clear whether they are directly responsible for the observed tissue damage (Shanahan 1994).

1.2.4.6 The role of inflammatory mediators

Despite these differences in the initiating mechanisms, both CD and UC have many ‘downstream’ inflammatory processes in common (MacDonald,TT and Murch 1994). These non-specific inflammatory responses include the production of cytokines, arachidonic acid metabolites and reactive oxygen and nitrogen radicals. In animal models, peroxynitrites, metabolites of NO, have been shown to produce
inflammatory changes similar to those seen in IBD (Rachmilewitz et al. 1993). Abnormalities in mast cells, which release a number of immunomodulatory substances, including histamine, 5-HT and platelet activating factor (PAF), have been identified in IBD (Nassif et al. 1996). 5-HT may be of particular importance in disturbances of blood flow in inflammation. In the cardiac circulation, 5-HT is released by platelets aggregating at sites of damaged endothelium. This leads to vasoconstriction and ischaemia. The 5-HT is also taken up by the sympathetic nerves and released on stimulation, perpetuating the vasospastic response (Shepherd et al. 1991).

PAF is thought to play a part in the pathogenesis of IBD (Rachmilewitz et al. 1992). It is liberated from biopsies from inflamed bowel and increased secretion from the tissues. Biopsies from patients with quiescent IBD, but not from normal bowel, could be stimulated to release PAF by 5-HT (Wardle et al. 1996). PAF can also induce NGF production by rat astrocytes (Brodie 1995). It is possible that it can also have this effect on peripheral glial cells.

Reactive oxygen metabolites or oxygen-derived free radicals produced by inflammatory leukocytes can cause tissue damage if their controlling mechanisms are defective (Grisham and Granger 1988; Rozga 1989). They may also promote carcinogenesis, which would account for the high incidence of colon carcinoma in IBD patients (Babbs 1992).

Leukocyte-endothelial reactions may account for some of the characteristics of the tissue damage in IBD and ischaemia-reperfusion injury, which is also associated with the release of ROM (Panes and Granger 1994). Cytokines may have a role in the pathogenesis of IBD, although there is no evidence for aberrant cytokine
secretion in the intestinal mucosa of patients with IBD (Pallone and Monteleone 1996). Thromboxanes (eicosanoids) are overproduced in CD, not only in inflamed mucosa, but also by uninvolved bowel and by isolated intestinal and peripheral blood mononuclear cells. This effect is mediated by PAF in vitro (Wardle et al. 1996). Eicosanoids have pro-inflammatory effects, both by direct action on leukocytes and by vasoconstriction and platelet activation (Rampton and Collins 1993). Mediators play an important role in the initiation of intestinal inflammation, but this does not help with understanding the aetiology. It does not seem likely that there is an underlying immunological disorder in IBD.

1.2.4.7 Intrinsic abnormalities of the intestine

**Enterocytes**

It appears that colonic epithelial abnormalities in UC occur independently of the presence of mucosal inflammation, which suggests that early events are more likely to involve the epithelium than immuno-inflammatory mechanisms (Gibson and Pavli 1992). An increase in intestinal permeability has been identified in both UC and CD (Franchimont et al. 1996). Some first-degree relatives of patients with CD have been demonstrated to have increased intestinal permeability and evidence of increased exposure to foreign antigens (Hilsden et al. 1996), although twin studies have questioned this (Lindberg et al. 1995). Exacerbations of CD are linked to exposure to agents which disrupt epithelial barrier function, and remission is induced by treatments which reduce antigenic load (Ma 1997). An increase in permeability precedes relapses of CD (Wyatt et al. 1993). In UC, the epithelial cells display
increased permeability, not only in active UC but also in remission and in unaffected areas of mucosa. There have been similar findings in CD (Peeters et al. 1994).

The colonic epithelium in UC also has a disturbed glycoprotein composition, and does not metabolise butyrate (de Dombal et al. 1993). However other studies have questioned the presence of permeability abnormalities in IBD (Munkholm et al. 1994), and in general, the evidence for a generalised abnormality in the intestinal epithelium is inconclusive.

**Abnormalities in colonic mucus**

Colonic mucus is important in maintaining mucosal integrity. IBD is associated with changes in this mucus (Clamp et al. 1981). In UC especially there are abnormalities in goblet cell mucus production. The mucus layer is thinner in UC than in controls, but thicker in CD (Dvorak and Dickersin 1980; Pullan 1996). The nicotine in cigarette smoke may increase this mucus layer, which would explain its protective role in UC (Pullan 1996).

**Enteric and autonomic nervous system**

Afferent fibres from the gut have been shown to exert an influence on inflammatory changes by means of activation of local effector mechanisms. The best evidence is in the stomach where afferent nerves initiate pro-inflammatory events in the gastric mucosa via an axon reflex mechanism, to prevent potential injury (Raybould and Mayer 1991).

Abnormalities in the ENS could be responsible for the prolongation of inflammation seen in IBD. An immunohistochemical study has demonstrated changes in the neurochemical coding of nerves in the myenteric plexus, with proliferation of some
of the nerve types (Belai et al. 1997). At the ultrastructural level, damage to the ENS has been identified in CD, even in areas not obviously involved by the disease (Dvorak et al. 1980). This damage does not seem to be present in UC, although the development of pouchitis is associated with axonal necrosis in the pouch (Dvorak and Silen 1985; Dvorak et al. 1993).

Mucosal lymphoid tissue is supplied by neuropeptide-containing nerves, and many cells involved with the immune system carry receptors to neuropeptides (Pascual et al. 1994). The neuroendocrine and immune systems work closely together to control inflammation in the gut, so this axis may be important in the control of IBD (Ottaway 1996). Abnormalities in this system may account for the abnormalities in the control of inflammation seen in IBD.

Of the neuropeptides, SP is most commonly associated with neurogenic inflammation, but other transmitters may also be involved. There is a pronounced up regulation of receptors to SP in small blood vessels and lymphoid follicles in the gut wall in IBD (Eysselein and Nast 1991). Hyperinnervation with SP-containing nerve fibres is seen in some cases, but a decrease in the number of nerves has also been observed (Miller, MJ et al. 1993). There is increasing evidence that SP from extrinsic sensory neurones in the gut have a role in neurogenic inflammation and wound healing, and may be important in the pathogenesis of these diseases.

In the rat colon, destruction sensorimotor nerves with capsaicin produced an increase in the inflammation produced by trinitrobenzene-sulphonic acid (Szepes et al. 1997). Another study, using a similar model, found worse inflammation and a decrease in the CGRP, but not SP, content of the colon following pre-treatment with immunoneutralising antibodies to the neurotrophins nerve growth factor (NGF) and
neurotrophin-3 (Reinshagen et al. 2000). SP is recognised as being involved in the immune response of the gut. Nerves with SP-LI are closely associated with GALT, and SP is involved with both T-lymphocyte activation and B-lymphocyte differentiation. In a study on a rat model of colitis, application of a SP antagonist prevented the development of inflammation (Di Sebastiano et al. 1999), indicating a potentially important role for this peptide in IBD.

CGRP is often colocalised in extrinsic neurones with SP, but there are also separate populations of intrinsic neurones containing these transmitters. The intrinsic nerves are thought to have a role in regulating motility, while the extrinsic neurones regulate intestinal blood flow and tissue repair. Studies of the concentration of these peptides in the bowel wall show differences in inflamed bowel, but it is unclear whether these are due to changes in the intrinsic or extrinsic neurones (Eysselein and Nast 1991). However, immunohistochemical studies of the extrinsic sensorimotor nerves show their transmitter content and number appear to be decreased in IBD (Eysselein et al. 1992).

Abnormalities have been found in nerve fibres containing other neuropeptides, such as VIP and NPY. A reduction of the density of nerves containing VIP has been noted in both human and animal models of intestinal inflammation (Kubota et al. 1992; Mazumdar and Das 1992), but other studies showed an increase (O'Morain et al. 1984) or no difference (Sjölund et al. 1983). Again, both intrinsic and extrinsic nerves contain VIP, which has functions such as stimulation of intestinal secretion and relaxation of smooth muscle. NPY is found in sympathetic nerves, colocalised with NA, and causes vasoconstriction. A marked increase in nerve fibres containing this peptide has been found in rectal biopsies in patients with UC. Many of these
patients responded to treatment with topical local anaesthetic (Bjorck et al. 1992). It is possible that hyperactivity of these nerves leads to vasospasm and contributes to the inflammation in IBD.

**Vascular abnormalities**

There is some evidence that ischaemia can cause inflammatory changes in the bowel mucosa. ‘Obstructive colitis’ has features that range from discrete ulceration to extensive fulminant colitis. This occurs proximal to obstructing lesions in the small and large bowel and has been postulated to be due to mural hypoperfusion (Levine, TS and Price 1994).

Abnormalities in the vasculature have been described in IBD, affecting both the microvasculature and larger vessels. There appear to be abnormalities in both the anatomical structure of the vessels and at the cellular level.

In both UC and CD, a system of microradiography found a significant reduction in the submucosal vascularity and occlusive changes in segmental CD in the affected areas only. In diffuse CD and UC, there was a generalised disruption of the microvasculature, but only in severe disease. Occlusive changes were not a feature (Carr et al. 1986). However, another study, using microcorrosion resin casting of vessels in CD, found severe damage to submucosal vessels even in areas not affected macroscopically by disease (Pounder 1994). In a rat model of colitis, microvascular changes were identified in the colonic mucosa, which preceded histological changes (Foitzik et al. 1999). In UC, the proximal demarcation of the affected bowel has been found to be associated with the territory of the inferior mesenteric artery, suggesting that some feature of this vessel, possibly of embryological origin, predisposes the dependent bowel to develop UC (Hamilton et al. 1995).
It has been proposed that the vessel damage in CD is due to a granulomatous vasculitis. Granulomatous vasculitis also affects areas affected by extra-intestinal CD, for example in cutaneous CD (Hackzell-Bradley et al. 1996). The presence of IgG anti-endothelial cell antibodies (AECA) has been determined in IBD. AECA levels were elevated in both UC and CD (Stevens et al. 1993), with higher levels in UC. This may support a vasculitic process, but may also be the result of an immune response to hidden endothelial self-antigen exposed after endothelial cell damage, or be a further marker of disturbed immunoregulation in IBD (Aldebert et al. 1995).

Leukocyte-endothelium interactions are also abnormal, and may lead to vascular damage (Panes and Granger 1994). There are also abnormalities of the endothelium and alterations in the blood that lead to a hypercoagulable state. There are changes in the expression of cell adhesion molecules in IBD in both involved and uninvolved areas of the gut and this endothelial activation may contribute to the early lesion in these diseases (Nakamura et al. 1993; Schuermann et al. 1993). Twenty-two percent of patients with CD were found to have anticardiolipin antibodies in both active and quiescent disease. These phospholipid-binding antibodies are thrombogenic, and may indicate changes in vessels which predispose to thrombosis (Chamouard et al. 1994). It has been found that in patients with IBD, over 85% have plasma concentrations of prothrombotic factors in excess of the limits regarded as posing an increased risk of occlusive vascular disease (Hudson et al. 1996). Both cigarette smoking and the oral contraceptive pill aggravate focal thrombosis and have been implicated as aggravating factors in CD (Wakefield et al. 1991).

There is an increased expression of cytokines such as TNFα in both CD and UC. Cells immunoreactive for TNFα were seen mainly deep to the epithelium in UC, and
throughout the lamina propria in CD. In CD, these cells were also seen clustering around arterioles and venules, infiltrating and disrupting the vascular endothelium. This TNFα production is thought to contribute to the pathogenesis of both CD and UC, by impairing the integrity of epithelial and endothelial membranes, increasing inflammatory cell recruitment, and by prothrombic effects on vascular endothelium (Murch et al. 1993).

Other cytokines produced in IBD are also prothrombotic (MacDonald, TT et al. 1993). The increased expression of cytokines in IBD supports activated coagulation and can lead to overt vascular thrombosis (Levine, JB and Lukawski-Trubish 1995). Some of the effects of PAF, released by platelets in thrombus, were mentioned earlier. These abnormalities lead to occlusion and thrombosis, which contribute to ischaemia.

Many abnormalities, in both the luminal contents and the vessel walls, have thus been identified in the mesenteric vasculature in IBD. In addition, the specialised microcirculation in the mucosa can lead to ischaemia and ulceration in the absence of vascular occlusion (Lundgren 1989). This evidence points to a potential role for abnormalities in the mesenteric circulation in the aetiology and pathophysiology of IBD.
1.3 Aims and Experimental Plan

1.3.1 Background

Understanding the mechanisms of the control of blood flow to the gut is important from a number of points of view. The gastrointestinal tract in man receives 15 - 20% of resting cardiac output but this proportion increases substantially following a meal. Local changes in mesenteric blood flow are important in the digestion and absorption of food, and have an influence on gut motility. The splanchnic veins form the largest bed of capacitance vessels in man, and can undergo significant active capacitance changes. This is of importance in maintaining systemic blood pressure during postural changes. In disease states such as IBD, total gut blood flow increases dramatically. However, the influence of local ischaemia and the disruption of normal vascular anatomy are increasingly recognised as being of pathophysiological importance. The unique vascular anatomy of the bowel wall creates conditions in which mucosal ischaemia and damage can occur in the absence of frank occlusion of the supplying vessels.

Mesenteric vessels are richly innervated. As with other areas of the ANS, the nerves were once considered either cholinergic or adrenergic. It is now known that the nerves release a variety of neurotransmitters, including purines, peptides and NO, and cotransmission commonly takes place. Vasodilatation and vasoconstriction are also mediated by endothelial cells. They are responsive to substances they produce themselves, to circulating vasoactive substances and to transmitters released by perivascular nerves.
Gross mesenteric blood flow changes are integrated centrally via the ANS. Smaller scale alterations in blood flow appear to be controlled largely by local factors, governed by the ENS. The ENS has been recognised as the 'third arm' of the ANS. It modulates many aspects of enteric function, including motility, nutrient absorption, water and electrolyte fluxes, intramural blood flow and the immune system.

Understanding the innervation of the mesenteric vasculature in health and disease is clearly of importance. Many aspects of the pathogenesis of IBD, specifically aspects of genetics, immunology and the role of non-specific mediators, are common to both UC and CD. However, some of these changes are non-specific and occur in other intestinal diseases, with known aetiology. This indicates that a great part of the pathogenesis of IBD may be due to a common syndrome of secondary mechanisms. The nature of these secondary mechanisms and the underlying physiological abnormality which gives rise to them remains to be elucidated.

The discovery of the great variety of neurotransmitters in the autonomic and enteric nervous systems, and their interactions with the immune system, indicates a potentially useful line of research. The perivascular nerve plexus is the intersection of the vascular and nervous systems, with the perivascular innervation being one of the 'dual control' mechanisms in controlling blood flow. The fibres supplying the vessels comprise motor nerves from the ANS and the ENS as well as primary afferent sensorimotor nerves. Abnormalities in this system may contribute to abnormalities in blood flow, vessel damage and ischaemia.

Much of the work on mesenteric perivascular nerves to date has been performed on animals. In this study, it is proposed to characterise the structure and function of the
control mechanisms in human mesenteric blood vessels obtained from patients undergoing surgery for non-inflammatory conditions. These will be compared with vessels obtained from subjects with the idiopathic inflammatory bowel diseases, CD and UC.

1.3.2 Immunohistochemical investigations

Confocal laser-scanning microscopy (CLSM) will be used to identify the presence and distribution of perivascular nerves containing NA, acetylcholine, 5-HT, NO and the neuropeptides VIP, SP, CGRP and NPY. Investigations will be carried out on whole-mount stretch preparations of the vessels. This provides a more comprehensive visual assessment of the density and distribution of the different perivascular nerve types than studies on cross-sections of the vessels. Immunohistochemical techniques will localise tyrosine hydroxylase (TH; a marker for NA), the neuropeptides, choline acetyltransferase (ChAT; a marker for acetylcholine), NOS and 5-HT. The general neuronal marker, PGP, will be used to identify the total number of nerve bundles present, in order to identify the proportion of nerves containing each marker. Computerised image analysis techniques will be used to assess the number and structure of nerves in the perivascular plexus. Control vessels will be compared with those from patients with IBD. The IBD group will be further subdivided into subjects with CD and UC and compared, to assess whether any differences can be identified between the two conditions.

1.3.3 Pharmacological investigations

Segments of isolated mesenteric vessels will be mounted between two platinum electrodes in an organ bath. Responses to transmural electrical field stimulation will
be assessed at submaximal frequency. Using the appropriate antagonists, the neurotransmitters released and the receptors involved in the response to EFS will be identified. Contractile responses to NA, ATP and NPY will be examined and the effects of NPY on the contractile response to NA assessed. The maximal contractility of the vessel to KCl will be established. Again, differences between UC and CD will be investigated, as well as those between the control group and IBD.

1.3.4 Summary

This work will contribute to the growing body of information on the role of the innervation of blood vessels in controlling blood flow in health and IBD. If anatomical changes in nerve distribution are identified, their functional importance will be related to the findings of the pharmacological investigations. Of importance is the use of human tissue. In spite of the difficulties with inter-subject variability, findings in human studies often have greater clinical applicability than animal studies. In studies on the ANS in particular, extrapolation from animal studies is of limited value, because of the wide interspecies variations in innervation.
Chapter 2  Quantitative analysis of the innervation of human mesenteric vessels in non-inflamed and inflamed bowel - a confocal study
2.1 Introduction

There is local control of vascular tone by perivascular nerves and endothelial cells (Burnstock and Ralevic 1994). Changes in the density of innervation and of expression of vasoactive agents coexisting in nerves and endothelial cells have been described in conditions such as ageing, (Mione et al. 1988; Kawasaki et al. 1991) hypertension, (Kawasaki et al. 1990; Kondo et al. 1997; Marin and Redondo 1999) and diabetes (Ralevic et al. 1993; Belai et al. 1996).

Studies of vessel innervation were previously assessed by semiquantitative methods that are open to subjectivity (Edvinsson et al. 1989; Jansen et al. 1992). The advent of new forms of microscopy, digital image manipulation and image analysis has allowed the development of more accurate, quantitative methods of assessment (Buwalda et al. 1997; Kondo et al. 1997).

Vascular abnormalities have been described in IBD, affecting both the microvasculature and larger vessels (Carr et al. 1986; Pounder 1994; Foitzik et al. 1999). Changes in the perivascular innervation of the vessels may contribute to these abnormalities. In this study, confocal microscopy and computerised image analysis have been used to assess quantitatively the density of nerves containing various neurotransmitters in the human mesenteric vasculature in control vessels and in IBD.

Quantitative differences in the density and pattern of innervation between arteries and veins were investigated with immunohistochemistry for protein gene product 9.5 (PGP; a general neuronal marker), tyrosine hydroxylase (TH; a marker for noradrenaline) and neuropeptide Y (NPY) for sympathetic nerves; calcitonin gene-
related peptide (CGRP) and substance P for sensory-motor nerves. The distribution of antibodies for vasoactive intestinal peptide (VIP), nitric oxide synthase (NOS; for nitric oxide), choline acetyltransferase (ChAT; for acetylcholine) and 5-hydroxytryptamine (5-HT) was also studied. A previous immunohistochemical study of human mesenteric vessels using PGP identified a greater number of nerve fibres using confocal laser scanning microscopy (CLSM) than with conventional epifluorescence microscopy (Buwalda et al. 1997). Few studies of the neurotransmitter subgroups have been made in human mesenteric vessels and only one immunohistochemical study for 5-HT-containing nerves has been reported previously (Griffith and Burnstock 1983).

2.2 Methods

Mesenteric vessels were obtained from specimens resected from patients undergoing surgery for inflammatory and non-inflammatory conditions. Vessels were dissected from the mesentery close to the resection margin, adjacent to the bowel wall. This avoided interference with mesenteric lymph nodes, which are histologically examined for prognostic information, in the case of carcinoma specimens. Informed consent for the procedures was obtained, using a standard consent form. Details of the age and sex of the patient, the nature of the operation, anatomical site of the vessels, comorbidity and current drug treatment were noted. Patient details are given in Appendix 1.

Segments of mesentery were removed in the operating theatre and immediately placed in cold Hanks’ balanced salt solution. The marginal vessels or vasa rectae of 1-3 mm diameter were then dissected free from excess fat and connective tissue and slit longitudinally. The relaxed dimensions of the vessel segment were then
measured. The vessel segment was stretched and pinned out on Sylgard silicone rubber, and re-measured. The longitudinal and transverse stretch factors were calculated. Segments were fixed for at least 2 h in 4% paraformaldehyde. When fixed the stretched dimensions were retained. Specimens were then washed in phosphate-buffered saline (PBS), and stored at 4°C until processed.

Whole-mount segments of mesenteric artery and vein were stained using a standard indirect immunofluorescence technique. Normal donkey serum (1:10) was applied for 2 h at room temperature to reduce background staining. Rabbit-derived polyclonal antibodies to TH, NPY, VIP, CGRP, 5-HT, NOS, ChAT or PGP were then applied to the tissue for at least 36 h in a humid chamber at room temperature, at the concentrations shown in Appendix 2. After washing three times in 0.1% Triton in PBS, biotinylated donkey anti-rabbit antibody was applied for 2 h. After further washing, streptavidin-fluorescein was applied for 1 h. Specimens were again washed prior to counterstaining in Pontamine sky blue. This has been shown to reduce background fluorescence (Cowen et al. 1985). Sections were then mounted on glass slides in Citifluor, an anti-fading compound.

The slides were viewed on a Leica TCS 4D confocal microscope, using an objective magnification of ×25 unless the nerves were too fine to be seen at the initial magnification, when ×40 objective was used. Three representative fields were then recorded, having set the depth of tissue scanned (‘z-series’) to include all visible nerves in each field. The z-series and the projected image were then stored digitally on optical disk.

The digital images were analysed for intercept density (ID) in both transverse and longitudinal orientations, and for total fluorescent area (TFA), using a Power
Macintosh computer running NIH Image software. The ID is proportional to the total length or number of nerve bundles present, whilst the TFA is proportional to the number of nerves within the bundles. The ratio of TFA to ID is therefore proportional to the average size of the nerve bundles (Cowen and Burnstock 1982), whilst the ratio of transverse and longitudinal ID gives an indication of nerve bundle orientation. These four features give a good indication of the structure and density of the nerve plexus.

After some minor initial editing to remove fluorescent artefacts, the images were converted to negative black-and-white format (Figure 2.1a). Modifications of described methods were then used to obtain values for the ID, TFA and TFA:ID ratio (Terenghi et al. 1991; Wooton et al. 1995). The greyscale level that demarcated the boundary between the background and the nerves was chosen individually for each image (Figure 2.1b). This image was then converted to a binary image which was subjected to the process of 'closing', which has the effect of removing small background speckles up to a predefined size (Figure 2.1c). The TFA of the nerves in the visual field was then measured. This value was expressed as a percentage of the total image area.

The nerve network was then 'skeletonised', reducing the nerves to a network no more than one pixel wide (Figure 2.1d). The ID was then measured using a superimposed array of 15 lines and counting the number of nerve intersections automatically. The mean transverse ID for the image was then calculated. The previously calculated transverse stretch factor was then applied to give the transverse intercept density.
For the ID, TFA and ID:TFA ratio of nerves containing the various neurotransmitters, differences both between arteries and veins and within each vessel type were examined. Control vessels were compared with vessels from patients with IBD, and any differences between the groups with CD and UC were investigated by paired or unpaired t-test as appropriate. Results are given as mean values ± 95% confidence interval (CI) and $P$ values of less than 0.05 were considered significant.

### 2.3 Results

#### 2.3.1 Qualitative observations

Sections of artery and vein were investigated to identify the position of the nerves within the vessel wall. In the arteries, nerves were distributed at the junction of the adventitia and the media, close to the external elastic lamina. In the veins, nerves were distributed throughout the muscular layer of the media. Large paravascular nerve bundles were identified in the outer adventitia in both arteries and veins (Figure 2.2). These bundles were not included in the investigation and were either removed during dissection or excluded during optical sectioning with the confocal microscope.

Nerves immunoreactive to PGP, TH and NPY were identified in all arteries and veins (Figure 2.3). Nerves displaying PGP, TH and NPY-LI in the control arteries appeared to be orientated longitudinally, whilst these nerves in the corresponding veins formed a reticular pattern. In the arteries of patients with IBD, the nerves appeared to be present at a higher density than in the controls, with a less organised
pattern (figure 2.4). The distribution in the veins appeared similar in IBD and control subjects.

Nerves immunoreactive to VIP were identified in all vessels studied in both the control (figure 2.5) and IBD groups (figure 2.6), although at a lower density than the TH and NPY nerves. SP immunoreactivity was identified in all control veins and in 4 out of 6 control arteries (figure 2.5). These nerves were very fine, often with well-defined varicosities, but were usually sparse or very sparse. Specimens from the 12 IBD patients displayed SP-LI in 9 arteries and 10 veins (figure 2.6).

Mesenteric veins from control specimens did not display nerves immunoreactive to CGRP, but these were identified in 5 out of 6 control and all the IBD arteries studied, with a higher density than that of SP immunoreactivity (figures 2.7, 2.8).

Nerves immunoreactive to 5-HT were not consistently observed, having been identified in one control artery and 3 of the 5 control veins studied (figure 2.7). In the IBD vessels, nerves with 5-HT-LI were identified in all 12 veins and in 6 out of 12 arteries (figure 2.8). The fluorescence appeared less intense than that of other transmitters. No nerves immunoreactive to NOS (n=7) or ChAT (n=3) were identified.

2.3.2 Quantitative observations

2.3.2.1 Control vessels

Total Fluorescent Area

The TFA is proportional to the total number or length of nerves within the bundles.

For arteries, the plane of focus was confined to the plexus at the adventitial/medial
border (about 20 μm thick) while in veins, the confocal analysis allowed nerves from the entire medial coat to be visualised (about 100 μm thick). The values for TFA are shown in Table 2.1, calculated according to the early quantitative methods developed by Cowen and Burnstock (Cowen and Burnstock 1980), using conventional light microscopy.

In the arteries, the TFA value of each immunomarker, relative to the total TFA as measured by the TFA to PGP, was 110%, 60%, 33%, 29% and 3% for NPY, TH, VIP, CGRP and SP respectively. For the veins, the values were 125%, 86%, 31% and 3% for NPY, TH, VIP and SP respectively. No nerves with CGRP-LI were identified in the veins.

The veins demonstrated a significantly higher TFA than the arteries for the immunomarkers PGP, NPY and TH. In the veins, the TFA value for NPY-immunoreactive nerves was significantly higher than that for PGP (NPY, 11.66 ± 0.41%; PGP, 9.31 ± 1.07%; mean ± 95% CI, P = 0.007, paired t-test). The value was also greater in the arteries, but this was not statistically significant. There were no significant differences between the arteries and the veins for the VIP and SP. Nerves with 5-HT-LI were identified inconsistently, and the figures given are the means for the vessels with visible nerves. These were not analysed statistically.

**Intercept density**

The ID is proportional to the total number of nerve bundles present. Transverse ID was significantly greater in veins than arteries for PGP and TH. The transverse ID for NPY in the veins was greater than the arteries, but the difference was not statistically significant (Table 2.2). The ID for each immunomarker, relative to the overall total (as measured by the transverse ID to PGP), followed the same pattern in
the arteries and veins as the TFA. The values given for 5-HT were not analysed statistically.

For most of the immunomarkers, the ratio of transverse ID to longitudinal ID was greater than one, indicating a longitudinal orientation of the nerve bundles. The exceptions were 5-HT and SP-LI, where the orientation tends to be transverse. The ratio was higher in the arteries than the corresponding veins for the markers PGP, TH, NPY and VIP (Table 2.2); the difference was statistically significant for TH and VIP. This confirms objectively the subjective impression that the nerve bundles in the arteries were more longitudinally orientated.

**TFA/ID ratio**

This ratio is proportional to the average size of the nerve bundles. The values are given in Table 2.3. The two vessel types were not significantly different in this respect. This indicates that the nerve bundles were of the same thickness in the arteries and the veins. The TFA:ID ratio in arterial SP-LI nerves was significantly smaller than that of PGP, confirming the impression of much finer nerve fibres in this nerve type. No other significant differences were identified between the immunomarkers for this parameter.

### 2.3.2.2 Inflamed vessels

**Total fluorescent area**

Values for the TFA for the arteries and veins from subjects with IBD are shown in the table (table 2.4). Relative to the TFA for PGP, the arterial TFA for both NPY and TH was 100%. In the veins, the values were 120% and 95% for NPY and TH
respectively. The proportion of VIP-LI nerves was 26% in the arteries and 24% in the veins. 5-HT was identified more consistently than in the control vessels, accounting for 14% of the total TFA in both the arteries and the veins. The proportion of nerves with SP-LI was 3% and 5% in the arteries and the veins respectively. CGRP was identified in vein specimens from IBD patients in small numbers. Relative to the total, the TFA for CGRP was 4% in the arteries and 2% in the veins.

Comparing the vessel types, there was a significantly greater TFA for TH in the arteries than in the veins. No other significant differences were identified between the vessel types.

**Intercept Density**

The pattern of the relative magnitude of the TID for the immunomarkers was similar to that of the TFA for both the arteries and the veins (table 2.5). There were no significant differences between the arteries and veins for any of the markers studied. The TID:LID ratio was also similar between the two vessel types (table 2.5). The ratio for SP was less than PGP for SP in both the arteries and the veins and for CGRP and 5-HT in the veins alone, indicating the transverse orientation of the fibres in these nerve types.

**TFA:ID ratio**

The values for the TFA:ID ratio are shown in table 2.6. Comparing the arteries and the veins, this ratio is higher in the arteries for PGP, indicating overall thicker nerve bundles in the arteries. There are no other significant differences between the two vessel types. Compared to the PGP value, the value for CGRP is lower in the veins.
alone, and that for SP and 5-HT is lower in both the arteries and the veins. The TFA:ID ratio is significantly higher in the TH-LI nerves in the veins.

2.3.2.3 Comparisons between IBD and control vessels

Total fluorescent area

The TFA values for PGP, TH and NPY were significantly greater in the IBD than control arteries (control arteries v. IBD arteries: PGP 6.94 ± 1.22 v. 19.83 ± 1.51, p=0.005; TH 4.19 ± 0.65 v. 19.78 ± 1.41, p=0.0005; NPY 7.64 ± 1.56 v. 19.81 ±1.01, p=0.0006; mean ± 95% CI, Student’s t test) and veins (control veins v. IBD veins: PGP 9.31 ± 1.07 v. 14.39 ± 0.59, p=0.01; TH 7.98 ± 1.07 v. 13.63 ± 0.35, p=0.0004; NPY 11.66 ± 0.41 v. 17.26 ± 0.95, p=0.03) (figure 2.9). There were no significant differences between the two groups in the TFA values for VIP, SP and, in the arteries, CGRP (figure 2.10). In the vessels from patients with IBD, 5-HT was identified much more consistently than in the control subjects. This was not tested statistically because of the paucity of nerves with 5-HT-LI in the control vessels, but it appears that, although sparse, there was a consistent population of nerves containing 5-HT, which were more numerous in the vessel from patients with IBD.

Intercept density

In the arteries, there was a greater TID in the vessels from patients with IBD for the immunomarkers PGP, TH and NPY (control arteries v. IBD arteries: PGP 3.43 ± 0.54 v. 6.80 ± 0.31, p=0.001; TH 2.20 ± 0.32 v. 6.96 ± 0.65, p=0.002; NPY 3.37 ± 0.68 v. 6.64 ± 0.28, p=0.003; mean ± 95% CI, Student’s t test) (figure 2.11). There were no significant differences for the other markers (figure 2.12). In the veins,
there were no statistically significant differences in the TID in any of the markers. The LID followed the same pattern in both the arteries and veins.

The TID:LID ratio was greater in the control arteries immuno-stained for PGP, TH, NPY, VIP and CGRP (control arteries v. IBD arteries: PGP 3.06 ± 0.66 v. 1.60 ± 0.14, \( p=0.04 \); TH 2.84 ± 0.35 v. 1.52 ± 0.10, \( p=0.004 \); NPY 2.33 ± 0.24 v. 1.46 ± 0.10, \( p=0.01 \); VIP 1.91 ± 0.13 v. 1.18 ± 0.10, \( p=0.02 \); CGRP 1.47 ± 0.25 v. 0.63 ± 0.07, \( p=0.008 \); mean ± 95% CI, Student's \( t \) test). This indicates that the orientation of the nerve bundles is not as regularly in the axis of the vessel in the IBD vessels.

In the veins, there were no significant differences in the values for the TID:LID ratio.

**TFA:ID ratio**

The TFA:ID ratio was significantly greater in the IBD arteries for the markers PGP, TH and NPY (figure 2.13), indicating thicker nerve bundles in these vessels (control arteries v. IBD arteries: PGP 0.50 ± 0.03 v. 0.74 ± 0.03, \( p=0.008 \); TH 0.47 ± 0.05 v. 0.75 ± 0.04, \( p=0.02 \); NPY 0.58 ± 0.04 v. 0.75 ± 0.02, \( p=0.01 \); mean ± 95% CI, Student's \( t \) test). In the IBD veins this value was significantly greater only for TH (control veins v. IBD veins: TH 0.44 ± 0.03 v. 0.67 ± 0.02, \( p=0.0005 \)). No significant differences were identified for the other markers (figure 2.14).

### 2.3.2.4 Differences between ulcerative colitis and Crohn's disease

IBD specimens were obtained from patients with both UC and CD. The results from patients in the two groups were examined to identify any differences between the disease types. There were no significant differences between the two groups.
2.4 Discussion

The control of blood flow to organs is an important function of the autonomic nervous system. The perivascular nerves supplying vascular smooth muscle modulate vascular tone. Perivascular nerves were previously thought to be primarily sympathetic vasoconstrictor fibres containing noradrenaline but are now recognized to utilize a variety of other neurotransmitters, usually ATP and NPY. (Burnstock 1990c) In addition, parasympathetic and sensory-motor nerve fibres releasing other transmitters are also present. The neuronal markers for this study were selected on the basis of the neurotransmitters commonly identified in the four classes of autonomic nerves found in perivascular nerves (Burnstock 1999a). These are: NA and NPY for sympathetic nerves; acetylcholine and VIP for parasympathetic nerves; SP and CGRP for sensorimotor nerves; and NO and VIP for intrinsic nerves from the ENS. 5-HT has previously been identified in human mesenteric perivascular nerves although the origins of the fibres containing it are debated (Griffith and Burnstock 1983). ATP is a cotransmitter in most, if not all of these nerve types, but there is no reliable immunohistochemical method for localising it. Detecting the presence of neuronal ATP relies on functional studies.

The perivascular nerves, in concert with signals from the endothelium, act on the smooth muscle and other components of the vessel wall to control the normal functioning of the vessel. This 'dual control' mechanism integrates local blood flow requirements with the overall needs of the organ. (Burnstock and Ralevic 1994; Remak et al. 1994)

The mesenteric circulation is highly specialised. In addition to supplying the metabolic needs of the gut and other organs, blood flow is also altered in response to
mechanical stimulation or food in the lumen (Meehan and Kreulen 1992), in mucosal protection from acid (Guth 1992), and as part of an inflammatory response (Alican and Kubes 1996). The mesenteric veins form the largest capacitance bed in man (Hainsworth 1986; Hogan et al. 1994), and control of venous tone is important in the effective maintenance of blood pressure (Nyhof et al. 1983).

Interest in analysing the structure of nerve plexuses is increasing with the recognition of the ways in which the autonomic nervous system can be affected in many disease states (Burnstock 1990a; Ralevic and Burnstock 1996).

2.4.1 Confocal microscopy

There are limitations to conventional two-dimensional (2D) microscopy. The plane of focus is relatively thick, even with the best lenses. In addition, light from just above and below the focal plane is collected by the objective, which contributes to out-of-focus blur. This can reduce the contrast and sharpness of the image, and decrease the 'signal to noise' ratio. Immunofluorescence microscopy, whilst benefiting from excellent contrast, suffers a great deal from out-of-focus interference with the images. Autofluorescence of structures that have not been specifically stained can also contribute to poor quality images.

When assessing three dimensional (3D) structures with conventional microscopy a clearer impression of the structure is often obtained by focusing up and down to obtain a rough idea of the 3D cellular structure. A 2D photomicrograph of the specimen only gives an incomplete impression of the structure. This is particularly important in structures such as nerve plexuses, which ramify through a tissue.
Human mesenteric vessels are relatively thick-walled compared with those from common laboratory animals. They also contain substantial amounts of elastic tissue, which autofluoresces in the light emitted by a mercury vapour lamp. These features make the study of perivascular innervation in these vessels difficult using conventional techniques. The background fluorescence from elastic tissue and muscle makes the nerves difficult to see and more difficult to photograph. The walls of the vessels are relatively thick, which makes it impossible to visualise the nerve network in one photographic optical plane using a reasonable aperture. Accurate image analysis under these conditions is difficult, as many nerves would be missed. In initial work for this study, using conventional microscopy, the images were not of a high enough quality for quantitative analysis (personal observation).

Confocal microscopy avoids these problems (Pawley 1995; Buwalda et al. 1997), by providing a thinner plane of focus with better quality 2D images than conventional light microscopy. Out-of-focus blur is also reduced, so that sharper and clearer images are obtained. This means that a series of in-focus optical sections can be obtained from scanning through a thick tissue section. These sections are called ‘z’ sections, as they are taken in the ‘z’ axis of the tissue.

These ‘tomographic’ images are stored digitally, so that, by using suitable software, they can be manipulated in a number of ways. A flat 2D projection of the z sections can be obtained, or 3D reconstructions of the tissue can be produced. In this study, a 2D projection gave sufficient information for our purposes. However, it is possible to produce exquisitely detailed reconstructions of, for example, a single neurone (Tekola et al. 1994).
2.4.1.1 Principles of confocal microscopy

The advantages of CLSM are due to three factors: the use of a pinhole, laser illumination and scanning of the subject. In modern machines, the use of powerful computerised image processing confers further advantages.

The confocal principle is a simple form of aperture processing. The pinhole at the crossover point of an illumination light path coupled with another at the corresponding point on the transmitted light path excludes information from out-of-focus planes in the specimen. In reflectance mode, the objective lens acts as its own condenser and the light paths of the illumination and reflected beams are reciprocal. The objective therefore focuses light on the detector. The pinhole (usually between 10 and 20 μm) excludes light that originates from the out-of-focus planes, so that only light from the in-focus plane is detected. This principle is not new, but the intensity of conventional light sources is too low to make use of it, as a great deal of the light used is cut away. The introduction of lasers has several advantages, the intensity of the light beam used being one of them.

Light emitted by a laser is coherent (i.e. has the same frequency and phase). This helps to focus the laser on the same spot, which improves resolution in the x, y plane. The monochromaticity also improves focus by eliminating chromatic aberration, in which light of different wavelengths focus in slightly different planes. These factors result in an improvement of the lateral resolution by a factor of 1.4 compared with the conventional microscope, which gives a lateral resolution of approximately 230 nm for wavelength of light (Hale and Matsumoto 1993).

The optical sectioning ability of the confocal microscope is another major advantage of this technique. The thickness of the optical section is dependent on the
wavelength of the laser, the numerical aperture of the objective and the effective
diameter of the objective (Tekola et al. 1994). The equation for depth of focus ($\Delta Z$)
in conventional microscopy is has two components, one dependent upon the
diffraction properties, the other determined by the geometric optics. In confocal
microscopy, this second term becomes negligible, so that $\Delta Z$ is dependent only on
the part of the equation that determines the diffraction property. This results in a
theoretical optimal $\Delta Z$ for conventional microscopy of approximately 0.46$\mu$m and
0.12$\mu$m for confocal microscopy (see Appendix 3 for details).

In fluorescence microscopy, lasers of different wavelengths can be chosen to excite
specific fluorescent dyes. In the present study, the fluorochrome used was
fluorescein isothiocyanate (FITC), which has an absorption maximum of 490nm and
an emission maximum of 520nm, which is well matched to the wavelengths emitted
by the argon/krypton laser (488, 514 and 647nm). In addition, multi-channel
investigations can be performed, using multi-line lasers, which split the emitted light
and filter the channels separately. This allows simultaneous assessment of structures
stained with different fluorescent dyes, or as in the case of this study, autofluorescent
structures with a different emission spectrum can be identified.

Scanning light microscopes build up an image by successively illuminating discrete
areas of the specimen. This improves resolution as the smaller the area illuminated,
the higher the resolution, as there is less 'confusing' information from light scattered
from neighbouring parts of the specimen. With laser illumination, the area
illuminated is reduced to a minimum. The disadvantage of this system is the time
taken to build up the image. Two types of scanning microscope exist. Stage
scanners move the specimen relative to the light source. This has the advantage of a
constant on-axis light-path, but they are expensive and scan slowly. Beam scanners use galvanometer mirrors to produce a full-screen image in less than two seconds. The disadvantage of this system is that it is more prone to optical artefacts, and thus resolution is reduced. The area to be scanned is also limited. The scanner used in this study was a beam scanner, as speed of image acquisition was of greater importance than resolution.

The light reflected from the specimen or produced by the fluorescence induced in the specimen is detected by a photomultiplier and converted into digital signals. The image is reconstructed after scanning the whole field, and displayed on a computer monitor. The digital nature of the data allows manipulation of the image by suitable software. Electronic zoom magnifies the image independently of the optical system. Objects of interest can be zoomed in on without changing the objectives. Software also allows the image to be displayed in a variety of forms, such as a standard x, y image, sectioning through the thickness of the specimen (x, z image) and a variety of projections and 3D reconstructions.

After capturing the series of optical sections, a composite or projected image of the 'stack' of images can then be produced. This allows the entire thickness of the three-dimensional plexus to be viewed in one image. The image produced is usually of high contrast, which is ideal for image analysis. This can be printed directly by the computer, photographed from the computer monitor or stored on disk or other mass storage medium for further manipulation.

### 2.4.2 Image analysis

Image analysis techniques have been developed for use with conventional microscopes (Cowen and Burnstock 1980; Mione et al. 1988; Di Sebastiano et al.
1995). The technique described above is an extension of these methods. Many of the problems encountered with the earlier technique are avoided with confocal microscopy. A common problem was that the illumination level varied across the visual field, owing to the characteristics of the mercury vapour lamp (Cowen and Burnstock 1982). This is overcome by confocal microscopy, in which the laser scans the entire field at the same intensity.

A problem with computerised techniques is the setting of a threshold level between the object to be analysed and the background. This introduces a level of subjectivity into the process. With a high contrast image the setting of the threshold is more reproducible, but the need to set each threshold level individually prohibits fully automated analysis. Future developments may make the automatic setting of a threshold possible.

2.4.3 Nerve density and orientation in human mesenteric vessels

2.4.3.1 Control vessels

Overall, there was a greater nerve density in mesenteric veins than in arteries. This is of interest because arteries are, in general, more densely innervated (Todd 1980). A previous study on vessels of the human greater omentum (which are part of the splanchnic circulation), demonstrated a lower nerve density in the veins (Edvinsson et al. 1985). In the present study, both the ID and TFA were higher in veins than arteries, indicating that both the number of nerve bundles and the number of nerves was greater. However, the nerve bundle size, as indicated by the TFA/ID ratio, was similar in the two vessel types.
The ratio between the transverse and longitudinal intercept densities indicates the orientation of the nerve bundles, a ratio above unity indicating a more longitudinal fibre orientation. The nerves in the arteries were largely longitudinally orientated, whilst those of the veins formed a more random lattice. The significance of this is unclear, but may reflect differences in nerve function.

It is well recognised that density of innervation in blood vessels is very variable, both in different vascular beds within species and in similar vascular beds between species (Tagawa et al. 1979; Griffith et al. 1982; Matsuyama et al. 1983). These variations presumably reflect differences in function of the vessels. In a study examining the innervation of the mesenteric vessels of the rat, the nerve density was found to be directly related to the contractile response (Nilsson et al. 1986; Bevan, RD et al. 1998). Human mesenteric veins have an important role as capacitance vessels. Precise control of venous tone is required to maintain constant blood pressure when posture changes. The relatively greater nerve density in mesenteric veins compared with the arteries may reflect this role.

### 2.4.3.2 IBD vessels

In both the arteries and the veins from patients with IBD, there was an increase in overall nerve density, measured as the TFA for PGP, compared with the control vessels. Within the IBD vessels, the TFA for PGP was greater for the arteries than the veins, whilst there were no differences in the transverse or longitudinal intercept densities between the two vessel types. This indicates a higher total number of nerves in the arteries, within thicker bundles than in the veins. The TFA:ID ratio was also greater in the IBD vessels than control. It is notable that the orientation of
the nerves in the arteries in IBD is of a more random orientation than in the control vessels, as shown by the TID:LID ratio.

Vessels from patients with IBD therefore display a hyperinnervation in IBD, which is relatively greater in the arteries than in the veins. The pattern of the innervation in the IBD arteries is also more disordered than that in the control arteries. Factors that may contribute to this are discussed in Chapter 5.

2.4.3.3 Noradrenaline and neuropeptide Y

All the vessels had a plexus of NA and NPY-containing nerves. As a proportion of the total number of nerves, 100% of the nerves were immunoreactive to NPY, whereas 60% of arteries and 85% of veins displayed TH-LI. NA and NPY coexist in sympathetic nerves (Bumstock 1987; Racchi et al. 1999). NA and ATP are the major vasoconstrictor transmitters of the sympathetic nervous system, acting via $\alpha_1$ and $\alpha_2$ post-junctional and $P_{2X}$ receptors in the human mesenteric bed (Bumstock 1999a). The role of NPY is less clear. Although in some blood vessels it acts as a vasoconstrictor, this is not consistent, and there are wide regional and species differences (Ekblad et al. 1984). In many vessels, NPY acts as a neuromodulator, postjunctionally enhancing the contraction produced by other transmitters and/or acting prejunctionally to reduce the release of transmitters (Bumstock 1990b).

NPY has been found in nerves other than those of the sympathetic nervous system. It is known to exist in the enteric nervous system (Nichols et al. 1994a), and in some nerves of the parasympathetic nervous system (Modin 1994). There was a consistently higher number of NPY-containing nerves than TH-containing nerves in
the vessels studied. This may represent a non-sympathetic population of nerves containing this peptide.

The TFA for NPY was also higher than that for PGP in both arteries and veins, although the difference was not statistically significant. PGP was originally thought to be a neuronal marker that identified all nerve fibres, but some studies suggest that the growth-associated protein GAP-43 identifies a consistently greater number of nerve fibres than PGP in the human intestine (Vento and Soinila 1999). However, expression of this protein is also affected by neuronal sprouting (Verze et al. 1999) and inflammation (Reinert et al. 1998). Whether the use of GAP-43 results in more accurate identification of the total number of nerves requires further investigation.

2.4.3.4 Vasoactive intestinal peptide

A moderately dense plexus of nerves containing VIP was identified in both vessel types. VIP usually acts as a vasodilator peptide and is a cotransmitter together with acetylcholine and other neuropeptides in some parasympathetic nerves (Modin 1994). We were unable to demonstrate ChAT-containing nerves in this study but this may have been due to the unreliability of the antibody (Rossier 1975). A novel marker for neuronal acetylcholine, vesicular acetylcholine transporter (VACHT) has recently been identified (Arvidsson et al. 1997). This may prove to be a more reliable marker than ChAT.

Alternatively, the VIP containing nerves may not in this case be markers for the parasympathetic system. The role of parasympathetic innervation of the human mesenteric circulation has been questioned (Bennett and Gardiner 1996a). A study using VACHT to investigate cholinergic innervation of human submucosal arterioles found the VACHT-LI nerves to be intrinsic (Li et al. 1998).

Furthermore,
colocalisation studies with VIP identified the two markers in different nerve populations. In mesenteric vessels of the pig, the VIP-LI nerves have been shown to derive from the ENS (Shen et al. 1993). Nerves with VIP-LI originating from the ENS have been identified following intramural blood vessels (Costa and Furness 1983; Bornstein and Furness 1988). In humans, the VIP-LI innervation may also derive intrinsically from the gut.

Studies on the central projections of ENS nerve fibres have identified transmitters in addition to those investigated in this study (Mann et al. 1995). Nerves containing calbindin or bombesin, as well as VIP and ChAT, have been identified in the celiac ganglion and are thought to be projections from the gut. Further studies to identify the presence of transmitters such as these in perivascular nerves may help to clarify the presence of intestinofugal nerves in the blood vessels.

In the IBD vessels, VIP-LI nerves accounted for 30% and 25% of the total in the arteries and veins, respectively. This was not significantly different from the control vessels. There were no differences between the two diseases. The lack of alteration in the VIP innervation of the vessels is perhaps surprising. VIP is a peptide with a major role in the immune system of the gut. It has been suggested that VIP is involved in the suppression of the chronic inflammatory response of the GI tract (Pascual et al. 1994). Studies on changes in VIP innervation in the bowel wall have been contradictory (Sjölund et al. 1983; Kubota et al. 1992; Mazumdar and Das 1992), and changes may only occur close to the site of mucosal inflammation (O'Morain et al. 1984).
2.4.3.5 Substance P and CGRP

Fewer than 5% of the nerve fibres in the control vessels were immunoreactive to SP and CGRP. These neuropeptides are among the most potent vasodilators known. In addition, they interact with the immune system and mast cells and may have a role in immune functions (Eysselein et al. 1991; Ottaway 1991; Pascual et al. 1994). Both SP and CGRP have potent effects on microvascular tone and permeability (Brain 1997), and are involved in the protection of the mucosa by increasing mucosal blood flow (Raybould and Mayer 1991). The origin of nerves containing these transmitters in mesenteric vessels is debatable. They may indicate primary afferent sensory-motor nerves in which they are thought to be cotransmitters (Aberdeen et al. 1992; Belai et al. 1996). In larger mesenteric vessels, transplantation experiments have shown both SP- and CGRP-containing nerves have an extrinsic origin (Shen et al. 1993). Alternatively, these nerves represent projections of intrinsic neurones of the enteric nervous system which have been shown to contain CGRP and SP in humans (Dhatt and Buchan 1994). The lack of CGRP in the veins suggests that there may be fewer, if any, sensory-motor nerves present in those vessels, in contrast to arteries. In submucosal vessels, nerves containing SP and CGRP derived from the enteric nervous system, are responsible for local vasodilatation in response to luminal stimuli (Meehan et al. 1991). The central extent of these centrifugal fibres is unknown. Abnormalities in this nerve type would have implications in mesenteric and transmural bowel inflammation.

There were no significant differences in the SP-LI perivascular nerve density between IBD and control vessels, with SP- and CGRP-LI in less than 10% of nerve fibres in IBD vessels. The detection of CGRP-LI in the IBD veins may indicate
changes in the populations of intrinsic and sensory-motor nerves. Whether small changes in innervation density, undetectable in this study, are present in IBD requires further study. Colocalisation experiments would help to define the origin of the nerves.

### 2.4.3.6 5-Hydroxytryptamine

5-HT is often described as a ‘false neurotransmitter’ in sympathetic nerves (Verbeuren et al. 1983). It is not produced by the neurone but is taken up from extracellular sources and stored in vesicles for subsequent release. However, subpopulations of enteric neurones have been shown to contain 5-HT as a principal transmitter (Furness and Costa 1982). 5-HT nerves were not identified consistently in the vessels studied and fluorescence was less intense than for the other transmitters. The role of these 5-HT-containing nerves is unclear, although vasodilator functions have been reported (Webster et al. 1991). 5-HT vasoconstrictor nerves have been identified in human mesenteric vessels (Griffith and Burnstock 1983) and those of other animals (Yıldız and Tuncer 1995) and may represent projections from the enteric nervous system. The net action of 5-HT on the vessel wall is thought to depend upon the pre-existing vascular tone and the integrity of the endothelium, and probably the level of circulating 5-HT.

In spite of the low density of innervation, there was a definite increase in the number of 5-HT-LI nerves in IBD. In only one control artery and three control vein specimens were any 5-HT-LI nerves identified. In the IBD group, six arterial specimens (three from each group) and all twelve of the vein specimens were positive for 5-HT-LI nerves. The TID was 3-4 times greater in the IBD than in the
control vessels, in both the arteries and the veins. The reasons for this and its probable effects are discussed in Chapter 5.

2.4.3.7 Nitric oxide

It was not possible to detect NOS-positive nerves in this study. This is consistent with a previous study on human infant mesenteric vessels, which demonstrated a lack of nitrergic nerves in the mesenteric perivascular plexus, whilst identifying these nerves in the enteric nervous system (Nichols et al. 1994a). In contrast, NOS has been shown to be present in perivascular nerves in other vascular beds, notably the cerebral circulation in dogs (Yoshida et al. 1993) and monkeys (Yoshida et al. 1994).
2.5 Tables

Table 2.1. Total fluorescent area of perivascular nerves in human mesenteric arteries and veins (% total image area) in control vessels. Nerves were demonstrated in whole mount stretch preparations by fluorescence immunohistochemistry. This value represents the total number of nerves present. Figures given are mean ± 95% CI.

<table>
<thead>
<tr>
<th>Immunomarker</th>
<th>Arteries</th>
<th>Veins</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGP</td>
<td>6.94 (5.72-8.16)</td>
<td>9.31 (8.24-10.37)</td>
<td>0.03</td>
</tr>
<tr>
<td>TH</td>
<td>4.19 (3.54-4.84)</td>
<td>7.98 (6.91-9.05)</td>
<td>0.02</td>
</tr>
<tr>
<td>NPY</td>
<td>7.64 (6.08-9.21)</td>
<td>11.66 (11.25-12.07)</td>
<td>0.04</td>
</tr>
<tr>
<td>VIP</td>
<td>2.28 (1.95-2.61)</td>
<td>2.90 (2.50-3.31)</td>
<td>0.44</td>
</tr>
<tr>
<td>SP</td>
<td>0.24 (0.15-0.32)</td>
<td>0.31 (0.24-0.39)</td>
<td>0.68</td>
</tr>
<tr>
<td>CGRP</td>
<td>2.00 (1.16-2.82)</td>
<td>0</td>
<td>0.008</td>
</tr>
<tr>
<td>5-HT</td>
<td>1.10 (n = 1)</td>
<td>1.13 (n = 3)</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 2.2. Transverse (T) and longitudinal (L) intercept density (ID) of perivascular nerves in human mesenteric arteries and veins (nerves / 100μm) from control vessels. The ID is proportional to the number of nerve bundles present. The ratio (R) between the LID and TID is an index of the orientation of the nerve plexus. Figures given are mean ± 95% CI.

<table>
<thead>
<tr>
<th>Immunomarker</th>
<th>Arteries</th>
<th>Veins</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>3·43 (2·89-3·97)</td>
<td>5·32 (4·61-6·02)</td>
<td>0·05</td>
</tr>
<tr>
<td>L</td>
<td>1·59 (1·22-1·96)</td>
<td>3·49 (3·30-3·69)</td>
<td>0·02</td>
</tr>
<tr>
<td>R</td>
<td>3·06</td>
<td>1·53</td>
<td>0·14</td>
</tr>
<tr>
<td>TH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>2·20 (1·88-2·52)</td>
<td>4·54 (5·22-6·27)</td>
<td>0·02</td>
</tr>
<tr>
<td>L</td>
<td>0·95 (0·76-1·14)</td>
<td>3·53 (3·14-3·92)</td>
<td>0·002</td>
</tr>
<tr>
<td>R</td>
<td>2·85</td>
<td>1·39</td>
<td>&lt;0·01</td>
</tr>
<tr>
<td>NPY</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>3·37 (2·69-4·06)</td>
<td>5·75 (5·22-6·27)</td>
<td>0·08</td>
</tr>
<tr>
<td>L</td>
<td>1·74 (1·33-2·16)</td>
<td>4·12 (3·64-4·61)</td>
<td>0·04</td>
</tr>
<tr>
<td>R</td>
<td>2·33</td>
<td>1·53</td>
<td>0·15</td>
</tr>
<tr>
<td>VIP</td>
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<td></td>
</tr>
<tr>
<td>T</td>
<td>1·15 (0·98-1·32)</td>
<td>1·87 (1·37-2·36)</td>
<td>0·36</td>
</tr>
<tr>
<td>L</td>
<td>0·60 (0·53-0·66)</td>
<td>1·36 (1·11-1·60)</td>
<td>0·09</td>
</tr>
<tr>
<td>R</td>
<td>1·91</td>
<td>1·38</td>
<td>0·09</td>
</tr>
<tr>
<td>SP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>0·15 (0·10-0·21)</td>
<td>0·25 (0·18-0·32)</td>
<td>0·53</td>
</tr>
<tr>
<td>L</td>
<td>0·12 (0·8-0·16)</td>
<td>0·36 (0·27-0·46)</td>
<td>0·15</td>
</tr>
<tr>
<td>R</td>
<td>0·69</td>
<td>0·75</td>
<td>0·91</td>
</tr>
<tr>
<td>CGRP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>1·04 (0·64-1·45)</td>
<td>0</td>
<td>0·09</td>
</tr>
<tr>
<td>L</td>
<td>0·55 (0·36-0·74)</td>
<td>0</td>
<td>0·07</td>
</tr>
<tr>
<td>R</td>
<td>1·47</td>
<td>0</td>
<td>&lt;0·01</td>
</tr>
<tr>
<td>5-HT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>0·76 (n = 1)</td>
<td>0·85 (n = 3)</td>
<td>-</td>
</tr>
<tr>
<td>L</td>
<td>1·29</td>
<td>0·90</td>
<td>-</td>
</tr>
<tr>
<td>R</td>
<td>0·10</td>
<td>0·36</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 2.3. The ratio of total fluorescent area to intercept density (for the entire optical field) in control vessels (TFA:ID ratio). This represents the relative thickness of the nerve bundles (the higher the number, the thicker the bundles). Figures given are mean ± 95% CI.

<table>
<thead>
<tr>
<th>Immunomarker</th>
<th>Arteries</th>
<th>Veins</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGP</td>
<td>0.50 (0.47-0.54)</td>
<td>0.47 (0.42-0.51)</td>
<td>0.64</td>
</tr>
<tr>
<td>TH</td>
<td>0.48 (0.43-0.53)</td>
<td>0.44 (0.42-0.47)</td>
<td>0.70</td>
</tr>
<tr>
<td>NPY</td>
<td>0.58 (0.54-0.61)</td>
<td>0.54 (0.49-0.60)</td>
<td>0.75</td>
</tr>
<tr>
<td>VIP</td>
<td>0.50 (0.45-0.54)</td>
<td>0.49 (0.45-0.54)</td>
<td>0.96</td>
</tr>
<tr>
<td>SP</td>
<td>0.23 (0.16-0.30)</td>
<td>0.55 (0.42-0.68)</td>
<td>0.08</td>
</tr>
<tr>
<td>CGRP</td>
<td>0.45 (0.37-0.52)</td>
<td>0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>5-HT</td>
<td>0.57 (n = 1)</td>
<td>0.38 (n = 3)</td>
<td>0.58</td>
</tr>
</tbody>
</table>
Table 2.4. Total fluorescent area of perivascular nerves in human mesenteric arteries and veins (% total image area) in inflammatory bowel disease (IBD). Figures given are mean ± 95% CI.

<table>
<thead>
<tr>
<th>Immunomarker</th>
<th>Arteries</th>
<th>Veins</th>
<th>p value</th>
</tr>
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<tbody>
<tr>
<td>PGP</td>
<td>19.83 (18.32-21.35)</td>
<td>14.39 (13.80-14.98)</td>
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<tr>
<td>TH</td>
<td>19.78 (18.37-21.19)</td>
<td>13.63 (13.28-13.99)</td>
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<tr>
<td>NPY</td>
<td>19.81 (18.81-20.82)</td>
<td>17.26 (16.32-18.21)</td>
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<tr>
<td>VIP</td>
<td>5.08 (4.34-5.81)</td>
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<tr>
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<td>0.66 (0.55-0.77)</td>
<td>0.93</td>
</tr>
<tr>
<td>CGRP</td>
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<td>0.31 (0.23-0.40)</td>
<td>0.06</td>
</tr>
<tr>
<td>5-HT</td>
<td>2.78 (2.18-3.37)</td>
<td>2.02 (1.73-2.31)</td>
<td>0.42</td>
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</table>
Table 2.5. Transverse (T) and longitudinal (L) intercept density (ID) of perivascular nerves in human mesenteric arteries and veins (nerves / 100μm) from IBD vessels. The ratio (R) between the LID and TID is an index of the orientation of the nerve plexus. Figures given are mean ± 95% CI.

<table>
<thead>
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<th>Immunomarker</th>
<th>Arteries</th>
<th>Veins</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGP</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>T</td>
<td>6.80 (6.49-7.11)</td>
<td>6.65 (6.27-7.03)</td>
<td>0.88</td>
</tr>
<tr>
<td>L</td>
<td>5.23 (4.81-5.65)</td>
<td>4.81 (4.53-5.09)</td>
<td>0.66</td>
</tr>
<tr>
<td>R</td>
<td>1.60 (1.46-1.74)</td>
<td>1.53 (1.42-1.65)</td>
<td>0.84</td>
</tr>
<tr>
<td>TH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>6.96 (6.47-7.44)</td>
<td>5.39 (5.18-5.59)</td>
<td>0.13</td>
</tr>
<tr>
<td>L</td>
<td>5.32 (4.92-5.71)</td>
<td>5.00 (4.74-5.25)</td>
<td>0.69</td>
</tr>
<tr>
<td>R</td>
<td>1.52 (1.43-1.62)</td>
<td>1.26 (1.13-1.39)</td>
<td>0.44</td>
</tr>
<tr>
<td>NPY</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>6.64 (6.35-6.92)</td>
<td>7.10 (6.69-7.51)</td>
<td>0.52</td>
</tr>
<tr>
<td>L</td>
<td>5.26 (4.88-5.63)</td>
<td>6.05 (5.72-6.38)</td>
<td>0.36</td>
</tr>
<tr>
<td>R</td>
<td>1.46 (1.36-1.55)</td>
<td>1.34 (1.23-1.45)</td>
<td>0.71</td>
</tr>
<tr>
<td>VIP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>2.09 (1.75-2.43)</td>
<td>1.61 (1.45-1.77)</td>
<td>0.38</td>
</tr>
<tr>
<td>L</td>
<td>1.72 (1.49-1.96)</td>
<td>1.58 (1.40-1.75)</td>
<td>0.62</td>
</tr>
<tr>
<td>R</td>
<td>1.18 (1.08-1.28)</td>
<td>1.23 (1.11-1.35)</td>
<td>0.88</td>
</tr>
<tr>
<td>SP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>0.28 (0.23-0.33)</td>
<td>0.49 (0.38-0.60)</td>
<td>0.37</td>
</tr>
<tr>
<td>L</td>
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<td>0.44 (0.38-0.50)</td>
<td>0.20</td>
</tr>
<tr>
<td>R</td>
<td>0.71 (0.62-0.80)</td>
<td>0.88 (0.76-1.00)</td>
<td>0.50</td>
</tr>
<tr>
<td>CGRP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>0.35 (0.29-0.41)</td>
<td>0.19 (0.14-0.23)</td>
<td>0.24</td>
</tr>
<tr>
<td>L</td>
<td>0.47 (0.40-0.54)</td>
<td>0.28 (0.25-0.31)</td>
<td>0.11</td>
</tr>
<tr>
<td>R</td>
<td>0.63 (0.56-0.70)</td>
<td>0.91 (0.76-1.07)</td>
<td>0.36</td>
</tr>
<tr>
<td>5-HT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>1.15 (0.91-1.38)</td>
<td>1.49 (1.33-1.66)</td>
<td>0.44</td>
</tr>
<tr>
<td>L</td>
<td>1.40 (1.06-1.74)</td>
<td>1.22 (1.11-1.32)</td>
<td>0.74</td>
</tr>
<tr>
<td>R</td>
<td>0.38 (0.29-0.48)</td>
<td>1.34 (1.21-1.47)</td>
<td>&lt;0.001</td>
</tr>
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</table>
Table 2.6. The ratio of total fluorescent area to intercept density (TFA:ID ratio) in IBD. Figures given are mean ± 95% CI.

<table>
<thead>
<tr>
<th>Immunomarker</th>
<th>Arteries</th>
<th>Veins</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGP</td>
<td>0.74 (0.71-0.76)</td>
<td>0.61 (0.58-0.63)</td>
<td>0.04</td>
</tr>
<tr>
<td>TH</td>
<td>0.75 (0.71-0.79)</td>
<td>0.67 (0.65-0.69)</td>
<td>0.20</td>
</tr>
<tr>
<td>NPY</td>
<td>0.75 (0.72-0.77)</td>
<td>0.67 (0.64-0.70)</td>
<td>0.17</td>
</tr>
<tr>
<td>VIP</td>
<td>0.63 (0.58-0.68)</td>
<td>0.57 (0.54-0.60)</td>
<td>0.51</td>
</tr>
<tr>
<td>SP</td>
<td>0.47 (0.40-0.54)</td>
<td>0.39 (0.37-0.43)</td>
<td>0.45</td>
</tr>
<tr>
<td>CGRP</td>
<td>0.73 (0.62-0.85)</td>
<td>0.39 (0.37-0.42)</td>
<td>0.15</td>
</tr>
<tr>
<td>5-HT</td>
<td>0.36 (0.27-0.45)</td>
<td>0.47 (0.43±0.51)</td>
<td>0.53</td>
</tr>
</tbody>
</table>
2.6 Figures

Figure 2.1. Illustration of the transformations performed on digital images in image analysis. The original image (a) is inverted and converted to a binary image at the selected threshold (b). Minor speckles are then removed by the process of ‘closing’ (c). The total fluorescent area is then measured. After skeletonising (d), the intercept density is calculated.
Figure 2.2. Confocal images of transverse sections of a human mesenteric artery (a) and vein (b). Perivascular nerves stained for PGP 9-5 (green) and elastic tissue (red) can be seen. The red colour represents autofluorescence identified on the second channel of the confocal microscope. In the artery, nerves are located at the adventitial/medial border (closed arrows). Note the well-defined external elastic lamina. In the vein, nerves are distributed throughout the media. In (a), a large paravascular nerve bundle can be seen (open arrow). These nerve bundles do not contribute to the innervation of the vessel, at least at this site, and are excluded from the analysis. L, lumen; M, media; A, adventitia. Scale bar: 100 μm.
Figure 2.3. Confocal images of typical whole-mount preparations of human mesenteric arteries (left column) and veins (right column) from control subjects. The longitudinal axis of the vessels is from top to bottom. Immunostaining is for: PGP (a, b), TH (c, d), NPY (e, f). Scale bar: 50 μm.
Figure 2.4. Confocal images of typical whole-mount preparations of human mesenteric arteries (left column) and veins (right column) from subjects with IBD. The longitudinal axis of the vessels is from top to bottom. Immunostaining is for: PGP (a, b), TH (c, d), NPY (e, f). There is an increase in the nerve density in the arteries compared to the control vessels. Scale bar: 50 μm.
Figure 2.5. Confocal images of typical whole-mount preparations of human mesenteric arteries (left column) and veins (right column) from control subjects. The longitudinal axis of the vessels is from top to bottom. Immunostaining for: VIP (a, b) and SP (c, d). Scale bar: 50 μm.
Figure 2.6. Confocal images of typical whole-mount preparations of human mesenteric arteries (left column) and veins (right column) from subjects with IBD. The longitudinal axis of the vessels is from top to bottom. Immunostaining for: VIP (a, b) and SP (c, d). Scale bar: 50 μm.
Figure 2.7. Confocal images of typical whole-mount preparations of human mesenteric arteries (left column) and veins (right column) from control subjects. The longitudinal axis of the vessels is from top to bottom. Immunostaining for: CGRP (a, b) and 5-HT (c, d). No nerves with CGRP-LI were identified in the control veins. Scale bar: 50 μm.
Figure 2.8. Confocal images of typical whole-mount preparations of human mesenteric arteries (left column) and veins (right column) from subjects with IBD. The longitudinal axis of the vessels is from top to bottom. Immunostaining for: CGRP (a, b) and 5-HT (c, d). Nerves with CGRP-LI were identified in the veins from patients with IBD. In addition, nerves with 5-HT-LI were identified more consistently than in the control vessels. Scale bar: 50 μm.
Figure 2.9. Image analysis of total fluorescent area (TFA) of perivascular nerves of human mesenteric arteries and veins in control subjects and patients with IBD. The FA is a measure of the total number of nerve fibres present. Nerves were demonstrated in whole mount stretch preparations by fluorescence immunohistochemistry using antibodies against protein gene product 9.5 (PGP; a general neuronal marker), tyrosine hydroxylase (TH; a marker for noradrenaline) and neuropeptide Y (NPY). Figures given are mean ± 95% confidence interval. * indicates significant difference between control and IBD vessels (p<0.05; see text for values).
Figure 2.10. Image analysis of fluorescent area of perivascular nerves of human mesenteric arteries and veins in control subjects and patients with IBD. The FA is a measure of the total number of nerve fibres present. Nerves were demonstrated in whole mount stretch preparations by fluorescence immunohistochemistry using antibodies against vasoactive intestinal peptide (VIP), substance P (SP), calcitonin gene-related peptide (CGRP) and 5-hydroxytryptamine (5-HT). Figures given are mean ± 95% confidence interval.
Figure 2.11. Image analysis of transverse intercept density (TID) of perivascular nerves of human mesenteric arteries and veins in control subjects and patients with IBD. The TID is a measure of the number of nerve bundles present. Nerves were demonstrated in whole mount stretch preparations by fluorescence immunohistochemistry using antibodies against protein gene product 9-5 (PGP; a general neuronal marker), tyrosine hydroxylase (TH; a marker for noradrenaline) and neuropeptide Y (NPY). Figures given are mean ± 95% confidence interval. * indicates significant difference between control and IBD vessels (p<0.05; see text for values).
Figure 2.12. Image analysis of transverse intercept density of perivascular nerves of human mesenteric arteries and veins in control subjects and patients with IBD. The TID is a measure of the number of nerve bundles present. Nerves were demonstrated in whole mount stretch preparations by fluorescence immunohistochemistry using antibodies against vasoactive intestinal peptide (VIP), substance P (SP), calcitonin gene-related peptide (CGRP) and 5-hydroxytryptamine (5-HT). Figures given are mean ± 95% confidence interval. * indicates significant difference between control and IBD vessels (p<0.05; see text for values).
Figure 2.13. Image analysis of the ratio of fluorescent area to transverse intercept density of perivascular nerves of human mesenteric arteries and veins in control subjects and patients with IBD. This ratio indicates the relative thickness of the nerve bundles. Nerves were demonstrated in whole mount stretch preparations by fluorescence immunohistochemistry using antibodies against protein gene product 9.5 (PGP; a general neuronal marker), tyrosine hydroxylase (TH; a marker for noradrenaline) and neuropeptide Y (NPY). Figures given are mean ± 95% confidence interval. * indicates significant difference between control and IBD vessels (p<0.05; see text for values).
Figure 2.14. Image analysis of the ratio of fluorescent area to transverse intercept density of perivascular nerves of human mesenteric arteries and veins in control subjects and patients with IBD. This ratio indicates the relative thickness of the nerve bundles. Nerves were demonstrated in whole mount stretch preparations by fluorescence immunohistochemistry using antibodies against vasoactive intestinal peptide (VIP), substance P (SP), calcitonin gene-related peptide (CGRP) and 5-hydroxytryptamine (5-HT). Figures given are mean ± 95% confidence interval.
Chapter 3  *In vitro pharmacology responses of vessels to vasoactive substances and electrical field stimulation of perivascular nerves*
3.1 Introduction

The pathogenesis of IBD remains unclear. Disturbances in blood flow have been described in the mesenteric circulation of patients with IBD (Bolondi et al. 1992; Maconi et al. 1996; Erden et al. 1997) and ischaemia of the gut has been proposed as an aetiological factor (Pounder 1994). Since local control of vascular tone is governed by perivascular nerves and endothelial cells (Burnstock and Ralevic 1994), changes in the function of mesenteric perivascular nerves may be involved in the disturbance of blood flow in IBD.

In Chapter 2, changes in the pattern and density of perivascular nerves in mesenteric arteries and veins from patients with IBD were described. In this study the contractile responses of human mesenteric vessels were investigated to identify functional changes in the vessels from patients with IBD, using organ-bath pharmacology techniques.

Section 1 of this chapter describes the response of ring segments of arteries and veins to the application of exogenous NA, ATP and NPY. Section 2 outlines experiments to determine the contractile response of ring segments of the vessels to electrical field stimulation of the perivascular nerves. The responses of the normal vessels are determined and differences are examined between the responses of vessels from control patients and those with IBD.
Section 1: Responses to vasoactive substances

3.2 Methods

3.2.1 Acquisition of tissue

Mesenteric vessels were obtained from specimens resected from both male and female patients undergoing surgery for inflammatory and a control group with non-inflammatory conditions. Vessels were dissected from the mesentery close to the resection margin, adjacent to the bowel wall. This avoided interference with mesenteric lymph nodes, which are histologically examined for prognostic information, in the case of carcinoma specimens. Details of the age and sex of the patient, the nature of the operation, anatomical site of the vessels, comorbidity and current drug treatment were noted. Patient details are given in Appendix 1. The specimens were immediately placed in ice-cold Hank’s solution and transported to the laboratory.

Segments of artery and vein between 3-5mm long were carefully dissected from the specimen using a dissecting microscope and immediately placed into cold Krebs’ solution. Corresponding sections of artery and vein were kept together, to allow comparison of vessels from the same part of the mesentery.

3.2.2 Equipment

The segments of HMA and HMV were suspended in a 5ml organ bath in oxygenated Bülbring modified Krebs’ solution of the following composition: (mM) NaCl 133, KCl 4-7, NaH2PO4 1·35, NaHCO3 16·3, MgSO4 0·61, CaCl2 2·52 and glucose 7·8, and maintained at 37°C. The solution was bubbled with 95% O2 and 5% CO2.
Isometric tension was measured with Grass force-displacement transducers (model FT 03C, Grass Instrument Co., Quincy, MA, USA) and a DC preamplifier and recorded on a Grass polygraph (model 79D, Grass). The vascular segments were given an initial passive load of 1 - 3mN (arteries) and 0.5 - 2mN (veins) and allowed to equilibrate for 90 minutes.

### 3.2.3 Contractile responses

Cumulative concentration-response curves (CRC) were constructed for NA, NPY and ATP. The effect of the presence of a sub-threshold dose of NPY (30nM) on the contractile response of NA was assessed. Finally, 200μl 3.0 M KCl was added to the organ bath (120mM final concentration) to provoke maximal contraction of the vessel.

### 3.2.4 Analysis of data

Contractile responses were calculated as the percentage contraction to 120mM KCl to account for differences in vessel size. In specimens in which a maximal response was achieved (the contraction reaches a plateau), the maximal response expressed as a percentage of the maximal contraction to KCl 120mM was noted (Emax). Linear regression of the values in the sigmoid CRC, between 16% and 84% of the plateau value, against the log-concentration, was calculated to obtain the log-concentration giving the 50% maximal response (EC50). For each drug and vessel type, the mean and standard deviation of the Emax and EC50 was calculated. In specimens in which maximal contraction was not achieved, the potency of the agonists was evaluated from the -log concentration that gave 25% of the maximal contraction of the vessel to KCl 120mM (p[A]25). Comparisons were made between the corresponding
arteries and veins, and between vessels from patients with inflammatory conditions and non-inflamed controls. Results were compared by using Student's t-test for paired or unpaired data as appropriate. Data shown in the text and figures are reported as means ± 95% confidence interval (CI). For all tests, a probability level of 0.05 or less was considered significant.

3.3 Results

3.3.1 Control vessels

3.3.1.1 Contractile responses to NA and ATP

NA and ATP produced concentration-dependent contractions in both the arteries and the veins (figures 3.1 and 3.2). The results, normalised to the maximal contraction to KCl 120mM, are summarised in table 3.1 and represented as graphs in figures 3.3 and 3.4. At the maximum concentration of ATP (3 mM), the contractions in neither the arteries nor veins had reached a plateau, so the figure given in the table is the p[A]25. The maximum contraction of the arteries was significantly greater than the veins (p=0.0008), but there was a significant left-shift of the response curve to NA (p<0.0001) and ATP (p=0.0054) of the veins in comparison to the arteries (two-sample t-test).

3.3.1.2 Effect of NPY and its effect on the contractile response to NA

In the veins (n=6), but not the arteries (n=6), administration of NPY resulted in concentration-dependent contraction in most (5/6) of the specimens (figures 3.5, 3.6a; table 3.2). There was no response at all in any of the arterial specimens.
The effect of a sub-threshold dose of NPY on the contractility to NA was assessed by first recording the CRC to NA. Following incubation with a sub-threshold dose of NPY (30nM), the contractile response to NA was reassessed. The arterial response was unaffected (figure 3.7; table 3.2). In the veins, the $E_{\text{max}}$ was significantly greater, with a significant left-shift of the response curve ($EC_{50}$), indicating an increased sensitivity to NA (figure 3.8; table 3.2).

### 3.3.2 IBD and comparison with control vessels

#### 3.3.2.1 Contractile responses to NA and ATP

In samples from patients with IBD, there was also a concentration-dependent contraction to NA and ATP in the arteries and the veins (figures 3.9 and 3.10). Again, the response to ATP did not reach a plateau, so the figure for the $p[A]_{25}$ is given (table 3.3). The responses are illustrated graphically in figures 3.11 and 3.12.

The $E_{\text{max}}$, normalised to the response to KCl 120 mM was greater in the arteries than the veins ($p=0.0007$), and the CRC was shifted to the left in the veins compared to the arteries for both NA ($p=0.0004$) and ATP ($p=0.0062$) (two-sample t-test).

There was a statistically significant right shift of the CRC to ATP in the arteries from patients with IBD compared with control ($p = 0.021$). The other responses were not significantly different from the responses of control vessels (table 3.4).

#### 3.3.2.2 Effect of NPY and its effect on the contractile response to NA

In the vessels of patients with IBD, there was a concentration-dependent contraction on the addition of NPY in 6/9 veins, but again, no response from the arteries (figure
There was no significant difference between the responses of the veins from patients in the IBD and control groups.

The contractile response to NA was assessed before and after incubation of the vessels with a sub-threshold dose of NPY (30nM). There was a significant reduction of the \( E_{\text{max}} \) in the arteries, in the absence of a shift in the curve (figure 3.13; table 3.5). There was no effect on the veins in these vessels (figure 3.14; table 3.5).

The pre-incubation responses of the vessels to NA were not significantly different between the control and IBD groups (table 3.6). In the presence of NPY, the arterial \( E_{\text{max}} \) in the control group was significantly higher (\( p=0.003 \)), with no change in the \( EC_{50} \). The \( E_{\text{max}} \) of the veins was no different in the two groups, but there was a significant left-shift of the CRC in the control group (\( p=0.045 \)).

### 3.4 Discussion

These data indicate that NA and ATP induce consistent vasoconstriction in HMA and HMV. NPY also produces vasoconstriction in HMV, but there was no response of the HMA at the physiological concentrations employed in these experiments. The absolute force of contraction was greater in the arteries than the veins, as would be expected due to the greater muscle content of the arterial wall compared with veins of a similar diameter. The maximum contraction (\( E_{\text{max}} \)) was therefore measured relative to the vasoconstriction produced by a single dose of 120mM KCl. The contraction evoked by KCl is not specific for a particular receptor. This comparison thus eliminates variations due to differences in the relative sizes and muscularity of the vessels studied, whilst allowing assessment of the effects of specific vasoactive agents.
Application of exogenous NA and ATP to HMA and HMV segments from control subjects caused a dose-dependent contraction. The effect of NPY was to contract the veins on its own, and to modulate the contraction of the veins to NA. Experiments to study the effect of relaxant transmitters had been planned, but it proved impossible to maintain an adequate pre-constriction in these vessels due to rapid fatigue of the tissues.

3.4.1 Control vessels

The $E_{\text{max}}$ to NA of the arteries was greater than that of the veins. In many cases, the $E_{\text{max}}$ of the arteries was equivalent to, or even slightly greater than the contraction induced by 120mM KCl, whereas the contraction induced in the veins by NA was only one-half to two-thirds of their potential maximum contraction. However, the veins have greater post-junctional sensitivity to the actions of NA and ATP, as shown by the relative left-shift of the CRC.

In these experiments, NPY failed to induce a contraction in any of the arterial specimens examined. A rather variable contraction was induced in the veins by NPY alone, but the effect of its presence on the contraction induced by NA was marked. It increased the post-junctional sensitivity of the vessels as indicated by the left-shift of the curve and increased the force of contraction to 100% of the contraction induced by KCl. In the arteries, a ‘sub-threshold’ dose could not be defined, so the same dose was used as in the veins. This had no effect on the contractions due to NA.

These results indicate that the veins studied are responsive to the presence of exogenous neurotransmitters, and are more sensitive to their post-junctional actions than the arteries. The response of the arteries was a strong contraction to relatively
high concentrations of NA and ATP, without the neuromodulatory effects of NPY being apparent. It is known that the blood supply to the mucosa of the gut is carefully controlled, with wide regional variations dependent on many factors, including the intraluminal contents of the gut. In rats, it has been found that almost half of the mesenteric vascular resistance resides in the mesenteric arterial arcades (Fenger-Gron et al. 1995). Whether these findings can be extrapolated to human mesenteric vascular anatomy is unclear. It may be that the relatively large vessels studied here are not responsible for the 'fine tuning' of blood flow to the gut, but are more concerned with large-scale changes, such as during haemorrhage, when there is a massive release of vasoconstrictive substances. On the other hand, human mesenteric veins form the largest capacitance bed in man, and must respond quickly to changes in posture. It may be for this reason that the veins in this study display more complex interactions with the exogenous transmitters than the arteries.

3.4.2 IBD and comparison with control vessels

In vessels from patients with IBD, CRC to ATP in the arteries of the IBD group displayed a right-shift, indicating a reduced post-junctional sensitivity to this transmitter. The inflammatory process results in the release of a large number of mediators, including ATP. The reduction in sensitivity of the arteries may be due to down-regulation of \( P_2 \) receptors in the face of persistently elevated levels of ATP due to the inflammatory process.

The effects of NPY on vessels of patients in the IBD group differed from those in the control group. The arteries again failed to respond to exogenous NPY. The contractile response of the IBD veins to NPY tended to be higher than the control veins, but the difference was not statistically significant.
In the veins in the IBD group, presence of NPY did not significantly alter the contractile responses to NA, although there was a non-significant increase in the $E_{\text{max}}$, from 58% to 70% of the contraction to 120mM KCl. The increase in post-junctional sensitivity of the veins shown in the control group was not demonstrated, as indicated by the lack any further left-shift of the CRC. The $E_{\text{max}}$ of the control veins pre-treated with NPY, 100% of the concentration to KCl 120mM, was also higher than that of the post-NPY IBD group, but again not statistically significant. This indicates a reduction in the neuromodulatory actions of NPY in the IBD vessels.

In the arteries, the result of incubation of the vessels in the IBD group with NPY was a reduction in the $E_{\text{max}}$ from 108% to 91% of the contraction to 120mM KCl. This resulted in a significant difference between the $E_{\text{max}}$ of the control and IBD arteries.

In studies on the guinea-pig vas deferens, the dominant effect of exogenous NPY was pre-junctional inhibition of release of NA and ATP (Ellis and Burnstock 1990). This effect overrode the post-junctional enhancement of the contractile response. Although the reduction in contraction to exogenous NA is not explained by reduction of pre-junctional release of the transmitter, it may be that there is an NPY-dependent post-junctional mechanism for inhibition of contraction in vessels from patients with IBD.

The significance of these differences is unclear. It does appear that there are complex differences in the response of mesenteric blood vessels to neurotransmitters in IBD. The damage to axons of the ENS described in CD in areas of the gut that were not macroscopically inflamed may be involved (Dvorak and Silen 1985). Whether changes in the response of blood vessels contribute to the pathogenesis of
the diseases, or whether they are directly due to the inflammatory process, requires further investigation.
### 3.5 Tables

Table 3.1 Responses to contractile agents noradrenaline (NA) and adenosine triphosphate (ATP) in human mesenteric arteries and veins obtained from patients with non-inflammatory conditions. The maximal contraction ($E_{\text{max}}$) is recorded as the % contraction to 120mM KCl (arteries: $6.45 \pm 1.24$; veins: $2.99 \pm 0.54$ mN). $EC_{50}$ represents the -log of the concentration producing half maximal contraction. $p[\text{A}]_{25}$ represents the -log of the concentration producing 25% of the maximal contraction to KCl 120mM. Values are expressed as mean $\pm 95\%$ confidence interval with the number of observations in parentheses. Comparisons are by two-sample Student's t-test.

<table>
<thead>
<tr>
<th>Vessel</th>
<th>$E_{\text{max}}$ (%)</th>
<th>p</th>
<th>$EC_{50}$</th>
<th>p</th>
<th>$p[\text{A}]_{25}$</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artery</td>
<td>$124.3 \pm 19.3$ (6)</td>
<td>0.0008</td>
<td>$5.23 \pm 0.12$</td>
<td>&lt;0.0001</td>
<td>$3.22 \pm 0.15$ (7)</td>
<td>0.0054</td>
</tr>
<tr>
<td>Vein</td>
<td>$64.1 \pm 19.0$ (11)</td>
<td>0.07</td>
<td>$6.07 \pm 0.08$</td>
<td></td>
<td>$4.31 \pm 0.55$ (8)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2 Responses to neuropeptide Y, noradrenaline and NA in the presence of a sub-threshold concentration of NPY in human mesenteric arteries and veins obtained from patients with non-inflammatory conditions. There was a significant increase in the $E_{\text{max}}$ and $EC_{50}$ in the veins in the presence of NPY, but not in the arteries (mean $\pm 95\%$ CI, paired t-test).

<table>
<thead>
<tr>
<th></th>
<th>$E_{\text{max}}$ (%)</th>
<th>p</th>
<th>$EC_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPY</td>
<td>NA</td>
<td>NA + NPY</td>
<td>p</td>
</tr>
<tr>
<td>Artery (n=6)</td>
<td>$124.3 \pm 19.3$</td>
<td>0.15</td>
<td>0.75</td>
</tr>
<tr>
<td>Vein (n=6)</td>
<td>$39.3 \pm 25.7$</td>
<td>$60.6 \pm 4.3$</td>
<td>0.016</td>
</tr>
</tbody>
</table>
Table 3.3 Responses to contractile agents noradrenaline (NA) and adenosine triphosphate (ATP) in human mesenteric arteries and veins obtained from patients with inflammatory bowel disease. The maximal contraction \( E_{\text{max}} \) is recorded as the % contraction to 120mM KCl (arteries: 7.05 ± 0.55; veins: 5.29 ± 1.12mN). Values are expressed as mean ± 95% confidence interval with the number of observations in parentheses. Comparisons are by two-sample Student’s t-test.

<table>
<thead>
<tr>
<th>Vessel</th>
<th>NA ( E_{\text{max}} ) (%)</th>
<th>ATP ( E_{\text{max}} ) (%)</th>
<th>p</th>
<th>( E_{C50} )</th>
<th>p</th>
<th>( p[A]_{25} )</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artery</td>
<td>110.2 ± 18.2 (15)</td>
<td>5.33 ± 0.18</td>
<td>&lt;0.0004</td>
<td>2.59 ± 0.45 (12)</td>
<td>0.0062</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vein</td>
<td>59.8 ± 17.8 (13)</td>
<td>5.97 ± 0.24</td>
<td>3.63 ± 0.50 (12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.4 Responses to contractile agents noradrenaline (NA) and adenosine triphosphate (ATP) in human mesenteric arteries and veins obtained from patients with inflammatory bowel disease (IBD) and controls. Comparisons are made of the response of vessel types between the groups by two-sample Student’s t-test ± 95%CI. Number of observations in parentheses.

<table>
<thead>
<tr>
<th>Vessel</th>
<th>NA ( E_{\text{max}} ) (%)</th>
<th>ATP ( E_{\text{max}} ) (%)</th>
<th>p</th>
<th>( E_{C50} )</th>
<th>p</th>
<th>( p[A]_{25} )</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artery Control</td>
<td>124.3 ± 19.3 (6)</td>
<td>5.23 ± 0.12</td>
<td>0.38</td>
<td>3.22 ±0.15 (7)</td>
<td>0.021</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IBD</td>
<td>110.2 ± 18.2 (15)</td>
<td>5.33 ± 0.18</td>
<td>2.59 ± 0.45 (12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vein Control</td>
<td>64.1 ± 19.0 (11)</td>
<td>6.07 ± 0.08</td>
<td>0.48</td>
<td>4.31 ±0.56 (8)</td>
<td>0.091</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IBD</td>
<td>59.8 ± 17.8 (13)</td>
<td>5.97 ± 0.24</td>
<td>3.63 ± 0.50 (12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.5 Responses to neuropeptide Y, noradrenaline and NA in the presence of a sub-threshold concentration of NPY in human mesenteric arteries and veins obtained from patients with IBD. There was a significant decrease in the $E_{\text{max}}$ of the arteries in the presence of NPY, but no effect on the veins (mean ± 95% CI, paired t-test).

<table>
<thead>
<tr>
<th></th>
<th>$E_{\text{max}}$ (%)</th>
<th>$E_{\text{max}}$ (%)</th>
<th>EC$_{50}$</th>
<th>EC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NPY</td>
<td>NA</td>
<td>NA + NPY</td>
<td>p</td>
</tr>
<tr>
<td>Artery (n=12)</td>
<td>0</td>
<td>108·2 ± 22·7</td>
<td>91·0 ± 9·8</td>
<td>0·043</td>
</tr>
<tr>
<td>Vein (n=9)</td>
<td>42·1 ± 25·2</td>
<td>57·9 ± 25·7</td>
<td>72·6 ± 25·8</td>
<td>0·24</td>
</tr>
</tbody>
</table>

Table 3.6 Responses to noradrenaline (NA) prior to and following incubation with NPY (30nM) in human mesenteric arteries and veins obtained from patients with inflammatory bowel disease (IBD) and controls. The $E_{\text{max}}$ of the control arteries is significantly higher in the presence of NPY, with no change in the position of the curve. In the veins, there is no change in the maximum contraction of the vessel, but there is a significant left-shift of the concentration-response curve compared to the inflamed vessels. Comparisons are made of the response of vessel types between the groups by two-sample Student's t-test ± 95% CI. Number of observations in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>$E_{\text{max}}$ (%)</th>
<th>$E_{\text{max}}$ (%)</th>
<th>EC$_{50}$</th>
<th>EC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NA</td>
<td>NA + NPY</td>
<td>p</td>
<td>NA</td>
</tr>
<tr>
<td>Artery</td>
<td>Control</td>
<td>124·3 ± 19·3 (6)</td>
<td>110·6 ± 4·3</td>
<td>0·003</td>
</tr>
<tr>
<td>IBD</td>
<td>108·2 ± 22·6 (12)</td>
<td>91·0 ± 9·8</td>
<td>5·27 ± 0·19</td>
<td>5·34 ± 0·14</td>
</tr>
<tr>
<td>Vein</td>
<td>Control</td>
<td>51·6 ± 23·0 (6)</td>
<td>100·8 ± 34·9</td>
<td>0·20</td>
</tr>
<tr>
<td>IBD</td>
<td>57·9 ± 25·7 (9)</td>
<td>72·6 ± 25·8</td>
<td>5·87 ± 0·32</td>
<td>5·94 ± 0·43</td>
</tr>
</tbody>
</table>
Section 2: Responses to electrical field stimulation of perivascular nerves

3.6 Methods

Segments of artery and vein between 3-5 mm long were carefully dissected from mesenteric specimens as in Section 1. Patient details are given in Appendix 1.

The vessel segments were suspended in a 5ml organ bath in oxygenated Bülbbring modified Krebs' solution, as before. The vascular segments were given an initial passive load of 1-3mN (arteries) and 0.5-2mN (veins) and allowed to equilibrate for 90 minutes.

Electrical stimulation of the perivascular nerves was delivered to the tissues via two platinum wire electrodes placed parallel to and on either side of the vessel segment using a Grass SD9 stimulator. The voltage (100V) and the pulse width (0.3ms) were kept constant throughout the experiments. Previous experiments had shown a frequency of 20Hz to produce maximal stimulation, but there had also been rapid fatigue of the tissue (Yianni 1994). In view of this, sub-maximal frequencies were selected for use in these experiments, and construction of full frequency-response curves was not possible. The vessels were stimulated at 5 and 15 Hz, with a 10-minute interval between each stimulation.

The aim of the investigation was to identify the noradrenergic and purinergic components of the contractions. Stimulation of the vessels was therefore repeated after incubation of the tissues with neuroreceptor antagonists or desensitisers. Those used included prazosin (α₁; 1μM), yohimbine (α₂; 1μM), α, β-methylene adenosine
triphosphate (α, β-meATP; P2X desensitiser; 1μM) and suramin (P2X, P2Y; 10μM). The details are listed in the table (table 3.7).

At the end of each experiment, if there was any remaining response from the specimens, tetrodotoxin 1μM was added to the organ bath for twenty minutes, after which they were re-stimulated. Tetrodotoxin is a general neuronal sodium channel blocker, which ensures that all the observed contraction is neuronal in origin and not due to direct stimulation of the muscle. Finally, 200μl 3·0M KCl was added to the organ bath (120mM final concentration) to provoke maximal contraction of the vessel.

Results were expressed as percentages of the maximal contraction to account for variations in vessel size. Comparisons were made between vessels from patients with inflammatory conditions and non-inflamed controls. Selective blockade of specific neuroreceptors allowed the estimation of the proportion of the contraction that was due to each transmitter. The order of application of the antagonists was reversed to allow interactions of the transmitters to be assessed. Responses to stimulation at 5 and 15Hz were analysed, as transmitter release increases at higher frequencies (Kennedy et al. 1986). Results were compared by using Student’s t-test for paired or unpaired data, as appropriate. Data shown in the text and figures are reported as mean values ± 95% confidence intervals and a probability value of 0·05 or less was considered significant.
3.7 Results

3.7.1 Arterial stimulation

None of the arterial specimens tested (n=11; 5 control, 3 CD, 3 UC) showed any response to nerve stimulation, whilst their corresponding veins did respond. This was a consistent finding; so, after demonstrating this in a number of subjects, further nerve stimulation experiments were not carried out on arterial specimens.

3.7.2 Control veins

Stimulation for 15s at 5 (n = 5) and 15 Hz (n = 10) resulted in contractions of 5·3 ± 3·5% and 9·1 ± 3·6% respectively. Having established that the vessels responded to nerve stimulation, the effects of the addition of neuroreceptor antagonists or desensitisers were investigated.

Following pre-incubation of the vessels for 20 minutes with prazosin (1μM), the vessels were re-stimulated. There was a significant reduction in the contraction at both frequencies. At 5Hz, the contraction was reduced by 75% from 6·3 ± 3·8 to 1·5 ± 1·7 (n = 4, p = 0·042, paired t-test). At 15Hz, there was a 63% reduction from 10·2 ± 4·2 to 3·8 ± 2·3 (n=7, p = 0·0029, paired t-test).

The vessel segments were then re-incubated with suramin (10 μM; 5Hz, n = 3; 15Hz, n = 4) or α,β-meATP (1μM; 5Hz, n = 1; 15Hz, n = 3). This resulted in a further reduction in the contraction to 6% of the initial contraction at 5Hz and 26% at 15Hz (table 3.8; figure 3.15).

Having blocked the contractions due to α1 adrenoceptors and P2 purinoceptors, yohimbine (1μM) was added to the organ baths in some cases, to assess the contribution of α2 adrenoceptors to the residual contraction. At 5Hz, yohimbine
abolished the residual contraction (n = 4). At 15Hz, a small residual contraction remained in 2/4 specimens following incubation with yohimbine. This was abolished by tetrodotoxin 1μM (figures 3.15, 3.16).

In some specimens, the order of application of the antagonists was reversed (5Hz, n= 1; 15Hz, n= 3). In these cases, the vessels were stimulated after incubation with suramin (10μM). This was followed by prazosin (1μM) after which they were re-stimulated. At 5Hz, the contraction was reduced by 67% after incubation with suramin. The addition of prazosin to the organ-bath resulted in complete abolition of the contraction. At 15Hz, suramin reduced the contraction by a mean of 40% and again, prazosin abolished the residual contraction (table 3.9; figures 3.17, 3.18).

### 3.7.3 Inflammatory bowel disease

In the absence of any neurotransmitter blockade, the mean contraction of the veins from patients with IBD was 11.8 ± 4.3 and 33.0 ± 14.3 at 5 and 15Hz respectively (mean ± 95% CI). Following incubation with prazosin the mean contraction was 4.3 ± 1.7 at 5 Hz and 26.6 ± 13.6% at 15Hz. The addition of P₂ purinergic blockade resulted in contractions of 3.8 ± 1.9 and 17.9 ± 8.5 at 5 and 15Hz respectively (figure 3.19, 3.20; table 3.10). Yohimbine abolished any residual contraction in 4/9 specimens at 5Hz and 3/9 at 15Hz. Any residual contraction was abolished by tetrodotoxin 1μM, indicating that it was neuronally mediated.

Reversing the order of incubation with the antagonists revealed a different pattern of response to the control vessels, with wide variability within the IBD group (figures 3.21, 3.22; table 3.11). Incubation with suramin did not result in such a large reduction in the contraction at either frequency; in fact, at 5Hz there was an increase in the size of the contraction in one specimen. The addition of prazosin to the organ...
bath did not abolish the contraction at either frequency in two cases. In these, the addition of yohimbine 1μM abolished the contraction (figure 3.22).

3.7.4 Comparison of control and IBD vessels

On comparison with control veins, the response of the IBD veins was not significantly different from that of the control vessels at 5Hz (p = 0.18). At this frequency, the mean reduction of the percentage contraction of the control vessels after α₁ antagonism was greater in control than IBD vessels (76% v. 63%), although the difference was not significant. Only 6% of the contraction in the control vessels was resistant to P₂ antagonism, compared with 32% of the contraction of IBD vessels (p=0.05; two sample t-test).

At 15Hz, the contraction of the IBD vessels was significantly greater than control (p = 0.04; two sample t-test). A significantly greater proportion of the contraction was resistant to α₁ antagonism (81% v 37%; p=0.03) and to P₂ blockade (54% v 26%; p=0.02) in the IBD vessels than the control vessels (table 3.12).
3.8 Tables

Table 3.7 List of receptor antagonists

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Concentration</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>prazosin</td>
<td>$\alpha_1$</td>
<td>1µM</td>
</tr>
<tr>
<td>yohimbine</td>
<td>$\alpha_2$</td>
<td>1µM</td>
</tr>
<tr>
<td>$\alpha,\beta$-meATP</td>
<td>$P_{2X}$</td>
<td>1µM</td>
</tr>
<tr>
<td>suramin</td>
<td>$P_{2X}, P_{2Y}$</td>
<td>10µM</td>
</tr>
</tbody>
</table>

Table 3.8 Contractions of control isolated human mesenteric vein segments to electrical field stimulation for 15s (15Hz, 0.3ms pulse width, 100V), given as the percentage of the maximal contraction to 120mM KCl. Effects of prazosin (1µM) followed by a combination of prazosin and a $P_2$ purinergic receptor antagonist (suramin or $\alpha,\beta$-meATP) on neurogenic contractions. Figures in parentheses indicate the percentage reduction in contraction.

<table>
<thead>
<tr>
<th>Frequency</th>
<th>No antagonist</th>
<th>+ prazosin</th>
<th>+ $P_2$ blockade</th>
<th>+ yohimbine</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 Hz</td>
<td>6.3 ± 3.8</td>
<td>1.5 ± 1.7 (75%)</td>
<td>0.4 ± 0.4 (94%)</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>15 Hz</td>
<td>10.2 ± 4.2</td>
<td>3.8 ± 2.3 (63%)</td>
<td>2.7 ± 1.7 (74%)</td>
<td>0.5 ± 0.5 (n=4)</td>
<td>7</td>
</tr>
</tbody>
</table>
Table 3.9 Contractions of isolated human mesenteric vein segments from control patients to electrical field stimulation for 15s (15Hz, 0.3ms pulse width, 100V), given as the percentage of the maximal contraction to 120mM KCl. Effects of suramin (10μM) followed by a combination of suramin and prazosin on neurogenic contractions. Figures in parentheses indicate the percentage reduction in contraction.

<table>
<thead>
<tr>
<th>Frequency</th>
<th>No antagonist</th>
<th>+ suramin</th>
<th>+ prazosin</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 Hz</td>
<td>1.5 ± 0.3</td>
<td>0.5 (67%)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>15 Hz</td>
<td>6.5 ± 3.6</td>
<td>3.9 ± 1.0 (40%)</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 3.10 Contractions of IBD isolated human mesenteric vein segments to electrical field stimulation for 15s (15Hz, 0.3ms pulse width, 100V), given as the percentage of the maximal contraction to 120mM KCl. Effects of prazosin (1μM) followed by a combination of prazosin and a P₂ purinergic receptor antagonist (suramin or α,β-meATP) on neurogenic contractions. Figures in parentheses indicate the percentage reduction in contraction.

<table>
<thead>
<tr>
<th>Frequency</th>
<th>No antagonist</th>
<th>+ prazosin</th>
<th>+ P₂ blockade</th>
<th>+ yohimbine</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 Hz</td>
<td>11.7 ± 4.2</td>
<td>4.4 ± 1.7 (63%)</td>
<td>3.8 ± 1.8 (67%)</td>
<td>1.8 ± 1.9</td>
<td>11</td>
</tr>
<tr>
<td>15 Hz</td>
<td>33.0 ± 14.3</td>
<td>26.7 ± 13.6 (19%)</td>
<td>17.8 ± 8.5 (46%)</td>
<td>5.9 ± 7.0</td>
<td>13</td>
</tr>
</tbody>
</table>
Table 3.11 Contractions of isolated human mesenteric vein segments from patients with IBD to electrical field stimulation for 15s (15Hz, 0.3ms pulse width, 100V), given as the percentage of the maximal contraction to 120mM KCl. Effects of suramin (10µM) followed by a combination of suramin and prazosin on neurogenic contractions. Figures in parentheses indicate the percentage reduction in contraction.

<table>
<thead>
<tr>
<th>Frequency</th>
<th>No antagonist</th>
<th>+ suramin</th>
<th>+ prazosin</th>
<th>+ yohimbine</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 Hz</td>
<td>10.7 ± 8.4</td>
<td>10.8 ± 13.3 (101%)</td>
<td>2.9 ± 5.1 (73%)</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>15 Hz</td>
<td>53.0 ± 38.0</td>
<td>43.6 ± 29.0 (18%)</td>
<td>31.7 ± 21.3 (40%)</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 3.12 Comparison of contraction of control v. IBD human mesenteric veins to EFS at different stimulation frequencies, expressed as a proportion of the maximal contraction to 120mM KCl ± 95% CI. Figures in parentheses are the proportion of the maximal contraction to EFS.

<table>
<thead>
<tr>
<th>Receptor blockade</th>
<th>Control (n=4)</th>
<th>IBD (n=11)</th>
<th>p</th>
<th>Control (n=7)</th>
<th>IBD (n=13)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>6.3 ± 3.8</td>
<td>11.9 ± 4.2</td>
<td>0.16</td>
<td>10.2 ± 4.2</td>
<td>33.0 ± 14.35</td>
<td>0.038</td>
</tr>
<tr>
<td>α1</td>
<td>1.5 ± 1.7 (24%)</td>
<td>4.4 ± 1.7 (37%)</td>
<td>0.10</td>
<td>3.8 ± 2.3 (37%)</td>
<td>26.7 ± 13.6 (81%)</td>
<td>0.029</td>
</tr>
<tr>
<td>α1 + P2</td>
<td>0.4 ± 0.4 (6%)</td>
<td>3.8 ± 1.8 (32%)</td>
<td>0.049</td>
<td>2.7 ± 1.7 (26%)</td>
<td>17.8 ± 8.5 (54%)</td>
<td>0.020</td>
</tr>
</tbody>
</table>

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3.9 Discussion

EFS of human mesenteric veins produces frequency-dependent contraction. This contraction is relatively small, but small reductions in vessel diameter have a large influence on both blood flow and the volume of the vascular bed. These neurogenic contractions were partially inhibited at both five and 15Hz by the receptor antagonists prazosin and suramin. The proportion of the contraction inhibited by prazosin was greater at 5Hz than at 15Hz (75% v. 63%). When $\alpha_1$ preceded $P_2$ blockade, a small residual contraction at 5Hz was abolished by yohimbine. At 15Hz, there was a larger residual contraction, which was almost completely abolished by yohimbine. These results suggest that NA and ATP are cotransmitters in HMV, and indicate the presence of post-junctional $\alpha_1$, $P_{2X}$ and $\alpha_2$ receptors. This supports previous work on human ovarian veins (Stones et al. 1994). Cotransmission of NA and ATP have been described previously in arteries of a variety of animals, but a purinergic response is less commonly found in veins (Sneddon and Burnstock 1984; Burnstock and Warland 1987; Machaly et al. 1988; McLaren et al. 1995).

The HMV from patients with IBD also displayed frequency-dependent contractions to EFS. At 5Hz, the magnitude of the contraction was not significantly greater than that of the controls, but at 15Hz, there was marked enhancement of the contractile response. The response at 5Hz differed from that of control vessels, in that prazosin only reduced the response by 63%, and the residual response was resistant to suramin or $\alpha_\beta$-meATP. On reversing the order of antagonist application, suramin again had no effect, although prazosin reduced the residual contraction by 73%.
At 15 Hz, prazosin only reduced the large EFS-induced contraction by 19%. At this frequency, P2X antagonism reduced the contraction by 46% of the initial response, leaving a considerable residual contraction. In some, but not all, this residual contraction was abolished by yohimbine, indicating that a much higher proportion of the contraction was mediated by α2 receptors. Any remaining contraction was abolished by tetrodotoxin, indicating that the response was due to nerve, and not direct muscle, stimulation. In one of the specimens demonstrating this augmented contraction, it was found to be resistant to ketanserin (a 5-HT S2 antagonist) and 1:10 rabbit anti-NPY antiserum. Further work is necessary to identify the agent responsible for the increased contraction in IBD.

Assessment of relaxant responses of pre-constricted specimens to nerve stimulation proved impossible, due to an inability of the tissue to sustain a reliable preconstriction due to fatigue of the tissues. Because of the scarcity of the clinical material, after a few attempts this part of the experiment was abandoned.

Non-responsiveness of arterial specimens has been noted in animal studies (Gallen et al. 1982; Cowen 1984), in which the ultrastructural characteristics of the vessels were defined. They indicated that in the non-responsive vessels, the nerves lay external to the EEL and were distant from the smooth muscle cells. Work to establish the ultrastructural relationships of the PVN to smooth muscle in human arteries is currently being carried out.

The arteries studied were relatively large. Although they possessed a muscular media, it may be that their main role is as distributing arteries. In the guinea pig, mesenteric arteries were very responsive to nerve stimulation (Cowen 1984). In the rat mesenteric circulation, one-third of the resistance to flow originated in the
arteries in the mesentery outside the bowel wall (Fenger-Gron et al. 1995). However, although these vessels are anatomically equivalent to the HMA studied, they are of a much smaller diameter. In the HMA, it is likely the resistance vessels are the smaller vessels within the bowel wall. Functional studies on these vessels would be difficult. Immunohistochemical studies of whole-mount preparations of small bowel have identified abnormalities of the intramural arterial innervation in CD (Belai et al. 1997). This may indicate differences in the contractility of these vessels.

These results suggest that vascular responses to nerve stimulation are abnormal in IBD. The arterial responses were not directly investigated, due to the lack of response in the arterial specimens. The vessels responsible for the majority of bowel wall blood flow control in man are likely to be the smaller arteries and arterioles of the intramural vascular plexus, which are inaccessible using these techniques. The enhanced response of the mesenteric veins in IBD may reflect a similar increased responsiveness of the resistance arteries within the bowel wall. If this were the case, it would help to explain abnormalities in intestinal blood flow, which are thought to contribute to the pathogenesis of IBD.
3.10 Figures

Figure 3.1 Trace examples of responses to noradrenaline (NA) in isolated ring preparations of human mesenteric vessels. (a) control mesenteric artery; (b) control mesenteric vein.
Figure 3.2 Trace examples of responses to adenosine 5'-triphosphate (ATP) in isolated ring preparations of human mesenteric vessels. (a) control mesenteric artery; (b) control mesenteric vein.
Figure 3.3 Responses of isolated ring preparations to NA in control mesenteric arteries (n=6) and control mesenteric veins (n=11).

Figure 3.4 Responses of isolated ring preparations to ATP in control mesenteric arteries (n=7) and veins (n=8).
Figure 3.5 Responses of isolated ring preparations to NPY in control mesenteric arteries (n=6) and veins (n=6).
Figure 3.6 Trace examples of responses to neuropeptide Y in isolated ring preparations of human mesenteric vessels. (a) control mesenteric vein; (b) IBD mesenteric vein. No response was obtained from the arteries to NPY.
Figure 3.7 Responses of isolated ring preparations of control mesenteric arteries veins to NA before and after incubation with a sub-threshold dose of NPY (10^{-5} M).

Figure 3.8 Responses of isolated ring preparations of control mesenteric veins to NA before and after incubation with a sub-threshold dose of NPY (10^{-5} M).
Figure 3.9 Trace examples of responses to noradrenaline (NA) in isolated ring preparations of human mesenteric vessels. (a) IBD mesenteric artery; (b) IBD mesenteric vein.
Figure 3.10Trace examples of responses to ATP in isolated ring preparations of human mesenteric vessels. (a) IBD mesenteric artery; (b) IBD mesenteric vein.
Figure 3.11 Responses of isolated ring preparations to NA in IBD mesenteric arteries (n=15) and IBD mesenteric veins (n=13).

Figure 3.12 Responses of isolated ring preparations to ATP in IBD mesenteric arteries (n=12) and veins (n=12).
Figure 3.13 Responses of isolated ring preparations of IBD mesenteric arteries (n=12) to NA before and after incubation with a sub-threshold dose of NPY ($10^{-8}$M).

Figure 3.14 Responses of isolated ring preparations of IBD mesenteric veins (n=9) to NA before and after incubation with a sub-threshold dose of NPY ($10^{-8}$M).
Figure 3.15 Trace examples of responses to electrical field stimulation. Contractions of isolated control human mesenteric vein (HMV) ring preparations to electrical field stimulation (EFS) at 5 and 15 Hz (5 s, 0.3 ms pulse width, 100 V). Neurogenic contractions produced in the absence of any receptor antagonist are compared with those obtained following α₁-adrenoceptor blockade by prazosin (1 μM), P₂ blockade by suramin (10 μM) and finally α₂-adrenoceptor blockade by yohimbine (1 μM).
Figure 3.16 Responses of control human mesenteric vein (HMV) ring preparations to electrical field stimulation at 5 and 15Hz (5s, 0.3ms pulse width, 100V). Neurogenic contractions produced in the absence of any receptor antagonist are compared with those obtained following α1-adrenoceptor blockade by prazosin (1μM), P2 blockade by suramin (10μM) and finally α2-adrenoceptor blockade by yohimbine (1μM). Each point represents the mean contraction as a percentage of the contraction induced by 120mM KCl. Error bars represent 95% confidence intervals.
Figure 3.17 Contractions of isolated control HMV ring preparations to EFS at 5 and 15Hz (5s, 0.3ms pulse width, 100V). Neurogenic contractions are compared with those obtained following P2 blockade by suramin (10μM) α1-adrenoceptor blockade by prazosin (1μM), and finally α2-adrenoceptor blockade by yohimbine (1μM).
Figure 3.18 Neurogenic contractions produced in the absence of any receptor antagonist are compared with those obtained following P2 blockade by suramin (10μM) blockade followed by α1-adrenoceptor by prazosin (1μM). Each point represents the mean contraction as a percentage of the contraction induced by 120mM KCl. Error bars represent 95% confidence intervals.
Figure 3.19 Trace examples of responses to electrical field stimulation. Contractions of isolated IBD human mesenteric vein ring preparations to electrical field stimulation (EFS) at 5 and 15 Hz (5s, 0.3 ms pulse width, 100 V). Neurogenic contractions produced in the absence of any receptor antagonist are compared with those obtained following $\alpha_1$-adrenoceptor blockade by prazosin (1 $\mu$M), $P_2$ blockade by suramin (10 $\mu$M) and finally $\alpha_2$-adrenoceptor blockade by yohimbine (1 $\mu$M).
Figure 3.20 Responses of IBD human mesenteric vein (HMV) ring preparations to electrical field stimulation at 5 and 15Hz (5s, 0.3ms pulse width, 100V). Neurogenic contractions produced in the absence of any receptor antagonist are compared with those obtained following $\alpha_1$-adrenoceptor blockade by prazosin (1µM), $\beta_1$ blockade by suramin (10µM) and finally $\alpha_2$-adrenoceptor blockade by yohimbine (1µM). Each point represents the mean contraction as a percentage of the contraction induced by 120mM KCl. Error bars represent 95% confidence intervals.
Figure 3.21 Contractions of isolated IBD HMV ring preparations to EFS at 5 and 15Hz (5s, 0.3ms pulse width, 100V). Neurogenic contractions are compared with those obtained following P2 blockade by suramin (10μM) α1-adrenoceptor blockade by prazosin (1μM), and finally α2-adrenoceptor blockade by yohimbine (1μM).
Figure 3.22 Neurogenic contractions produced in the absence of any receptor antagonist are compared with those obtained following $P_2$ blockade by suramin (10μM) blockade followed by $\alpha_1$-adrenoceptor by prazosin (1μM). Each point represents the mean contraction as a percentage of the contraction induced by 120mM KCl. Error bars represent 95% confidence intervals.
Chapter 4  The ultrastructure and function of human mesenteric arteries and veins
4.1 Introduction

Perivascular nerves supply the smooth muscle of human mesenteric blood vessels, working with the endothelium as part of the 'dual control' mechanism to control blood flow to the organs (Burnstock and Ralevic 1994). The nerves are primarily sympathetic, containing noradrenaline, adenosine 5'-triphosphate and neuropeptide Y as cotransmitters (Saville et al. 1990). Sensory-motor nerves (Maynard et al. 1990) and projections of nerve fibres from the enteric nervous system may also be present (Costa and Furness 1983; Ekblad et al. 1988).

It has previously been shown that human mesenteric arteries were non-responsive to transmural electrical stimulation of the perivascular nerves, whilst the corresponding veins responded with contraction (Chapter 3). Studies of vessels from a number of vascular beds in the guinea-pig have shown a wide variability in the response to nerve stimulation in vitro (Vanhoutte 1978; Bevan, JA and Brayden 1987; Morris and Gibbins 1990; Ralevic and Burnstock 1996). For example, guinea-pig mesenteric arteries were responsive, whereas the renal arteries were not. Ultrastructural differences were identified between the vessels which may account for the difference in response, (Cowen 1984) including a wide neuromuscular gap and the presence of interposed connective tissue in the non-functional group.

There are surprisingly few published morphological studies on the innervation of human vessels (Luff 1996). Thus, the purpose of this study was to investigate differences in the ultrastructural relationships of the nerves and smooth muscle in these vessels that may relate to the effects of nerve stimulation. Comparisons were also made between vessels from patients with IBD and controls.
4.2 Methods

4.2.1 Acquisition of tissue

Mesenteric vessels were obtained from specimens resected from patients undergoing surgery for inflammatory (n=3) and non-inflammatory (n=3) conditions. Vessels were dissected from the mesentery close to the resection margin, adjacent to the bowel wall. This avoided interference with mesenteric lymph nodes, which are histologically examined for prognostic information, in the case of carcinoma specimens. Informed consent for the procedures was obtained, using a standard consent form. Details of the age and sex of the patient, the nature of the operation, anatomical site of the vessels, comorbidity and current drug treatment were noted. Patient details are given in Appendix 1.

Corresponding segments of mesenteric artery and vein were carefully dissected from the specimen using a dissecting microscope. The vessels were those directly entering the bowel wall (the vasa recta). These segments were fixed in a freshly made solution of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer, prior to processing for electron microscopy.

4.2.2 Electron microscopy

Specimens were transferred to a solution of 1% osmium tetroxide in 0.1M cacodylate buffer for secondary fixation, after which they were stained en bloc with 2% uranyl acetate in distilled water and embedded in Araldite resin. Ultrathin sections were then cut. Secondary staining was performed with 4% uranyl acetate and Reynolds lead citrate. The sections were viewed on a JEM 1010 transmission electron microscope. The location of perivascular nerves was determined using both
low and high power to determine the relationship of the nerves to the vascular smooth muscle and other components of the vessel wall. To quantify the nerve densities of the vessels, each image was examined and the number of nerves counted. The total area of the adventitia and the media was then determined (in mm²) and the number of nerves per square millimetre was calculated. Corresponding arteries and veins were compared within the patient groups, and differences between control and IBD groups were assessed. Results are given as mean values ± 95% confidence interval (CI) and P values of less than 0.05 were considered significant.

4.3 Results

4.3.1 Assessment of images

Low-power light micrographs confirmed the structure of the vessels (figure 4.1). Each showed three identifiable layers comprising the outer fibrous adventitia, the muscular media and the endothelial monolayer supported on a basement membrane. In the arteries, the layers were defined by two elastic laminae: the inner elastic lamina (IEL) between the endothelium and the media and the external elastic lamina (EEL) between the media and the adventitia. In the veins, the EEL is not seen as a discrete layer; the elastic tissue within these vessels was scattered throughout the vessel wall.

Electron micrographs identified the nerves. In both the arteries (figure 4.2) and the veins (see below), they appeared as unmyelinated fibres, with a number of neurones enveloped within a single Schwann cell sheath. Within each nerve fibre, a number of vesicles can be seen. There is a well-developed fenestrated EEL separating the
medial smooth muscle cells from the adventitial connective tissue (figure 4.2a). Perivascular nerves in the arteries were all external to the EEL (figure 4.2b). None of the nerve profiles seen came closer than 2000μm to the smooth muscle. Many nerve bundles are surrounded by collagen and do not appear to be associated with any structure (figure 4.2c).

The smooth muscle cells of the arterial media are orientated in a circular fashion, with little intercellular connective tissue (figure 4.3a, b). The intima in these vessels was rather thickened, due to sub-endothelial connective tissue deposition. Within the connective tissue, randomly orientated intimal smooth muscle cells were identified (figure 4.3c).

In contrast to the arteries, the nerves in the veins were situated throughout the muscular media. In many of the images, the nerve fibres approach the muscle cells and are in close apposition to them (figures 4.4b, c, d; 4.5b).

In some of the veins, capillaries of the vasa venorum were noted. These appeared to be related to nerve bundles in some cases (figure 4.4a). As in the arteries, the intima was thickened, with sub-intimal connective tissue and smooth muscle cells (figure 4.5a).

In the vessels from patients with IBD, the distribution of perivascular nerves was similar to the control vessels, with nerve bundles confined to the adventitial/medial border in the arteries, and distributed throughout the media in the veins. However, in the arteries, closer apposition of the nerve bundles to the smooth muscle cells was seen in places, compared to the control vessels, although they still appeared to be external to the EEL (figure 4.6a). In the veins, nerve bundles approach the smooth muscle cells closely, with little intervening tissue (figure 4.6b).
In the vein specimens from one of the IBD subjects, nerve fibres were found in the sub-endothelial layer of the intima (figure 4.6c). Nerve fibres at this site were not seen in the other vessels from patients in the IBD group, or in any of the control vessels. The unmyelinated fibres contained small granular and agranular vesicles, although they were distant from the vascular smooth muscle of the vessel wall.

4.3.2 Nerve density

The nerve densities of the two vessel types differed significantly. In the control arteries, the mean nerve density was 41mm$^{-2}$ whereas in the control veins, the density was 227mm$^{-2}$ ($P=0.03$, paired $t$-test; 95% CI of difference: 122.5 – 250.7). This difference was not seen in the IBD vessels. The mean nerve density in the IBD vessels was 367mm$^{-2}$ in the arteries and 346mm$^{-2}$ in the veins ($P=0.78$, paired $t$-test; 95% CI of difference: -144.9 – 103.7). The greater nerve density in the IBD arteries was statistically significant ($P=0.0008$, unpaired $t$-test), although the increase in the veins was not ($P=0.21$).

4.4 Discussion

The veins were much more densely innervated than the corresponding arteries, confirming the immunohistochemical findings in Chapter 2. This is of interest, since in general arteries are more heavily innervated (Todd 1980; Ralevic and Burnstock 1996). These vessels also had a thick muscular media. Human mesenteric veins have an important role in controlling mesenteric capacitance in man (Hainsworth 1986; Hogan et al. 1994). The high nerve density may reflect the need for fine control of their tone.
This study confirms the marked increase in nerve density in the IBD arteries observed using immunohistochemical methods (Chapter 2). Ultrastructural evidence of autonomic nerve proliferation has been described in the enteric nervous system in Crohn's disease and pouchitis (Dvorak et al. 1993). This proliferation was associated with axonal degeneration. The perivascular nerves in this study did not show evidence of degeneration, suggesting that the nerve damage in the previous study was due to the inflammatory processes in the bowel wall.

The presence of intimal nerve fibres in one of the vein specimens is of interest. Nerves in this position have not been described before in blood vessels, although nerve fibres have been identified close to endothelium in bladder lymphatics (Ji and Kato 2000). The proximity to the endothelium suggests a direct signalling role to the endothelial cells. The nature of these signals is impossible to determine in this study, although it is interesting to speculate that they may have an immune function in IBD. Further work is required to determine whether these nerves are a feature of IBD, and if so, which neurotransmitters they contain.

The nerves in the IBD arteries were observed to approach the smooth muscle more closely, in some places, than those in the control vessels. In spite of this, no response to nerve stimulation was obtained from arterial IBD specimens. The lack of response to nerve stimulation by the arteries (see Chapter 3) and the vessel structure on electron microscopy is of interest. It has previously been noted that some blood vessels, whilst possessing varicose noradrenergic nerve fibres, are not responsive to nerve stimulation in vitro, using standard stimulation parameters (Gallen et al. 1982; Cowen 1984). The guinea-pig renal artery is an example of a vessel displaying 'non-functional' innervation. In these vessels, the nerve
varicosities were separated from smooth muscle cells by a wide neuromuscular space containing cellular and other connective tissue elements. Responsive vessels, such as the guinea-pig mesenteric artery, had narrow neuromuscular spaces, with little more than the basement membrane separating the varicosities and muscle cells.

It appears that human mesenteric arteries belong to the 'non-functional' group of vessels. Although the walls of the arteries are thick-walled and muscular, typical of resistance arteries, nerves were in all cases external to the external elastic lamina. There were often large amounts of connective tissue, fibroblasts and other cell types between the nerves and the muscle. As mentioned above, these are features of non-functional arteries such as guinea-pig renal arteries. In the guinea-pig, mesenteric arteries have a substantial response to nerve stimulation (Cowen 1984) and are responsible for about one-third of the total mesenteric vascular resistance (Fenger-Gron et al. 1995). One may expect the human vessels studied to have the same function, as they are the anatomical equivalent of these vessels. However, because of the difference in size, the physiological equivalent may be the smaller diameter resistance vessels in the submucosa.

The lack of response to nerve stimulation and the anatomical relationships to fibroblasts and smooth muscle cells in the mesenteric arteries studied raises questions about the role of the nerves. A trophic role has been proposed. Some mature tissues are known to be under trophic control of their nerves. In the heart, the normal development of cardiac muscle is dependent upon an intact sympathetic nerve supply (Renick et al. 1997). It appears that ATP, not noradrenaline, is the transmitter responsible for this effect (Osswald 1991). In blood vessels, collagen synthesis increases in blood vessel walls after chemical sympathectomy (Fronek et
al. 1978) indicating that nerve activity has an influence on vessel wall structure. There is also evidence that the sensory innervation of blood vessels affects the expression of vasoactive substances by the endothelium (Milner and Burnstock 1996). Sensory denervation increases the sympathetic vasoconstriction in rat mesenteric arteries, an effect which is thought to be due to long-term trophic changes in the vessels (Ralevic et al. 1995). Much has yet to be learned about the possible trophic effects of neurotransmitters but such effects may explain the presence of nerves containing substances that appear to have little direct effect on the innervated smooth muscle.
4.5 Figures

Figure 4.1. Low power light micrographs showing the structure of the artery (a) and vein (b). Three distinct layers are shown: adventitia ($A$), media ($M$), and endothelium ($E$). Note the two well-defined elastic layers in the artery: the inner (iel) and outer elastic laminae ($eel$). In the vein, the elastic tissue is scattered throughout the media. Scale bar 140 μm
Figure 4.2. (a) Electron micrograph showing the adventital/medial junction in a human mesenteric artery. Smooth muscle fibres (sm) in the media are separated from the fibrous adventitia by a well-defined external elastic lamina (eel). The adventitia contains collagen (col), bundles of longitudinally-orientated elastic tissue and capillaries (cap). (b) Electron micrograph showing a nerve fibre (arrow) largely enveloped by a Schwann cell process in the adventitia close to the arterial external elastic lamina. The external elastic lamina is fenestrated, but the nerve fibre is not on the medial side. A fibroblast-like cell (f) is interposed between the connective tissue and the medial muscle coat. (c) Axon fibres (Ax) within a nerve bundle in the arterial adventitia with its associated Schwann cell (Sch) are embedded in collagen. Scale bars (a) 4 μm, (b) 2 μm, (c) 1 μm
Figure 4.3. (a and b) Electron micrograph of arterial smooth muscle from the media orientated in a circular fashion. Cells are separated by collagen except for small areas of close apposition, probably punctate gap junctions (arrows). (c) Intima of artery, exhibiting slight thickening due to intimai smooth muscle cell (sm) and collagen between the endothelial cell (E) and the internal elastic lamina. L, lumen. Scale bars (a) 2 μm, (b) 2 μm, (c) 1 μm.
Figure 4.4. (a-d). Electron micrograph of media of vein. (a) The wall of the vein appears to have a less organized orientation of the smooth muscle (sm) compared to the artery, though this may relate to the state of contraction of the vessel at the time of fixation or because of the presence of the vasa vasorum (vv). Within the blocks of smooth muscle cells there is less collagen and elastic material than found between smooth muscle cells in the artery. Capillaries of the vv are often found in the inner media, which is sometimes associated with small nerve bundles and associated Schwann cells (see higher magnification micrographs in c and d). (b) A section through the media showing substantial collagen between the blocks of smooth muscle cells. A nerve bundle (arrow) is present and enlarged in (c). (c) Shows in higher magnification a small nerve bundle with associated Schwann cell in relatively close proximity to smooth muscle cells. (d) High magnification of nerve fibres containing granular vesicles (gv) in close apposition to smooth muscle cell with no Schwann cell processes interposed. Scale bars (a) 2 μm, (b) 3 μm, (c) 1 μm, (d) 1 μm.
Figure 4.5. (a) High power view of an endothelial cell (E) in a vein in close association with underlying smooth muscle cells (sm) in the intima. A dense connective tissue matrix is present. L, lumen. (b) Medial-adventitial border of a vein showing the lack of an external elastic lamina and the close apposition of nerves, some containing granular vesicles (gv), within the adventitia to the medial smooth muscle without collagen (col) interposed. Sch, Schwann cell and process. Scale bars (a) 2 μm, (b) 1 μm.
Figure 4.6. (a). Electron micrograph of the medial-adventitial border of an IBD artery. Small nerve bundles can be seen within the adventitia (arrows), external to the external elastic lamina (eel). The nerve fibres are enveloped by a Schwann cell process (Sch) and approach a protrusion of an arterial smooth muscle cell (sm) through a fenestration in the EEL. No nerves were seen within the muscular media. A process from a fibroblast-like cell can be seen in the adventitia (f). (b). High-power electron micrograph showing nerve bundles (arrows) in the media of an IBD vein. Nerve bundles can be seen in close proximity to smooth muscle cells (sm) in the media. A considerable amount of intercellular collagen is present (col). (c). Endothelial cells (E) of an IBD vein with closely associated nerve bundles (arrows) containing small granular and agranular vesicles. There is little collagenous material (col) between the nerve bundles and the endothelium. No smooth muscle cells are visible. Scale bars (a) 1µm, (b) 1µm, (c) 1µm.
Chapter 5  General discussion
5.1 Introduction

It has been suggested that ischaemia plays a part in the pathogenesis of IBD (Pounder 1994). Recent experimental evidence in mice indicates that microcirculatory disturbances precede histological abnormalities (Foitzik et al. 1999). This work set out to identify whether abnormalities exist in the perivascular innervation of human mesenteric vessels. The perivascular nerves are one arm of the dual control system for controlling blood flow (Burnstock and Ralevic 1994; Remak et al. 1994), and changes in this system in patients with IBD may be important in its pathogenesis.

5.2 Changes in perivascular nerves in IBD

The majority of the perivascular nerves were of sympathetic origin. It is likely that NPY and TH were colocalised in all the nerves. However, there was a tendency to a higher NPY- than TH-LI nerve density. One-third of the nerve bundles were positive for VIP-LI. The discrepancy between the sum of the nerve densities for these markers raises the possibility of colocalisation of VIP with one of the other markers, possibly NPY. Colocalisation studies may clarify the distribution of the transmitters. Nerves immunoreactive to the other markers studied were identified at a much lower density. Relative to the density of PGP, the pattern of innervation in the arteries and veins was the same. The only exception to this was that no nerves immunoreactive to CGRP were identified in the veins. The origin of these nerves requires further study.

In IBD, there was a greater nerve density for the sympathetic cotransmitters NPY and NA in arteries and veins from patients with IBD. A more ‘disordered’ plexus
was found in the arteries. The number of nerves immunoreactive to 5-HT had also increased. These are likely to represent nerves of sympathetic origin. CGRP was absent in the control veins, but present in all the veins from patients with IBD, which may indicate a change in the sensorimotor innervation in HMV in IBD. For the other neuropeptides studied, no differences were identified between the control and IBD vessels.

These neurochemical changes are similar to those identified in the myenteric plexus of the ileum from patients with CD (Belai et al. 1997), where an increase in the number of nerves with TH-, NPY- and 5-HT-LI was observed. Other studies do not entirely agree with these findings. In a study comparing biopsies from normal colon with those from treated CD and resected specimens from grossly inflamed Crohn's colitis, a decrease in the number of sympathetic nerves was identified in the grossly inflamed bowel. However, there was an increase in cases treated with mesalazine over control specimens (Feher et al. 1997). It is likely that in grossly inflamed bowel, nerve damage masks any increase in nerve density that pre-dated the damage.

Ultrastructural studies in CD identified axonal proliferation accompanied by necrosis, both in the inflamed region of the bowel and the resection margins (Dvorak et al. 1980). Changes have been identified in inflamed continent ileo-anal pouches (Dvorak et al. 1993), and were associated with anaerobic infection in the pouch (Onderdonk et al. 1992). Axonal necrosis may be the result of inflammation, but the abnormalities distant from the active inflammation indicate other factors may have a role. In this study, nerve proliferation was identified but there was no evidence of axonal degeneration at the ultrastructural level.
Nerve proliferation appears to be a widespread feature in chronic inflammation of the gut, and has been noted in conditions other than IBD (Di Sebastiano et al. 1995). Although qualitative changes have been recognised for several years, only recently has the neurochemical coding of the nerves involved been identified. The increase in sympathetic innervation, if associated with an increase in neurotransmitter release, would contribute to the vasospasm, non-synchronised bowel contraction and oedema observed in IBD. Abnormalities of the perivascular nerves of the mesenteric vessels supplying the gut are likely to be a further factor in the pathophysiology of IBD, as vasospasm and consequent ischaemia would perpetuate the destructive inflammation. Whether the nerve proliferation is the result of the inflammatory process, due to an underlying abnormality of the ANS and ENS, or to an unknown environmental factor, has yet to be determined.

This study has shown that in addition to sympathetic nerve proliferation in blood vessels, functional differences were also present. The in the IBD arteries, the response to exogenous NA was unchanged, but the vessels were less responsive to ATP. As in the control arteries, there was no contraction to extrinsic NPY. The result of incubating the HMA with NPY only resulted in a lower $E_{\text{max}}$ than in its absence.

In the veins, the neuromodulatory effect of NPY was not demonstrated, but the CRC to NA alone in IBD group was already equivalent to that for the control group after incubation with NPY. This would indicate that the vascular smooth muscle had already been sensitised to produce its maximal contraction to NA alone, without the need for NPY to induce this. Changes in the contractility of smooth muscle in IBD
have been noted previously (Vermillion et al. 1993), but the causative agent was not clear.

As in the control vessels, the arteries from patients with IBD were non-reactive to nerve stimulation. In the veins, there was a significant increase in the contractility of the vessels to electrical nerve stimulation. This is likely to be explained by the increase in nerve density. It was also noted that the proportion of the response due to stimulation of different receptors varied in IBD. This may reflect differences in the expression of receptors in IBD. It was notable that a significant proportion of the response in IBD vessels was not blocked by $\alpha_1$, $\alpha_2$, $\text{P}_{2X}$ or $\text{P}_{2Y}$ antagonists. In one of the specimens, the contraction was found to be resistant to the $5\text{-HT}_2$ antagonist ketanserin. The contraction was abolished by tetrodotoxin, indicating that it was of nerve origin. Further immunohistochemical studies using different markers, coupled with nerve stimulation work, is necessary to identify the agent responsible for the augmented contraction in IBD.

The alteration in contractility of the veins is likely to be of clinical importance. Localised venous contraction would contribute to engorgement and oedema of the gut, which is a prominent feature of CD. Furthermore, non-invasive tests of ANS function have identified systemic abnormalities in UC and CD. Changes in the contractility of the splanchnic veins would be reflected in tests such as these (Lindgren et al. 1991, 1993). Abnormalities in blood flow in the SMA and IMA have been identified using Doppler sonography (Ludwig et al. 1999a, b). These observed increases in flow are difficult to explain if the abnormalities in the mesenteric circulation result in an increase in contractility. Differential blood flow in the layers of the bowel wall may divert blood away from the mucosa, leading to
paradoxical ischaemia. Increased flow in the face of contraction of the post-capillary resistance vessels may also contribute to vascular engorgement and oedema. Further work on the precise anatomical abnormalities in IBD is required.

The abnormal venous contraction was only identified at 15Hz, the higher frequency of stimulation. In vivo, it may require a prolonged period of high-frequency stimulation to trigger any clinical features caused by these anatomical changes. It is interesting that there is an association between 'stress' and the onset or recurrence of IBD. The increased sympathetic stimulation observed under stressful conditions may be of significance. Whatever the cause of the abnormalities in the nerve plexus, if they are irreversible, a suitable stimulus may be able to trigger further episodes.

The vessels studied are anatomically quite distant from the site of inflammation, indicating that the stimulus is not restricted to the immediate vicinity of the inflamed tissues. The finding of an increased number of nerves with 5-HT-LI in the vessels from patients with IBD may have clinical significance. 5-HT has many physiological actions and is active in the inflammatory process. Human (El-Salhy et al. 1997) and animal (Oshima et al. 1999) studies have shown an increase in the number of 5-HT-containing mucosal enterochromaffin and mast cells in inflamed bowel. 5-HT is also released by platelets in damaged vessels, causing constriction of the vascular smooth muscle. This 5-HT is also thought to be taken up by perivascular nerves and subsequently released, causing further vasoconstriction (Shepherd et al. 1991). The increased number of perivascular nerves displaying 5-HT-LI in this study may be due to a population of nerves using 5-HT as a 'false transmitter'. This could contribute to vasospasm and disturbances in blood flow, perpetuating the inflammatory response. These changes may all be secondary to
active inflammation. However, abnormalities in the release of inflammatory mediators, which may be dependent on 5-HT, have been identified in non-inflamed bowel from patients in remission from IBD (Wardle et al. 1996). This may indicate an underlying abnormality in 5-HT release or metabolism in the gut of patients with IBD.

In IBD, over-expression of NGF and its high affinity receptor Trk has been identified in PMN cells of the lamina propria, mast cells and ganglia in the myenteric plexus (Di Mola et al. 2000). NGF may be an important factor in the increase in mast cell numbers, nerve proliferation and subsequent tissue damage observed in IBD, and may be one of the linking factors between the nerves and immune system in this complex condition. Furthermore, increased circulating levels of NGF have been identified in autoimmune and allergic conditions, and in subjects under stress (Aloe et al. 1997). A link between the immune, neuronal and psychological aspects of IBD is therefore possible.

A further factor that may be of importance is ATP, which is a cotransmitter in sympathetic nerves. Immunohistochemical techniques for localisation are unreliable, but the organ-bath pharmacology studies show that it is present in the perivascular nerves of the vessels studied. In addition to vasoconstriction, ATP has many actions in the inflammatory process, including mast cell degranulation, leukocyte adhesion to the endothelium and potentiation of the oxidative burst via P2 receptors. The response to ATP is determined by the receptors present in the tissues, which have been found to be altered in chronically inflamed tissues.
5.3 Difficulties encountered

5.3.1 Poor visualisation of nerves due to high background fluorescence

One of the aims of this study was a quantitative assessment of the nerve plexus density and structure. For this reason, the technique of whole-mount immunohistochemistry was chosen to identify the nerves. This allows the plexus structure to be seen en-face, to allow assessment of the way the nerves are arranged. Individual nerve fibres can be seen in some cases, and structures such as varicosities identified. Immunofluorescence can provide images with excellent contrast, unlike some histochemical methods in which the staining is rather subtle. Image analysis requires a clean image of high contrast and uniform illumination intensity (Cowen and Thrasivoulou 1992). It is also necessary to have all the structures of interest in focus in the image to be analysed, or detail will be missed. In the early stages of this work, conventional microscopy was used. It was found that a high level of background fluorescence prevented clear visualisation of the perivascular nerves. In addition, in many of the specimens, the entire nerve plexus was only visible by focusing up and down through the tissue. Photomicrographs of these specimens were not suitable for image analysis as a proportion of the fibres were out of the plane of focus. In animal studies, the vessels are thinner-walled and this is less of a problem.

One remedy for this situation would have been to abandon whole-mount studies and revert to studying sections of the vessels. However, it would only have been possible to assess the transverse intercept density, and information on structural
detail would have been lost. CLSM was found to overcome these problems, and was therefore used for this study.

CLSM produced images with high contrast and low background fluorescence. The digital images were ideal for computerised image analysis and easily stored and transferred to different computers. The files are large, so a high-powered computer is necessary to manipulate the images, although these are now more easily available.

The main drawback of the technique was that it was very time-consuming, with each image taking several minutes to build up from a series of optical sections through the thick tissue of the whole-mount specimens. Over 800 images were obtained, which required manipulation and analysis. The time necessary for this was considerable. This makes the technique unwieldy for large studies. Faster computers with more memory and microscopes that scan more quickly will reduce the time required for this technique and make it more applicable to other studies. The potential for investigating the innervation of the mesenteric vessels and the ENS is enormous. With the availability of multiple channels, simultaneous scanning of double- or multi-labelled specimens may give rise to interesting results.

The image analysis method itself had one main disadvantage. A threshold had to be set individually for each image to distinguish between the nerves and the background. This was not difficult because of the high contrast of the images, but had to be performed manually, as there were differences between the images in the brightness of the background. This introduced an element of subjectivity into the analysis, was time-consuming and prevented fully automated analysis. Having the threshold set by performed by a blinded third party would have reduced the subjectivity, was not practical in this study.
5.3.2 Tissue availability

Studies on human tissue have the benefit of being directly applicable to clinical conditions, but have a number of difficulties, the main one being lack of availability of suitable material. Informed consent for the research was obtained from the patients prior to their operations, but the operative technique was not modified for the purposes of the research. Material had therefore to be collected very carefully so as not to interfere with the pathological examination of the specimen, and was done with the collaboration of the pathologists. In the case of the non-inflamed specimens, the vessels were taken from the bowel close to the normal resection margins, well away from the tumour. This was important, because there is evidence that cancer can affect the perivascular nerves of the vessels supplying it (Kennonin et al. 1994). In IBD, much less of the mesentery is removed at operation, and vessels in these specimens were often scarce. In view of these problems, it was decided to concentrate on the contractile responses of the tissues, as persevering with attempts to obtain consistent pre-constriction would have wasted tissue. Further work, concentrating on the relaxant responses, would complement this study.

5.3.3 Patient variability

Another concern in studies on human tissue is the variability between individuals. In these studies, the control groups were on average older than the IBD group. This is to be expected from the natural history of the diseases involved, but may reduce the comparability of the groups. In some animal studies, changes have been identified in perivascular innervation with age (Dhall et al. 1986; Geppetti et al. 1988). However, most of these studies are comparing immature with mature
animals. In this study, all the subjects were adults, so developmental changes would not be important. In the more elderly subjects, degenerative effects may become significant. A study on human cutaneous resistance arteries has identified an age-related reduction in responsiveness to adrenergic stimulation (Nielsen et al. 1992). However, other studies have questioned whether age-related changes occur (Mione et al. 1988; Stones et al. 1994; Bevan, RD et al. 1998). In this study, there was no difference in the response to exogenous NA between the two groups. Further study is required to investigate alterations in mesenteric perivascular nerves and the response of innervated tissues with ageing (Cowen 1993). Addressing this question to compare mesenteric vessels from adults of different ages would be difficult as the availability of non-inflamed mesenteric vessels from younger patients would be extremely limited.

5.3.4 Smoking

Smoking is further potential confounding factor. In the functional experiments, five of the subjects were current smokers, two in the IBD group (both with CD). Nicotine impairs endothelium-dependent vasodilatation (Chalon et al. 2000), and has been shown to potentiate NA-induced vasoconstriction (Mayhan 1999). Immediately after smoking there is an increase in plasma NA and NPY, giving rise to elevation in blood pressure and tachycardia (Rudehill et al. 1989). The duration of these effects may be short-lived (Moreno et al. 1998). The effects of smoking are unlikely to have influenced the results of this study, as the patients would not have had the opportunity to smoke for several hours prior to surgery.
5.3.5 Drug treatments

Many drugs may be expected to alter the functioning of nerves and smooth muscle. The prescribed drugs taken by the subjects of this study are detailed in Appendix 1. Two of the control patients were taking selective serotonin-reuptake inhibiting (SSRI) antidepressants. Neither was included in the nerve stimulation experiments. Non-steroidal anti-inflammatory drugs (NSAID) are cyclo-oxygenase inhibitors and have many physiological actions. Cyclo-oxygenase produces many vasoactive substances and its inhibition has been found to have a number of different actions (Rapoport and Murad 1983; Katusic et al. 1987). Of the patients taking regular NSAID, none were included in the functional experiments. Two of the control patients were taking atenolol, a β1 adrenergic antagonist: neither of these patients contributed specimens to the nerve stimulation experiments.

The main difference between the groups in terms of drug therapy was that 21/23 of the patients in the IBD group were taking steroids in one form or another. Glucocorticoids have been shown to have the effect of increasing the responsiveness of vascular smooth muscle to NA by increasing the number of α1 receptors (Sakaue and Hoffman 1991) and intracellular second-messenger production (Liu et al. 1992). The effect of exogenous NA on the IBD arteries in this study are not in keeping with a steroid-induced increase in contractility, but the responses of the veins are. The IBD venous samples did not show the same neuromodulatory effect of NPY to exogenous NA, as it appeared that NA alone had achieved the maximum contraction possible for the vessels, consistent with an up-regulation of the α1 receptors in the vein. However, the augmented response to nerve stimulation was largely resistant to blockade of NA receptors. An increase in second messenger production would
result in enhanced contractions to other second messenger-coupled stimuli, as observed in this study. The nature of this stimulus remains to be determined.

The increase in innervation density is unlikely to be due to steroid treatment of the IBD patients. Corticosteroids have been demonstrated to cause neuronal death in vitro (Chan et al. 1996). The increased innervation has therefore to be explained by another mechanism, as discussed above. However, if the vessels were already primed to have a greater contraction than usual, the increased neurotransmitter response would contribute to the vasospasm.

5.4 Conclusion

In multifactorial diseases such as IBD, the contributions of abnormalities primarily due to the disease and the effects of the body’s defence mechanisms and pharmacological manipulations in its treatment are almost impossible to disentangle. CD and UC have different pathological characteristics, but the bowel inflammation has some common features. The precise mechanisms producing the inflammation are obscure, but appear to include tissue ischaemia. Aphthoid ulceration, an early change in CD, can be identified on endoscopy. These lesions may indicate early ischaemic damage (Lundgren 1989).

This work identifies changes in the perivascular nerves of mesenteric vessels in IBD, which are likely to contribute to vascular hyper-responsiveness and mucosal ischaemia. Whether the changes observed in the nerves and in the response of the vascular smooth muscle is an intrinsic abnormality, or secondary to the
inflammatory response is not addressed by this study. Further studies on the vessels supplying the gut may help to clarify this aspect of the pathophysiology of IBD.
Appendices
## Appendix 1: Table of patient details

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<th>Chapter</th>
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<th>Diagnosis</th>
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<th>Tobacco</th>
<th>Steroid</th>
<th>NSAID</th>
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Appendix 2: Details of antisera

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\(^1\) Marker for noradrenaline; \(^2\) general neuronal marker; \(^3\) marker for acetylcholine; \(^4\) marker for nitric oxide.

NPY=neuropeptide Y; VIP=vasoactive intestinal peptide; SP=substance P; CGRP=calcitonin gene-related peptide; 5-HT=5-hydroxytryptamine.
Appendix 3: Optical theory for confocal microscopy

Theoretical lateral resolution: \( R = 1 \cdot \frac{2\lambda}{2NA} \); where \( R \) = lateral resolution, \( \lambda \) = wavelength (e.g. 442 nm for blue light, 520 nm for fluorescein-conjugated antibodies, \( NA \) = numerical aperture of objective lens - \( \times 2 \) for epi-illumination (Hale and Matsumoto 1993).

Equation for depth of focus (\( \Delta Z \)): \( \Delta Z = n_s \left[ \frac{4\lambda}{(NA)^2}H \right] \); where \( n_s = 1.52 \), the refractive index of the immersion medium (oil), \( n_o = 1.33 \), the refractive index of the object, \( \lambda = 0.488 \) (the wavelength of the argon laser), \( NA = 1.30 \) (the numeric aperture of the objective lens) and \( H \) is an empiric constant with a value of 0.125.

For confocal microscopy, the equation is: \( \Delta Z = n_s \frac{4\lambda}{(NA)^2}H + \frac{S}{(NA)M}W \); where \( n_s = 1.52 \), the refractive index of the immersion medium (oil), \( n_o = 1.33 \), the refractive index of the object, \( \lambda = 0.488 \) (the wavelength of the argon laser), \( NA = 1.30 \) (the numeric aperture of the objective lens), \( H \) and \( W \) are empiric constants with values of 0.125 and 0.00136 respectively, \( S = 250,000 \) \( \mu \text{m} \), the normal viewing distance, which has the same units as the wavelength, and \( M = 1,000 \) (the magnification of the microscopic image).
Bibliography


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Dvorak AM and Dickens GR (1980). Crohn's disease: transmission electron microscopic studies. I. Barrier function. Possible changes related to alterations of


polypeptide-containing nerves on the wall of cerebral arteries: an immunohistochemical study using whole-mounts. *Neuroscience*, **10**: 89-96.


Yianni J (1994) Neural and endothelial control of vascular tone in the human splanchnic circulation. B.Sc , University College London.


Publications arising from this thesis
