Inflammation in Auerbach's Plexus in Motility Disorders of the Oesophagus

by

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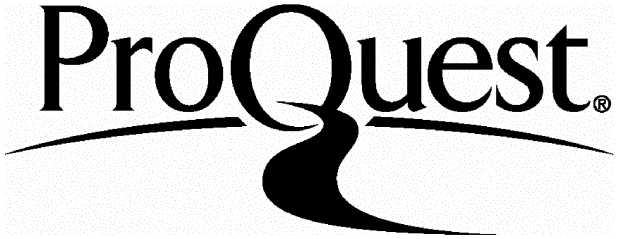

ABSTRACT

Inflammation in Auerbach's plexus has been reported in motility disorders of the oesophagus. An eosinophil infiltrate has occasionally been described. Tissue eosinophilia is often pathogenic in diseases where it is present. Eosinophil granule proteins, such as eosinophilic cationic protein, are cytotoxic and neurotoxic.

This thesis examines the inflammatory response, with specific attention to the role of eosinophils, in Auerbach's plexus in motility disorders of the oesophagus.

Oesophageal muscle biopsies obtained at thoracotomy (including Auerbach's plexus) were stained with haematoxylin and eosin and examined.

A new staining combination was developed to demonstrate eosinophils and mast cells in the same tissue section. Using computer-aided area measurement and manual counting, the number of cells per unit area was assessed in normal and diseased oesophagus.

In biopsies with inflammation, eosinophil activation, and the distribution of T and B lymphocytes and cell-bound IgE were assessed using immunohistochemical staining. Nerve distribution and structure, and HLA-DR expression were similarly established.

Peripheral blood eosinophil counts, serum IgE and eosinophilic cationic protein were estimated in patients with various motility disorders.

Eosinophils were absent in normal oesophagus. Although present in the mucosa in severe reflux disease, they were uncommon in Auerbach's plexus. In some patients with disordered motility, eosinophil infiltration with degranulation and cellular activation was found. This was commonest in the vigorous contraction abnormalities diffuse oesophageal spasm and vigorous achalasia but was also seen less frequently in achalasia and nutcracker oesophagus.

Peripheral blood eosinophil counts and eosinophilic cationic protein levels were normal in the vigorous contraction abnormalities. In some cases raised serum IgE, with IgE bearing cells and tissue eosinophilia in Auerbach's plexus were found.

Eosinophil infiltrates in Auerbach's plexus are only seen in the motility disorders and are likely to be pathogenic. The inflammatory response has many of the features of an allergic disorder.

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ABBREVIATIONS

ABC	Avidin Biotin Complex
ABC-AP	ABC- alkaline phosphatase
ABVNR	Astra blue / vital new red
AP	Auerbach's plexus
B cells	'Bursa-derived' lymphocytes
CCF	Congestive cardiac failure
CD	Cluster designation (classification of inflammatory cells)
CLC	Charcot Leyden Crystals
COAD	Chronic obstructive airways disease
CVA	Cerebrovascular accident
DAB	Diaminobenzamine
DKA	Diabetic ketoacidosis
DOS	Diffuse oesophageal spasm
ECF-A	Eosinophil chemotactic factor of anaphylaxis
ECP	Eosinophil cationic protein
EDN	Eosinophil-derived neurotoxin
EDTA	Ethylene diamine tetracetate
EG1	Monoclonal antibody against ECP
EG2	Monoclonal antibody against ECP(secreted)
EPO	Eosinophil peroxidase
EPX	Eosinophil protein X
GALT	Gut-associated lymphoid tissue
HCl	Hydrochloric acid
HETE	Lipo-oxygenase product of arachidonic acid
H&E	Haematoxylin and eosin
HH	Hiatus hernia
HLA-DR	Human leucocyte antigen D region
IHD	Ischaemic heart disease
IL-5	Interleukin 5
LN	Lymph node
LOS	Lower oesophageal sphincter
MBP	Major basic protein
MC	Mast cell

NANC	Non-adrenergic, non-cholinergic
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PE	Pulmonary embolus
PMN	Polymorphonuclear leucocyte
PVC	Polyvinyl chloride
SD	Standard deviation
SEM	Standard error of the mean
SRS-A	Slow reacting substance of anaphylaxis
T cell	Thymus derived lymphocyte
UOS	Upper oesophageal sphincter
VIP	Vasoactive intestinal peptide

DEDICATION

This work is dedicated to Jane, Sean, Thomas, Jack and my parents.

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This work could not have been undertaken without the help and co-operation of the individual patients who were the subjects of this research.

Written informed consent was obtained from patients who had oesophageal muscle biopsies or blood tests during the period of the study. The study had approval from the local hospital ethics committee.

STATEMENT OF ORIGINALITY OF THE WORK

1) All the basic laboratory work was performed by myself. This included the cutting of sections, histochemical and immunohistochemical staining.

2) All the quantification studies were performed by myself.

3) The manometric records were reviewed personally in association with Mr.I.Adams.

4) The eosinophil counts and serum IgE levels were performed routinely in the departments of haematology and immunology but the radioimmunoassay for serum ECP levels was performed by myself.

5) The data have not been submitted for any other degree.

The work for this thesis was performed in the Thoracic Surgery Department of Birmingham Heartlands Hospital (formerly East Birmingham Hospital), Birmingham, England.

1.INTRODUCTION

1.1 INTRODUCTION TO MOTILITY DISORDERS OF THE OESOPHAGUS.

Although recognised clinically since the 17th century, primary motility disorders of the oesophagus have only been fully characterised with the advent of radiological assessment, manometry and endoscopy. They are now recognised as an heterogeneous group which include achalasia, diffuse oesophageal spasm (DOS), nutcracker oesophagus and other related disorders.

Some believe that they should be considered as a spectrum of disease since there are individual patients who have features of both achalasia and DOS but do not meet the strict criteria for either disorder (Vantrappen et al. 1979, Castell 1979). There are also reports of transition over time of one motility disorder to another (Narducci et al. 1985, Anggiansah et al. 1990, Vantrappen et al. 1979). Recent experience with 24 hour ambulatory manometry suggests that the distinction between DOS and nutcracker oesophagus may be artificial, one study having shown the two conditions to be indistinguishable when 24 hour manometric assessment was performed (Stein et al. 1989).

Whilst there have been many studies of the histopathology of the oesophagus in achalasia, there has been very little work on the the pathological changes found in the other motility disorders.

To understand the changes seen in the motility disorders of the oesophagus it is necessary to review what is known about the anatomy and control of normal oesophageal function. This will be discussed before an appraisal of the knowledge relating to the individual disorders.

1.2 FEATURES OF THE NORMAL OESOPHAGUS

Anatomy

The oesophagus is a muscular tube lined for the majority of its length by squamous epithelium. It transports ingested solids and liquids from the pharynx to the stomach. The muscularis propria is in two layers: an outer longitudinal and an inner circular layer. It is protected at each end by sphincters. The upper oesophageal sphincter (UOS) is a clearly defined band of striated muscle that is attached to the cricoid cartilage anteriorly. Cricopharyngeus forms the major part of the sphincter with a small contribution from some fibres of the inferior constrictor. The smooth muscle lower oesophageal sphincter (LOS) can be identified clearly during manometry but is not distinguishable on microscopic or macroscopic examination.

The cricopharyngeus muscle and the muscle of the upper third to one half of the oesophagus are predominantly striated muscle. The muscle of the lower half to two thirds of the oesophagus and the LOS is predominantly smooth muscle (Ingelfinger 1958, Meyer et al. 1986).

Nerve supply
The upper oesophagus is supplied by the recurrent laryngeal nerves and by sympathetic fibres from cell bodies in the middle cervical ganglion on the inferior thyroid arteries. The lower oesophagus is supplied by the oesophageal plexus which encircles the oesophagus below the lung roots. The sympathetic supply is by way of fibres from the upper four thoracic ganglia of the sympathetic trunk.

Physiology

Swallowing

The pharyngeal phase of swallowing delivers the food bolus to the upper oesophagus. The oesophageal phase commences with a coordinated relaxation of the upper oesophageal sphincter following which the bolus is propelled down the oesophagus by a peristaltic wave until it reaches the lower oesophageal sphincter which relaxes and allows the bolus to pass into the stomach.

Oesophageal peristalsis

Primary peristalsis is the pattern of muscular contraction that occurs in response to the voluntary act of swallowing. This is usually initiated by the passage of a bolus into the pharynx and consequent pharyngeal contraction. A wave of circular muscle contraction then passes down the oesophagus propelling the bolus towards the stomach. In a series of experiments on dogs at the turn of the century Meltzer demonstrated that this response could be bolus independent (Meltzer 1898).

Secondary peristalsis is a contraction sequence that originates in the oesophagus itself as a result of distension. This functions as a mechanism whereby food material not cleared by the primary peristaltic wave or material refluxed from the stomach is cleared from the oesophagus (Roman and Gonella 1987).

A third contraction pattern is also described called tertiary activity. This may be evident on barium swallow as simultaneous contractions at several levels in the oesophagus which produce segmentation of the barium. They may also be evident on manometry as simultaneous, non-progressive contractions. The relationship between this pattern which can be seen in the apparently normal oesophagus and pathological non-progressive contractions, as seen in diffuse oesophageal spasm, is unclear.

Deglutitive inhibition

If repetitive swallowing is performed (<5 secs between swallows) then this will result in inhibition of peristalsis. A large clearing wave will occur at the cessation of the swallows.

Nervous control of swallowing.

Extrinsic innervation.

Extrinsic control of oesophageal motor function is exerted by the swallowing centre which lies in the brain-stem. It has three main areas of influence: the oropharynx, the oesophageal body and the lower oesophageal sphincter. It controls the swallowing sequence and coordinates swallowing with respiration and other activities.

The swallowing centre receives information in afferent nerve fibres from the oropharynx carried in cranial nerves V, VII, IX, X, and XII and from the oesophagus and lower oesophageal sphincter in sensory fibres carried in the vagus. Some sensory information is carried from the oesophagus in sympathetic nerves to spinal levels T3 to T12 (Diamant 1989).

Motor neurones involved in the swallowing sequence lie mainly in the motor nuclei of cranial nerves V, VII, IX, XII, the nucleus ambiguus (for oesophageal striated muscle) and the dorsal motor nucleus of the vagus (for oesophageal smooth muscle) (Roman and Gonella 1987). The vagus nerve receives fibres from both these nuclei and innervates the whole oesophagus.

Efferent sympathetic fibres from the cervical and paravertebral ganglia reach the oesophagus primarily through its vascular supply but also to a lesser extent in the vagus nerves. Extensive sympathectomy does not appear to alter human oesophageal motility (Ingelfinger 1958).

Intrinsic innervation.

There is a nerve plexus lying between the two outer muscle layers (outer longitudinal and inner circular) of the oesophagus called the myenteric or Auerbach's plexus. This is less well developed in the upper striated oesophagus. There is evidence that the innervation of the upper striated muscle differs from that of the lower smooth muscle and therefore these merit separate discussion.

Striated muscle.

It is believed that efferent vagal fibres do not synapse in myenteric plexus ganglion cells but end directly on neuromuscular junctions of the striated muscle cells as occurs in skeletal muscle elsewhere (Diamant 1989). Transmission is via cholinergic, nicotinic receptors (Roman and Gonella 1987). It is unclear whether the myenteric plexus has any direct influence on these striated muscle cells or whether it merely subserves a sensory role (Roman and Gonella 1987).

There is little evidence that sympathetic nerves have any motor function in the striated muscle of the oesophagus.

Smooth muscle.

There are two important effector neurones innervating the body of the oesophagus and the lower oesophageal sphincter. One mediates cholinergic excitation of both the longitudinal and circular muscle layers via muscarinic receptors. The other consists of nonadrenergic, noncholinergic (NANC) inhibitory neurones that inhibit the circular muscle layer. The neurotransmitter for these neurones is unknown although many putative candidates have been suggested from amongst the nucleotide, peptide and other proteins present in the oesophagus (Diamant 1989, Goyal et al.1980, Christensen 1987). It has recently been suggested that the transmitter might be nitric oxide (Tottrup 1993). The smooth muscle of the lower sphincter is also sensitive to a wide variety of substances, including peptide hormones, drugs, histamine, prostaglandins, dopamine, serotonin and others (Castell 1975, 1987).

The role of the sympathetic innervation of the smooth muscle of the oesophagus is unclear. There is extremely rare contact between sympathetic neurones and muscle cells and it seems that their role may be to modulate the contractile response by influencing the motor neurones of the vagus and altering parameters such as contraction velocity and amplitude (Diamant 1989).

Control of oesophageal function.

In response to a normal swallow, a peristaltic wave progresses smoothly down the oesophagus with no apparent transition or holdup between the upper striated muscle and the smooth muscle of the lower half to two thirds of the oesophagus. Although the intermixing of smooth muscle and striated muscle cells in the area of transition may be important, the basic mechanism by which the smooth transition is achieved is poorly understood.

There is general agreement that the peristaltic wave in the striated muscle oesophagus is controlled by the swallowing centre. Sequential firing of vagal neurones directly innervating small motor units results in a progressive wave of contraction (Roman and Gonella 1987, Janssens 1973). Sensory information relayed to the swallowing centre from the oesophagus can alter the force and speed of oesophageal contraction.

There is more debate about the mechanism of peristalsis in the smooth muscle oesophagus. Although some favour the idea that the peristaltic sequence is

programmed centrally (Diamant 1989), there is evidence to suggest that peristalsis in the smooth muscle segment is programmed in the oesophagus itself. Thus a peristaltic sequence can be produced by a mass stimulation of the divided vagus (i.e. without a central program) (Dodds 1978a, 1978b, Mukhopadhyay 1975). Local stimulation of the oesophagus (e.g. by balloon distension) after high vagotomy or in the isolated organ in vitro has been shown to result in peristaltic contractions. The model suggested by Christensen suggests that swallowing induces a simultaneous excitation of the nonadrenergic, noncholinergic neurones throughout the whole smooth muscle oesophagus. This results in a period of inhibition of contraction followed, after a latent period, by a rebound or "off" contraction. This latent period appears to increase distally in the oesophagus and may thus be responsible for the peristaltic sequence (Christensen 1987). There is some evidence that there may be a craniocaudal gradient in the intracellular milieu of the smooth muscle cells in the oesophagus and this may be responsible for the gradient in latency (Schulze et al. 1978).

Although neural involvement in oesophageal contraction is well established, there is also some evidence to suggest that there may be a myogenic component to peristalsis. Thus direct electrical stimulation of muscle in the opossum oesophagus produces a peristaltic sequence when neural activity is blocked with tetrodotoxin. It is postulated that a myogenic mechanism may occur and be modulated by the extrinsic and intrinsic innervation (Sarna et al.1977, Helm et al.1991).

Although the precise mechanism of control and coordination of peristalsis in the smooth muscle oesophagus is not clear, it appears that both a central input from the swallowing centre and local involvement of the myenteric plexus are required for normal oesophageal contraction.

Control of lower oesophageal sphincter

Basal tone.

Unlike the oesophageal body, the lower oesophageal sphincter is normally tonically contracted. This may result from intrinsic muscle tone since in animals it is not abolished by the neurotoxin tetrodotoxin. In humans the resting pressure is unaffected by truncal vagotomy. Calcium-channel blockers have a direct effect on smooth muscle and can produce relaxation of the lower oesophageal sphincter.

However there is some cholinergic influence on the lower sphincter since atropine has been shown to reduce lower oesophageal sphincter pressure.

Mechanism of relaxation of the lower oesophageal sphincter.

This appears to be mediated primarily by nonadrenergic, noncholinergic neurones. It has been shown that vasoactive intestinal peptide (VIP) is present in the intramural neurones of the LOS and that stimulation of the vagus can release VIP. A study with an antiserum to VIP showed that the LOS relaxation in response to intravenous VIP and to intramural stimulation could be reduced by the antiserum suggesting VIP has a significant role (Goyal 1980). More recently, evidence that nitric oxide is the transmitter has been put forward (Tottrup 1993). The exact neural pathway has not been established and it seems likely that both these agents are involved.

However there are many substances that have been shown to influence the tone of the lower oesophageal sphincter including other neural mediators, hormones and drugs (Castell 1975). Which of these plays a major part in vivo is unclear.

1.3 MOTILITY DISORDERS OF THE OESOPHAGUS

Introduction

Disordered oesophageal motility is recognised in association with many pathological disorders of the oesophagus. Thus disordered motility may be secondary to oesophageal neoplasia, stricture or reflux disease (Olsen and Schlegel 1965). It can also occur in a variety of systemic diseases such as diabetes and scleroderma (Hollis et al. 1977).

This discussion will concentrate on the primary motility disorders where the underlying aetiology has not been identified.

Achalasia.

Introduction

Sir Thomas Willis is credited with the first description of achalasia in the 17th century. Hurst introduced the term achalasia (derived from a Greek word meaning failure to relax) to describe the lower sphincter disorder in 1915 but acknowledged that failure of relaxation had been proposed initially by Einhorn in 1888 (Hurst and Rake 1930).

Although achalasia can present at any age it classically presents between the third and fifth decade. Its annual incidence is between 1 in 100,000 to 1 in 200,000 and it is equally common in both sexes (Earlam 1969, Mayberry and Atkinson 1985a).

Pathophysiology.

The most consistent pathological finding in achalasia is a reduction in numbers, or absence of, ganglion cells in Auerbach's plexus (Hurst and Rake 1930, Lendrum 1937, Cassella 1964, Csendes et al. 1985). However ganglion cell loss is not complete in all cases and may be more prominent in the body of the oesophagus (Adams et al. 1976). Inflammation in Auerbach's plexus, with increased numbers of small round cells (presumed to be lymphocytes) has often been described but does not occur in all cases. In some cases an eosinophil infiltrate in Auerbach's

plexus has been described and this has recently received specific attention since the potential of these cells for causing tissue damage has been realised (Tottrup et al. 1989).

As well as clear evidence of neuronal damage occurring locally within the myenteric nerve plexus (Auerbach's), there have been several reports showing damage to vagal nerve fibres innervating the oesophagus. Cassella et al.(1964) found ultrastructural changes similar to Wallerian degeneration in vagal nerve fibres and also evidence of smooth muscle damage (similar to that expected after denervation). It has also been demonstrated in postmortem studies of a small number of patients that there is damage to the motor nuclei of the vagus in the brain stem (Cassella 1964). Experiments on cats and dogs have shown that ablation of these nuclei can produce a picture not dissimilar to achalasia of the oesophagus (Higgs 1965).

Despite pathological evidence of vagal nerve fibre damage, clinical tests of vagal nerve function have only demonstrated damage to the main vagal trunks in a few cases (Atkinson 1986, Eckardt 1989). Woolam et al. studied gastric secretory responses to insulin-induced hypoglycemia in 32 patients with achalasia and demonstrated that eight patients satisfied Hollander's criteria for a complete vagotomy (Woolam et al. 1967). However 19 of these patients were studied after oesophagomyotomy and it is not stated in their report whether the eight patients with evidence of complete vagotomy had had surgery or not. This may be important since the results could reflect vagal damage at the time of surgery or as a secondary feature of postoperative healing and fibrosis rather than an inherent defect in the vagus. In contrast, Atkinson et al showed that vagal function was normal in the majority of patients they studied (Atkinson et al. 1987). Atkinson studied ten patients and assessed lower oesophageal sphincter pressure rise in response to raised abdominal pressure - a vagally mediated reflex. In eight cases the response was unimpaired (Atkinson et al. 1987). In contrast, a study of direct stimulation of the vagus at thoracotomy in achalasia showed an absent oesophageal response (as assessed by oesophageal shortening) to vagal stimulation in all seven cases studied (Matthews et al. 1985). It should be emphasised that these clinical tests of vagal function assess the overall function of the vagus as shown by preservation of vagally mediated cardiac or gastric secretion reflexes and these may not be relevant to the vagal innervation of the oesophagus itself.

Pharmacological investigations, both in vivo and in vitro, have shown that the normal lower oesophageal sphincter (LOS) circular muscle receives an excitatory

cholinergic innervation and a postganglionic inhibitory noncholinergic nonadrenergic innervation. The longitudinal muscle receives an excitatory cholinergic innervation (Tottrup et al. 1990b).

In a manometric study it has been demonstrated that atropine decreased the LOS pressure in achalasic patients and edrophonium, an anticholinesterase, increased LOS pressure in patients with achalasia but not in controls. These findings suggest that the postganglionic cholinergic innervation of the LOS is preserved in achalasia (Holloway et al. 1986). However previous studies had demonstrated a supersensitivity of the LOS to cholinergic agonists suggesting the presence of postganglionic denervation (Kramer 1951, Cohen et al. 1971). The findings of these studies are at variance with each other and the discrepancy is as yet unexplained.

Isolated circular smooth muscle strips from the LOS in achalasic patients have been studied infrequently. Misiewicz et al. (1969) studied one strip and found it did not relax in response to the ganglion stimulant nicotine as would normal oesophagus. Adams et al. treated isolated circular smooth muscle from patients with achalasia with electrical field stimulation and showed a contraction could be produced which was blocked by atropine. This finding suggests an intact postganglionic cholinergic innervation (Adams et al. 1961). The largest study to date of LOS circular muscle showed that in controls the muscle relaxed in response to field stimulation and this appeared to be mediated by noncholinergic, nonadrenergic nerves. In achalasia the circular muscle contracted in response to field stimulation, an effect that was blocked by atropine. Longitudinal muscle strips demonstrated contraction in both controls and achalasics in response to field stimulation, an effect also blocked by atropine. These results suggest that in achalasia the excitatory cholinergic innervation of longitudinal muscle and circular muscle is preserved. However the inhibitory nonadrenergic, noncholinergic innervation of circular muscle fibres is impaired (Tottrup et al. 1990a).

There is evidence to suggest that the nonadrenergic, noncholinergic neurotransmitter involved in control of lower oesophageal sphincter pressure might be vasoactive intestinal peptide (VIP). VIP has been shown to relax the lower oesophageal sphincter and significant decreases in the number of VIP nerves has been demonstrated in achalasia (Aggestrup et al. 1983). As noted earlier, nitric oxide has recently been implicated as the nonadrenergic, noncholinergic transmitter but its role in achalasia has not yet been defined.

Theories of aetiology.

The findings of ganglion cell loss in association with inflammation in Auerbach's plexus have suggested to some that achalasia could be an inflammatory disorder, perhaps resulting from infection with a neurotropic virus (Smith 1970). A report of an association with antibodies to measles virus has not been confirmed and may have arisen by chance (Jones et al. 1983). It has recently been shown that there is an increase in incidence of varicella-zoster antibodies in the serum of patients with achalasia and also varicella-zoster virus DNA in the oesophageal myenteric plexus (Robertson et al 1993). Unfortunately the investigation did not encompass measles or other viruses.

In a postmortem study, 2 of 8 achalasic patients were shown to have intracytoplasmic inclusion bodies, Lewy bodies, in ganglion cells in Auerbach's plexus. These are characteristically found in the brainstem in Parkinson's disease (Qualman et al. 1984). One of the two achalasic patients with Lewy bodies had similar changes in the brainstem as would be seen in Parkinson's disease. Of 22 patients with Parkinson's disease, 3 had dysphagia and in one Lewy bodies were found in the ganglion cells of the myenteric plexus. The authors suggested that there may be a common mechanism for neuronal degeneration in those achalasic patients with AP Lewy bodies and the patient with dysphagia and Parkinson's disease. However Lewy bodies have not been described in subsequent reports of the pathology of achalasia and the association must be regarded as a rare occurrence.

Changes similar to achalasia are seen in the oesophagus in Chagas' disease, but although the oesophageal features are similar, unlike achalasia this disorder affects the whole of the gut and other organs including the heart (Earlam 1972b). There is evidence to suggest that the damage resulting in Chagasic oesophagus may arise from an autoimmune reaction with antibodies to T. Cruzi crossreacting with the antigens present on Schwann sheaths of myelinated somatic and unmyelinated autonomic nerves (Khoury 1979). In achalasia, however, a recent study could find no evidence of an autoimmune disorder. Serum immunoglobulin levels and results of standard autoantibody screening tests were no different to controls and there were no antibodies in serum reacting with myenteric plexus or ganglion cells (Robertson et al. 1991). Similarly Thorpe et al found no evidence of autoantibodies on standard autoantibody screening in serum from patients with achalasia (Thorpe et al. 1988).

It has been suggested that this might be an hereditary disorder based on sporadic reports of its occurrence in multiple family members and also in association with the HLA phenotype HLA DQW1 (London et al. 1977, Nagles et al. 1963, Mackler et al. 1978, Kilpatrick et al. 1972, Westley et al. 1975, Wong et al. 1989). However in Mayberry and Atkinson's study of 1012 first degree relatives of patients with achalasia there was not a single case of achalasia (Mayberry and Atkinson 1985b). This suggests that in the majority of cases achalasia cannot realistically be regarded as a hereditary disorder.

It has been demonstrated in animals that oesophageal ischaemia can produce ganglion cell loss in Auerbach's plexus associated with an achalasia-like manometric picture (Earlam 1967). Although evidence to suggest this could result from an in utero ischaemic injury has been put forward, and might therefore explain some cases of achalasia in childhood, it seems unlikely as a cause in those cases which become apparent only in later life (Earlam 1972a).

Clinical

The most common symptoms are dysphagia, chest pain and regurgitation. Dysphagia occurs for both solids and liquids. The oesophagus dilates as the disease progresses and as this occurs pain becomes less of a feature and dysphagia far more of a problem. Aspiration of stagnant oesophageal contents becomes a real risk.

Manometric features

The two manometric features required for the diagnosis of achalasia are (McCord et al. 1991):

1. LOS fails to relax in response to deglutition.
2. Absence of distal body peristalsis.

Other features may also be found but are not a prerequisite for the diagnosis to be made:

3. Hypertensive LOS.
4. Elevated intraoesophageal pressure.
5. Low amplitude simultaneous contractions throughout the smooth muscle body.

Radiological features.

On chest radiography, in severe disease, a wide mediastinal shadow with a fluid level or absent gastric air bubble may be seen. On barium examination, in early cases, tertiary contractions may be seen. As the disease progresses the oesophagus dilates with a characteristic 'bird's beak' appearance at the lower oesophageal sphincter.

Treatment.

Laboratory studies have shown that lower oesophageal sphincter function can be modified by a variety of drugs, including nitrates and calcium channel antagonists. There is some evidence to suggest that nifedipine may be effective in the treatment of mild or moderate achalasia (Goccia et al. 1991) but in many cases this does not offer a long term solution. Forceful dilatation of the cardia using bougies or pneumatic dilatation is effective and can be expected to produce good results in up to 75% of patients (Reynolds and Parkman 1989).

Surgery in the form of oesophageal myotomy is required for those patients not responding to drugs or dilatations. This produces good results in most reported series (Vantrappen and Hellemans 1980).

Chagas' disease affecting the oesophagus

Introduction

Chagas' disease is an endemic disease confined to South America caused by the protozoan Trypanosoma cruzi. It is a multisystem disease affecting the myenteric plexus of the gastrointestinal tract, the heart and the salivary and sweat glands (Earlam 1972, Bettarello and Pinotti 1976).

Oesophageal involvement in Chagas' disease produces a very similar disorder of oesophageal motility to that found in classical achalasia. This account will concentrate on the oesophageal manifestations of the disease.

Pathology

The disease is carried by a reduviid bug infected with trypanosomes. An initial bite results in inoculation of the skin and is followed by local multiplication of the parasite. There follows a transient parasitemia with systemic illness during which the parasite is deposited in the target organs. Not all individuals progress to the later stages of the disease. In these the parasite is destroyed and the only evidence of infection is a positive Machado-Guerreiro complement fixation test (Koberle 1963, Bettarello et al.1976, Earlam 1972).

It is likely that eosinophils are important in the initial response to the parasite since one of their major roles is in the defence of man from parasitic infestation (Spry 1988). In vitro experiments have shown that eosinophils can kill parasites using toxic proteins contained in their cytoplasmic granules (see section on eosinophils). Eosinophils have been described in Chagasic lesions and recent in vitro evidence has shown that they are activated for secretion shortly after interacting with the parasite (Kierszenbaum et al. 1986).

Those individuals in which the parasite remains in the tissues subsequently develop manifestations of the disease. Once in the tissues, in its leishmanial form, it multiplies to form pseudocysts in the tissues laden with trypanosomal forms. When the pseudocyst ruptures the trypanosomal forms invade adjacent structures particularly small arteries and the myenteric plexus (Bettarello and Pinotti 1976). Progressive damage to the ganglion cells occurs which results in the development of megaoesophagus some years later.

What causes the ganglion cell loss is unclear. The possibilities include direct damage to the neurones by the Trypanosoma, possibly with secretion of a neurotoxin or an inflammatory process. The latter is supported by the frequent finding of a lymphocytoplasmic infiltrate in the region of degenerating ganglion cells.

Recent studies suggest an autoimmune reaction may play a part. T. cruzi-immune lymphocytes can bind to and kill parasympathetic ganglion cells (Teixeira et al. 1980). It appears that the Trypanosoma shares common antigenic determinants with peripheral neurones - a monoclonal antibody to neurones was shown to cross-react with antigens on T. cruzi parasites (Wood et al. 1982). The finding of antibodies in the serum from patients with both the acute and chronic forms of the disease lends further support to the concept that an autoimmune reaction might be involved (Khoury et al. 1979).

Physiology

It has been shown that more than 50% of the myenteric plexus neurones must be destroyed before abnormalities in oesophageal motility can be detected (Koberle 1963). More than 90% must be destroyed before marked dilatation occurs. Initially there is hypertrophy of the circular muscle of the oesophagus but as the disease progresses marked dilatation of the oesophagus occurs with dilatation and thinning of the muscular wall.

In the early stages there is alteration of the motility of the body of the oesophagus and as the disease progressses the disordered function of the LOS becomes more prominent. Stationary manometry reveals the following features.
1. Disordered function of the LOS. Incomplete relaxation of the sphincter in response to a swallow. In the later stages of the disease the sphincter fails to relax in response to deglutition.
2. Reduction in amplitude of oesophageal contractions and subsequent aperistalsis.
3. Presence of spontaneous and repetetive contractions.

Radiology

Barium swallow reveals evidence of tertiary waves, spastic contractions with stasis of barium and failure of relaxation of the LOS.

Clinical features

The commonest complaint is of dysphagia. This is often progressive and can result in cachexia in the untreated case. Regurgitation of oesophageal contents also occurs. Retrosternal pain is not a common feature but occurs occasionally in the early stages.

The diagnosis is established on the clinical findings, radiology, manometry and should only be entertained in patients coming from an area where the disease is endemic. A positive complement fixation test will help to confirm the diagnosis as will the finding of parasites in oesophageal tissues in those cases undergoing surgery (these can be absent since the oesophageal disease can present many years after the initial infection).

Treatment

Drug treatment of the disease is seldom effective. The mainstays of treatment, as for achalasia, are oesophageal dilatation, myotomy and in severe cases oesophageal resection.

Diffuse oesophageal spasm.

Introduction

'A peculiar form of oesophagismus' was the title given to a paper written by Osgood more than one hundred years ago (Osgood 1889). This is credited as the first clinical description of the disease we now know as diffuse oesophageal spasm (DOS). However the aetiology of this condition remains unknown today.

Of all the motility disorders, diffuse oesophageal spasm is perhaps the most difficult to define accurately. The definition will depend on manometric techniques used and these may therefore differ from laboratory to laboratory. Although not a common disorder, in patients investigated for non-cardiac chest pain it accounts for 5 to 15% of the motility disorders identified (Clouse and Staiano 1983, Katz et al. 1987).

DOS has been reported in all adult age groups but its prevalence appears to increase with age. It is equally common in both sexes.

Pathology and pathophysiology.

This disorder affects primarily the smooth muscle oesophagus. Thickening of the distal oesophageal wall has been reported in some cases (Gillies et al. 1967) and histological examination has shown both hypertrophy and hyperplasia in the two outer muscle layers of the oesophagus. The ganglion cells of Auerbach's plexus are not significantly reduced in numbers when assessed at light microscopy. There is conflicting evidence of neural damage in studies performed using the electron microscope (Friesen et al. 1983, Cassella RR et al. 1965). In some reported cases there is some inflammation in Auerbach's plexus and on occasions this has been described as containing eosinophils (Nicks et al. 1968, Marston and Bradshaw 1959).

Manometric findings

Since the early days of manometric assessment the presence of simultaneous contractions at different levels in the oesophagus has been recognised as a characteristic feature of DOS. Other features may be associated: spontaneous contractions, high pressure contractions of prolonged duration and abnormalities

of the lower oesophageal sphincter; however these are not essential for the diagnosis of the disorder (Richter 1984). Simultaneous contractions after both wet and dry swallows can occur in patients with an apparently normal oesophagus but these are more frequent after dry swallows (Richter 1987a).

Clinical

It is important to distinguish symptomatic diffuse oesophageal spasm from those cases which have features of diffuse oesophageal spasm on radiology or manometry without symptoms. The fact that this can occur serves to highlight the difficulties in making the diagnosis. Thus the clinical, radiological and manometric features must all be taken into account in making the diagnosis of DOS.

Chest pain is a common symptom in DOS and may actually mimic cardiac chest pain as it typically occurs retrosternally and may radiate up into the neck or down the arm. The pain is intermittent and may arise spontaneously and may be instigated or exacerbated by swallowing hot or cold liquids, hyperventilation, stress or may arise spontaneously.

Dysphagia is also intermittent and typically patients complain of dysphagia for both liquids and solids. In some cases dysphagia can be exacerbated by stress. Regurgitation is not a usual feature of DOS though may be present in some cases.

Radiology

On barium examination peristalsis is seen in the proximal oesophagus but in the distal oesophagus the normal peristaltic wave may be interrupted by lumen obliterating tertiary contractions. These give the characteristic radiological corkscrew appearance. It is noteworthy that the finding of tertiary contractions on a barium examination is not diagnostic of diffuse oesophageal spasm. These radiographic findings can occur in normal people with no evidence of oesophageal disease and conversely patients who have diffuse oesophageal spasm diagnosed by other criteria may not have tertiary contractions on the barium swallow although there is usually some abnormal motility (Bennett and Hendrix 1970, Chen 1989).

Treatment

Many patients can tolerate their symptoms once they are reassured that no sinister disease underlies their swallowing difficulty. Drugs, e.g. nifedipine, have been tried with variable success (Editorial Lancet 1987, Vantrappen and Hellemans 1982). Dilatation may be effective in some cases but often has to be repeated. In those cases where symptoms are severe and simpler treatment has failed, surgical treatment in the form of a long oesophageal myotomy may be required (Ellis et al.1964, Eypasch et al. 1992).

Nutcracker oesophagus.

Like DOS, nutcracker oesophagus is a well recognised cause of noncardiac chest pain (Benjamin et al.1979). In this condition, although contractions are all peristaltic, they are of an excessively high amplitude. It is currently defined as a manometric abnormality characterised by an average distal oesophageal peristaltic pressure greater than 2 standard deviations above the mean for the normal population (Castell 1987). It was first described by Brand and Pope in a manometric study of patients with angina-like chest pain (Brand and Pope 1977). Clinically it presents with episodic chest pain and dysphagia. It is the most frequent abnormality found in manometric studies on patients with oesophageal chest pain, making up 38% to 48% of the motility abnormalities detected (Herrington et al. 1984, Katz et al. 1987). The pathogenesis and pathological features of the disorder are unknown.

Treatment of the disorder with drugs such as nitrates, anticholinergics, apresoline or nifedipine may be effective but no controlled trials are available to assess their effectiveness. Simple reassurance may lead to an improvement with time. The place of oesophageal myotomy in treating the disorder is contentious (Richter and Castell 1987b).

Vigorous Achalasia

This is a condition, first described by Olsen, in which there are clinical and manometric features characteristic of both achalasia and DOS (Olsen 1957).

Dysphagia, pain and regurgitation are the predominant symptoms. Patients usually have a shorter history of disease than that normally found in achalasia.

Pain can be quite a distressing symptom - a problem found less frequently in classical achalasia.

Radiological assessment shows a moderate degree of oesophageal dilatation in most cases but features suggestive of DOS are found in nearly half of the cases (Sanderson et al. 1967).

Manometry reveals that patients with vigorous achalasia share features of both achalasia and diffuse oesophageal spasm. It has thus been described as a variant of DOS (Creamer et al. 1958, DiMarino and Cohen 1974) and also as a variant of achalasia (Editorial Brit Med J 1974). Like achalasia, there is absence of peristalsis in the body of the oesophagus associated with failure of relaxation of the LOS. Simultaneous contractions in response to a swallow which may be repetetive are found and these are of higher amplitude than classical achalasia. It is this latter feature that is said to distinguish the two conditions manometrically. However recent studies using modern manometric techniques suggest that there is a range of amplitude of simultaneous contractions in achalasia and that vigorous achalasia merely represents the upper end of this range (Goldenberg et al.1990, Todorczuk et al.1991). No differences in clinical features or response to treatment in those patients with higher amplitude contractions were found.

Treatment consists of dilatation in the first instance, but, unless a lasting response is achieved, oesophagomyotomy is usually required to produce relief of symptoms.

Hypertensive lower oesophageal sphincter.

The more recent widespread use of manometry in the assessment of oesophageal disease has led to the recognition of a condition with an excessively high LOS pressure (Code et al.1960, Katz et al.1987, McCord et al. 1991). Some cases have other abnormalities of contraction in addition, e.g. DOS, but in a proportion of cases the LOS hypertension is an isolated finding (Clouse and Staiano 1992). These patients often have very large and prolonged contractions occurring after sphincter relaxation (Garrett and Godwin 1969).

It is not clear why patients with a hypertensive LOS but with normal oesophageal peristalsis should have the presenting symptoms of pain and dysphagia. It is possible that this is a marker of a more severe but intermittent underlying motility

disorder. The advent of 24-hour manometric assessment may help to clarify the situation.

Nonspecific oesophageal motility disorders.

With the increased use of manometry in the investigation of oesophageal disease it has become apparent that there are patients who have manometric features that are clearly abnormal but do not fit into any of the current diagnostic classifications as detailed above (Castell 1987).

These include the following:-

Increased nontransmitted contractions (>20% wet swallows).
Triple-peaked contractions.
Retrograde contractions.
Low-amplitude contractions.
Isolated incomplete LOS relaxation.
Prolonged-duration peristaltic waves (>6seconds).

The clinical significance of these findings is not clear but it is thought that some may represent early forms of one of the classical motility disorders (Castell 1987).

Conclusions

The aetiology of the motility disorders as a group is unknown. Pathological studies have demonstrated ganglion cell loss in achalasia but this is not a feature of diffuse oesophageal spasm. An inflammatory exudate has been described in both DOS and achalasia and in some instances an associated eosinophil component has been found. These cells may have a significant pathogenic role.

1.4 INFLAMMATION

Inflammation is the local tissue response to injury. The injury may be physical, chemical or may occur in response to a foreign antigen. There are three components of an inflammatory reaction:- increased blood flow to the area; increased capillary permeability of local blood vessels; increased migration of cells from the blood vessels to the local area.

The cellular characteristics of an inflammatory response depend upon a number of factors. The antigenic stimulus, the site of the inflammatory response, and the duration of the response all influence the nature of the cellular infiltrate.

In acute inflammatory lesions neutrophils are very numerous. When a lesion becomes chronic there is a relative reduction in the number of neutrophils and mononuclear cells become more prominent. In delayed-type hypersensitivity large numbers of basophils are seen. It is well known that in asthma and some parasitic diseases eosinophils are frequently found in the inflammatory infiltrate.

1.5 THE EOSINOPHIL POLYMORPHONUCLEAR LEUCOCYTE

Introduction

Since Ehrlich's first description of the characteristic staining properties of the eosinophil in 1879 many different functions have been attributed to this readily recognisable cell (Spry 1988). Accummulation of eosinophils in the tissues has been associated with parasitic and allergic diseases since the turn of the century. However their role in these disorders has only become more clear with advances in the understanding of the immune response at a cellular and biochemical level.

The cytoplasm of the eosinophil contains granules which are responsible for its particular staining properties (the granules stain a red colour with eosin). The nucleus is lobulated in a similar fashion to that of the neutrophil. These cells are however derived from very different precursor cells within the bone marrow and have very different functions. The eosinophil granules contain toxic proteins which are responsible for many of the local effects that can result from eosinophil degranulation.

Eosinophil granules.

There are three types of granules present in the cytoplasm of eosinophils (Gleich and Loegering 1984):

1. Primary granules.
These are found in immature eosinophils and are thought to develop into secondary granules.

2. Secondary granules.
These are the predominant type in mature cells and consist of a matrix with a crystalline core.

3. Small granules.
These are less dense and contain arylsulphatase and acid phosphatase.

It is only relatively recently that the main component proteins of the secondary granules have been characterised.

Major basic protein (MBP).

The crystalline core of the secondary granule is a crystal of major basic protein (Gleich and Loegering 1984). This protein has a molecular weight of 14,000 Daltons. MBP is also present in the granules of basophils, which might be expected since they both appear to arise from a common precursor cell in the bone marrow. Since eosinophils are found in the tissues in parasitic infestations it is of interest that they have been found to have the ability to damage parasites in vitro. Major basic protein has been shown to be directly toxic to schistosomules (an intermediate stage in the life cycle of the Schistosoma) in vitro. It has also been shown to have the potential to damage mammalian cells in experiments on murine ascites tumour cells in vitro. Thus it may have a role in tissue damage associated with hypersensitivity reactions. As well as its direct toxic effects it is also able to stimulate mast cells to degranulate.

Eosinophilic cationic protein (ECP).

This protein was first isolated from the matrix of the granule by Olsson (Olsson and Venge 1974). Its molecular weight is 18-21,000 Daltons. ECP is cytotoxic to both mammalian and nonmammalian cells such as parasites. It can also induce

the Gordon phenomenon. (In 1933 M.H.Gordon injected extracts of eosinophil-rich lymph nodes from patients with Hodgkin's disease into the brains of rabbits and produced paralysis. The active ingredient was shown to be produced by the eosinophils and was subsequently called eosinophil-derived neurotoxin (Gordon 1933, Fredens 1982).). Two antigenically distinct forms exist depending on whether the ECP is found intracellularly or extracellulary (Tai et al. 1984a). Like MBP it has the ability to stimulate histamine release from mast cells but it can also inhibit T lymphocyte responses.

Eosinophil-derived neurotoxin (EDN).

This 18,000 Dalton protein, like ECP, is found in the granule matrix. It is neurotoxic as shown in the Gordon phenomenon (Durack et al. 1981).

Eosinophil peroxidase (EPO).

EPO is formed of two chains: a heavy chain of 52,000 Daltons and a light one of 15,000 Daltons. It is found in the granule matrix. In conjunction with hydrogen peroxide and halide it can kill bacteria, parasites and mammalian cells. It can also initiate mast cell secretion.

Charcot-Leyden Crystals (CLC)

These crystals can be extracted from eosinophils and may be shed in the sputum of asthmatics. They are pure crystals of a lysophospholipase which is located in the plasma membrane of the eosinophil. Lysophospholipase may have a role in removing potentially cytotoxic lysophospholipids formed by lysophospholipase A2.

Other granule-associated enzymes include the following (Kay 1985):

Arylsulphatase
Phospholipase
Histaminase
Acid phosphatase
Beta glucuronidase
Acid beta glycerophosphatase
Ribonuclease
Cathepsin

Eosinophils can also release lipid mediators which include platelet activating factor (PAF) and leukotriene C4. The latter is a potent vasoactive mediator which causes smooth muscle spasm and mucus secretion (Weller et al. 1983).

In addition to generating inflammatory mediators themselves, eosinophils can induce mediator release from mast cells and basophils thus amplifying the inflammatory response.

Cell surface receptors and proteins

Interactions between different inflammatory cells and between inflammatory cells and locally released mediators are determined by receptor expression. There has been much study of such receptors in relation to the eosinophil (Kay 1985). Eosinophils express receptors for IgG, IgE and IgA on their cell membranes. There are also receptors for complement components C1q, C3b/C4b (CRI), iC3b (CR3) and C5a (Weller 1991). There are receptors for the cytokines interleukin 3 (IL-3), interleukin 5 (IL-5) and granulocyte monocyte colony stimulating factor (GM-CSF).

Recent interest in leucocyte adhesion molecules (which are involved in regulating the sites of leucocyte tissue migration) has been extended to the eosinophil. Although these glycoproteins appear to be expressed on the cell membrane of the eosinophil, the cell still has the ability to migrate into the tissues in an independent fashion (Weller 1991).

It has also been shown that eosinophils express CD4 which acts as a signal transducer which on binding promotes migration rather than degranulation. The eosinophil can also synthesise HLA-DR and express it on its cell surface allowing it to present antigen to accessory inflammatory cells.

Eosinophil production and activation occurs principally under the influence of three cytokines for which it has receptors:- GM-CSF, IL-3 and IL-5. GM-CSF and IL-3 can stimulate other cells such as neutrophils, but IL-5 appears to have an effect that is relatively specific for eosinophils.

Functions of the eosinophil

Originally the eosinophil was thought to have a role that was primarily one of modulating or dampening down inflammation occurring subsequent to the release of mast cell and other cell products in inflammation. The presence of enzymes that could deactivate mast cell products lent support to this idea (e.g. histaminase, arylsulphatase) (Goetzl et al. 1979). However the increased understanding of the structure and properties of the granule proteins, and the finding of granule protein products in the tissues and secretions of patients with eosinophil-associated diseases, has resulted in the view that these cells are highly cytotoxic and may be producing much of the tissue damage in their own right (Weller 1991, Kay 1985).

It seems that the primary role of eosinophils is host defence. Although the eosinophil is capable of phagocytosis it is much less efficient at this than neutrophils. Its toxic granule proteins and other mediators are capable of extracellular cytotoxicity and it seems that the eosinophil is best suited to this role. Thus they form an important defence mechanism against parasites which are too large to be phagocytosed.

Eosinophil-associated diseases

Apart from those diseases caused by parasitic infestation, eosinophils have also been shown to accumulate in the tissues in a wide variety of diseases (for review see Spry 1988) A significant role for eosinophils has been demonstrated in the pathogenesis of disorders as diverse as asthma, allergic rhinitis, endomyocardial fibrosis, allergic skin disorders and the idiopathic hypereosinophilic syndrome.

Since mast cells are often intimately involved in reactions involving eosinophils their role in the inflammatory response will be discussed next.

1.6 THE MAST CELL

Introduction

The mast cell is ubiquitous in the body. It is found in the mucosa lining the gastrointestinal tract, in the mucosa of the bronchial tree and is present in connective tissue usually in perivascular sites (Wershil and Galli 1991, Lemanske et al. 1983).

A single mast cell contains several hundred metachromatically staining granules each surrounded by a two-layered membrane.

There is evidence to suggest that mast cells are a heterogeneous group. In rats and man, mucosal and connective tissue mast cells appear quite dissimilar with regard to staining properties and secreted products.

Mucosal mast cells occurring in the gastrointestinal tract appear to have granules which all contain tryptase so that these cells are also called MC^T cells. In vitro experiments have shown they are dependent on the presence of interleukin-3 for their growth and development.

In contrast, connective tissue mast cells contain the enzymes tryptase and chymase (hence designated MC^{TC}) in all their granules and are independent of interleukin-3 for their growth and development (Bernstein 1990).

Mast cells can be stained with a number of different histochemical stains or their contents can be targetted by antibodies for immunohistochemical staining. Most histochemical stains rely upon metachromasia to demonstrate the mast cells. Toluidine blue, alcian blue, astra blue, thionine and azure A have all produced satisfactory staining of these cells.

The mast cell plays an important role in immediate-type hypersensitivity. It possesses Fc receptors on its membrane with high affinity for the Fc component of IgE. Thus IgE is passively bound to mast cells and acts as a trigger for mast cell degranulation when these IgE molecules are crosslinked by the appropriate antigen. This results in release of preformed mediators from the mast cell granules and also the synthesis of other mediators for release.

Mast cells can also be stimulated to release their contents by non-IgE-mediated mechanisms. There is much evidence to suggest a nerve-mast cell association

providing a link between the nervous and immune systems (Church et al. 1989, Russell et al. 1984, Shanahan 1985, Goetzl et al. 1990, Greene et al. 1988).

Mast cells play a significant role in tissue inflammation since release of their preformed or newly formed inflammatory mediators into the tissues contribute to the vascular and cellular changes seen. The range of substances that may be secreted by mast cells is detailed in Table 1.4.1 (modified from Wasserman 1980).

Table 1.4.1.

Mediators derived from mast cells(* = preformed in granules)

Vasoactive/smooth muscle reactive mediators

Histamine*
SRS-A (LTC4 and LTD4 are the most important components)
Serotonin
PAF (platelet activating factor)
Arachidonic acid metabolites: PGD, PGE, PGI, PGF2alpha,
HETE

Chemotactic mediators

Eosinophil chemotactic factor of anaphylaxis. ECF-A*
ECF-oligopeptides
Neutrophil chemotactic factor of anaphylaxis. NCF-A
Lipid chemotactic factors
Histamine*

Structural proteoglycans

Heparin*
Chondroitin 4 and 6 sulphates and Dermatan sulphate

Enzymes
Chymase
Arylsulphatase
N-acetyl-beta-D-glucosaminidase
Kallikrein
Plasmin

Mast cells have a central role in allergic responses. They secrete substances that produce the characteristic features of inflammation and also substances chemotactic for other cells including eosinophils.

Role of mast cells in human disease .

Mast cells have been shown to be involved in the pathogenesis of a wide variety of diseases (Zweiman 1983, Bernstein 1990).

Skin.

The population of dermal mast cells is increased in a number of skin diseases and they appear to play a pathogenic role in mastocytosis and urticaria pigmentosa.

Nasal diseases.

Allergic rhinitis is associated with a prominent eosinophilic exudative response but there are conflicting reports as to whether mast cell numbers are increased or not. Nasal polyposis provides a rich source of mast cells.

Asthma.

Mast cells have a central role in acute asthma and will be discussed more fully in the next section.

Gastrointestinal disorders.

There is evidence that mast cells may have a role in a number of gastrointestinal disorders including ulcerative colitis, Crohn's disease and other disorders.

1.7 EOSINOPHIL AND MAST CELL ASSOCIATED DISEASES

Asthma

Asthma is perhaps the most extensively studied disorder associated with a tissue eosinophilia. As such, much of what has been learned of its pathogenesis may be applicable to other disorders associated with eosinophil accumulation.

Asthma is a clinical syndrome characterised by episodes of reversible airway obstruction which resolve spontaneously or in response to appropriate treatment. Asthma can be thought of as either extrinsic, where a clearly defined allergen is associated, or as intrinsic where it is not. The pathological changes have been more extensively studied in extrinsic asthma but are similar in both (Johnston et al. 1991).

Inhalation of the appropriate allergen in an extrinsic asthmatic will result in an acute asthmatic response. These patients frequently have a raised level of IgE in the blood which is specific for the allergens to which they are allergic. These IgE antibodies are passively bound to mast cells in the airways. Encounter with the appropriate antigen (allergen) will result in crosslinking of these antibodies and will stimulate the mast cells to release histamine and other mast cell products which produce an early asthmatic response (Johnson et al. 1991). This occurs within 15 minutes of allergen inhalation and is characterised by smooth muscle contraction (bronchospasm), plasma exudation and mucus production in the airways. After a recovery period of 1 - 2 hours there is a further decrease in respiratory function with maximal effect at 6 -12 hours and further recovery at 24 -36 hours. There is a release of inflammatory mediators into the blood and pathological studies have demonstrated an infiltrate of eosinophils, neutrophils and lymphocytes in the airways with evidence of their activation (Bradley et al. 1991, Moqbel et al.1992) In association with this inflammatory infiltrate there appears to be a bronchial hyperreactivity to nonspecific stimuli as assessed by response to inhaled histamine or methacholine (Kay 1987, Ferguson et al. 1992). Of interest, a recent study of biopsies taken from mild asthmatics has shown mast cell degranulation and a widespread eosinophil infiltrate in the mucosa and submucosa of the bronchial tree in these patients. Eosinophils, mast cells and mononuclear cells were present in increased numbers and were frequently found to be in contact with vascular endothelium (Beasley et al.1989).

Eosinophils are thought to have a major pathogenic role in asthma. Their cytotoxic potential has been discussed earlier and evidence of activation of these cells and the finding of products of secretion in the airways in asthmatics lend support to this hypothesis (Filley et al.1982,De Monchy et al.1985).

What attracts the eosinophil to the airways is as yet unclear. It appears that the mechanism may differ in intrinsic and extrinsic asthmatics (Kay 1993). In extrinsic asthmatics it seems that the patients have increased numbers of IgE antibodies in the blood with specificity for the offending antigens. Degranulation of mast cells when their passively bound IgE is crosslinked results in the release of mediators that attract eosinophils to the site of inflammation. In intrinsic asthma it seems that mast cells and IgE are not involved in attracting eosinophils and it may be that a subgroup of T cells (TH2 cells) may be involved. In response to an as yet unknown stimulus these cells secrete IL-5 and other substances that attract eosinophils to the site of inflammation. This results in a dense eosinophil infiltrate in the airways.(Johnson et al.1991).

Much of what has been learnt from the study of asthma is relevant to other eosinophil-associated diseases.

Eosinophilic Gastroenteritis

Eosinophilic gastroenteritis can affect any part of the gastrointestinal tract and is characterised by oedema of the bowel wall with an associated infiltrate of eosinophils. The stomach and small intestine are most frequently reported as being affected by the disease, but colon and oesophagus can also be involved.

The condition can affect adults and children of either sex. The aetiology is unclear. In some reports there is evidence of an allergic cause; patients giving a strong history of allergic phenomena (asthma, eczema, allergic rhinitis, food allergy). Some cases are associated with elevation of serum IgE.

The clinical features depend on the part of the gastrointestinal tract involved and the depth of involvement of the wall (Klein et al.1970).

Three patterns emerge:-

1. Predominantly mucosal disease
   These patients have a protein-losing enteropathy with faecal blood loss and iron-deficiency anaemia.

2. Predominantly muscle disease
   There is marked hypertrophy and rigidity of the bowel wall producing obstructive symptoms. This most commonly occurs at the pylorus producing gastric outlet obstruction. The condition is often diagnosed at surgery.

3. Predominantly serosal disease.
   In this variant, patients present with ascites which is heavily infiltrated with eosinophils.

Treatment of eosinophilic gastroenteritis depends on the individual variant type. Many of the patients with obstructive symptoms will come to surgical relief of their obstruction. Supportive therapy will be needed for the other forms. Corticosteroid therapy has proved effective in treating the initial disease and any further relapses (Scudamore et al.1982, Zora et al. 1984). A proportion of patients require long term steroid treatment. A recent report described a patient with predominant mucosal involvement who responded well to oral sodium cromoglycate (Moots et al. 1988).

Eosinophilic gastroenteritis affecting the oesophagus.

This was first described by Dobbins et al in 1977 (Dobbins et al. 1977). Their patient, who had a long history of asthma and hay fever, presented with dysphagia. He was found to have a heavy eosinophil infiltrate in the mucosa of the oesophagus and also in that of the small bowel, but there was no evidence of gastric involvement. Oesophageal pH studies were normal. Manometry showed features suggestive of diffuse oesophageal spasm with simultaneous contractions of high pressure and increased duration (Dobbins et al. 1977).

It is unclear whether this case was truly a case of eosinophilic gastroenteritis since there was no eosinophilic infiltrate in the stomach during the illness.

A further report is of a 3 month old child presenting with vomiting in whom many eosinophils were found in the oesophageal mucosa. No other part of the gastrointestinal tract was biopsied and manometry was not performed. This child

did not have pH studies and although barium swallow and endoscopy did not demonstrate reflux, the possibility that the child had reflux was not excluded (Forget 1978). It is interesting that this child had an elevated eosinophil count and raised IgE.

Landres described a man who presented with dysphagia having had two previous gastric operations. He had a peripheral eosinophilia and raised IgE in the serum. Radiological and manometric assessment revealed features of vigorous achalasia. Oesophageal biopsies showed chronic inflammation. Jejunal biopsies were normal. When symptom relief from dilatation was not successful a myotomy was performed. Biopsies from the muscularis propria revealed a marked eosinophilic infiltrate. Ganglion cells were not seen but nerve fibres were normal (Landres 1978).

Picus investigated a 16 year old boy and found him to have an oesophageal stricture. An oesophageal mucosal biopsy showed an eosinophil infiltrate. He had an associated eosinophilia. This report could easily be explained by reflux oesophagitis, which was not excluded by their investigations (Picus and Frank 1980). Matzinger reported a further case in which a 15 year old boy had a nine year history of illness and was found to have eosinophil infiltrates in his oesophagus and stomach. There was radiological evidence of small bowel damage with evidence of excess protein loss. No manometry was performed. Treatment with sodium cromoglycate was commenced but clinical improvement was only achieved when oral prednisone was started (Matzinger and Daneman 1983).

The above cases would appear to be an heterogeneous group. It is likely that the cases of Dobbins and Landres suffered with the motility disorders diffuse oesophageal spasm and vigorous achalasia rather than eosinophilic gastroenteritis. The case described by Picus did not have complete investigations, but did not have strong evidence of a gastroenteritis. Of the papers listed, Matzinger's case is probably the only true case of eosinophilic gastroenteritis affecting the oesophagus.

1.8 AIMS OF THIS RESEARCH.

The myenteric plexus has a pivotal role in the control of normal oesophageal contraction. It is possible that the primary abnormality in motility disorders of the oesophagus could lie in this plexus. This work aims to study the pathological changes within Auerbach's plexus in these motility disorders. The presence of inflammation in relation to the plexus and the definition of its cellular characteristics will be specifically addressed.

2.GENERAL METHODS

2.1 MANOMETRIC ASSESSMENT.

Manometric assessment was performed at the Oesophageal Function Laboratory at Birmingham Heartlands Hospital (East Birmingham Hospital). All the manometric assessments were to the same protocol and were performed or supervised by a single clinical physiologist (Mr.I Adams). The manometric records were analysed retrospectively by J.Duffy in association with I. Adams.

Equipment

Manometry catheter.

A 4 lumen PVC manometry catheter (Portex Ltd.- modified). The internal diameter of each channel was 1mm. The catheter assembly had an elliptical cross-section with dimensions of 3mm x 5mm. Each channel had one side hole and these were radially orientated at 5 cm. intervals with the lowest being 5 cm. from the catheter tip. There was no end hole in the catheter.

Perfusion.

Each lumen was separately perfused with distilled water at 0.6 mls/min. from an Arndorfer pneumohydraulic infusion pump.

Recording.

Each lumen was perfused through a separate transducer (PDCR 75, Druck Ltd.) A throat microphone (Lectromed) recorded voluntary swallows. A belt pneumograph monitored respiration. All the above parameters were recorded simultaneously using a Lectromed M19 8-channel recorder at a paper speed of 2 mm./second. The system was calibrated against a manometer every 6 months.

Clinical procedure

Patient preparation.

All drugs known to affect oesophageal function were stopped for 24 hours prior to the study. The patients were not allowed to eat food in the 6 hours prior to the study but could drink freely.

Standard protocol.

After spraying the nose internally with lignocaine spray the manometry catheter was passed pernasally into the stomach. The belt pneumograph and throat microphone were attached. After a 10 - 20 minute settling period the manometric recording was started.

A station pull-through technique was used with the catheter being withdrawn at 1 cm. steps. At each station the trace was allowed to settle and the response to at least one dry swallow recorded (the patient was asked to dry swallow at 30 second intervals). These 1 cm. steps were repeated until the bottom sidehole reached the cricopharyngeus. The procedure was only repeated if the quality of the recording was deemed inadequate.

Normal findings.

Using the above techniques and equipment a set of limits for what is regarded as normal has been established. These are set out below.

Body of the oesophagus.

At least 80% of contractions should be peristaltic after a swallow. There should be no repetitive activity although there may be some double-peaked contractions. Spontaneous activity is uncommon.

Lower oesophageal sphincter (LOS).

Pressure:
End-expiratory, mean of 3-4 measurements (asymmetric pressure profile) mmHg. (Normal range 12-25 mmHg).

Relaxation:
The LOS should relax in response to a swallow. Full relaxation is defined as within 3 mmHg of gastric baseline.

Upper oesophageal sphincter.

Pressure:
Variable. Much greater radial asymmetry than LOS. Range : 40 - 175 mmHg.

Relaxation:
Full to pharyngeal pressure.

Manometric definitions of the motility disorders.

The widespread use of stationary manometry has allowed more accurate definition of the motility disorders of the oesophagus. The techniques used in our laboratory are well recognised and comparable to those used in many other laboratories throughout the world.

Achalasia

It is generally recognised that there are two essential features for the diagnosis of achalasia to be made. These are a lower oesophageal sphincter (LOS) that relaxes incompletely in response to swallowing and absence of peristalsis in the oesophageal body. (see Fig 2.1.1 for an example of a typical manometric tracing).

Other features are also present and support the diagnosis

Lower oesophageal sphincter (LOS):
Non-relaxing in response to swallowing.
Pressure >25 mmHg.

Hypercontraction with/without previous partial
relaxation of LOS.

Oesophageal body:
No peristaltic pressure waves.
Contractions < 50 mmHg. in the majority of contractions.
Raised intraoesophageal resting pressure (> gastric).
Simultaneous pressure waves, mirror image, common cavity effect throughout the entire oesophagus.

Upper oesophageal sphincter:

Normal.

Vigorous achalasia:

The criteria for vigorous achalasia are essentially those of achalasia except that there is high pressure contractile activity in the majority of contractions (> 50 mmHg). A typical manometric record is shown in Fig 2.1.2

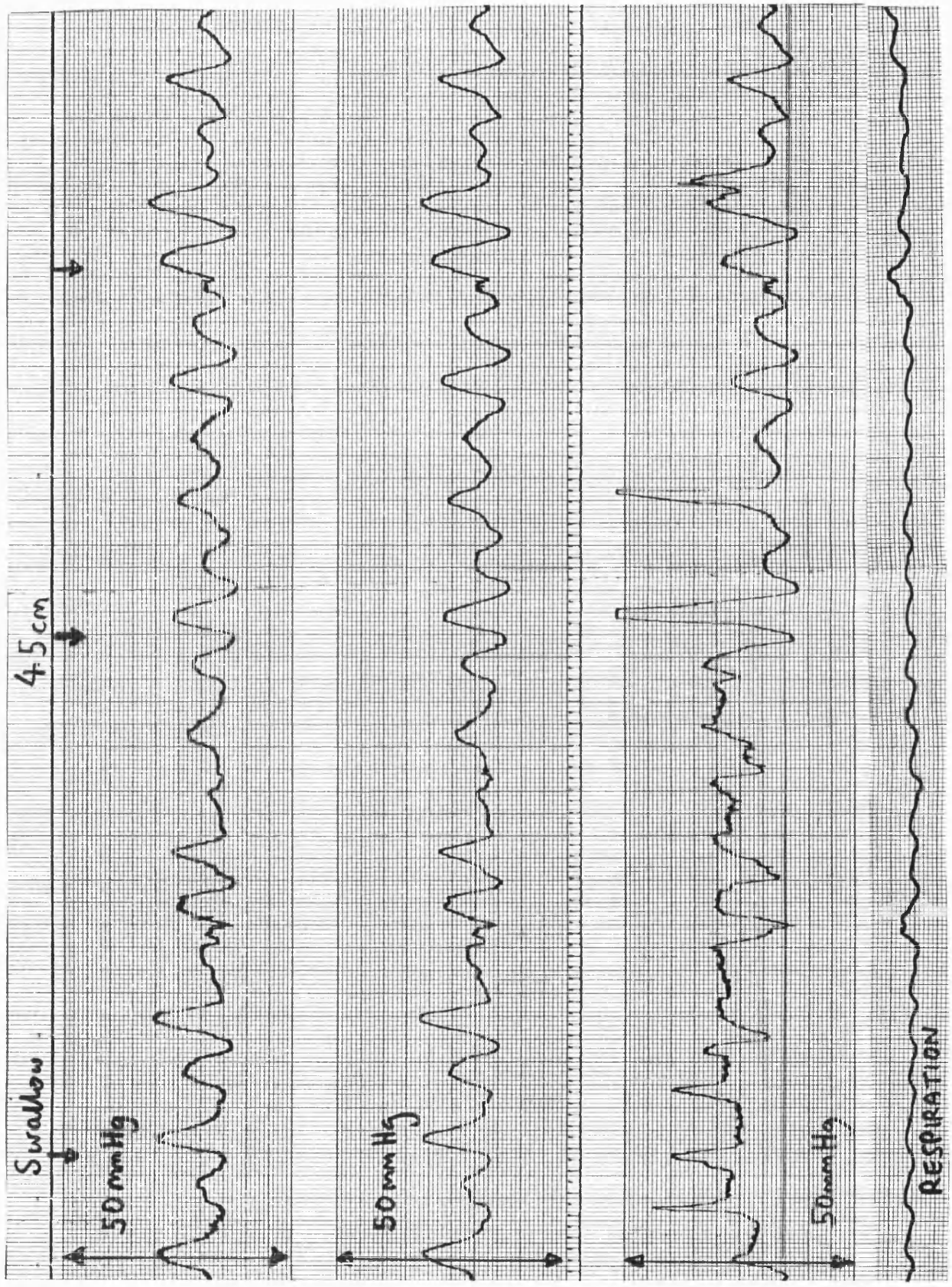


Fig 2.1.1 Typical manometric recording of achalasia (4249/81)

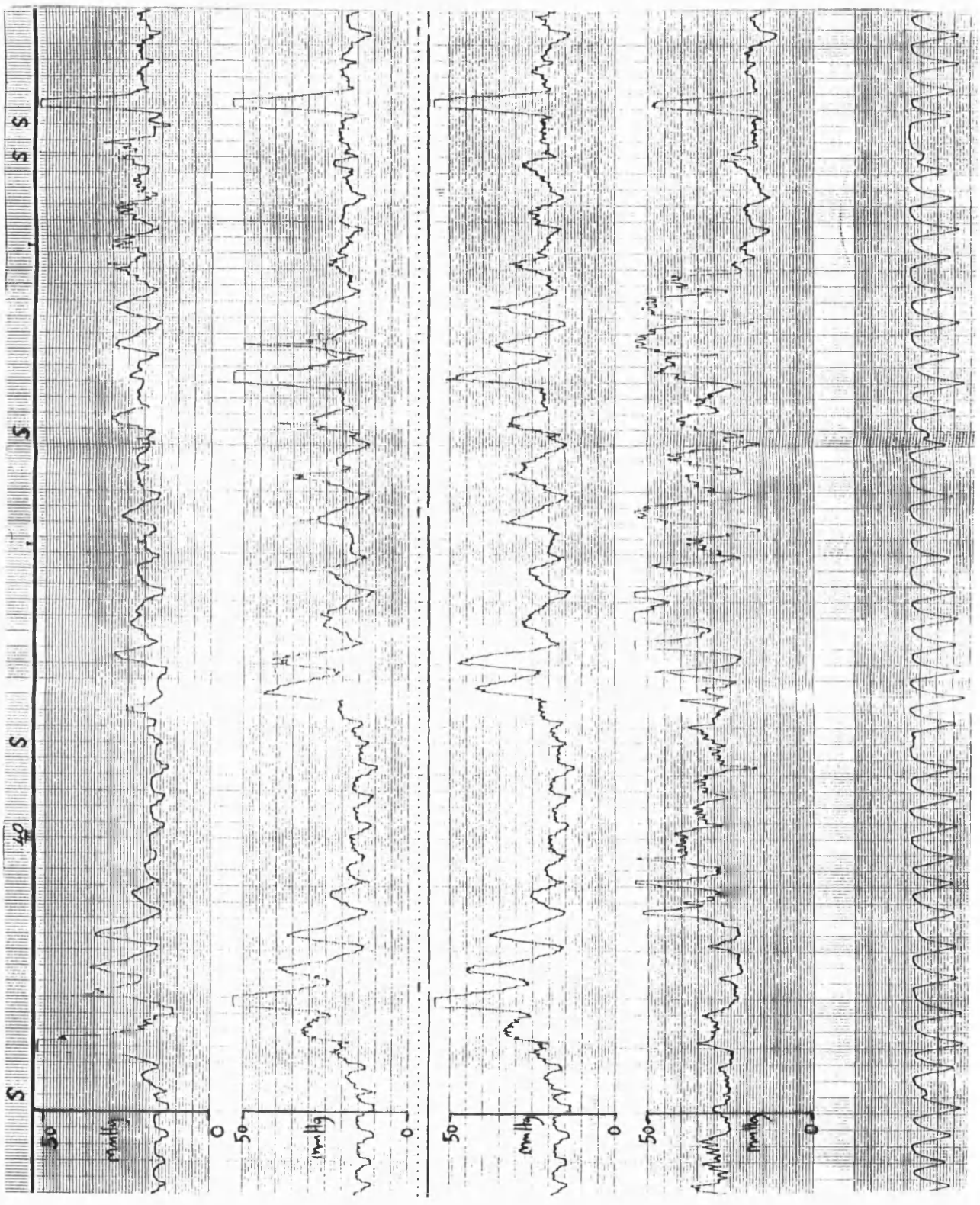


Fig 2.1.2 Typical manometric recording of vigorous achalasia (11/92)

Diffuse oesophageal spasm.

For the diagnosis of diffuse oesophageal spasm there has to be evidence of an increased number of simultaneous contractions occurring at different levels in the oesophagus. Some normal peristalsis should take place. Although other features such as spontaneous activity and multipeaked contractions may be present these are not prerequisites for the diagnosis. (see Fig 2.1.3 for a typical manometric tracing).

Lower oesophageal sphincter:
The lower oesophageal sphincter in DOS has variable characteristics.
LOS pressure can be normal, decreased or increased.
LOS may or may not relax on swallow.

Oesophageal body:
Peristalsis present in the upper third of oesophagus.
In the lower two thirds of the oesophagus:
Nonprogressive contractions.
Repetitive contractions (more than two peaks are required).
Increased frequency of spontaneous contractile activity.
Contractile pressure normal or high (ie >25 mmHg)

Upper oesophageal sphincter:
Normal.

Nutcracker oesophagus

Nutcracker oesophagus is a condition characterised by excessively high amplitude contractions which are peristaltic in nature and often painful. See Fig. 2.1.4 for a typical manometric tracing.

Lower oesophageal sphincter:
Variable response to swallowing, as in DOS
Variable pressure.

Oesophageal body:
High pressure peristaltic activity in the lower two thirds of the oesophagus:
Pressure waves of mean amplitude of >130 mmHg.
Peak pressures of >200 mmHg.
Duration of contractions extended (>5.5 seconds).
(High pressure contractions tend to be longer anyway).

Upper oesophageal sphincter:
Normal.

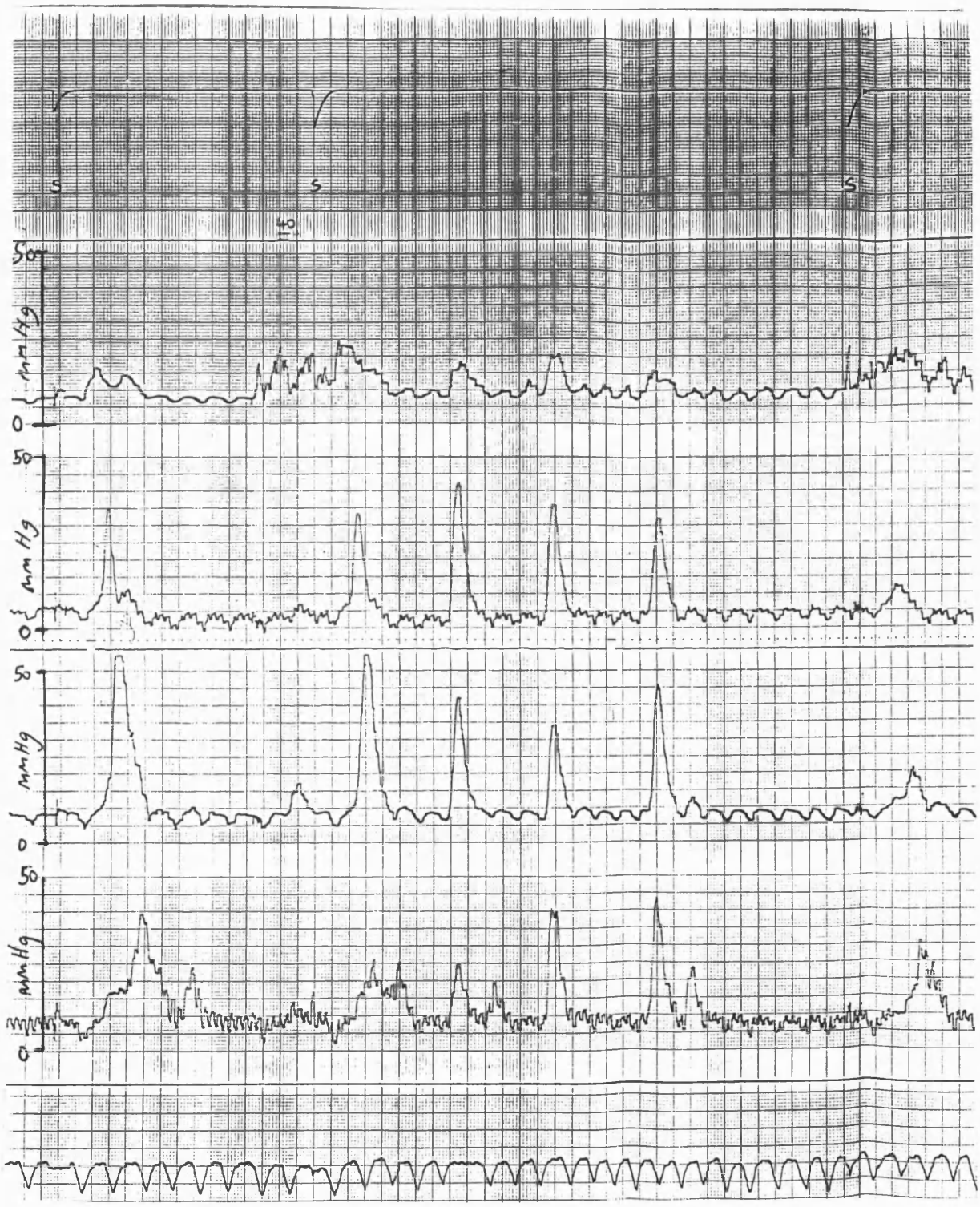


Figure 2.1.3 Typical manometric tracing of diffuse oesophageal spasm. (4335/83)

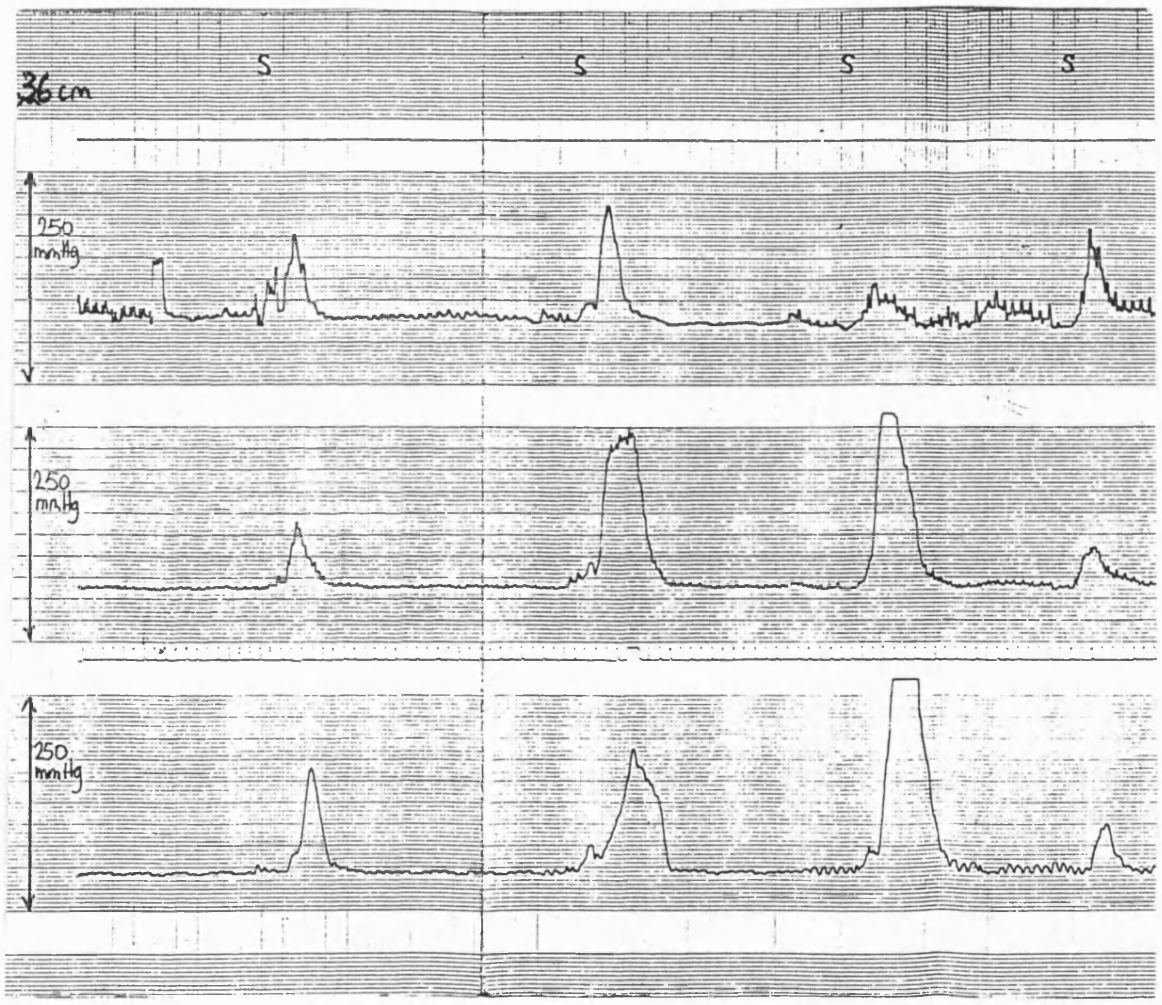


Figure 2.1.4 Typical manometric tracing of nutcracker oesophagus. (8135/82)

2.2 OPERATIVE TECHNIQUES.

The tissue studied in this thesis has come from a number of different sources. A brief outline of the operations performed is given below.

Oesophageal resection.

Specimens were obtained at the time of surgery for both benign and malignant disease. The most frequent approach at Birmingham Heartlands Hospital for oesophageal resection was a left thoracolaparotomy and subtotal oesophagectomy. The stomach was used as an intrathoracic conduit being placed in the posterior mediastinum and anastomosed to the cervical oesophagus through a left cervical incision. Thus at each operation two separate specimens were obtained. The main specimen comprised a cuff of stomach attached to the distal oesophagus. At the time of the cervical anastomosis a segment of proximal oesophagus was also obtained.

Removal of transplant donor oesophagus.

Transplant donor oesophagi were all harvested by myself. Full informed consent was obtained from the next of kin. The oesophagus was harvested after the heart, lungs, liver and kidneys had been removed. The oesophagus was exposed in the posterior mediastinum and dissected as far proximally as possible (within 2 - 3 cm of cricopharyngeus). Distally two ligatures were placed around the gastric fundus and the stomach divided between them. Thus the oesophagus had a cuff of stomach attached allowing for easy orientation.

Myotomy

The approach for those patients undergoing myotomy (for achalasia, diffuse oesophageal spasm or nutcracker oesophagus) differed only in the extent of the myotomy itself. A left thoracotomy was performed through the 7th intercostal space. The oesophagus was mobilised up to the aortic arch. A longitudinal myotomy was performed using a Pott's scissors. The outer two layers of the oesophagus were divided so that the oesophageal mucosa bulged through. The myotomy in achalasia extended proximally for 10 cm. from the oesophagogastric junction and distally was carried a short distance onto the stomach. The myotomy

extended from oesophagogastric junction to the aortic arch in patients with DOS and nutcracker oesophagus. A specimen was taken of the edge of the divided muscle so that both outer muscular layers and the myenteric plexus were included. A Belsey Mark IV hiatus hernia repair was then performed.

Hiatus hernia repair.

A standard Belsey Mark IV hiatus hernia repair was performed through a left thoracotomy. Mobilisation of the oesophagus was performed up to the aortic arch. 1 - 2 cm. above the gastrooesophageal junction a vertical cut was made in the outer muscle layers using a Pott's scissors. In no case was the oesophageal mucosa breached during this procedure. Following this the small muscular defect was closed with one or two interrupted silk sutures. Where possible the subsequent wrap of stomach enclosed the site of the biopsy.

Full informed consent was obtained from the patients and the study had local Ethical Committee approval.

2.3 SPECIMEN FIXATION AND PREPARATION

Resected specimens.

The oesophagus was obtained fresh from the operating theatre and was opened out longitudinally. It was then pinned out flat on a cork board. It was immersed in a tank of 10% formol-saline for 20 - 24 hours before being trimmed into pieces which were then embedded in paraffin wax blocks.

Biopsy specimens.

These specimens were obtained fresh and were immediately placed in 10% buffered formaldehyde for 20-24 hours before being embedded in paraffin wax.

Sectioning of specimens.

The paraffin wax blocks were placed in a microtome and 3 micrometre sections cut. These were floated onto slides in a water bath at 40^0 C. The slides were then placed on a hot plate at 50^0 C for 10 minutes to cause adherence of the tissue to the slide. When dry, the slides were ready for staining with histochemical dyes. For immunohistochemistry the slides were dried by incubation at 37^0C for 14 - 16 hours.

2.4 STAINING METHODS

The basic staining methods will be described. In later chapters details of any variation in the staining technique or specific details of the immunohistochemical techniques used will be given.

Routine histochemical staining.

All the tissue examined in the thesis was stained initially using haematoxylin and eosin on the standard laboratory automatic staining run.

Specific histochemical staining.

A number of different stains are available to stain specifically for eosinophils and mast cells. However for the purposes of this work a staining technique was sought that would demonstrate eosinophils and mast cells in the same tissue section. Minimal background staining was a prerequisite for ease of counting the cells.

Recent quantitative studies of eosinophils and mast cells in other tissues have used separate stains for each cell type thus requiring staining of two sections to study their relative distribution (Benfield et al. 1990, Lozewicz et al.1988). This approach increases the workload and also makes assessment of their relative distribution difficult to appreciate.

Others have used different staining techniques to stain both mast cells and eosinophils on the same section. Fisher et al.(1989) used the Giemsa stain to look for these cells in rectal cancer but had to count the cells at high magnification (oil immersion, X1000). In a complex staining technique Ball and Hay (1990) used alcian blue, naphthalene black, chromotrope 2R and nuclear fast red to demonstrate mast cells and eosinophils in tissue sections containing helminths. This staining technique is too complicated for routine use and when staining a large volume of tissue as in this thesis.

Therefore a specific stain for eosinophils, vital new red (Smith et al. 1984), was combined with a specific stain for mast cells, astra blue (Blaies and Williams 1981). After repeated experiments with the two stains it was found that the staining schedule detailed below produced the best results (Duffy et al. 1993).

Astra blue / Vital new red (ABVNR)

1.Dewax tissue sections in xylene and bring to 95% ethanol.
2.Stain with astra blue solution for 30 minutes. (for preparation see below)
3.Rinse in tap water to remove excess stain.
4.Stain with vital new red solution for 30 minutes (for preparation see below).
5.Rinse in tap water to remove excess stain.
6.Counterstain with Mayer's haemalum for 5 - 10 seconds.
7.Blue in Scott's tap water substitute for 10 seconds.
8.Dehydrate, clear and mount in synthetic mounting medium.

Preparation of astra blue

(Blaies et al. 1981)
1.Dissolve 2 g. $MgCl_2$.6H20 and 0.1 g. pararosaniline hydrochloride C.I. 42500 (Sigma Chemical Co.Ltd. P.O. Box 14508, St.Louis, MO 63178, USA) in 80 mls 95% ethanol in an Erlenmeyer flask.
2.Dissolve 0.5 g. astra blue (Product no.34195. Merck Ltd., Merck House, Poole, Dorset BH15 1TD England.) in 10mls glass distilled water.
3.Add the astra blue solution slowly to the alcoholic mixture while stirring constantly.
4.Add 12 N HCl dropwise, dispensing from a burette, until the colour changes from purple to violet then to royal blue. The amount needed is approximately 9 ml.; however adding several drops more will not affect the stain adversely because in the weaker ionising ethanol environment low hydrogen ion concentrations are less liable to fluctuate wildly than in wholly aquaeous solutions.
5.After allowing the solution to settle for 1 hour, filter. The stain should always be refiltered before use. If the colour shifts toward violet on storage then a few drops of 12N HCl will restore the royal blue tint.

Preparation of vital new red.

(Smith et al. 1984)
A 10 g./l solution in 50% ethanol of vital new red (C.I.25380) (Pfaltz and Bauer, Inc., Division of Aceto Corporation, 172E Aurora St., Waterbury, CT 06708, UK Distributors - Phase Separations Sales, Deeside Industrial Park, Deeside, Clwyd CH5 2NU). The solution is filtered then stored at room temperature in an airtight bottle.

Using this combination, eosinophils stained bright red and mast cells blue. (see figure 2.4.1) There was minimal background staining and other inflammatory cells were not stained. This allowed counting of cells at relatively low magnification.

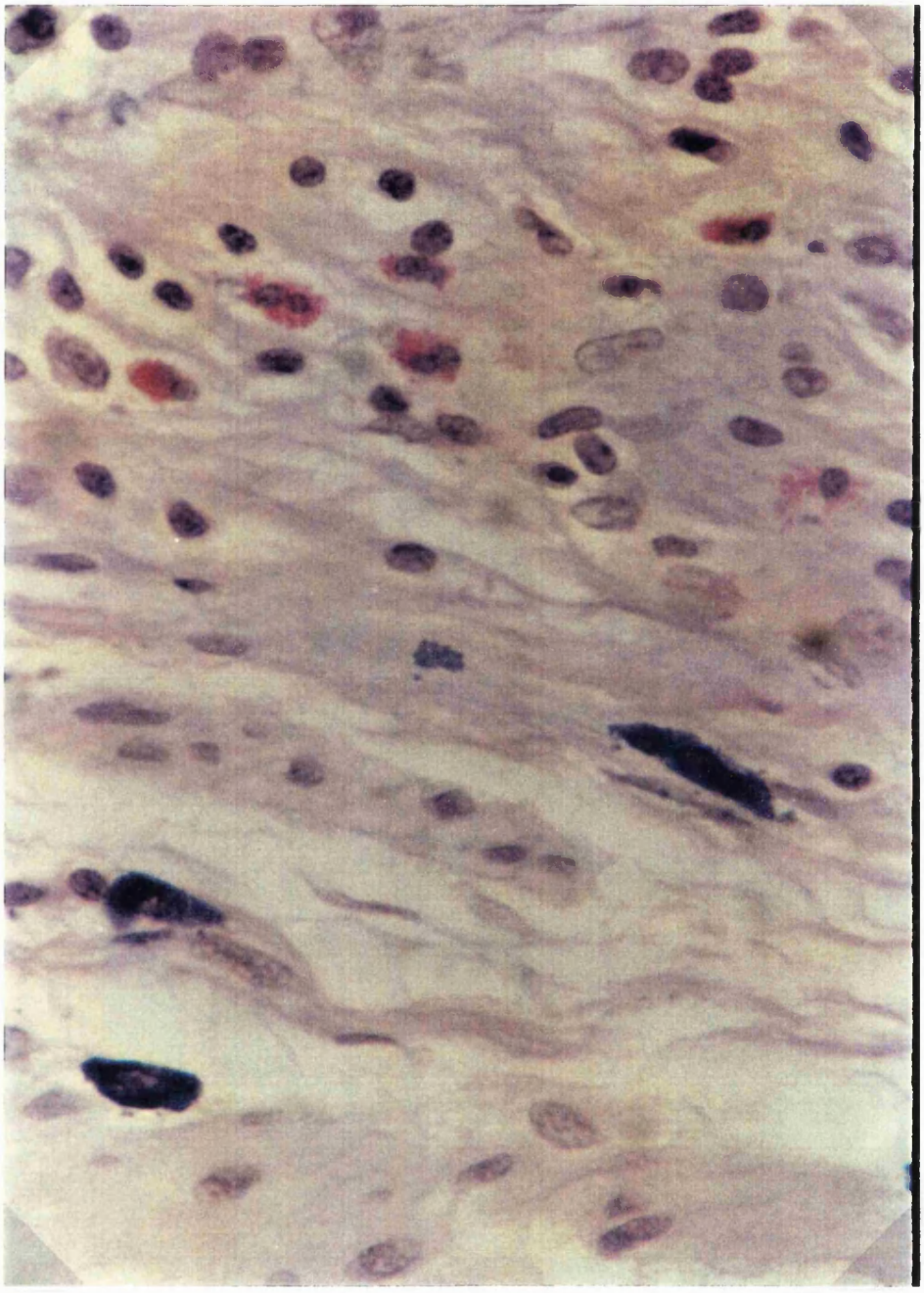


Figure 2.4.1
Connective tissue in lamina propria of the oesophagus stained with ABVNR (Duffy et al.1993). Eosinophils stain red and mast cells dark blue. Original magnification X800

Immunohistochemical staining

The immunohistochemical technique used depended on the specific antibody being used to detect a specific tissue antigen. Most of the staining was performed using a standard ABC-immunoperoxidase technique with diaminobenzamine as a chromogen (Naish 1989).

One of the problems of this technique is that red blood cells, eosinophils and neutrophils can exhibit endogenous peroxidase activity. This was blocked by incubation of the sections with hydrogen peroxide prior to staining. Nonspecific binding of the antibody to the tissue section was blocked by preincubation of the section with serum from the same animal in which the primary antibody was raised. Haematoxylin acts as a blue counterstain for the nuclei.

With some antibodies the resulting staining was weak and therefore the use of an amplifying staining technique using alkaline phosphatase with Vector red SK100 as a chromogen was used.

For immunohistochemical staining the slides were mounted in a 'Sequenza' staining station (Shandon, UK) for the majority of the staining process. Each slide was in contact with a chamber of 100 microlitres into which the various antibodies and reagents were sequentially placed by hand.

Controls for immunohistochemistry.

Positive and negative controls were used, processed in parallel with the test slides. From each block of tissue two sections were used in each antibody run. On the test section the primary antibody was used whereas the control section was incubated with an antibody of similar class but which was directed against a different target antigen. The rest of the steps in the staining process were the same.

Immunoperoxidase technique

1. Take sections through xylene and alcohols to water.
2. Trypsinise where appropriate with 0.1% Trypsin in Tris-buffered saline for 15 minutes.
3. Wash in running tap water.
4. Block endogenous peroxidase with 0.3% - 0.5% hydrogen

peroxide in methanol for 15 minutes.
5. Load sections into a Sequenza.
6. Add appropriately diluted primary antibody (diluted with phosphate-buffered saline) and leave at room temperature for 30 - 45 minutes or overnight at 4^0 C.
7. Wash with phosphate buffered saline (PBS) for 5 minutes.
8. Add secondary antibody (biotinylated) at 1 in 200 dilution in PBS for 30 - 45 minutes.
9. Wash with PBS for 5 minutes.
10. Add avidin-biotin complex (ABC) and leave for 30 minutes.
11. Wash with PBS for 5 minutes.
12. Remove slides from Sequenza and place in slide rack.
13. Immerse in diaminobenzamine and hydrogen peroxide solution for 5 minutes.
14. Rinse in running tap water for 5 minutes.
15. Counterstain with Mayer's Haemalum for 30 seconds.
16. Wash in running water.
17. Dehydrate, clear and mount in nonaquaeous mounting medium.

ABC-alkaline phosphatase staining technique.(Vector Laboratories)

1. Dewax tissue sections.
2. Rinse in tap water for 5 minutes.
3. Load into Sequenza.
4. Wash in phosphate buffered saline(PBS) twice.
5. Add dilute normal serum for 20 minutes.
6. Wash in PBS.
7. Add primary antibody (appropriately diluted in PBS) and leave for 30 minutes or overnight at 4 degrees centigrade.
8. Add biotinylated antibody and leave for 30 minutes.
9. Wash in PBS.
10. Add Vectastain ABC-AP reagent and leave for 30 - 45 minutes.
11. Wash with PBS.

12. Add freshly prepared alkaline phosphatase substrate solution for 30 minutes.
13. Wash in running water for 5 minutes.
14. Counterstain with Mayer's haemalum.
15. Dehydrate, clear and mount.

Preparation of working solutions.
Blocking serum.- Add three drops of stock solution to 10mls of buffer.
Biotinylated antibody. - Add one drop of stock solution to 10 mls of buffer.
Vectastain ABC-AP reagent - Add 2 drops of reagent A to 10 mls of buffer in mixing bottle. Add 2 drops of solution B to the same mixing bottle and allow to stand for 30 minutes before use.

Alkaline phosphatase substrate kit.
Vector Red SK 5100

1. Immediately before use add 2 drops of reagent 1 to 5 mls of Tris buffer pH 8.2 and mix well.
2. Add 2 drops of reagent 2 and mix well.
3. Finally add 2 drops of reagent 3 and mix well. Allow to stand for 30 minutes before use.

Results
DAB as a chromogen produced brown staining at the site of the antigen of interest when the section was viewed under the light microscope. Vector Red produced a red stain.

2.5 COUNTING TECHNIQUES AND VALIDATION

A high resolution monochrome video camera (Western Sound Visual Ltd., Bristol, UK) was attached to the research microscope (Leitz Laborlux D) so that the image from the microscope slide could be converted to a video image (see Fig. 2.5.1). The video image was digitised using a digitiser (Archimedes Video Digitiser, Jessa House, Watford) fitted into an Archimedes 340 computer. The image was then manipulated using the software package Revelation2 (Longman Logotron, 124, Cambridge Science Park, Milton Rd., Cambridge).

Initially the system was calibrated using a slide with a graticule of known length and subdivisions. The test slide was then examined and an area for measurement chosen. The contrast of the image was adjusted and the image saved as a 'sprite file'. The latter could be manipulated using Revelation2; using an electronic crayon, the area to be measured was circumscribed and then filled to the margins. This area was then measured by the computer and the result displayed in square millimetres on the screen (see Fig. 2.5.2).

The number of cells staining positively within this area was then counted using a manual counter and the final result expressed as cells/square millimetre of tissue section.

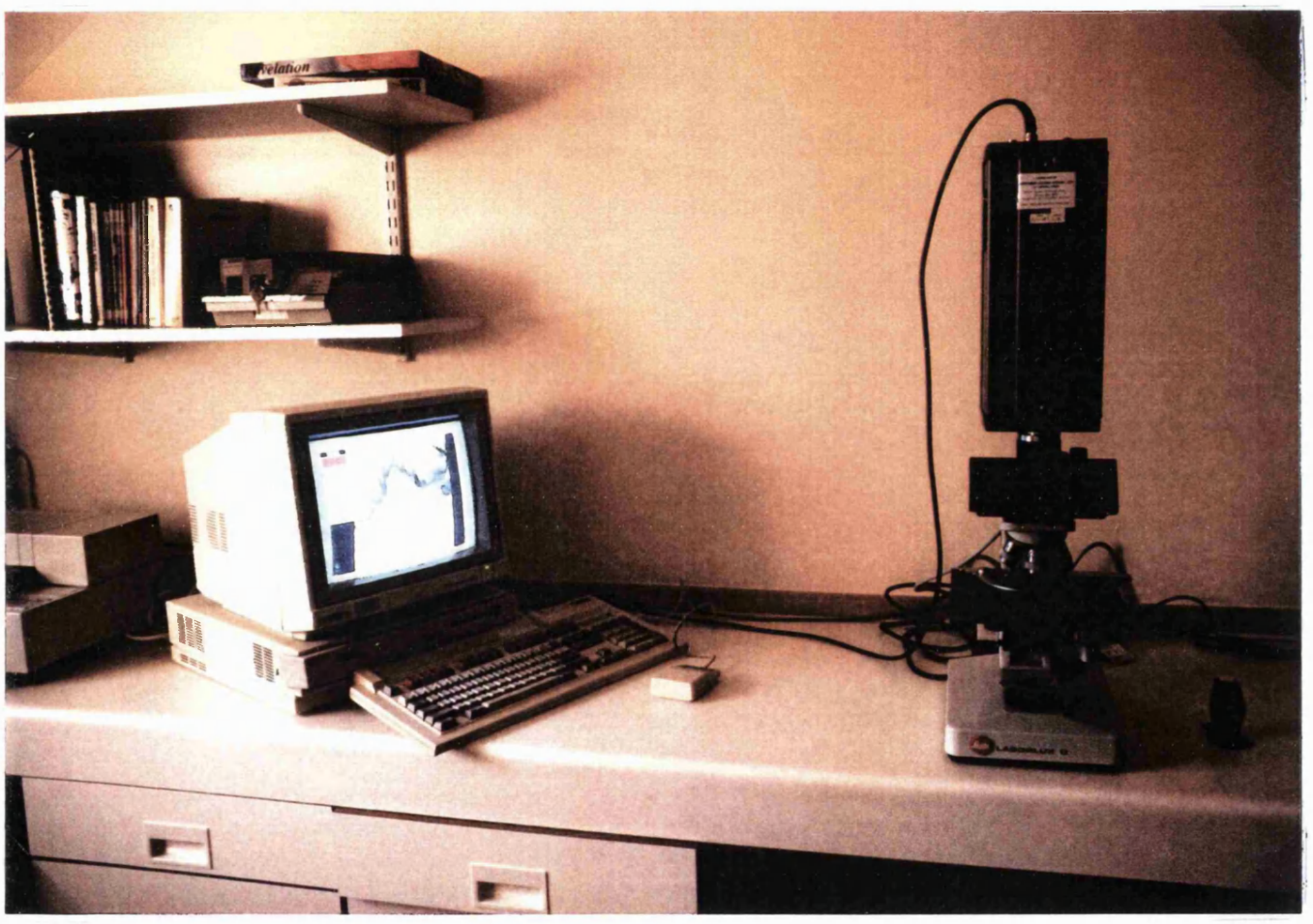


Fig. 2.5.1. Equipment used in computer aided cell counting. A video camera is attached to the microscope allowing a digitised image to be projected on the television screen.

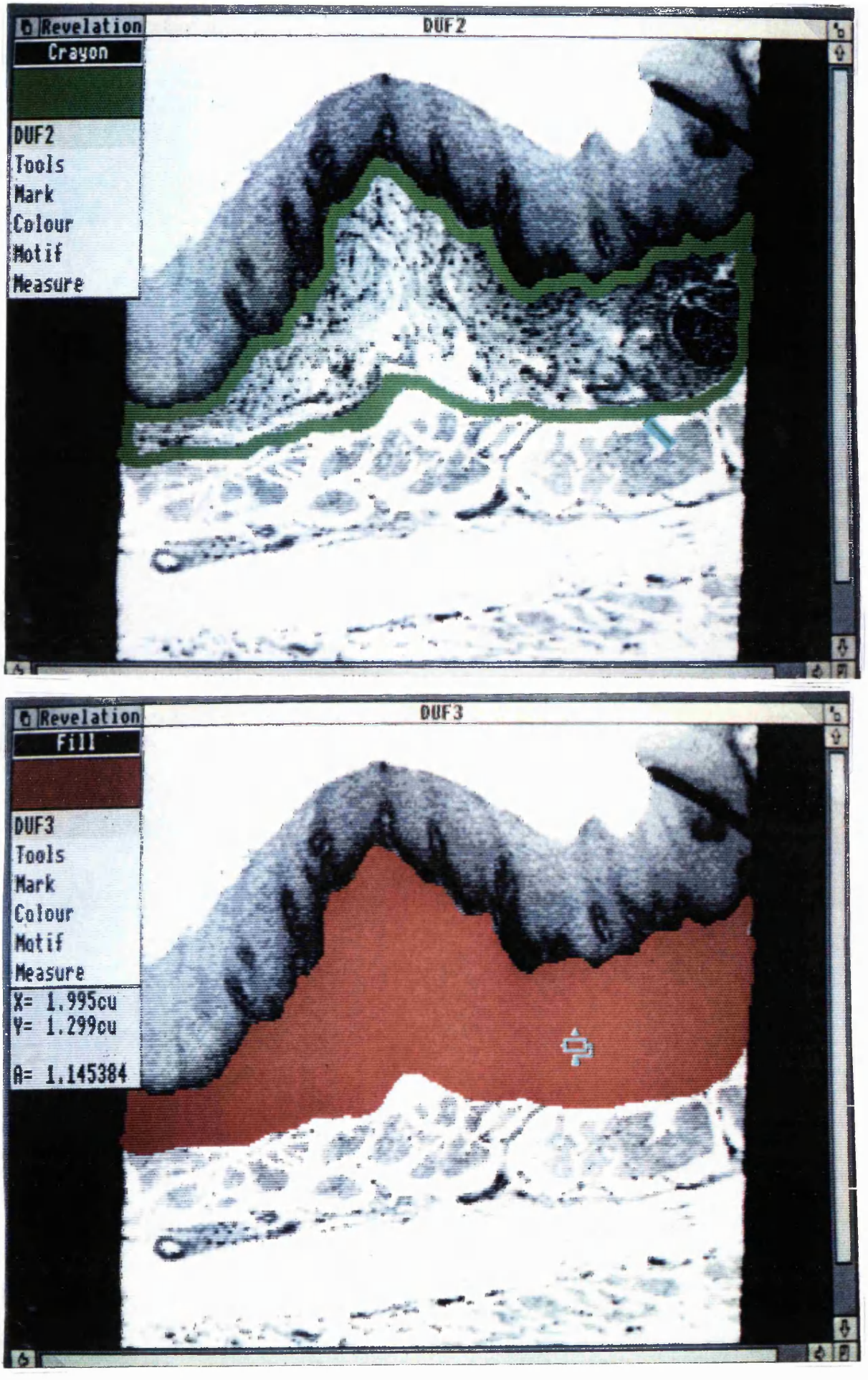


Fig. 2.5.2. Photographs taken from the television screen illustrating the technique of area measurement. In the upper picture an area of lamina propria (LP) has been outlined in green. In the lower picture the area bounded by this line has been filled in in red. The area is then displayed on the screen (A =1.145 mm2)

Repeatability of cell counts

The repeatability of the counting technique was tested by the following method. A piece of oesophagus 2 mm X 2mm was embedded in a paraffin wax block so that each section cut from the block was a transverse section. 20 sections were all stained in the same fashion with astra blue / vital new red and then the numbers of eosinophils (red staining) and mast cells (blue staining) in the lamina propria were counted. The lamina propria was defined as that area bounded by the epithelium above and the muscularis mucosa below. Where there were gaps in the muscularis mucosa then an imaginary line was drawn across from adjacent muscularis mucosa and the cells counted between this line and the epithelium. Although the majority of cells were nucleated, if the characteristic staining pattern was seen shaped like a cell but without nucleus this was counted. Counting was performed at a magnification of X400.

The counts were repeated 6 months apart. The differences between each pair of results were plotted against their mean so that if the difference were related to the absolute number of cells counted this would be apparent. The coefficient of repeatability was calculated according to the method described by Bland and Altman (Bland and Altman 1986).

Eosinophil counts.

The difference between the counts was not influenced by the absolute number of cells counted (see Table. 2.5.1. and Figure 2.5.1.). All repeated counts were within 10% of previous counts. The coefficient of repeatability was 3.79 and none of the differences between counts exceeded this value.

Table 2.5.1

Repeated counts of eosinophils six months apart

Count 1	Count 2	MEAN	Difference between counts	Difference squared
53	50	51.5	3	9
28	26	27	2	4
26	28	27	-2	4
16	14	15	2	4
56	55	55.5	1	1
58	59	58.5	-1	1
39	40	39.5	-1	1
11	12	11.5	-1	1
21	19	20	2	4
23	25	24	-2	4
33	34	33.5	-1	1
28	28	28	0	0
24	25	24.5	-1	1
40	43	41.5	-3	9
32	34	33	-2	4
29	28	28.5	1	1
27	24	25.5	3	9
53	55	54	-2	4
26	23	24.5	3	9
22	23	22.5	-1	1
			0	72

STANDARD DEVIATION
1.90
COEFFICIENT OF REPEATABILITY
3.79

Table 2.5.1
Repeated counts of eosinophils six months apart

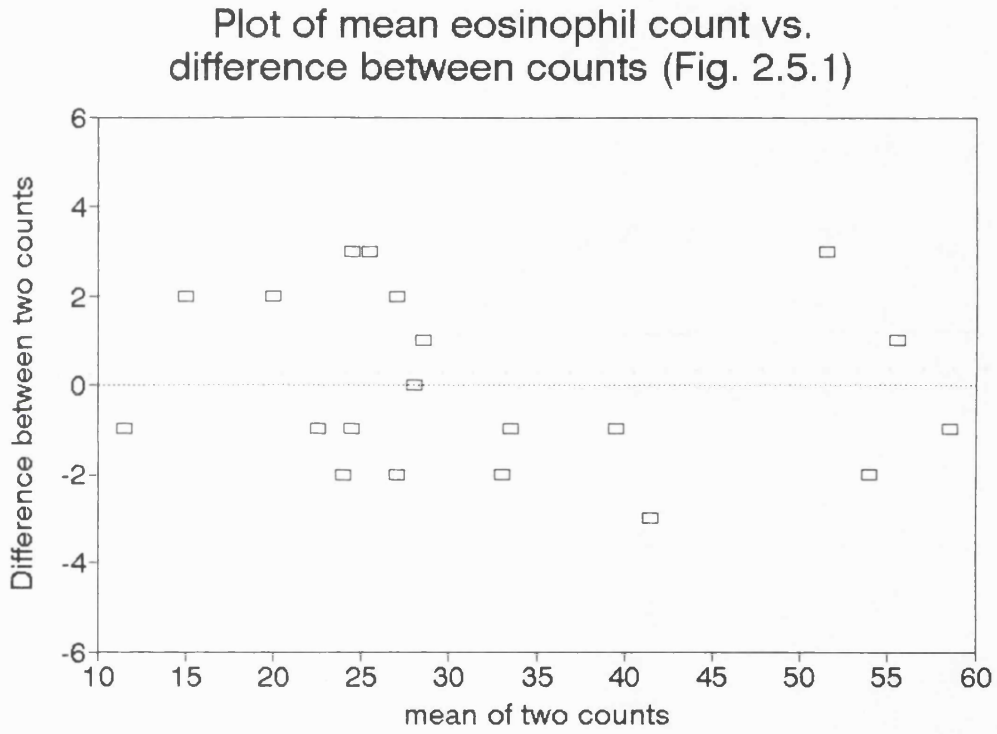


Figure 2.5.1

Plot of mean eosinophil count against difference between counts.

Mast cell repeated counts.

The difference in the counts was not influenced by the absolute number of cells counted (see Fig 2.5.2. and Table 2.5.2.). All repeated counts were within 10% of previous counts. The difference between counts was greater than the coefficient of repeatability (9.92) in two instances.

The major source of error was judged to be in clear definition of the area in which cells were to be counted rather than in deciding whether a cell was staining positive or not.

Table 2.5.2

Repeated counts of mast cells six months apart

Count 1	Count 2	Mean	Difference between counts	Difference squared
117	125	121	-8	64
116	111	113.5	5	25
138	137	137.5	1	1
132	129	130.5	3	9
115	115	115	0	0
87	82	84.5	5	25
89	92	90.5	-3	9
92	93	92.5	-1	1
114	119	116.5	-5	25
118	115	116.5	3	9
115	125	120	-10	100
105	112	108.5	-7	49
125	136	130.5	-11	121
127	127	127	0	0
111	113	112	-2	4
131	126	128.5	5	25
93	90	91.5	3	9
119	122	120.5	-3	9
114	112	113	2	4
119	117	118	2	4
			-1.05	493

STANDARD DEVIATION
4.96
COEFFICIENT OF REPEATABILITY
9.93

Table 2.5.2
Repeated counts of mast cells performed six months apart.

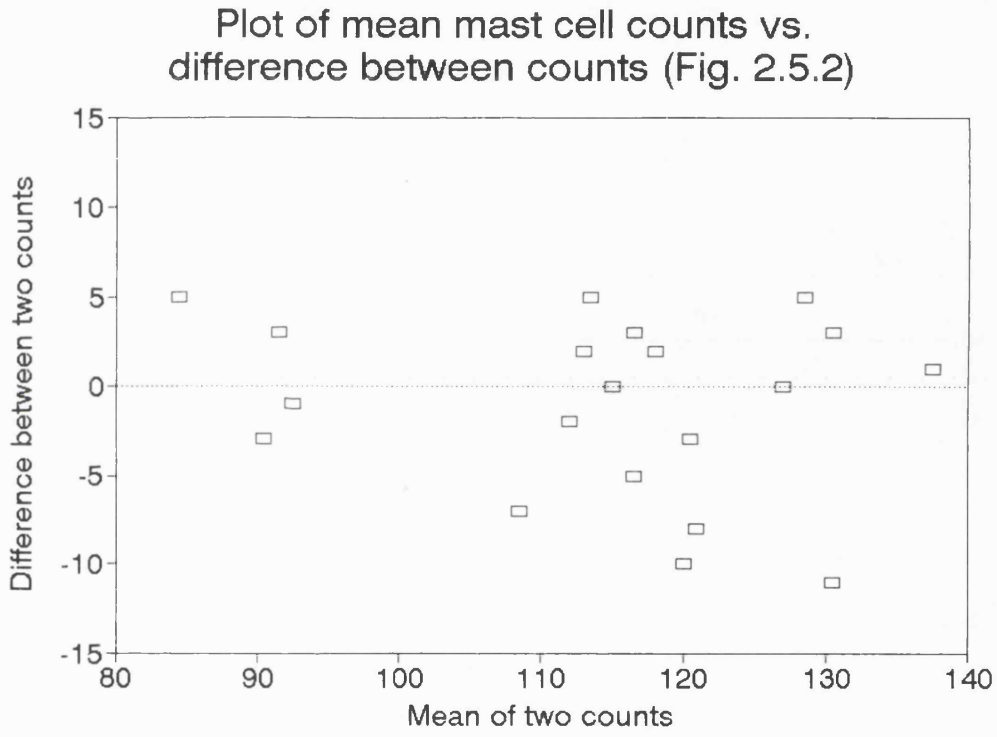


Figure 2.5.2
Plot of mean mast cell counts against the difference between counts.

2.6 MATERIALS AND SOLUTIONS

Alkaline phosphatase substrate kit.(Vector Red)
Cat. No. SK-5100. Vector Laboratories, Burlingame, CA 94010, USA.

Astra blue
Chroma 1B163, Chroma-Gesellschaft Schmid Co., W.Germany.

Avidin-biotin complex
DAKO Strept ABComplex HRP. Lot 050, Code no.: K377
DAKO Ltd., High Wycombe, Bucks.

Biotinylated goat anti-mouse antibody
Biotin-SP-Affinipore Goat Antimouse IgG F(ab')2 fragment.(min X Hu, Bov, Hrs serum proteins, Jackson Immunoresearch.

Biotinylated sheep anti-rabbit antibody
Sheep anti-rabbit IgG (H+L) Biotin.
Code PB311. Batch G1895
Sheep anti-rabbit IgGAM (H+L) Biotin, Code:PB310.
The Binding Site Ltd., Vincent Drive, Birmingham, B15 2SQ, UK.

Calcium chloride (fine granular)
Prod.27587 BDH Ltd., Poole, England.

Diaminobenzamine solution
150 mg diaminobenzamine
300 mls of TBS
30 drops of 60% hydrogen peroxide

Eosin yellowish.
Prod. 34197. BDH Chemicals Ltd., Poole, England.

Ethanol
Absolute alcohol 100. Ethyl alcohol 99.86% v/v SIN 1170. Hayman Ltd., Witham, Essex.

Haematoxylin (Harris) with acetic acid.
Prod. 35162. BDH Chemicals Ltd., Poole, England.

Hydrochloric acid
Sp.Gr. 1.18. Prod. 10125. BDH Chemicals Ltd., Poole, England.

Hydrogen peroxide solution
BDH Ltd, Poole, England.

Magnesium chloride 6-hydrate.
Prod 101494 V. BDH Chemicals Ltd., Poole, England.

Mayer's haemalum
Haemalum (Mayer's) Prod. 35060. BDH Chemicals Ltd., Poole, England.

Nonaquaeous mounting medium
DPX mounting medium. Diachem. Diagnostic Developments, Southport, UK.

Normal sheep serum
The Binding Site, BP013, Birmingham
Batch R1804.

Pararosaniline HCl
Sigma chemical co.,C.I. 42500. PO Box 14508, St.Louis, USA.

Phosphate-buffered saline
Dulbecco's phosphate buffered saline. D5652. Sigma Chemical Co., PO Box 14508, St. Louis, MO. 63178. USA

Primary antibodies.
The majority of these were obtained from DAKO Ltd., High Wycombe, Bucks, UK. Specific details of antibodies used may be found in the text.

Scott's tap water substitute
3.5 g. Sodium Bicarbonate,
20 g. Magnesium Sulphate
1000 cc. Water with thymol added.

TRIS
Tris(hydroxymethyl)methylamine. Prod.27119. BDH Chemicals Ltd., Poole, England.

TRIS buffer
3 volumes of 0.1 M hydrochloric acid.
3 volumes of 0.15 M sodium chloride.
2 volumes of 0.2 M TRIS

Trypsin
Trypsin from beef pancreas.(3.4.21.4) Prod. 39041 447269 OM. BDH Chemicals Ltd., Poole, England.

Vectastain ABC
Vectastain Elite ABC Kit. Vector Laboratories, Burlingame CA 94010 USA.

Vectastain ABC-AP Kit
Vector Laboratories, Burlingame, CA 94010, USA

Vital new red
CI 25380 Pfaltz and Bauer Inc., Division of Aceto Corporation, 172E Aurora St., Waterbury, CT 06708 UK
Distributors: Phase separations sales, Deeside, Clwyd, 3.

Xylene (Xylol) Liquid
UN No. 1307. Genta Medical, Tockwith, Yorks, England.

2.7 STATISTICAL METHODS

A variety of statistical methods have been used throughout the thesis using standard parametric and nonparametric tests as described in a standard text (Altman 1991). Many of the calculations were performed using the statistical computer program Oxstat II (Version 1.1, release 1.2, Microsoft Corporation).

3.HISTOLOGICAL FEATURES OF THE NORMAL OESOPHAGUS

INTRODUCTION

The basic structure of the oesophagus conforms to the general pattern of the rest of the gastrointestinal tract. The wall of the oesophagus consists of a mucosa, submucosa, muscularis propria and adventitia.

Mucosa

The mucosa consists of a stratified squamous epithelium, an underlying lamina propria and muscularis mucosa.

Epithelium

The epithelium consists of basal, prickle and functional cell layers. Argyrophilic endocrine cells and melanocytes have been found scattered among the basal cells (DeNardi and Riddell 1991).

A variety of inflammatory cells have been described in the epithelium. These include occasional lymphocytes and Langerhans cells (Geboes et al.1983).

Lamina propria

The lamina propria consists of areolar connective tissue and contains blood vessels, scattered inflammatory cells and mucous-secreting glands. Lymphocytes of predominantly OKT4 type and plasma cells are frequently seen. The mucous secreting glands are of the cardiac type and are present in less than one fifth of oesophagi examined (Enterline 1984).

Muscularis mucosa

This is composed of smooth muscle bundles that are orientated longitudinally.

Submucosa

This consists of loose connective tissue containing vessels, nerves of Meissner's plexus (not well developed in this part of the gastrointestinal tract), lymphatics and submucosal glands.

Muscularis propria

The muscularis of the oesophagus consists of both striated and smooth muscle. Only the upper 5% is entirely striated muscle, distal to this there is a mixture of both striated and smooth muscle and in the distal 50% of the oesophagus the muscle layers are entirely composed of smooth muscle (Meyer et al.1986).

The inner circular layer is separated from the outer longitudinal layer by connective tissue which runs through Auerbach's plexus.

Adventitia

Only short lengths of the thoracic and intraabdominal oesophagus have a true serosal layer. The greater part of the oesophagus is covered by a loose adventitia only.

Inflammatory Cells

The distribution of inflammatory cells including eosinophils and mast cells within the layers of the oesophagus has not been specifically studied before. Several studies have looked for eosinophils in relation to the motility disorders of the oesophagus and the distribution in normal oesophagus has been mentioned. Tottrup et al.(1989) studied myotomy biopsies from achalasics and compared these with tissue taken either at postmortem (time after death unspecified) or from oesophagus obtained during surgery for adenocarcinoma of the stomach. Since it is well established that there is an inflammatory response to malignant tissue, using adjacent oesophagus as a normal control to study the normal complement of inflammatory cells may be open to error. Also if the distal malignancy results in stasis of oesophageal contents then this can also result in mucosal inflammation secondary to the stasis.

In the present study the aim has been to establish the distribution of eosinophils and mast cells within the normal oesophagus by studying tissue obtained from a number of different sources. It is hoped that by collating the data obtained it will be possible to draw some conclusions about the distribution of these cells in the normal oesophagus.

Methods

Three sources of resected oesophagus have been used for the purpose of this study:

Group 1 : normal oesophagus obtained from patients at malignant oesophageal resection.

Oesophagus obtained from 5 patients undergoing oesophagectomy for cancer were obtained (see Table 3. for clinical details). Patients having chemotherapy for oesophageal cancer (the majority of patients treated in our unit) were excluded because of the potential effects of chemotherapy on inflammatory cells in the oesophagus.

Group 2: oesophagus obtained at postmortem examination.

Oesophagus was obtained at the time of postmortem examination in 7 patients. See Table 3 for clinical details. One patient was excluded since the oesophagus was found to contain an oesophageal diverticulum.

Group 3: oesophagus obtained at the time of organ harvest for transplantation.

Oesophagus was obtained from 5 transplant donors at the time of organ harvest. None of these patients had any history of gastrointestinal symptoms nor had they any allergies. See Table 3 for further clinical details. These specimens were obtained and fixed as detailed in the general methods section.

Groups 1 and 2 were stained with H & E and vital new red only. Eosinophil counts were obtained. In those patients with carcinoma only a small segment of oesophagus some distance from the tumour-bearing oesophagus was studied.

In Group 3 the oesophagus was stained with H&E and ABVNR. Counts of eosinophils and mast cells in all layers of the oesophagus and at different levels along the length of the oesophagus were made.

Table 3.1 Clinical data for control oesophagus
(tissue from tumour-bearing oesophagus)

Lab.No.	Age	Sex	Tumour	Site of tumour	Site of 'normal'
OE10/91	57	F	Adenocarcinoma	Lower third	Upper third
OE16/91	71	M	Adenocarcinoma	Lower third	Upper third
OE7/91	68	F	Squamous	Middle third	Upper third
			Carcinoma		Lower third
OE5/91	56	M	Adenocarcinoma	Lower third	Upper third
OE9/91	65	F	Adenocarcinoma	Lower third	Upper third

Table 3.2 Clinical data for control oesophagus
(tissue obtained at autopsy)

Lab.No.	Age	Sex	Primary diagnosis	Hours from death	Notes
OE4/91	77	M	Carcinomatosis	48	
OE14/91	86	M	ischaemic Heart d.	8	
OE18/91	76	F	COAD	72	Diverticulum
OE21/91	73	M	Ischaemic Heart d.	24	
OE26/91	69	M	IHD, PE	96	
OE29/91	76	F	Endometrial Ca, CV	40	
OE33/91	93	F	CCF, Mitral stenosis	48	

Table 3.3 Clinical data for control oesophagus
(tissue obtained from transplant donor)

Lab. No.	Age	Sex	Diagnosis	Ventilation (hours)	Nasogastric tube	Steroids
OE44/91	39	F	Subarachnoid H.	24	no	no
OE49/91	11	M	Head Injury	36	yes	no
OE59/91	9	F	Diabetic KA	66	yes	no
OE10/92	48	F	Subarachnoid H.	29	no	yes
OE35/92	42	F	Subarachnoid H	48	no	yes

Table 3.

Clinical data for control oesophagus is detailed in the three tables above.

Results

1) Group 1 (normal oesophagus from tumour-bearing oesophagus)

The results from this group are shown in Figure 3.1. In one patient, (OT), counts were obtained above and below a mid-third tumour of the oesophagus. In all patients studied the oesophageal specimens sampled had squamous epithelium and were at least 5 cm. away from the tumour. In three cases eosinophils were present in the lamina propria of the oesophagus. In the other layers of the oesophagus eosinophils were less frequent. In no case was eosinophil degranulation seen.

2) Group 2 (Postmortem oesophagus)

Suitable postmortem oesophagus proved difficult to obtain because of the inevitable time-lag between death and autopsy. Although oesophagus obtained from one patient at 8 hours after death appeared morphologically intact, oesophagus obtained at more than 24 hours showed significant postmortem changes. In all cases the epithelium was lost and there was widespread muscle necrosis. No eosinophils were seen in any of these oesophagi. The best-preserved oesophagus (specimen OE14/91) was stained with ABVNR and the mast cell counts in the layers at different levels in the oesophagus are detailed in Appendix 1. No eosinophils were seen but there were mast cells staining in all layers of the oesophagus.

3) Group 3 (transplant donor oesophagus)

a) Eosinophil counts

Only 3 eosinophils were seen in all the oesophageal tissue examined (multiple blocks were taken from each case.).

b) Mast cell counts.

Mast cells were most frequently seen in the lamina propria with a mean count of 120 cells per sq.mm. Within the tissues they appeared most frequently associated with blood vessels. Mast cells were also frequently seen in the region of Auerbach's plexus. In Fig. 3.2 mast cell counts at 1-2 cm. above the oesophagogastric junction are shown.

c) Distribution of eosinophils and mast cells with respect to level of the oesophagus.

Since there were virtually no eosinophils in any of the oesophageal tissue examined a gradient in counts down the oesophagus could not be seen.

There was no apparent difference in mast cell counts between upper, middle and lower thirds of the oesophagus. The counts for OE44/91 are shown graphically in Fig. 3.2.

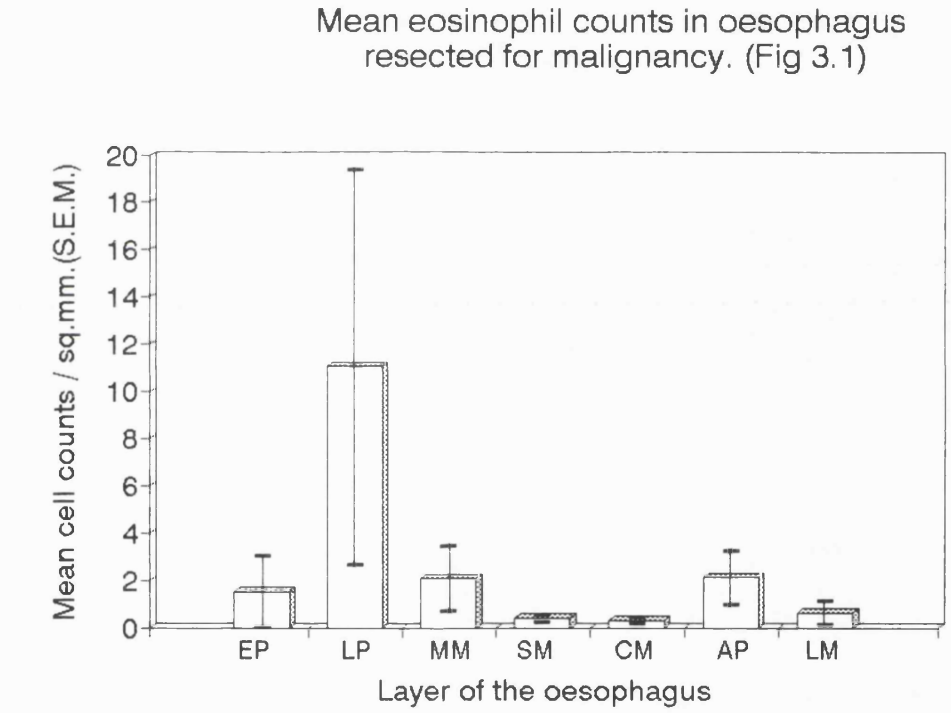


Figure 3.1

Mean eosinophil counts in oesophagus resected for malignancy. Error bars represent the standard error of the means (S.E.M.)

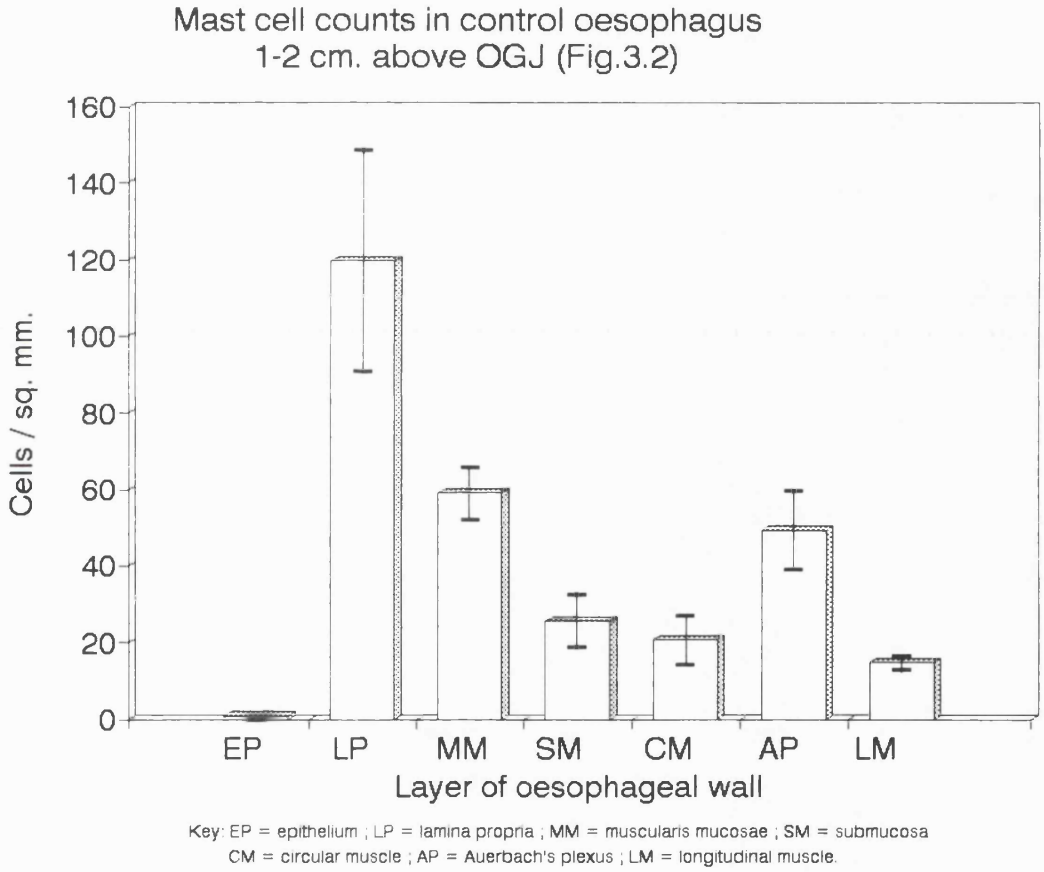


Figure 3.2

Mast cell counts in control oesophagus 1 - 2 cm. above the oesophagogastric junction (OGJ).

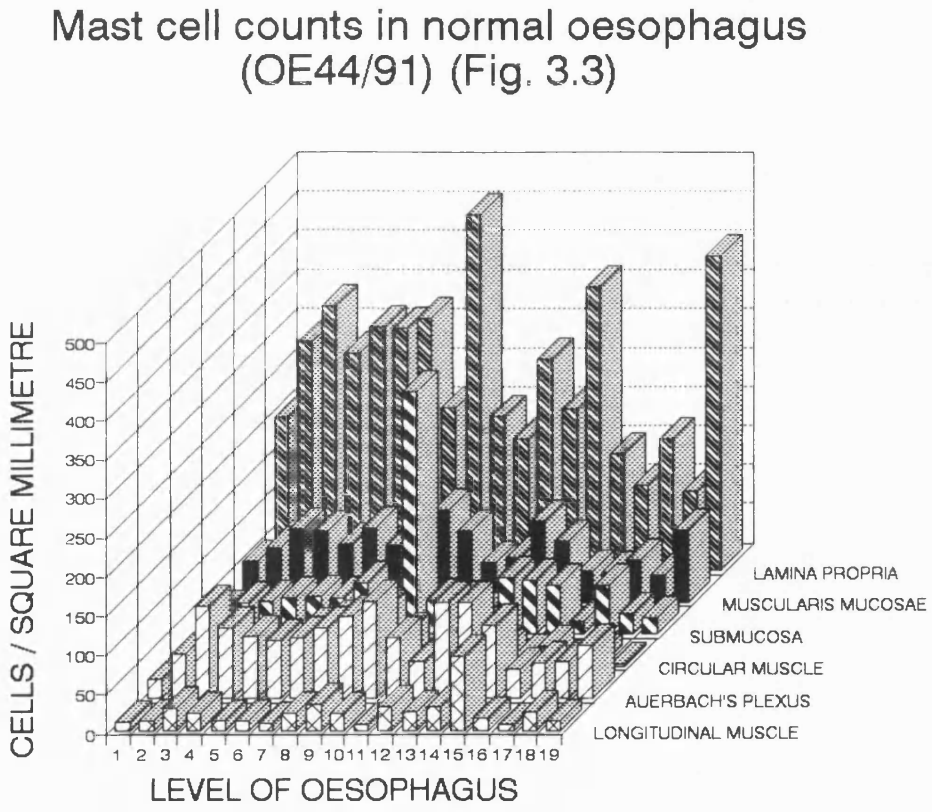


Figure 3.3

Mast cell counts in normal oesophagus. (OE 44/91)

Mast cell counts in various levels in the oesophagus are detailed. The level of the oesophagus is the number of centimetres from the oesophagogastric junction.

Discussion

The finding of inflammatory cells in pathological tissue can only be properly interpreted against a background knowledge of the normal distribution of the inflammatory cells concerned. Although it is relatively easy to obtain control mucosal tissue, obtaining full thickness oesophageal wall is more difficult.

Previous histological studies have used oesophageal tissue obtained from patients with benign oesophageal disease, malignant oesophageal, gastric or pulmonary disease undergoing resection or from tissue obtained at postmortem examination (Adams et al. 1976, Csendes et al. 1985, Tottrup et al. 1989).

Our studies have shown that oesophageal tissue from tumour-bearing oesophagus may contain eosinophils in the lamina propria and scattered throughout the other layers of the oesophagus. Whether this is an effect of stasis proximal to an obstruction or is related to tumour effects on the oesophagus is unclear. However it is well known that tumours can provoke an eosinophilic response in the tissues in which they arise. This has been shown in cervical, gastric and colonic carcinoma (Yoon 1959, Lowe et al. 1981, Iwasaki et al. 1986). In one of our cases there were no eosinophils seen at all.

Our experience with autopsy oesophagus was disappointing. The local pathology practice means that obtaining a postmortem within 12 hours of death is a rarity. Whether other studies using postmortem oesophagus obtained fresher tissue is uncertain. Although Csendes et al. (1985) managed to obtain postmortem tissue within 2 to 3 hours of death, in most publications the time from death is not stated.

For the above reasons it was decided to examine fresh oesophagus obtained at transplant organ retrieval. The specimens were removed, opened longitudinally and fixed immediately. This resulted in a well-preserved specimen in all cases.

The eosinophil was extremely rarely seen in all five cases. The mast cell distribution tended to reflect the vascularity of the respective layers of the oesophagus. Two of the five specimens were obtained from children. Although it would be preferable to have all the control oesophagus from similar age-groups to the patients studied, it was not possible due to the small number of opportunities we had to harvest fresh oesophagus. However it is likely that a child of 9 has the full complement of inflammatory cells in the oesophagus by that age and may still relevant information on the normal oesophagus.

Steroid administration is known to affect tissue eosinophilia and steroids were in fact administered to 2 out of the five donors studied as part of the transplant protocol. Whether these will have had any effect on tissue eosinophils is unknown.

In 2 of the 5 cases a nasogastric tube was present. Nasogastric intubation has been shown to be closely associated with reflux damage to the oesophagus (Lodge 1955). However no evidence of any inflammation in the oesophagi studied was found. It may be that the short period of nasogastric intubation and the lack of underlying gastrointestinal pathology (a common reason for nasogastric intubation) were important factors.

Conclusions

1. The eosinophil is a rare cell in the tissues of the normal oesophagus.

2. Mast cells are distributed in all layers of the oesophagus being more numerous in the more vascular layers.

3. Oesophageal tissue from oesophagi containing tumours may not be the best control when studying inflammatory cells in the oesophagus.

4.HISTOPATHOLOGICAL CHANGES IN REFLUX DISEASE OF THE OESOPHAGUS

INTRODUCTION

Pathologists have been interested in the pathological changes associated with reflux oesophagitis for some time. They have attempted to correlate histological changes seen on mucosal biopsies with the degree of severity of reflux as assessed by symptoms, endoscopy and pH studies. Behar et al.(1975) studied 40 patients with heartburn and abnormalities in two out of three investigations: acid perfusion studies, endoscopy and pH studies (not 24 hour). When comparing mucosal biopsies from these cases with biopsies from 15 control patients they found no polymorphonuclear cells in control patients but they were present in 5% of those with mild oesophagitis and in 40% of those with severe disease. Eosinophils were present in 33% of the control group compared with 15% of those with mild and 60% of those with severe disease.

Similarly, Seefeld et al.(1977) compared 24 normal volunteers with 36 symptomatic patients. Neutrophils were present in 29% of the reflux group but not in controls. Eosinophils were present in 10% of controls and 62% of the patients with proven reflux (a cell was judged to be present in significant numbers if at least one cell was present in at least 50% of all visual fields examined at high power (450X)).

In 1982 Winter et al. reported that the presence of intraepithelial eosinophils should be regarded as a marker for reflux oesophagitis. The patients however were in the paediatric age group (ages 3 months to 19 years) and the biopsies were compared with 3 controls only. Eosinophils were present in 18 of the 46 study patients and in 6 of these patients there was more than one eosinophil per high power field. 17 of 35 patients with an abnormal acid clearance time had intraepithelial eosinophils compared to one of 11 patients with a normal acid clearance. They thus correlated the presence of intraepithelial eosinophils with delayed acid clearance and suggested that this could be a marker of reflux oesophagitis.

The pathological changes of oesophagitis have been found in studies of oesophagus from unselected postmortem examinations (Peters et al.1955, Lodge et al.1955) These have shown a spectrum of changes from acute inflammation in

the superficial layers to more severe inflammation with oesophageal ulceration. In about 5% of 500 postmortems studied by Lodge et al. (1955) there was evidence of chronic oesophagitis. Fibrosis extending through the wall of the oesophagus was a prominent feature. A diffuse infiltration with inflammatory cells especially lymphocytes and plasma cells was found. Many of these patients had other associated pathology and may have developed oesophagitis as a part of the terminal stages of their disease. What relationship these patients have to those who have isolated well-documented acid reflux into the oesophagus and who develop chronic oesophagitis is unclear. Since most studies in life are of mucosal biopsies often containing just the epithelium, information about changes occurring in the muscle layers is scant.

Aims of this study.

This study was undertaken to evaluate the extent and characteristics of inflammation in all the layers of the oesophageal wall in severe reflux disease in patients undergoing resection and in moderately severe reflux disease in patients undergoing hiatus hernia repair.

Methods.

Resected group.

Five patients undergoing oesophageal resection for Barrett's oesophagus or reflux stricture were studied. These patients were chosen from the operation records. Patients in whom there were insufficient details as to the location of the various blocks taken from the resected oesophagus were excluded. Clinical details are shown in table 4.1. Staining was with ABVNR and counting was as detailed in Chapter 2.

Hiatus hernia group

11 patients with hiatus hernia and proven moderately severe reflux disease had oesophageal muscle biopsies taken at the time of hiatus hernia repair. Clinical details are shown in table 4.2. Staining was with ABVNR and counting performed as detailed in Chapter 2.

Table 4.1

Clinical details of patients undergoing resection for Barrett's oesophagus or reflux stricture.

Clinical data for patients undergoing resection for reflux disease. (Table 4.1)

Lab. No.	Age	Sex	Indications for resection	Previous treatment
3691	55	M	Mid third stricture	Dilatations
7391	41	M	Haematemesis. Oesophagitis. GOR	Hiatus hernia repair 1986
691	17	M	Stricture. Severe GOR	Dilatations
1991	37	M	Stricture	Dilatations
7191	50	M	Ulceration in Barrett's oesophagus.	Gastroplasty and hiatus hernia repair 1988

Table 4.2

Clinical details of patients undergoing hiatus hernia repair (with muscle biopsy) for reflux disease.

Clinical data for patients undergoing hiatus hernia repair

Table 4.2

Lab. No.	Age	Sex	Length of history months	% time pH < 4	Manometric assessment			Barium swallow	Endoscopic findings	Allergy/ Atopy
					Motility of body of oesophagus	LOS Pressure	Relaxation			
7091	37	M	24	16.6	normal	8	complete	HH	oesophagitis	penicillin
1492	37	F	84	23.5	low pressure	4	complete	HH + GOR	oesophagitis	anaphylactic shock
4391	37	F	48	23.3	weak	8	complete	HH + GOR	oesophagitis	no
6391*	64	F	24	not assessed	not assessed			HH,?tumour	oesophagitis, (dysplasia)	no
4791	45	M	24	19.5	weak	1	complete	no HH, free reflux	oesophagitis	no
1592	62	M	36	15.5	weak	6	complete	HH + GOR	no oesophagitis	no
5591	66	M	120	17.2	not assessed			HH	GOR	no
3791	32	M	36	19.4	disordered	7	complete	GOR + HH	Reflux, no oesophagitis	no
5391	40	M	12	18.7	not assessed			HH	oesophagitis	no
1692	46	M	72	23.7	not assessed	no	no	GOR	normal	asthma extrinsic
5191	37	M	24	11.2	weak, some spontaneous activity	6	complete	HH + GOR	no ulceration/stricture	no

Key: HH = hiatus hernia GOR = gastrooesophageal reflux

* This patient had known reflux but was suspected to have a lower oesophageal tumour. At operation no tumour was found.

Results

Resected specimens

a) General comments

In all specimens there was good preservation of all the layers of the oesophagus. The epithelium contained an infiltrate of inflammatory cells which included neutrophils, eosinophils, lymphocytes and mast cells. In places the epithelium was lost (i.e. an oesophageal ulcer) and in others the normal squamous epithelium was replaced with columnar epithelium.

b) Eosinophil counts

It was not possible to assess the epithelium in these patients since quantitation is very difficult in Barrett's mucosa or in those cases with ulceration. The lamina propria contained an eosinophil infiltrate in all cases. (mean 115 cells/sq.mm. SEM 42.8)

The infiltrate did not extend across all layers of the oesophagus. In no case however were there more than 20 cells/sq.mm. in Auerbach's plexus (mean 8.6 cells/sq.mm. SEM 6.7) There were no focal collections of eosinophils nor evidence of degranulation. Mean eosinophil counts in each layer of the oesophagus are detailed in Fig. 4.1

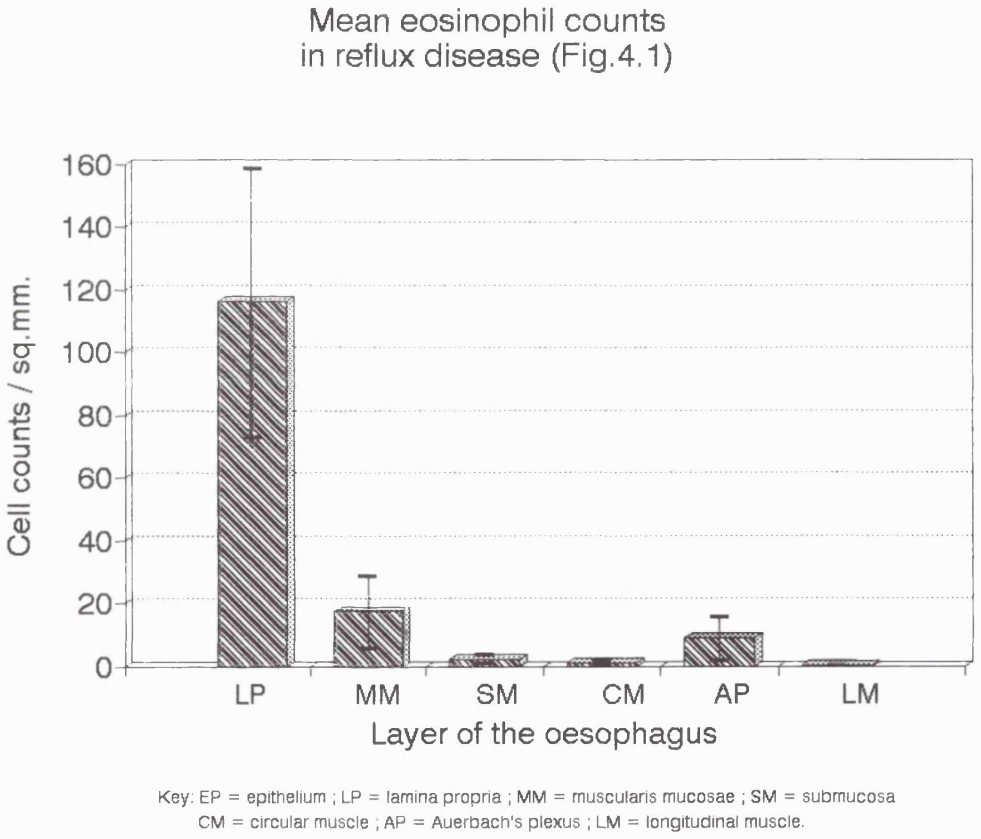


Figure 4.1
Mean eosinophil counts in oesophagus resected for severe reflux damage to the oesophagus.

c) Mast cell counts.
Mast cells were found in all layers of the oesophagus and although similar numbers to controls were found in the lamina propria (153.4 cells/sq.mm. compared with 119.8 cells/sq.mm.), there were increased numbers in all the other layers of the oesophagus (see Fig 4.2).

Myotomy biopsies.

a) General histology
In all cases nerves and ganglion cells were seen in Auerbach's plexus. In only one case was there a moderate infiltrate of polymorphonuclear cells and mononuclear cells. See table 4.3.

b) Eosinophil counts
Occasional eosinophils were seen (mean 2.3 cells/sq.mm.) In one case 9 cells/sq.mm. were seen. There was no evidence of focal collections or eosinophil degranulation.

c) Mast cell counts
The mean mast cell count was 84.3 cells/sq.mm. (range 69 - 136 cells/sq.mm.). This compares with a count of 49.2 cells/sq.mm. in transplant controls.

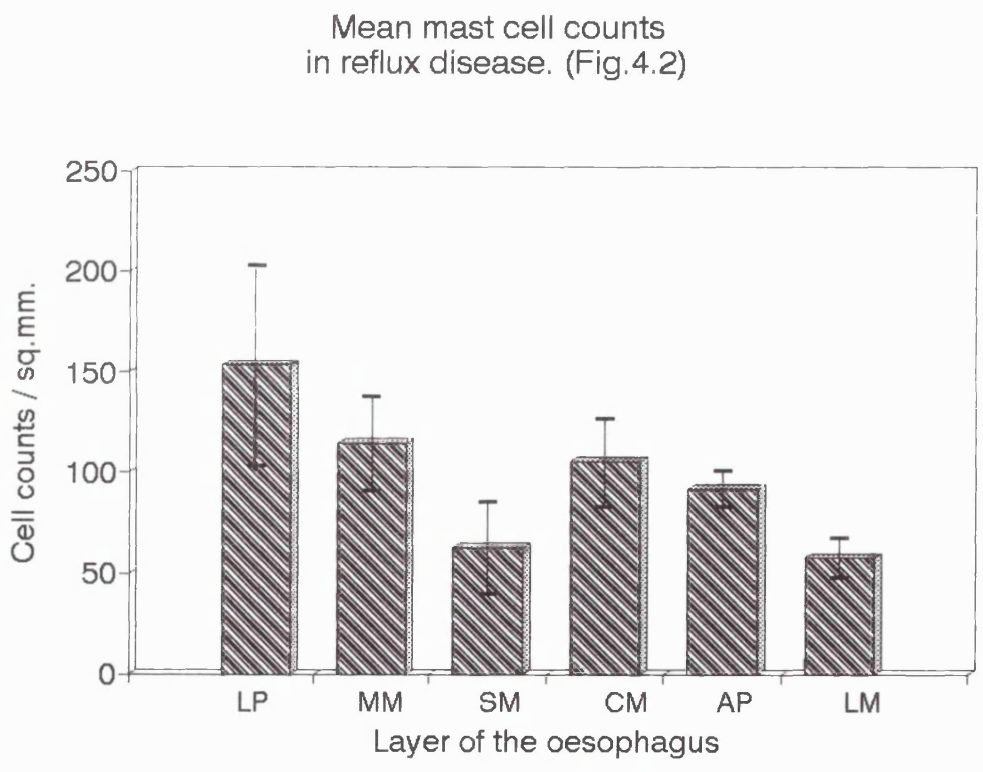


Figure 4.2

Mean mast cell counts in oesophagus resected for reflux disease.

Histopathological findings in Auerbach's plexus in myotomy biopsies from patients with hiatus hernia. (Table 4.3)

Patient	Counts / sq.mm.			Ganglion	Neutrophil	Mononuclear
Lab. no.	Eosinophils	Mast cells	Nerves	cells	PMN	cells
7091	1.6	71.4	YES	YES	0	0
1492	0.01	71.2	YES	YES	0	0
4391	5.95	69	YES	YES	0	0
6391	0.01	27.3	YES	YES	0	0
4791	0.55	101.7	YES	YES	0	0
1592	9	90.7	YES	YES	0	+
5591	0.01	72	YES	YES	0	0
3791	1.9	74.7	YES	YES	0	0
5391	0.96	135.6	YES	YES	+	+
1692	1.5	97	YES	YES	0	0
5191	4	117	YES	YES	0	0

Key: 0 = no cells seen, + = 1-5 cells/HPF, ++ = 5 - 20 cells/HPF, +++ = >20 cells/HPF

Table 4.3

Histopathological findings in Auerbach's plexus in muscle biopsies from patients with hiatus hernia.

Discussion

Previous studies have looked at mucosal biopsies in reflux and have demonstrated the presence of eosinophils in the mucosa. Little is known of mast cell distribution in the oesophagus.

Study of oesophagus resected for end-stage reflux disease is likely to identify the most severe effects of long-term acid reflux and stasis of oesophageal contents in those cases with stricture. In order that less advanced disease could be assessed, oesophageal muscle biopsies from patients having hiatus hernia repair were studied also.

Eosinophils were prominent in the oesophageal mucosa in the resected oesophagus. Mast cells were distributed throughout the layers of the oesophagus in increased numbers.

An infiltrate of eosinophils and mast cells has been described in other conditions affecting the gastrointestinal tract. It has been described in jejunal biopsies from patients with tropical sprue and also in ulcerative colitis (Marsh and Hinde 1985, Sarin et al.1987, Benfield et al.1990). It is likely that there is an allergic component to these disorders.

Auerbach's plexus in reflux disease contains scattered eosinophils and a similar number of mast cells to the normal oesophagus.

Conclusions.

1. Longstanding severe reflux requiring resection is associated with a significant eosinophilic infiltration of the oesophageal mucosa. It is also associated with an increase in the number of mast cells in all layers of the oesophagus.

2. There are very few eosinophils in Auerbach's plexus and other layers of the wall even when there is a heavy mucosal infiltrate.

3. An eosinophilic infiltrate in Auerbach's plexus is not a feature found in patients with uncomplicated reflux coming to hiatus hernia repair.

4. There were increased numbers of mast cells in Auerbach's plexus when compared with transplant controls.

5.HISTOPATHOLOGY OF OESOPHAGEAL MOTILITY DISORDERS

5.1 ACHALASIA

Introduction

The aetiology of achalasia has remained elusive for many years. It is well established that there is loss of ganglion cells in Auerbach's plexus (Lendrum 1937, Cassella et al.1964, Misiewicz et al.1969, Smith 1970a, Adams et al.1976, Csendes et al.1985).

Previous papers have alluded to an inflammatory infiltrate in Auerbach's plexus with predominantly round cells (Cassella et al.1964, Misiewicz et al.1969, Smith 1970a). These cells have been presumed to be lymphocytes but it has also been suggested that some of these cells might be proliferating ganglion-supporting or satellite cells - cells that support the function of the ganglion cells (Lendrum 1937, Adams et al. 1976).

It has been suggested that eosinophils may have a pathogenic role in achalasia (Tottrup et al. 1989, Man et al. 1993). In an immunohistochemical study of biopsies from 9 patients with manometrically proven achalasia Tottrup et al. demonstrated an infiltrate of eosinophils in Auerbach's plexus with evidence of degranulation in some cases (Tottrup et al. 1989).

The present study was undertaken to examine inflammatory changes in Auerbach's plexus in achalasia and to quantify eosinophils and mast cells in the plexus.

Methods

Oesophageal tissue was obtained from 4 patients who had had oesophageal resection for end-stage achalasia. A further 20 patients with achalasia were also studied - biopsies being taken at the time of myotomy. In 16 cases the diagnosis of achalasia was confirmed by a combination of clinical characteristics, radiology, manometric findings and operative appearances. In 4 cases achalasia was diagnosed without the benefit of manometry but radiology and clinical features were classical.

All the patients undergoing myotomy for achalasia had had previous attempts at improving their symptoms with oesophageal dilatation. Only those patients who did not improve with the first dilatation or who required multiple dilatations were considered for surgery. As can be seen in Table 5.1.1, three of the five patients undergoing resection for achalasia had had previous surgery for their disease. Their pathological picture will therefore reflect not only the advanced stage of their disease process but also the effects of the previous surgery on the oesophagus. The clinical data for the two groups is shown in Table 5.1.1.and Table 5.1.2

The tissues were prepared as in the General Methods section. The presence of inflammation and its characteristics was assessed on sections stained with haematoxylin and eosin. An estimate was made of the number of ganglion cells in the the section and the number of inflammatory cells present.

Eosinophils and mast cells were formally counted as described in the General Methods section.

Table 5.1.1

Clinical data for patients undergoing oesophageal resection for endstage achalasia.

Clinical data for patients with oesophageal resection for end-stage achalasia

Table 5.1.1

Lab. No.	Age	Sex	Length of history years	Indications for resection	Previous treatment
662383	24	M	10	Atonic, reflux-damaged oesophagus	Heller's myotomy + gastroplasty 1977 Repeated dilatations
432186	9	M	5	Dysphagia, choking attacks Immotile oesophagus, nonrelaxing LOS	Repeated dilatations
308484	39	F	22	Dysphagia Atonic, absent LOS, no reflux	Heller's myotomy 1968, redo 1969. Redo Heller's myotomy + Belsey 1977
646184	63	F	2	Immotile oesophagus Reflux stricture, ulceration	Heller's 1983

Data for patients undergoing myotomy for achalasia

Lab. No.	Age	Sex	LOS Pressure(mmHg)	LOS Relaxation	Motility of oesophageal body	Intraoesophageal pressure	Mean contraction amplitude
33581	58	m	No manometry				
50386	42	m	No manometry				
53784	29	m	No manometry				
328841	64	m	No manometry				
124882	71	m	Not passed	Not passed	Almost immotile	positive	10
61684	23	f	Not passed	Not passed	S	positive	26
69686	39	f	24	N	S,Sp	positive	25
297082	41	f	27	N	S	positive	12
69983	19	m	38	N	S	positive	28
492384	38	f	50	N	S,Sp, rhythmic	positive	20
116083	21	f	23	I	S	positive	22
424981	47	m	20	I	S,R	positive	20
374181	42	f	22	i	S	positive	18
962680	28	f	20	N	S	positive	15
54913	41	m	20	N	S,R	positive	24
350581	39	f	34	N	S	positive	12
523483	58	m	41	I	S,R	positive	30
91488	22	m	17	I	S,R,multipeaked	positive	17
3291	43	m	26	I	S,Sp,R	positive	22
772690	30	m	25	N	S,Sp	positive	30
Means	39.8		27.6				20.7

Abbreviations: N = nonrelaxing S = simultaneous contractions, Sp = spontaneous contractions
I = incomplete relaxation, R = repetetive contractions, IOP = intraoesophageal pressure

Table 5.1.2
Clinical data for patients having oesophageal muscle biopsy at the time of oesophageal myotomy.

Results

a) Resected oesophagus.

1) Inflammatory changes.

In all cases there was a marked inflammatory response in the mucosa. This contained lymphocytes, polymorphonuclear cells and a heavy lamina propria infiltration with eosinophils. Although most prominent in the mucosa, there were inflammatory cells present in all layers of the oesophagus. There was also fibrosis of the muscular wall and within the connective tissue. Ganglion cells were seen infrequently in three of the specimens and not at all in one.

2) Eosinophil counts

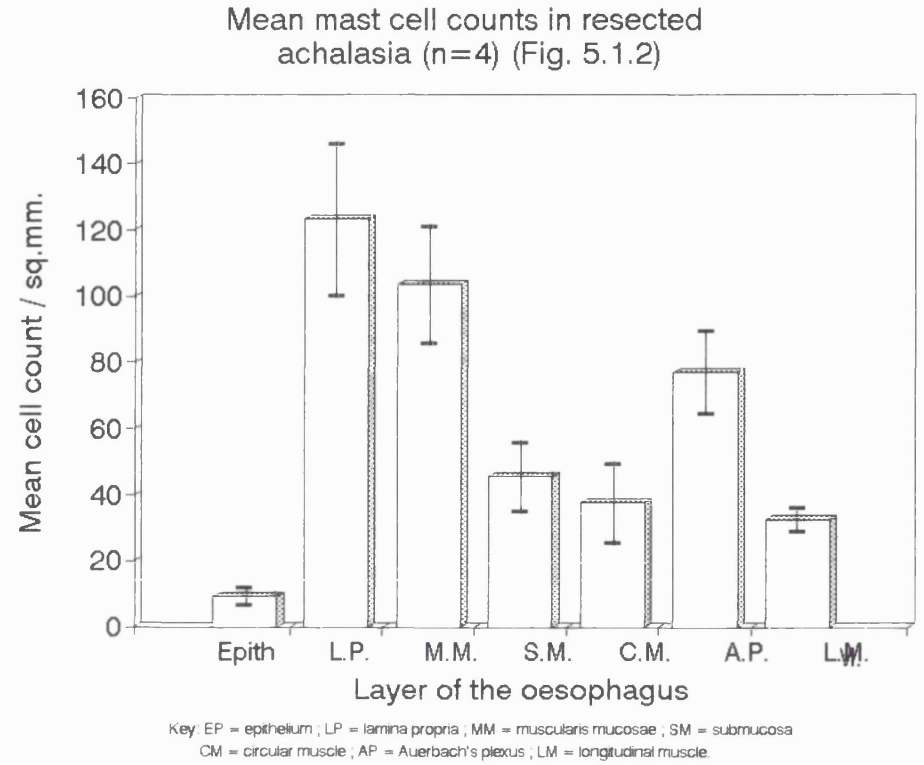
Eosinophils were present in greatest numbers in the lamina propria with much smaller numbers present in the other layers (see Fig 5.1.1.).

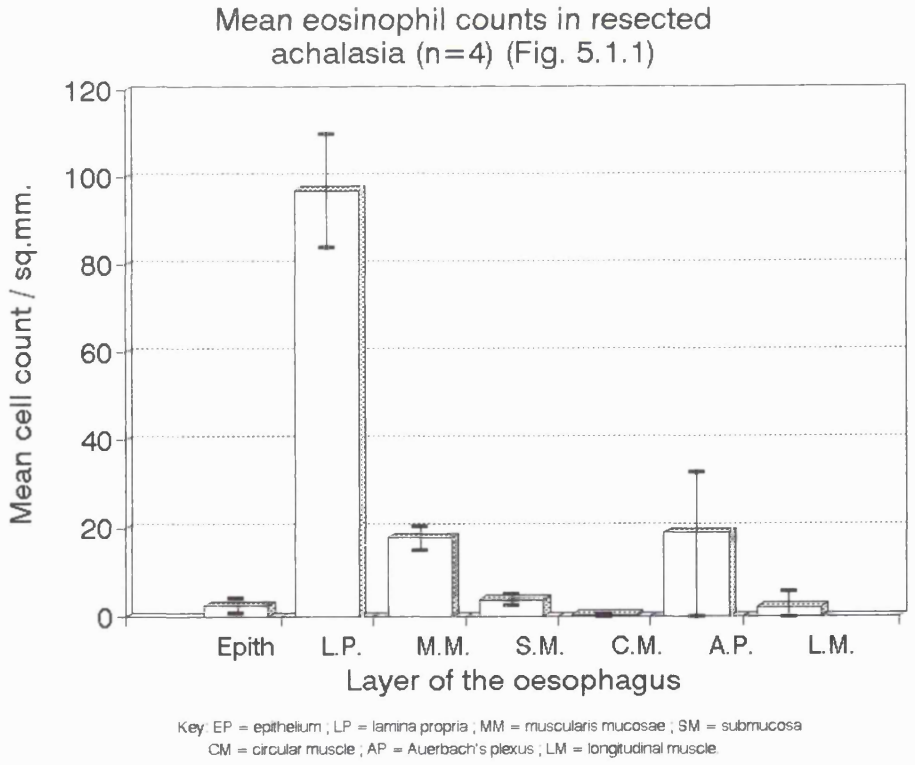
The counts of eosinophils in Auerbach's plexus in resected achalasia (median 14.75, range 0-26.1) were similar to those in resected reflux disease (median 2.1, range 0-36.2). The groups were too small for meaningful statistical analysis. Absolute counts are detailed in appendix 4.

3) Mast cell counts

Mast cells were more common cells in all the layers, being most numerous in the lamina propria and muscularis mucosae (see Fig. 5.1.2.). The original counts from which this data is derived are detailed in Appendix 3.

Mast cells counts in AP in resected achalasia (mean 77.16, SEM 12.6) were similar to those in resected reflux disease (mean 92.2, SEM 9.2). Again the groups are too small for meaningful statistical analysis.





Figures 5.1.1 and 5.1.2

Mean eosinophil and mast cell counts in resected achalasia.

b) Myotomy biopsies.

1) Ganglion cell numbers.
Ganglion cells were absent in all but one of the biopsies evaluated.

2) Inflammation in Auerbach's plexus.
There was no significant polymorphonuclear infiltration in any case. There was a mononuclear infiltrate in 5 of the 20 cases, in 2 of which it appeared quite marked. Data detailed in Table 5.1.3.

3) Eosinophil counts.
The majority of the biopsies contained few eosinophils. The median eosinophil count for achalasia was 2.95 cells / sq.mm.(range 0-192.9). The median eosinophil count for hiatus hernia was 1.5 (range 0-9). This difference was not significant ($P > 0.05$, Mann-Whitney test). The counts are compared in Fig 5.1.3. Two cases had more than 100 eosinophils per square millimetre with evidence of degranulation. See Figure 5.1.5.

4) Mast cell counts
There were significantly more mast cells in AP in the achalasia biopsies (mean 125.56, SD 53.83) than in those from patients with hiatus hernia (mean 84.33, SD 28.76) Unpaired t test, $p < 0.05$. The mast cell counts are detailed in Table 5.1.3 and compared with those in hiatus hernia in figure 5.1.4.

Table 5.1.3

Histological findings in myotomy biopsies from patients with achalasia.

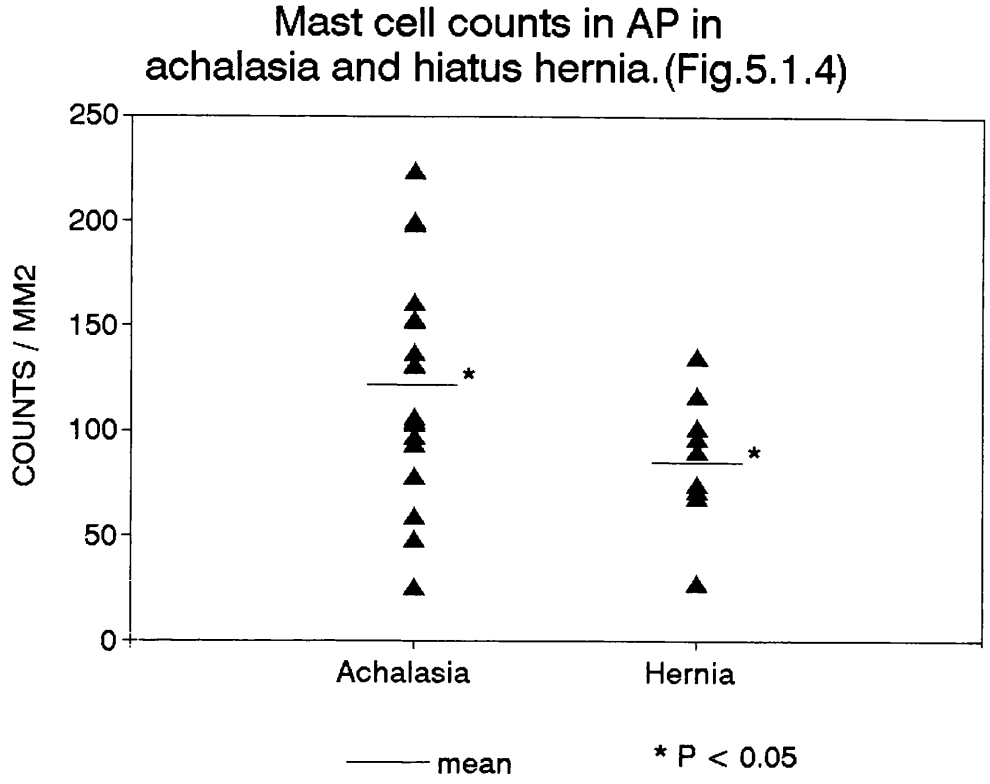
EOSINOPHIL AND MAST CELL COUNTS IN EXTERNAL MUSCLE BIOPSIES IN ACHALASIA

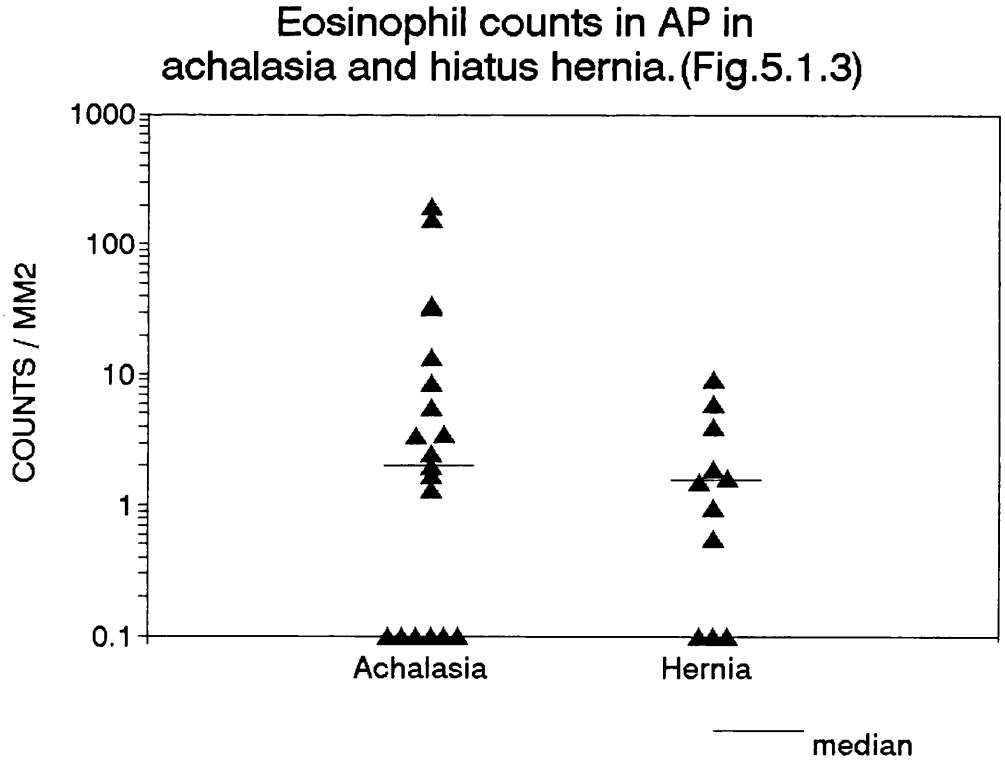
Lab. No.	Diagnosis	Cell counts		Area	Eosinophils	Mast cells		Ganglion		Mononuclear
		Eosinophils	Mast cells	(sq.mm.)	per sq.mm.	per sq.mm.	Nerves	cells	PMN	cells
328841	Achalasia*	0	8	0.31	0.00	25.81	YES	NO	+	+
335581	Achalasia*	1	170	0.76	1.32	223.68	YES	NO	+	++
50386	Achalasia*	81	44	0.42	192.86	104.76	YES	NO	++	+
53784	Achalasia*	0	49	0.32	0.00	153.13	YES	NO	+	+
124882	Achalasia+	4	44	0.47	8.51	93.62	YES	NO	+	++
61684	Achalasia+	1	53	0.51	1.96	103.92	YES	NO	+	+
69696	Achalasia	25	101	0.77	32.47	131.17	Degenerating	NO	+	+
297082	Achalasia	0	18	0.23	0.01	78.26	NO	NO	+	++
699831	Achalasia	3	92	0.86	3.49	106.98	YES	NO	+	+++
492384	Achalasia	4	116	0.72	5.56	161.11	YES	NO	+	+
116083	Achalasia	2	30	0.15	13.33	200.00	NO	NO	+	++
424981	Achalasia	3	175	0.88	3.41	198.86	YES	YES	+	+
374181	Achalasia	1	35	0.59	1.69	59.32	YES	NO	+	++
962680	Achalasia	0	59	0.45	0.01	131.11	YES	NO	+	+
54913	Achalasia	0	37	0.38	0.01	97.37	YES	NO	+	+
350581	Achalasia	0	56	0.28	0.01	200.00	YES	NO	+	++
523483	Achalasia	0	19	0.39	0.01	48.7	YES	NO	0	+
91488	Achalasia	2	83	0.8	2.50	103.75	YES	NO	+	+++
3291	Achalasia	18	74	0.54	33.3	137	NO	NO	+	+++
772690	Achalasia	59	58	0.38	155.3	152.6	YES	NO	+	++

Key: Achalasia* = diagnosed without manometry = Eosinophil degranulation

Achalasia+ = manometry catheter would not enter stomach

0 = no cells seen, + = 1 - 5 cells/HPF, ++ = 5 - 20 cells/HPF, +++ = >20 cells/HPF





Figures 5.1.3 and 5.1.4

Comparison of eosinophil and mast cell counts in achalasia and hiatus hernia muscle biopsies

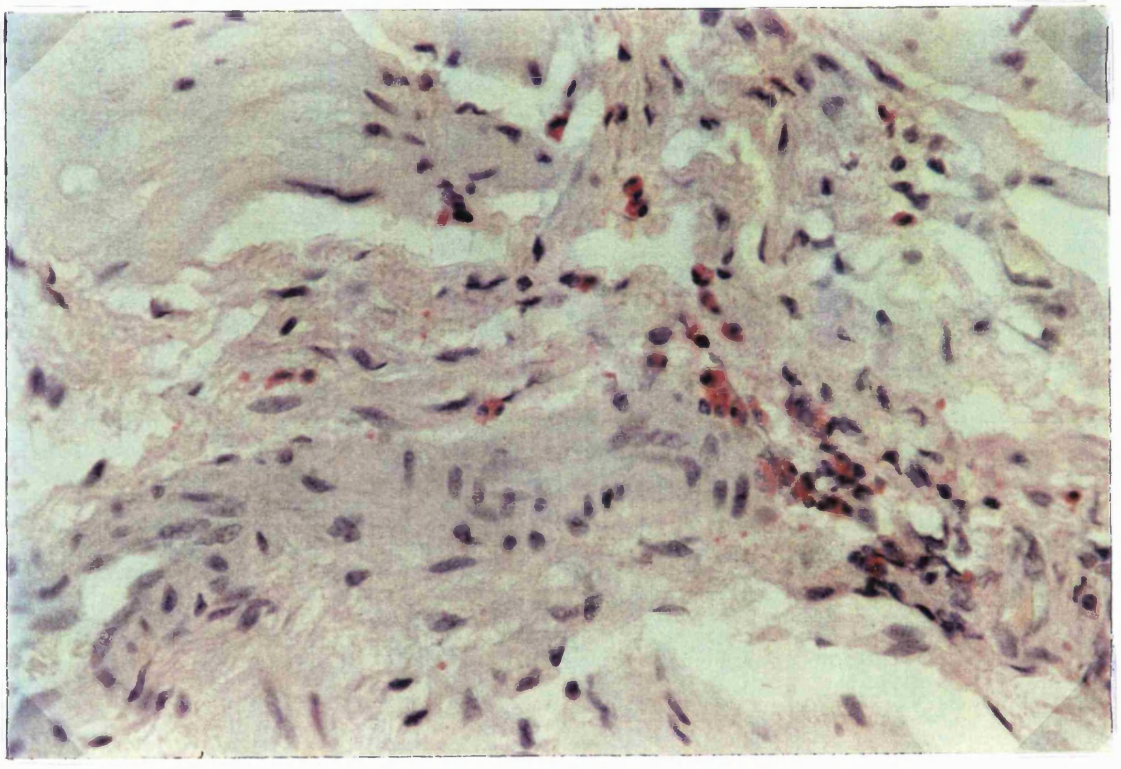


Figure 5.1.5

Connective tissue close to Auerbach's plexus stained with ABVNR. Numerous red-staining eosinophils are seen with staining of granules some distance from the cells. Achalasia: 503/86. Magnification X400

Discussion

As in oesophagus resected for reflux disease, the resected achalasic oesophagus showed severe inflammatory changes. Such changes have recently been described in a study of 42 resected specimens from patients with achalasia (Goldblum et al.1994). Inflammation in AP with reduction or absence of ganglion cells was noted in all cases. The infiltrate was found to be a mixture of lymphocytes and eosinophils. No quantitation was performed. Hypertrophy of the muscular wall of the oesophagus with degeneration and fibrosis was also a feature. A heavy inflammatory infiltrate in the mucosa was considered secondary to stasis of food contents within the oesophageal lumen. These are features of end-stage disease and are very similar to those found in the resected cases in this thesis. The relevance of histological findings at this stage of the disease to pathological changes occurring earlier in the disease, as seen in the myotomy biopsies, is open to question. However, even in those resected cases with very heavy eosinophil infiltration in the mucosa, heavy eosinophil infiltration of Auerbach's plexus was not a concomitant feature, being seen only in part of one specimen where 330 cells per square millimetre were found.

Of the 20 myotomy biopsies studied, only 2 had significant numbers of eosinophils in Auerbach's plexus, and only one had evidence of degranulation. Tottrup et al studied biopsies from 9 patients and found an eosinophil infiltrate in Auerbach's plexus (Tottrup et al.1989). However the cells were not quantified and it is not clear in how many cases this occurred to a significant degree. In two of their 8 controls there were many eosinophils in the outer muscular layers. Although these cells rarely stained positively for the activated secretion product ECP (using the antibody EG2), it should be noted that the biopsies obtained from the achalasia patients were processed differently to those of their controls. Immunohistochemical staining is known to vary considerably in tissues that have different methods of fixation and therefore their comparisons should be regarded with caution.

Tottrup et al state that use of haematoxylin and eosin to stain for eosinophils can result in an eosinophil infiltrate being overlooked. Thus using immunohistochemical staining they found eosinophil infiltrates and extracellular secretion which was not apparent on haematoxylin and eosin staining. In this study, with the use of the histochemical staining combination ABVNR, it was found that eosinophils were identified with ease since there was very little background staining. However review of H&E staining of the same tissues

revealed that, with experience, the characteristic staining characteristics and typical granularity of the cytoplasm allow eosinophils to be identified with little difficulty. It was only when eosinophils were present very infrequently that using a specific stain made their identification easier. It is unlikely that a significant infiltrate of eosinophils would be missed.

Csendes et al.(1992) studied 34 patients with achalasia and compared them with 10 postmortem controls. In 31 of the achalasic patients oesophageal biopsy showed absence of neurones in Auerbach's plexus (present in all controls). In two cases lymphocytic infiltration of the plexuses was noted and only one had 'minor eosinophilic infiltration' despite specific examination for this. The findings of Csendes et al. appear to be more in keeping with those described in this thesis. It is likely therefore that an eosinophilic infiltrate in Auerbach's plexus is only an occasional finding in achalasia.

Conclusions

1) A prominent round cell inflammatory infiltrate of Auerbach's plexus occurs in 20% of patients with achalasia.

2) Mast cell counts in Auerbach's plexus are higher in achalasia than in hiatus hernia or controls.

3) Significant eosinophil infiltration of Auerbach's plexus occurs in 10% of patients with achalasia.

5.2 DIFFUSE OESOPHAGEAL SPASM AND RELATED DISORDERS.

Diffuse Oesophageal Spasm.

Introduction

It is over 100 years since Osgood, in his paper entitled 'a peculiar form of oesophagismus', described the clinical features of the condition we know as diffuse oesophageal spasm (DOS). However reports of the histopathology of this condition are scant. Many of the reported cases were described prior to the advent of manometry with the diagnosis based on clinical and radiological grounds. Studies of the correlation between radiological features and manometry have shown that when radiological features of DOS were found motility was always abnormal but did not always conform to a manometric diagnosis of DOS (Chen et al. 1989, Hewson et al. 1990). Thus studies in which manometry was not been performed may actually have been looking at a different group of patients.

Nerve and muscle abnormalities in diffuse oesophageal spasm.

In DOS the outer muscular wall of the oesophagus is often grossly thickened (Craddock et al. 1966, Henderson et al. 1974, Flye and Sealy 1975, Friesen et al. 1983, Nicks et al. 1968). Light microscope studies of DOS have demonstrated that, unlike in achalasia, ganglion cells are present in Auerbach's plexus (Gillies et al. 1967). However one study suggested they might be reduced in number (Adams et al. 1976). It has also been shown that in some cases of DOS there is oesophageal hypersensitivity to Mecholyl, as is seen in achalasia, suggesting that this may be a result of denervation (Kramer et al. 1967b).

Cassella et al. (1965) studied 6 patients with clinical, radiological and manometric DOS (no manometric definition was given). Electron microscopy was performed on muscle biopsies obtained at long myotomy and also on biopsies of vagal nerves. Most of the muscle cells appeared normal but in all cases there were isolated groups of cells that showed detachment, fragmentation and clumping of myofilaments. In some cases intercellular neutrophils were found. Electron microscopy showed generalised degenerative changes in the oesophageal branches of the vagus nerve, possibly representing involvement of the vagal afferent system

in diffuse oesophageal spasm. However their findings were not confirmed in a subsequent study of 9 patients by Friesen et al.(1983). They found some electron microscopical changes in a proportion of muscle fibres but not in all cases. Quantitation of muscle fibres suggested that the increased muscle thickness was due to hyperplasia and not hypertrophy. They found no evidence of neural abnormality in DOS.

Inflammation in Auerbach's plexus in DOS.

Marston in 1959 described the pathology from one patient with DOS in whom an eosinophilic infiltrate was found in the perivascular and perineural tissue with some 'lymphangiectasis'. However it was concluded that the lack of fibrosis and inflammatory exudate in previously reported cases of DOS ruled out an inflammatory cause for the condition. Gillies in 1967 studied 4 myotomy biopsies from patients with DOS and demonstrated chronic inflammatory changes with eosinophilic infiltration. Ganglion cells were present in all cases. In a further report from the same centre in 1968 (Nicks et al. 1968) similar findings were described. Lymphocytes and plasma cells were present in varying density and eosinophils were present in variable numbers. The presence of eosinophils was thought to be a nonspecific response to operative trauma and was not thought to be an essential feature of the pathology of diffuse oesophageal spasm.

Idiopathic muscular hypertrophy of the oesophagus.

Since muscular hypertrophy of the oesophagus is a prominent feature in DOS, the finding of similar thickening of the oesophageal wall as an incidental finding at postmortem examination has received much attention (Sloper 1954, Demian et al. 1978, Iyer et al. 1986). A similar infiltrate of inflammatory cells, including eosinophils, has been described in this condition. However the relationship of this condition to DOS is unclear. The majority of the patients had no dysphagia or other gastrointestinal symptoms during life. Sloper et al.. described 7 cases diagnosed at postmortem (Sloper 1954). In only one case were the clinical features and barium swallow prior to death compatible with DOS. In 5 patients microscopy was performed and showed a lymphocytic and eosinophilic infiltrate in Auerbach's plexus. This was also found in the patient diagnosed before death. Sloper reviewed the literature and found 25 cases of idiopathic muscular hypertrophy of which 12/25 had lymphocytes in Auerbach's plexus and 7/25 had eosinophils present also. One patient had loss of ganglion cells. Demian et

al.(1978) reported 6 cases found in a series of 1360 autopsies over 3 years. Only one of these cases had had symptoms referrable to the oesophagus prior to death. The patient had suffered with dysphagia for 4 years and a barium swallow had demonstrated dilatation of the oesophagus with some distal narrowing compatible with a diagnosis of achalasia. 4 patients had muscle thickening of the lower two thirds of the oesophagus, one in the midportion only and one in the entire thoracic oesophagus. The ganglia and nerves were present in all cases in Auerbach's plexus. There was no mention of any inflammation in AP. In two cases there was ulceration in the oesophagus. Iyer 1986 described 6 cases of postmortem muscular thickening of the oesophagus. In one patient ,who died of pneumonia and a lung abscess, a barium swallow had shown tertiary contractions and the typical appearances of achalasia in the distal oesophagus. One patient, a diabetic, had ulceration in the lower oesophagus as well. Histology showed a lymphocytic infiltration of Auerbach's plexus.

Vigorous achalasia and nutcracker oesophagus.

There is only one reported case to my knowledge of vigorous achalasia in which the pathological changes in Auerbach's plexus have been examined (Landres et al. 1978). The muscle wall was found to be thickened and there was an infiltrate of AP with eosinophils. Ganglion cells were not seen but nerve bundles were present in normal numbers. Since vigorous achalasia is regarded by some as a variant of achalasia it is possible that other cases of vigorous achalasia have been included in studies of classical achalasia.

Nutcracker oesophagus rarely requires surgical intervention and there are no reports to my knowledge of pathological studies of the condition.

Aims of current study.

This study aims to look at changes present in Auerbach's plexus in myotomy biopsies taken from a series of patients undergoing long myotomy for a diagnosis of diffuse oesophageal spasm, vigorous achalasia or nutcracker oesophagus. Within this group some patients had classical manometric features but others were more difficult to classify. Therefore the group will be studied as a whole and the manometric features of each case will be detailed.

Methods.

Myotomy biopsies were taken over a 10 year period at the time of long myotomy for disordered oesophageal motility. Essentially these were biopsies from patients who had DOS ,vigorous achalasia or nutcracker oesophagus and who were not part of the study on pure achalasia.

The patients' clinical details are shown in Table 5.2.1. Their manometric features are detailed in Table 5.2.2. Manometry was performed and the patients classified as described in the General Methods section. The manometric record for each patient was reviewed and the details recorded in the table.

From each biopsy numerous sections were taken. Only sections in which the connective tissue lying between the outer longitudinal and inner circular muscle (normally containing Auerbach's plexus) could be clearly identified were used.

The slides from each biopsy were initially assessed using the standard stain haematoxylin and eosin. The presence or absence of ganglion cells and nerves was noted. The area of Auerbach's plexus was also examined for the presence or absence of inflammatory exudate, characterised depending upon the presence of small round cells (presumed lymphocytes) or neutrophils. The sections were stained with the staining technique astra blue/vital new red (Duffy et al. 1993) and examined for mast cells and eosinophils. These were quantified using the technique described in the General Methods section.

Table 5.2.1 Clinical features of patients with DOS and related disorders.

Clinical features of patients with DOS and related disorders Table 5.2.1

Lab. No.	Diagnosis	Age	Sex	Length of history (months)	Dysphagia	Pain	Regurgitation	Allergy	Relevant PMH	Barium swallow	Oesophagoscopy
8192	DOS	70	F	48	yes	yes	no	no	no	diverticula, tertiary contractions	diverticulum
7092	DOS	46	F	24	yes	yes	yes	asthma	no	normal	?
5792	DOS	55	F	60	yes	no	yes	eczema	angina	occasional retrograde	repeated dilatations, normal
158687	DOS	45	F	17	yes	yes	no	no	no	normal	normal
238786	DOS	50	M	72	yes	yes	yes	no	no	tertiary contractions	meat bolus
883785	DOS	54	F	72	yes	no	yes	aspirin	irritable bowel	diverticulum	normal
433583	DOS	33	M	30	yes	yes	no	no	no	tertiary waves	normal
6591	DOS	44	M	108	yes	yes	yes	no	no	holdup of Ba	normal
1591	DOS	53	M	30	no	yes	no	no	HSV	hiatus hernia	normal
5691	DOS	45	F	60	yes	yes	no	septrin	no	normal	normal
608885	DOS	57	M	36	yes	yes	no	penicillin	no	normal	normal
306286	DOS	39	F	1	yes	yes	yes	no	no	diverticulum	normal
773281	VIG ACHALASIA	50	M	36	yes	yes	no	no	no	dilated, distal holdup, tertiary contns	narrow distally, mucosa normal
525390	VIG ACHALASIA	45	M	60	yes	no	yes	no	no	tertiary contractions, diverticulum	normal
815990	VIG ACHALASIA	67	M	36	yes	no	yes	no	colitis 1944	diverticulum	food residue, mucosa normal
715786	VIG ACHALASIA	47	F	14	yes	yes	no	no	no	delayed transit	normal
125590	VIG ACHALASIA	38	M	24	yes	yes	no	asthma	on steroids	oscillation of Ba, not dilated	normal, dilated X1
1192	VIG ACHALASIA	56	F	48	yes	yes	yes	no	no	distal holdup	normal
792	NUTCRACKER	40	F	96	yes	yes	yes	no	no		minimal oesophagitis
393	NUTCRACKER	67	F	9	yes	yes	no	no	no	tertiary, retrograde, holdup midthird	tortuous, mild dilatn, mucosa nad
813582	NUTCRACKER	19	F	18	yes	yes	no	grass pollen	no	tertiary contractions	no record
2293	NUTCRACKER	34	F	48	yes	yes	yes	no	no	normal	normal
1048490	NUTCRACKER	68	F	120	yes	yes	no	penicillin	no	dyskinesia	normal

Table 5.2.2 Manometric features of patients with DOS and related disorders.

MANOMETRIC CHARACTERISTICS OF DOS AND RELATED DISORDERS

Table 5.2.2

Laboratory Number	Working diagnosis	Total swallows	% peristaltic contractions	% simultaneous	Interrupted/ aborted	No. spontaneous	Repetetive runs	Resting IOP	LOWER OESOPHAGEAL SPHINCTER		% time pH <4
									Pressure(mmHg)	Relaxation	
433583	DOS	36	41.60	47.20	4	5	8	<gastric	9	F(i)	
158687	DOS	38	60.50	31.60	3	4	4	<gastric	16	I	0
1591	DOS	22	63.40	36.40	0	8	3	<gastric	21	F(i)	3.2
6591	DOS	16	0.00	68.70	5	9	6	<gastric	18	I(f)	7.1
5691	DOS	52	25.00	55.80	10	9	4	<gastric	9	F	5.9
306286	DOS	26	57.70	38.50	1	4	2		catheter would not pass		
608885	DOS	31	35.50	64.50	0	10	7	<gastric	15	I(f)	0
5792	DOS	23	26.10	52.20	5	8	3	<gastric	25	I(f)	nopH
7092	DOS	28	14.30	67.90	5	23	3	<gastric	11	F	0
8192	DOS	25	36.00	64.00	0	7	3	<gastric	12	I(f)	0
2387786	DOS	18	44.40	50.00	1	0	0	<gastric	12	I	2
883785	DOS	28	14.30	75.00	3	0	0	<gastric	40	F	*
815990	VIG ACHALASIA	25	0.00	100.00	0	6	0	<gastric	48	F	
525390	VIG ACHALASIA	14	0.00	100.00	0	1	0		catheter would not pass		
125590	VIG ACHALASIA	37	0.00	97.30	1	5	1	<gastric	14	N	0
1192	VIG ACHALASIA	23	0.00	91.30	2	2	7	gastric	30	N	
715786	VIG ACHALASIA	23	0.00	100.00	0	6	3	>gastric	18	N	
773281	VIG ACHALASIA	13	0.00	100.00	0	1	2	>gastric	24	N	
813582	NUTCRACKER	28	64.30	35.70	0	0	0	<gastric	18	F(i)	?
1048490	NUTCRACKER	20	100.00	0.00	0	0	0	<gastric	11	I	0
792	NUTCRACKER	20	100.00	0.00	0	0	0	<gastric	11	N(i)	2.2
393	NUTCRACKER	17	70.60	29.40	0	0	0	<gastric	13	I	8.7
2293	NUTCRACKER	19	100.00	0.00	0	0	0	<gastric	13	F	0

Results

1. Characteristics of the inflammatory response.

The characteristics of the inflammatory response are detailed in Table 5.2.3. It can be seen that polymorphonuclear cell infiltration in the region of Auerbach's plexus is not a prominent feature in this group of disorders. In only 2 of the cases was there a prominent mononuclear cell infiltrate. Ganglion cells were present in all biopsies except for 2 cases with vigorous achalasia where they were absent in multiple tissue sections.

2. Mast cell counts

Mast cell counts in DOS (mean 82.5 cells/mm^2, SD 46.8) were not significantly different to those found in hiatus hernia biopsies (mean 84.33 cells/mm^2, SD 28.8) ($P > 0.05$, unpaired t test) Similarly, counts in vigorous achalasia (mean 103.65 cells/mm^2, SD 57.1) and nutcracker oesophagus (79.9 cells/mm^2, SD 15.9) were not significantly different to those in hiatus hernia.

3. Eosinophil counts

In four of the cases of DOS, 4 with vigorous achalasia and one case with nutcracker oesophagus there was prominent eosinophil infiltration of Auerbach's plexus (see Figure 5.2.1, 5.2.2). In 7 of those cases there was evidence of eosinophil degranulation (see Fig 5.2.1). In the patient with nutcracker oesophagus, (22/93), there was an extremely extensive eosinophil infiltrate in Auerbach's plexus extending into the muscular layers of the oesophagus (see Fig 5.2.2). The counts in each condition (DOS: median 3.7, range 0 - 206.5, vigorous achalasia: median 64.8, range 7.9 - 361 and nutcracker oesophagus: median 0.41 , range 0 - 1619) were compared with those in AP in hiatus hernia. There were no significant differences ($P > 0.05$, Wilcoxon test on unpaired samples).

EOSINOPHIL AND MAST CELL COUNTS IN
EXTERNAL MUSCLE BIOPSIES Table 5.2.3

Patient Lab No	Clinical diagnosis	Eosinophils per sq mm	Mast cells per sq mm	Ganglion cells	Neutrophil PMN cells	Mononuclear cells
5792	DOS	3.7	141	YES	+	+
238786	DOS	2.4	87	YES	+++	++
5691	DOS	5.26	63.1	YES	+	+
608885	DOS	26.9	89.5	YES	+	+
883785	DOS	0.49	34.00	YES	+	++
8192	DOS	0	65.4	YES	+	+
7092	DOS	0	52.63	YES	+	+
6591	DOS	206.5	38	YES	++	++
433583	DOS	143	189	YES	++	+++
1591	DOS	32.7	105.7	YES	++	++
158687	DOS	0.01	61	YES	+	+
306286	DOS	1	68.3	YES	+	+
1192	VIG ACHALASIA	16.1	112	YES	+	++
525390	VIG ACHALASIA	361	203	YES	++	++
815990	VIG ACHALASIA	7.9	46	YES	+	+++
773281	VIG ACHALASIA	100.00	120.69	NO	+	+
715786	VIG ACHALASIA	29.6	86	NO	+	+
125590	VIG ACHALASIA	322	54.2	YES	+	+
813582	NUTCRACKER	0	80	YES	+	+
792	NUTCRACKER	0	79	YES	+	+
393	NUTCRACKER	0	59	YES	+	+
1048490	NUTCRACKER	0.83	69.00	YES	+	++
2293	NUTCRACKER	1619	105.5	YES	+	++

Key: 0 = no cells seen, + = 1 - 5 cells/HPF, ++ = 6 - 20 cells/HPF, +++ = >20 cells/HPF

Table 5.2.3
Characteristics of the inflammatory response in myotomy biopsies in DOS and related disorders.

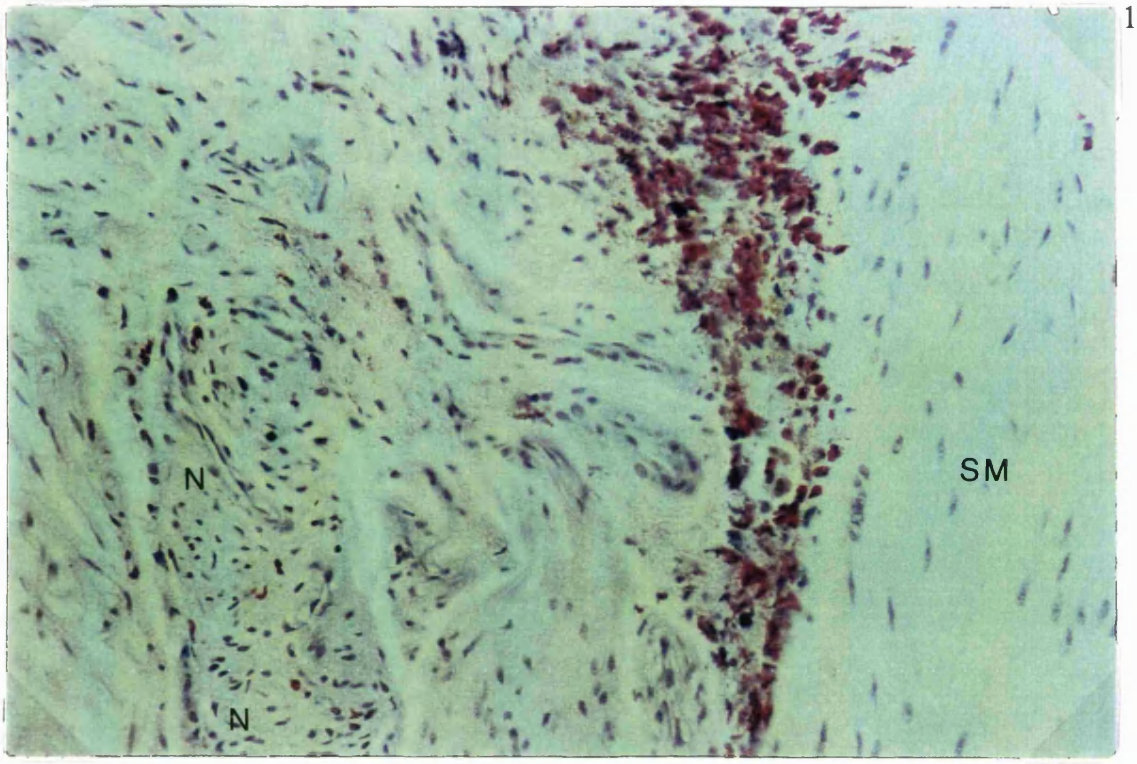


Figure 5.2.1 (a) Myotomy biopsy from patient with vigorous achalasia (5253/90) stained with ABVNR (Magnification X250). A florid eosinophil infiltrate is present (red-staining). Scattered eosinophils are seen within the nerve (N). Smooth muscle is labelled (SM).

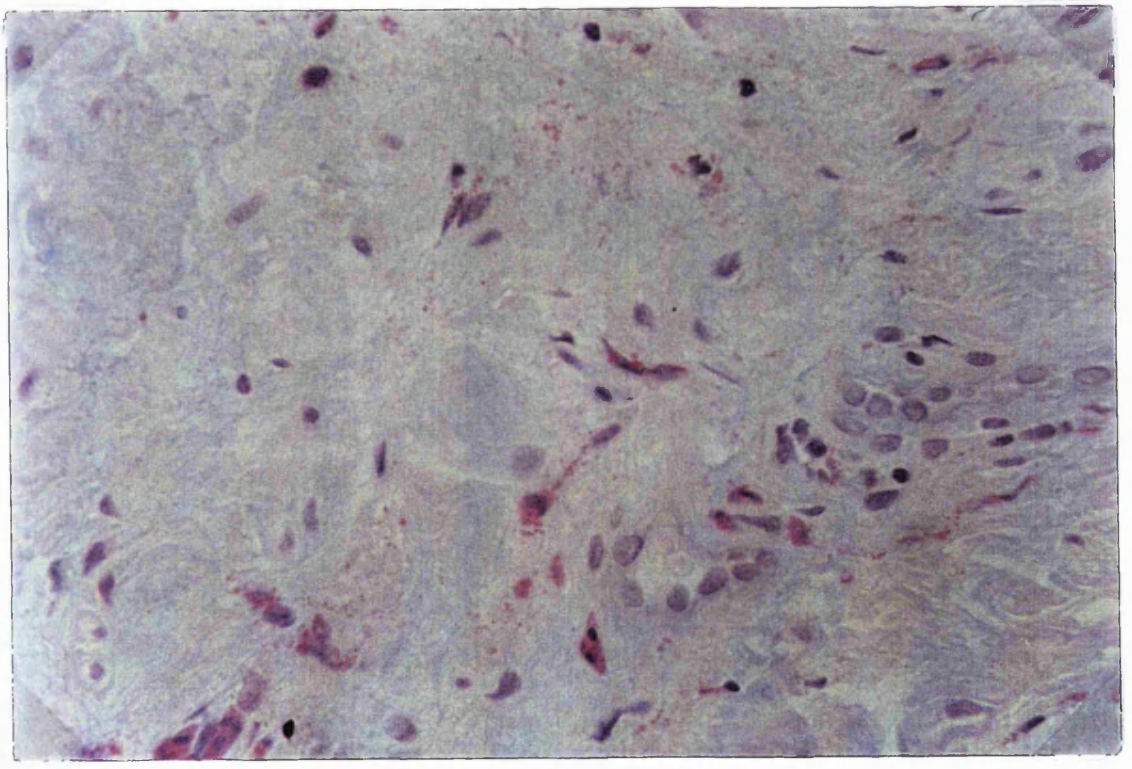


Figure 5.2.1 (b) Myotomy biopsy from patient with DOS (OE 65/91) stained with ABVNR. Numerous eosinophils (red-staining) with degranulation are seen. (Magnification X400)

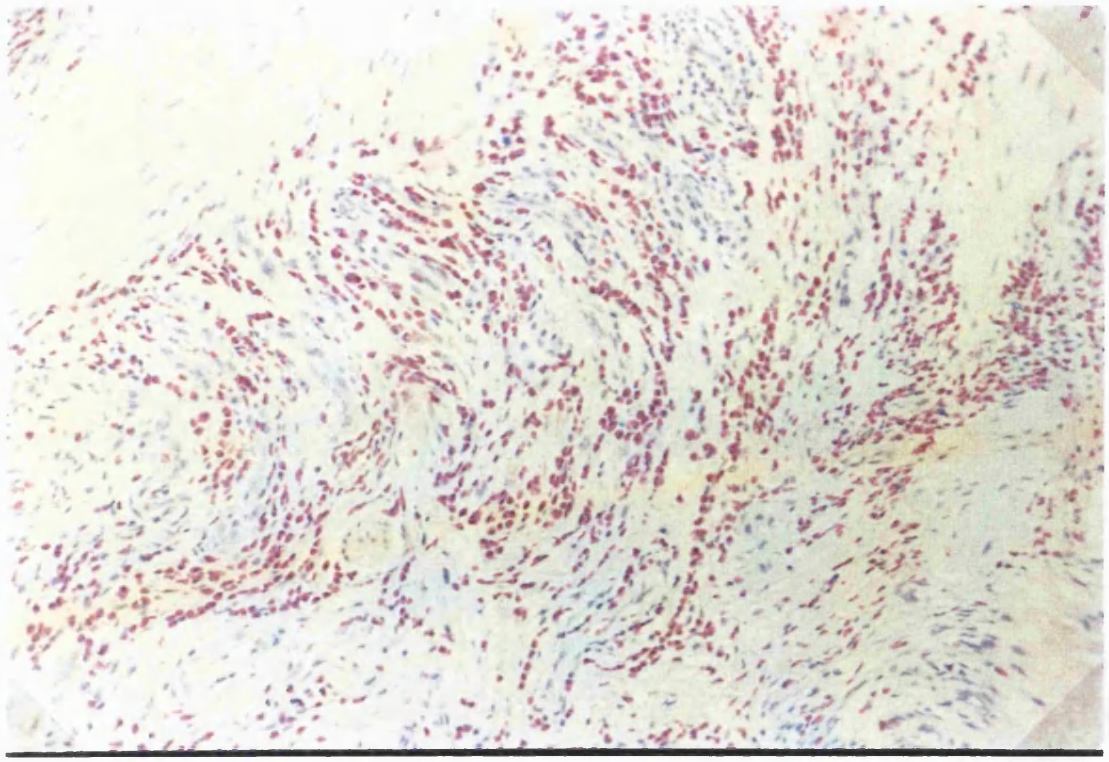


Figure 5.2.2 (a) Magnification X100

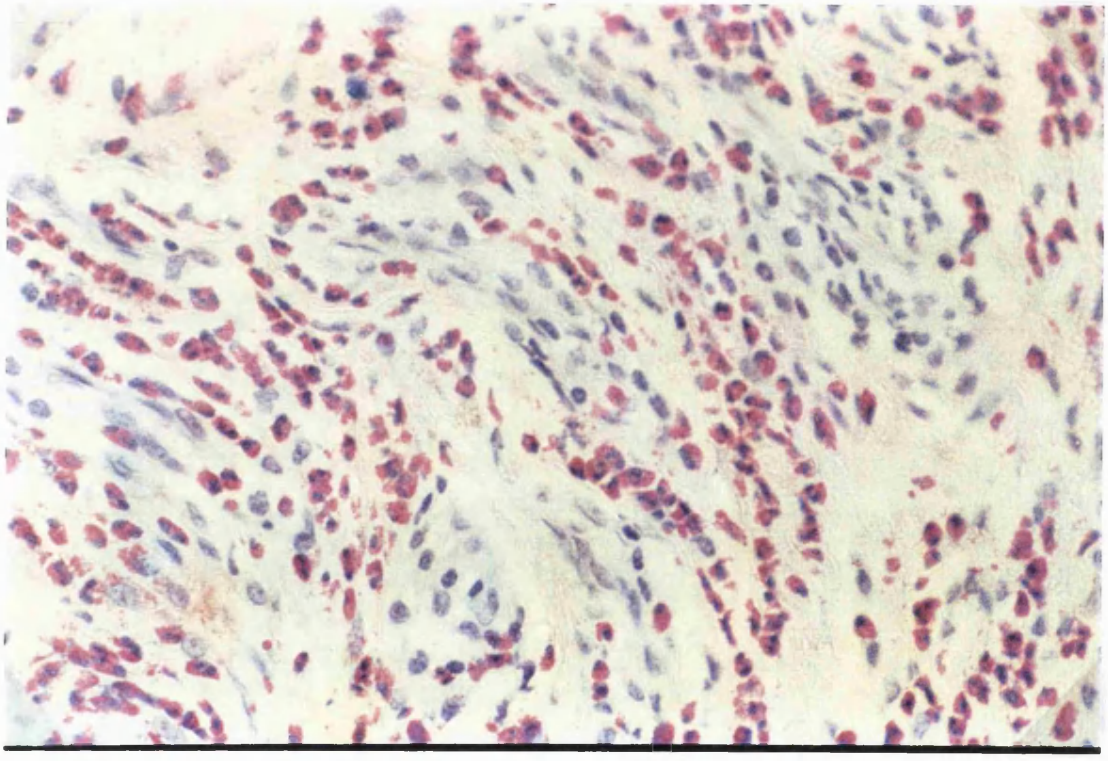


Figure 5.2.2 (b) Magnification X350

Figure 5.2.2 Myotomy biopsy from patient with nutcracker oesophagus (22/93) stained with ABVNR. There is a florid infiltrate of eosinophils (staining red) in the intermuscular connective tissue with some degranulation.

Discussion

This study has attempted to evaluate pathological changes in a range of motility disorders all of which have been classified with stationary manometry. It is generally accepted that the diagnosis of diffuse oesophageal spasm and related disorders can be difficult. Even in those studies where manometry has been used, patient classification may differ due to variation in manometric techniques used and in the criteria used for the diagnosis. Although it is generally accepted that diffuse oesophageal spasm is characterised by the presence of simultaneous contractions in the lower two thirds of the oesophagus, it has been shown that such simultaneous contractions can occur in normal individuals and also in other disorders such as reflux disease and diabetes (Bennett 1970). Using wet swallows and water perfused manometry catheters, it has been suggested that for the diagnosis of DOS more than 10% of swallows should result in nonperistaltic contractions but also that some swallows should result in a peristaltic sequence (Richter 1987). Studies using dry swallows have shown that there is a higher percentage of nonperistaltic contractions in normal individuals and therefore to diagnose DOS more than 30% of swallows should be nonperistaltic (DiMarino et al. 1974, Mellow et al. 1977). What are less clearly established are the criteria for diagnosis of simultaneous contractions. Hewson et al. (1990) in a simultaneous radiological and manometric study found intervals between manometric contraction onsets rather than peaks at different levels were better at assessing primary peristalsis and disordered bolus transit. Vantrappen et al. have used contraction onset as assessed by first steep upstroke after a swallow in assessing oesophageal contractions (Vantrappen et al. 1979). Others have used simultaneous peaks of contraction at different levels within the oesophagus (Craddock et al. 1966). For the purposes of this study contractions were judged to be simultaneous if the first steep upstroke of the contraction occurred simultaneously at different levels in the oesophagus.

As can be seen from the results, the finding of an eosinophil infiltrate in AP was commonest in DOS and vigorous achalasia. In most of these cases there was widespread eosinophil degranulation. In a few cases the inflammatory response was characterised by the presence of numerous polymorphonuclear cells or round cells (presumed to be lymphocytes). It is noteworthy that, of the six patients with vigorous achalasia, 2 had absence of ganglion cells in Auerbach's plexus in the sections studied.

The finding of a dense eosinophil infiltrate across the whole range of diagnostic categories studied (including 2 cases in the achalasic group described in the previous chapter) is very interesting. It appears most common in the intermediate disorder vigorous achalasia. The fact that eosinophils are a rare cell in AP, and also that infiltrates of eosinophils in other diseases are invariably pathogenic, suggests they may have a pathogenic role in these disorders.

Why an eosinophil infiltrate is not found in all the cases is not clear. There are a number of possible explanations for this.

Firstly, it is possible that these are just biopsies taken at different phases of the same disease. However there was no obvious difference between length of symptoms in relation to the time of biopsy and the presence or absence of eosinophils. If an eosinophil infiltrate were pathogenic, it might be that once the infiltrate had produced its damage in AP that the stimulus for eosinophil accummulation would disappear and they would not be detected at a later date. Many people consider the motility disorders to be a spectrum of disease and it is interesting to postulate that if it is accepted that nutcracker oesophagus and DOS are not entirely separate entities then vigorous achalasia could represent an intermediate stage in progression of these conditions to achalasia.

Secondly, it is possible that some of the biopsies were taken from patients without florid disease. The decision to operate on these patients was taken not just on the basis of manometric records alone, but in association with all the information gleaned from clinical and radiological examination. They had all been seen on numerous occasions prior to surgery and standard practice involved use of oesophageal dilatation as a temporising measure prior to definitive surgery. Only those cases who had persistent symptoms were submitted to surgery. This explanation seems unlikely.

Thirdly, it is possible that an eosinophil infiltrate is a pathogenic agent in some patients and some other agent is responsible for the disease in the rest.

Lastly, it is possible that the disorder may be patchy throughout the oesophagus. Thus there could be areas within parts of the oesophagus where there was no apparent inflammation and others with quite florid inflammation. The only way to prove this would be to study complete resected oesophagus from these patients. Since resection for the primary motility disorder itself is almost unheard of, such a specimen would only arise very infrequently. In later chapters an attempt has been made to establish whether there is any evidence of an eosinophil infiltrate in

paraoesophageal lymph nodes or whether there are any changes in the peripheral blood suggestive of eosinophil inflammation.

Since this group of vigorous contraction abnormalities has shown evidence of a high frequency of eosinophilic inflammation further studies will be undertaken in this group to study this in more detail. These will be presented in subsequent chapters.

Conclusions

The vigorous contraction abnormalities of the oesophagus can all be associated with an eosinophilic infiltrate in Auerbach's plexus. It appears most common in DOS and vigorous achalasia but can occur in nutcracker oesophagus. There was evidence of eosinophil degranulation in many of the cases with large numbers of eosinophils present.

5.3 STUDIES OF LYMPH NODES IN MOTILITY DISORDERS OF THE OESOPHAGUS.

Introduction

The initial route by which an antigen enters the body is important in determining the type of immune response which results. This depends on which antigen presenting cells take up the antigen and process it. For example an antigen applied to the skin will be picked up by the Langerhans cells (antigen presenting cells) and will be transported to the regional lymph nodes where it may induce a T-cell mediated hypersensitivity reaction. In similar fashion the same antigen entering the gut will be transported to the gut-associated lymphoid tissue (GALT) where a predominantly IgA-producing response will be elicited. In the oesophagus there are patches of lymphoid tissue in the lamina propria but these are by no means as well developed as the Peyer's patches seen further along the intestine. Given that the oesophagus is lined by squamous epithelium and also that the epithelium also contains Langerhans cells, it is possible that any antigen entering the epithelium would be taken to the regional lymph nodes by these cells and there initiate an immune response.

Thus if there is a florid eosinophilic response in the oesophagus it is possible that signs of such a response might be apparent on examination of the regional lymph nodes. Whether eosinophils migrate into tissues direct from the blood or gain access via lymphatics is unclear. It is possible that both may occur. The following study was undertaken to establish if there was any change in the numbers of eosinophils and mast cells in lymph nodes draining oesophageal tissue in motility disorders of the oesophagus.

Methods

Lymph nodes were obtained from patients at the time of hiatus hernia repair and at transthoracic myotomy for motility disorders. Full informed consent was obtained with local ethical committee approval. Clinical details of the patients studied are given in previous chapters.

Paraoesophageal lymph nodes were sought. When found, all the lymph nodes seen were dissected out. They were then removed and immediately fixed in

formaldehyde solution. They were subsequently stained with astra blue/vital new red as described in the General Methods section.

Counting of eosinophils and mast cells was performed under high power (X400). Follicular areas were excluded since eosinophils and mast cells were hardly ever found there. The number of cells was counted in 5 randomly placed high power fields and the mean number of cells established. These counts were performed without knowledge of the diagnosis to avoid prejudicing the results.

Results

The number of patients studied is relatively small. In some cases no obviously enlarged lymph nodes were found. Lymph nodes were also only obtained from the prospective part of the study.

In lymph nodes from patients with hiatus hernia the median result was 10.9 eosinophils / HPF (range 3 - 260) and 15.3 mast cells / HPF (range 2.8 - 151). In one case, (5591), there were markedly increased numbers of eosinophils and mast cells in the lymph node. Comparison with the results of the myotomy biopsy count showed there was no corresponding increase in these cells in Auerbach's plexus (0 eosinophils / mm^2, 72 mast cells / mm^2).

In DOS/vigorous achalasia the median count was 13.5 eosinophils / HPF (range 2.2 - 308) and median mast cell count was 19.3 mast cells / HPF (range 23 - 122.4). Only 3 cases were assessed in the achalasia and nutcracker groups respectively and their results are detailed in the table. In those cases of motility disorders in which large numbers of eosinophils were found (1591, 5691, 2293) the myotomy biopsy counts were raised in 2 (1591 - 32.7 eosinophils / mm^2, 2293 - 1619 eosinophils / mm^2) Figure 5.3.1 shows a lymph node from one of these patients and shows the florid infiltrate of eosinophils found (note the lack of similar infiltrate in a typical lymph node from a hiatus hernia patient). The counts of eosinophils and mast cells in paraoesophageal lymph nodes are shown in Table 5.3.1 and Figure 5.3 2 respectively. Statistical analysis has not been performed because of the small numbers involved.

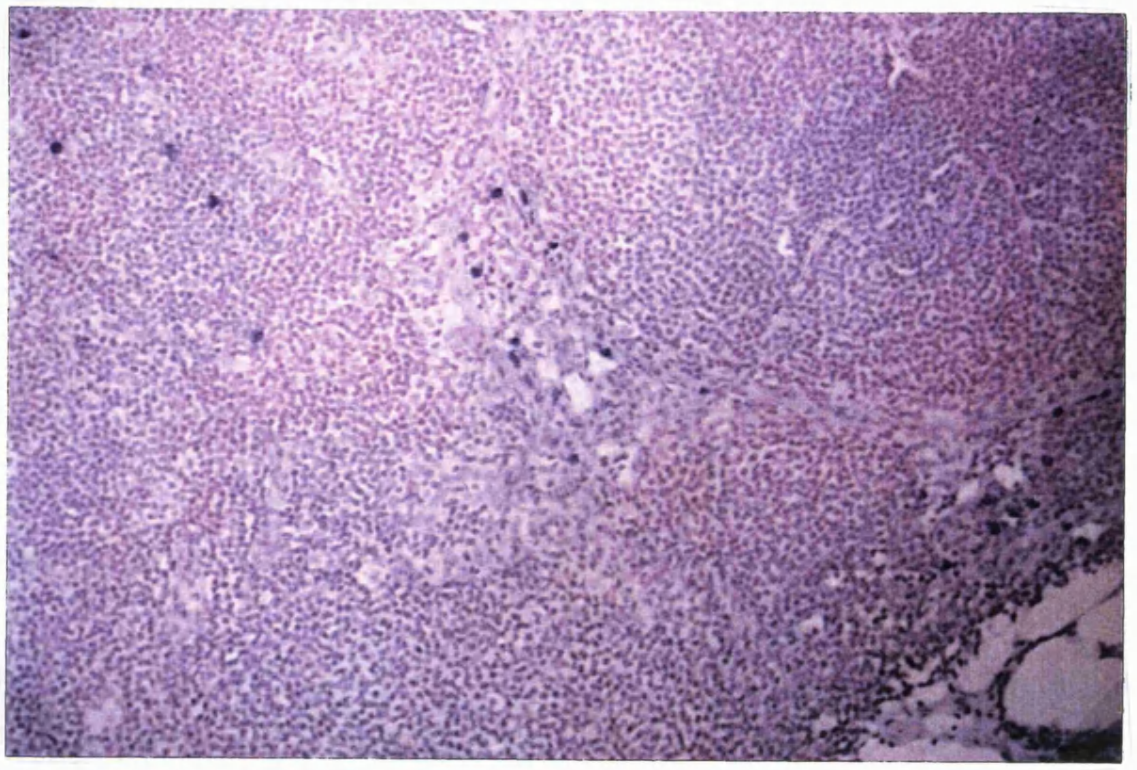


Fig. 5.3.1 (a)

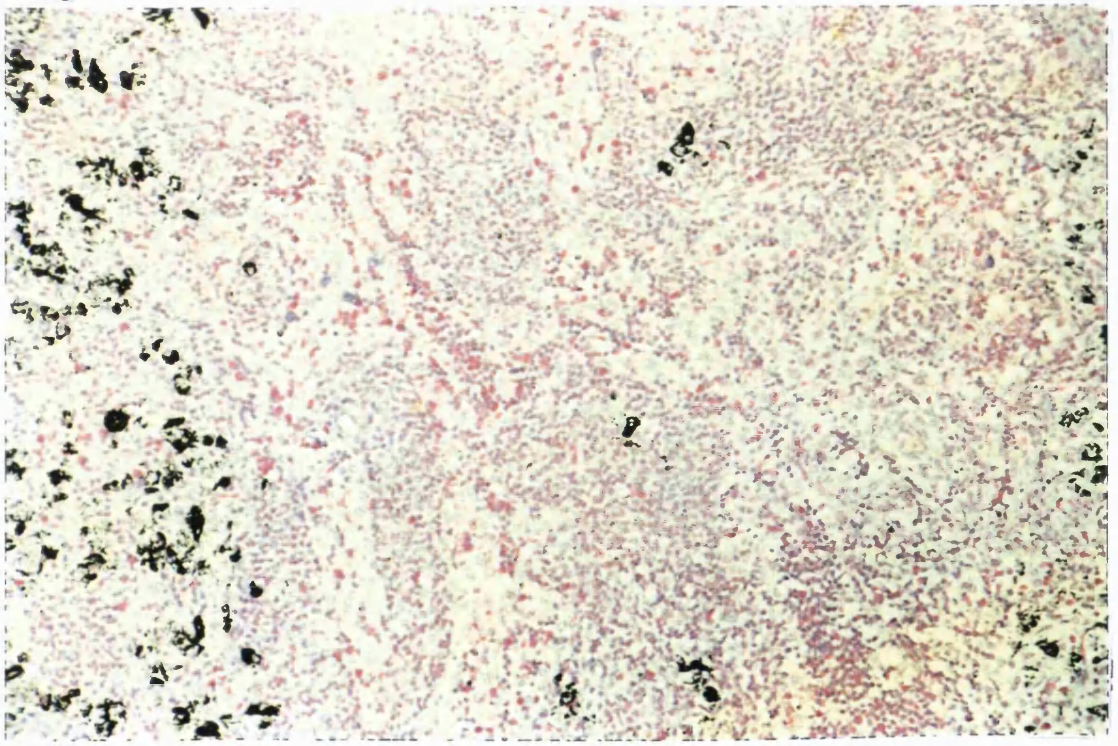


Fig. 5.3.1 (b)

Figure 5.3.1

Sections of paraoesophageal lymph nodes from patients with hiatus hernia (5.3.1 (a)) (53/91) and nutcracker oesophagus (5.3.1 (b)) (22/93) stained with ABVNR and showing eosinophils in increased numbers in the latter. (Magnification X 150)

Eosinophil and mast cell counts in paraoesophageal LN (Table 5.3.1)

Diagnosis	Lab.No.	Eosinophils	Mast cells
Hiatus hernia	4891	37	43
"	5391	3	151
"	5191	9	10
"	5591	260	94
"	5291	12	18
"	4391	5	36
"	6391	20.8	19.2
"	7091	6.6	22.8
"	992	11.2	9.4
"	1392	10.6	7.2
"	1592	22.2	11.4
"	1692	5	2.8
"	6691	4.2	8.4
"	1992	11.2	9.4
DOS/vigorous achalasia	1591	308	42
"	6591	19.6	4.6
"	5691	69.3	53.3
"	5792	7.4	6.4
"	7092	2.2	3.2
"	8192	19	6.4
Achalasia	692	6.4	5.6
"	8692	14	6.4
"	5491	35	4
Nutcracker	792	23	12.8
"	393	28	5.2
"	2293	122.4	10.6

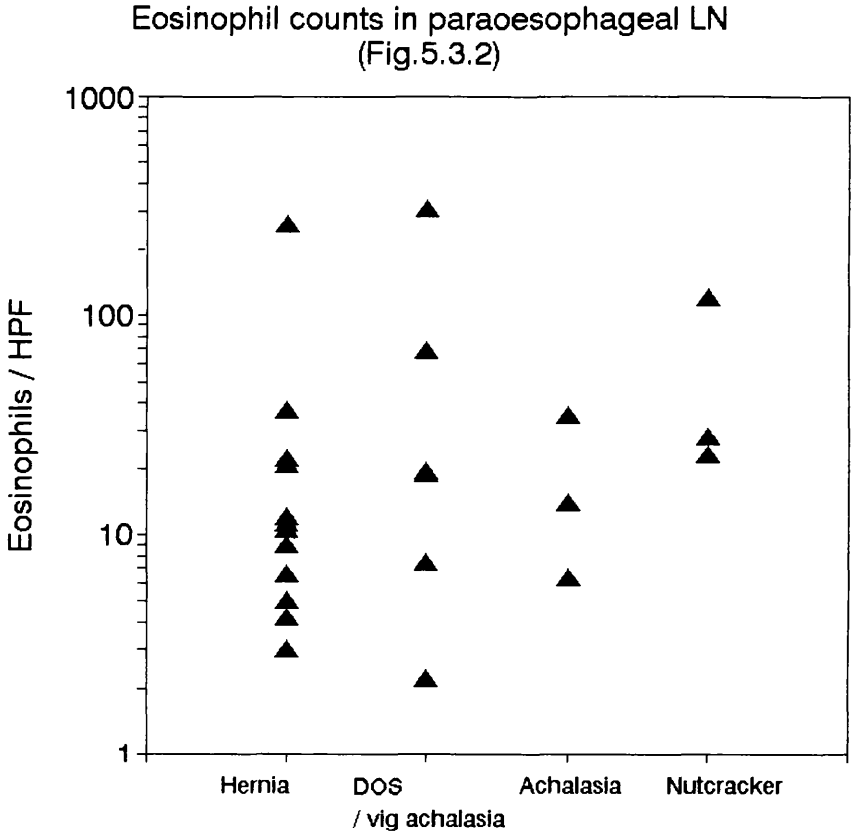


Figure 5.3.2 Graph of eosinophil counts in paraoesophageal lymph nodes in hiatus hernia and motility disorders.

Discussion

Eosinophil and mast cell counts have not been previously performed in paraoesophageal lymph nodes. In fact little is known of counts in any lymph node group. Horny and Horst (1986) studied the frequency distribution of eosinophils and mast cells in tumour-draining axillary and paracolic lymph nodes. Using Giemsa staining they found counts of 5 eosinophils / 0.55 sq.mm. (range 0 - 17) and 2 mast cells / 0.55 sq.mm. (range 1 - 31) in tumour-free paracolic lymph nodes. When the counts found in this thesis are expressed in counts/mm^2 (One HPF is approximately 0.2mm^2) they appear to be greater by a factor of ten.

Lymph nodes from patients with hiatus hernia may not be the best controls with which to compare counts in the motility disorders. It is known that patients with reflux disease have increased numbers of eosinophils in the oesophageal mucosa and this eosinophilic inflammation may be accounting for what is found in the draining lymph nodes. The numbers of eosinophils and mast cells in lymph nodes draining normal oesophagus would be an interesting fact to determine. However, in the specimens taken from transplant donors it proved very difficult to find any lymph nodes at all. Unfortunately lymph nodes were not specifically dissected out at the time of harvest. Future work should include specific lymph node sampling from paraoesophageal tissue.

The fact that a high eosinophil and mast cell count occurred in lymph nodes from one patient with hiatus hernia with very small numbers of similar cells in a sample of Auerbach's plexus suggests that lymph node counts may not accurately reflect cell counts in the Auerbach's plexus itself. However this could be a result of eosinophil infiltration of another layer of the oesophagus e.g. the mucosa.

Conclusions

1.Eosinophils and mast cells have been found in paraoesophageal lymph nodes in both reflux disease and motility disorders of the oesophagus.

2.Some of the patients with a florid eosinophil infiltrate in Auerbach's plexus had markedly increased numbers of eosinophils in the draining lymph nodes.

3.Counts of eosinophils and mast cells are needed from normal paraoesophageal lymph nodes in future studies.

6 IMMUNOHISTOCHEMICAL STUDIES

6.1 STAINING WITH THE MONOCLONAL ANTIBODY EG2 TO DEMONSTRATE ACTIVATED EOSINOPHILS

Introduction

Eosinophil granules contain several toxic basic proteins that are secreted into the tissues when the cells degranulate. Eosinophilic cationic protein (ECP) is one of these proteins and it has both cytotoxic and neurotoxic properties.

Tai et al. (1984a) have demonstrated that ECP exists in two antigenically distinct forms. They raised two different antibodies against ECP, namely EG1 and EG2. EG1 reacted with both the storage and secreted forms of ECP but EG2 only bound to ECP during secretion. Thus EG2 detects cells that have been activated for secretion and can also stain secreted extracellular ECP. In their study Tai et al demonstrated the potential value of these antibodies by demonstrating that activation and secretion of eosinophils was found in the skin in chronic urticaria.

Subsequently EG2 has been used to detect activated eosinophils in a wide variety of disorders. In asthma an increase in the numbers of activated eosinophils has been found in the airways of asthmatics when compared with controls (Bousquet et al. 1990, Bradley et al. 1991, Azzawi et al. 1990). Similarly, activated eosinophils have been demonstrated in other diseases associated with eosinophil infiltration. Thus EG2 positive cells have been demonstrated in Churg-Strauss syndrome, coeliac sprue , eosinophilic gastroenteritis and eosinophilic endomyocardial fibrosis (Tai et al. 1984b, Keshavarzian et al. 1985, Hallgren et al. 1989). In achalasia increased numbers of EG2-staining cells were found in Auerbach's plexus in a small group of patients (Tottrup et al. 1989).

In order to establish whether activated eosinophils have a role in motility disorders of the oesophagus the following study was undertaken.

Methods

Patients in whom quantitation of eosinophils and mast cells had been performed were the subjects of this study. Their clinical details are described in earlier

chapters. Some patients were excluded because insufficient tissue remained. The technique of preparation and staining has been described in the General Methods section. The immunoperoxidase technique was used. The tissue sections were not trypsinised nor incubated with blocking serum. However they were incubated in a solution of 1.5% hydrogen peroxidase in methanol for 15 minutes prior to staining to block endogenous peroxidase. The correct dilution of EG2 (EG2 Pharmacia, batch no.31233, code no.10-9196-01) was found by titration and best results were obtained when EG2 was used in a dilution of 1 in 200 and was incubated for 12 hours 4°C. The secondary antibody was a biotinylated goat anti-mouse serum. Appropriate positive and negative tissue controls were used and a duplicate of each tissue section was incubated with an antibody of the same class directed against another antigen (Monoclonal mouse anti-human carcinoembryonic antigen. DAKO-CEA,A5B7).

Results

Positive staining resulted in dark brown staining of eosinophils. No positive staining was seen in the controls. The results are shown in table 6.1.1. It was not feasible to formally quantify the cells as had been done with ABVNR. However the connective tissue around Auerbach's plexus was carefully examined under the microscope in each case. An estimate of the numbers of cells staining positively per high power field was made and the results detailed in Table 6.1.1. The area of one high powered field was calculated to be 0.2 mm^2

From this table it can be seen that there is reasonable agreement in most cases between the number of eosinophils seen using ABVNR for staining as using EG2. In 3 cases out of the 44 examined there were more eosinophils apparent with EG2 than ABVNR. In all cases with a florid eosinophil infiltrate with ABVNR a similar distribution of activated cells was seen using EG2 (see Fig. 6.1.1) This is illustrated graphically in Fig. 6.1.2. It was also apparent that in those cases where degranulation was seen on ABVNR staining, the extracellular material stained positively with EG2 suggesting it contained secreted ECP.

EG2 staining in Auerbach's plexus Table 6.1.1

Lab No	Diagnosis	Eosinophils ABVNR, cells/mm2	EG2 positive cells
2293	NUTCRACKER	1619	+++
238786	DOS	2.4	+
5691	DOS	5.3	0
608885	DOS	26.9	++
1591	DOS	32.7	++
158687	DOS	0	0
306286	DOS	1	0
6591	DOS	206.5	+++
433583	DOS	143	+++
883785	DOS	0.5	0
815990	VIG ACHALASIA	7.9	+
525390	VIG ACHALASIA	361	+++
715786	VIG ACHALASIA	29.6	0
125590	VIG ACHALASIA	322	+++
1192	VIG ACHALASIA	16.1	++
424981	ACHALASIA	3.4	+
61684	ACHALASIA	2	+
374181	ACHALASIA	1.7	0
492384	ACHALASIA		0
350581	ACHALASIA	0	0
297084	ACHALASIA	0	0
962680	ACHALASIA	0	+++
69686	ACHALASIA	32.5	+
492384	ACHALASIA	5.6	0
69983	ACHALASIA	3.5	++
91488	ACHALASIA	2.5	+++
3291	ACHALASIA	33.3	+
5491	ACHALASIA	0	0
50386	ACHALASIA	192.9	+++
32884	ACHALASIA	0	+
53784	ACHALASIA	0	+
335581	ACHALASIA	1.3	+
5391	HH	1	0
4391	HH	5.9	+
1692	HH	1.5	+
5591	HH	0	0
4791	HH	0.5	0
1492	HH	0	+
6391	HH	0	0
7091	HH	1.6	+
1592	HH	9	++
5191	HH	4	0
4991	CONTROL	0	0
4491	CONTROL	0	0

*KEY: 0 = 0 EG2-positive cells / HPF
+ = 1 - 5 EG2-positive cells/HPF
++ = 5 - 20 EG2-positive cells/HPF
+++ = >20 EG2-positive cells/HPF

Table 6.1.1 Activated eosinophils in Auerbach's plexus stained with the monoclonal antibody EG2.

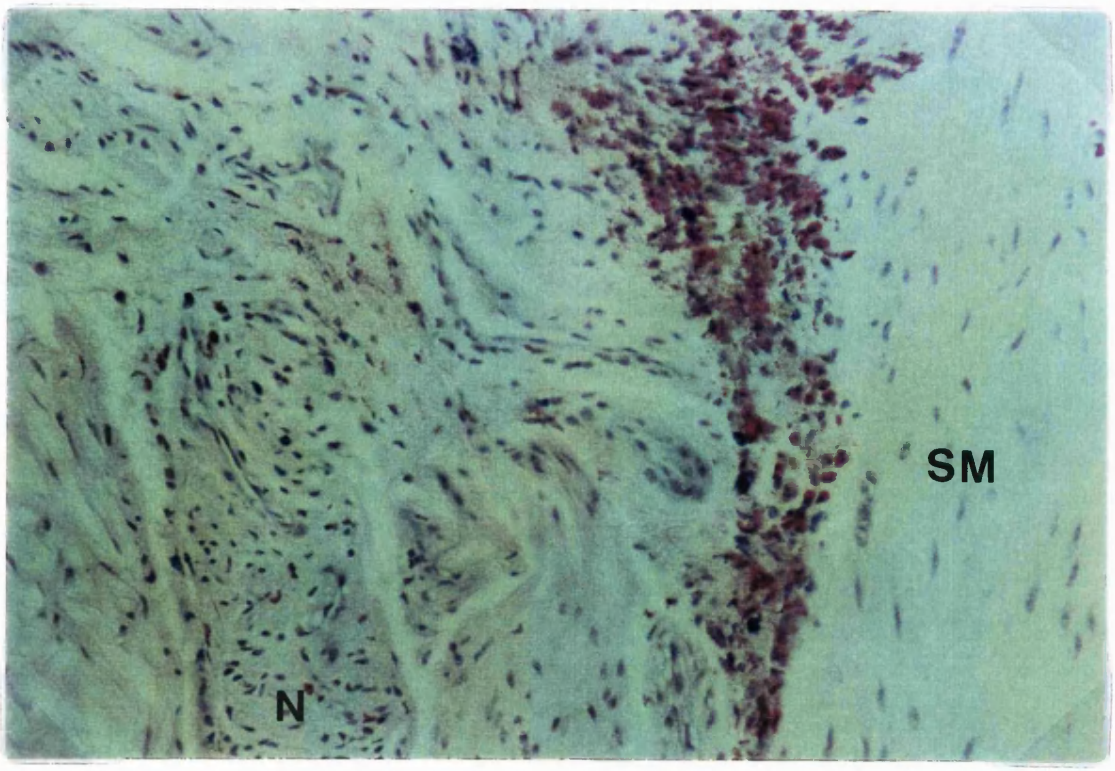


Figure 6.1.1 (a)

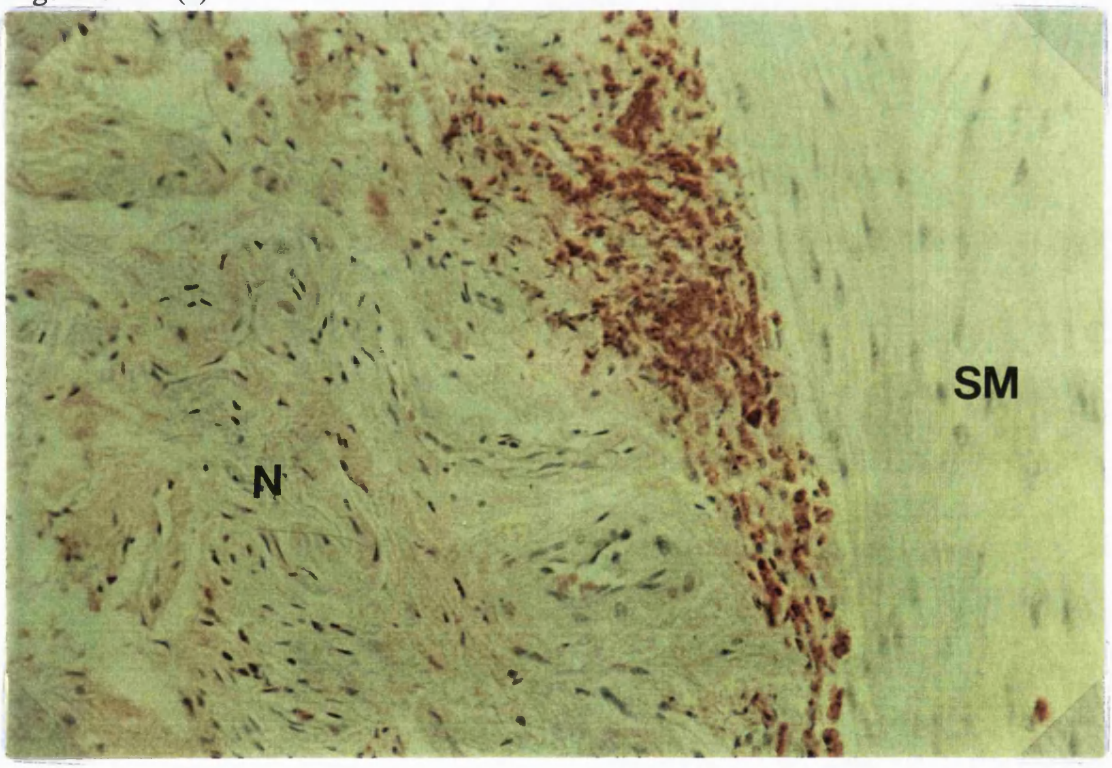


Figure 6.1.1 (b)

Figure 6.1.1 Myotomy biopsy from a patient with vigorous achalasia (5253/90) stained with ABVNR (Fig. 6.1.1 (a)) and using an immunoperoxidase technique to demonstrate EG2 positive cells. There is an infiltrate of positively-stained cells (brown) in a similar distribution to that seen when stained with ABVNR (Fig. 6.1.1.(b)). Magnification X250. N = nerve, SM = smooth muscle.

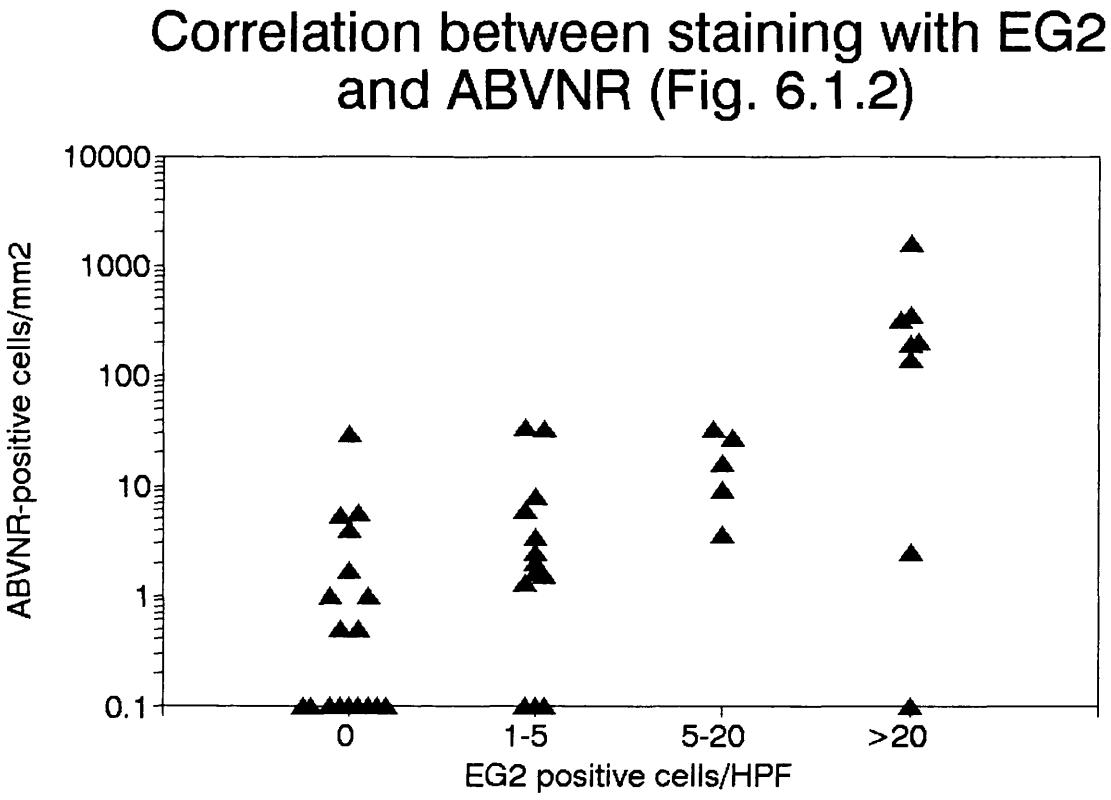


Figure 6.1.2

Graph comparing counts of eosinophils stained with ABVNR and with EG2.

Discussion

The stain ABVNR was used for the purposes of detecting and quantifying eosinophils in tissue sections both because of its ease of handling and also because of its distinct staining characteristics. Although it has been shown that the eosinophil is an unusual cell to find in normal oesophageal tissue, the finding of eosinophils in increased numbers in the connective tissue might just represent these cells passing through the tissues on their way to other sites of inflammation in the oesophagus (it is sometimes difficult to determine whether an eosinophil is intravascular or extravascular on light microscopy). Thus it was decided that immunohistochemical staining with EG2 would allow identification of cells that were activated and that were secreting ECP. Further evidence to suggest that the eosinophils were playing a pathogenic role would be if degranulation of eosinophils was demonstrated in the tissues concerned.

The results of our study have demonstrated that in those cases where eosinophils were present in large numbers or in which eosinophil degranulation had been demonstrated using the stain ABVNR, there were many cells staining positively for EG2. There was general agreement between the two stains but given they were quantified using different techniques statistical analysis was not performed.

Tottrup et al also used the stain EG2 to stain for activated eosinophils in achalasia and found them in variable numbers in Auerbach's plexus (Tottrup et al. 1989). Of the 17 cases of achalasia evaluated in this study there were a significant number of eosinophils staining with EG2 in AP in 3 cases. In one of these a similar number had been demonstrated using ABVNR. Quite why there were positive staining cells in the other cases is unclear. It was not always possible to stain adjacent sections with ABVNR and EG2 so that it is possible that this represents a sampling error within the tissue blocks used. Unlike Tottrup et al. we did not find many eosinophils in control tissue (from transplant donor and hiatus hernia biopsies) even when EG2 was used. Quite why in their experiments EG2 demonstrated eosinophils which were not obvious on histochemical staining is unclear. My experience suggests that although ABVNR made eosinophils very easy to identify in tissues, it was unusual not to be able to identify them using standard histochemical staining such as haematoxylin and eosin.

Conclusions

1. There was good correlation in most cases between positive staining with ABVNR and EG2.

2. In those cases with large numbers of eosinophils staining positively with ABVNR in Auerbach's plexus the same cells stained positively with EG2.

3. In those cases showing evidence of degranulation using ABVNR there was extracellular staining with EG2 suggesting the extracellular material contained ECP.

6.2 IGE STAINING CELLS IN AUERBACH'S PLEXUS IN MOTILITY DISORDERS

Introduction

The finding of increased numbers of eosinophils in the tissues in some patients with motility disorders is very similar to that found in asthma. In asthma the mast cell has been shown to be involved in the early asthmatic response and secretes mediators that are reponsible for the immediate bronchospastic response and also for attracting other cells such as eosinophils to the site of tissue inflammation. It has been shown that mast cells release their granule contents when specific cell-bound IgE is crosslinked by its appropriate antigen or by an anti-IgE immunoglobulin (Caulfield 1990). Thus mast cell bound IgE may have a role in triggering off the local tissue infiltration with eosinophils.

In a study of patients with proctitis, a condition known to be associated with increased numbers of eosinophils in the rectal mucosa, Heatley et al. (1975) stained tissue specifically for IgE using a fluoroscein conjugated anti-IgE antiserum on frozen tissue sections. They demonstrated that there very few IgE positive cells in the mucosa of normal controls but increased numbers of IgE positive cells were found in the mucosa in active proctitis. Serum IgE levels were in the normal range.

This study was undertaken to establish the number of IgE staining cells found in AP in patients with DOS and vigorous achalasia.

Methods

7 patients with DOS, and 2 cases with vigorous achalasia were compared with 8 patients with hiatus hernia acting as controls. 5 of the test cases had been shown to have eosinophil counts above 25 cells/mm^2 in a previous study. The clinical details of these patients have been detailed earlier. Procurement and preparation of tissue was as detailed in the General Methods section.

Initial attempts at staining with a monoclonal IgE antiserum (Monoclonal mouse anti-human IgE (DAKO-IgE) M 793) proved unsuccessful. Despite alteration in the dilution of the antibody and change in immunohistochemical technique, staining could not be reliably obtained on the routinely processed paraffin-

embedded oesophageal tissue. Therefore a polyclonal IgE antiserum (Rabbit anti-human IgE, specific for epsilon chains, code no. A094, lot no. 036, DAKO Ltd., High Wycombe, Bucks.) was used. Staining was found to be better using a Vectastain ABC-AP Kit using Vector Red as a substrate, than with an immunoperoxidase technique. Thus a Vector alkaline phosphatase kit was used with a red substrate. The tissue was treated with Trypsin 0.1% + Calcium chloride in Tris-buffered saline pH 7.6 at 37^0C for 10 minutes. After application of blocking serum for 20 minutes primary antibody was applied at a dilution of 1:400 and left for 15 hours at 4^0C. The rest of the methods used were as described in the General Methods section. Duplicates of each slide were similarly stained but the antibody was substituted with an unrelated antibody raised in the same species (Rabbit anti-Human Prostate Specific Antigen (PSA) DAKO A 562). Standard positive controls (nasal polyps) and negative controls were used.

The cells were quantified using the same technique as was used for the ABVNR counts described in the General Methods section.

Results

In the hiatus hernia controls staining of cells was infrequent. However in the test group bright red staining cells were seen scattered throughout the layer of intermuscular connective tissue that includes Auerbach's plexus. These cells were of a similar size to mast cells and had round/oval nuclei (see Fig. 6.2.1). Results of quantification are shown in Table 6.2.1 and are graphically represented in Figure 6.2.2. It can be seen that positive staining cells were very uncommon in the hiatus hernia group but were frequent in the DOS/Vigorous achalasia group. One patient in the hiatus hernia group had 27.3 IgE staining cells per square millimetre but it is interesting to note that she had a history of repeated hospital admissions with anaphylactic shock. The numbers of eosinophils found in sections taken from the same tissue blocks (not necessarily adjacent tissue sections) is also detailed in the table and graphically represented in Figure 6.2.3. Although there appeared to be a weak relationship between the level of IgE staining and ABVNR-staining cells in Auerbach's plexus this did not reach statistical significance.

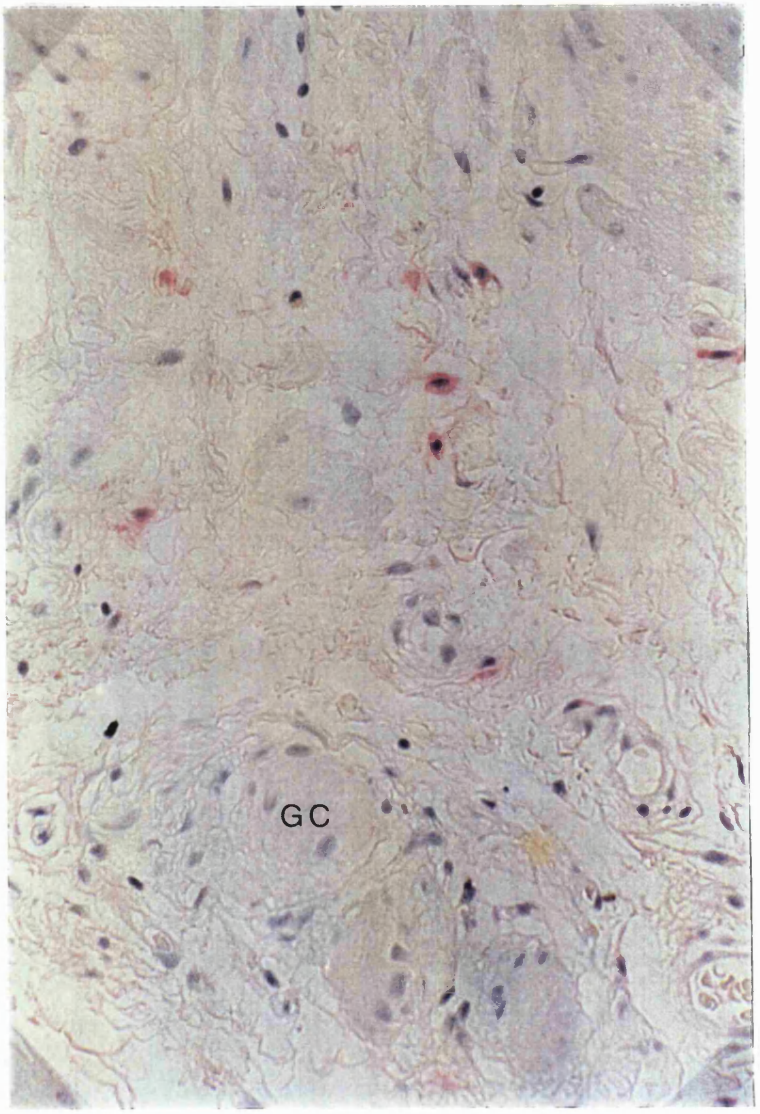


Figure 6.2.1
Myotomy biopsy from a patient with DOS (15/91) stained with polyclonal antibody to IgE. Using an ABC-AP staining technique, the positively stained cells are shown as bright red. Adjacent ganglion cells (GC) can be seen. Magnification X375

Counts of IgE positive cells around Auerbach's plexus Table 6.2.1

Laboratory No.	Diagnosis	AREA	COUNT	CELLS/MM	Eosinophils count/mm2
815990	VIG ACHALASIA	1.25	47	37.6	7.9
525390	VIG ACHALASIA	0.48	15	31.2	361
43912	HERNIA	0.45	1	2.2	5.9
1592	HERNIA	0.52	0	0	9
1692	HERNIA	0.34	0	0	1.5
47912	HERNIA	0.34	0	0	0.5
37912	HERNIA	0.45	0	0	1.9
63912	HERNIA	0.42	1	2.4	0.1
14921	HERNIA	0.31	9	27.3	0.1
70912	HERNIA	0.33	0	0	1.6
306286	DOS	0.4	16	40	1
5691	DOS	0.51	0	0	5.26
65913	DOS	0.76	20	26.3	206.5
608885	DOS	0.2	12	60	26.9
433583	DOS	0.27	23	85	143
158687	DOS	0.44	0	0	0.1
1591	DOS	1.23	100	81.3	32.7

Table 6.2.1
Results of quantitation of IgE positive cells in connective tissue in the region of Auerbach's plexus.

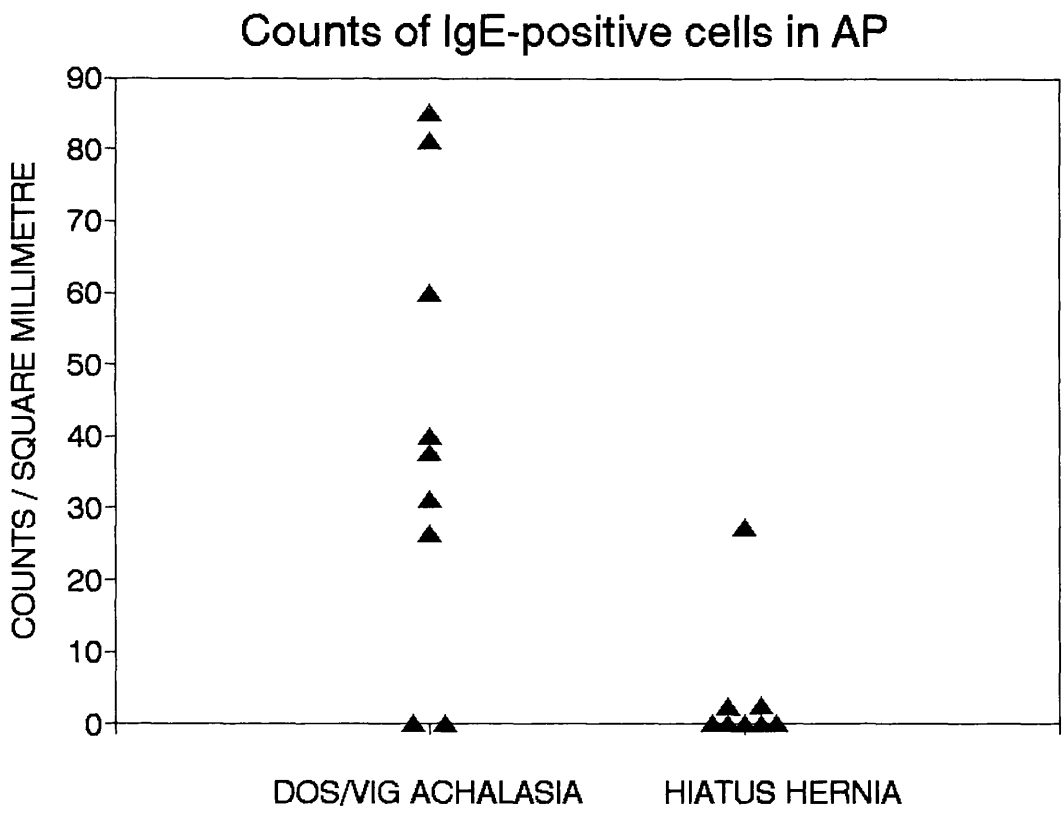


Figure 6.2.2
Graph comparing counts of IgE-positive cells in AP in DOS and vigorous achalasia with those in AP in hiatus hernia.

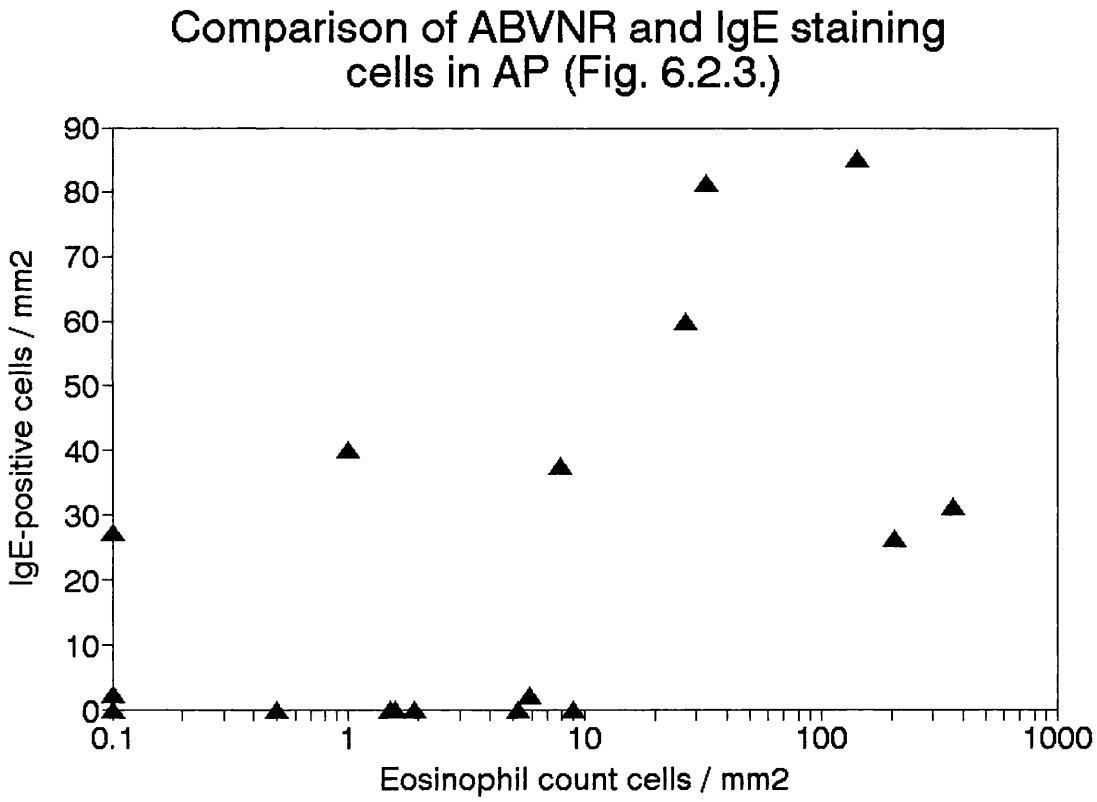


Figure 6.2.3
Graph of eosinophil counts in Auerbach's plexus in those cases stained with antibody to IgE.

Discussion

This study is the first to have looked at IgE staining cells in the vigorous contraction abnormalities DOS, nutcracker oesophagus and vigorous achalasia. There appears to be a significant increase in these cells in the Auerbach's plexus when compared to hiatus hernia controls. It is difficult to assess precisely which cells are staining positively but it is clear from their morphology that they are not eosinophils (the nucleus is not bilobed) and they are not lymphocytes (cells look much bigger than lymphocytes). Mast cells and basophils possess a high affinity receptor for IgE on their surface membrane. A low affinity receptor is present on about 30% of T cells and 1 - 2% of T cells and mononuclear phagocytes. Although there is an antibody directed against the low affinity receptor it does not work well on formalin-fixed, paraffin-embedded sections. Given the above facts, and the appearance of the cells, it is likely that the antibody is labelling IgE bound to tissue mast cells. Further study will be needed to confirm this.

It is interesting to note that these findings are in agreement with those found in a study of proctitis which appears to have an allergic aetiology (Heatley et al.1975). Although in their study Fluorescein-conjugated anti-IgE was used on frozen tissue, in this study routine formalin-fixed paraffin embedded tissue was used. Despite this good staining was obtained. In a later chapter serum levels of immunoglobulins will be examined across the range of oesophageal motility disorders.

The finding of these IgE-bearing cells in AP lends further support to the idea that an active inflammatory process, possibly allergic in origin, is responsible for the disordered motility observed in some cases of disturbed oesophageal motility.

Conclusions

Increased numbers of IgE-bearing cells are found in Auerbach's plexus in oesophageal biopsies from patients with DOS and vigorous achalasia, in whom an eosinophil infiltrate was often present.

6.3 HLA-DR EXPRESSION IN AUERBACH'S PLEXUS.

Introduction

Although the major histocompatibility complex (MHC) was first recognised for its role in graft rejection, the true physiological function of the complex is in the process of recognition of antigen by T cells. The MHC is made up of three classes of molecules: class I, class II and class III. Three separate class I loci (HLA-A, HLA-B and HLA-C) which encode the classical transplantation antigens have been found. Class II genes encoded in the HLA-D region are identical to the immune response genes which control mouse responses to different antigens. Class III genes, although located in the MHC, do not play a large part in the control of the immune response unlike their counterparts.

The majority of nucleated cells express MHC class I antigens whereas MHC class II antigen expression is more limited. MHC class II antigens are expressed predominantly on B cells and antigen presenting cells. Other cells can express MHC class II antigens and these include endothelial cells, lymphatics, activated T cells and epithelial cells in a number of tissues (Natali et al. 1981). In order for T cells to respond to an antigen (a T-dependent antigen) the antigen must be presented to the T cell along with either MHC class I or II molecules by an antigen presenting cell. CD8 (T suppressor/cytotoxic cells) only recognise antigen when it is presented in association with MHC class I molecules. CD4 T cells (T helper cells) recognise antigen only in association with MHC class II molecules. It is interesting to note that at sites of inflammation there can be increased MHC class II antigen expression. Thus resting macrophages express relatively low levels of MHC class II antigens until they are activated by cytokines such as interferon gamma released by T cells at the sites of inflammation.

MHC class II antigen can also be expressed on other cells under pathological conditions. It has been shown that gamma interferon released at the site of inflammation can induce the expression of MHC class II antigens on epithelial cells and neurones. There is evidence to suggest that certain autoimmune diseases are associated with abnormal MHC class II antigen expression. Aberrantly expressed HLA-DR has been found on thyroid epithelium in thyroiditis, pancreatic beta cells in diabetes mellitus, bile duct epithelium in primary biliary cirrhosis and gut epithelium in inflammatory bowel disease (Foulis et al. 1986).

It is thought that these cells may present endogenous antigens in association with MHC class II antigens and will elicit a T-cell mediated autoimmune response.

The purpose of this study was to examine the staining characteristics with HLA-DR in the connective tissue containing Auerbach's plexus in motility disorders of the oesophagus. The recent availability of a monoclonal antibody that will detect HLA-D region antigen on formalin-fixed, paraffin-embedded tissue sections allowed the distribution of HLA-DR antigen expression to be studied (Epenetos et al.1985) Abnormal expression of class II antigens on nerves and ganglion cells in Auerbach's plexus was specifically sought.

Methods

The pattern of HLA-DR expression was studied in two groups of biopsies. One group was from patients with DOS or vigorous achalasia and another from HH acted as controls. The antibody used was DAKO monoclonal mouse antihuman HLA-DR, CR3/43 (DAKO-HLA-DR, CR3/43) Code no.: M775, Lot no.: 049.

The immunohistochemical staining technique was as described in the General Methods section for immunoperoxidase staining. The following notes detail any departures from the standard technique.

Trypsin - no
Block endogenous peroxidase - 1.5% for 15 mins
Blocking serum - Vector 20 mins
Primary antibody - antihuman HLA-DR 1:50 45 mins at 20^0 C (or 15 hours at 4^0C)
Control antibody - NF 1:50
Secondary antibody -Biotinylated Vector 30 mins
ABC - Vector 30 mins
DAB - 5 mins

Duplicates of each slide were incubated with a primary antibody of the same class as the antihuman HLA-DR, but of an unrelated specificity. Antibody to neurofilament was used (DAKO monoclonal mouse anti-human neurofilament protein, DAKO-NF, 2F11 Code no.:M 762, Lot no: 119) as a control antibody. Positive controls were lymph nodes and also resected oesophagus where strong staining macrophages could be seen.

The number of positively stained cells in each high powered field was estimated from a sample of at least 5 high powered fields examined in the intermuscular connective tissue surrounding AP.

Results

In lymph nodes there were HLA-DR positive staining cells with the appearance of macrophages in the medulla. In postmortem oesophagus there were scattered cells in the epithelium and scattered large mononuclear cells with appearance of macrophages throughout the tissues. Endothelial cells stained positively.

The results of the staining for HLA-DR are shown in Table 6.3.1. There appeared to be quite a variation in the intensity of staining even within the same batch (20 - 30 slides were stained at one time). In those biopsies of AP that stained well, numerous positive staining mononuclear cells with the appearance of macrophages were found scattered in the connective tissue. Figure 6.3.1 shows extensive HLA-DR positive staining in the intermuscular connective tissue in a case of nutcracker oesophagus, (22/93). There was no evidence of any neural staining in AP with anti-human HLA-DR.

HLA-DR Staining in Auerbach's plexus Table 6.3.1

Lab No	Diagnosis	Eosinophils ABVNR, cells/mm2	HLA-DR staining cells
1591	DOS	32.7	++
608885	DOS	26.9	0
158687	DOS	0	+++
433583	DOS	143	++
5691	DOS	5.3	+
6591	DOS	206.5	+++
238786	DOS	2.4	++
306286	DOS	1	+
2293	Nutcracker	1619	+++
815990	Vigorous achalasia	7.9	+++
1192	Vigorous achalasia	16.1	++
525390	Vigorous achalasia	361	+
125590	Vigorous achalasia	322	++
903887	Resected achalasia		+++
432186	Resected achalasia		0
308484	Resected achalasia		0
378878	Resected achalasia		+++,all layers
856184	Resected achalasia		+++,all layers
892	Achalasia		++
3291	Achalasia	33.3	+
962680	Achalasia	0	++
97683	Achalasia		+
69983	Achalasia	3.5	+++
3791	Hiatus hernia	1.9	0
5391	Hiatus hernia	1	0
4391	Hiatus hernia	5.9	0
5591	Hiatus hernia	0	+
1991	Resected reflux		++
4991	Control		+
4491	Control		+
1491	Control		+++
491	Control		++

Key: 0 = no positive staining cells
+ = 1 - 5 positive cells/HPF
++ = 5 - 20 positive cells/HPF
+++ = > 20 positive cells/HPF

Table 6.3.1
HLA-DR staining in Auerbach's plexus.

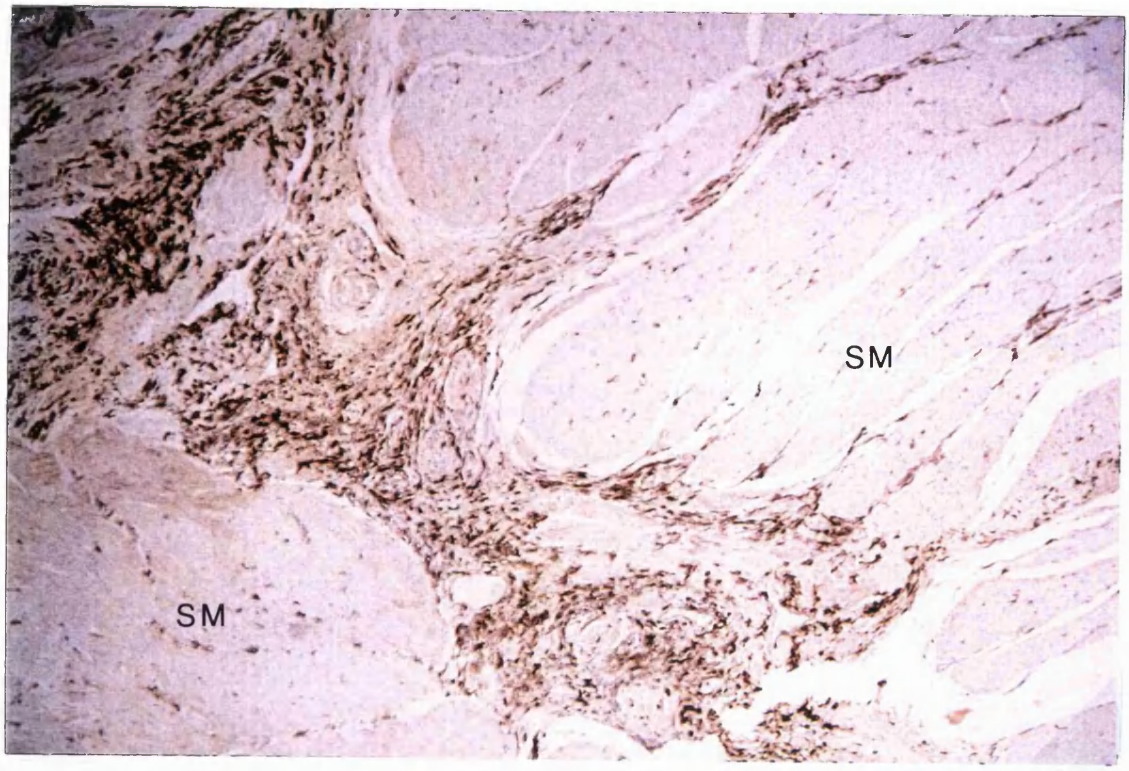


Figure 6.3.1 (a) Magnification X75

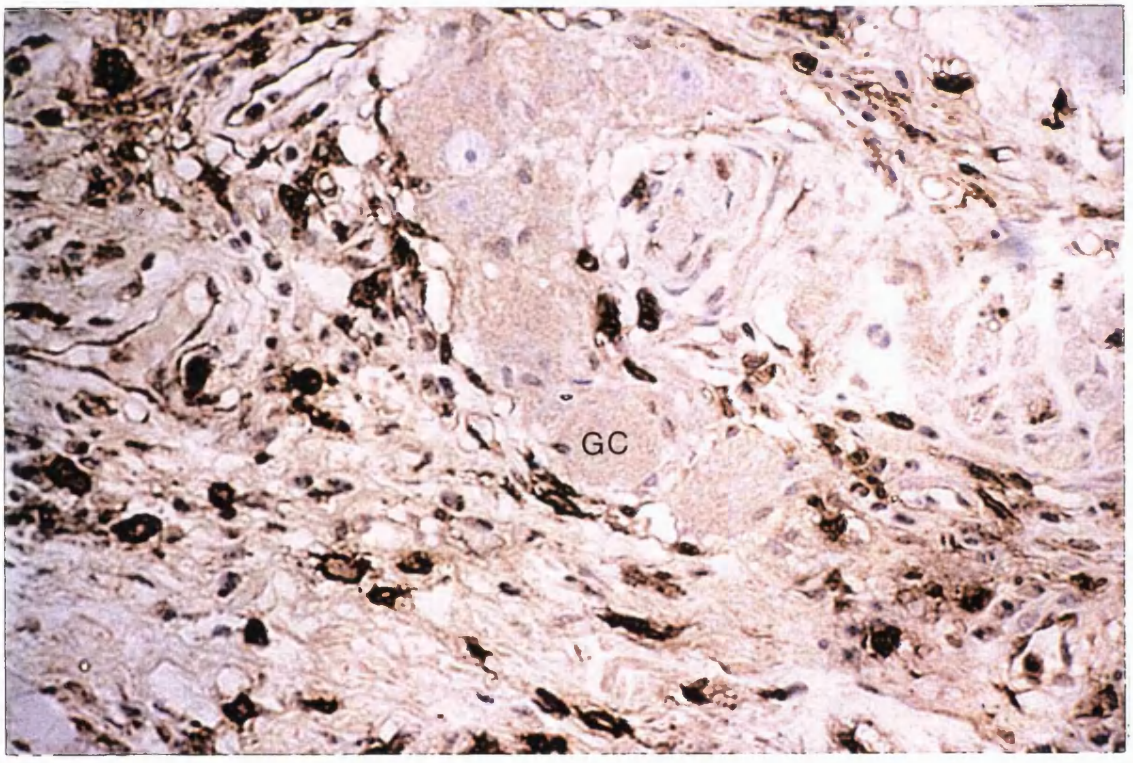


Figure 6.3.1 (b) Magnification X 375

Figure 6.3.1 Myotomy biopsy from patient with nutcracker oesophagus showing extensive staining for HLA-DR in the intermuscular connective tissue close to Auerbach's plexus. An immunoperoxidase staining technique was used and positive cells stained dark brown. GC = ganglion cell, SM = smooth muscle.

Discussion

HLA-DR antigen expression was studied across the whole range of motility disorders of the oesophagus, concentrating on DOS and vigorous achalasia. The staining obtained with the positive control slides was specific and appropriate. Thus the antibody stained macrophages and interdigitating reticulum cells in the medulla of the lymph nodes as expected.

In each antibody run some of the slides stained very well and others more faintly. As a result, it has not been possible to perform quantitative comparisons between slides with different staining intensity. Many sections were stained again in subsequent runs and their staining characteristics remained. Of the five resected achalasia specimens, all stained in the same batch of slides, 3 had strong staining and 2 had very weak staining. This is almost certainly accounted for by some difference in specimen preservation occurring between cases. It is well recognised that immunohistochemical staining is very sensitive to fixation techniques.

Very few cells in AP stained positively in the hiatus hernia or control specimens. In the DOS/vigorous achalasia group, positively-staining cells were seen throughout the intermuscular connective tissue. Similar cells were seen in close relationship to Auerbach's plexus. None of the positively staining cells had the morphological features of small lymphocytes or eosinophils.

It has been shown in previous chapters that in AP and surrounding connective tissue in motility disorders an inflammatory response can be found. The finding of positively staining cells, which are likely to be macrophages (the commonest form of antigen presenting cells), scattered throughout the tissues and in AP is not unexpected.

It has been suggested in achalasia, which has been shown to be associated with HLA-DQw1, that aberrant expression of MHC class II antigens on myenteric nerves might provide an autoimmune explanation for the disorder (Wang et al 1989). Positive staining for HLA-DR was not found on nerves or ganglion cells in any of the patients studied in this thesis.

Conclusions

1. In many cases of DOS/vigorous achalasia there are many HLA-DR positive cells in Auerbach's plexus. These cells are likely to be antigen-presenting cells involved in a local inflammatory response.

2. There was no evidence of abnormal HLA-DR expression on nerves or ganglion cells in the plexus.

6.4 T AND B CELLS IN AUERBACH'S PLEXUS IN MOTILITY DISORDERS OF THE OESOPHAGUS.

Introduction

An inflammatory response in Auerbach's plexus has been described in many studies of both DOS and achalasia. As described earlier, this contains eosinophils in some cases but in others a mononuclear cell infiltrate has been described (Cassella et al.1964, Nicks et al.1968, Adams et al.1976). A mononuclear cell infiltrate is not a universal finding - occurring in less than 50% of cases of both DOS and Achalasia (Adams et al.1976).

Although these mononuclear cells have the appearance of lymphocytes, it has been suggested that the infiltrate may represent proliferation of satellite cells (ganglion-supporting cells)(Lendrum 1937, Cross 1952, Adams et al.1976). In a recent study of 42 resected specimens of achalasia inflammation was found in AP in all cases - typically a mixture of lymphocytes and eosinophils (Goldblum et al.1994). Although immunohistochemical staining of T and B cells was performed on mucosal cells it was not stated in their report whether these cells in AP were B cells or T cells. In no study to date have the small round cells in AP from myotomy biopsies been stained specifically for lymphocyte markers so that the controversy is still unresolved.

This study was undertaken to assess the frequency of mononuclear cell infiltration in Auerbach's plexus in the motility disorders and to stain specifically for T and B lymphocytes in those sections with an infiltrate.

Methods

Myotomy biopsies from patients with motility disorders of the oesophagus were stained initially with H&E and the numbers of inflammatory cells present in AP assessed for each biopsy. In those biopsies in which an infiltrate of small round cells was found, together with a selection of cases without, further sections were stained for T cells using CD3 antibody and B cells using L26 antibody.

The sections were stained using the immunoperoxidase technique described in the General Methods section. Basic data for each staining procedure are detailed below.

Staining for L26 positive cells.

Dako monoclonal mouse anti-human B cell, CD20
(DAKO-CD20, L26) code no.: M 755, Lot no.: 091
Trypsin - no
Block endogenous peroxidase - 1% hydrogen peroxide in methanol for 15 mins
Blocking serum - Vector 20 minsPrimary antibody - L26 1:100 30 mins at 200C (or 12 hours at 40C)
Control antibody - EMA 1:100 Monoclonal mouse antihuman epithelial membrane antigen (EMA) E29 Code No. M 613 DAKO
Secondary antibody - Vector biotinylated 30 mins
ABC - Vector 30 mins
DAB - 5 mins

Staining for CD3 positive cells

DAKO Rabbit anti-human T cell, CD3 (code no.: A 452, Lot no: 100)
Trypsin - 0.1% Trypsin + 0.1% CaCl2
Block endogenous peroxidase - 1.5% for 15 mins
Blocking serum - N Sheep serum 20 mins
Primary antibody - CD3 1:50 30 mins
Control antibody - PSA 1:50 Rabbit-antihuman prostate specific antigen (PSA) Code no. A 562, Lot no.079 DAKO.
Secondary antibody -Biotinylated 30 mins
ABC - DAKO 30 mins
DAB - 5 mins

Duplicates of each slide were stained with the test antibody and a control antibody of the same antibody type but irrelevant specificity. With each run sections of tonsil or lymph node were stained to confirm the specificity of the antibody.

The areas containing collections of small round cells were then examined and an estimate of the percentage of these cells staining positively with each antibody was made.

Results

The results are detailed in table 6.4.1. Collections of mononuclear cells were found in 5 of the 20 achalasia biopsies and in 3 of the biopsies from patients with nutcracker/DOS/vigorous achalasia. It was not possible to formally quantify the cells since they were not stained simultaneously (and therefore in some cases the percentages of T and B cells adds up to more than 100%).

In figure 6.4.1 and 6.4.2 Auerbach's plexus from a normal transplant donor is seen stained with H&E, L26 and CD3. Although not as prominent as the collections present in the motility disorders, CD3 positive cells were found amongst the cells surrounding ganglion cells in AP. In Figure 6.4.3 staining in the intermuscular connective tissue close to AP is seen. In most cases the cells stain positively for both CD3 and L26. CD3-positive cells appeared to make up a larger proportion of cells in AP than did L26-positive cells.

Mononuclear cells in Auerbach's plexus Table 6.4.1.

Lab No	Diagnosis	Number of mononuclear cells	CD3 % positive	L26 % positive
1048490	Nutcracker	few	60	0
2293	Nutcracker	few	60	50
5691	DOS	none	<10%	<10%
238786	DOS	few	<10%	0
433583	DOS	many	40	60
1591	DOS	few	70	30
6591	DOS	few	50	0
125590	Vigorous achalasia	none	40	0
815990	Vigorous achalasia	many	70	10
525390	Vigorous achalasia	few	<10%	0
773281	Vigorous achalasia	few	60	no tissue
1192	Vigorous achalasia	few	20	<10%
69983	Achalasia	many	70	50
91488	Achalasia	many	70	50
3291	Achalasia	many	60	<10
61684	Achalasia	many	60	0
116083	Achalasia	few	20	0
772690	Achalasia	few		none(few monos)
124882	Achalasia	few		<10
374181	Achalasia	few	50	0
1692	Hiatus hernia	few	80	0
4791	Hiatus hernia	none	0	0
3791	Hiatus hernia	none	0	0
4491	Control	few	100	0

Table 6.4.1

Staining of mononuclear cells in Auerbach's plexus. The number of mononuclear cells was first assessed using H&E staining. These cells were further stained with the monoclonal antibodies CD3 and L26.

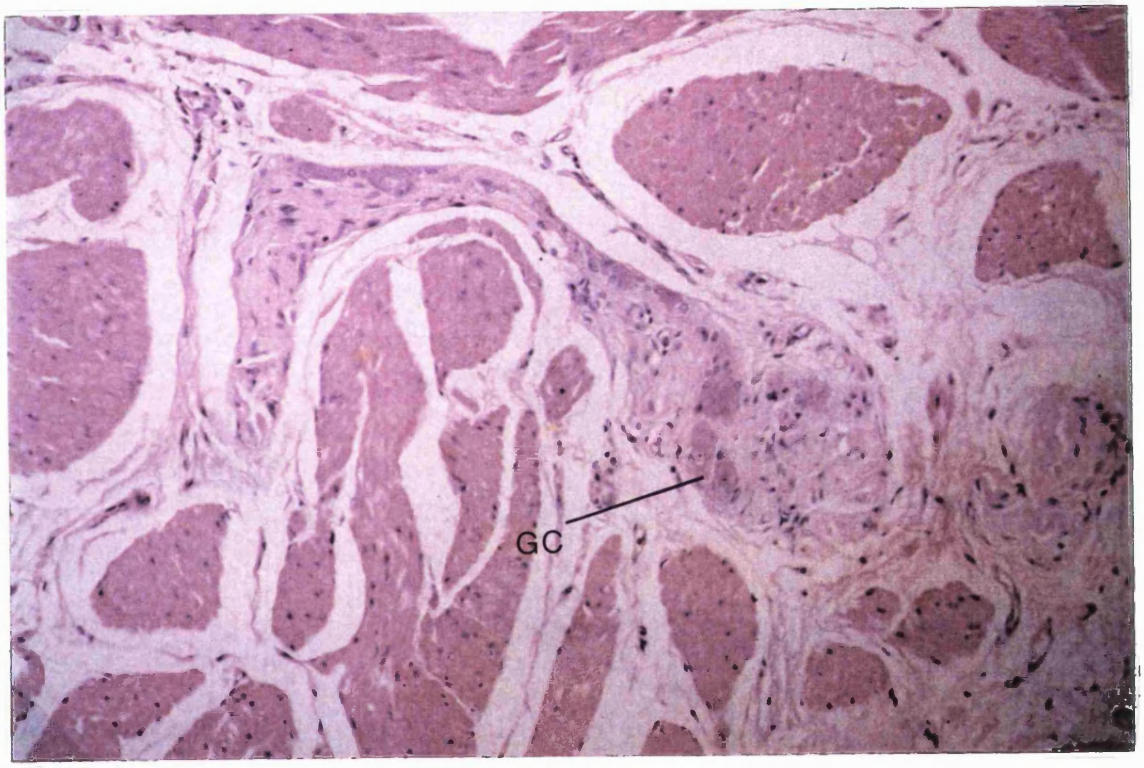


Figure 6.4.1
Section of normal oesophagus from transplant donor (44/91) stained with haematoxylin and eosin. Ganglion cells (GC) are clearly seen. Magnification X 150

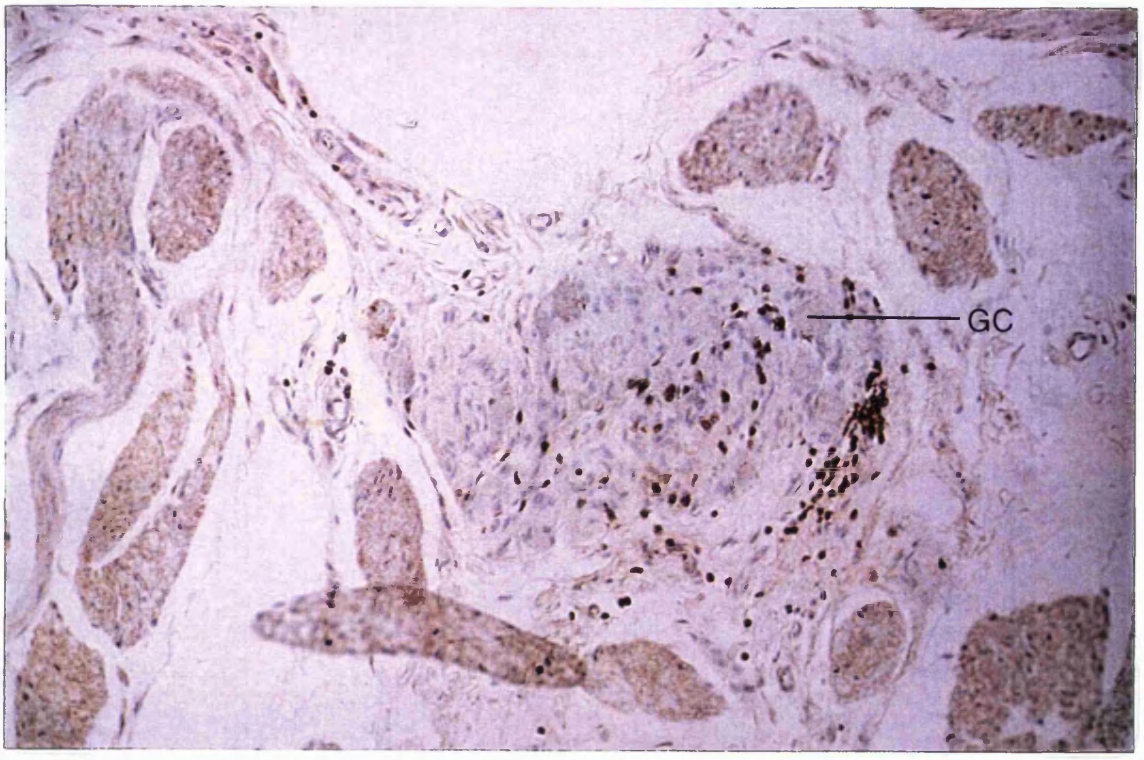


Figure 6.4.2 (a) Auerbach's plexus from a transplant donor oesophagus (44/91) stained with CD3 demonstrating positively staining cells (brown) in close relationship to ganglion cells (GC). Magnification X150.

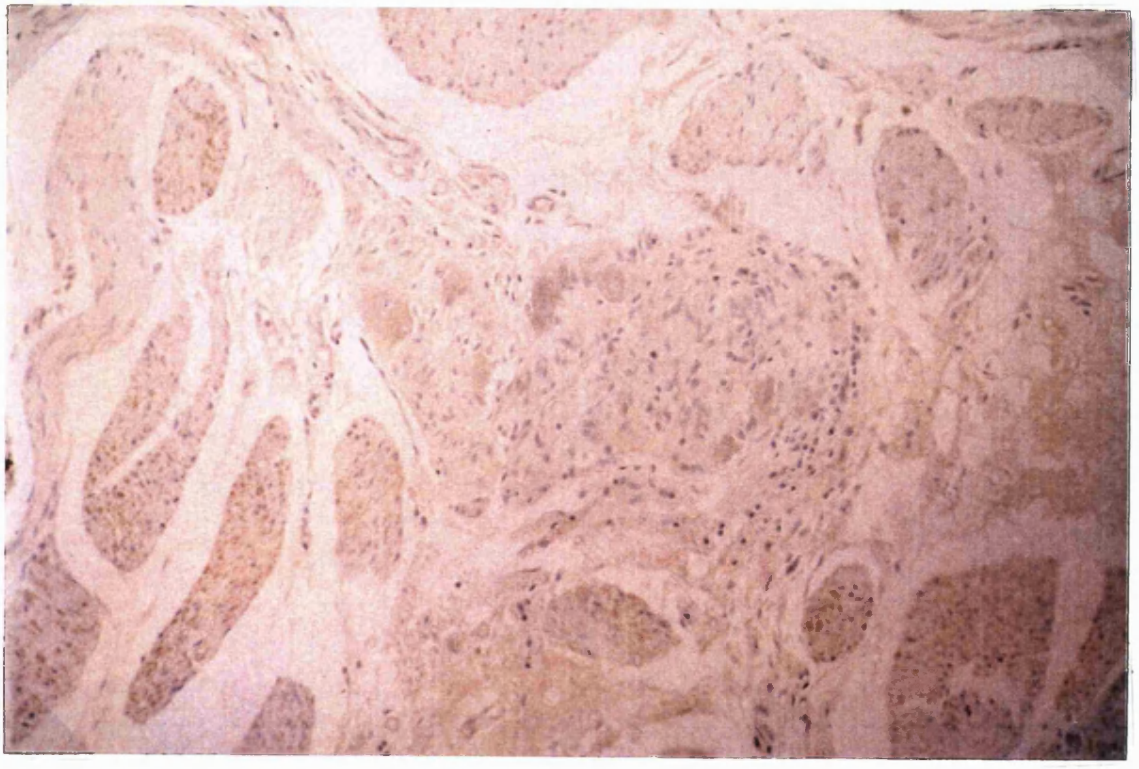


Figure 6.4.2 (b) Auerbach's plexus from the same patient as (a) stained with monoclonal antibody L26. Magnification X 150.

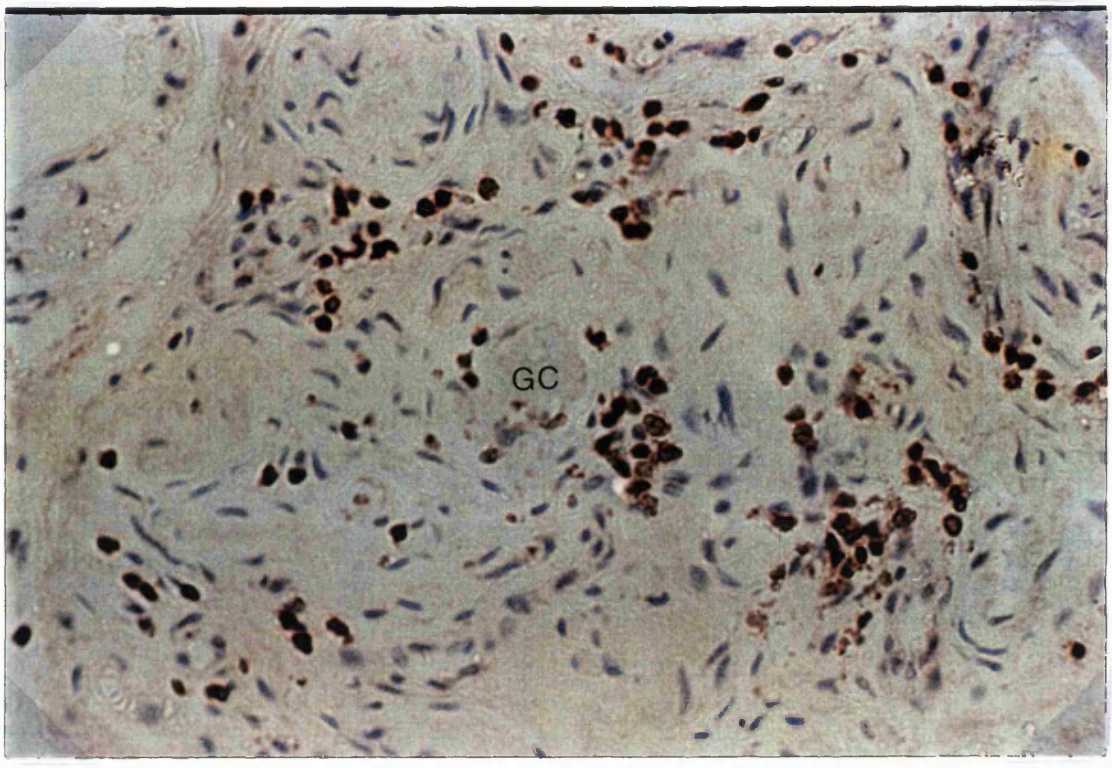


Figure 6.4.3 (a) Connective tissue close to a ganglion cell (GC) in patient with achalasia (669/83). Using monoclonal antibody CD3 (immunoperoxidase staining technique) positively staining cells are brown in colour. Magnification X 300

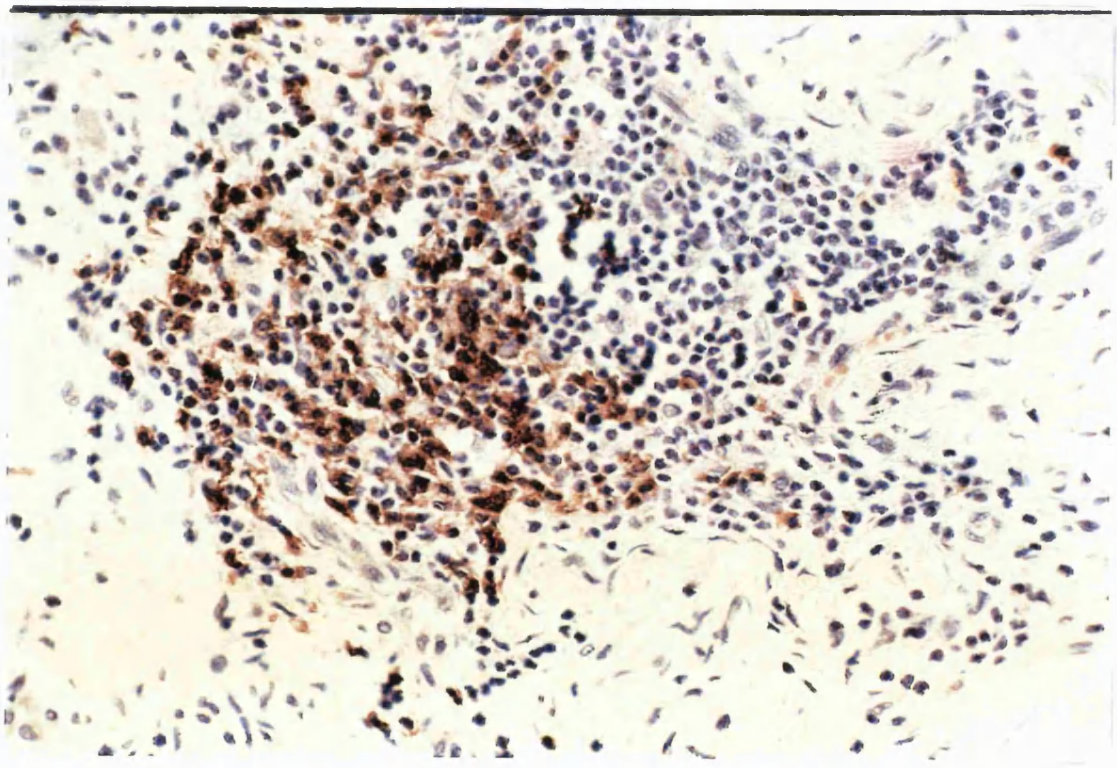


Figure 6.4.3 (b) Inflammatory focus in intermuscular connective tissue from patient with achalasia (699/83). Stained using immunoperoxidase technique with L26 antibody. Positively staining cells are dark brown. Magnification X250

Discussion

Since Lendrum first looked at the cells in AP of the oesophagus the finding of small round cells in association with the ganglion cells has been frequently described. In many instances they have been reported as lymphocytes but no specific staining tests have been performed. In the transplant control stained in this study CD3 positive cells were found in AP.

The finding of both T and B lymphocytes in AP in motility disorders is not entirely unexpected given the complementary roles these cells have in the immune response. Whether all the cells are lymphocytes cannot be established from our findings - simultaneous staining of B and T cells would be required to show this.

Previous studies in asthma have shown that there are increased numbers of T lymphocytes in the airways of patients with asthma compared with controls. These T cells were of both CD4+ and CD8+ phenotypes (Bradley 1991). There is evidence that these T cells are activated - expressing increased levels of the antigen CD25 (IL-2 receptor) (Azzawi 1990). It has been shown in cutaneous allergic reactions that there are increased numbers of CD4+ lymphocytes (Frew and Kay 1988).

In order to stain for specific subtypes of T cells and to assess their activation in the tissues requires the use of antibodies that work only on frozen tissue. The vast majority of the tissue used in this thesis was formalin-fixed, paraffin-embedded so it has not been possible to categorise these cells any further. Future studies are planned to look at T cell markers on stored frozen tissue from the more recent examples.

Conclusions

1. An infiltrate of small mononuclear cells occurs in 25% of cases of achalasia and similarly frequently in the vigorous group of contraction abnormalities.

2. This infiltrate appears to consist mainly of lymphocytes with CD3-positive cells (T cells) being the most common.

6.5 NEURAL STAINING IN MOTILITY DISORDERS OF THE OESOPHAGUS.

Introduction

In achalasia there is neural damage in Auerbach's plexus as shown by the absence or loss of ganglion cells on routine histochemical staining (Hurst and Rake 1930, Lendrum 1937, Cross 1952, Cassella 1964, Misiewicz 1969). Silver staining of thick sections has demonstrated decreased or absent argyrophil neurones in the plexus (Smith 1970a). Electron microscopy has shown normal numbers of large nerve bundles but decreased numbers of small nerve fibres in the plexus (Friesen 1983). Specific immunohistochemical staining has demonstrated decreased numbers of nerves with immunoreactivity to antibodies raised against VIP and substance P (Aggestrup 1983, Tottrup et al. 1989). VIP is known to relax smooth muscle and is believed to be responsible for smooth muscle relaxation of the lower oesophageal sphincter.

In DOS routine histochemical staining in most reports has suggested that ganglion cells are still present in AP. However in one paper it has been suggested these are reduced in number but quantitation was rather crude and this has not been subsequently confirmed (Adams et al. 1976). Electron microscopy has revealed conflicting results. Friesen et al. (1983) found no evidence of neural damage but Cassella et al. (1965) found diffuse damage of both myelinated and unmyelinated fibres.

Hirschsprung's disease, like achalasia, is associated with ganglion cell loss in AP in the colon. In a study on formalin-fixed, paraffin embedded tissue using antineurofilament antibodies, Kluck et al. (1984) demonstrated differences in staining characteristics between AP in Hirschprung's disease and controls. The nerve fibres stained in normal colon showed only partial staining of some of the axon bundles whereas the tissue from the Hirschsprung's patients showed heavy staining of hyperplastic axon bundles.

In this study it was decided to use a commercially available monoclonal antibody (antihuman neurofilament protein DAKO-NF) to stain oesophageal tissue. The antibody reacts with the 200 kD and the 70kD component of the three major polypeptide subunits generally present in neurofilaments. It does not recognise other intermediate filament proteins.

This study was undertaken to look primarily at DOS and vigorous achalasia to establish if neural damage could be demonstrated using an immunohistochemical technique.

Methods

Two groups of tissue samples were studied. In the first, resected specimens from patients with achalasia was compared with control oesophagus obtained postmortem and at transplant harvest. In the second group, biopsies from 11 patients with DOS or vigorous achalasia were compared with similar biopsies from 5 patients with hiatus hernia. Clinical details for these patients can be found in previous sections.

Tissue was sectioned, as described previously, and stained using the immunoperoxidase technique as described in the General Methods section. Specific details of the staining technique are listed below.

Primary antibody:
DAKO monoclonal mouse anti-human neurofilament protein
(DAKO-NF, 2F11) Code no.: M 762, Lot no.: 119
Trypsin - no
Block endogenous peroxidase - 1.5% for 15 mins
Blocking serum - Vector blocking serum 20 mins
Primary antibody - NF 1:200 over 12 hours at 40C
Control antibody - HLA-DR
Secondary antibody -Biotinylated Vector 30 mins
ABC - Vector 30 mins
DAB - 5 mins

Once stained, the tissue was examined under the light microscope and the extent of neural staining established.

Results

The pattern of neural staining appeared to vary from specimen to specimen in the same staining run. There was variation in staining intensity that could not be altered by changing antibody dilution. In some cases the staining was quite marked with good delineation of the fibres (see Figure 6.5.1). In others the fibres were fragmented and less densely stained (see Figure 6.5.2). In others staining

was minimal even though neural fibres could be seen on H&E staining of the tissue section.

The pattern of staining in the patients studied is detailed in Table 6.5.1. As can be seen, no clear difference in staining emerged in comparing either achalasia and transplant controls or DOS / Vigorous achalasia and hiatus hernia.

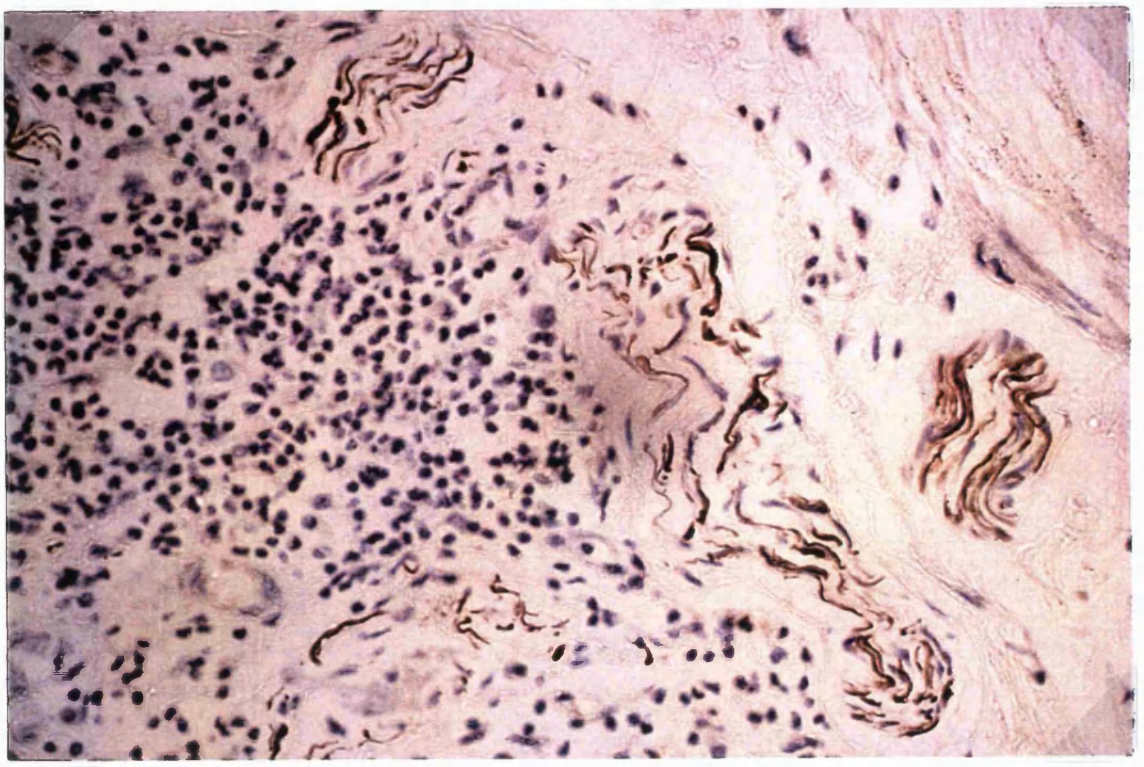


Figure 6.5.1 Myotomy biopsy from patient with achalasia (699/83) stained with antineurofilament antibody using and immunoperoxidase technique. The nerve fibres can be seem staining dark brown. Magnification X 150

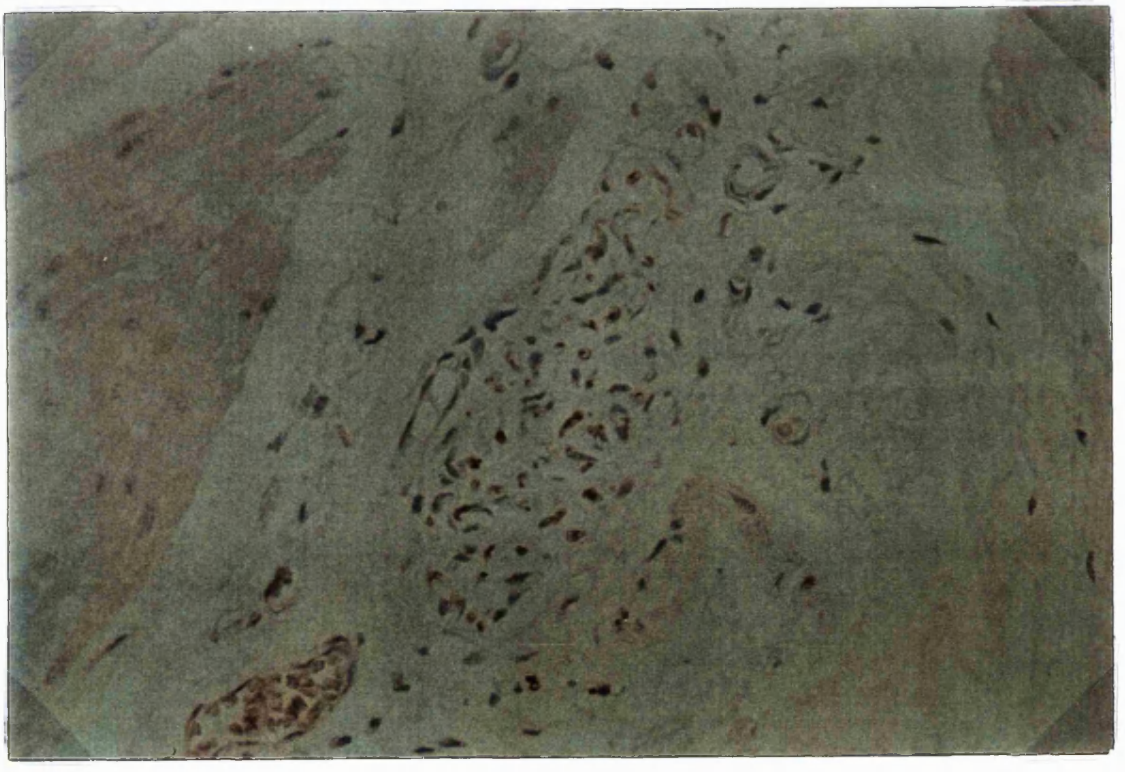


Figure 6.5.2 Myotomy biopsy from patient with vigorous achalasia (5253/90) stained with antineurofilament antibody using an immunoperoxidase technique. The nerve fibres can be seen staining dark brown. They appear somewhat fragmented. Magnification X 150

Neurofilament staining in motility disorders Table 6.5.1.

Specimen	Lab. Number	Neural staining			
		No	Fragmented	Weak	Strong
Control	49915	X			
	44915		X		
	1491	X			
	491			X	
Achalasia					
resected	856184		X		
	308484				X
	662583		X		
	432186	X			
Hernia	5191		X		
	43912			X	
	3791			X	
	5591				X
	5391				X
DOS	608885			X	
	6591				X
	5691				X
	433583		X		
	1591				X
	158687				X
vig achalasia	525390			X	
	815990			X	
	125590			X	
	1192				X
	306286			X	

Table 6.5.1

Patterns of neurofilament staining in motility disorders of the oesophagus

Discussion

The staining with antineurofilament protein was very specific and little background staining was observed using the above technique. As described in the results section above, the pattern of neural staining was very variable between the different diagnostic groups. Staining of neural elements was seen in all cases except 2 of the resected achalasic specimens. It is interesting to note that, in the study on Hirschsprung's disease, normal neurones appeared to stain weakly whilst the pathological neurones stained strongly and were judged to be hyperplastic (Kluck et al. 1984). The two different appearances described are very similar to those we have observed. It is possible that the differences observed may result from small differences in fixation or preservation technique since in all the prospective cases these techniques were standardised. Kluck's study was partly retrospective and partly prospective - it is conceivable that the majority of the pathological specimens were in the retrospective material and the majority of the control material in the prospective group. As far as the oesophagus is concerned, what is needed is a prospective study with standard fixation and preservation techniques of this antibody with a parallel study of cryopreserved tissue.

Study of three dimensional structures such as a nerve plexus can be difficult pathologically. It has been recommended that for proper assessment of Auerbach's plexus, sections in the plane of the plexus, not at right angles to the plexus (as in a transverse section) should be used (Smith 1970b). The small biopsies taken at myotomy are not ideally suited for this sort of study, but larger samples especially in DOS and vigorous achalasia are rarely acquired. Since many of the biopies used in our study were very small and tissue was at a premium, it was decided that only thin (3 micrometre) sections could be assessed.

In conclusion, it can be said that antineurofilament antibody can produce very good staining of neural elements in the oesophagus but rigorous prospective specimen fixation and preservation is needed before any conclusions can be drawn from such findings as above.

Conclusions

1. Antineurofilament antibody can produce good staining of neural elements in Auerbach's plexus.

2. Inconsistency of results may be a result of differences in fixation technique which could not be altered in what is largely a retrospective study.

6.6 SIMULTANEOUS STAINING OF NERVES, EOSINOPHILS AND MAST CELLS.

Introduction

When eosinophil infiltrates are identified using ABVNR, eosinophils are often seen close to nerves in Auerbach's plexus. However, many of these cells are scattered in the connective tissue in AP not obviously related to nerves and in the dense collections it is difficult to identify neural elements without specific staining. If these areas of dense infiltration could be shown to contain neural elements, this would provide support for the hypothesis that the nerves are being directly damaged by the eosinophil infiltrate.

There are no stains available which will stain for mast cells, eosinophils and nerves in the same tissue section. In order to do this it has been necessary to stain sequentially with the histochemical stain ABVNR and use immunohistochemical staining to demonstrate nerves. This staining combination has not been described before.

Methods

Sections known to contain significant numbers of eosinophils were used for this study. Various sequences of staining were tried. It was found that quenching endogenous peroxidase first, staining with ABVNR and then using immunohistochemistry produced a good result. Two different nerve stains were used - S100 and antineurofilament antibodies. Rabbit anti-cow S100 labels glial cells and ependymal cells in the brain and Schwann cells in the peripheral nervous system. Mouse anti-human neurofilament protein labels the 200kD and 70kD components of the three major polypeptide subunits present in neurofilaments. The staining techniques are detailed in full below.

1. Sections were taken through xylene and alcohols to water.
2. Endogenous peroxidase was blocked using 0.5% hydrogen peroxidase in methanol for 30 minutes.
3. Stain with astra blue solution for 30 minutes
4. Wash in running water.
5. Stain with vital new red for 30 minutes.
6. Wash in running water.

7. Incubate with primary antibody
   a) Rabbit anti-cow S-100 (DAKO , Z 311) dilution 1 in 400 incubated for 1 hour at 20^0C
or b) Mouse anti-human neurofilament protein (DAKO-NF, 2F11, code no.: M 762) dilution 1 in 50 incubated for 12 hours at 4^0C.
8. The rest of the steps are the same as described in the General Methods section for immunoperoxidase staining using biotinylated sheep anti-Rabbit IgG for the S100 run and using biotinylated horse anti-mouse IgG for the NF run.

Results

Using both techniques, eosinophils, mast cells and nerve elements stained in the same tissue section. Eosinophils stained red, mast cells blue and neural elements brown (see Fig. 6.6.1). In most areas of extremely dense eosinophil infiltration neural elements no neural staining could be seen. Elsewhere both eosinophils and mast cells could be seen in close proximity to nerves (see Fig. 6.6.2.). However in some places there was no obvious association between the stained cells and stained nerves.

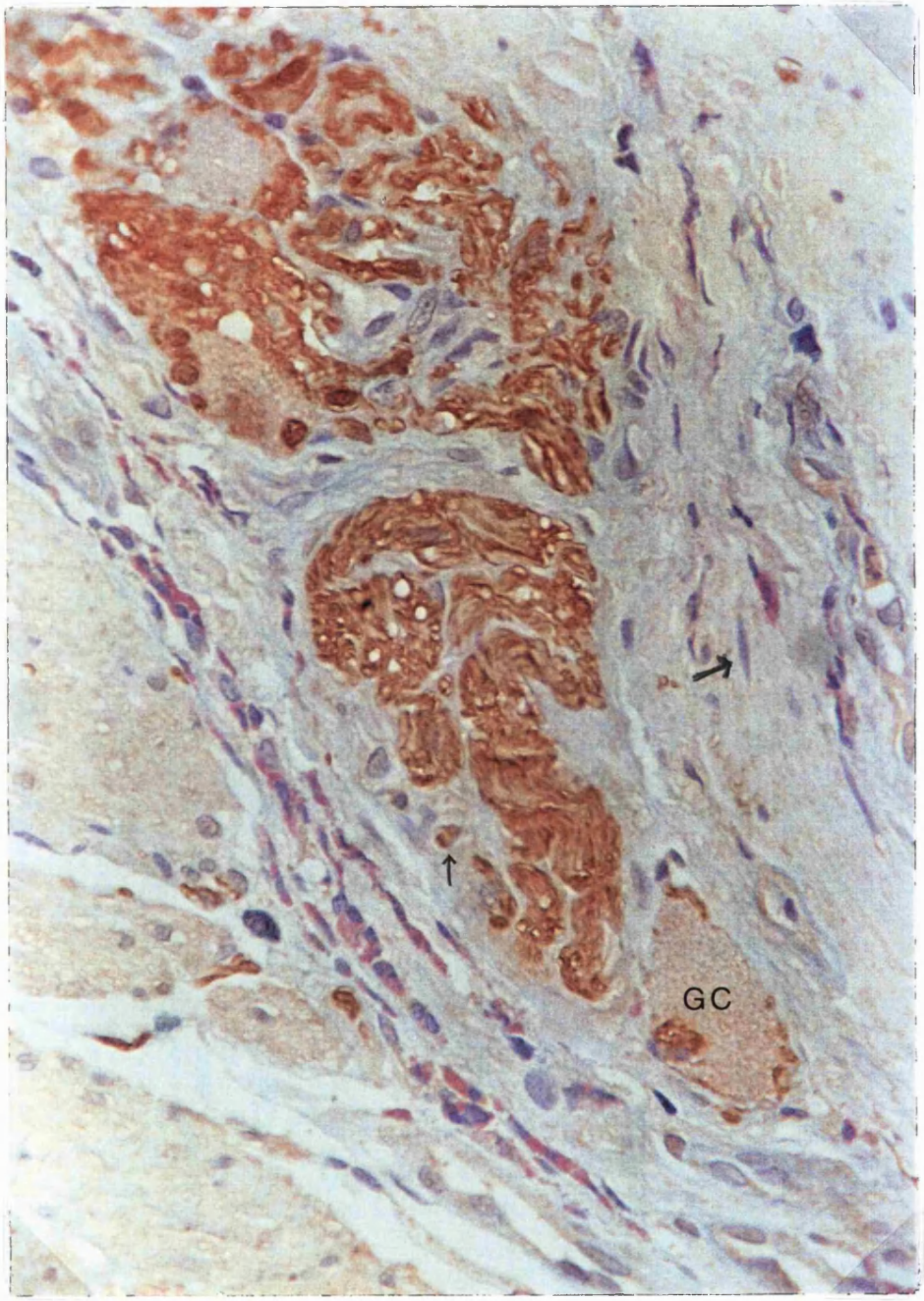


Figure 6.6.1
Myotomy biopsy from patient with DOS (65/91). Stained with a combination of ABVNR and S100. An immunoperoxidase technique was used to demonstrate S100 positive staining producing a brown colour. Eosinophils stained red (small arrow) and mast cells blue (large arrow). A ganglion cell is labelled GC. Magnification X 500.

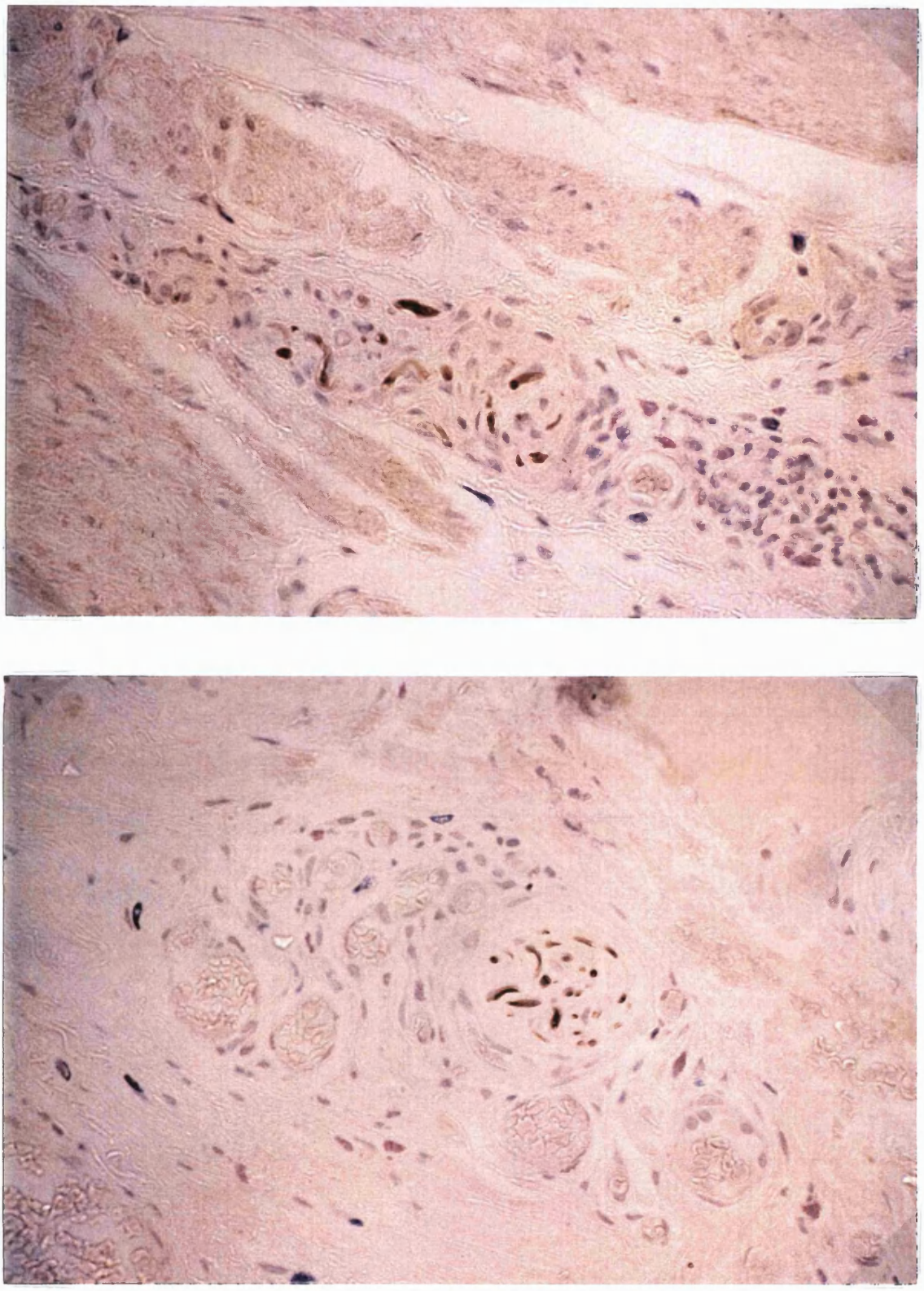


Figure 6.6.2 Myotomy biopsy from patient with DOS (4335/83) stained with a combination of ABVNR and antineurofilament antibody (using an immunoperoxidase technique). Eosinophils (red) and mast cells (blue) can be seen close to the nerve bundles (brown). Magnification X 150

Discussion

This is the first time this staining combination has been described. The technique worked well with good staining of both the eosinophils and mast cells and clear delineation of the stained nerve fibres.

There was frequent association between mast cells and nerves throughout the AP. There has been much recent interest in the relationship between mast cells and nerves (Goetzl et al. 1990). It has been shown in a study combining a specific immunohistochemical stain for nerves (PGP 9.5) and a histochemical stain for mast cells (alcian blue) that intestinal mucosal mast cells in the rat are in intimate contact with peptidergic nerves (Stead et al.1987). It has been shown that neuroenteric peptides can stimulate histamine release from mast cells (Shanahan et al.1985, Church et al.1989). Whether this nerve/mast cell association is significant in motility disorders of the oesophagus is unclear, but our findings lend support to the concept that nerves and mast cells can be intimately associated.

Although it was not possible to find damaged neural elements in areas of very dense eosinophil infiltration, eosinophils were often seen in close association with neural elements. The lack of neural staining in these dense collections of eosinophils does not mean that nerves are not the focus of this inflammation since it is likely when there is dense infiltration that the neural elements are too damaged to stain.

Conclusions

1.This new staining combination produced good staining of eosinophils, mast cells and nerves in the same tissue section.

2.Both eosinophils and mast cells were often found associated with positively stained nerves.

3.Staining of nerves was only occasionally observed in very dense collections of eosinophils and this could be explained if neural damage was so severe that the neural elements no longer stained.

7. STUDIES ON PERIPHERAL BLOOD

7.1 BLOOD EOSINOPHIL COUNTS IN OESOPHAGEAL DISEASE.

Introduction

In many diseases associated with tissue eosinophilia there is an associated peripheral blood eosinophilia. This has been shown in most parasitic diseases and also in some fungal infections (Spry 1988). The association between intrinsic asthma and peripheral blood eosinophilia has been known for some time but is not invariable. Peripheral blood eosinophil counts have been shown to correlate well with clinical severity of asthma and pulmonary function (Bousquet et al. 1990). It has also been shown that there is a link between the eosinophil count and bronchial hyperresponsiveness (Wardlaw and Kay 1987). Peripheral blood eosinophilia has also been described in allergic gastroenteritis, allergic proctitis and eosinophilic gastroenteritis (Goldman et al.1986). It is often associated in these disorders with raised IgE. The peripheral blood eosinophil count may not correlate well with the extent of tissue eosinophilia. Thus in the acute phase of development or of recovery of a tissue infiltrate of eosinophils there can be a disparity between bone marrow, circulating blood and tissue eosinophil levels (Nutman 1988). Thus, early on in the development of the tissue infiltrate in pulmonary eosinophilia, there may be few eosinophils in the tissues whilst the peripheral eosinophil count may be quite high. Conversely, at a later stage when the tissue infiltration is well established the peripheral blood eosinophil count may be normal.

Since an eosinophil infiltrate has been observed in the tissues in some cases of motility disorders of the oesophagus it was decided that blood eosinophil counts should be measured over the whole spectrum of motility disorders.

Methods

Eosinophil counts were obtained from normal controls and patients suffering with the following disorders: reflux disease, diffuse oesophageal spasm, vigorous achalasia and achalasia. The clinical data for all these patients are shown in Appendix 4 and summarised in table 7.1.1.

The blood was placed in standard EDTA bottles and the eosinophil count established using a standard laboratory method of detection using a Coulter counter (Coulter VCS). At this hospital eosinophil counts are not routinely performed on full blood count samples unless requested or unless the Coulter STKR counter identifies more than 0.7 X 10^9 cells/litre of similar size to eosinophils. In this situation or if an eosinophil count is specifically requested, then the more sensitive Coulter VCS counter is used to give an eosinophil count.

Some of the blood samples were obtained from patients undergoing surgery who were studied with the techniques described in the previous chapters. Those patients who were not undergoing surgery, or who had previously been operated on, were individually interviewed to assess the prevalence of other factors that might explain an eosinophilia if this was found subsequently. Thus a history of asthma, allergic skin disease, drug and other allergic responses was sought. Enquiry was made with respect to travel to tropical countries and associated illnesses.

Results

The results are detailed in Appendix 5 and shown graphically in Fig.7.1.1. They demonstrate that the eosinophil count in the vast majority of patients with motility disorders of the oesophagus was normal (<0.44 X 10^9/l.) Similarly, control patients and patients with hiatus hernia had counts within the normal range. One patient with hiatus hernia had a high eosinophil count. He was a West Indian man and he also had a significant elevation of his serum IgE.

Summary of clinical data for patients undergoing blood tests

Table 7.1.1 (Full details to be found in Appendix 4)

Diagnosis	No.patients	Sex: Male / Female	Mean age (yrs)	Mean LOSP (mmHg)	Mean % pH < 4
Achalasia	17	7/10	43	25.5	-
Vigorous achalasia	2	0/2	44	31.5	-
DOS	25	12/13	51	16.4	-
Nutcracker oesophagus	5	3/2	56	11	-
Hiatus hernia	19	15/4	46	4.6	18.5
Controls	12	4/8	56	-	-

Table 7.1.1

Clinical details for patients undergoing blood tests.

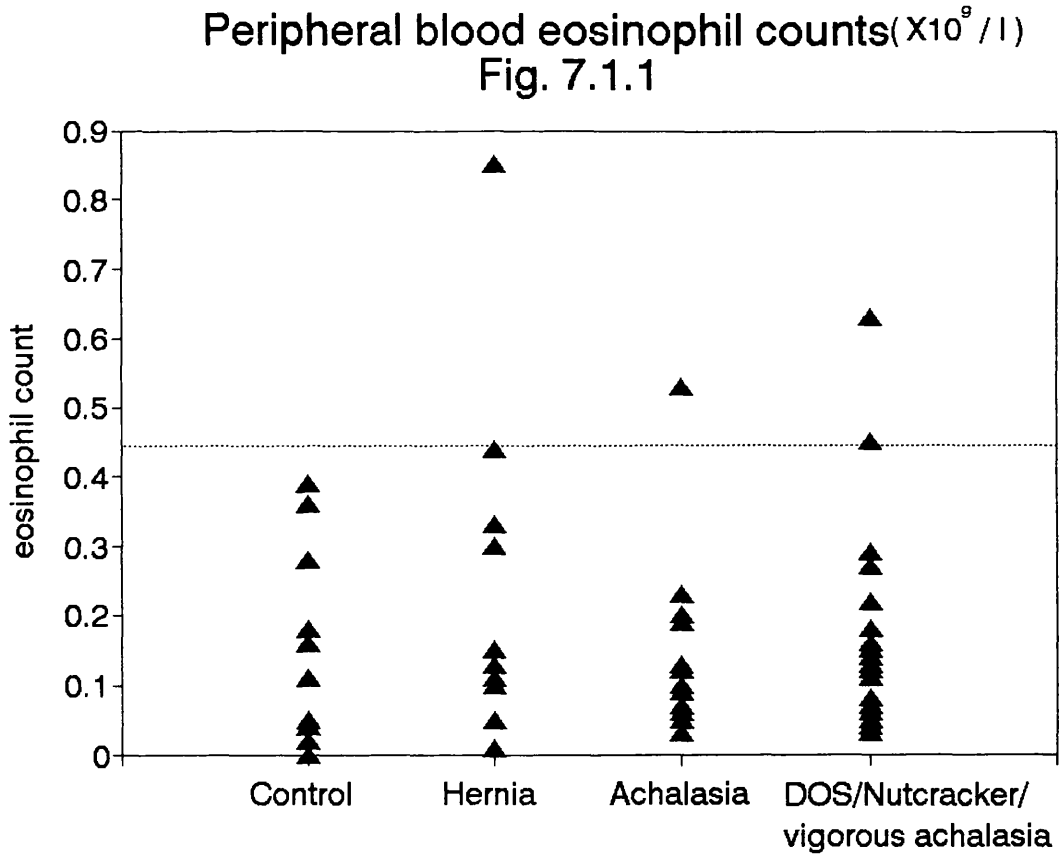


Figure 7.1.1

Peripheral blood eosinophil counts in controls and patients with reflux disease or motility disorders of the oesophagus.

Discussion

It is possible to stain eosinophils in the blood using specific stains and then to count them in special counting chambers. However this is time consuming and not readily applicable to clinical practice. Therefore the routine, automated counting method used at East Birmingham Hospital was used for this study.

The finding of a significantly elevated blood eosinophil count in the motility disorders would have been of great interest. If it had been restricted to those cases with associated AP eosinophilia then it would have been a useful marker of an eosinophil association for the disorder. On the other hand, if significantly elevated eosinophil counts were found in all the motility disorders it would have suggested that eosinophils were involved in the pathogenesis in all cases and that the lack of tissue eosinophils in AP might be a result of sampling at inappropriate times in the development of the disease or at a relatively uninvolved site in the oesophagus.

However neither of these were found. The vast majority of patients had eosinophil counts within the normal range for the laboratory. This does not mean that eosinophils are not involved in the pathogenesis of the disease. Although asthma is commonly associated with eosinophilia it is well established that eosinophil counts can be normal in asymptomatic asthmatics and some patients during severe attacks can demonstrate eosinopenia (Wardlaw and Kay 1987)

Conclusions

The peripheral blood eosinophil count is not pathologically raised in reflux disease, achalasia or the other motility disorders DOS, nutcracker oesophagus and vigorous achalasia.

7.2 SERUM IGE LEVELS IN OESOPHAGEAL DISEASE.

Introduction

In the normal individual the serum level of IgE is low relative to the levels of the other immunoglobulins. However in patients who are atopic or with parasitic disorders the levels of IgE can be markedly elevated in the blood. This IgE can be passively bound to mast cells in the tissues, and when exposed to the specific antigen, crosslinking of IgE molecules occurs and mast cell degranulation is initiated. This can then result in release of vasoactive mediators and chemotactic factors. Eosinophils are often found in the resultant infiltrate.

In asthma, serum IgE levels can be normal or raised. Although the tissue infiltrate of eosinophils is similar irrespective of the serum IgE, there are thought to be two separate mechanisms underlying the asthmatic tissue response. One is IgE dependent and the other is not.

In a previous chapter increased numbers of cells staining positively with an anti-IgE antibody have been found in DOS and vigorous achalasia. Large numbers of cells staining with anti-IgE antibody have also been found in a study of the rectal mucosa in patients with proctitis (Heatley et al.1975). However, serum immunoglobulin E levels were within the normal range for the majority of patients.

Serum IgE was measured in a variety of oesophageal disorders to assess how frequently serum IgE was elevated in the motility disorders of the oesophagus. In those cases with marked elevation of serum IgE a radioallergosorbent test (RAST) was performed on the serum looking for IgE antibodies directed against common food allergens.

Methods

The patients studied included 10 controls, 14 patients with reflux disease, 17 patients with achalasia and 28 patients with a diagnosis of DOS, vigorous achalasia or nutcracker oesophagus. Their clinical details can be found in Appendix 4. Informed consent was obtained from all the subjects.

Blood was obtained by peripheral venepuncture and placed in plain glass tubes. It was then spun at 25,000 rpm for 5 minutes and the serum pipetted off. It was

frozen to -70^{0}C and stored in a batch prior to analysis. Analysis of serum IgE using a standard radioimmunoassay (using the Pharmacia CAP system) and the specific RAST tests (Pharmacia) were performed in the Regional Immunology Dept., Birmingham Heartlands Hospital. RAST testing was performed on the following common food allergens: cod, egg, milk, wheat, nuts and soya.

Results

The results of the serum IgE estimations are detailed in Appendix 6 and displayed graphically in Figure 7.2.1. The upper limit of normal for the laboratory is 200 KU/l. It can be seen that all the control results are within the normal range. In 7 of 28 patients (25%) in the DOS/vigorous achalasia group, 2 of 16 in the achalasia group (12.5%), and 4 of 14 patients in the hernia group (28.5%) IgE levels were above the normal range. Statistical analysis using the Mann Whitney U test showed no difference in IgE levels between the vigorous contraction abnormalities and either control patients (P= 0.39) or patients with hiatus hernia (P= 0.88).

In table 7.2.1 those patients who had both biopsies and serum IgE estimations are detailed. Although there was no statistically significant correlation between blood IgE levels and AP IgE positive cells, it should be noted that 3 of the 5 cases of DOS\vigorous achalasia who had high serum IgE levels (195-535 KU/l) had increased numbers of both AP eosinophils and AP IgE-positive cells in AP. Two of the hernia patients wtih biopsies had raised IgE levels. In neither of these were AP eosinophils raised and in one, a patient with repeated attacks of anaphylaxis, there was a raised level of AP IgE-positive cells.

Table 7.2.2 details the results of the RAST testing on those samples obtained from patients with very high immunoglobulin E levels. In the patients studied, no clear evidence of an IgE response to these common food allergens emerged.

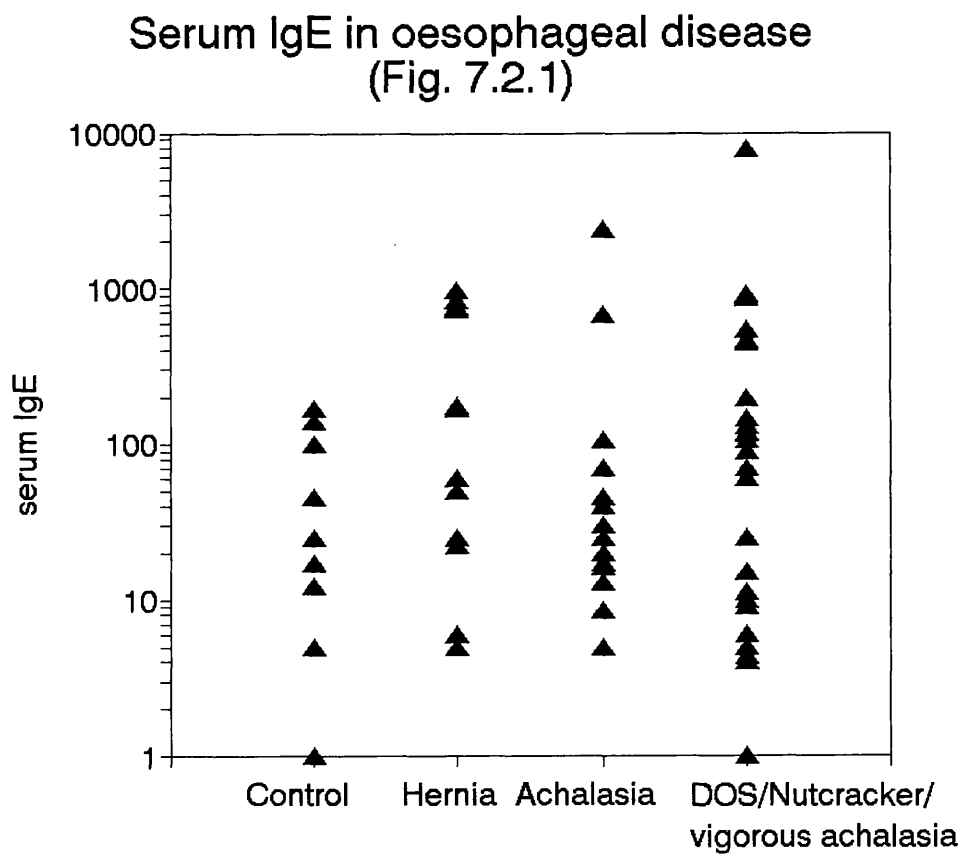


Figure 7.2.1
Serum IgE levels in controls and patients with reflux disease or motility disorders of the oesophagus.

Patients with biopsies of Auerbach's plexus and blood samples Table 7.2.1

		Blood tests		Biopsy cell counts / sq.mm.		
Lab. No.	Diagnosis	Eosinophil counts	Serum IgE	Eosinophil count	Mast cell count	IgE positive cells
5491	Achalasia	0.1	13	0	97.4	
158687	DOS	0.11		0	61	
238786	DOS		0	2.4	87	
433583	DOS	0.18	465	143	189	85
6591	DOS		535	206.5	38	26.3
1591	DOS	0.45	145	32.7	105.7	81.3
306286	DOS	0.22	195	1	68.3	40
5691	DOS	<0.44	10	5.3	63	0
5391	Hernia	0.44	5	1	135.6	
5191	Hernia	0.3	175	4	117	4
1692	Hernia	0.33	25	1.5	97	0
7091	Hernia		5	1.6	71.4	
4391	Hernia	<.44	22	6	69	2.2
1492	Hernia	0.01	970	0	71.2	27.3
1592	Hernis	0.85	760	9	90.7	
792	Nutcracker	0.12	70	0	79	
1192	Vig. Achalasia		10	16.1	112	

Table 7.2.1.
Results of blood tests and tissue cell counts in patients who had both blood tests and biopsies.

Table 7.2.2

Details of the results of RAST tests to common food allergens in those patients with elevated serum IgE levels.

RAST testing to common food allergens

Hospital No.	Diagnosis	IgE (KU/L)	Cod	Egg	Milk	Wheat	Nuts	Soya
291405	DOS	915	negative	negative	negative	negative	negative	negative
418918	DOS	465	negative	negative	negative	negative	negative	negative
385126	DOS	910	negative	weak positive	negative	negative	negative	negative
631814	DOS	455	negative	negative	negative	positive	negative	negative
508581	DOS	535	negative	negative	negative	negative	negative	negative

Discussion

These results show that an elevated IgE level is found in the blood in about 25% of cases of DOS\vigorous achalasia. Since blood tests were performed on only a small percentage of patients with myotomy biopsies, a statistical correlation between raised IgE and AP IgE and eosinophilia could not be obtained. However, in a small number of cases there appeared to be a link between all three. A large prospective study over a period of years would be needed to establish a firm association.

It is interesting to note that a raised IgE was found in 4 of the cases of hiatus hernia. Two of these were cases of Barrett's oesophagus, one was a man brought up in the West Indies and the fourth a lady with repeated episodes of anaphylactic shock. It may be that there is an eosinophilia in the mucosa of those cases of Barrett's oesophagus (as demonstrated in Chapter 4) and that this is associated with elevated IgE levels in the serum.

There have been no similar studies of IgE levels in motility disorders of the oesophagus. In a study looking for immunological abnormality in achalasia, Robertson et al. measured serum levels of IgG, IgA and IgM and found no significant difference to controls (Robertson et al. 1991). However, no attempt was made to study IgE levels. In a study of patients with eosinophilia, Takenaka et al. (1975) found that the eosinophilia was not necessarily accompanied by a raised IgE. Since peripheral eosinophil counts in the group we studied were nearly always normal, no attempt was made in this study to correlate eosinophil counts and IgE levels. It is of interest to note that in eosinophilic gastroenteritis total serum IgE levels are often elevated (Zora et al.1984).

The RAST tests performed on a small group with grossly raised IgE levels failed to reveal significant food allergy to common food allergens. It was decided to just look at this small group first as a pilot study and if positive results were obtained to look at all the patients subsequently (all the serum is stored at -70^0C).

This study has thus demonstrated that a raised IgE level can occur in motility disorders of the oesophagus. However because of the small numbers of patients analysed it is not possible to say with certainty whether a raised serum IgE could be a marker of eosinophilic inflammation in the oesophagus in motility disorders of the oesophagus.

Conclusions

1. Raised serum IgE was found in 25% of cases of DOS/vigorous achalasia and in 12.5% of cases of achalasia.

2. There was no statistically significant correlation between elevated serum IgE and the presence of eosinophils or IgE-positive cells in Auerbach's plexus. Further studies are needed to establish this.

3. There was no evidence for food allergy either on history or, in those cases with grossly elevated IgE, on RAST testing to common food allergens.

7.3 ASSESSMENT OF SERUM ECP LEVELS IN PATIENTS WITH DOS.

Introduction

An eosinophil infiltrate has been found in Auerbach's plexus in motility disorders as described in previous chapters. There is no evidence of peripheral blood eosinophilia in these disorders. When activated, eosinophils have been shown to secrete their toxic granule proteins into the tissues and into body fluids. It has been shown that in the late asthmatic reaction both ECP and MBP can be found in bronchoalveolar lavage fluid (DeMonchy et al. 1985, Wardlaw et al. 1988). Similarly raised levels of ECP and EPX have been found in the blood of patients exhibiting a late asthmatic response. It has been shown that in response to an acute inhalation challenge asthmatics will have an early rise in serum ECP levels falling 3-4 hours after the challenge and then slowly rising again (Dahl et al. 1978). These changes were found to be out of phase with changes in blood eosinophil counts.

This study was undertaken to see if patients with a diagnosis of DOS had an increased level of ECP in the serum. ECP can be detected by radioimmunoassay (Venge et al. 1977).

Methods

The assay was performed on 10 control patients and 14 patients with DOS (clinical details in Appendix 7). Peripheral venous blood was allowed to clot at room temperature for 60 minutes. Serum was then collected by centrifuging at 1350 g for 10 minutes. Samples were stored as a batch at -20^0C until they were analysed.

A double antibody radioimmunoassay was performed. In this technique ECP in the sample competes with a fixed amount of 125I-labelled ECP for the binding sites of specific antibodies. Bound and free ECP are separated by addition of a second antibody immunosorbent followed by centrifugation and decanting. The radioactivity in the pellet was then measured and is inversely proportional to the quantity of ECP in the sample.

Test procedure:

Standards, control sera and unknowns were assayed in duplicate.
50 microlitres of standard or test serum was placed in a test tube. 50 microlitres of ECP-125I was added. 50 microlitres of Anti-ECP antibody was then added and the mixture incubated for 3 hours at room temperature. Then 2 mls of decanting suspension was added and the tubes incubated for 1/2 hour at room temperature. The tubes were centrifuged at 1500g for 10 minutes. The tubes were decanted and left upside down on filter paper for 30 seconds. The radioactivity left in the pellets was determined using an Innotron Hydragamma 16 gamma counter (Oakfield instruments, Witney, Oxfordshire) and counting over 60 seconds. Background counts prior to analysis were acceptable (within the range 50 - 90). For each individual studied 2 samples were concurrently analysed and the average of the 2 results used for further analysis.

A graph was then plotted between the known concentrations of ECP in the standard sera and the counts obtained on the counter. The counts for the control sera and the unknown sera were then converted to concentrations of ECP using this graph. This was performed automatically by the computer in the counter after entering the data for the standard sera. The laboratory counter used for these estimations was able to produce results for the control and unknown sera both as absolute counts and as a concentration of ECP based on the results obtained with the known standards.

The materials were all supplied in a Pharmacia ECP RIA kit.(manufactured by Pharmacia Diagnostics AB S-571, 82 Uppsala, Sweden.)

Results

The calibration curve is shown in Figure 7.3.1. In Figure 7.3.2. the results for controls are compared with those of the patients with DOS. There was no significant difference in serum ECP between the two groups ($P > 0.05$ using Wilcoxon rank sum test).

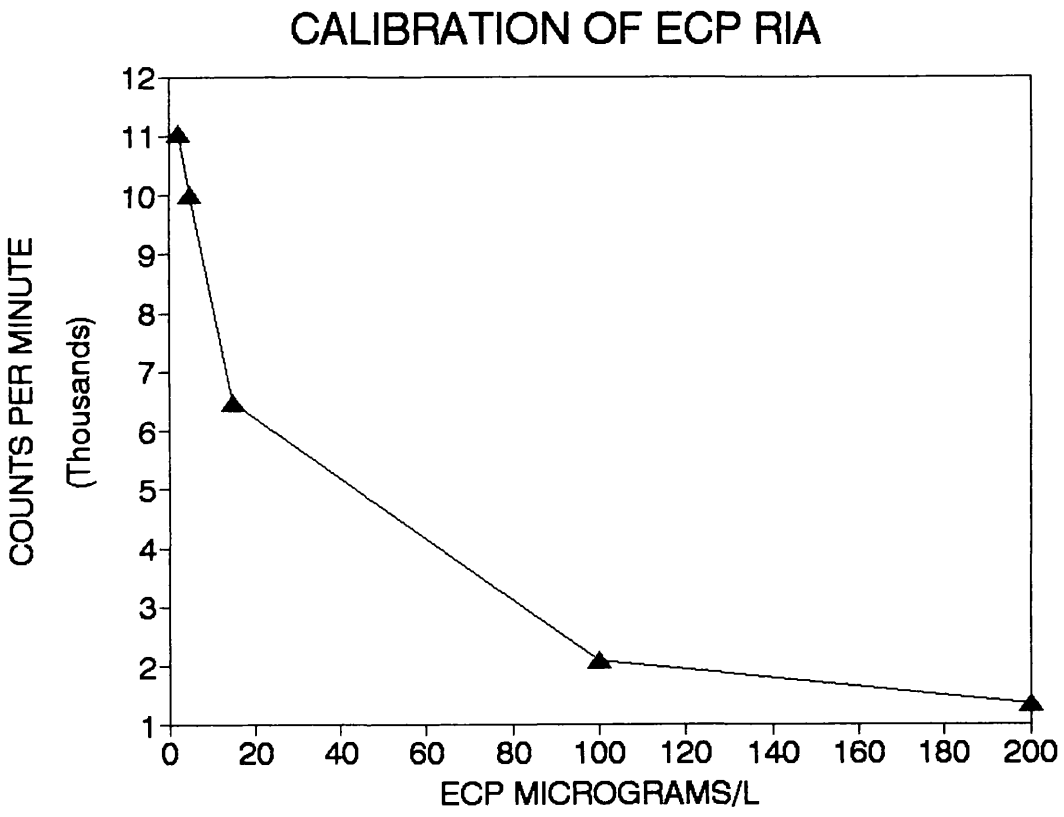


Figure 7.3.1
Calibration curve for serum ECP radioimmunoassay.

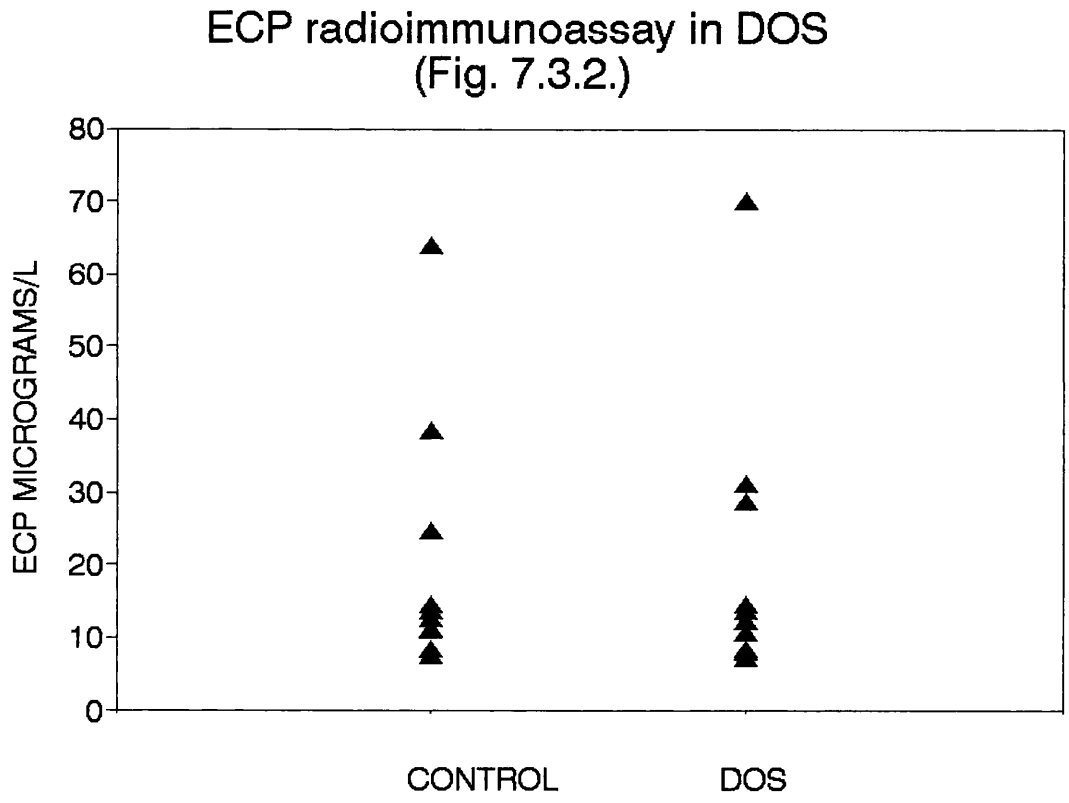


Figure 7.3.2
Comparison of results of serum ECP radioimmunoassay in controls and patients with DOS.

Discussion

Raised serum ECP has been demonstrated in the acute phase in some asthmatic patients. The level of serum ECP has correlated well with the severity of the illness and also the peripheral eosinophil count.

In the motility disorders of the oesophagus the peripheral eosinophil count is normal. Unlike asthma, although there are times when symptoms are more severe than others, the motility disorders do not have acute attacks comparable to an acute asthmatic attack. Thus one might not expect to find high levels of ECP as found in acute asthmatic attacks.

When an eosinophilic infiltrate in the myenteric plexus has been demonstrated, the infiltrate appears to be patchy even within one biopsy. Thus, even in the most florid case, the actual numbers of eosinophils actively secreting may be relatively low compared to the very large numbers of eosinophils that are likely to be activated in an acute asthmatic reaction. It is thus not too surprising, given the above facts, that the serum ECP is not grossly elevated in diffuse oesophageal spasm. This is unfortunate since it might have supplied a marker for disease activity allowing different drug treatments to be assessed.

Conclusions

Serum ECP levels were not elevated in serum samples from the patients with DOS examined in this study.

8. DISCUSSION

8.1 CLASSIFICATION OF MOTILITY DISORDERS

The motility disorders are currently classified on the basis of a combination of clinical, radiological and manometric features. Since many of these disorders can only be diagnosed with manometry then this investigation is crucial in the classification. Are the boundaries set by manometry merely arbitrary or are they valid in distinguishing the different disorders?

For a classification of disease to be meaningful then, after initial classification, there should either be a difference in subsequent behaviour or pathological features should differ in each diagnostic group. The diagnosis should be reproducible in the same patient on different occasions.

The diagnosis of achalasia on the basis of stationary manometry is the least contentious. The features of achalasia found on manometry are distinctive and are not found in the normal population. In a 24 hour manometric study all 7 patients with a diagnosis of achalasia on stationary manometry had the diagnosis confirmed on 24 hour manometry (Barham et al.1992). Secondly, the manometric diagnosis is supported by radiology which has distinct features in this disorder. If left untreated a progressive oesophageal dilatation occurs (McCord et al.1991). Perhaps one of the greatest supports to this diagnostic category is that there is a distinct pathological change detectable in the wall of the oesophagus. Absence or reduction in the number of ganglion cells in Auerbach's plexus is invariably found in achalasia (Lendrum 1937, Hurst and Rake 1930, Cassella et al.1964, Csendes et al.1985).

However the other diagnostic categories are less rigorously supported. The differences between DOS and nutcracker oesophagus, as far as the clinical features are concerned, are minimal. Pain and dysphagia are both common symptoms which occur to a greater or lesser degree in individual patients.

The boundaries between normality and the possession of a diagnosis of nutcracker or DOS are arbitrary. The classical definition of nutcracker oesophagus by stationary manometry is that the patient exhibits essentially normal peristalsis but the strength of the contractions are outside 2 standard deviations of the normal and this is associated with pain (Castell 1987). Similarly in DOS the main

diagnostic feature of simultaneous contractions can occur in normal individuals as can many of the other features. It is only the manometric boundaries that separate these conditions from normality. It should be remembered that stationary manometry is performed under artificial conditions, with the patient starved, lying on a couch for a short period of time. It may not be appropriate to assess a disorder of swallowing in such an artificial manner.

It is interesting to note that recent experience with 24 hour manometry has suggested that these 2 conditions may not in fact be separate since it has been shown that individuals can exhibit features of both conditions when studied with stationary and 24 hour manometry (Barham 1992, Eypasch 1990). This is in keeping with reports of transition from nutcracker oesophagus to DOS using stationary manometry (Anggiansah 1990, Narducci 1985). It has been suggested that these disorders form a spectrum of 'spastic disorders of the oesophagus' (Castell 1987).

Neither is the separation of these disorders justified by subsequent behaviour - neither disease progresses in a predictable separate fashion. Pathological examination using light microscopy, as shown in this study, does not reveal any abnormality in Auerbach's plexus in a large proportion of cases. Certainly there is no evidence of grossly different pathology. In both disorders muscular hypertrophy occurs but this is not an invariable feature at operation (Ellis FH et al.1964, Gillies et al.1967). If they are all suffering with the same disease then one would expect uniform findings at operation. Inflammation in Auerbach's plexus is only found in less than 50% of reported cases of DOS and although there are no reports of histological findings in nutcracker oesophagus, this study has suggested that the incidence of inflammation in nutcracker oesophagus is similar.

Vigorous achalasia is a disorder that shares features of DOS and classical achalasia. The presence of high amplitude nonperistaltic contractions is the main feature distinguishing vigorous achalasia from classical achalasia. This distinction has recently been questioned since there appears to be no difference between classical and vigorous achalasia with respect to symptoms or outcome (Todurczuk et al.1991). However in this study it has been demonstrated that an eosinophil infiltrate is commonly associated with this manometric picture.

In this thesis achalasia has been recognised as a separate entity and the whole spectrum of vigorous contraction abnormalities have been studied as a single group. Eosinophil infiltration was commonest amongst the vigorous contraction

abnormalities but was also found in 4 patients with achalasia, in 2 of which degranulation could be demonstrated. There are 2 possible explanations for this.

Firstly the disorders may form a spectrum of manifestations of a single disease. Progression from nutcracker oesophagus and DOS to achalasia, if and when it occurs, could involve a stage of eosinophil infiltration commonest in the intermediate disorder vigorous achalasia. The eosinophil infiltrate would destroy neurones in AP until the full blown picture of achalasia results. Thus one might expect to find an eosinophil infiltrate most commonly in the intermediate conditions and least commonly in the others. However reports of transition of DOS and nutcracker to achalasia are uncommon - certainly less commonly described than one would expect if the natural progression of disease was towards achalasia (Kramer et al. 1967, Vantrappen et al. 1979 Narducci et al. 1985, Anggiansah et al.1990). The fact that not all patients with DOS or nutcracker oesophagus have an eosinophil infiltrate begs the question what is causing their disturbance of motility.

An alternative hypothesis to explain the finding of an eosinophil infiltrate in different manometric categories is that there is a discrete disease process characterised by eosinophil infiltration in Auerbach's plexus which has different manometric manifestations at different stages of the disease. This would seem a far more acceptable explanation.

8.2 IS THERE JUSTIFICATION IN ATTACHING SIGNIFICANCE TO FINDING OF EOSINOPHILS IN AUERBACH'S PLEXUS?

The role of AP in control of normal oesophageal function has been examined in the Introduction to this thesis. There is further supporting evidence that damage to the AP nerves interferes with motility of the gut. As stated above, the loss of ganglion cells in achalasia has shown that neural damage is associated with abnormal oesophageal motor function in this disease. In Chagas' disease there is evidence of damage to the myenteric neurones causing disordered motility in all affected parts of the gastrointestinal tract. In chronic laxative abuse damage to myenteric plexus neurones occurs with resulting alteration in colonic motility (Smith 1970b). Thus there is much evidence to support the contention that neurological damage in Auerbach's plexus alters gut motility.

In this thesis it has been shown that in a group of patients with motility disorders of the oesophagus eosinophils are present in large numbers in AP with evidence

of degranulation and cellular activation. Similar infiltration was not seen in normal, neoplastic or any other benign oesophageal disease. This finding therefore appears to be unique to the motility disorders of the oesophagus.

In other diseases associated with eosinophilia such infiltrates are regarded as pathogenic. Thus in asthma there are increased numbers of eosinophils in the bronchi with evidence of activation (Bousquet et al.1990, Bradley et al.1991, Azzawi et al.1990). Eosinophils have also been shown to have a significant role in the pathogenesis of other gastrointestinal disorders such as coeliac disease, Crohn's disease and allergic gastroenteritis (Hallgren et al.1989, Zora et al.1984) In a recent study of intestinal biopsies from 7 patients with connective tissue diseases, perineural and intraneural inflammatory infiltrates were found in the submucosal plexus in all patients and in 2 patients with full thickness biopsies in Auerbach's plexus also (DeSchryver-Kecskemeti 1989). Mast cells and eosinophils were found in clusters in close proximity to the neural plexuses in all patients. There was electron microscopical evidence of activation. Although no manometry was performed, it is interesting to note that in 6 of the 7 patients barium swallow demonstrated aperistalsis or hypomotility. Whether the infiltrate involved the oesophagus is unknown but clearly is of great interest given the findings in this thesis.

This study has also demonstrated that eosinophils and mast cells can be found in close proximity to the nerve bundles and ganglion cells within AP. They thus are in a situation where their released granule constituents can affect these neural structures directly. As yet convincing pathological evidence of nerve damage in DOS and nutcracker oesophagus is lacking.

In conclusion, there is evidence to support the contention that the disordered motility seen in this group of patients is caused by the eosinophil infiltration in Auerbach's plexus.

8.3 HOW COULD THE PATHOGENIC ROLE OF EOSINOPHILS BE PROVEN?

To prove this a number of basic requirements would need to be satisfied:
1. Eosinophils should be present in AP in the motility disorders.
2. Eosinophils should not be present in AP in other diseases not associated with disordered motility.
3. Evidence of eosinophil activation and local secretion of granule contents

should be obtained.
4. A similar infiltrate in the AP in an animal model should produce a similar disease.
5. Interfering with eosinophil action or accummulation should result in clinical improvement.

To achieve all these requirements would be quite difficult. In this thesis data has been presented to support tenets 1, 2 and 3. With regard to requirement number 4, no satisfactory animal model exists of DOS, vigorous achalasia or achalasia. Megaoesophagus does occur in animals including dogs, cats and rats and it is possible to induce megaoesophagus by producing distal oesophageal obstruction or using neural poisons (Satchell 1990, Sons et al.1986) However these interventions did not result in inflammation in Auerbach's plexus. It would be extremely difficult to induce an eosinophil infiltrate in AP without introducing other variables (for example it might be possible to induce tissue eosinophilia using a parasitic infestation but then other effects of the parasitic infestation could not be discounted). It should be possible to investigate the effects of eosinophil and mast cell secretion products on the oesophagus by studying isolated preparations of the muscular wall in vitro as has been used to measure the effects of drugs (Adams et al.1961).

There are a number of potential ways in which eosinophil and mast cell function can be altered clinically (as suggested in tenet number 5). This will be discussed later in this chapter.

This thesis has produced evidence of an association between eosinophil infiltration in AP and disordered motility. The eosinophils have been shown to be activated and such findings have not been demonstrated in any of the many benign and malignant tissues examined. An animal model of the disease is needed for further study and drug treatment directed against the eosinophilia needs further investigation.

8.4 IF WE ACCEPT THAT EOSINOPHILS ARE PATHOGENIC IN SOME PATIENTS WITH DISORDERED OESOPHAGEAL MOTILITY A NUMBER OF QUESTIONS ARISE.

8.4.1 Is the process confined to Auerbach's plexus?

Eosinophilic gastroenteritis can affect the entire GI tract. As specified earlier, it can be predominantly mucosal, serosal or muscular in its distribution (Klein et al.1970). The fact that only in the muscular distribution does disordered muscle function occur, lends further support to the idea that AP is involved in the process.

In a study of 12 patients with high counts of intraepithelial eosinophils in oesophageal biopsies, Attwood demonstrated an associated motility disorder in 4 patients (Attwood et al.1993). Two had manometric features of DOS and 2 others the features of nutcracker oesophagus. 6 patients had concurrent gastric mucosal biopsies and in only one of these, who also had eosinophils in his jejunal mucosa, was there an eosinophil infiltrate in the gastric mucosa.

In this regard this study is lacking. Firstly no attempt was made to define the extent of the tissue eosinophilia in the muscle wall of the rest of the gastrointestinal tract. In practice this would be difficult to investigate. It would necessitate taking biopsies from the muscular wall of stomach, jejunum and colon. Stomach and jejunal biopsies would be difficult to obtain at thoracotomy and would have to be taken transhiatally. Secondly, biopsies were not taken from the mucosa from patients with motility disorders. This was felt to be a risky procedure just prior to a long myotomy since the very loose connective tissue in the submucosa means that at the time of long myotomy a potential full thickness perforation could result. For logistical reasons it was difficult to organise mucosal biopsies from these patients on a separate admission (many had had endoscopy prior to establishing the diagnosis).

8.4.2 Is this an allergic disorder and, if it is, what is the allergen?

Atopic individuals are patients who react to a foreign antigen (allergen) and commonly develop asthma, hay fever or eczema. They often have raised levels of IgE in the serum and can be shown on RAST testing to have IgE directed against

specific allergens. The factors that suggest we may be dealing with an allergic phenomenon are as follows. Firstly, eosinophils are common cells at sites of allergic inflammation. Secondly, a raised number of IgE positive cells was found in AP in a group of patients with DOS and vigorous achalasia. Thirdly, there was a raised serum IgE in some cases also. However there was little in the way of a positive history of allergy or atopic disease in these patients. There was no history of allergy to particular foods and even when performed on patients with grossly raised IgE no evidence of the presence of allergy to common foods could be identified using RAST testing.

On balance, the findings in this thesis are compatible with this being an allergic disorder although the allergen is as yet unknown.

8.4.3 If the eosinophil infiltrate is not a result of an allergic disorder what could be causing the infiltrate?

Eosinophil infiltrates occur in parasitic disorders and are often associated with a raised level of IgE in the serum. There was no history of parasitic disease in any of the patients with motility disorders. Parasitic disorders and infestation with worms is common in tropical countries but very few of the patients studied had travelled to tropical countries. Histological evidence of parasitic infestation was not found in any case we have studied. However no specific serological tests for parasite infestation nor examination of the stools for ova, cysts and parasites were performed in this study.

Mast cells can secrete factors responsible for eosinophil infiltration. In the early asthmatic response mast cell degranulation is an important feature. Whether the subsequent infiltrate of eosinophils is a direct result of secretion of chemotactic factors by mast cells or whether substances secreted by T cells are responsible is not clear. It is known that mast cells can be stimulated to secrete their granule contents by methods other than cross-linking of passively bound IgE. Thus neuropeptides such as substance P, VIP and somatostatin can cause mast cells to release their granule contents (Lowman et al.1988, Goetzl 1990). It has also been shown that eosinophils contain substance P and vasoactive intestinal peptide (VIP) (Aliakbari et al. 1987).

Several studies have shown that there are links between the nervous system and inflammation. In the skin the red flare phase of the classical triple response to trauma is mediated by an axon reflex. Antidromic stimulation of cutaneous

nerves results in reflex arteriolar vasodilatation in the surrounding area. It has been shown that the vasodilatation results from the action of antidromically stimulated sensory nerves on dermal mast cells (Kiernan et al.1975). These sensory nerves have neuropeptides in their nerve endings. It has been shown that substance P can be released following antidromic stimulation (Church et al. 1989). Other neuropeptides are associated with sensory nerves including somatostatin, neurokinin A and calcitonin gene related peptide (CGRP). Human mast cells from skin can be stimulated to release histamine and other mediators by application of substance P, vasoactive intestinal peptide (VIP) and somatostatin (Church et al.1989). It is of interest that mast cells from colonic mucosa, lung and other sites are not stimulated by substance P, crosslinking of cell-bound IgE being a more effective stimulus (Lowman et al.1988). How mast cells behave in the oesophagus is not known.

Thus there is strong evidence to link antidromic stimulation of sensory nerves, release of substance P and other mediators from the nerve endings and subsequent mast cell degranulation in the tissues. Recent interest in 'neurogenic inflammation' has emerged in research into the pathogenesis of asthma (Barnes 1992). It is postulated that epithelial shedding results in stimulation of sensory nerves with activation of an axon reflex with release of neuropeptides. It has recently been shown that there is an increase in substance P immunoreactivity in nerves in the airways of asthmatics and patients with atopic dermatitis (Ollerenshaw et al. 1991, Tobin et al. 1992). Also substances released from IgE stimulated mast cells have been shown in vitro to directly excite vagal sensory neurones (Greene et al. 1988).

An intimate anatomical association between mast cells and nerves in the bowel wall has been demonstrated (Stead et al. 1987). It has been suggested that this implies a special association between mast cells and nerves but a recent study has found that other cells such as eosinophils and plasma cells can also be found in close association with nerves (Arizono et al. 1990). This does not mean that nerve/mast cell communications do not occur but possibly that other cells are also in a situation where they can influence or be influenced by nerves.

In a classical conditioning experiment in which an immunological challenge was paired with presentation of an odour, guinea pigs showed a plasma histamine increase when presented with the odour alone (Russell et al.1984). This experiment demonstrated the influence the CNS has on immunological function. This may explain the links between stress and severity of asthma and is interesting

to note with regard to the motility disorders since it is well recognised that psychiatric disorder is common in these patients (Clouse et al.1983).

To summarise, there is no clinical or histological evidence that this is a parasitic disorder. There is increasing evidence of interactions between the nervous system and inflammatory cells making the concept of 'neurogenic inflammation' one that could also apply to the oesophagus. The possibility that neurogenic inflammation plays a significant role in asthma with its associated tissue eosinophilia suggests that a similar mechanism could be involved in those cases of disordered oesophageal motility associated with tissue eosinophilia. There is also evidence of an increased frequency of psychiatric disturbance in motility disorders of the oesophagus. Certainly many patients interviewed in this study admitted that stress and anxiety exacerbated their symptoms. Thus there may be a neurogenic component to the disorder. Whether this could result in mast cell degranulation and subsequent eosinophil infiltration is open to question.

8.4.4 How could eosinophils affect oesophageal motility?

It has been shown in the introduction that eosinophils can secrete a number of toxic materials from their granules. Amongst these there are several candidates for substances that might interfere with nerve function. Firstly there is a group of substances that can cause direct cell damage such as ECP, MBP, EPO and EDN. Both ECP and EDN have direct neurotoxic effects (Fredens et al.1982).

Other mediators released by eosinophils can directly affect smooth muscle contraction. Thus lipid mediators such as the prostaglandins PGD2 and PGF2a, thromboxane A2 ,leukotriene LTC4 and platelet activating factor (PAF) can all be generated de novo and can all cause smooth muscle contraction (Kroegel et al.1992).

It is possible that the eosinophil infiltrate is merely a marker and other cells in the infiltrate are responsible for the disordered function. Thus mast cell products including histamine and leukotrienes could be the predominant influence on nerves or smooth muscle contraction (Frigas et al.1986). It is interesting to note a recent report of an animal model of achalasia where marked mast cell and basophil proliferation with evidence of activation was found in the oesophageal wall (Tung et al.1992). No comment was made on eosinophil infiltration and the infiltration was attributed to restructuring of connective tissue during oesophageal distension and hypertrophy. In this regard it is interesting to note that this thesis

has demonstrated there were increased numbers of mast cells in AP in patients undergoing myotomy for achalasia.

8.4.5 Is it possible to identify patients with eosinophil infiltration in AP without resorting to muscle wall biopsy at myotomy?

If treatment is to be directed against the inflammatory infiltrate in AP then clearly it is essential that those patients who have an eosinophil infiltrate in the plexus are identified prior to surgery. The possible means by which this could be achieved are detailed below.

Analysis of the manometry records of those patients with a prominent eosinophilic infiltrate in AP suggested that this finding was most prevalent in the disorders classified as DOS and vigorous achalasia. However eosinophils were also found in increased numbers in occasional cases of achalasia and one case of nutcracker oesophagus. Thus it is possible to say that an eosinophil infiltrate is most common in the vigorous contraction abnormalities of the oesophagus but that within this group eosinophilia is not invariable.

Unfortunately synchronous mucosal biopsies were not taken from patients undergoing long myotomy. If it were shown in subsequent studies that there was an infiltrate of eosinophils in the mucosa in cases with AP inflammation this could be a very useful marker.

It was hoped that study of peripheral blood might provide a marker of eosinophil infiltration in the oesophagus. However study of eosinophil counts failed to reveal a significant eosinophilia. It should be noted that in asthma, in which eosinophil infiltration of the airways occurs, peripheral blood eosinophil counts can be normal.

Serum IgE levels were raised in most cases who had eosinophil infiltrates but not in all and the numbers who had both an infiltrate and a blood test were very small. Thus serum IgE may be the best marker available at the moment but further studies linking serum IgE and eosinophil counts in Auerbach's plexus are needed to confirm this association.

8.5. WHAT ARE THE IMPLICATIONS FOR TREATMENT OF THE MOTILITY DISORDERS?

If one accepts that there is a group of patients with disordered motility associated with eosinophil infiltration of AP then this group may benefit from pharmacological intervention to either stop the infiltration or reduce its effects. Several drugs are available that can influence the process of such inflammation.

Steroids are used in the management of severe asthma. The precise mode of action of these drugs is unknown. It has been shown that eosinophil secretion can be decreased in vitro by administration of steroids (Spry 1988). There is further evidence to suggest that at therapeutic concentrations steroids may be having their effect more on T lymphocytes and antigen presenting cells than eosinophils themselves (Corrigan and Kay 1992). Steroids have been effective in the treatment of eosinophilic gastroenteritis, and in one of the cases described by Attwood (Zora et al.1984, Matzinger and Daneman 1983, Attwood et al.1992).

Antihistamines work by blocking histamine receptors on target organs. Clearly if these worked in motility disorders it might suggest that the eosinophil infiltrate was more of a marker of disease which was mediated largely by mast cell secretion of histamine.

Cromoglycate is a drug that can inhibit IgE-triggered mediator release from human mast cells and although this may be responsible for its clinical effect in diseases such as asthma there is evidence to suggest that it may also have an effect by preventing eosinophil recruitment in the airways (Holgate 1989). Although its effects have been most extensively studied in asthma it has been shown to be effective in many disorders associated with eosinophil infiltration. It has been shown to be effective in eosinophilic gastroenteritis (Moots et al.1988).

Increasing knowledge of the mechanisms of eosinophil recruitment into the sites of inflammation and identification of the intercellular adhesion molecules responsible for this has opened a further area that may be amenable to pharmacological intervention. It has been shown in non-human primates that accumulation of eosinophils in the lungs after antigen challenge involves ICAM-1 mediated interactions between eosinophils and endothelial cells. Anti-ICAM-1 antibodies may be able to prevent eosinophil accumulation in the lungs and their consequent effects.

Thus there are a number of potential drugs for use in eosinophil-associated diseases. Since surgical treatment of motility disorders is a major undertaking, there is an urgent need for further study of the role of eosinophils in motility disorders of the oesophagus and of drugs that can prevent their accumulation or modify their effects.

8.6 WHAT CAN BE SAID ABOUT THE PATIENTS WITH NO OBVIOUS INFLAMMATION IN AUERBACH'S PLEXUS?

It is of interest that the many of the patients studied did not have an eosinophil infiltrate or inflammation in AP. The reasons for this are not clear. It is possible that this group are either at a different stage of the same disease or have a different underlying aetiology.

It is also possible that the eosinophil infiltrate is patchy within the oesophagus. Thus the small biopsies taken from AP might miss the areas of eosinophil infiltration so that this was simply a sampling error. However, in the cases studied in this thesis in which there were eosinophil infiltrates, eosinophils were present in all parts of the plexus studied in increased numbers, although some areas were much more densely infiltrated than others.

8.7 DIRECTIONS OF FUTURE RESEARCH.

The advent of 24 hour manometry should allow clearer definitions of the motility disorders to be made and may allow distinction between those with an eosinophil infiltrate from those without. They have already suggested that the distinction between nutcracker oesophagus and DOS may be artificial. Thus further research in this area must include 24 hour manometric assessment of the patients studied.

Further studies are needed on the histology of normal oesophagus. The immunohistochemical profiles of cells in AP are still only very sketchy although this study has shown T lymphocytes occur in close relationship to ganglion cells in the plexus. Further details are needed of the types of nerve fibres present in the normal oesophagus and should include staining for neuropeptides such as substance P and VIP.

Further studies of the effects of drugs on strips of smooth muscle from the body of the oesophagus and lower oesophageal sphincter are needed. Using such techniques it should be possible to study the effect of eosinophil and mast cell products on smooth muscle contraction.

It is clear from earlier studies that at the time of long myotomy longer and larger biopsies of the muscular wall are needed both for histological study and pharmacological studies of muscle strips. This may be difficult to achieve in the future if thoracoscopic techniques of long myotomy are universally adopted. Since the range of antibodies reacting with routinely processed tissue is limited some tissue obtained in this way will need to be snap-frozen so that the range of antigens studied can be expanded.

As evident from the studies on lymph nodes, knowledge of changes in paraoesophageal lymph nodes in disease of the oesophagus is limited. Lymph nodes need to be obtained from a larger group of normal transplant donors and also from a variety of benign oesophageal diseases to study the relationship between changes in the lymph nodes and changes in the oesophageal mucosa and Auerbach's plexus. Staining for specific cells involved in the immune response would be appropriate and should include staining specifically for antigen-presenting cells, T and B cells, markers of cell activation such as CD23 and IgE. This is potentially a very large area of research.

Also blood should be taken prospectively on patients undergoing myotomy to try and establish links between changes in immunoglobulins in the blood (including IgE) and changes in AP. Recent evidence has suggested that IgG4 may have a significant role in eosinophilic inflammation and this should be studied further (Feldman et al.1992). Studies to look at autoantibodies reacting against neural tissue in the oesophagus also need to be undertaken (as was performed in the study by Robertson et al.1991).

An animal model of both achalasia and the vigorous contraction abnormalities is needed. Several studies have shown that it is possible to produce an achalasia-like syndrome in animals but its relationship to true achalasia is uncertain (Satchell 1990, Sons et al.1986). To my knowledge there is as yet no animal model of diffuse oesophageal spasm.

8.8 SUMMARY

The manometric distinctions between the various vigorous contraction abnormalities DOS, nutcracker oesophagus and vigorous achalasia may be artificial. This is suggested by recent findings on 24 hour manometry and also by the fact that in DOS and nutcracker oesophagus there is little evidence of any difference in disease progression or pathology.

The finding of eosinophils in Auerbach's plexus is significant. Infiltration and degranulation, as seen in the motility disorders in this study, was not seen in any other disease studied. Many studies have shown that eosinophil infiltrates have pathogenic potential. Their secretion products can damage or alter the function of nerve and smooth muscle cells. To prove that the eosinophils caused the disease would require the development of an animal model and demonstration that drugs affecting eosinophil function produced clinical improvement.

Finding eosinophil infiltrates and IgE-staining cells in AP in association with raised IgE in the blood suggests that this could be an allergic disorder. No allergen has been identified. Further studies of the link between serum immunoglobulins and tissue eosinophilia in the motility disorders is needed since serum IgE is a potential marker of eosinophil infiltration. .

The possibility that 'neurogenic inflammation' may play a role in asthma, a disease sharing many similarities with the vigorous contraction abnormalities, may prove to be important in the pathogenesis of both diseases. Further research in this area is needed.

It is an exciting prospect that if those patients with an eosinophil infiltrate could be identified without biopsying the muscle wall then treatment with the large number of drugs available to impair eosinophil action might obviate the necessity for major surgery.

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APPENDICES

Appendix 1 Cell counts in normal oesophagus.

1.1 Eosinophil counts in normal oesophagus from tumour-bearing oesophagus.

Eosinophil counts for control oesophagus (cells/sq.mm.)
(tissue from tumour-bearing oesophagus)

Lab. No.	Epithelium	Lamina Propria	Muscularis mucosae	Submucosa	Circular muscle	Auerbach's Plexus	Longitudinal muscle
OE10/91	7.48	44.2	2.49	0.68	0.68	6.12	0.23
OE16/91	0	0	0	0	0	0.56	0.11
OE7/91	0.1	5.59	0.68	0.3	0.3	0.76	0.1
OE5/91	0	0	0	0	0	0	0
OE9/91	0	5.45	7.27	0.91	0	3.18	2.73
Mean	1.51	11.05	2.09	0.38	0.31	2.12	0.63

1.2 Eosinophil counts in single oesophagus taken postmortem (OE14/91)

EOSINOPHILS AND MAST CELLS IN RESECTED OESOPHAGUS

		DIAGNOSIS	POSTMORTEM			
	SLIDE:-	OE14/91 1	OE14/91 5	OE14/91 10	OE14/91 20	
	CM ABOVE GOJ	2.00	4.00	8.00	12.00	MEAN
EPITHELIUM	AREA	0.17	0.23	0.15	0.07	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	1.00	0.00	0.00	1.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	5.88	0.00	0.00	14.29	5.04
LAMINA PROPRIA	AREA	0.09	0.29	0.30	0.05	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	6.00	13.00	9.00	10.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	66.67	44.83	30.00	200.00	85.37
MUSCULARIS MUCOSA	AREA	0.27	0.57	0.27	0.13	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	12.00	56.00	18.00	34.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	44.44	98.25	66.67	261.54	117.72
SUBMUCOSA	AREA	0.68	0.69	0.49	0.53	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	19.00	25.00	8.00	22.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	27.94	36.23	16.33	41.51	30.50
CIRCULAR MUSCLE	AREA	0.76	0.60	0.52	0.54	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	0.48	14.00	7.00	30.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	0.63	23.33	13.46	55.56	23.25
AUERBACH'S	AREA	0.22	0.33	0.12	0.21	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	34.00	19.00	10.00	18.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	154.55	57.58	83.33	85.71	95.29
LONGITUDINAL M.	AREA	0.68	0.86	0.34	0.56	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	63.00	53.00	17.00	10.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	92.65	61.63	50.00	17.86	55.53

1.3 Eosinophil and mast cell counts from transplant donor oesophagi.
OE10/92

EOSINOPHILS AND MAST CELLS IN RESECTED OESOPHAGUS

		DIAGNOSIS	TRANSPLANT CONTROL			
	SLIDE:-	OE49/91 17	OE49/91 10	OE49/91 4	OE49/91 1	
	CM.ABOVE GOJ	1.00	5.00	8.00	11.00	MEAN
EPITHELIUM	AREA	0.26	0.28	0.10	0.17	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	0.00	9.00	0.00	4.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	0.00	32.14	0.00	23.53	13.92
LAMINA PROPRIA	AREA	0.37	0.16	0.13	0.16	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	25.00	23.00	24.00	33.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	67.57	143.75	184.62	206.25	150.55
MUSCULARIS MUCOSA	AREA	0.34	0.34	0.11	0.42	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	15.00	27.00	33.00	95.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	44.12	79.41	300.00	226.19	162.43
SUBMUCOSA	AREA	0.79	0.60	0.44	0.73	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	7.00	17.00	18.00	43.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	8.86	28.33	40.91	58.90	34.25
CIRCULAR MUSCLE	AREA	0.84	0.78	0.37	0.47	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	28.00	10.00	14.00	22.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	33.33	12.82	37.84	46.81	32.70
AUERBACH'S	AREA	0.14	0.13	0.19	0.24	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	12.00	13.00	14.00	21.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	85.71	100.00	73.68	87.50	86.72
LONGITUDINAL M.	AREA	0.55	0.66	0.49	0.58	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	6.00	4.00	9.00	4.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	10.91	6.06	18.37	6.90	10.56

OE49/91

EOSINOPHILS AND MAST CELLS IN RESECTED OESOPHAGUS

DIAGNOSIS TRANSPLANT CONTROL

	SLIDE:-	OE49/91 17	OE49/91 10	OE49/91 4	OE49/91 1	
	CM.ABOVE GOJ	1.00	5.00	8.00	11.00	MEAN
EPITHELIUM	AREA	0.26	0.28	0.10	0.17	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	0.00	9.00	0.00	4.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	0.00	32.14	0.00	23.53	13.92
LAMINA PROPRIA	AREA	0.37	0.16	0.13	0.16	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	25.00	23.00	24.00	33.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	67.57	143.75	184.62	206.25	150.55
MUSCULARIS MUCOSA	AREA	0.34	0.34	0.11	0.42	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	15.00	27.00	33.00	95.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	44.12	79.41	300.00	226.19	162.43
SUBMUCOSA	AREA	0.79	0.60	0.44	0.73	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	7.00	17.00	18.00	43.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	8.86	28.33	40.91	58.90	34.25
CIRCULAR MUSCLE	AREA	0.84	0.78	0.37	0.47	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	28.00	10.00	14.00	22.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	33.33	12.82	37.84	46.81	32.70
AUERBACH'S	AREA	0.14	0.13	0.19	0.24	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	12.00	13.00	14.00	21.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	85.71	100.00	73.68	87.50	86.72
LONGITUDINAL M.	AREA	0.55	0.66	0.49	0.58	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	6.00	4.00	9.00	4.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	10.91	6.06	18.37	6.90	10.56

EOSINOPHILS AND MAST CELLS IN RESECTED OESOPHAGUS

OE44/91 DIAGNOSIS

	SLIDE:-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	MEAN
	CM ABOVE GOJ																				
EPITHELIUM	AREA	1.22	1.37	1.15	1.13	1.05	1.76	2.12	1.15	1.37	0.75	0.32	1.16	0.82	1.33	0.58	1.21	0.44	0.26	1.01	
	EOSINOPHILS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
	MAST CELLS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.38	0.00	0.02
	MC/MM2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.72	0.00	0.00	0.00	0.00	0.09
LAMINA PROPRIA	AREA	0.66	0.76	0.56	0.79	0.67	0.71	0.48	1.11	1.09	0.84	0.38	0.60	0.61	0.67	0.21	1.21	0.34	0.38	0.73	
	EOSINOPHILS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	
	MAST CELLS	108.00	219.00	189.00	220.00	177.00	220.00	154.00	230.00	495.00	165.00	63.00	161.00	125.00	243.00	31.00	131.00	57.00	38.00	293.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.92	0.00	0.00	0.00	0.00	1.49	0.00	0.00	0.00	0.00	0.00	0.13
	MC/MM2	196.36	292.00	337.50	278.48	310.53	309.86	320.83	207.21	454.13	196.43	165.79	268.33	204.92	362.69	147.62	108.26	167.65	100.00	401.37	254.21
MUSCULARIS MUCOSA	AREA	1.86	1.17	1.84	1.44	0.99	1.69	1.07	1.05	0.77	1.26	0.57	1.33	0.62	0.91	0.18	1.62	0.28	0.24	2.26	
	EOSINOPHILS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
	MAST CELLS	98.00	81.00	170.00	129.00	73.00	150.00	77.00	74.00	90.00	112.00	29.00	75.00	64.00	71.00	7.00	60.00	15.00	8.00	206.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.95	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05
	MC/MM2	52.69	69.23	92.39	89.58	73.74	94.34	71.96	70.48	116.88	88.89	50.88	56.39	103.23	78.02	38.89	37.04	53.57	33.33	91.15	71.72
SUBMUCOSA	AREA	2.76	2.27	4.35	2.87	1.63	0.84	0.84	1.65	0.53	3.34	0.79	2.71	2.25	1.79	0.21	4.37	0.32	0.62	1.64	
	EOSINOPHILS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
	MAST CELLS	77.00	92.00	180.00	131.00	78.00	39.00	55.00	57.00	164.00	141.00	31.00	67.00	161.00	123.00	13.00	66.00	20.00	15.00	33.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	MC/MM2	27.90	40.53	41.38	45.64	47.85	46.43	65.48	34.55	309.43	42.22	39.24	24.72	71.56	68.72	61.90	15.10	62.50	24.19	20.12	57.34
CIRCULAR MUSCLE	AREA	2.49	2.30	3.49	2.48	2.85	5.18	5.59	4.98	4.77	4.48	0.75	5.50	1.91	5.15	0.41	7.29	0.68	0.38	4.72	
	EOSINOPHILS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
	MAST CELLS	27.00	11.00	53.00	54.00	29.00	235.00	318.00	50.00	329.00	181.00	10.00	59.00	62.00	100.00	5.00	179.00	2.00	5.00	10.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	MC/MM2	10.84	4.78	15.19	21.77	10.18	45.37	56.89	10.04	68.97	40.40	13.33	10.73	32.46	19.42	12.20	24.65	2.94	13.16	2.12	21.86
AUERBACH'S	AREA	0.71	0.82	0.58	0.50	0.45	0.69	0.54	0.63	0.80	0.47	0.38	0.94	0.61	0.62	0.26	1.25	0.20	0.26	1.09	
	EOSINOPHILS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
	MAST CELLS	17.00	45.00	68.00	44.00	36.00	50.00	41.00	56.00	83.00	58.00	29.00	43.00	74.00	75.00	24.00	47.00	9.00	12.00	73.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	MC/MM2	23.94	54.88	117.24	88.00	77.78	72.46	75.93	88.89	103.75	123.40	76.32	45.74	121.31	120.97	92.31	37.60	45.00	46.15	66.97	77.82
LONGITUDINAL M.	AREA	2.10	3.68	1.79	3.92	3.41	3.77	4.24	3.01	3.71	3.94	0.90	5.29	2.65	3.29	0.26	5.23	0.62	0.59	5.32	
	EOSINOPHILS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
	MAST CELLS	22.00	39.00	50.00	79.00	40.00	41.00	33.00	64.00	120.00	82.00	6.00	152.00	61.00	99.00	24.00	72.00	4.00	14.00	60.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	MC/MM2	10.48	10.60	27.93	20.15	11.73	10.88	7.78	21.26	32.35	20.81	6.67	28.73	23.02	30.09	92.31	13.77	6.45	23.73	11.28	21.58

OE59/91

EOSINOPHILS AND MAST CELLS IN RESECTED OESOPHAGUS

DIAGNOSIS TRANSPLANT CONTROL (OE 5991)

	CM.ABOVE GOJ	1.00	5.00	10.00	15.00	MEAN
EPITHELIUM	AREA	0.21	0.10	0.12	0.21	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	0.00	9.00	0.00	4.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	0.00	0.00	0.00	0.00	0.00
LAMINA PROPRIA	AREA	0.33	0.20	0.10	0.18	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	28.00	10.00	13.00	14.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	84.85	50.00	130.00	77.78	85.66
MUSCULARIS MUCOSA	AREA	0.21	0.22	0.12	0.22	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	14.00	10.00	9.00	21.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	66.67	45.45	75.00	95.45	70.64
SUBMUCOSA	AREA	0.58	0.58	0.50	0.41	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	15.00	4.00	4.00	19.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	25.86	6.90	8.00	46.34	21.78
CIRCULAR MUSCLE	AREA	0.35	0.48	0.59	0.57	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	2.00	4.00	7.00	7.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	5.71	8.33	11.86	12.28	9.55
AUERBACH'S	AREA	0.12	0.08	0.10	0.07	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	6.00	4.00	4.00	9.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	50.00	50.00	40.00	128.57	67.14
LONGITUDINAL M.	AREA	0.25	0.47	0.46	0.54	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	5.00	13.00	6.00	7.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	20.00	27.66	13.04	12.96	18.42

OE35/92

EOSINOPHILS AND MAST CELLS IN RESECTED OESOPHAGUS

DIAGNOSIS Transplant donor OE3592

	SLIDE:-				
	CM ABOVE GOJ	2	8	14	MEAN
EPITHELIUM	AREA	0.24	0.13	0.14	
	EOSINOPHILS	0.00	0.00	0.00	
	MAST CELLS	1.00	0.00	0.00	
	EOS/MM2	0.00	0.00	0.00	0.00
	MC/MM2	4.17	0.00	0.00	1.39
LAMINA PROPRIA	AREA	0.12	0.19	0.17	
	EOSINOPHILS	0.00	0.00	0.00	
	MAST CELLS	8.00	11.00	32.00	
	EOS/MM2	0.00	0.00	0.00	0.00
	MC/MM2	66.67	57.89	188.24	104.27
MUSCULARIS MUCOSA	AREA	0.08	0.15	0.26	
	EOSINOPHILS	0.00	0.00	0.00	
	MAST CELLS	4.00	11.00	23.00	
	EOS/MM2	0.00	0.00	0.00	0.00
	MC/MM2	50.00	73.33	88.46	70.60
SUBMUCOSA	AREA	0.44	0.50	0.37	
	EOSINOPHILS	0.00	0.00	0.00	
	MAST CELLS	7.00	10.00	10.00	
	EOS/MM2	0.00	0.00	0.00	0.00
	MC/MM2	15.91	20.00	27.03	20.98
CIRCULAR MUSCLE	AREA	0.36	0.49	0.65	
	EOSINOPHILS	0.00	0.00	0.00	
	MAST CELLS	5.00	12.00	6.00	
	EOS/MM2	0.00	0.00	0.00	0.00
	MC/MM2	13.89	24.49	9.23	15.87
AUERBACH'S	AREA	0.11	0.10	0.16	
	EOSINOPHILS	0.00	0.00	0.00	
	MAST CELLS	4.00	12.00	8.00	
	EOS/MM2	0.00	0.00	0.00	0.00
	MC/MM2	36.36	120.00	50.00	68.79
LONGITUDINAL M.	AREA	0.44	0.76	0.75	
	EOSINOPHILS	0.00	0.00	0.00	
	MAST CELLS	7.00	14.00	26.00	
	EOS/MM2	0.00	0.00	0.00	0.00
	MC/MM2	15.91	18.42	34.67	23.00

Appendix 2 Cell counts in oesophagus resected for reflux disease.

2. OE6/91

EOSINOPHILS AND MAST CELLS IN RESECTED OESOPHAGUS

	DIAGNOSIS							
	SLIDE:-	OE6/91 1A	OE6/91 TS	OE6/91 S4	OE6/91 S3	OE6/91 S2	OE6/91 S1	
	CM.ABOVE GOJ	0.50	3.50	12.00	12.50	13.00	13.50	MEAN
EPITHELIUM	AREA			0.09	0.24	0.34	0.45	
	EOSINOPHILS			0.00	0.00	0.00	0.00	
	MAST CELLS			13.00	4.00	6.00	10.00	
	EOS/MM2			0.00	0.00	0.00	0.00	0.00
	MC/MM2			144.44	16.67	17.65	22.22	50.25
LAMINA PROPRIA	AREA	0.26	0.60	0.25	0.17	0.33	0.39	
	EOSINOPHILS	25.00	38.00	6.00	8.00	9.00	27.00	
	MAST CELLS	34.00	37.00	19.00	10.00	37.00	67.00	
	EOS/MM2	96.15	63.33	24.00	47.06	27.27	69.23	54.51
	MC/MM2	130.77	61.67	76.00	58.82	112.12	171.79	101.86
MUSCULARIS MUCOSA	AREA	0.16	0.20	0.14	0.29	0.40	0.79	
	EOSINOPHILS	0.00	0.00	0.00	5.00	4.00	24.00	
	MAST CELLS	22.00	11.00	4.00	13.00	21.00	49.00	
	EOS/MM2	0.00	0.00	0.00	17.24	10.00	30.38	9.60
	MC/MM2	137.50	55.00	28.57	44.83	52.50	62.03	63.40
SUBMUCOSA	AREA	0.66	0.65		0.54	0.90	0.76	
	EOSINOPHILS	0.00	2.00		0.00	0.00	0.00	
	MAST CELLS	45.00	8.00		10.00	9.00	24.00	
	EOS/MM2	0.00	3.08	ERR	0.00	0.00	0.00	3.08
	MC/MM2	68.18	12.31	ERR	18.52	10.00	31.58	140.59
CIRCULAR MUSCLE	AREA	0.57	0.76	0.46	0.76	0.79	0.85	
	EOSINOPHILS	1.00	0.00	0.00	0.00	0.00	0.00	
	MAST CELLS	23.00	25.00	29.00	48.00	65.00	29.00	
	EOS/MM2	1.75	0.00	0.00	0.00	0.00	0.00	0.29
	MC/MM2	40.35	32.89	63.04	63.16	82.28	34.12	52.64
AUERBACH'S	AREA	0.26	0.09	0.29	0.16	0.35	0.12	
	EOSINOPHILS	0.00	0.00	2.00	0.00	2.00	0.00	
	MAST CELLS	34.00	8.00	13.00	15.00	7.00	20.00	
	EOS/MM2	0.00	0.00	6.90	0.00	5.71	0.00	2.10
	MC/MM2	130.77	88.89	44.83	93.75	20.00	166.67	90.82
LONGITUDINAL M.	AREA	0.74	0.45	0.34	0.41	0.36	0.70	
	EOSINOPHILS	0.00	0.00	0.00	0.00	0.00	0.00	
	MAST CELLS	11.00	16.00	20.00	5.00	21.00	6.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	MC/MM2	14.86	35.56	58.82	12.20	58.33	8.57	31.39

OE19/91

EOSINOPHILS AND MAST CELLS IN RESECTED OESOPHAGUS

	SLIDE:-	OE19/91 S1	OE19/91 S2	OE19/91 S3	OE19/91 S4	
	CM.ABOVE GOJ					MEAN
EPITHELIUM	AREA	0.19	0.19	0.20	0.06	
	EOSINOPHILS	0.00	0.00	0.00	1.00	
	MAST CELLS	4.00	3.00	2.00	1.00	
	EOS/MM2	0.00	0.00	0.00	16.67	4.17
	MC/MM2	21.05	15.79	10.00	16.67	15.88
LAMINA PROPRIA	AREA	0.03	0.08	0.10	0.13	
	EOSINOPHILS	0.00	1.00	1.00	13.00	
	MAST CELLS	25.00	16.00	24.00	14.00	
	EOS/MM2	0.00	12.50	10.00	100.00	30.63
	MC/MM2	833.33	200.00	240.00	107.69	345.26
MUSCULARIS MUCOSA	AREA	0.23	0.18	0.27		
	EOSINOPHILS	3.00	0.00	0.00		
	MAST CELLS	68.00	36.00	31.00		
	EOS/MM2	13.04	0.00	0.00	Damaged	Damaged
	MC/MM2	295.65	200.00	114.81	Damaged	Damaged
SUBMUCOSA	AREA	0.20	0.28	0.57		
	EOSINOPHILS	4.00	0.00	0.00		
	MAST CELLS	25.00	20.00	37.00		
	EOS/MM2	20.00	0.00	0.00	Damaged	Damaged
	MC/MM2	125.00	71.43	64.91	Damaged	Damaged
CIRCULAR MUSCLE	AREA	0.40	0.28	0.70	0.43	
	EOSINOPHILS	0.00	0.00	0.00	6.00	
	MAST CELLS	40.00	15.00	96.00	37.00	
	EOS/MM2	0.00	0.00	0.00	13.95	3.49
	MC/MM2	100.00	53.57	137.14	86.05	94.19
AUERBACH'S	AREA	0.20	0.11	0.28	0.55	
	EOSINOPHILS	1.00	0.00	1.00	0.00	
	MAST CELLS	38.00	8.00	35.00	58.00	
	EOS/MM2	5.00	0.00	3.57	0.00	2.14
	MC/MM2	190.00	72.73	125.00	105.45	123.30
LONGITUDINAL M.	AREA	0.45	0.40	0.68	0.43	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	8.00	4.00	41.00	27.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	17.78	10.00	60.29	62.79	37.72

OE73/91

EOSINOPHILS AND MAST CELLS IN RESECTED OESOPHAGUS

DIAGNOSIS **BARRETTS**

	SLIDE:-	OE73/91 A1	OE73/91 A2	OE73/91 A3	OE73/91 A4	
	CM. ABOVE OGJ	5	4	3	2	MEAN
EPITHELIUM	AREA	0.26	0.35	0.11		
	EOSINOPHILS	0.00	0.00	1.00		
	MAST CELLS	0.00	0.00	0.00		
	EOS/MM2	0.00	0.00	9.09	No tissue	2.27
	MC/MM2	0.00	0.00	0.00	No tissue	0.00
LAMINA PROPRIA	AREA	0.29	0.34	0.65	0.60	
	EOSINOPHILS	28.00	10.00	85.00	62.00	
	MAST CELLS	36.00	24.00	20.00	53.00	
	EOS/MM2	96.55	29.41	130.77	103.33	90.02
	MC/MM2	124.14	70.59	30.77	88.33	78.46
MUSCULARIS MUCOSA	AREA	0.70	0.36	0.78	0.52	
	EOSINOPHILS	1.00	2.00	25.00	2.00	
	MAST CELLS	90.00	15.00	116.00	46.00	
	EOS/MM2	1.43	5.56	32.05	3.85	10.72
	MC/MM2	128.57	41.67	148.72	88.46	101.85
SUBMUCOSA	AREA	0.67	0.81	0.78	0.73	
	EOSINOPHILS	0.00	1.00	0.00	0.00	
	MAST CELLS	9.00	37.00	6.00	23.00	
	EOS/MM2	0.00	1.23	0.00	0.00	0.31
	MC/MM2	13.43	45.68	7.69	31.51	24.58
CIRCULAR MUSCLE	AREA	0.66	0.58	0.61	0.62	
	EOSINOPHILS	0.00	1.00	0.00	1.00	
	MAST CELLS	46.00	132.00	64.00	145.00	
	EOS/MM2	0.00	1.72	0.00	1.61	0.83
	MC/MM2	69.70	227.59	104.92	233.87	159.02
AUERBACH'S	AREA	0.31	0.48	0.20	0.55	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	19.00	20.00	22.00	33.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	61.29	41.67	110.00	60.00	68.24
LONGITUDINAL M.	AREA	0.64	0.54	0.67	0.68	
	EOSINOPHILS	0.00	0.00	1.00	0.00	
	MAST CELLS	34.00	56.00	55.00	60.00	
	EOS/MM2	0.00	0.00	1.49	0.00	0.37
	MC/MM2	53.13	103.70	82.09	88.24	81.79

OE36/91

EOSINOPHILS AND MAST CELLS IN RESECTED OESOPHAGUS

DIAGNOSIS

	SLIDE:-	OE36/91 B1	OE36/91 B2	OE36/91 B3	OE36/91 B4	OE36/91 B5	
	CM ABOVE GOJ	16	12	9	5	0	MEAN
EPITHELIUM	AREA						
	EOSINOPHILS						
	MAST CELLS						
	EOS/MM2	The epithelium in this resected specimen was predominantly					
	MC/MM2	columnar lined making accurate quantitation difficult					
LAMINA PROPRIA	AREA	0.56	0.49	0.36	0.35	0.18	
	EOSINOPHILS	36.00	69.00	79.00	62.00	10.00	
	MAST CELLS	94.00	81.00	53.00	61.00	19.00	
	EOS/MM2	64.29	140.82	219.44	177.14	55.56	131.45
	MC/MM2	167.86	165.31	147.22	174.29	105.56	152.05
MUSCULARIS MUCOSA	AREA	0.22	0.31	0.45	0.68	0.12	
	EOSINOPHILS	0.00	0.00	0.00	1.00	0.00	
	MAST CELLS	22.00	38.00	48.00	46.00	8.00	
	EOS/MM2	0.00	0.00	0.00	1.47	0.00	0.29
	MC/MM2	100.00	122.58	106.67	67.65	66.67	92.71
SUBMUCOSA	AREA	0.82	0.50	0.63	0.18	0.73	
	EOSINOPHILS	1.00	0.00	0.00	0.00	0.00	
	MAST CELLS	15.00	8.00	15.00	9.00	18.00	
	EOS/MM2	1.22	0.00	0.00	0.00	0.00	0.24
	MC/MM2	18.29	16.00	23.81	50.00	24.66	26.55
CIRCULAR MUSCLE	AREA	0.53	0.67	0.49	0.57	0.62	
	EOSINOPHILS	0.00	1.00	0.00	0.00	0.00	
	MAST CELLS	136.00	130.00	84.00	77.00	9.00	
	EOS/MM2	0.00	1.49	0.00	0.00	0.00	0.30
	MC/MM2	256.60	194.03	171.43	135.09	14.52	154.33
AUERBACH'S	AREA	0.49	0.37	0.25	0.30	0.59	
	EOSINOPHILS	0.00	1.00	0.00	3.00	0.00	
	MAST CELLS	28.00	47.00	29.00	47.00	19.00	
	EOS/MM2	0.00	2.70	0.00	10.00	0.00	2.54
	MC/MM2	57.14	127.03	116.00	156.67	32.20	97.81
LONGITUDINAL M.	AREA	0.45	0.55	0.53	0.63	0.49	
	EOSINOPHILS	0.00	0.00	0.00	0.00	0.00	
	MAST CELLS	43.00	19.00	53.00	61.00	10.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00	0.00
	MC/MM2	95.56	34.55	100.00	96.83	20.41	69.47

OE71/91

EOSINOPHILS AND MAST CELLS IN RESECTED OESOPHAGUS

DIAGNOSIS BARRETTS OESOPHAGUS

	SLIDE:-	A11	A6	A1	
	CM ABOVE GOJ	2	5	9	MEAN
EPITHELIUM	AREA		0.20	0.46	
	EOSINOPHILS		8.00	30.00	
	MAST CELLS		2.00	1.00	
	EOS/MM2	ERR	40.00	65.22	52.61
	MC/MM2	ERR	10.00	2.17	6.09
LAMINA PROPRIA	AREA	1.08	0.27	0.18	
	EOSINOPHILS	127.00	59.00	87.00	
	MAST CELLS	121.00	8.00	23.00	
	EOS/MM2	117.59	218.52	483.33	273.15
	MC/MM2	112.04	29.63	127.78	89.81
MUSCULARIS MUCOSA	AREA	0.35	0.11	0.16	
	EOSINOPHILS	9.00	4.00	20.00	
	MAST CELLS	36.00	13.00	18.00	
	EOS/MM2	25.71	36.36	125.00	62.36
	MC/MM2	102.86	118.18	112.50	111.18
SUBMUCOSA	AREA	0.75	0.11	0.75	
	EOSINOPHILS	0.00	0.00	0.00	
	MAST CELLS	12.00	8.00	12.00	
	EOS/MM2	0.00	0.00	0.00	0.00
	MC/MM2	16.00	72.73	16.00	34.91
CIRCULAR MUSCLE	AREA	0.78	1.23	0.54	
	EOSINOPHILS	1.00	0.00	0.00	
	MAST CELLS	80.00	86.00	14.00	
	EOS/MM2	1.28	0.00	0.00	0.43
	MC/MM2	102.56	69.92	25.93	66.14
AUERBACH'S	AREA	0.23	0.19	0.54	
	EOSINOPHILS	25.00	0.00	0.00	
	MAST CELLS	39.00	9.00	14.00	
	EOS/MM2	108.70	0.00	0.00	36.23
	MC/MM2	169.57	47.37	25.93	80.95
LONGITUDINAL M.	AREA	0.80	1.33	1.18	
	EOSINOPHILS	1.00	0.00	0.00	
	MAST CELLS	126.00	49.00	9.00	
	EOS/MM2	1.25	0.00	0.00	0.42
	MC/MM2	157.50	36.84	7.63	67.32

Appendix 3 Cell counts in oesophagus resected for achalasia.

3. 4321/86

EOSINOPHILS AND MAST CELLS IN RESECTED OESOPHAGUS

DIAGNOSIS ACHALASIA 4321 86

	SLIDE:-				
	CM ABOVE GOJ	2	5	16	MEAN
EPITHELIUM	AREA	0.30	0.19	0.50	
	EOSINOPHILS	0.00	2.00	0.00	
	MAST CELLS	2.00	8.00	0.00	
	EOS/MM2	0.00	10.53	0.00	3.51
	MC/MM2	6.67	42.11	0.00	16.26
LAMINA PROPRIA	AREA	0.58	0.11	0.31	
	EOSINOPHILS	56.00	22.00	15.00	
	MAST CELLS	76.00	33.00	44.00	
	EOS/MM2	96.55	200.00	48.39	114.98
	MC/MM2	131.03	300.00	141.94	190.99
MUSCULARIS MUCOSA	AREA	0.46	0.12	0.37	
	EOSINOPHILS	3.00	1.00	17.00	
	MAST CELLS	50.00	29.00	42.00	
	EOS/MM2	6.52	8.33	45.95	20.27
	MC/MM2	108.70	241.67	113.51	154.63
SUBMUCOSA	AREA	0.30	0.56	0.59	
	EOSINOPHILS	0.00	5.00	0.00	
	MAST CELLS	32.00	24.00	47.00	
	EOS/MM2	0.00	8.93	0.00	2.98
	MC/MM2	106.67	42.86	79.66	76.39
CIRCULAR MUSCLE	AREA	0.70	0.55	0.46	
	EOSINOPHILS	0.00	0.00	0.00	
	MAST CELLS	97.00	7.00	19.00	
	EOS/MM2	0.00	0.00	0.00	0.00
	MC/MM2	138.57	12.73	41.30	64.20
AUERBACH'S	AREA	0.15	0.12	0.20	
	EOSINOPHILS	0.00	1.00	0.00	
	MAST CELLS	34.00	6.00	10.00	
	EOS/MM2	0.00	8.33	0.00	2.78
	MC/MM2	226.67	50.00	50.00	108.89
LONGITUDINAL M	AREA	0.62	0.45	0.37	
	EOSINOPHILS	0.00	0.00	0.00	
	MAST CELLS	28.00	16.00	5.00	
	EOS/MM2	0.00	0.00	0.00	0.00
	MC/MM2	45.16	35.56	13.51	31.41

3084/84

EOSINOPHILS AND MAST CELLS IN RESECTED OESOPHAGUS

DIAGNOSIS ACHALASIA 3084/84

	SLIDE:-	DP	NP	M	AP	DN	
	CM.ABOVE GOJ	1	3	5	7	10	MEAN
EPITHELIUM	AREA	0.40	0.32	0.19	0.11	0.30	
	EOSINOPHILS	7.00	2.00	1.00	0.00	0.00	
	MAST CELLS	11.00	3.00	0.00	0.00	0.00	
	EOS/MM2	17.50	6.25	5.26	0.00	7.25	7.25
	MC/MM2	27.50	9.38	0.00	0.00	9.22	9.22
LAMINA PROPRIA	AREA	0.07	0.20	0.12	0.09	0.10	
	EOSINOPHILS	19.00	0.00	9.00	5.00	4.00	
	MAST CELLS	6.00	11.00	10.00	14.00	19.00	
	EOS/MM2	271.43	0.00	75.00	55.56	40.00	88.40
	MC/MM2	85.71	55.00	83.33	155.56	190.00	113.92
MUSCULARIS MUCOSA	AREA	0.21	0.35	0.22	0.25	0.38	
	EOSINOPHILS	8.00	2.00	0.00	0.00	3.00	
	MAST CELLS	26.00	15.00	20.00	15.00	22.00	
	EOS/MM2	38.10	5.71	0.00	0.00	7.89	10.34
	MC/MM2	123.81	42.86	90.91	60.00	57.89	75.09
SUBMUCOSA	AREA	0.30	0.58	0.20	0.22	0.39	
	EOSINOPHILS	1.00	0.00	1.00	0.00	0.00	
	MAST CELLS	19.00	9.00	6.00	4.00	11.00	
	EOS/MM2	3.33	0.00	5.00	0.00	0.00	1.67
	MC/MM2	63.33	15.52	30.00	18.18	28.21	31.05
CIRCULAR MUSCLE	AREA	0.54	0.60	0.41	0.48	0.48	
	EOSINOPHILS	0.00	0.00	0.00	0.00	0.00	
	MAST CELLS	1.00	7.00	2.00	16.00	26.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00	0.00
	MC/MM2	1.85	11.67	4.88	33.33	54.17	21.18
AUERBACH'S	AREA	0.29	0.18	0.13	0.18	0.36	
	EOSINOPHILS	1.00	1.00	0.00	0.00	1.00	
	MAST CELLS	13.00	7.00	7.00	11.00	23.00	
	EOS/MM2	3.45	5.56	0.00	0.00	2.78	2.36
	MC/MM2	44.83	38.89	53.85	61.11	63.89	52.51
LONGITUDINAL M.	AREA	0.63	0.62	0.37	0.45	0.39	
	EOSINOPHILS	0.00	0.00	0.00	1.00	0.00	
	MAST CELLS	12.00	30.00	1.00	7.00	13.00	
	EOS/MM2	0.00	0.00	0.00	2.22	0.00	0.44
	MC/MM2	19.05	48.39	2.70	15.56	33.33	23.81

6625/83

EOSINOPHILS AND MAST CELLS IN RESECTED OESOPHAGUS

DIAGNOSIS ACHALASIA

	SLIDE:-	662583.00				
	CM ABOVE GOJ	2.00	3.00	4.00	5.00	MEAN
EPITHELIUM	AREA	0.30	0.18	0.45	0.27	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	3.00	2.00	0.00	1.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	10.00	11.11	0.00	3.70	6.20
LAMINA PROPRIA	AREA	0.13	0.26	0.15	0.16	
	EOSINOPHILS	21.00	8.00	8.00	2.00	
	MAST CELLS	5.00	24.00	16.00	22.00	
	EOS/MM2	161.54	30.77	53.33	12.50	64.54
	MC/MM2	38.46	92.31	106.67	137.50	93.73
MUSCULARIS MUCOSA	AREA	0.13	0.10	0.08	0.27	
	EOSINOPHILS	9.00	0.00	0.00	1.00	
	MAST CELLS	12.00	11.00	8.00	23.00	
	EOS/MM2	69.23	0.00	0.00	3.70	18.23
	MC/MM2	92.31	110.00	100.00	85.19	96.87
SUBMUCOSA	AREA	0.37	0.21	0.40		
	EOSINOPHILS	0.00	2.00	0.00		
	MAST CELLS	11.00	14.00	3.00		
	EOS/MM2	0.00	9.52	0.00		3.17
	MC/MM2	29.73	66.67	7.50		34.63
CIRCULAR MUSCLE	AREA	0.41	0.62	0.49	0.46	
	EOSINOPHILS	0.00	0.00	0.00	0.00	
	MAST CELLS	23.00	39.00	26.00	15.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00
	MC/MM2	56.10	62.90	53.06	32.61	51.17
AUERBACH'S	AREA	0.23	0.26	0.30	0.24	
	EOSINOPHILS	6.00	4.00	0.00	0.00	
	MAST CELLS	12.00	22.00	17.00	13.00	
	EOS/MM2	26.09	15.38	0.00	0.00	10.37
	MC/MM2	52.17	84.62	56.67	54.17	61.91
LONGITUDINAL M.	AREA	0.63	0.49	0.31	0.46	
	EOSINOPHILS	0.00	0.00	0.00	1.00	
	MAST CELLS	37.00	16.00	10.00	17.00	
	EOS/MM2	0.00	0.00	0.00	2.17	0.54
	MC/MM2	58.73	32.65	32.26	36.96	40.15

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EOSINOPHILS AND MAST CELLS IN RESECTED OESOPHAGUS

DIAGNOSIS ACHALASIA 8461/84

	SLIDE:-	A	C	D	E	F	G	DA	MEAN
	CM ABOVE GOJ	1	7	14	18	23	25	26	
EPITHELIUM	AREA	0.13	0.23	0.21	0.25	0.15	0.16	0.24	
	EOSINOPHILS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
	MAST CELLS	1.00	0.00	0.00	1.00	2.00	0.00	1.00	
	EOS/MM2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	MC/MM2	7.69	0.00	0.00	4.00	13.33	0.00	4.17	4.17
LAMINA PROPRIA	AREA	0.18	0.12	0.22	0.04	0.07	0.09	0.07	
	EOSINOPHILS	5.00	13.00	119.00	1.00	7.00	1.00	2.00	
	MAST CELLS	4.00	5.00	10.00	8.00	3.00	10.00	14.00	
	EOS/MM2	27.78	108.33	540.91	25.00	100.00	11.11	28.57	120.24
	MC/MM2	22.22	41.67	45.45	200.00	42.86	111.11	200.00	94.76
MUSCULARIS MUCOSA	AREA	0.16	0.10	0.18	0.09	0.08	0.07	0.12	
	EOSINOPHILS	2.00	2.00	23.00	0.00	0.00	0.00	0.00	
	MAST CELLS	2.00	2.00	15.00	1.00	12.00	9.00	25.00	
	EOS/MM2	12.50	20.00	127.78	0.00	0.00	0.00	0.00	22.90
	MC/MM2	12.50	20.00	83.33	11.11	150.00	128.57	208.33	87.69
SUBMUCOSA	AREA	0.36	0.18	0.47	0.45	0.51	0.51	0.42	
	EOSINOPHILS	0.00	0.00	17.00	1.00	5.00	0.00	2.00	
	MAST CELLS	7.00	5.00	18.00	37.00	27.00	11.00	16.00	
	EOS/MM2	0.00	0.00	36.17	2.22	9.80	0.00	4.76	7.57
	MC/MM2	19.44	27.78	38.30	82.22	52.94	21.57	38.10	40.05
CIRCULAR MUSCLE	AREA	0.43	0.30	0.38	0.51	0.46	0.41	0.25	
	EOSINOPHILS	0.00	0.00	2.00	1.00	0.00	0.00	0.00	
	MAST CELLS	1.00	1.00	2.00	11.00	10.00	14.00	2.00	
	EOS/MM2	0.00	0.00	5.26	1.96	0.00	0.00	0.00	1.03
	MC/MM2	2.33	3.33	5.26	21.57	21.74	34.15	8.00	13.77
AUERBACH'S	AREA	0.44	0.35	0.18	0.10	0.10	0.09	0.21	
	EOSINOPHILS	0.00	0.00	9.00	33.00	3.00	0.00	3.00	
	MAST CELLS	15.00	30.00	12.00	20.00	13.00	6.00	3.00	
	EOS/MM2	0.00	0.00	50.00	330.00	30.00	0.00	14.29	60.61
	MC/MM2	34.09	85.71	66.67	200.00	130.00	66.67	14.29	85.35
LONGITUDINAL M.	AREA	0.20	0.52	0.23	0.32	0.43	0.17	0.27	
	EOSINOPHILS	0.00	0.00	0.00	5.00	5.00	0.00	7.00	
	MAST CELLS	9.00	16.00	13.00	14.00	6.00	7.00	3.00	
	EOS/MM2	0.00	0.00	0.00	15.63	11.63	0.00	25.93	7.60
	MC/MM2	45.00	30.77	56.52	43.75	13.95	41.18	11.11	34.61

Appendix 4

Clinical details for patients with blood samples.

Key to abbreviations used in the tables.

S = Simultaneous contractions

R = Repetetive contractions

Sp = Spontaneous contractions

P = Peristaltic contractions

Lower oesoophageal sphincter (LOS)

Relaxation

F = Full

I = Incomplete

N = Nonrelaxing

( ) = feature occurs occasionally

All pressures quoted are in mmHg

PATIENTS WITH BLOOD SAMPLES TAKEN FOR EOSINOPHIL COUNT AND IgE

Hospital Number	DIAGNOSIS	Age	Sex	History months	Pain	Dysphagia	Regurgitation	Allergy/ atopy	Barium swallow	Manometry Body	Manometry Amplitude	LOS Pressure	LOS Relaxation	pH STUDIES	ENDOSCOPY
609796	Achalasia	45	m	5	no	yes	yes	no	distal narrow, prox dilatation	S,R	weak	26	N	no	no
629495	Achalasia	36	f	15	yes	yes	yes	no	poor motility	S,R	weak	44	N	no	
615124	Achalasia	52	f	24	no	yes	yes	no	achalasia	S,R		27			
613135	Achalasia	42	f	18	no	yes	yes	no	dilated	S,R	weak	32			
243752	Achalasia	28	m	60	yes	yes	yes	no	bird's beak	S,R		31	N	?	?
508123	Achalasia	72	m	36	no	yes	yes	?	dyskinesia, reflux	S,Sp	<40	13	I	no	food residue, no oesophagitis
615129	Achalasia	40	m	11	yes	yes	yes	keflex	rat-tailed stricture	S,R	weak	20	N	?	residue, oesophagitis
227454	Achalasia	31	f	48	no	yes	yes	no	dilated, atonic	S, rhythmic		21	N	no	
Z9137971	Achalasia	20	f	24	no	yes	no	no	achalasia	S,R	weak	35	N	no	normal
z9136439	Achalasia	42	m	9	yes	no	no	asthma	distal taper, dilated	S,R	weak	40	N	?	?
616992	Achalasia	39	m	18	no	yes	no	no	dilated, distal holdup	no	no		no	no	normal mucosa
622805	Achalasia	57	m	48	no	yes	yes	no	dilated, spasm, diverticulum	S,R	weak	18	N	no	?
471451	Achalasia	21	f	11	no	yes	yes	no	holdup, fluid level	immotile		33	N		normal
Z182310	Achalasia	49	f	20	no	yes	no	no	achalasia	no					
605102	Achalasia	47	f	30	no	yes	yes	eczema	achalasia	S,R	weak	13	N	no	normal
613730	Achalasia	56	f	24	yes	yes	no	no	diverticulum	S,	weak	22	N	no	midoesophageal diverticulum
397980	Achalasia postop	49	f	49	yes	yes	yes	no	distal narrow,no holdup			7			oesophagitis
	MEAN	43										25.4667			
AU	Control	62	f		no	no	no	no							
LM	Control	41	m		no	no	no	no							
MB	Control	64	f		no	no	no	no							
IS	Control	63	m		no	no	no	no							
JD	Control	48	f		no	no	no	no							
PW	Control	56	f		no	no	no	hay fever							
SH	Control	54	f		no	no	no	no							
IB	Control	56	f		no	no	no	no							
442392	Control	38	f		no	no	no	no							
DB	Control	64	m		no	no	no	no							
SL	Control	56	f		no	no	no	no							
602767	Control	57	m		no	no	no	no							
		55													

PATIENTS WITH BLOOD SAMPLES TAKEN FOR EOSINOPHIL COUNT AND IgE

Hospital Number	DIAGNOSIS	Age	Sex	History months	Pain	Dysphagia	Regurgitation	Allergy/ atopy	Barium swallow	Manometry Body	Manometry Amplitude	LOS Pressure	LOS Relaxation	pH STUDIES	ENDOSCOPY
559921	DOS	28	f	36	yes	yes	no	no		S,Sp,R		24	F(l)		normal
616994	DOS	74	m	6	no	yes	yes	no	dilated,tertiary contractions	S	high	29	N	1.3	normal, HH
76976	DOS	60	f	32	yes	yes	yes	hay fever	normal	S,Sp,	normal/low	23	I	3.4	normal
DOB26134	DOS	57	m	56	yes	yes	yes	no	distal narrow, incoordination	S,Sp,R		8	I	no	normal
593841	DOS	66	m	6	no	yes	yes	no	diverticulum, incoordination	S,Sp	250	16	F(l)	no	normal
556307	DOS	66	m	36	no	yes	yes	mould, dust mite	diverticulum,spasm	S		48	I	no	residue, normal mucosa
631814	DOS	24	f	24	no	yes	yes	no	tertiary contractions	S,P	some high	14	I(F)	0	normal
508263	DOS	53	m	72	yes	no	no	no	HH, tertiary contractions	S	100	12	(l)	0	oesophagitis, ulcer
	DOS	53	f	48	yes	no	no	no		S,(Sp)		10	F	3	normal
385126	DOS	46	f	36	yes	yes	yes	asthma	normal	S,R,Sp		11	F	no	no
DOB71130	DOS	62	f	48	yes	yes	no	no	normal	S,R		12			
446821	DOS	56	f	60	yes	no	no	no	incoordination	P,R,S	250	11	(l)		
540462	DOS	61	f	48	yes	yes	no	no	normal	S,	110	10	(l)	1.1	normal
508581	DOS	44	m	108	yes	yes	no	no	holdup mid third	S,R,P		18		7.1	normal
578234	DOS	36	f	60	no	yes	yes	eczema	retrograde peristalsis	S,R		25	I	no	normal
Z9140791	DOS	52	m	24	yes	yes	yes	yes	normal	S,Sp		5	F	no	dilated,corkscrew
599508	DOS	45	f	60	yes	yes	no	septrin	normal	S, Sp		6	F	5.9	normal
574214	DOS	59	f	42	yes	yes	no	no	diverticula	S,Sp		14	F(l)	no	norml
595642	DOS	53	m	30	yes	no	no	no	HH, reflux	R,S		21	I	3.2	normal
608559	DOS	51	m	32	no	yes	no	no	HH, reflux	P,S,Sp,R		12	I	2.6	normal,distal narrow
291405	DOS POSTOP	59	m	96	no	yes	yes	no	normal	S,R		19	I	no	?
482490	DOS POSTOP	62	m	60	yes	yes	yes	no	normal	S,R		28	I	no	normal
505495	DOS POSTOP	45	f	17	yes	yes	no	no	normal	S,Sp,R		16	F(l)	0.6	normal
424868	DOS POSTOP	39	f	6	yes	yes	no	no	midoesophageal diverticulum	S,Sp	up to 250	9	I	failed	normal
418918	DOS POSTOP	33	m	30	yes	yes	no	no	HH, tertiary contractions	S,R,Sp		9	F	no	normal
		51										15.4762			

PATIENTS WITH BLOOD SAMPLES TAKEN FOR EOSINOPHIL COUNT AND IgE

Hospital Number	DIAGNOSIS	Age	Sex	History months	Pain	Dysphagia	Regurgitation	Allergy/ atopy	Barium swallow	Manometry Body	Manometry Amplitude	LOS Pressure	LOS Relaxation	pH STUDIES	ENDOSCOPY
577343	HH	38	m	24	yes	no	no	penicillin	HH	P	normal	8	F	16.6	oesophagitis
593208	HH	59	m	19	yes	yes	yes	no	normal	P	normal	11	F	10.5	normal
159718	HH	61	f	24	yes	yes	no	no	HH, reflux	no	no		no	4	oesophagitis, ulcer, stricture
609526	HH	40	m	12	yes	yes	yes	no	HH, reflux	P	weak	1	F	25	oesophagitis
359881	HH	37	f	84	yes	no	yes	anaphylaxis	HH, reflux	P	low distal	4	F	23.5	oesophagitis
491464	HH	37	m	36	yes	no	no	no	HH,reflux	no record					
562154	HH		m	48	yes	yes	no	no	HH, reflux	P	low	0		24.2	ulceration
504977	HH	72	f	60	yes	yes	yes	no	paraoesophageal H	no	no		no		no
558154	HH	38	f	48	yes	no	yes	no	HH	P	normal	8	F	23.3	
316970	HH	40	m	12	yes	no	yes	no	?	P	low	2		18.7	oesophagitis
348842	HH	45	m	24	yes	no	yes	hay fever	reflux, no HH	S	weak	1	normal	19.5	oesophagitis
255434	HH	37	m	24	yes	no	yes	asthma	HH,reflux	P,Sp distal	weak	6	F	11.2	normal
491414	HH	66	M	120	yes	yes	yes	no	HH, reflux	no	no		no	17.2	Barrett's ulcer
605007	HH	32	m	60	yes	yes	yes	no	reflux	yes				19.4	HH, no oesophagitis
432781	HH	62	m	36	no	no	yes	no	reflux,HH	P	weak	6	normal	15.5	normal
629493	HH	47	m	60	yes	no	yes	no	reflux	no	no		no	23.7	normal
290366	HH (Barretts)	53	m	12	yes	no	no	aspirin	HH,reflux	P	normal	4	F	24.4	Barrett's,oesophagitis
599699	HH (Barretts)	45	m	48	yes	yes	yes	no	HH	no	no		no	19	oesophagitis
661343	HH (Barretts)	18	m	60	yes	yes	yes	no	mid third stricture	no	no		no		laser Rx
		46										4.63636		18.48125	
379058	Nutcracker	54	m	48	yes	yes	yes	no	normal	P	300	36	I	no	normal
532987	Nutcracker	39	f	96	yes	yes	yes	no	?	P	High	11	F	2.2	normal
31400	Nutcracker	68	m	36	yes	no	yes	no	reflux	P	high	normal		3.6	normal
368960	Nutcracker postop	50	m	72	yes	yes	yes	eczema	tertiary,HH	P,S	200	13	F		normal
291518	Nutcracker postop	68	f	120	yes	yes	no	no	HH, reflux,dyskinesia	P	175	11	I(F)	3.2	normal
		56										17.7		3	
584854	Vigorous achalasia	56	f	48	yes	yes	no	no	distal holdup	S,Sp		33	I	no	normal
669296	Vigorous achalasia	32	f	40	no	yes	yes		dilated,poor emptying,spasm	S,Sp	weak?35	30	N	no	normal
		44										31.5		no	

Appendix 5

Eosinophil counts in oesophageal motility disorders

Identification/ Hospital No.	DIAGNOSIS	Diagnostic category	Eosinophil counts
LM	CONTROL	1	0.16
AU	CONTROL	1	0
DB	CONTROL	1	0.18
SH	CONTROL	1	0.02
RM	CONTROL	1	0.04
MB	CONTROL	1	0.28
JD	CONTROL	1	0.11
PW	CONTROL	1	0.39
IB	CONTROL	1	0.05
442392	CONTROL	1	0.36
661343	HERNIA(BARRETTS)	2	0.05
432781	HERNIA	2	0.85
255434	HERNIA	2	0.3
316970	HERNIA	2	0.44
359881	HERNIA	2	0.01
504977	HERNIA	2	0.05
491414	HERNIA	2	0.33
629493	HERNIA	2	0.33
159718	HERNIA	2	0.11
609526	HERNIA	2	0.15
593208	HERNIA	2	0.13
599699	HERNIA(BARRETT'S)	2	0.1
616992	ACHALASIA	3	0.09
626227	ACHALASIA	3	0.23
612128	ACHALASIA	3	0.06
615129	ACHALASIA	3	0.1
613135	ACHALASIA	3	0.07
613730	ACHALASIA	3	0.12
609796	ACHALASIA	3	0.05
471451	ACHALASIA	3	0.19
605102	ACHALASIA	3	0.13
Z182310	ACHALASIA	3	0.53
629495	ACHALASIA	3	0.03
615124	ACHALASIA	3	0.03
227454	ACHALASIA POSTOP	3	0.1
243752	ACHALASIA	3	0.2
7/11/30	DOS	4	0.08
559921	DOS	4	0.08
446821	DOS	4	0.07
508581	DOS	4	0.15
540462	DOS	4	0.06
Z9140791	DOS	4	0.05
608559	DOS	4	0.04
574214	DOS	4	0.03
578234	DOS	4	0.08
595642	DOS	4	0.45
385126	DOS	4	0.45
76976	DOS	4	0.27
593841	DOS	4	0.22
26/1/34	DOS	4	0.29
30/1/39	DOS	4	0.18
505495	DOS POSTOP	4	0.11
418918	DOS POSTOP	4	0.18
291405	DOS POSTOP	4	0.11
424868	DOS POSTOP	4	0.22
482490	DOS POSTOP	4	0.08
556307	DOS	4	0.63
631814	DOS	4	0.16
532987	NUTCRACKER	4	0.12
379058	NUTCRACKER	4	0.14
11/7/23	NUTCRACKER	4	0.14
368960	NUTCRACKER POSTOP	4	0.13
584854	VIG ACHALASIA	4	0.13
669296	VIG ACHALASIA	4	0.22
508263	VIG ACHALASIA	4	0.04

Appendix 6

Results of Serum IgE estimations.

Identification HOSP.NO.	DIAGNOSIS	Diagnostic category	Eosinophil count	IgE
DB	CONTROL	1	0.18	100
IS	CONTROL	1		170
RM	CONTROL	1	0.04	140
AU	CONTROL	1	0	1
MB	CONTROL	1	0.28	25
442392	CONTROL	1	0.36	45
SH	CONTROL	1	0.02	0.1
SL	CONTROL	1		17
IB	CONTROL	1	0.05	12
JD	CONTROL	1	0.11	5
255434	HERNIA	2	0.3	175
609526	HERNIA	2	0.15	6
629493	HERNIA	2	0.33	25
316970	HERNIA	2	0.44	5
491464	HERNIA	2	<.7	5
577343	HERNIA	2		5
359881	HERNIA	2	0.01	970
562154	HERNIA	2		60
432781	HERNIA	2	0.85	760
593208	HERNIA	2	0.13	50
558154	HERNIA	2	<.7	22
599699	HERNIA(BARRETT'S)	2	0.1	170
661343	HERNIA(BARRETTS)	2	0.05	840
290336	HERNIA(BARRETTS)	2		725
Z9137971	ACHALASIA	3	<.7	16
605102	ACHALASIA	3	0.13	5
243752	ACHALASIA	3	0.2	30
615129	ACHALASIA	3	0.1	13
616992	ACHALASIA	3	0.09	17
613135	ACHALASIA	3	0.07	25
612128	ACHALASIA	3	0.06	30
615124	ACHALASIA	3	0.03	30
Z182310	ACHALASIA	3	0.63	30
609796	ACHALASIA	3	0.05	40
613730	ACHALASIA	3	0.12	40
629495	ACHALASIA	3	0.03	20
471451	ACHALASIA	3	0.19	2390
z9136439	ACHALASIA	3	<.7	675
626227	ACHALASIA	3	0.23	105
508123	ACHALASIA	3	<.7	70
227454	ACHALASIA POSTOP	3	0.1	8.5
397980	ACHALASIA POSTOP	3	<.7	45
593841	DOS	4	0.22	15
446821	DOS	4	0.07	60
599508	DOS	4	<.7	10
385126	DOS	4	0.45	910
616994	DOS	4	<.7	90
76976	DOS	4	0.27	11
559921	DOS	4	0.08	105
30/1/39	DOS	4	0.18	120
540462	DOS	4	0.06	6
595642	DOS	4	0.45	145
Z9140791	DOS	4	0.05	6
26/1/34	DOS	4	0.29	875
508581	DOS	4	0.15	535
578234	DOS	4	0.08	4
631814	DOS	4	0.16	455
574214	DOS	4	0.03	25
7/11/30	DOS	4	0.08	4.9
21/11/38	DOS	4	0.04	115
608559	DOS	4	0.04	7840
424868	DOS POSTOP	4	0.22	195
482490	DOS POSTOP	4	0.08	9
291405	DOS POSTOP	4	0.11	915
418918	DOS POSTOP	4	0.18	465
379058	NUTCRACKER	4	0.14	130
L31400	NUTCRACKER	4	0.14	1
532987	NUTCRACKER	4	0.12	70
368960	NUTCRACKER POSTOP	4	0.13	1
291518	NUTCRACKER POSTOP	4	<.7	4.3
684854	VIG ACHALASIA	4	0.13	9.9

Appendix 7

Clinical details and data for ECP RIA.

Patient ID	Diagnosis	Age	Sex	Gamma Count	ECP concentration micrograms/l
IS	Control	63	M	7849	11
JD	Control	48	F	8789	8.3
SL	Control	56	F	9083	7.5
DB	Control	52	M	3923	64
MB	Control	64	F	6922	13.6
LM	Control	41	M	5242	38.4
BD	Control	64	M	5955	24.6
IB	Control	56	F	7765	11.2
RM	Control	57	M	6649	14.4
SH	Control	54	F	7241	12.7
Mean		55.5			20.57
418918	DOS	42	M	5742	28.7
7/11/30	DOS	62	F	9225	7.1
291405	DOS	59	M	7375	12.3
446821	DOS	57	F	9221	7.1
Z914071	DOS	59	M	8921	8
JW	DOS	53	F	8001	10.6
578234	DOS	56	F	8760	8.4
593841	DOS	66	M	6638	14.4
631814	DOS	25	F	6912	13.6
76976	DOS	47	F	3668	70.1
26/1/34	DOS	56	M	5617	31.1
368960	DOS	57	M	5899	25.7
424868	DOS	42	F	3156	78.9
508851	DOS	45	M	4139	59.8
Mean		51.86			36.73
CONTROLS					
				11024	2
				9980	5
				6451	15
				2069	100
				1336	200

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Each year a paper is chosen for this award from all those published in the Journal of Histotechnology. The selected paper is judged to be original in concept and of interest to the readers.

Combined Staining Method for the Demonstration of Tissue Eosinophils and Mast Cells

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Abstract

Quantitation of mast cells and eosinophils is of value in studies of allergic or parasitic tissue inflammation. We describe a new method that combines two reliable stains, astra blue and vital new red, to stain both mast cells and eosinophils in the same tissue section. The mast cells stain violet-blue and the eosinophils bright red. Because this method stains both cells in tissue that has been fixed with formol saline or buffered formaldehyde, it can be used on archival tissue that has undergone standard fixation. The minimal staining of the background makes quantitative studies of both cells simple, even at low magnification. (*The J Histotechnol* 16:143, 1993)

Key words: astra blue stain, eosinophils, mast cells, vital new red stain

Introduction

Both mast cells and eosinophil polymorphonuclear leukocytes (eosinophils) are important effector cells in the tissue response to parasitic infestation and in allergic inflammation. Both mast cells and eosinophils may accumulate in the tissues under such circumstances, and it is of interest to demonstrate both cell types in the same tissue section. In quantitative studies, specific staining of mast cells and eosinophils with minimal background staining is required.

Astra blue is a highly specific and reliable stain for mast cells with virtually no background staining (1). Eosinophils can be stained specifically with vital new red, again with minimal background staining (2,3). In studies of the inflammatory infiltrate in benign esophageal disease, we have combined both stains to produce a technique that is of value in studies of these cells in routinely processed tissue.

Materials and Methods

Solutions

Astra Blue (1)

1. Dissolve 2 gm magnesium chloride (hydrated) and 0.1 gm pararosaniline hydrochloride (CI 42500; Sigma Chemical Co, St Louis, MO; potential carcinogen—handle with appropriate precautions) in 80 ml of 95% ethanol.
2. Dissolve 0.5 gm astra blue (#34195; Merck Led, Poole, England) in 10 ml distilled water; mix with the pararosaniline solution.
3. Add 12 N hydrochloric acid drop by drop until the color changes from purple to violet, then to royal blue. The amount needed is approximately 9 ml.
4. Allow the solution to settle for 1 hr, then filter. If the color shifts toward violet on storage, add a few drops of 12 N hydrochloric acid to restore the royal blue tint. We have found the solution can be used for up to 6 mo when stored at room temperature in an airtight container.

Vital New Red (2)

A 0.1 gm/L solution of vital new red (CI 25380; Pfaltz and Bauer, Waterbury, CT; UK Distributors—Phase Separations sales, Clwyd) is made in 50% ethanol (deionized water). The solution is filtered and will remain stable for more than 6 mo if stored at room temperature in an airtight bottle.

Staining Procedure

The following method is used for formalin fixed, paraffin-embedded tissue. Sections are cut at 3 μm thickness.

1. Dewax tissue sections in xylene and bring to 95% ethanol.
2. Stain with astra blue solution for 30 min.
3. Rinse in tap water to remove excess stain.
4. Stain with vital new red solution for 30 min.
5. Rinse in tap water to remove excess stain.
6. Counterstain with Mayer hemalum for 5–10 sec.
7. Blue in Scott tap water substitute for 10 sec.
8. Dehydrate, clear, and mount in synthetic mounting medium.

Results

Eosinophils stained a bright red, and mast cells stained a violet/dark blue color (Figure 1). They were easily distinguished from each other and from other tissue cells that do not take up the stains. Both cells stood out against the minimal

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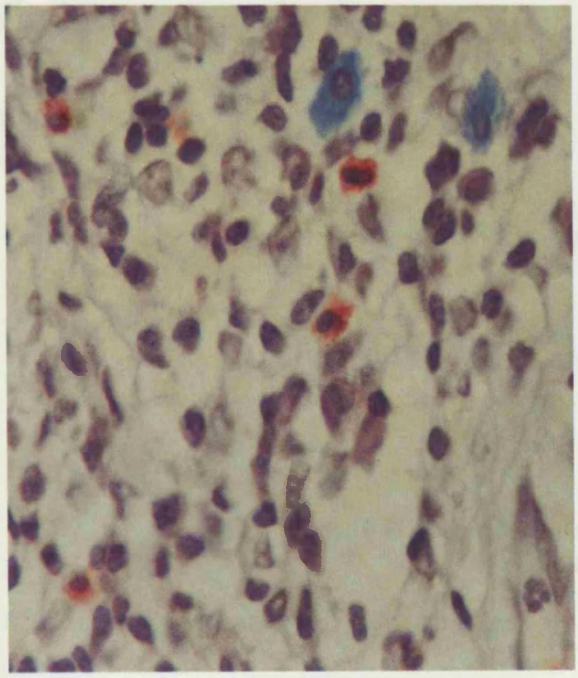

Figure 1. Eosinophils (red) and mast cells (blue) in the lamina propria of human esophagus (stained with astra blue and vital new red with hemalum counterstain). Original magnification × 800.

background staining, making counting possible at low magnification, × 400 and less.

Discussion

Although mast cells and eosinophils can be stained specifically with immunohistochemical stains, these techniques can be expensive, difficult, and time consuming. To the best of our knowledge, sequential immunohistochemical staining of both cells has not been described. Recent quantitative studies of mast cells and eosinophils in ulcerative colitis and asthma have used standard histochemical stains on separate sections of tissue (4,5). This increases the workload associated with the studies significantly and does not allow the tissue distribution of the two types of cell to be compared in the same section.

In two recent studies both cell types were stained and quantitated on the same tissue sections. Fisher et al, in a study on eosinophils and mast cells in rectal cancer, used the Giemsa stain and counted under oil immersion (× 1000) (6). Marsh et al used toluidine blue to stain glutaraldehyde fixed tissue from patients with celiac sprue (7). The cell staining depended on metachromasia of the dye, and the cells were again counted under oil immersion (× 1000). In both these methods the cells were not easily distinguished except at high magnification. The method we described results in easily distinguishable cells and a pale background, so counting can be performed at much lower magnification (× 400 and below), making quantitation easier.

Ball and Hay used alcian blue, naphthalene black, chromotrope 2R, and nuclear fast red to demonstrate eosinophils and mast cells in tissue sections containing helminths (8). Eosinophils stained blue-black, mast cells turquoise, and connective tissue grey to light pink. However, their staining technique is more complex than the one we describe.

Fixation is critical in the staining characteristics of mast cells, with mucosal mast cells more sensitive to the fixation technique than connective tissue mast cells. Carnoy solution has been recommended for fixation of mast cells for studies in the human (9). However, eosinophils do not stain well with Carnoy solution, and tissue fixed this way is not suitable for simultaneous staining of both cells. For staining of both mast cells and eosinophils in the same section, Newlands et al found 4% paraformaldehyde as a fixative allows both populations to be stained in rat tissue (10). However, their method precludes examination of retrospective material that has not been processed with paraformaldehyde. Our initial experience suggests that the astra blue/vital new red stain is just as effective on tissue fixed with formaldehyde based fixatives as it is when the tissue is fixed with Carnoy solution.

We believe the combination of astra blue and vital new red has many advantages over the stains described. The staining technique is simple and reliable and the reagents are stable at room temperature. The staining is apparently specific and the background staining minimal so that the cells can be counted at magnifications of × 400 and less. The distinct colors of the two cells make it easy to distinguish one from another. This is an ideal sequential staining technique for quantitative studies of eosinophils and mast cells in routinely processed tissue and can be used on both retrospective and prospective material.

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