ACQUIRED IMMUNE RESPONSES IN THE LUNG:

THEIR RELEVANCE TO BRONCHIECTASIS

by

JOSE' ROBERTO LAPA E SILVA

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

in the

UNIVERSITY OF LONDON

This work was carried out at the Host Defence Unit, Department of Thoracic Medicine, National Heart and Lung Institute, Royal Brompton National Heart & Lung Hospital Chelsea, and at the Academic Department of Clinical Immunology, Royal Free Hospital School of Medicine, London.

March 1991.

This thesis is dedicated to

MOYSES AND EDITH, my parents

JUCELIA, my beloved wife

ALESSANDRA, LEANDRO, IURI, NATALIA, my children

COLIN, MARCELO, FABIO, GUSTAVO, son-in-law and nephews

for their love and support during the years of hard work

PROF. NEWTON BETHLEM, model of physician, scientist, and academician, for his lifelong incentive.

A B S T R A C T

Bronchiectasis consists of irreversible dilatation of one or more bronchi, usually manifested as persistent expectoration of purulent, infected sputum. The question as to whether local cellular immune responses in the bronchial wall might contribute to the inflammation associated with bronchiectasis was addressed by comparing samples of bronchial mucosa from bronchiectasis patients with those from controls by immunohistochemical methods. Intense infiltration of mononuclear cells was seen in bronchi from all bronchiectasis samples and was organised into follicles in half of them. The major component of the infiltrates was the T-lymphocyte but increments of monocyte/macrophages were also seen. B-lymphocytes were only present in samples displaying follicles. In the majority of samples the predominant T cell subset was the CD8+. The T cells co-expressed activation markers and CD45RO antigens. These findings suggest that in bronchiectasis a bronchial cellular immune reaction forms part of the inflammatory response.

Further studies were performed using a new experimental model of bronchiectasis in the pseudomonas infected rat, in which the bronchial dilatation and histopathology are similar to human bronchiectasis. An intense inflammatory reaction in the bronchial wall preceeded and accompanied the development of the bronchiectasis, the main component of which at the height of the inflammation was the T-lymphocyte. Initially, infiltration of T cells was seen in the bronchus-associated lymphoid tissue. In peribronchial areas the number of T cells increased up to 8 weeks after the induction, with predominance of the CD8+ subset. The number of macrophages also increased, particularly in the peribronchial areas. Increments in the expression of activation markers on T cells also reflected the human disease. None of these changes were

seen in control rats, suggesting that, as in the human disease, a cell-mediated immune reaction paralleled the development of experimental bronchiectasis. In rats with bronchiectasis, significant increase in footpad thickness was seen 48 hours after injection of pseudomonas antigens suggestive of a type IV hypersensitivity reaction. Mononuclear cells were isolated from rat lungs and tested in cytotoxic assays against target cells and exhibited increased specific cytotoxicity when compared with controls.

The findings described in this thesis suggest that acquired immune mechanisms could play a role in the establishment and progression of bronchiectasis.

A C K N O W L E D G E M E N T S

I am grateful to the Brazilian Research Council (CNPq) and the Federal University of Rio de Janeiro, Brazil, for their financial support.

It has been a privilege to work under the supervision of Prof. Peter J. Cole at the Host Defence Unit, Department of Thoracic Medicine, National Heart and Lung Institute, Royal Brompton National Heart & Lung Hospital Chelsea, from where all specimens employed in this research originated, and under the supervision of Dr. Leonard W. Poulter at the Academic Department of Clinical Immunology, Royal Free Hospital School of Medicine, where all bench work was performed. I am also indebted to Prof. George Janossy, Head of the Dept. of Immunology, for allowing me to use all the resources of the Department.

I am grateful to Drs. M. Smith, M. Finnegan, J. Goldman, Monica Spiteri, and Margaret Johnson for performing the bronchoscopic biopsies used in the study, and also to Drs. C. Steinfort, D. Currie, N. Munro, and Mr. P. Goldstraw for allowing access to tissue specimens.

I am grateful to Dr. E. Sheffield for performing the dissection of bronchiectasis samples obtained by lung resection.

I am grateful to Mr. D. Guerreiro, who devised the model of experimental bronchiectasis used in this research, for allowing me to use his model and for operating on most of the animals used in this study, and also to Drs. N. Munro and Philippa Roberts for operating on the remaining animals.

I am also grateful to Mr. H. Todd for performing the bacteriological studies and preparation of pseudomonas antigens used in the animal model, and to Mrs. Juliet Rohde for staining the rat lungs

for elastin and preparing the conventional histology of footpad biopsies.

I am indebted to Prof. Bernice Noble for invaluable advice in the early stages of preparation and staining of tissue from the experimental model, to Ms. Deirdre Brennan for advice regarding collagenase digestion of tissue, to Dr. A. Akbar for advice regarding cytotoxic assay, and to Dr. D. Campana for advice regarding the intraepithelial lymphocyte study. I am also grateful to Mr. K. Sullivan for advice regarding statistical analysis.

Finally, I am grateful to all technical staff of the Dept. of Immunology of the Royal Free Hospital School of Medicine, especially to Ms. Aida Condez and to all colleagues from both hospitals for their constant support, and to all colleagues from the Thoracic Medicine Unit at the University Hospital, Federal University of Rio de Janeiro, for their extra work during my years of absence.

I hereby declare that, apart from the assistance acknowledged above, all scientific procedures, studies and statistical analysis used in this thesis were performed by me.

C O N T E N T S

ABBREVIATIONS

ACP - acid phosphatase

BALT - bronchus-associated lymphoid tissue

CD - cluster of differentiation

CF - cystic fibrosis

CFU - colony-forming unit

Cr - sodium chromate

DAB - diaminobenzidine

DNA - deoxyribonucleic acid

DNAase - deoxyribonuclease

DPX - distrene-plasticizer-xylene

EP - epithelium

FCS - fetal calf serum

FITC - fluorescein isothiocyanate

HBSS - Hanks balanced salt solution

HLA-DR - human leukocyte antigen - D region

IEL - intraepithelial lymphocyte

Ig - immunoglobulin

IL-2R - interleukin-2 receptor

LCA - leucocyte common antigen

LP - lamina propria

M (cell) - microfold

MNC - mononuclear cell

MoAb - monoclonal antibody

N - age-matched normal control group of rats

n - number of samples

ND - not done

NS - statistically not significant

OCT - optimum cutting tmperature

P. aeruginosa - Pseudomonas aeruginosa

Pa + LIG - partial bronchial ligation and distal intrabronchial injection of P. aeruginosa

Pa + NOLIG - intrabronchial injection of P aeruginosa without bronchial ligation

PBS - phosphate buffered saline

PBS + LIG - partial bronchial ligation and injection of sterile PBS

PCD - primary ciliary diskinesia

PHA - phytohaemgglutinin

RSV - respiratory syncytial virus

S - sham-operated animals

SD - standard deviation

SPF - specific-pathogen free

TCR - T cell antigen receptor

TRITC - tetramethylrhodamine isothyocianate

u - micron

ul - microlitre

v/v - volume to volume

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

C H A P T E R 1

A BACKGROUND TO BRONCHIECTASIS

1.1 - HISTORY AND DEFINITIONS

Bronchiectasis is a chronic bronchopulmonary disease characterized by irreversible dilatation of one or more bronchi, usually associated with chronic purulent sputum production. The affected bronchi are the seat of persistent inflammation, which may lead to scarring and shrinkage of the lung and ultimately, in some cases, to cor pulmonale and death or transplantation. The first clinicopathological description of the disease was produced by Rene Laennec, the famous French physician, in his classic treatise "De l'Auscultation Mediate ou Traite du Diagnostique des Maladies des Poumon et du Coeur", published in 1819, which is generally regarded as the point of departure of Chest Medicine as a subspeciality. In this work, apart from describing his invention of a tubular, wooden instrument (forerunner of the modern stethoscope) to be used as an aid to the auscultation of breath and cardiac sounds, he described in chapter II, "Of Dilatation of Bronchi", "...an organic lesion which I am now to notice, seems to have been hitherto entirely overlooked both by the anatomist and the practitioner". The first two cases of what we now know of as bronchiectasis had been brought to his attention in 1808 by Prof. Cayol, to which he added another two cases. In the second edition of his book, published in 1826, the year of his premature death from tuberculosis, a more extended description of the pathology and clinical correlations of the disease was produced. Laennec described the two major types of bronchial dilatation recognized by modern pathologists: cylindrical and saccular.

The term "bronchiectasis" was only introduced in 1846 by Swaine's translation of Hasse's book on diseases of the organs of circulation and respiration (Cole, 1990). Most of the second half of the 19th

century did not see any major advancement in knowledge or management of the disease. In 1896, Killian reported the first successful use of bronchoscopy and in 1907 Chevalier Jackson's improved instruments and techniques allowed the retrieval of foreign bodies lodged in the airways and implicated in the development of bronchiectasis (Lindskog, 1986). The bronchoscope was then also used as a supplement for postural drainage, aiding the aspiration of respiratory secretions. In 1901, Heidenhaim in Germany reported the first successful partial lobectomy for bronchiectasis, opening the field of surgical treatment for the disease (Lindskog, 1986). However, the greatest improvement in the diagnosis and management of bronchiectasis came in 1922 with the introduction of bronchography, the contrasted radiography of the bronchial tree with iodized poppy seed oil (Lipiodol) by Sicard and Forestier. The method allowed for the first time in vivo examination of affected lobes and helped in planning surgical and conservative treatments.

The three decades after the introduction of bronchography may be considered the Golden Age of bronchiectasis, with the publication of a string of classic papers covering all major aspects of the disease. However, the end of the Second World War heralded general improvement in the living standards of the population. The introduction of widespread vaccination against many viral and bacterial diseases implicated in the development of bronchiectasis, and the advent of chemotherapy against tuberculosis and nonspecific bacterial infections, led to a steady decline both in the incidence of the disease and in the interest of physicians and scientists (Barker & Bardana, 1988). Since the classic paper of Whitwell in 1952, no major advance in the

understanding of the pathogenesis of the disease occurred until the early 1980s, when a renewed interest in the disease took place. The identification of a progressive form of the disease (Cole, 1984) which despite the most careful management was sometimes fatal prompted a revival of the interest. More recently, a newly recognized association between bronchiectasis and obliterative bronchiolitis in patients undergoing heart-lung transplantation (Burke, Theodore, Dawkins et al, 1985) has helped to focus more attention on this condition.

The worldwide and local prevalence of the disease in 1990 is unknown. The general impression is that since the 1950s the disease has decreased dramatically. At least the so-called old-fashioned bronchiectasis characterized by copious, foetid purulent sputum, breathlessness and physical disability appears in decline. Lindskog described a steady decline of admissions to the Department of Thoracic Surgery at Yale University due to bronchiectasis from 8.7% of total admissions in the five-year period 1947-1952 to 1.3% in 1967-1968 (Lindskog, 1986). The same was concluded from the data from five paediatric hospitals described by Field in 1969, and was also discussed by Ellis and colleagues (1981). This has led to a low index of clinical suspicion and perhaps to underdiagnosis (Cole, 1989). However, the disease has never ceased to be an important clinical problem in the Third World (Le Roux, Mohlala, Odell et al, 1986).

1.2 - PATHOLOGY OF BRONCHIECTASIS

Since the days of Laennec, bronchiectasis has been defined mainly on morphological grounds, and the pathological finding of irreversible dilatation of a bronchus provides the ultimate diagnosis of the condition. In practical terms, however, the clear correlation between the pathological changes and the radiological findings of bronchography, carefully described by Lynne Reid (1950), and more recently the use of less invasive high-resolution computed tomography (Cooke, Currie, Morgan et al, 1987; Munro, Cooke, Currie et al, 1990), has allowed the management of bronchiectasis without the need for pathological studies.

For the purposes of this thesis, however, the conventional pathology of bronchiectasis is of great importance and this thesis will begin by reviewing the classical ideas and descriptions of the pathology of the condition. In his description of the dilatation of the bronchi, Laennec in 1826 clearly describes the two major types of bronchiectasis as recognized today by modern pathologists - cylindrical and saccular. Over the years many other classifications of bronchiectasis were attempted, mainly based on the shape of the dilated bronchi, with some variations around the two basic types described by Laennec. Ewart, in 1898, proposed three types: regular or cylindrical, fusiform, globular or sacculated (cited by Reid, 1950). Roles and Todd (1933) proposed five types: tubular, early fusiform, late fusiform, fusosaccular, and saccular. In 1950, Lynne Reid simplified these classifications to three basic types by correlating the pathological changes with bronchographic findings: cylindrical, saccular or cystic, and varicose. This classification, although very useful in practical terms, did not correlate the morphology with possible aetiologies and

histopathological changes.

The definitive study of the pathology of bronchiectasis was published in 1952 by Whitwell. More than just a description of forms and sizes, the author concentrated on a comprehensive approach to macropathology and histopathology and their possible correlation with aetiology, and with the pathogenesis of the condition. The large series he studied, with 200 consecutive resection specimens, of which 180 were carefully investigated pathologically and 20 were prepared as neoprene bronchial casts, allowed him to quantitate the main changes and group them according to their main characteristics. One of his important observations was that bronchiectasis is not a single pathological entity but a group of different conditions having bronchial dilatation as a common factor. He was in some way ahead of his time when he stated that, contrary to the belief of many of his contemporary physicians, he regarded bronchiectasis as a destructive inflammatory process. The majority of cases in his series were classified into three types of bronchiectasis: follicular, saccular, and atelectatic. A large number of cases, however, failed to fit into those three types and were left unclassified.

His main contribution to the understanding of the pathogenesis of bronchiectasis was the proposal of a new subgroup - follicular bronchiectasis. Previous authors have mentioned the presence of bronchial follicles in bronchiectasis but their significance has not been studied (Whitwell, 1952). He also noted that the lymphoid tissue, although prominent, was only part of an extensive mural inflammation. The basic histopathological picture of follicular bronchiectasis is of excessive vascularity of the subepithelial tissues, which are thicker

than normal due to oedema, dilatation of the vessels, and aggregation of lymphoid cells. The epithelium is usually ciliated columnar, with various degrees of surface ulceration or squamous metaplasia. The chronic inflammatory infiltration is composed mainly of plasma cells and lymphocytes, and the lymphoid aggregates have the appearance of the follicles of lymph nodes. Some lymphocytic aggregates are found around the opening of mucus gland ducts and, when subepithelial, they cause distortion and partial occlusion of the lumen, and stenosis of the bronchial branchings. Bronchial supporting tissues such as the elastic tissue, muscle and cartilage are affected and sometimes extensively damaged, particularly near the follicles. The elastic tissue is the first of the supporting structures to suffer and this is a fundamental lesion in follicular bronchiectasis.

The second type described by Whitwell, saccular bronchiectasis, is well recognized because its anatomical and bronchographic appearances are very similar. One of his important observations about this type of bronchiectasis was the presence of severe inflammatory changes in the pre-saccular bronchi which, although not bronchiectatic, exhibit striking histopathological changes. The saccular walls show less marked inflammation and are composed of dense fibrous tissue which contains no elastic tissue, muscle or cartilage. He proved that these sacs are a direct continuation of large bronchi.

The third type he called atelectatic bronchiectasis. The pathological findings were varied, from moderate inflammation without any destruction of supporting tissues to severe inflammatory changes with epithelial ulceration and destruction of the supporting tissues.

1.3 - EVOLUTION OF IDEAS ABOUT THE AETIOLOGY AND PATHOGENESIS OF BRONCHIECTASIS

Of all aspects of bronchiectasis, its pathogenesis is the one promoting most confusion and disagreement between authors. Since the early days of Laennec to modern times, conflicting ideas of how bronchiectasis evolves have been put forward. Laennec devoted only a few lines to this theme in his classic book. He apparently conceived the so called dilatation theory, according to which retention of secretions would provoke the bronchus to dilate. He also had some idea about the so called traction theory, which presumes that the organization and resulting contraction of parenchymal exudates, which occur in certain types of pneumonitis, exert a relentless pull and dilate contiguous bronchi (Lindskog, 1986). The dilatation theory proposed by Laennec was discredited by Williams as early as 1838 who argued that most patients with copious sputum do not develop dilatation, and many patients with bronchiectasis have little sputum.

The 20th Century saw many different ideas advanced to explain the formation of bronchiectasis. The real turning point was the now classic dissertation by Whitwell published in 1952, in which he reviewed the main concepts, and proposed a system of ideas based upon the observation of clinical and pathological changes of a large series of cases. Whitwell could not find a pathological basis for the traction theory. He also ruled out the possibility that peripheral bronchial obstruction and alveolar absorption collapse, a much cherished theory of pathogenesis, was implicated in the formation of the bronchial dilatations, because obstruction of peripheral bronchi was always present in cases of follicular and saccular bronchiectasis but absorption collapse was absent in 90% of specimens. In the 10% of

specimens where absorption collapse was found, it affected whole lobes, not lobules or segments.

Whitwell related the advent of follicular bronchiectasis to the occurrence of viral and/or bacterial infections in early childhood, a sequel to pertussis, measles or primary bronchopneumonia. In the case of saccular bronchiectasis, he combined the "traction" and "dilatation" theories to explain the advent of the saccules. Persistent inflammation leads to the replacement of the original bronchial structures by fibrosis and destruction of the supporting tissues. The saccules are then distended by the retained pus, their internal pressure raised by the partial occlusion of the pre-saccular bronchi, thus resulting in permanent damage. In the pathogenesis of atelectatic bronchiectasis he suggested a role for mechanical factors, such as bronchial obstruction.

In summary, according to Whitwell acquired bronchiectasis, which was responsible for more than 90% of cases of his series, would be generated by the combination of different factors, such as infection and obstruction, inflammation, and to a lesser degree, fibrosis associated with traction, and dilatation.

1.4 - MODERN IDEAS ABOUT THE PATHOGENESIS OF BRONCHIECTASIS: THE "VICIOUS CIRCLE" HYPOTHESIS

Between 1980 and 1984 a new hypothesis for the pathogenesis of the disease was proposed by Cole. Over a period of five years, he observed more than 300 patients complaining of daily production of infected sputum and coined the term chronic bronchial sepsis for this condition. Many of these patients proved to have bronchiectasis and the clinical data originating from this group were very intriguing: the patients were relatively young, slightly more frequently women, mainly never- or ex-smokers and often had associated sinusitis and purulent posterior nasal discharge. Culture of their sputa showed the presence of relatively avirulent microorganisms, such as non-capsulated Haemophilus influenzae. The majority of these patients responded to the presence of these microbes in the airways by mounting an exuberant local and systemic immune response, with more than 75% of them possessing raised levels of serum immunoglobulins. He concluded that these patients, rather than presenting a syndrome of classical immunity deficiency, were mounting an appropriate immune response to normaly absent intrabronchial microorganisms, or even an over-response, as suggested by the appearance of auto-antibodies in some or association with autoimmune diseases. A considerable number of patients presented a progressive form of the disease that led to scarring and shrinkage of the lung, and finally cor pulmonale and death (Cole, 1980; 1984).

Based on these observations, Cole disputed the dogma that the microorganisms present in the affected bronchial tree were infecting the patient in an invasive manner such as in pneumonia. He asked the question as to whether the microorganisms in this condition were in fact directly damaging the lung or whether the damage was mediated

indirectely by the host's normally protective inflammatory response to the colonising microbes (Cole, 1984). He then proposed a "vicious circle" hypothesis (Fig. 1.1), by which the lesions seen in bronchiectasis are the result of a sequence of host-mediated biological events stimulated by the avirulent colonising microbes which, although intended to protect the lung's integrity against insults, in fact damage the lung and impair its defence mechanisms. Thus increasing colonisation by microorganisms in turn elicits more, and chronic, host-mediated inflammation - and hence a "vicious circle" with resulting progressive lung damage.

This hypothesis set out a convenient framework for investigating the various components that might play a role in the pathogenesis of the disease. In the few years that have followed the publication of this hypothesis, data has accrued which, in the main, support it.

1.4.1 - INITIAL INSULTS

The "vicious circle" hypothesis supposes that an initial event compromises the first-line sinobronchial defence mechanism of mucociliary clearance, resulting in mucus and microorganisms remaining within the respiratory tract for longer than normal (Cole, 1990). This initial insult could be environmental, including infection, or a primary genetic condition - or an environmental trigger event on the background of a genetic condition.

1.4.1.1 - Infection

For a long time an initial episode of infection has been suspected as associated with bronchiectasis. Large numbers of patients relate the

beginning of their symptoms to a severe episode of viral or bacterial infection, often in the early years of life. This could directly damage bronchial supporting tissues, well exemplified in the case of post-tuberculosis bronchiectasis (Rosenzweig & Stead, 1966) or could act by triggering inflammatory processes in susceptible patients, resulting in progressive disease. Whitwell correlated the onset of the follicular form of bronchiectasis to a previous episode of measles, pertussis or primary pneumonia (Whitwell, 1952), and stressed its histopathological similarity to some forms of adenoviral infections in animals. Becroft (1971) studied the relationship of an outbreak of adenovirus infection with the emergence of bronchiectasis in a group of children in New Zealand. Recent studies employing in situ hybridization failed to find adenovirus in bronchiectasis samples (Hogg, Irving, Porter et al, 1989). However, they used relatively insensitive molecular techniques and use of the polymerase chain reaction is required before this possibility is discounted.

Cole (1990) describes at least five ways by which microorganisms can directly damage host mucociliary clearance. These mechanisms can contribute both to the initial insult and/or to the aggravation of previous damage, with progression of the lesions: 1) inhibition of ciliary function by bacterial compounds such as the phenazine pigments of Pseudomonas aeruginosa (Wilson, Pitt, Taylor et al, 1987) or pneumolysin from Streptoccocus pneumoniae (Steinfort, Wilson, Mitchell et al, 1989); 2) direct toxic effects by some bacterial compounds leading to destruction of ciliated epithelium (Read, Wilson, Rutman et al, 1991); 3) inhibition of mucus transport (Munro, Barker, Rutman et al, 1989); 4) inhibition of ion transport in ciliated epithelium (Stutts, Schwab, Chen et al, 1986); and 5) stimulation of mucus

secretion (Somerville, Rutmsn, Wilson et al, 1991). All these impair the first-line defence mechanism of mucociliary transport, inducing a kind of mechanical obstruction in a similar way to other mechanisms to be discussed below.

1.4.1.2 - Primary Genetic Conditions

In some genetic conditions mucociliary clearance of the bronchial tree is impaired by a defect in a particular component of this first line defence mechanism. The best studied conditions are primary ciliary dyskinesia and cystic fibrosis.

Primary Ciliary Dyskinesia (PCD) is an inherited disorder characterised by a defect in the axonemal structure of the cilia, leading to ciliary dysfunction throughout the body (Rubin, 1988). In approximately half of the patients with PCD, the ciliary defect is associated with bronchiectasis, sinusitis, dextrocardia or situs inversus totalis, which is known as Kartagener's syndrome (Kartagener, 1933). The male patients usually suffer from infertility since the sperm tail structure is similar to that of cilia.

Cystic Fibrosis (CF) is a well studied genetic abnormality, the defective gene responsible for which has been recently cloned (Rommens, Ianuzzi, Kerem et al, 1989). The clinical picture is almost certainly caused by abnormal regulation of epithelial cell ion transport which causes very viscid secretions in the respiratory and gastrointestinal tracts. The abnormally viscid mucus impedes mucociliary transport (Rutland & Cole, 1981), and appears to facilitate microbial colonisation of the bronchial tree, particularly with Pseudomonas aeruginosa, leading to severe bronchiectasis. At birth CF patients have

virtually normal bronchopulmonary structures, but with the passage of time a large proportion develop bronchiectasis. Ultimately, cardiopulmonary failure supervenes, with death, or heart-lung transplantation at a relatively young age.

Other conditions which generate bronchiectasis are: the aspiration of foreign bodies followed by their impaction in the bronchial tree (Denney, Berkas, Snider et al, 1968; Kurklu, Williams & Le Roux, 1973), and other mechanical obstructions provoked by endobronchial tumours, such as lipomas (MacArthur, Cheung & Spiro, 1977), teratomas (Bateson, Hayes & Woo-Ming, 1968), and carcinomas (Chiu & Campbell, 1973). It is stressed by all authors, though, that superimposed infection is essential for the subsequent development of the bronchial abnormality.

In these conditions, as well as in the others described above, it is clear that the association of obstruction, generated by the defective mucociliary clearance, and infection are of paramount importance for the development of bronchiectasis. Since the pioneering experimental work of Tannenberg and Pinner (1942) it is known that it is possible to induce bronchiectasis in laboratory animals by inducing a combination of these two factors, bronchial obstruction and infection.

1.4.2 - THE HOST'S INFLAMMATORY RESPONSES

The "vicious circle" hypothesis suggests that failure to eliminate bacteria from the bronchial tree by such mechanisms as those referred to above facilitates colonisation of the bronchi by microorganisms. This bacterial load stimulates a host response of inflammation which is unsuccessful in eliminating the microbes and therefore becomes chronic, leading to more and permanent damage to the bronchial wall and

surrounding lung tissues. Hence, Cole (1984) proposed that the host mediated inflammatory reactions were an integral part of the chain of events leading to the generation of bronchiectasis. The host-mediated inflammatory reaction can be studied with respect to its nonspecific and its immunologic components.

The nonspecific component of inflammation has been carefully studied over the past decade. The basis for the understanding of this component lies in the fact that microbial products (Ras, Wilson, Todd et al, 1990) but also products of the host's inflammatory response, such as C5a (Jose, Forrest & Williams, 1983) have the capacity to attract polymorphonuclear neutrophil leukocytes to sites of inflammation. Often bronchiectasis is associated with production by the patient of large amounts of sputum, usually purulent - this represents the debris of the neutrophil influx attracted to the bronchial lumen. It has been demonstrated that large numbers of neutrophils are attracted to the affected bronchus in severe cases of active bronchiectasis by radiolabelling granulocytes from the blood of bronchiectatic patients with 111-Indium and reinjecting them into the circulation (Currie, Peters, George et al, 1988; Currie, Peters, Garbett et al, 1990). To fulfil their role as professional phagocytes, neutrophils possess a number of potent proteolytic enzymes and generate a variety of oxygen radicals. Presumably it would require only small amounts of those agents released from each cell to produce severe damage to host tissue in view of the large cell traffic involved.

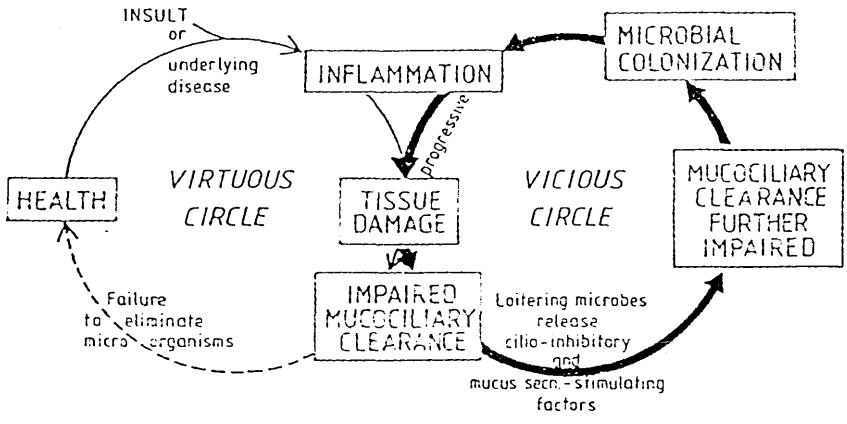
One of the best studied of these proteolytic enzymes is neutrophil elastase. It has been shown that this enzyme can produce mucus gland hyperplasia in animals (Snider, Lucey, Christensen et al,

1984; Snider, Stone, Lucey et al, 1985; Snider, 1986), damage to epithelium (Tegner, Ohlsson, Toremalm et al, 1979; Amitani, Wilson, Rutman et al, 1991), reduction of ciliary beat frequency (Smallman, Hill & Stockley, 1984; Sykes, Wilson, Greenstone et al, 1987) and that these effects can be abolished by the addition of specific inhibitor of the enzyme. The fact that neutrophil elastase activity is usually found in the secretions of patients with bronchiectasis (Stockley, 1984; Stockley, Shaw, Afford et al, 1988) prompts these authors to suggest that it is the main pathogenetic component of bronchiectasis. The finding of increased degradation products of elastin in the sputa of patients with bronchiectasis associated with cystic fibrosis was also taken as evidence of the role of neutrophil elastase in the genesis of the condition (Bruce, Poncz & Klinger, 1985).

In contrast, the cellular immunologic component of the inflammation associated with bronchiectasis has received practically no attention and is the subject of this thesis.

FIG. 1.1

THE "VICIOUS CIRCLE" HYPOTHESIS



The diagram represents the "vicious circle" of events occurring when the mucociliary clarance mechanisms are initially compromised, leading to bronchiectasis (Cole, 1990)

1.5 - THE BRONCHUS IS PART OF THE MUCOSAL IMMUNE SYSTEM

The concept of a common mucosal immune system is relatively recent. Its starting point was the work of Hanson (1961) who described the presence in milk of a novel immunoglobulin, then called serum beta2-alpha globulin. Some years later, Tomasi and co-workers (1965) discovered that this same molecule was found in combination with a polypeptide, now called secretory component, secreted locally by mucosal surfaces, and forming the so called secretory immunoglobulin A. They found this molecule to be the main immunoglobulin present in secretions and on mucosal surfaces.

Much of the knowledge in this field is derived from observations in the gut, eg. the morphology and function of Peyer's patches and other gut-associated lymphoid tissue, the phenotype of the intraepithelial lymphocytes, etc. A substantial contribution to the concept of the common mucosal immune system derives, however, from the observations of Bienenstock and associates in the respiratory tract. Analysing the effect of the introduction of immune complexes into the airways of rabbits they noticed lymphoid tissue within the bronchial wall that bore a striking morphological resemblance to the Peyer's patches within the intestine. They called it bronchus-associated lymphoid tissue or BALT (Bienenstock, Johnson & Perey, 1973a, b; Bienenstock & Johnson, 1976; Bienenstock, McDermott & Befus, 1979; Bienenstock & Befus, 1980; Bienenstock, McDermott & Befus, 1982; Bienenstock & Befus, 1984). In the years that followed, other mucosal surfaces were shown to possess similar structures and the concept of a common mucosal immune system, pioneered by Bienenstock and others, was clearly reinforced. The main components of this system are: gut, upper and lower respiratory tract, mammary glands, conjunctiva, middle ear,

parts of the urogenital tract, and salivary glands.

1.5.1 - DISTRIBUTION AND ORGANIZATION OF IMMUNOCOMPETENT CELLS IN THE BRONCHUS

The lymphoid tissue in the mucosal sites consists of both loosely distributed lymphocytes and those organized into follicles and lymphoid aggregates. A certain compartmentalisation can be established, with a very distinctive lymphoid population found in the epithelium. It is mainly composed of T lymphocytes with suppressor/cytotoxic function (Selby, Janossy & Jewell, 1981; Ernst, Befus & Bienenstock, 1985) and has been considered to play a key role in the regulation of the epithelium itself, considering the observations by Miller and Nawa (1979) that T cells can regulate goblet cells numbers in the epithelium and their secretory activity. Recently many observations have been published on the structure and function of the T Cell Antigen Receptor (TCR) isotypes (Brenner, Mclean, Dyalinas et al, 1986; Brenner, Mclean, Scheft et al, 1987; Fitch, Lancki & Havran, 1988; Campana, Janossy, Counstan-Smith et al, 1989; Strominger, 1989), particularly on the characterization of the gamma/delta TCR isotype (Campana, 1989; Fallini, Flenghi, Pileri et al, 1989; Spencer, Isaacson, Diss et al, 1989; Ullrich, Schieferdecker, Ziegler et al, 1990; Inghirami, Zhu, Chess et al, 1990), with emphasis on their distribution in the gut epithelium. As yet, no information is available on the phenotype of the bronchial intraepithelial lymphocyte regarding the distribution of TCR isotypes.

In the bronchial lamina propria and submucosa, B and T cells are loosely distributed, with a predominance of the latter. The B cells

express predominantly IgA (60%) but IgG, IgM, and IgE can also been found (Bienenstock, 1984).

The lymphoid tissue for which Bienenstock coined the term bronchus-associated lymphoid tissue or BALT (Bienenstock, 1984) is a highly organized structure present in various amounts in different avian and mammalian species. It is easily distinguishable in the lungs of birds, rabbits and rats, less so in guinea-pigs, dogs and humans. Controversy still exists about the very existence of these structures in man (Berman, 1990; Pabst & Gehrke, 1990) but it is likely that they can play a role in the human species (Bienenstock, 1984). The magnitude of development of the BALT appears to correlate with the degree of antigenic stimulation of the bronchial mucosa (Kaltreider, 1984).

The BALT consists of lymphoid follicles that lack germinal centres and capsules. It is covered by a specialised epithelium called lymphoepithelium, composed of a flat epithelium devoid of cilia or goblet cells, and heavily infiltrated by lymphocytes. The presence of the lymphoepithelium is essential to distinguish the BALT from other lymphoid aggregates. The M cells described in Peyer's patches can also be found in the lymphoepithelium of the BALT (Bienenstock, 1984). Both arterioles and venules can be seen forming an extensive capillary network but the main vascular component is the high endothelium post-capillary venule through which lymphocytes and lymphoblasts migrate. Circulating lymphocytes have special homing receptors for this endothelium (Otsuki, Ito & Magari, 1989). Efferent but not afferent lymph vessels can be identified in BALT (Plesch, 1982). The BALT itself consists of a reticulin network filled with lymphocytes and macrophages. The follicles contain mainly B cells with varying proportions of immunoglobulins according to the species and antigenic

exposure. The parafollicular area lateral to the follicles is a T-dependent area, consisting of about 20% of the total number of BALT cells (Bienenstock, 1984).

1.5.2 - BRONCHIAL IMMUNITY

It is known that epithelial surfaces play a key role in the interaction between the external and internal milieu and the bronchus is no exception. The bronchial mucosa is constantly challenged and must develop appropriate and effective immune defences in order to limit invasion and also to preserve the anatomical and functional integrity of the fragile mucosal tissues they protect. A breach in this equilibrium may result in widespread and permanent damage to the mucosa. Local immune mechanisms interact with responses which occur at other mucosal and systemic sites. Mucosal antibodies and cell-mediated immunity may provide local resistance to bacteria, viruses, parasites, toxins, allergens, etc. These immune responses are initiated upon antigenic stimulation and the specialized lymphoepithelium plays a major role in sampling the antigen and transfering it to antigen presenting cells in the BALT, where it is presented to T cells, thus initiating the cascade of events that results in the immune response (Kaltreider, 1984). The products of B lymphocyte mediated immunity are specific antibodies that enhance phagocytosis, promote microbial killing, and neutralize toxins. The T lymphocyte effector cells can be cytotoxic for neoplastic and virus- infected cells or may act through the secretion of lymphokines that mediate subacute or chronic inflammatory responses. It appears that immune effector functions expressed at mucosal surfaces of the airways right the way down to the

bronchoalveolar junction involve predominantly responses generated locally by resident lymphoid cells, whereas the parenchymal immune responses depend upon the recruitment of immunocompetent cells from outside sources (Kaltreider, 1984).

In the airways it is likely that under certain conditions the control mechanisms of the immune response are defective and local immune reactions become a decisive part of the immunopathogenesis of bronchopulmonary diseases. In these situations, the normal populations of lymphocytes, mainly T cells, present in the bronchial mucosa change in number and type in response to the inflammation. The changes occurring during the inflammatory process in the airways may be better reflected by these local cells than by sampling from other sites, such as the blood (Bienenstock, 1986).

1.6 - IS THERE EVIDENCE FOR THE INVOLVEMENT OF THE IMMUNE SYSTEM IN THE PATHOGENESIS OF BRONCHIECTASIS?

The association of progressive sinobronchiectasis with different forms of immunodeficiencies, especially hypogammaglobulinaemia, caught the attention of many researchers during the 1970s, when those deficiencies were considered a major aetiological factor for the development of bronchiectasis. However, in his large series of patients presenting with chronic bronchial sepsis, mainly associated with bronchiectasis, Cole (1984) found that only a small proportion of cases had hypogammaglobulinaemia or other immunodeficiency. Hilton & Doyle (1978) also investigated bronchiectasis patients and found that 79% had at least one abnormality of immunoglobulins, usually elevation of IgA, IgG, and IgM but only 2 patients had selective IgA deficiency. Varpela investigated 178 patients presenting with chronic bronchial inflammation, of whom 49 had proven bronchiectasis, and found that the large majority had raised levels of serum IgA, with only two cases of IgA deficiency (Varpela, Savilahti & Lokki, 1977). Murphy did not find any immunoglobulin deficiency in 23 patients with proven bronchiectasis (Murphy, Reen & Fitzgerald, 1984). Noda and colleagues (1989) reported the levels of serum secretory IgA in 33 patients with diffuse panbronchiolitis, 13 with chronic bronchitis, and 24 with bronchiectasis. In all three types of chronic bronchial inflammation they found raised levels of sIgA, especially in those patients infected with Pseudomonas aeruginosa. In a subsequent study of 412 patients with chronic persistent respiratory infections mainly due to bronchiectasis, Cole also found a low prevalence of immune deficiencies, around 9%, whilst 83% of the patients had one or more of the serum immunoglobulin classes raised by more than two standard deviations above the mean

(Cole, 1989). Horan and colleagues (1984) correlated the increased serum levels of immunoglobulin in bronchiectasis patients with raised levels of serum endotoxin. In summary, there is large evidence from the literature correlating bronchiectasis with raised levels of serum immunoglobulins.

Relatively few studies have concentrated on the local production of immunoglobulins in the respiratory tree. Brogan reported the composition of bronchopulmonary secretions from patients with bronchiectasis. 24-hour collections of sputum were obtained from 17 patients with bronchiectasis and the level of IgA and other proteins were determined by immunoelectrophoresis of the sol phase of the sputum. They failed to see raised levels of IgA or IgG (Brogan, Davies, Ryley et al, 1980). Plusa determined the levels of immunoglobulins in the sera and bronchoalveolar lavage supernatants of 27 patients with proven bronchiectasis and found higher levels of IgG and IgM but not IgA in the supernatants compared with controls (Plusa & Wasek, 1987).

Rivasi stained bronchial tissue sections from five bronchiectasis patients with antisera against the main classes of immunoglobulins and found marked fluorescence for IgA but less marked fluorescence for IgG and IgM, suggesting that in this condition an increased local production of IgA was present (Rivasi, Rivasi, Botticelli et al, 1975). Burnett studied necropsy specimens from 10 bronchiectasis patients and also found an increased number of IgA positive cells in the bronchi of the patients compared with controls, both IgA1 and IgA2 being affected (Burnett, Crocker & Stockley, 1987). More recently, Hill studied the composition of sputa from 28 bronchiectatic patients and found an increase in all four subtypes of IgG compared with controls, mainly in

patients producing large amounts of purulent sputum (Hill, Mitchell, Burnett et al, 1990).

Another aspect of the humoral immunity that has been explored is the presence of immune complexes in the serum of patients with bronchiectasis (Hilton, Moore, Howat et al, 1978; Hilton, Hasleton, Bradlow et al, 1984). However, no conclusive evidence of the presence of immune complexes in bronchiectatic bronchial wall or of their role in the pathogenesis of the disease has as yet been provided.

No published data appear to be available concerning the possible involvement of cell-mediated immune mechanisms in the pathogenesis of bronchiectasis. Despite the strong evidence provided by Whitwell in his classic paper of the presence of massive lymphocytic infiltration of the affected bronchial walls in bronchiectasis, this aspect of the immune system seems not to have caught the imagination of researchers working in the area of chronic bronchial sepsis.

C H A P T E R 2

ONE APPROACH TO INVESTIGATION OF THE PATHOGENESIS OF BRONCHIECTASIS

The hypothesis that acquired immune mechanisms, particularly those mediated by cellular components of the immune response, are implicated in the pathogenesis of bronchiectasis was proposed. This proposal was based on the observations of Whitwell that an extensive bronchial infiltration of lymphocytes, plasma cells and histiocytes, organized or not as follicle-like structures is the main histopathological feature of bronchiectasis. Also, upon Cole's "vicious circle" hypothesis for the pathogenesis of bronchiectasis, according to which host-mediated chronic inflammatory responses to bacterial colonisation of the bronchi are an integral part of the process leading to the disease.

To test this hypothesis, a protocol was devised to study biopsy specimens from patients with bronchiectasis and to study a novel animal model of experimental bronchiectasis, using immunohistochemical and other immunological methods.

2.1 - IMMUNOHISTOCHEMISTRY AS A TOOL FOR THE STUDY OF THE BRONCHUS

Immunohistochemistry is essentially a chemical technique where the basic reagents are immunologic products used for the detection and localization of antigens in tissue sections. The combination of different histochemical and immunological techniques provides a powerful methodological tool to explore the nature of a local immunologic milieu. Such methods have been used extensively over the past decade to ascertain the character of immunological processes in different pulmonary disorders, especially the interstitial lung diseases, where the use of bronchoalveolar lavage as the method of obtaining immunocompetent cells from the lungs has proved extremely

useful. Only in the past few years has such methodology been applied in the study of chronic bronchial conditions such as asthma, chronic bronchitis, bronchiolitis, bronchiectasis, allograft rejection and infection in heart-lung transplantation, etc. The reliability and simplicity of fibreoptic bronchoscopy as the means of obtaining the bronchial samples have allowed a significant technical advance. The only major disadvantage of this endoscopic method is the small size of the biopsy but this problem can be overcome by increasing the number of biopsies (Janin-Mercier, Mignot, Delage et al, 1985). Bronchial samples can also be obtained from surgically resected lobes or lungs (and even from necropsies) provided that the time interval between the procedure and the handling of the bronchial sample is kept to a minimum in order to preserve the integrity of the molecules present on the cell surfaces.

The increase in information about the immune system obtained in the last decade concerning its cellular composition, the nature of molecules on the cell surface, the cellular interactions, and many other aspects, has been made possible chiefly by the advent of highly specific immunological reagents, the monoclonal antibodies.

Monoclonal antibody is by definition an immunoglobulin produced by the progeny of a single antibody-producing cell, called a clone. The antibodies produced by this clone are chemically, physically, and immunologically homogeneous. The production of monoclonal antibodies was achieved in 1975 by Kohler and Milstein, who were awarded the Nobel Prize for their discovery which has caused a revolution in immunology, as well as in virtually all biological and medical fields.

The discovery came as a by-product of research into the

structure and synthesis of immunoglobulins and into the mechanisms for generation of antibody diversity (Clark & Waldmann, 1983). Two main concepts, the clonal selection hypothesis and the production of antibody with a single specificity by each B-lymphocyte or plasma cell, provided the idea that a single clone of cells would secrete antibodies of a monoclonal nature. Tumours of immunoglobulin secreting cells in certain strains of mice were adapted to in vitro culture, and this allowed the observation that cell fusion between two immunoglobulin-producing cell lines resulted in the co-dominant expression of the immunoglobulin chains. Following this observation, Kohler & Milstein fused myeloma cell lines with lymphocytes obtained from a mouse immunized with sheep red blood cells and obtained hybrid cell clones, some of which secreted antibodies specific for the immunizing agent. The somatic hybridization of antibody-forming cells with continually replicating cell lines provided the immortalisation of cells secreting antigen-specific antibodies in an almost unlimited way.

During the years since the publication of this seminal paper, an endless number of monoclonal antibodies has been produced and characterized, their applications ranging from basic research to the therapy of different conditions (Beverley, 1983). A concerted international effort, coordinated by the World Health Organization, to achieve some uniformity in the nomenclature of the monoclonal antibodies recognizing similar human leukocyte antigens resulted in the proposition of Clusters of Differentiation (Knapp, Dorken, Rieber et al, 1989).

In the research for this thesis a combination of different immunological and immunohistological techniques employing monoclonal antibodies against human and rat antigenic determinants was used.

C H A P T E R 3

AIMS OF THE INVESTIGATION

3.1 - INVESTIGATION OF THE IMMUNOPATHOLOGY OF HUMAN BRONCHIECTASIS

- To identify the cells/subsets present in the lesions of bronchiectasis;

- To determine whether local immunological reactivity (as determined by the expression of activation markers) can be demonstrated in the pathology of bronchiectasis.

3.2 - INVESTIGATION OF THE IMMUNOPATHOGENESIS OF EXPERIMENTAL BRONCHIECTASIS: IN VIVO STUDIES

- To ascertain whether there is any similarity between the immunopathological changes of experimental bronchiectasis and those seen in the human disease;

- To investigate the cells/subsets present in the rats with experimentally induced bronchiectasis;

- To study the time relationships of the development of the lung lesions and the involvement of immunocompetent cells;

- To study changes in local immunological reactivity (as determined by the expression of activation markers) in relation to the development of experimental bronchiectasis;

- To determine whether cellular sensitisation to bacterial antigens employed in the development of experimental bronchiectasis was present in rat footpads;

3.3 - INVESTIGATION OF THE IMMUNOPATHOGENESIS OF EXPERIMENTAL BRONCHIECTASIS: IN VITRO STUDIES

- To obtain single cell suspensions of immunocompetent cells from affected and control rat lungs;

- To study the cytotoxic activity of immunocompetent cells present in lungs developing experimental bronchiectasis.

C H A P T E R 4

THE IMMUNOPATHOLOGY OF HUMAN BRONCHIECTASIS

4.1 - INTRODUCTION - THE HISTOLOGY OF THE NORMAL BRONCHUS

The understanding of the immunopathological changes in bronchiectasis depends on the study of the normal structure of the bronchial tree, with particular reference to its histological organisation. The bronchus is widely considered to be a mere conducting airway connecting the trachea to areas of gas exchange deeper in the substance of the lungs. However, far from this, the bronchus is a highly complex organ, with a unique role to play. The trachea and bronchi are derived as a bud from the foregut. At birth the basic formation of cartilaginous airways is complete and growth occurs not by additional branching but by increase in length and diameter. The number of branchings varies according to the length from the segmental bronchi to the alveoli throughout the lungs. The diameter of each new generation of airways decreases progressively but the total cross-sectional area of the lumen steadily increases at successively lower levels throughout the tracheobronchial tree (Murray, 1986). The airways possess different and complex functions, including the secretion of many different substances (the detailed analysis of which is beyond the objectives of this thesis), as well as active and passive dilatation and contraction in response to numerous stimuli, and as a result of nonspecific and specific respiratory defence mechanisms. All these functions are accomplished by the elements that form the bronchial wall: epithelium, lamina propria, smooth muscle, glands, nerves, vessels, immunocompetent and inflammatory cells, connective and elastic tissue, and cartilage.

The most complex of these structures is the epithelium, a continuous lining of the respiratory tract from the trachea to the respiratory bronchioli and of the naso-sinus areas. There are at

least eight different types of cells forming the bronchial epithelium. The ciliated cell is the major cell in the epithelium, playing a vital role as the drive mechanism for the first line defence mechanism of the respiratory tract, the mucociliary escalator.

The bronchial epithelium rests on the lamina propria, a loose structure of connective tissue between the epithelium basement membrane and the muscle coat. It normally contains a rich network of capillaries and nonmyelinated nerve fibres. Mast cells, lymphocytes and neutrophils are present in this region. Sometimes the lymphocytes form aggregates (the so-called bronchus-associated lymphoid tissue, reviewed in section 1.5) but in the human lamina propria only occasionally can such formations be seen, with the lymphocyte population usually loosely distributed. Another important component of the lamina propria is the elastic tissue, which is arranged in longitudinal bundles.

External to the elastic layer of the lamina propria is the smooth muscle coat, sandwiched between the elastic sheet forming the deep part of the lamina propria and an external sheet of elastic tissue on the outer surface of the muscle. The smooth muscle is penetrated in all directions by intercommunicating elastic fibres, linking the two elastic laminae. This complex is of paramount importance in the regulation of the bronchial calibre and tonus, which provide another important non-specific defence mechanism of the respiratory tract.

Outside the muscle and elastic coat lies a variable amount of connective tissue and the submucosa, in which are the mucus glands. Together with the epithelial goblet cells these glands secrete the complex array of substances essential for bronchial homeostasis. Lymphocytes and mast cells can also be seen among the cellular

constituents of the glands. The glands are especially numerous in medium-sized bronchi, less prevalent in smaller bronchi, and absent in bronchioli.

The last main component of the bronchial wall is the cartilage. In the large bronchi it retains the tracheal shape in its classical U. As the bronchus continues towards the periphery the cartilage is composed of irregular plates, some of which completely encircle the bronchial lumen. The smallest bronchi contain less cartilage and this no longer forms complete rings, and is absent in the bronchioli. The cartilage plates are surrounded by perichondrium, which blends with the fibrous peribronchial tissue. This peribronchial sheath is continuous with the adventitia of the pulmonary artery accompanying the bronchus. In this sheath also lies the bronchial arteries, bronchial nerves, and peribronchial lymph nodes.

The complex structure of the bronchus is the seat of the histopathological and immunopathological changes seen in bronchiectasis.

4.2 - PATIENTS

The samples of bronchial wall included in this study were obtained from patients undergoing resection or fibreoptic bronchoscopy between November 1986 and July 1988, and were handled by the Department of Lung Pathology of the Royal Brompton National Heart & Lung Hospital Chelsea. Frozen samples from bronchiectatic bronchial wall stored in the Department of Lung Pathology were also included in the study by kind permission of Dr. C. Steinfort and Dr. E. Sheffield. Most of the resections were performed by Mr. P. Goldstraw.

The selection of patients depended mainly on two factors: the availability of samples and the desire to include as varied a population in terms of aetiopathogenetic factors related to bronchiectasis. The first factor also applied to the selection of non-bronchiectatic controls, and to the matching of vital characteristics of the bronchiectatic population. The controls were as normal as possible in terms of macropathology, and any sample was discarded if the histological study suggested the presence of gross abnormality.

All patients gave informed written consent before the procedures and the study had the approval of the Ethics Committee of Royal Brompton Hospital.

4.3 - METHODS

4.3.1 - TISSUE SAMPLING AND PREPARATION

Resected organs were transported from the operating theatre to the pathology laboratory within 60 minutes. No preservative of any kind was added to the organ. The organ was dissected to find an area of macroscopically visible bronchiectasis or, in the case of potential controls, an area as far from the tumour (or other pathology for which the resection was performed) as possible. Segments of bronchi about 1 cm long were dissected from the chosen area. The samples were placed in sterile tubes and taken immediately to the Host Defence Unit laboratory to be frozen.

Endoscopic bronchial biopsy was used to obtain specimens from patients with diffuse bronchiectasis, which was not treatable by resection. The bronchoscopy was performed by the clinician in charge of the patient following the standard procedures in use at the Royal Brompton National Heart & Lung Hospital Chelsea. The patient was pre-medicated with diazepam (Dumex Ltd, Bucks, UK) and atropine, local anaesthesia of the nose and throat was obtained with Lignocaine 2% (Astra Pharmaceuticals, UK), and an Olympus BF10 fibreoptic bronchoscope introduced in the respiratory tract. Bronchial biopsies were obtained with aligator forceps. At least five biopsies were obtained from previously chosen sites and placed directly into sterile universals containing sterile phosphate buffered saline (PBS, see Appendix 2), taken immediately to the Host Defence Unit laboratory and frozen. The size of the biopsies varied between 1 and 2 mm^3.

The bronchial segments were trimmed and rings of not more than 0.5 cm long were orientated lumen upwards. In the case of endoscopic

biopsies the fragments of bronchial mucosa were collected in sterile gauze and when visible the luminal side was orientated laterally in order to allow the sections to include all compartments of the bronchial biopsy. The rest of the procedures were similar for both kind of samples. They were placed on round cork discs (Bright Instrument Co, Huntington, UK), clearly marked on the opposite side with the name of the patient and covered by Tissue-Tek Optimum Cutting Temperature (OCT) compound (Miles Laboratories, Inc., Illinois, USA). Isopentane (BDH Ltd, Poole, UK) was placed in a plastic beaker suspended by a metallic frame and cooled in a bath of liquid nitrogen until the bottom of the beaker presented an icy aspect due to freezing of the isopentane. The cork discs containing the samples were then immersed in the cooled isopentane and kept there for five minutes to ensure complete freezing of the tissue. The samples were stored in liquid nitrogen until use.

Six micron sections were cut in a cryostat (Bright Instrument) at -30^{0} C. The sections were placed on glass slides previously coated with poly-l-lysine (Sigma Chemical Company, Inc., St. Louis, USA - see Appendix 2) with two consecutive sections placed on each slide. At least 50 consecutive slides were processed from each block. The sections were left to dry for one hour at room temperature, and then fixed in a solution containing equal volumes of chloroform (BDH) and acetone (BDH) for 10 minutes. After allowing the surplus to evaporate at room temperature, the slides were wrapped in cling film (Handywrap, Payne Scientific Ltd., Berks., UK) and stored at -20^{0} C until use.

Sections to be stained with the monoclonal antibody Ki67 (Campana, Counstan-Smith & Janossy, 1987) were stored unfixed but immersed in

cold methanol immediately prior the staining procedure.

Some representative sections of different areas of the block were kept for immediate conventional histological staining. Sections to be stained for elastin were fixed in 10% formalin in saline, left to dry, wrapped in cling film, and stored at room temperature until use.

Blocks of human palatine tonsil obtained at tonsillectomy were processed in the same way as the bronchial samples to provide positive controls for the staining procedures.

4.3.2 - CONVENTIONAL HISTOLOGICAL STAINING METHODS

Three conventional histological stains were used for routine histopathological analysis (see Appendix 2 for solutions):

a) Toluidine blue: sections were fan dried at room temperature for 10 minutes, covered in 0.1% Toluidine Blue for 10 seconds, washed in running tap water, dried and examined under the light microscope.

b) Haematoxylin-eosin: sections were air dried at room temperature for one hour, and fixed in 10% formalin in saline for one minute. After a quick rinse in running tap water, the sections were put in Harris' haematoxylin for six minutes, and again rinsed in tap water until the water was clear. The slides were then placed in acid-alcohol for one minute, quickly rinsed, and left in alkaline water for 2 minutes. The sections were placed in 1% eosin for six minutes, rinsed in running water, quickly dehydrated in grade alcohol, cleared in xylene (BDH) or Citroclear (HD Supplies, Aylesbury, UK), and mounted with DPX (BDH) and coverslip.

c) Miller's Elastic Van Gieson: the sections were placed in Miller's solution (BDH) for 1 hour, then rinsed in 70% alcohol to remove the excess stain, washed in tap water and counterstained in Van Gieson's

solution, dehydrated, cleared and mounted.

4.3.3 - IMMUNOHISTOCHEMISTRY

4.3.3.1 - Immunoperoxidase

Immunoperoxidase was used as the method of choice for staining the tissue sections. The principle of the method is the property of horseradish peroxidase to catalyse, in the presence of small amounts of hydrogen peroxidase, a reaction with 3,3'-diaminobenzidine (DAB), producing an insoluble golden brown product (Janossy & Amlot, 1987; Janossy, Campana, Counstan-Smith et al, 1989).

The indirect immunoperoxidase method was used throughout this research, and as all monoclonal antibodies employed here were derived from mouse (mouse anti-human monoclonal antibodies), the horseradish peroxidase was conjugated to rabbit anti-mouse immunoglobulin, known as P161 (Dakopatts a/s, Copenhagen, Denmark) (Poulter, Seymour, Duke et al, 1982; Munro, Mitchell, Poulter et al, 1986; Janossy & Amlot, 1987):

- The sections were taken from the freezer 30 minutes before the start of the staining procedures and allowed to reach room temperature before unwrapping;
- The sections were ringed with the water-repellent polysiloxane;
- All sections were placed in "wet-boxes" to prevent drying out;
- The sections were rehydrated with 50 ul of a solution containing 1% v/v normal rabbit serum in PBS to block nonspecific binding of the antibodies to Fc receptors, left for 20 minutes and drained without letting the sections dry;
- The monoclonal antibodies were diluted appropriately in PBS and

50 ul of the solution added to the test slide, while the negative control received the same amount of PBS, then incubated for 60 minutes at room temperature;

- The slides were washed with PBS for 10 minutes;

- A second layer consisting of 50 ul of a solution of 1% v/v rabbit anti-mouse immunoglobulin conjugated to horseradish peroxidase (P161, Dakopatts a/s, Denmark) and 0.5% v/v normal human serum in PBS was applied for 45 minutes;

- The slides were then washed in PBS and developed with a solution containing 3,3'-diaminobenzidine (Sigma Chemicals Ltd., St. Louis, USA) and hydrogen peroxide (Sigma - Appendix 2) for two minutes. After development slides were washed in running water for five minutes, in distilled water for two minutes, counterstained in Harris' haematoxylin for 20 seconds, washed, dehydrated in graded alcohols, cleared in xylene or Cytroclear, mounted in DPX and examined under a light microscope (Plate 4.1).

4.3.3.2 - Single Immunofluorescence

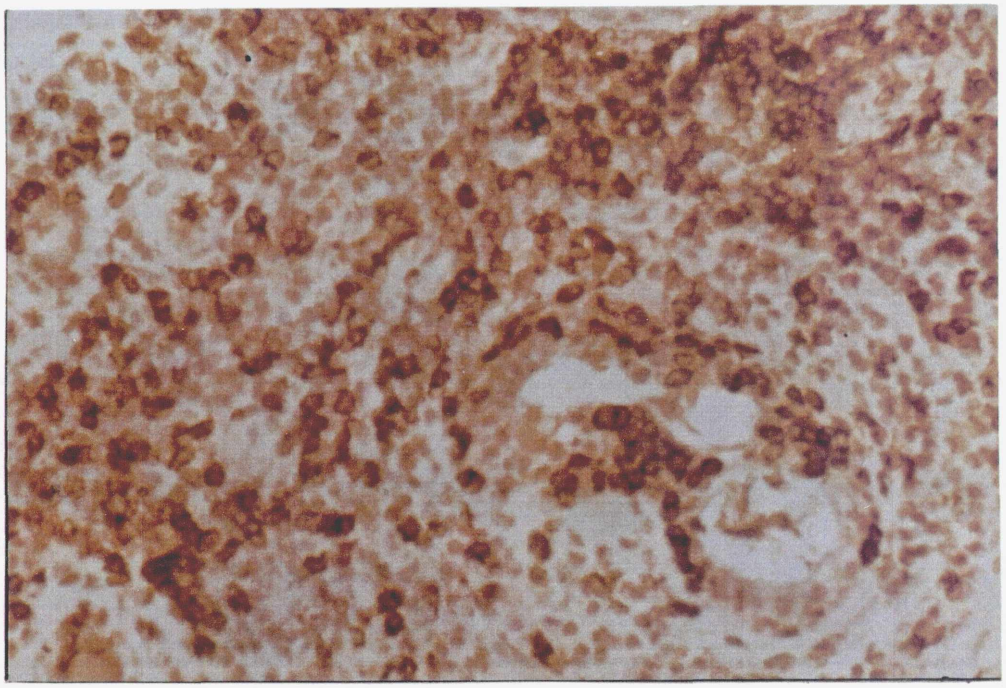
Single immunofluorescence could be used with directly conjugated monoclonal antibodies:

- The slides were prepared as previously described for immunoperoxidase;

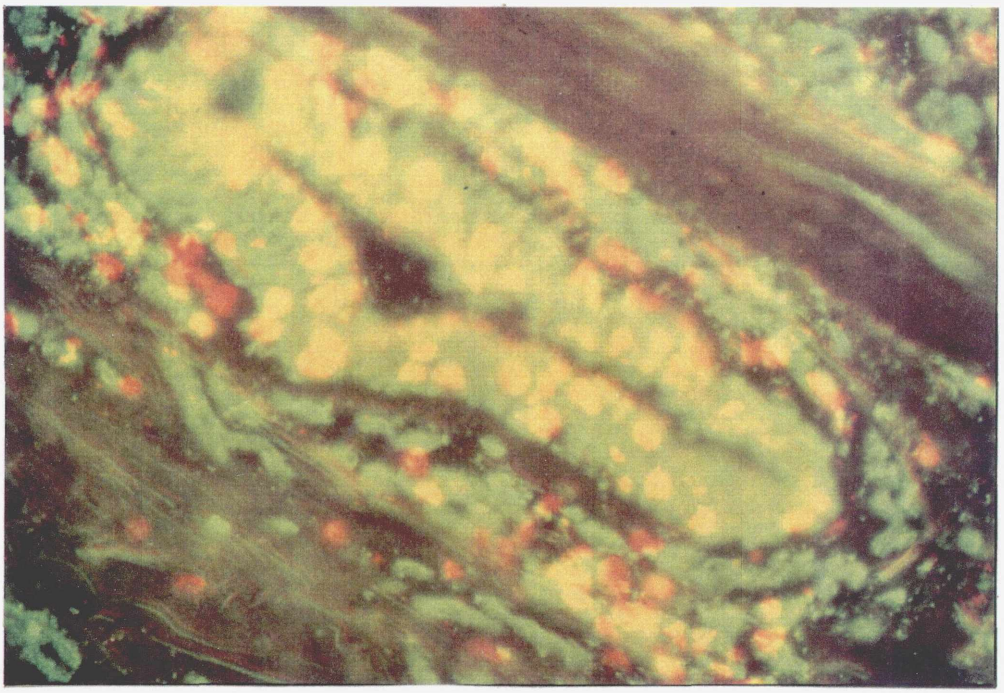
- The monoclonal antibody was diluted appropriately, or used neat to provide a brighter reaction, and 50 ul added to the sections and incubated for 45 minutes;

- The slides were washed in three changes of PBS, mounted in 10% glycerol-PBS and coverslip, and read in a Zeiss fluorescence microscope equipped with epi-illumination and the appropriate barriers for the

PLATE 4.1



a: Indirect immunoperoxidase staining of human bronchial mucosa with a T-lymphocyte marker. The positive cells appear in brown (bronchiectasis, CD3, original magnification x250)



b: Double immunofluorescence staining of human bronchial mucosa with a CD8 marker stained in red and a MHC Class II marker stained in green. Cells co-expressing the markers appear in yellow (bronchiectasis, CD8/HLA-DR, original magnification x250)

fluorochrome employed.

More frequently, however, the indirect method was used:

- The slides were prepared as for immunoperoxidase;

- The monoclonal antibody was diluted appropriately and 50 ul added to the sections, and incubated for 45 minutes;

- The slides were washed in three changes of PBS;

- The second layer was a solution of 1-2% v/v of goat anti-mouse immunoglobulin in PBS directed against the isotype of the monoclonal antibody conjugated to a fluorochrome, incubated for 30 minutes;

- The slides were washed in three changes of PBS, mounted in glycerol-PBS and coverslip, and read as before.

Four types of goat anti-mouse immunoglobulins were employed both for single as well as for double immunofluorescence, and chosen according to the heavy-chain specificity of the first-layer monoclonal antibody and the desired colour of the end-products, all supplied by SBA (Southern Biotechnology Associates, Inc., Alabama, USA):

- IgG heavy chain-specific antiserum conjugated with fluorescein isothiocyanate (FITC), giving a green colour;

- The same with IgM specificity;

- IgG heavy chain-specific antiserum conjugated with tetramethylrhodamine isothiocyanate (TRITC), giving a red colour;

- The same with IgM specificity.

4.3.3.3 - Double Immunofluorescence

The indirect two-colour immunoflorescence was used (Poulter, Chilosi, Seymour et al, 1983; Janossy, Bofill & Poulter, 1986; Janossy & Amlot, 1987; Janossy, Campana, Counstan-Smith, 1989):

- The sections were prepared as for immunoperoxidase;

- A combination of the two monoclonal antibodies to be tested were prepared in such way that the working dilution for each of the individual monoclonal would be achieved, and 50 ul of the mixture added to the sections and incubated at room temperature for 45 minutes;

- The slides were washed with three changes of PBS;

- The second layer was composed of a combination of goat anti-mouse IgM conjugated with TRITC and IgG conjugated with FITC, in a proper dilution to provide good staining by each monoclonal, usually 4% v/v in PBS, and 50 ul added to the sections and incubated for 30 minutes;

- The slides were washed with three changes of PBS, mounted in glycerol-PBS and covered with a coverslip, then examined under the Zeiss fluorescence microscope. The first section to be examined was the tonsil to make sure that the staining procedure was performed correctly. In some studies a combination of direct/indirect methods were employed.

An important part of the immunofluorescence studies in this research was the use of photography as a tool to provide a permanent record of the results, considering the transient nature of the staining. The fluorescence microscope was equipped with a photographic device which allowed a given microscopic field to be double exposed to a film. Each filter allows only a certain colour emitted by the fluorochrome, red or green, to be registered by the film, the system working like two superimposed pictures of the same object reflecting two different light wavelengths. The end result is a colour film where cells co-expressing the two antigens tested (one red plus one green) appear in yellow, and the cells expressing only one of the antigens

appear in red or green, giving a three-colour picture (Plate 4.1).

4.3.3.4 - Other Histochemical Methods

Two other histochemical methods were employed in this research. The first one was actually a combination of haematoxylin-eosin and staining for endogenous peroxidase in consecutive sections, in order to study the possible distribution of polymorphonuclear leukocytes in situ, a method particularly useful considering the elusive nature of neutrophils in their passage from the bronchial vessels to the bronchial lumen, where they can be seen in large numbers in bronchiectasis samples. The method consisted of staining consecutive sections with the two methods as explained before. The sections stained for endogenous peroxidase were examined first under the microscope, the areas with black dots denoting high endogenous peroxidase activity recorded. The same areas in the consecutive slide were then examined and the presence of neutrophils determined by the typical nuclei morphology.

The second method was the staining of the lysosomal hydrolase acid phosphatase with the pararosaniline method coupled with immunoperoxidase staining of tissue macrophages. The method starts with the indirect immunoperoxidase staining of the bronchial sections as described previously, using monoclonal antibodies that recognise different subsets of macrophage-like cells. The sections were then incubated in the substrate solution (Appendix 2) at 37^{0} C for 60-90 minutes, then washed, counterstained with haematoxylin, dehydrated and mounted as previously described. The sections were examined under the light microscope and cells positive for the macrophage marker presented

a brown precipitate, and cells with high content of cytoplasmic acid phosphatase bore a pink colour. The 'activated' macrophages co-expressing the brown precipitate and the pink colour were enumerated and expressed as a percent of the total number of positive macrophages.

4.3.4 - PANEL OF MONOCLONAL ANTIBODIES

The panel of monoclonal antibodies directed against leukocyte and macrophage antigens used in this research is presented in Table 4.1.

4.3.4.1 - Combinations of Monoclonal Antibodies Used in Double Immunofluorescence

1) To study the proportions of T cell subsets, particularly CD3+, in the intraepithelial compartment: CD3/CD8; CD3/CD4; CD3/CD4+CD8; CD3/CD5; CD8/CD5; CD3/TCRδ1; CD3/βF1 (the last two performed in immunoperoxidase staining of consecutive slides).

2) To study the ratio of CD4:CD8 positive cells in the bronchial mucosa: CD4/CD8.

3) To identify the proportions and locations of CD8 positive T cells expressing activation markers: CD8/CD7; CD8/CD25; CD8/CD38; CD8/RFDR.

4) To determine the proportions of unprimed and primed CD8 positive cells in the bronchial mucosa: CD8/CD45RA; CD8/CD45RO.

5) To identify the proportions and locations of CD4 positive cells expressing activation markers: CD4/CD7; CD4/CD25; CD4/CD38; CD4/RFDR.

6) To determine the proportions of unprimed and primed CD4 positive T cells in the bronchial mucosa: CD4/CD45RA; CD4/CD45RO.

7) To determine if cells in the follicular aggregates of bronchiectatic bronchial mucosa co-expressed different epitopes of the Leukocyte Common Antigen: CD45RA/CD45RO.

TABLE 4.1

PANEL OF MONOCLONAL ANTIBODIES EMPLOYED IN THE RESEARCH

CD	NAME	ISOTYPE	SPECIFICITY	SOURCE	REFERENCE
2,3,7,8	RFTx	IgG	All T cells	RFH	Janossy et al,1982
3	UCHT1	IgG	All T cells	UCH	Beverley et al,1981
4	Leu3a	IgG	Helper/inducer T cell	BD	Reinherz et al,1980
8	RFT8	IgM	Suppr./cytotoxic T cell	RFH	Janossy et al,1982
5	RFT1	IgG	Most T cells	RFH	Batory et al,1984
7	RFT2	IgG	T cell blasts	RFH	Janossy et al,1982
-	TCR 1	IgG	Gamma/delta TCR	TCS	Brenner et al,1986
-	F1	IgG	Alpha/beta TCR	TCS	Brenner et al,1987
57	Leu7a	IgM	Natural Killer cells	BD	Abo et al,1981
22,37	RFBx	IgG	All B cells	RFH	Collings et al,1984
-	RFD6	IgG	Plasma cells	RFH	Ling et al,1987
-	IgA	IgG	IgA Fc fragment	SL	Raphael et al,1985
-	IgG	IgG	IgG Fc fragment	SL	Raphael et al,1985
-	RFD1	IgM	Interdigitating cells	RFH	Poulter et al,1986
-	RFD7	IgG	Mature macrophages	RFH	Poulter et al,1986
-	RFD9	IgG	Epithelioid cells	RFH	Munro et al,1987
-	UCHM1	IgG	Monocytes	UCH	Hogg et al,1984
25	RFT5	IgG	Interleukin-2 receptor	RFH	Uchyiama et al,1981
-	RFDR1	IgM	MHC Class II	RFH	Janossy et al,1986
-	RFDR2	IgG	MHC Class II	RFH	Janossy et al,1986
38	RFD10	IgG	Activated T/B cells	RFH	Janossy et al,1981
45RA	SN130	IgG	LCA, virgin T cells	RFH	Akbar at al,1988
45RO	UCHL1	IgG	LCA, primed T cells	UCH	Smith et al,1986
-	Ki67	IgG	Proliferating cells	DAKO	Gerdes et al,1984

RFH = Royal Free Hospital School of Medicine, London, UK; UCH = University College Hospital, London, UK; BD = Becton Dickinson Ltd, Oxford, UK; TCS = T Cell Sciences Inc., Cambridge, USA; SL = Sera-Lab, Crawley, UK; DAKO = Dakopatts a/s, Copenhagen, Denmark; LCA = leucocyte common antigen; TCR = T cell receptor; MHC = major histocompatibility complex.

8) To determine if CD8 positive T cells presented evidence of proliferation in situ in the bronchial mucosa: CD8/Ki67.

9) To determine if tissue macrophages in the bronchial mucosa co-expressed differentiation antigens of mononuclear phagocyte series: RFD1/RFD7.

10) To study other co-expression of molecules by CD8 positive cells: CD8/Anti-Leu7a.

4.3.5 - QUANTITATION AND STATISTICS

The quantitation of the immunoperoxidase positive cells present in the bronchial mucosa sections was performed with the Solitaire Plus Image Analysis System (Seescan Imaging PLC, Cambridge, UK). Consecutive sections of bronchial samples were stained with each of the monoclonal antibodies described previously and analysis of technically satisfactory fields, ie. fields where the histological structure have not been disrupted by the lengthy staining procedure, performed. The sections were coded to allow "blind" examination. The positive cells were counted in at least 15 different fields in each of the bronchial mucosa compartments analysed, as defined below. In the case of bronchial biopsies obtained by fibreoptic bronchoscopy, the count was performed in all technically satisfactory fields. A cell was considered positive when brown staining different from the negative controls was seen.

The morphometric system has a series of different commands that allows the count of positively stained cells to be expressed per unit area:

a) Frame Analysis: a precise frame was drawn with the computer's

"mouse" around the compartment to be analysed (Plate 4.2). In this study three "compartments" of the bronchial mucosa were analysed:

- Epithelium: situated between the luminal aspect of the epithelial cells and the epithelial basement membrane;

- Lamina Propria: situated between the epithelial basement membrane and the muscle coat;

- Submucosa: situated between the outer layer of the muscle coat and the perichondrium.

b) Frame Area: this command allows determination of the area of the frame defined above. The device was calibrated in microns and the area of the frame was expressed in square microns. The reference unit area used in all measurements was 10^4 u^2;

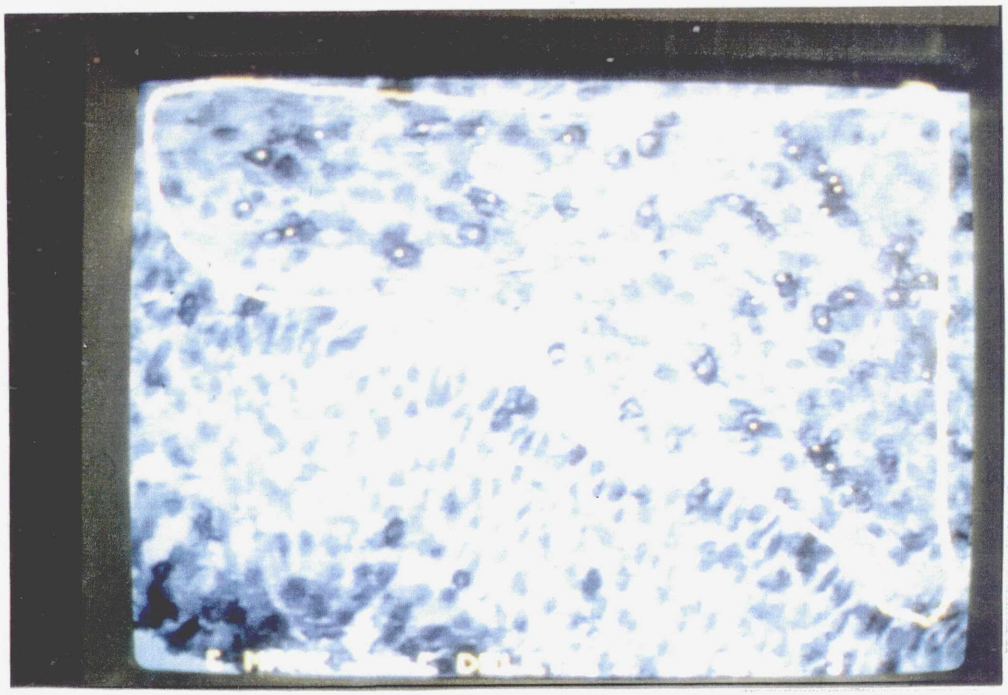
c) Manual Colour Counting: this enabled the positive cells present in the area defined by the frame to be enumerated with the computer's pointer;

d) Colour Enhancement: this provided a best definition of the colours in the image captured by the system, particularly useful in the analysis of three-colour staining.

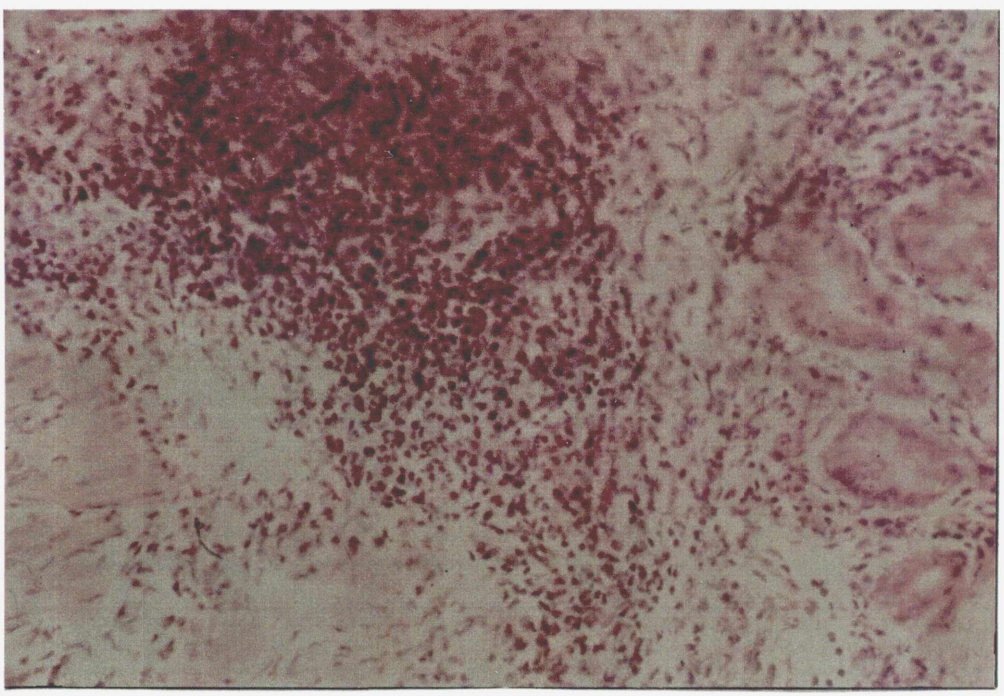
The results for each sections were recorded as the number of positively stained cells per unit area (10^4 u^2) of each compartment analysed.

The quantitation of immunofluorescence positive cells in the two-colour procedures was performed as follows: in the case of CD4:CD8 ratios, at least 200 positive cells were counted in 10 different fields of the bronchial mucosa and the results expressed as the ratio between CD4 and CD8 positive cells. In the case of further definition of the phenotype of a given cell type, at least 150 cells expressing this type were counted in 10 different fields and, by changing the filters, the

PLATE 4.2



a: Counting positive cells with the Solitaire Plus Image Analysis System: the frame delineates an area of lamina propria, allowing positive cells to be enumerated and expressed by unit area



b: Intense infiltration of positive cells in human bronchial mucosa, with the formation of a follicular structure (bronchiectasis, haematoxylin-eosin, original magnification x250)

number of those cells co-expressing the other molecule was recorded. The results were expressed as the percent of the total number of positive cells co-expressing the second molecule studied. In most cases of two-colour immunofluorescence, results were expressed as the overall percentage of double-labelled cells throughout the bronchial mucosa, without taking into consideration their distribution by compartments of the bronchial wall. In the analysis of the phenotype of intraepithelial lymphocytes, however, only cells present in this compartment, as defined above, were enumerated.

The statistical analysis was performed with an Opus Technology PC V Turbo computer, employing the statistical package Minitab (Ryan, Joiner & Ryan, 1985). Results for each parameter analysed were compared between bronchiectasis and control groups. Descriptive statistics (arithmetic mean, median, and standard deviation of the mean) were calculated for each group, as well as 95% confidence intervals of mean and median. Significance testing between the two groups was performed by Student's two-sample t-test. Nonparametric statistics were also performed by Mann-Whitney two-sample rank test. The results were considered statistically significant when $p = < 0.05$.

4.4 - RESULTS

Twenty two patients with bronchiectasis were included in this research on the basis of the criteria presented previously, ie. surgical material available (mostly composed of localised disease) and fibreoptic bronchoscopic biopsies on consecutive patients presenting with diffuse bronchiectasis. Eleven non-bronchiectatic control patients were also studied and the clinical details of both populations are shown in Table 4.2. This study was not designed as a clinical investigation so no attempt was made to correlate the clinical details with the immunopathologic findings.

Due to differences in the clinical data between the control population and the bronchiectatic group, an intra-population analysis of the immunopathological data obtained from the control group was performed. The data was grouped according to sex, age group, site and mode of biopsy, and smoking status. No differences could be established in any of these intra-population analyses (data not shown).

4.4.1 - HISTOPATHOLOGY

Results of the histology of the bronchial samples are shown in Table 4.3. A simple digital scoring system was devised to correlate these data between the groups: a given parameter would score 1 (one) point when present, and when absent it would score 0 (zero); the means of both groups were then compared. All bronchiectasis samples showed infiltration of mononuclear cells in the lamina propria and submucosa, compared with 18.2% of the controls in the lamina propria, and only 9.1% in the submucosa, confirming that in the bronchiectasis population this was the main histopathological feature (Plate 4.2). In almost half of the bronchiectasis samples, these infiltrates showed a follicular

TABLE 4.2

CLINICAL DETAILS

		BRONCHIECTASIS (n=22)	CONTROLS (n=11)	p
Age*		40.8±14.7	57.0±15.8	<0.02
Duration of Symptoms(years)*		22.5±16.6	0.27±0.41	<0.0001
		%	%	
Sex:	Male	27.3	45.5	
	Female	72.7	54.5	
Smoking:	Smokers	12.5	40	
	Non-smokers	75	40	
	Ex-smokers	12.5	20	
Mode of sampling:	Surgical	81.2	72.7	
	Endoscopy	18.2	27.3	
Area of sampling:	LLL	50	27.2	
	RLL	13.6	36.4	
	RUL	-	36.4	
	RML	18.2	-	
	LUL	13.6	-	
	LING	4.6	-	
Aetiology:	Idiopathic	77.5	-	
Primary ciliary dyskinesia		4.5	-	
	Cystic fibrosis	4.5	-	
	ABPA	9	-	

(cont.)

TABLE 4.2 (cont.)

		bronchiectasis	controls
	Foreign body	4.5	-
	Cancer	-	72.7
	Pneumonia	-	27.7
Bronchography:	Cylindrical	63.6	-
	Saccular	18.2	-
	Mixed	18.2	-
Distribution:	Localized	68.2	-
	Diffuse	31.8	-
Pathology:	Follicular	45.5	-
	Non-follicular	55.5	-

Results expressed as percent of patients in both groups presenting each of the charateristics or * as means plus or minus standard deviation. LLL = left lower lobe; RUL = right upper lobe; RML = middle lobe; LUL = left upper lobe; RLL = right lower lobe; LING = lingula; ABPA = allergic bronchopulmonary aspergillosis.

arrangement. None of the controls showed similar changes.

Also relevant was the presence of mucopurulent secretions in the bronchial lumen (68.2% versus 9.1% in the controls). Other pathological changes in the epithelium were also noted in the bronchiectasis group, with 45.2% of samples showing shedding, 50% squamous metaplasia and/or abnormal mucosal folding, and 27.2% goblet cell hyperplasia. Mucous gland hyperplasia was noted in one third of the bronchiectasis samples. Changes in the muscle coat or disruption of the bronchial cartilage were not frequent, neither were changes in the perichondrium or in the peribronchial stroma. Only two samples showed heavy infiltration by eosinophils, both originated from patients with bronchiectasis associated with allergic bronchopulmonary aspergillosis. When the above mentioned scoring system was applied to the sections stained with haematoxylin-eosin, the bronchiectatic population scored 6.0±2.2 (mean ± standard deviation) of a possible total of 14, while the control population scored 0.72±1.3, with a highly significant statististical difference ($p = < 0.0001$). The scoring system also confirmed that the control population displayed a relatively normal histology, which made it a reliable group for comparing with the bronchiectasis group.

4.4.2 - DISTRIBUTION AND PHENOTYPE OF THE IMMUNOCOMPETENT CELL POPULATION

Table 4.4 presents the distribution and phenotype of the immunocompetent cell population in the bronchial epithelium of the bronchiectasis and control groups, Table 4.5 shows the results for the lamina propria of both populations, and Table 4.6 shows the results for

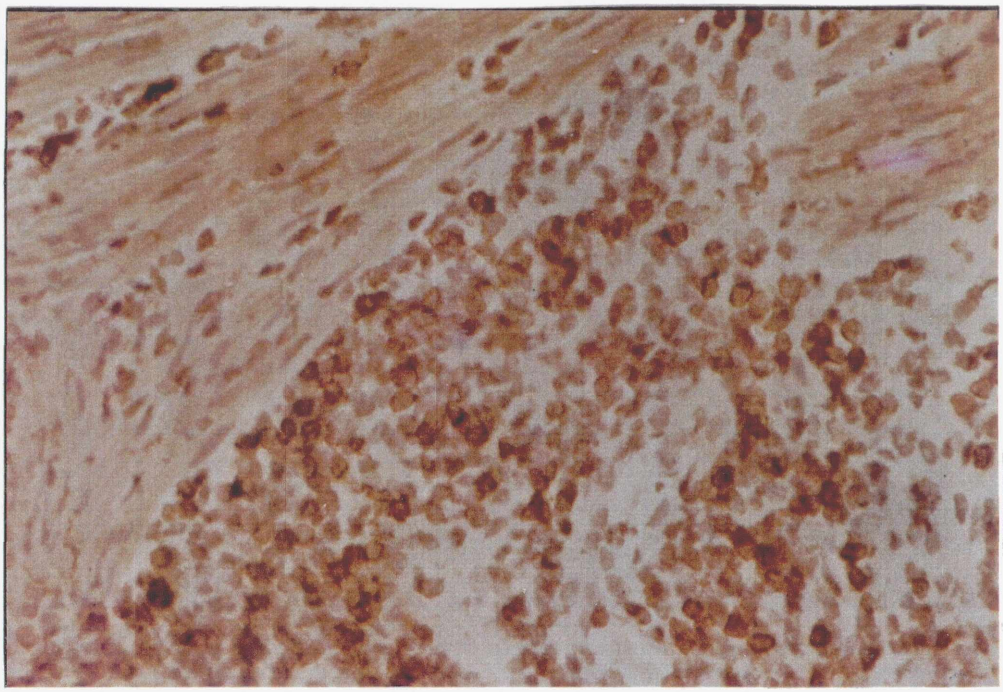
TABLE 4.3

HISTOPATHOLOGICAL CHANGES

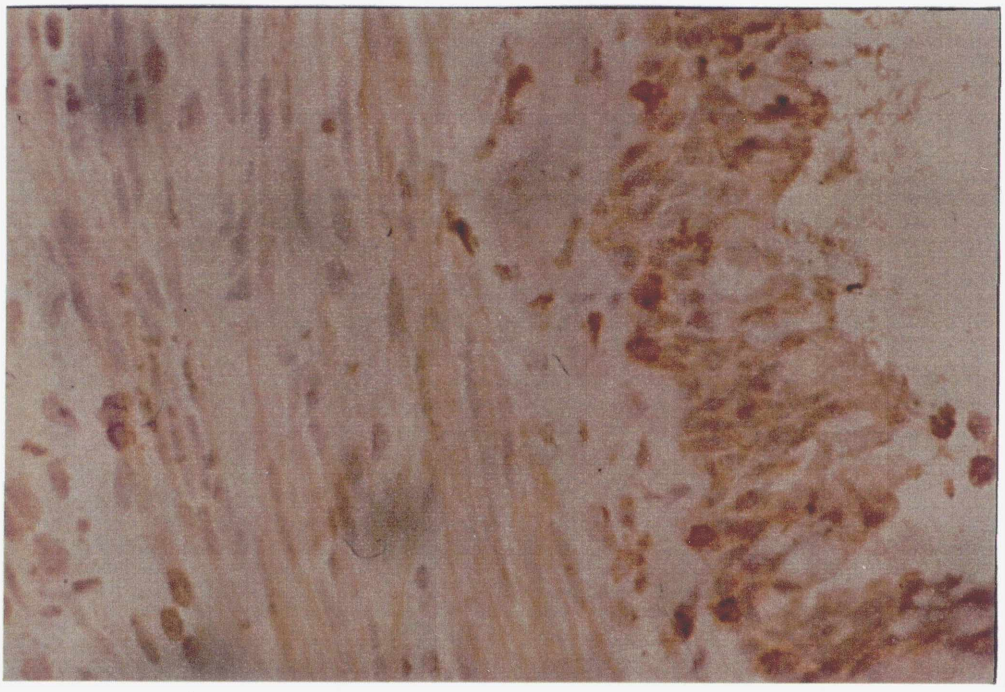
	BRONCHIECTASIS (n=22) %	CONTROLS (n=11) %
Mucopus in lumen	68.2	9.1
Disruption of epithelium	45.5	18.2
Goblet cell hyperplasia	27.3	9.1
Other EP changes	50.0	0
Fibrosis in LP	59.1	0
Neovascularisation in LP	22.7	0
Infiltration of MNC in LP	100.0	18.2
Disruption of muscle coat	27.3	0
Presence of follicles	45.5	0
Eosinophils	9.1	0
Mucous gland hyperplasia	31.8	0
MNC in submucosa	100.0	9.1
Disruption of cartilage	15.8	0
Peribronchial stroma	5.2	0

Results expressed as percentage of samples from each group showing the change. MNC = mononuclear cell; EP = epithelium; LP = Lamina propria.

PLATE 4.3



a: Intense infiltration of T-lymphocytes in human bronchial mucosa (bronchiectasis, CD3, immunoperoxidase, original magnification x250)



b: Scant T-lymphocytes in normal human bronchial mucosa (control, CD3, immunoperoxidase, original magnification x250)

the bronchial submucosa. The main characteristics of the chronic inflammatory reaction of all bronchiectasis cases was the large increment in the number of T-lymphocytes infiltrating all compartments of the bronchial wall (Plate 4.3). These were diffusely distributed in all compartments of the bronchial wall but in some cases a follicular pattern was seen. There was a highly significant difference between the numbers of T cells in bronchiectatic and control samples in all compartments but in the lamina propria this difference was most prominent ($p = < 0.00005$).

Large numbers of B-lymphocytes and plasma cells were present in about half the samples but in the other half none or very few of these cells were seen, with a large standard deviation almost precluding statistical significance. Another interesting aspect was the complete absence of B cells in the intraepithelial compartment, even in those samples with large numbers of such cells in other compartments.

The frequency of accessory cells in the bronchiectatic wall was also very different from the controls. The RFD7+ macrophage was seen in large numbers in all compartments and was the most frequent of the three types of accessory cells studied (Plate 4.4). The RFD1+ interdigitating dendritic cells were also present in great numbers, mainly in the epithelium.

The bronchiectasis cases were subdivided in two groups, according to the presence of follicular aggregates. The immunopathologic data was compared between the two groups (Table 4.7). Differences were seen in the numbers of T cells present in the lamina propria and submucosa of cases with follicular arrangement compared with the other subgroup: a highly significant difference was seen in both compartments, with the "follicular" subgroup showing a prominent increase in the absolute

TABLE 4.4

PHENOTYPE OF IMMUNOCOMPETENT CELLS IN THE BRONCHIAL EPITHELIUM: BRONCHIECTASIS AND CONTROLS

MoAb	BRONCHIECTASIS (n=22)	CONTROLS (n=11)
RFTmix	7.64±4.15(19)	2.61±1.18(11)
p	0.0001 [0.0003]	
Leu7	0.96±1.45(14)	0.21±0.37(11)
p	0.08 [< 0.03]	
RFBmix	0(19)	0(11)
p	--	
RFD7	2.36±1.43(17)	0.66±0.44(11)
p	0.0002 [0.0001]	
UCHM1	1.51±0.83(17)	0.11±0.16(11)
p	0.00005 [0.0002]	
RFD1	1.99±1.08(17)	0.48±0.58(11)
p	0.00005 [0.0001]	

Results expressed as arithmetic mean ± standard deviation (no. of samples) of numbers of positively stained cells per 10^4 u^2 of epithelium; MoAb = monoclonal antibody; p values - Student's t-test [Mann-Whitney test]. For descriptive statistics see Appendix 1.

TABLE 4.5

PHENOTYPE OF IMMUNOCOMPETENT CELLS IN THE BRONCHIAL LAMINA PROPRIA : BRONCHIECTASIS AND CONTROLS

MoAb	BRONCHIECTASIS (n=22)	CONTROLS (n=11)
RFTmix	7.35±4.47(22)	1.67±0.68(11)
p	< 0.00005 [< 0.00005]	
Leu7	0.50±0.60(14)	0.15±0.22(11)
p	0.056 [0.04]	
RFBmix	1.86±3.72(21)	0.012±0.04(11)
p	0.03 [0.02]	
RFD6	3.56±4.02(18)	1.02±0.46(11)
p	0.02 [0.03]	
RFD7	3.10±1.61(21)	0.79±0.60(11)
p	< 0.00005 [< 0.00005]	
UCHM1	2.25±1.33(20)	0.22±0.19(11)
p	< 0.00005 [0.0001]	
RFD1	1.63±0.90(19)	0.66±0.49(11)
p	0.0008 [0.04]	

Results presented as arithmetic mean± SD (number of samples) of numbers of positively stained cells per 10^4 u^2 of lamina propria;
MoAb = monoclonal antibody; p values = Student's t-test [Mann-Whitney test]. For descriptive statistics see Appendix 1.

TABLE 4.6

PHENOTYPE AND DISTRIBUTION OF IMMUNOCOMPETENT CELLS IN THE BRONCHIAL SUBMUCOSA: BRONCHIECTASIS AND CONTROLS

MoAb	BRONCHIECTASIS (n=22)	CONTROLS (n=11)
RFTmix	6.90±4.02(21)	1.77±0.6(10)
p	< 0.00005 [< 0.00005]	
Leu7	0.56±0.58(12)	0.09±0.15(10)
p	< 0.02 [< 0.02]	
RFBmix	2.25±4.62(20)	0.01±0.02(10)
p	0.03 [0.07]	
RFD6	4.09±4.09(17)	1.36±1.13(10)
p	0.02 [0.02]	
RFD7	3.19±1.39(19)	1.12±0.71(10)
p	< 0.00005 [0.0002]	
UCHM1	2.74±1.51(19)	0.20±0.24(10)
p	< 0.00005 [< 0.00005]	
RFD1	1.86±1.21(18)	0.77±0.61(10)
p	0.005 [0.005]	

Results presented as arithmetic mean± SD (number of samples) of numbers of positively stained cells per 10^4 u^2 of submucosa;

MoAb = monoclonal antibody; p values = Student's t-test [Mann-Whitney test]. For descriptive statistics see Appendix 1.

TABLE 4.7

COMPARISON BETWEEN FOLLICULAR AND NON-FOLLICULAR BRONCHIECTASIS

MoAb / COMP.		FOLLICULAR (n=10)	NON-FOLLICULAR (n=12)
Tx	LP	10.91±3.59(10)	4.63±2.34(12)
	p	0.0003 [0.0007]	
	SM	9.98±3.64(10)	4.09±1.46(11)
	p	0.0006 [0.0003]	
Bx	LP	2.53±1.85(9)	0.01±0.04(12)
	p	0.004 [<0.00005]	
	SM	3.08±2.92(9)	0.01±0.06(11)
	p	0.02 [0.004]	
D6	LP	4.22±2.78(9)	1.22±0.64(8)
	p	0.02 [0.02]	
	SM	4.72±2.07(9)	1.39±0.53(7)
	p	0.002 [0.003]	

Results presented as arithmetic mean±SD (number of samples) of the numbers of positively stained cells per unit area (10^4 u^2) of each compartment. LP = lamina propria; SM = submucosa; MoAb = monoclonal antibody; COMP = compartment; Tx = RFTmix; Bx = RFBmix; D6 = RFD6; p values = Student's t-test [Mann-Whitney test]. For descriptive statistics see Appendix 1.

numbers of T cells.

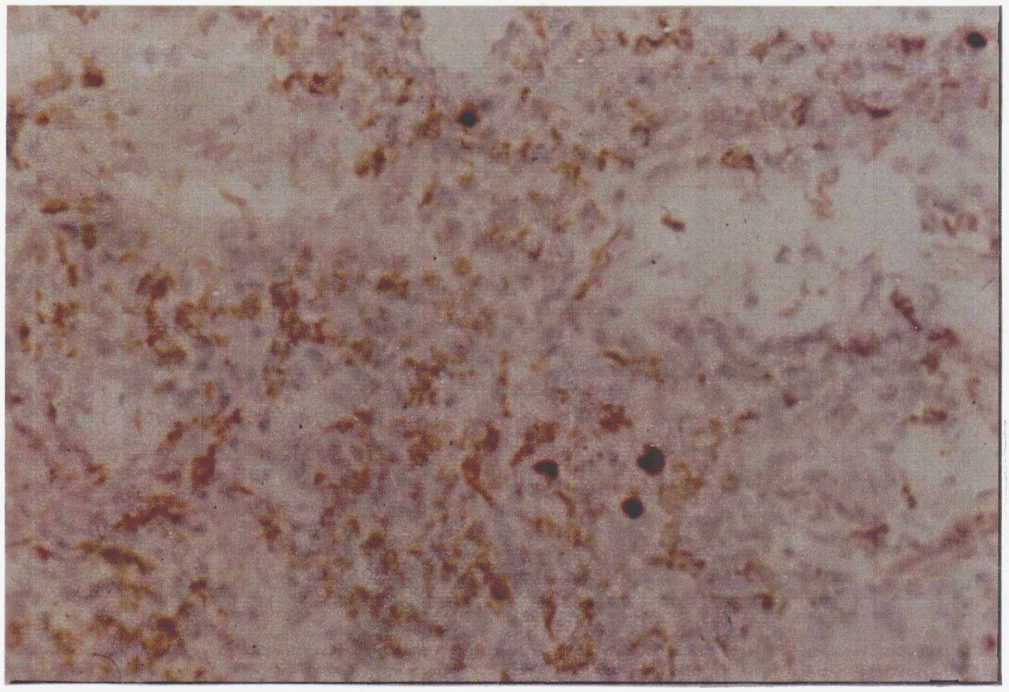
B-lymphocytes were only found in the "follicular" subgroup, with a significant difference ($p = < 0.004$) in B cells present in the lamina propria (Plate 4.4). The numbers of B-lymphocytes and plasma cells in the "non-follicular" group were similar to that in controls. No other differences could be established between the two subgroups with respect to the numbers and distribution of accessory cells. The B-lymphocytes were almost totally confined within the follicles, which were also composed of large numbers of T cells and some accessory cells. Plasma cells were also seen within the follicles, many were diffusely present in the lamina propria, but most were seen in the submucosa in close association with the mucus glands.

4.4.3 - EXPRESSION OF FUNCTIONALLY RELEVANT MOLECULES BY BRONCHIAL T-LYMPHOCYTES

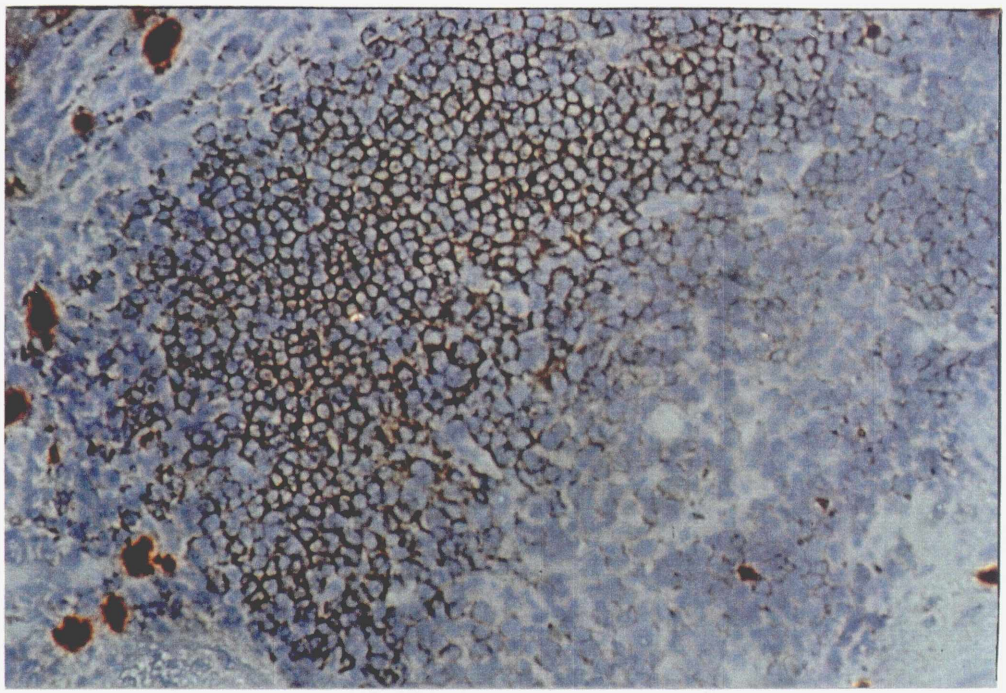
4.4.3.1 - Ratios of CD4 and CD8 Positive T-Lymphocytes in the Bronchial Mucosa

The ratios of the two main subsets of T-lymphocytes - the CD4 positive helper/inducer cells, and the CD8 positive suppressor/ cytotoxic cells were first examined. The CD4 : CD8 ratio of the control samples showed the number of CD8+ suppressor/cytotoxic cells to exceed the number of CD4+ helper/inducer cells [CD4 : CD8 = 1 : 2.27 ± 0.47 (11)], mainly due to the larger number of CD8+ cells in the intraepithelial compartment. In the lamina propria and submucosa, the number of CD4+ cells slightly exceeded the number of CD8+ cells but the overall ratio for the bronchial mucosa (used here and in all other measurements presented in this section) showed predominance of the CD8+

PLATE 4.4



a: Intense infiltration of macrophages in human bronchial mucosa (bronchiectasis, RFD7, immunoperoxidase, original magnification x250)



b: B-lymphocytes arranged in a follicular structure in human bronchial mucosa (bronchiectasis, RFBmix, immunoperoxidase, original magnification x250)

TABLE 4.8

EXPRESSION OF FUNCTIONALLY RELEVANT MOLECULES BY CD8 POSITIVE SUPPRESSOR/CYTOTOXIC T-LYMPHOCYTES IN THE BRONCHIAL MUCOSA

	BRONCHIECTASIS (n=22)	CONTROLS (n=11)	p
CD25	4.6±3.9(17)	0(10)	0.001
CD38	12.3±8.9(13)	0(9)	0.0005
CD7	78.2±17.2(19)	23.5±21.7(10)	<0.00005
HLA-DR	19.2±7.3(19)	2.8±2.2(10)	<0.00005
CD45RO	73.5±9.9(13)	46.0±13.5(10)	0.0001
CD45RA	18.5±10.3(17)	20.5±4.4(10)	NS
Leu7a	9.3±8.4(15)	0(5)	0.03
Ki67	0(16)	0(5)	-

Results expressed as means ± SD (number of samples) of percents of doubly labelled cells in all compartments of the bronchial mucosa; p value = Student's t-test. For descriptive statistics see Appendix 1.

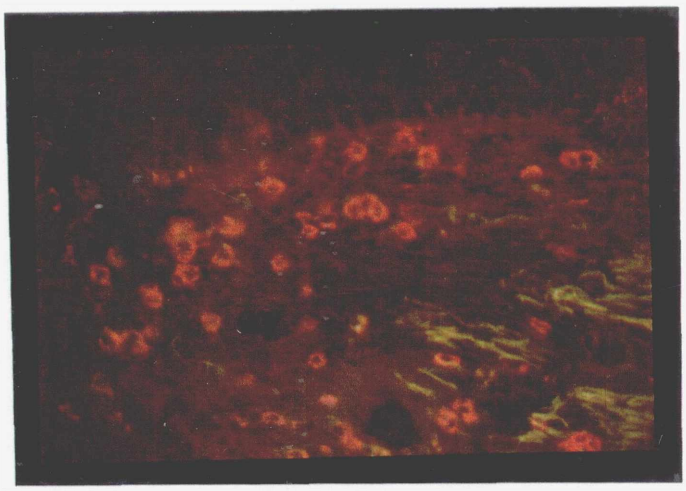
subset.

The bronchiectasis samples showed an overall predominance of the CD8 positive subset. In a majority of specimens (15 out of 22), the CD8+ cells predominated over the CD4+, with ratios ranging from 2 : 1 to 10 : 1 (mean CD4 : CD8 ratio+SD = 1 : 6.4±3.2), and a highly significant difference when compared with the ratios in controls ($p = < 0.0003$). However, in six samples a large predominance of CD4+ cells was noted, also ranging from 1 : 2 to 1 : 10 (mean CD4 : CD8 ratio±SD = 5.33±2.6 : 1; $p = < 0.004$) (Plate 4.5). In one specimen, the numbers of CD4+ and CD8+ T cells were similar.

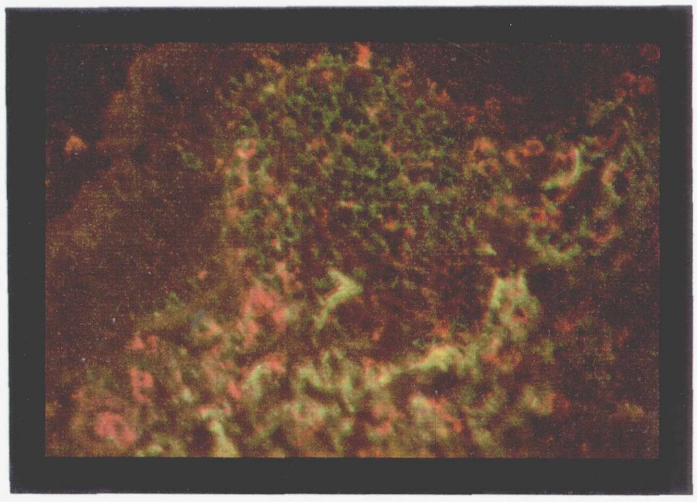
4.4.3.2 - Expression of Activation Markers by the CD8 Positive Suppressor/Cytotoxic T-Lymphocytes in the Bronchial Mucosa

Further analysis of the CD8 positive cells was performed by means of indirect double imunofluorescence to determine the co-expression of activation markers and other relevant molecules. The results were expressed as percentages of double labelled cells shown in Table 4.8. Statistically significant differences between bronchiectatic and control samples were noted in the co-expression of CD8 and different cell activation markers. Almost 80% of the CD8+ cells present in bronchiectatic samples co-expressed CD7, a T-cell antigen expressed in large amounts by lymphoblasts, compared with less than 25% in the controls ($p = < 0.00005$). Also very significant was the co-expression of CD8 and Class II Major Histocompatibility Complex HLA-DR antigens, with almost 20% of double labelled cells in the bronchiectatic wall, compared with only 3% in controls ($p = < 0.00005$). Small but statistically significant populations of double positive CD8/CD38 (p =

PLATE 4.5



a: Predominance of CD8+ T cells, stained in red, over CD4+ cells, stained in green, in human bronchial lamina propria (bronchiectasis, CD4/CD8, double immunofluorescence, original magnification x250)



b: Predominance of CD4+ T cells, stained in green, over CD8+ cells, stained in red, in a follicular structure seen in human bronchial lamina propria (bronchiectasis, CD4/CD8, double immunofluorescence, original magnification x250)

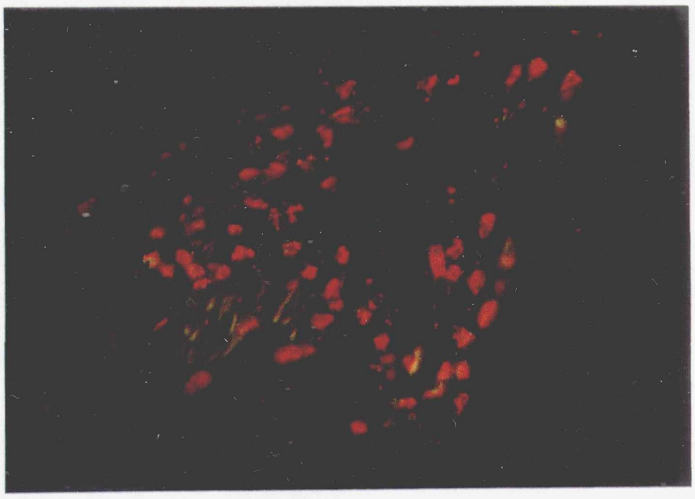
TABLE 4.9

EXPRESSION OF FUNCTIONALLY RELEVANT MOLECULES BY CD4 POSITIVE HELPER/INDUCER T-LYMPHOCYTES IN THE BRONCHIAL MUCOSA

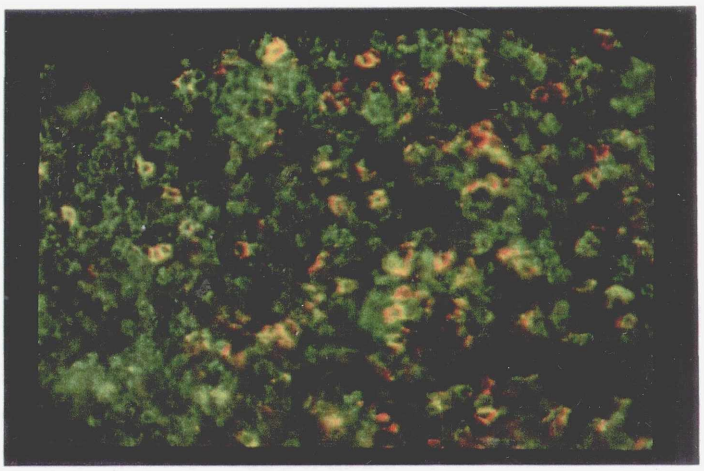
	BRONCHIECTASIS	CONTROLS	p
CD25	1.23±1.5(17)	0(10)	NS
CD38	6.1±6.7(18)	1.1±2.1(10)	<0.01
CD7	27.6±26.1(17)	15.5±12.3(10)	NS
HLA-DR	24.0±26.1(17)	3.0±3.6(10)	<0.01
CD45RO	63.1±18.9(16)	46.1±13.8(9)	0.02
CD45RA	46.1±29.6(13)	28.3±10.6(9)	NS

Results expressed as means ± SD (number of samples) of percents of double labelled cells in all compartments of the bronchial mucosa; p value = Student's t-test. For descriptive statistics see Appendix 1.

PLATE 4.6



a: CD8+ cells, stained in red, double stained with CD45RA in green, fail to show positivity for the second marker, and appear in red in the doubly exposed film (bronchiectasis, CD8/CD45RA, double immunofluorescence, original magnification x250)



b: CD8+ cells, stained in red, double stained with CD45RO in green, showing almost 100% co-expression of the markers (bronchiectasis, CD8/CD45RO, double immunofluorescence, original magnification x250)

0.0005) and CD8/CD25 ($p = 0.001$) were also seen in samples from bronchiectatic patients.

4.4.3.3 - Expression of CD45 and Other Relevant Molecules by the CD8 Positive Suppressor/Cytotoxic T-Lymphocytes in the Bronchial Mucosa

The proportions of CD8+ cells in the bronchial wall expressing antigens of the CD45 superfamily were investigated by double immunofluorescence and the results shown in Table 4.8. Bronchiectatic specimens possessed a larger population of CD8+/CD45RO+ T cells (a phenotype of primed, memory cells) than the controls ($p = 0.0001$), whereas the number of CD8+/CD45RA+ T cells (a phenotype of naive, virgin cells) was similar in both populations (Plate 4.6).

Other relevant molecules were also studied and a small but significant proportion of CD8+ cells co-expressing Leu7, an antigen present in cells with natural killer capacity, was found in all compartments of bronchiectatic bronchial wall ($p = 0.03$).

No cells with the double phenotype CD8/Ki67 were seen in either bronchiectasis or control specimens, suggesting that the CD8+ cells were not dividing <u>in situ</u>, despite displaying signs of cell activation and immunological commitment in bronchiectatic cases.

4.4.3.4 - Expression of Activation Markers by the CD4 Positive T-Lymphocytes in the Bronchial Mucosa

The phenotype of CD4+ T cells was investigated in a similar manner. Table 4.9 shows the results for bronchiectasis group and controls, and statistical comparison. A significant population of CD4+ cells co-expressing the activation marker HLA-DR was seen in the bronchiectatic wall ($p = 0.006$), mainly in the large follicular

aggregates. Also significantly increased was the number of double positive CD4/CD38 T cells in the bronchiectatic samples but the populations co-expressing CD4/CD7 or CD4/CD25 did not differ significantly from the controls. However, in the case of CD4+ T cells, a clear correlation was found when the bronchiectatic samples were divided into two groups according to the presence or absence of follicular aggregates. In follicular bronchiectasis samples, a much larger and statistically significant proportion of CD4+ cells co-expressing activation markers was found, as presented in Table 4.10.

4.4.3.5 - Expression of CD45 Antigens by the CD4 Positive T-Lymphocytes in the Bronchial Mucosa

Large numbers of CD4+ cells bore the double phenotype CD4/CD45RO, in the bronchiectasis samples (Table 4.9), differing significantly from controls ($p = <0.02$). Despite a larger number of CD4+ cells co-expressing CD45RA, no significant difference could be established from the controls. An interesting phenomenon was observed in five samples from follicular bronchiectasis: large numbers of CD4+ cells in the follicles co-expressed CD45RA and CD45RO.

4.4.4 - EXPRESSION OF OTHER MOLECULES BY CELLS OF MONOCYTE/MACROPHAGE LINEAGE IN THE BRONCHIAL MUCOSA

Co-expression of the molecules RFD1 and RFD7 by the macrophage population (the phenotype of a subset of macrophage-like cells with suppressor functions of pathogenetic significance in lung diseases where cell-mediated immune mechanisms are thought to be important) was investigated by means of double immunofluorescence. Twelve

TABLE 4.10

EXPRESSION OF FUNCTIONALLY IMPORTANT MOLECULES BY CD4 POSITIVE T-LYMPHOCYTES IN THE BRONCHIAL MUCOSA OF FOLLICULAR AND NON-FOLLICULAR BRONCHIECTASIS

	FOLLICULAR	NON-FOLLICULAR
CD7	45.5±14.2(9)	7.5±21.2(8)
p	0.002 [0.005]	
CD25	2.62±1.06(8)	0.01±0.01(9)
p	0.0002 [0.001]	
CD38	10.0±4.33	2.22±6.66(9)
p	0.02 [0.003]	
HLA-DR	33.5±26.4(8)	10.0±13.3(9)
P	< 0.05 [< 0.05]	

Results expressed as mean±SD (number of samples) of percents of double labelled cells in all bronchial compartments.

p values = Student's t-test [Mann-Whitney test]. For descriptive statistics see Appendix 1.

bronchiectasis samples and five controls were stained but no double phenotype was found in either group.

No RFD9 positive cells were seen in bronchiectasis or control samples.

The increased expression of the lysosomal hydrolase acid phosphatase in macrophage-like cells was investigated in both populations using a double imunohistochemical procedure: sections were stained with RFD1 and RFD7 in immunoperoxidase staining, followed by staining of acid phosphatase (ACP) with the hexazonium pararosaniline. Eleven bronchiectasis and five control samples were double stained: 18.6±5.1 % of the RFD1+ cells in the bronchiectasis samples were also positive for acid phosphatase staining (controls: 14±6.5%; p = not significant), while 45±10.7% of the RFD7+ cells in bronchiectasis specimens were acid phosphatase positive as well (controls: 24±8.5%; p = <0.002).

4.4.5 - DISTRIBUTION OF SURFACE IMMUNOGLOBULIN ISOTYPES ON THE CELLS OF B-LYMPHOCYTE LINEAGE IN THE BRONCHIAL MUCOSA

In cases of bronchiectasis where the distribution of surface immunoglobulin positive cells was investigated, increased proportion of IgA and IgM bearing cells was seen in the submucosa (Table 4.11).

4.4.6 - THE BRONCHIAL INTRAEPITHELIAL LYMPHOCYTE

In most human mucosae, intraepithelial lymphocytes are composed almost exclusively of a heterogeneous population of T cells whose differentiation pathways and functions remain largely unknown. It has been proposed that these cells may be important in epithelial surveillance. To accomplish this function, T cells use the T Cell

TABLE 4.11

DISTRIBUTION OF SURFACE IMMUNOGLOBULIN POSITIVE CELLS IN THE BRONCHIAL MUCOSA

		BRONCHIECTASIS	CONTROLS	p
IgA	LP	1.08±0.99(6)	0.48±0.24(8)	NS
	SM	1.63±0.84(6)	0.60±0.44(7)	0.03
IgM	LP	0.51±0.20(7)	0.33±0.34(7)	NS
	SM	1.16±0.79(7)	0.34±0.25(7)	0.04
IgG	LP	0.75±0.49(7)	0.40±0.23(5)	NS
	SM	1.12±0.83(7)	0.48±0.48(7)	NS

Results expressed as means±SD (number of samples) of numbers of positively stained cells per unit area (10^4 u^2) of each bronchial compartment. LP = lamina propria; SM = submucosa; NS = statistically not significant; p values = Student's t-test. For descriptive statistics see Appendix 1.

Antigen Receptor, or for short the T cell receptor (TCR), as the antigen-recognizing element. This receptor has two isotypes, the TCR-alpha/beta and the TCR-gamma/delta. Very little is known about the phenotype of intraepithelial lymphocytes and the distribution of the isotypes of the T cell receptor in the bronchial epithelium either in normal or pathological conditions. Fifteen samples from patients with bronchiectasis and seven samples from controls were included in this study to determine the phenotype of bronchial intraepithelial lymphocytes.

Table 4.12a shows the results for the CD3+ intraepithelial lymphocytes and the distribution of the two isotypes of the TCR. The number of CD3+ intraepithelial lymphocytes increased almost three-fold in the bronchiectatic group and both isotypes of TCR also increased significantly. When the number of cells expressing the alpha/beta isotype was expressed as a percent of the CD3+ intraepithelial lymphocytes, no differences between bronchiectasis and controls were noted. However, the percentage of cells exhibiting the phenotype of gamma/delta TCR in relation to the CD3+ T cells was slightly but statistically significantly different between the two populations.

Table 4.12b shows the distribution of the main T cell subsets. The large majority of CD3+ intraepithelial lymphocytes in both bronchiectasis and controls were CD8+ suppressor/cytotoxic T cells. The large majority of CD8+ intraepithelial lymphocytes failed to express CD5, a T-lymphocyte marker usually not expressed by the resident intraepithelial population, and no differences were seen in this respect between the bronchiectasis group and controls. However, significant differences between bronchiectasis and controls were

established in the numbers of CD8+ cells co-expressing CD45RO and HLA-DR, the bronchiectasis samples exhibiting large increments of both markers (Plate 4.7).

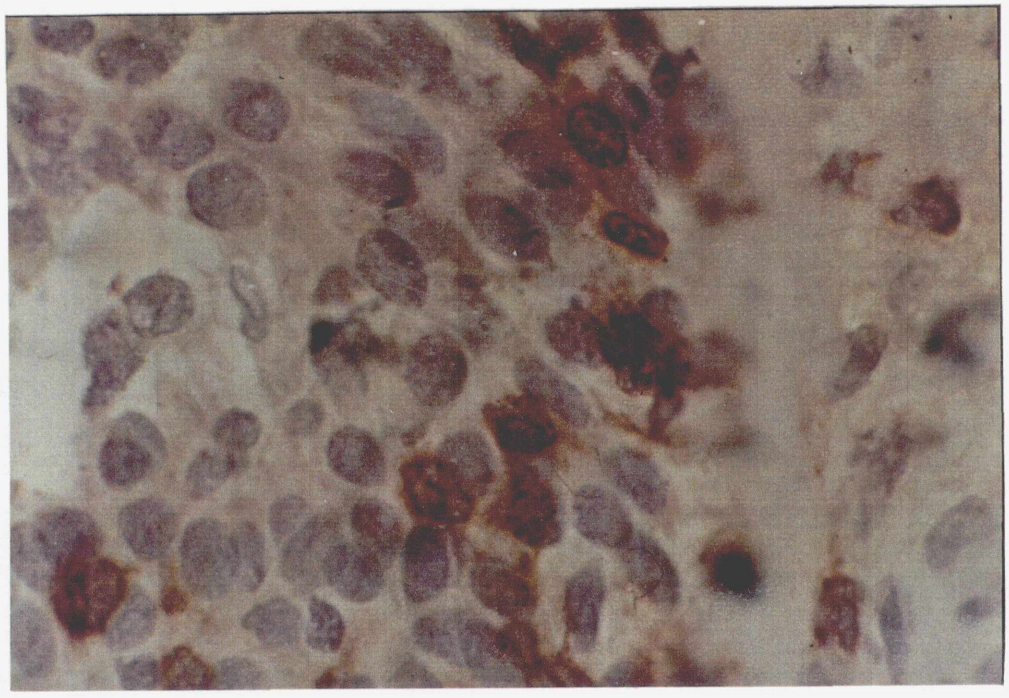
TABLE 4.12

PHENOTYPE OF BRONCHIAL INTRAEPITHELIAL LYMPHOCYTES IN BRONCHIECTASIS AND CONTROLS

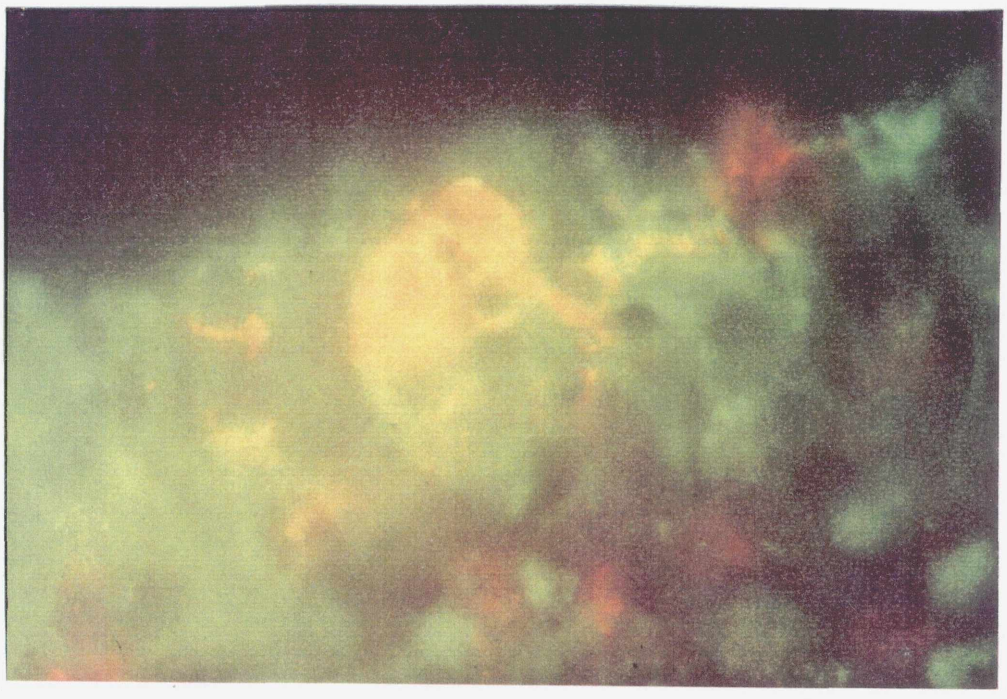
	BRONCHIECTASIS	CONTROLS	p
A) Distribution of T-cell receptor isotypes			
CD3*	6.71±2.85(15)	2.74±1.05(7)	0.0001
TCRδ1*	1.14±0.54(15)	0.28±0.18(7)	<0.00005
βF1*	4.36±2.24(15)	2.05±0.55(7)	<0.002
δ1/CD3	19.6±11.6(15)	10.7±5.1(7)	<0.03
βF1/CD3	66.4±24.6(15)	78.6±17.6(7)	NS
B) distribution of other relevant molecules			
CD3+/CD8+	84.9±10.8(14)	78.8±8.8(7)	NS
CD3+/CD4+	15.3±10.6(14)	10.7±6.3(7)	NS
CD3+/CD4-CD8-	6.6±3.8(12)	3.4±1.9(7)	NS
CD8+/CD5+	9.5±3.4(14)	13.6±6.4(7)	NS
CD8+/CD45RO+	75.5±8.8(10)	52.7±11.1(7)	0.001
CD8+/HLA-DR+	15.9±7.3(13)	3.8±2.03(7)	0.0001

Results expressed as means ±SD (number of samples) of percents of double labelled cells per unit area of bronchial epithelium (10^4 u^2) or *means+SD (number of samples) of numbers of positively stained cells per same unit area; p values = Student's t-test; NS = statistically not significant. For descriptive statistics see Appendix 1.

PLATE 4.7



a: CD3+ intraepithelial lymphocytes in human bronchus (bronchiectasis, immunoperoxidase, original magnification x600)



b: CD8+ intraepithelial lymphocytes, stained in red, double labelled with MHC Class II in green. Cells co-expressing the markers appear in yellow (bronchiectasis, CD8/HLA-DR, double immunofluorescence, original magnification x600)

4.5 - DISCUSSION

Bronchiectasis is an understudied chronic respiratory disease (Barker & Bardana, 1988), particularly from the immunopathogenesis aspect. The results reported in this thesis represent the first comprehensive study of the immunopathological contribution to the inflammatory host response occurring in the bronchiectatic bronchial wall (Lapa e Silva, Cole, Jones et al, 1987; Lapa e Silva, Jones, Cole et al, 1988; Lapa e Silva, Jones, Noble et al, 1988; Lapa e Silva, Cole & Poulter, 1989; Lapa e Silva, Jones, Cole et al, 1989; Lapa e Silva, Cole & Poulter, 1990; Lapa e Silva, Poulter & Cole, 1990). The information here forms an essential basis for work aiming to extend knowledge about how host-mediated inflammatory mechanisms, particularly those of immunologic nature, may be implicated in pathogenesis of bronchiectasis.

The use of immunohistochemical techniques allowed characterization of the phenotype of the immunocompetent cells that populate the normal bronchus and demonstration of the changes in these which occur in bronchiectasis. Despite increasing interest in immunopathogenic aspects of a variety of lung diseases, particularly interstitial diseases of unknown aetiology (cryptogenic fibrosing alveolitis, sarcoidosis) or of established cause (hypersensitivity pneumonitis, occupational lung diseases), it is only comparatively recently that publications regarding the phenotype of bronchial immunocompetent cells have started to appear (Fournier, Lebargy, Ladurie et al, 1989; Poulter, Power & Burke, 1990).

There was an increase in the number of all major types of immunocompetent cells in most of the bronchiectasis samples. The most

consistent finding throughout the investigation was the increased numbers of T-lymphocytes affecting all samples and seen in all compartments. However, a large variation was seen, with increases from 3 to 10-fold in T cells numbers over controls. The infiltrating T cells were loosely distributed in the bronchial wall of all samples and, in almost a half, clusters or even follicle-like structures were present in the lamina propria and in the submucosa. A large number of infiltrating T cells has been described in other lung diseases, especially with involvement of the terminal lung structures and interstitium. A lymphocytic alveolitis is a hallmark of sarcoidosis (Campbell, Poulter & Du Bois, 1985) and of hypersensitivity pneumonitis (Barrios, Selman, Franco et al, 1985) where the lymphocytes are thought to perform a role in the immunopathogenesis of the conditions. However, much wider information is available about the role of T cells, particularly of activated T cells, in the development of chronic inflammatory diseases of the intestine (MacDonald & Spencer, 1988; MacDonald, 1990; Allison, Poulter, Dhillon et al, 1990) and in the emergence of auto-immune diseases (Janossy, Duke, Poulter et al, 1981). As far as bronchiectasis is concerned, the major question is whether T-lymphocytes are implicated in the genesis of the chronic lesions of the bronchial mucosa or are merely secondary consequences of the disease. The simple presence of large numbers of those cells associated with the lesions does not indicate their primary role in the development of the disease.

Lymphocytes exhibiting the phenotype of natural killer cells were also seen in large numbers in the lamina propria of bronchiectasis samples. These cells are recognised to be implicated in the pathogenesis of chronic inflammatory bowel disease (Shanahan, Brogan &

Targan, 1989) and therefore could also play a role in the development of bronchiectasis.

The number of cells of monocyte/macrophage lineage was also increased in the lesions of bronchiectasis. Large and highly significant increments in numbers of monocytes, mature histiocytes and interdigitating dendritic cells were noted in all compartments of the bronchial wall They occurred in close association with the lymphocytic infiltration in situations reminiscent of Type IV hypersensitivity cell-mediated immune reactions, as those seen in sarcoidosis, leprosy (Munro, Campbell, Collings et al, 1987), and the Mantoux reaction (Poulter, Seymour, Duke et al, 1982). However, no granulomata were ever seen, apart from in a very advanced case of bronchiectasis occuring in a case of allergic bronchopulmonary aspergillosis evolving to bronchocentric granulomatosis, a rare complication of this condition.

The distribution of B-lymphocytes was an enigma. In more than half of the samples, none or very few B-lymphocytes were seen. However, plasma cells were seen in all samples. In those where few or no B cells were present, there were similarly scant numbers of plasma cells. In the other samples, B-lymphocytes were seen in very variable numbers. Coincidentally, all these samples presented follicular aggregates. The follicles were composed of large numbers of T cells (mainly arranged in the periphery), B cells (centrally orientated), and fewer macrophages and dendritic cells interspersed between them all. The structure is reminiscent of any peripheral lymphoid organ, such as lymph node or spleen.

Correlation of the immunopathological data with the presence of follicular aggregates indicated that in the "follicular group" the

numbers of T-lymphocytes in lamina propria and submucosa were significantly greater than those present in the "non-follicular" group and even greter than those present in the whole bronchiectatic group. The CD4 positive phenotype formed the main T cell subset in the large follicular aggregates. Many of those CD4+ cells bore the double phenotype of CD45RO+/CD45RA+ cells.

Overall, both T cell subsets displayed the phenotype of activated, immunologically committed cells in bronchiectasis samples. The CD8+ population co-expressed statistically significant numbers of different activation markers, such as CD25, CD7, CD38, and mainly HLA-DR. Differences between bronchiectatics and controls were also noted in the proportions of CD8+/CD45RO+, the phenotype of primed, memory cells (Akbar, Terry, Timms et al, 1988). The CD4 positive population in the bronchiectatic samples expressed significant levels of some activation markers too, especially of HLA-DR, and differences could be found in the proportions of CD4+/CD45RO+.

The activation of T-lymphocytes in an immune process results in the expression of cell surface molecules, such as the Major Histocompatibility Complex Class II antigen HLA-DR and the receptor for the lymphokine interleukin-2 (IL-2R), the expression of IL-2R being one of the first events (Janossy, Campana & Akbar, 1989). The expression of this receptor is transient, whereas the expression of HLA-DR appears later and lasts longer. This could explain the findings of a small but significant expression in the T cells of CD25 antigen in the bronchiectasis samples and of larger numbers of T cells expressing HLA-DR.

The predominance of the phenotype CD45RO+/CD45RA- in the T-lymphocytes infiltrating the bronchiectatic areas was remarkable. It

has been demonstrated that the unprimed, "virgin" T cell is CD45RA+/CD45RO- and, upon activation by antigen, stimulation by phytohaemaglutinin or in a mixed lymphocyte reaction, this T cell loses the CD45RA and gains the CD45RO molecule. This CD45RO+/CD45RA- T cell shows a much greater capacity of proliferation when rechallenged by the original antigen than its counterpart with inverse phenotype, suggesting that CD45RO identifies a primed population of T cells which may include memory cells (Akbar, Timms & Janossy, 1989). The fact that the large majority of T cells in the bronchiectatic lesions was CD45RO+/CD45RA- suggested that these cells were actively engaged in the immunological process. In other situations, such as rejecting kidneys after transplantation also show a large number of CD8+/CD45RO+ cells (Akbar, Amlot, Timms et al, 1990). The presence of CD4+ cells exhibiting the double positive phenotype CD45RA/CD45RO in the follicular aggregates seen in some bronchiectatic specimens suggests that those cells were actively proliferating inside the follicles, because this phenotype is found in an intermediate stage of cell activation (Janossy, Campana & Akbar, 1989). This was not seen within the CD8+ population, neither was the presence of Ki67 positivity, (typical of the mytotic phase of the cell cycle) observed (Gerdes, Lemke, Baisch et al, 1984).

The distribution of these phenotypically distinct populations of T cells in the normal bronchial tissue is still open to debate. In the present study, a difference could be established between the CD8+/CD45RO+ or CD4+/CD45RO+ T cell populations in bronchiectasis and control tissues. Recent reports showed that T cells obtained by bronchoalveolar lavage in normal volunteers are predominantly

CD45RO+/CD45RA- (Dominique, Bouchonnet, Smiejan et al, 1990). One can argue that the T cells present in the broncho-alveolar compartment are exposed to a large number of antigens, resulting in a phenotype of immunological commitment. Similarly, in the normal gut the T cell population within the lamina propria is almost exclusively composed of CD45RO+/CD45RA- cells (Harvey, Jones & Wright, 1989; Allison, Poulter, Dhillon et al 1990).

Compared with other mucosae, bronchial intraepithelial lymphocytes did not express surface antigens of B-lymphocyte lineage and lacked the expression of certain T-lymphocyte markers, such as CD5, which is considered the phenotype of resident intraepithelial population. They expressed the CD3 antigen, a group of surface molecules in close association with the T cell antigen receptor, and of those CD3+ intraepithelial lymphocytes, almost 90% co-expressed the phenotype of CD8 positive suppressor/cytotoxic T-lymphocytes, either in bronchiectasis or control samples. However, a significant increase in the absolute number of these CD3 positive bronchial intraepithelial lymphocytes was seen when compared with controls. These findings were in line with the data from Fournier and colleagues (1989) in chronic bronchitis.

The present study went further in revealing the distribution of T cell receptor isotypes in the normal bronchial epithelium: about 90% of them exhibited the alpha/beta phenotype, while 10% were of gamma/delta isotype. A large increment in absolute numbers of cells expressing either TCR isotype was seen in bronchiectasis, together with a small but statistically significant increase in the relative contribution of the TCR gamma/delta isotype. Striking increase in the gamma/delta TCR isotype has been described in gut inflammatory diseases

such as coeliac disease (Spencer, Isaacson, Diss et al, 1989; Haltensten, Scott & Brantzaeg, 1989). However, no differences could be established between bronchiectasis and controls in terms of the relative proportions of CD4+ or CD8+ cells, and of CD8+/CD5+ cells. An important difference between bronchiectasis and controls was seen, with a raised percentage of cells co-expressing CD8 and the molecules HLA-DR and CD45RO in the former.

These results strongly suggest that the immunocompetent cell population taking part in the inflammatory processes resulted from the expansion of the resident cell population: the main phenotype of these cells in bronchiectasis samples were not substantially different from controls. It also suggests that, if cell-mediated immune mechanisms were to be involved in the immunopathogenesis of the condition, they represent a local rather than a systemic reaction. This could be evidence of compartmentalization of the immune response via recruitment and activation of immunocompetent cells.

In the context of the "vicious hypothesis" for the pathogenesis of the disease, proposed by Cole in 1984, host-microbial interrelationship during the process of chronic colonisation of the bronchial tree lead to a circle of events whose end result is permanent, and sometimes progressive bronchopulmonary damage (Cole, 1984; Cole, 1986; Cole & Wilson, 1988; Cole, 1990). To date most attention has focussed on nonspecific mechanisms of lung inflammation, mediated by neutrophil polymorphonuclear leukocytes and their biochemical products. There is evidence that neutrophils participate in the chronic inflammatory process associated with bronchiectasis. The work by Currie and associates (Currie, Peters, George et al, 1988; Currie, Garbett, Chan

et al, 1990) labelling neutrophils with 111-Indium and reinjecting them into the blood of patients with bronchiectasis demonstrated that a large proportion of these cells to traffic to the sites of this disease during the inflammatory process. Considerable evidence about the role of neutrophils also derives from the work of Stockley and colleagues (Stockley, 1984; Stockley, Shaw, Hill et al, 1988; Stockley, Shaw, Afford et al, 1990), exploring the mechanisms of neutrophil chemotaxis in bronchiectasis, the composition of lung secretions, the presence of elastolytic activity associated with the episodes of acute exacerbations of bronchial infections, and the experimental effects of inhibitors of the action of some of the neutrophil products. Snider and associates (1984) have also showed the direct action of elastase, a neutrophil proteinase, on the bronchial mucosa of animals, resulting in goblet cell hyperplasia, mucous gland hypertrophy and other effects. However, none of these studies was able to demonstrate the development of bronchial dilatation and other histopathological features of bronchiectasis as being a a result of neutrophils or their products. The use of neutrophil elastase has been clearly associated with the development of anatomically demonstrated emphysema and chronic bronchitis, but not bronchiectasis (Sibille & Reynolds, 1990).

The results reported here raise a new possibility in terms of the pathogenesis of bronchiectasis. The presence of large number of lymphocytes and macrophages in sites of bronchiectatic lesions, some of them exhibiting signs of cellular activation and immunological commitment, suggests that these cells may be involved in the development of the condition. The active involvement of immunocompetent cells in the pathogenesis of chronic inflammatory conditions has been exaustively studied in other situations. Many authors have used

immunohistochemical methods to investigate the immunopathogenesis of chronic inflammatory diseases of the intestinal tract. Some of these results are contradictory but most researchers agree that immune reactions are involved in the genesis of the disease. In recent report, Trejdosiewicz and colleagues (1989) studied the phenotype of colonic mucosal T-lymphocytes in ulcerative colitis and found a large number of them exhibiting increased expression of the activation marker CD7, in close association with increased expression of HLA-DR by colonic enterocytes during the inflammatory process. This was taken to suggest the existence of local immunostimulation of T cells. One of the consequences of this immunostimulation of T cells is the production of interferon-gamma, which leads to augmentation of nonconstitutive expression of MHC Class II antigens by colonic enterocytes and acquisition of antigen-presenting capacity by these cells. In those circumstances, HLA-DR positive epithelial cells might inappropriately present other antigens, exogenous or autoantigens, and broaden the spectrum of local immune response thus contributing to the perpetuation of the chronic inflammation. MacDonald (1990) discussed recently the role of activated T-lymphocytes in intestinal disease, showing that these cells are capable to produce crypt cell proliferation, villous atrophy, and increased HLA-DR expression by enterocytes in different experimental systems, such as jejunal allograft rejection. This picture is very similar to the one seen in coeliac disease, where a large number of T-lymphocytes are known to infiltrate the intestinal mucosa. Similar findings can be seen in explants of foetal human intestine kept in organ culture over extended periods of time. When T cells in the explants are artificially stimulated by different mitogens, the mucosa

presents atrophy and other changes also seen in human diseases. Allison and co-workers (1990) also demonstrated the presence of activated, CD45RO positive colonic lymphoid cells in the inflamed intestinal mucosa. Similar mechanisms could also play a role in bronchiectasis, induced mainly by the large numbers of activated T cells seen in the condition. In fact, association of inflammatory bowel disease, particularly ulcerative colitis, with bronchiectasis have been described (Butland, Cole, Citron et al, 1981).

Recently, some publications disclosed the association of obliterative bronchiolitis and bronchiectasis in patients undergoing heart-lung transplantation (Burke, Theodore, Dawkins et al, 1985). This complication is also found in recipients of bone marrow transplantation (Holland, Wingard, Beschorner et al, 1988). The involvement of active immune processes in the genesis of the condition was suggested by apparent arrest of the process by augmented immunosuppression (Glanville, Baldwin, Burke et al, 1987). Griffith and co-workers (1988) have proposed an immune-mediated basis for the obliterative bronchiolitis occurring in patients subjected to heart-lung transplantation. It presumably begins with epithelial injury induced by activated lymphocytes, resulting in epithelial necrosis with sloughing of cells into the lumen. Injury to the small airways causes them to dilate, due to the loss of smooth muscle support, and terminates in cylindrical bronchiectasis with mucopurulent secretions retained within the airways. Glanville and colleagues (1989) suggested that an increased expression of Class II antigens by the bronchial epithelial cells during the rejection process made them a likely target for cytotoxic cells directed against the allo-antigens and could explain the fact that lung allograft rejection is bronchocentric and has as

consequences the development of obliterative bronchiolitis and bronchiectasis. Similar findings were reported by Clelland and associates (1990), using transbronchial biopsies taken during episodes of acute rejection. Perivascular and bronchiolar lymphocytic infiltrates of similar composition were the main features of the rejection process. Holland and colleagues recently studied the composition of these lymphocytic infiltrates in a patient presenting post-transplant bronchiolitis obliterans and found a large preponderance of CD8+ cells, mainly in peribronchial and interstitial areas (Holland, Cagle, Windsor et al, 1990).

Hence, evidence from inflammatory intestinal disease and lung allograft rejection suggests that activated T cells and macrophages can play a role in aggravating and perpetuating inflammation in the respective organ system. In the results described in this dissertation, activated T-lymphocytes, B-lymphocytes, and macrophage-like cells are spatially associated with bronchiectasis lesions and they may play a major role in the inflammatory process. However, immunohistochemical methods, although important to draw a clear picture of the distribution and nature of the immunocompetent cells involved in the lesions, cannot provide cause-effect evidence for any possible primary role of this immune reaction in the development of the disease.

C H A P T E R 5

IMMUNOPATHOGENESIS OF EXPERIMENTAL BRONCHIECTASIS: *IN VIVO* STUDIES

5.1 - INTRODUCTION: WHY AN EXPERIMENTAL MODEL?

Because of the difficulty in obtaining sufficient relevant tissue, at different stages in the natural history of the condition, from homogeneous groups of bronchiectasis patients with respect to aetiology (and in many cases aetiology is not known), there is a need for an experimental model of the disease to allow dissection of basic pathogenic mechanisms, including their time-course. To develop such a model an understanding of different aspects of the pathogenesis of bronchiectasis is required eg. the importance of impaired mucociliary clearance mechanisms and the involvement of particular strains of colonising bacteria with potential for damaging bronchial epithelium.

One of the first animal models of the condition was that in rabbits described by Tannenberg and Pinner (1942), examining the relationship between atelectasis and bronchiectasis. The first to use rats in the production of experimental bronchiectasis was Cheng (1954). Other important observations derived from the work of Ventura and Domaradzki (1967a; 1967b) who also used the rat. At present the most popular model of chronic bronchial inflammation and infection is that devised by Cash and co-workers (1979; 1983) as a model of cystic fibrosis (CF) and chronic lung colonisation by Pseudomonas aeruginosa, a bacterium known to infect CF patients (Hoiby, 1977; Pier, 1985; Hata & Fick, 1988) and also patients with bronchiectasis due to non-CF causes (Cole, 1989) in a chronic manner. Colonisation with P. aeruginosa is considered, especially in CF, a marker of poor prognosis: those patients undergoing chronic colonisation by Pseudomonas aeruginosa usually sufferring more rapidly progressive disease. Cash and colleagues (1979) tried to mimic the situation of inspissated secretions seen in cystic fibrosis by injecting into the trachea of

rats a solution containing agar beads of appropriate size, cultured together with a strain of mucoid Pseudomonas aeruginosa isolated from a CF patient. The beads stuck in the smaller bronchi and caused bronchial obstruction and infection. Their main objective was to obtain a model of chronic bronchopulmonary infection by Pseudomonas aeruginosa but they also found the presence of bronchiectasis, which was only referred to superficially in the paper. Despite still widely used in cystic fibrosis and chronic Pseudomonas infection research (Freihorst, Merrick & Ogra, 1989; Konstan, Vargo & Davis, 1990; Iwata, Sato, Chida et al, 1990) the Cash model presents several problems, such as the difficulty in predicting where the beads will go, the therefore patchy nature of the lesions due to heterogeneous distribution of the infected beads in the bronchial tree, the difficulty assessing the infecting dose, the potential effect of the agar bead itself in the chronic inflammation, and the lack of clear control normal areas in the lung. This renders the model less than ideal for the study of bronchiectasis.

Advances in understanding of the pathogenesis of bronchiectasis clearly indicate the need for a new animal model of experimental bronchiectasis incorporating the information from the Tannenberg and Pinner experiments with concepts of pathogenesis of the disease developed in the past decade, particularly the "vicious circle" hypothesis discussed in Chapter 1. This requires restriction of the bronchial lumen to induce defective mucociliary clearance, chronic infection by a pathogen known to be important in human disease - and should result in histopathological changes similar to those seen in the human disease. Guerreiro has achieved this model (Guerreiro, Rohde, Todd et al, 1990):

- Young growing rats were verified as Specific Pathogen-Free (SPF) and were kept in clean conditions so as to avoid cross-contamination;

- The surgical technique chosen allowed the animals to withstand the trauma of the procedure and subsequent infection. It was a more localised form of bronchial ligation than the ligation of a whole lung as described in the past. The apical lobe of the right lung was chosen and the bronchial ligation was partial;

- Partial but permanent bronchial ligation was chosen, to achieve a localised reduction in mucociliary clearance without producing atelectasis of the lobe, allowing the remaining lung and opposite lung to act as control tissue;

- The bacteria chosen to infect the apical lobe were those known to be associated with human bronchiectasis and to produce cilio-inhibitory factors: Pseudomonas aeruginosa (strain P455), and Haemophilus influenzae (strain SH 9), both isolated from bronchiectatic patients. The quantity of each bacterium required to produce bronchiectasis was established in preliminary controlled experiments;

- Experiments were then performed with partial ligation of the apical lobe bronchus and distal intrabronchial injection of adequate doses of bacterial suspension (test group). Controls were prepared for comparison: partial bronchial ligation with distal intrabronchial injection of dead bacteria; partial bronchial ligation with distal intrabronchial injection of sterile PBS; intrabronchial injection of the same inoculum of bacteria, viable or dead, without previous ligation of the bronchus; sham operation only; and age-matched normal controls.

Quantitative histology was performed (Guerreiro, Rohde, Todd et al, 1990a; Guerreiro, Rohde, Todd et al, 1990b; Guerreiro, Heard, Rohde

et al, 1990). Clear bronchial dilatation with all the histologic hallmarks of human bronchiectasis were observed only in the test group. None of the controls developed any form of the disease. Of the bacteria employed, Pseudomonas aeruginosa provoked the most florid bronchiectatic changes. Thus the Pseudomonas - partial ligation model appeared suitable for investigating the mechanisms responsible for the development of this disease under conditions not available when studying human samples. From the narrower point of view of the objectives of this thesis, the model has allowed detailed study of the immunopathological changes occurring in bronchiectasis.

The rat lung is essentially similar to that of man and other mammals (Jennings, 1970). There are some differences in the bronchial tree, such as the fact that all airways within the lung lack cartilage. The mucus gland in the rat and other common laboratory rodents are only found in the trachea but the epithelial goblet cells have similar distribution to that of man. In the rat, as well as in other rodents, the bronchus-associated lymphoid tissue (BALT) is a prominent feature, with its lymphoepithelial structure easily seen in the intrapulmonary bronchi at points of branching. In addition to those aggregates of lymphocytes and non-lymphoid cells, lymphocytes can also be seen in the epithelium. The specialised epithelium overlying the BALT is non-ciliated and seems to be particularly active in antigen sampling (Bienenstock, 1984). The number of BALT correlates with the amount of antigen to which the animal has been exposed: the cleaner the animal, the less BALT (Gregson, Davey & Prentice, 1979b). These authors also suggested (1979a) that BALT first appeared in SPF rats around the second week of life. In a careful study of BALT in rats, Plesch (1983)

concluded that BALT occurs at fixed sites between the pulmonary artery and the bronchial epithelium, probably determined by genetic factors. But even in germ-free rats BALT can be found (Bienenstock, Johnson & Perey, 1973a). Post-capillary venules characterised by the presence of high endothelium are an important feature of the BALT, through which small lymphocytes, and occasionally lymphoblasts migrate. The lamina propria is usually a narrow band of connective tissue, with few loose mononuclear cells interspersed between the mesenchymal cells. The pulmonary arteries accompany bronchi and bronchioles while the veins drain separately.

Lymphocytes and macrophages are also important constituents of the lung cellular pool. There is evidence that, in the rat and several other species, large numbers of circulating blood lymphocytes reside in a large intravascular pulmonary pool and are retained there by means of specific homing receptors (Pabst, Binns, Licence et al, 1987). Holt and colleagues (1986) have shown, by using collagenase digestion of previously perfused and lavaged lung slices, that the parenchymal lymphoid cell pool appears to be considerably larger than recognised, and about the same size as the blood pool (Holt, Robinson, Reid et al, 1986). Crawford and Miller (1984) examined the distribution of lymphocyte subpopulations in rat lungs and compared it with Peyer's patches. They found differences in the lymphocyte subpopulations, with more B cells present in the Peyer's patches than in the lung and, conversely, there were more T cells in the lung than in the Peyer's patches. They also found a large predominance of CD4 positive T cells in the Peyer's patches whereas in the lungs 46% of T cells were CD8 positive. In 1986, Simecka and colleagues found the BALT to be divided into three regions of lymphoid tissue: the central zone was composed of

surface immunoglobulin positive cells, probably B-lymphocytes, surrounded by a peripheral region of T cells which included both T helper and T suppressor/cytotoxic cells. The majority of BALT cells were Ia positive, including the lymphoepithelial cells.

In 1987, Holt and Schon-Hegrad used monoclonal antibodies against a range of surface markers seen in T-lymphocytes, macrophages, and dendritic cells in cryostat sections of rat trachea and peripheral lung. They found a population of Ia-bearing cells with characteristic dendritic morphology within the epithelium, closely associated with the basement membrane, and with Ia-positive dendritic processes reaching the luminal fluid layer. More recently, the authors confirmed that these Ia-positive dendritic cells form a tightly meshed network within the epithelium, by cutting sections through the epithelium parallel to the basement membrane and perpendicular to the long axis of the epithelial cells (Holt, Schon-Hegrad, Phillips et al, 1989). Epithelial cells of rat airways are otherwise Ia-negative.

This new information and technology have been extensively used in the past few years to extend knowledge of the immunopathogenesis of several lung disease, using animal models, including those situations where cell-mediated immune mechanisms are thought to be important, such as allograft rejection (Romaniuk, Prop, Petersen et al, 1987; Kirby, Parfett, Reader et al, 1988) pulmonary vasculitis (Harbeck, Launder & Staszak, 1986), occupational lung diseases (Kumar, 1989), delayed-type hypersensitivity (Enander, Ahlstedt & Nygren, 1984; Garssen, Van Loveren, Van Der Vliet et al, 1990), and asthma (Frew, Moqbel, Azzawi et al, 1990). In a similar manner it allows those aspects involved in the pathogenesis of bronchiectasis to be studied. One of the first

questions to be asked is the nature and distribution of the cellular immune response in the disease - as a prelude to determining whether such immunopathology contributes to pathogenesis.

5.2 - MATERIALS AND METHODS

5.2.1 - ANIMALS

Bronchiectasis was induced in male albino Wistar outbred rats (Charles Rivers Ltd., Margate,UK). They were used in all experiments, and were specially screened for Mycoplasma pulmonis and other common rodent contaminants (ie. specific pathogen-free, SPF). Young rats, 6-8 weeks old, weighing around 150 g, were received 3-4 days before the procedure and housed in standard cages in the Biosciences Unit. Two or three days were allowed for acclimatization of the animals before the surgical procedures. They were fed with Purina Chow and allowed to drink water ad libitum. All personnel handling the animals, as well as the protocols of the research, were appropriately licenced by the Home Office, under the Animals (Scientific Procedures) Act, of 1986. Before the surgery, the animals were fasted for 12 hours but allowed water freely. All procedures that involved possible pain were performed under anaesthesia.

5.2.2 - SURGICAL INDUCTION OF EXPERIMENTAL BRONCHIECTASIS

The animals were operated using the technique devised by Guerreiro (Guerreiro, Rohde, Todd et al, 1990). In brief, 160 g animals were anaesthetised with an intramuscular injection of 20 ul of Hypnorm (a mixture of fentanyl acetate and fluanisone; Janssen Pharmaceutical Ltd., Marlow, U.K.). When the animals did not respond to sound stimuli and lost pedal reflex, they were placed in supine position and intubated with a 4FG cannula (Portex Ltd., Hythe, U.K.) under direct vision. They were then placed on their left side and connected to a small animal ventilator (Harvard Apparatus Ltd., Edenbridge, U.K.). The

rats were ventilated with 0.05% halothane (Fluothane, ICI Pharmaceuticals (UK), Cheshire, UK) in 100% oxygen, and a tidal volume of 1.6-2.0 ml/breath at a rate of 96 inflations per minute. The chest wall was shaved and the skin cleaned (Hibiscrub, ICI Pharmaceuticals). A right thoracotomy was performed aseptically through the 5th intercostal space. The apical lobe was then retracted towards the diaphragm to expose the apical bronchus, which was partially ligated with surgical suture (7/0 prolene, Ethicon Ltd, UK) as near as possible to its origin from the main bronchus, together with a needle from an insulin-injecting micro-syringe (Terumo Europe N.V., Leuven, Belgium). The suture was tightened, ligating the bronchus to an internal diameter of 0.45 mm, corresponding to the external diameter of the needle. A volume of 20 ul of bacterial suspension was then injected into the ligated bronchus towards the periphery of the lung using the micro-syringe, the lungs inflated manually with a 5 ml syringe, and the chest wall closed in layers with 4-0 Dexon surgical suture (Dexon Ltd, Gosport, UK). The air in the pleural space was then extracted via an intercostal drain through the wound. The rats were injected intramuscularly with 30 ul of Naloxone (Du Pont (UK) Pharmaceuticals Ltd, Herts, UK), and placed in their anatomical position. Once spontaneous breathing was resumed, the animals were extubated and allowed to breath a high oxygen concentration via a modified face mask for five minutes before being returned to their own environment to recover.

5.2.3 - BACTERIA

Pseudomonas aeruginosa (P455) was isolated from a bronchiectasis

patient (see Appendix 3 for additional data). Frozen organisms were cultured in Oxoid Nutrient Broth no. 2 for 18 hours at 37^{0} C, centrifuged at 3000 rpm for 20 minutes at 4^{0} C, the cell pellet washed twice and finally resuspended in sterile Phosphate Buffered Saline (PBS). The purity of the isolates was confirmed by subculture. Viable counts were undertaken and the suspension adjusted by nephelometry (Evans Electrosellenium LTD., Halstead, UK) to contain 1×10^{9} viable colony-forming units per ml.

5.2.4 - EXPERIMENTAL DESIGN

Four in vivo studies on the immunopathogenesis of experimental bronchiectasis were performed:

A) Comparison of histopathology and immunopathology of experimental and human bronchiectasis - five groups of animals were studied:

- Test group: partial apical lobe bronchus ligation followed by distal intrabronchial injection of live P. aeruginosa (Pa + LIG), as described in section 5.2.2;

- Control group I: animals received intrabronchial injection of the same number of live bacteria without bronchial ligation (Pa + NOLIG):

- Control group II: partial ligation of the apical lobe bronchus followed by intrabronchial injection of 20 ul of sterile PBS (PBS + LIG);

- Control group III: sham operation (S), achieved by thoracotomy under the same conditions as described in section 5.2.2, without any bronchopulmonary manipulation;

- Normal controls (N): a group of rats from the same batch used for the preparation of the above groups was kept under the same housing

conditions, in order to allow comparisons between the littermates.

This study evaluated 13 animals in the test group, 11 in the Pa + NOLIG group, five in each of the other two control groups, and 10 age-matched normal controls. They were killed between two and three months after the surgery.

B) Development of experimental bronchiectasis - this was the main experiment, studying histopathology and immunopathology of the experimental condition. The same test and control groups were prepared as described above, involving a total of 135 rats. Five animals of each group were killed at the following time points:

GROUPS	TIME SINCE SURGERY (WEEKS)							TOTAL
	2	4	8	12	16	20	24	
Pa + LIG	5*	5	5	5	5	5	5	35
Pa + NOLIG	5	5	5	5	ND	ND	5	25
PBS + LIG	5	5	5	ND	ND	ND	ND	15
SHAM	5	5	ND	5	ND	ND	5	20
NORMAL	5	5	5	5	5	5	5	35
TOTAL	25	25	20	20	10	10	20	130

* = number of animals used ND = not done

One additional set of five normal controls were killed at time zero to establish baseline parameters.

C) Changes in local cellular reactivity: markers of cell activation - the same groups of animals described above were employed, with the exception of the PBS + LIG control group. Immunohistochemical markers of cellular activation were employed to investigate whether the development of pathology described in the previous experiment resulted in changes in cellular phenotype denoting activation.

D) Study of delayed-type hypersensitivity to pseudomonas antigens: rat

footpad tests with antigens from Pseudomonas aeruginosa were used in a pilot study to determine whether partial bronchial ligation and injection of the bacterium results in the emergence of cell-mediated immunity to pseudomonas antigens in the rat. At this stage of the research only rats from the test group (n=11) and age-matched normal littermates (n=8) were tested at the following time intervals after the surgery: 12, 16, and 20 weeks.

5.2.5 - HANDLING OF SAMPLES

Animals were killed with an overdose of 0.5 ml of Hypnorm (Jannsen Pharmaceutical Ltd.,Marlow,UK) and exsanguinated by cardiac puncture. The clotted blood was centrifuged at room temperature at 2000 rpm for five minutes, the serum collected and stored at -20^{0} C for future biochemical analysis. Lung lavage was performed by instilling 5 ml sterile PBS through a 4FG cannula (Portex Ltd., Hythe,UK) inserted into the pulmonary tree through a small incision in the trachea. Six aliquots of 5 ml of sterile PBS were introduced and the liquid recovered from the first aliquot was cultured bacteriologically, the remainder stored for future determinations. A midline incision from the cervical region to the abdomen was then performed, the contents of the thorax were excised "en bloc". The apical lobe of the right lung was dissected, a small cannula was inserted into the lobar bronchus and the lobe was inflated gently with 1 ml of Histocon (Polysciences, Inc., Warrington, USA). The lung was then mounted on cork with the hilar aspect upwards, covered with OCT compound (Miles Laboratories Inc.,Illinois, USA) and snap frozen in isopentane cooled by liquid nitrogen. The block was stored in liquid nitrogen until use. The spleens of normal controls were also dissected and frozen to provide a

positive control lymphoid organ for the immunohistochemical studies. All blocks were cut in a cryostat as described in section 4.3.1. Sections were prepared from each of the following levels: central, intermediate, and peripheral. Serial sections were used to evaluate co-expression of different antigens by a given cell type. Conventional histology stains were performed, as described in section 4.3.2.

5.2.6 - BACTERIOLOGICAL STUDIES

The lung lavage liquid was placed in a 5 ml sterile vial and 50 ul placed onto P. aeruginosa Isolation Agar (Oxoid, Basingstoke, UK) and 7% Blood Agar (Oxoid) plate for aerobic culture growth. They were incubated at 37^{0} C overnight for the former and for 48 hours at room temperature for the latter, after which they were examined for growth of the organisms. Another 50 ul aliquot of the lavage fluid was also plated in 7% Chocolate Agar (Oxoid), placed in an anaerobic jar, and examined at 48 hrs. The number of colonies present on the Blood Agar were counted. Colonies on Isolation Agar which were Gram-negative bacilli were subcultured for oxidase tests to confirm the presence of P. aeruginosa. Tissue samples were homogenized in a volume of 5 ml of sterile PBS by vortex mixing with sterile glass spheres until no tissue fragments remained. The homogenates were then treated in the same manner as the lavage fluid.

Three animals from each group were also tested for the presence of Mycoplasma, using two different methods: DNA hybridization assay (Gen Probe, Inc., San Diego, USA) and a cell culture sensitive to the organisms (Denny, Taylor-Robinson & Allison, 1972).

5.2.7 - IMMUNOHISTOCHEMICAL PROCEDURES

Indirect immunoperoxidase was the main method used for the immunohistochemical studies of immunocompetent cells present in the rat lung. Consecutive sections were used in all staining procedures, in order to allow correlation with histologic parameters. Slides were prepared as described in section 4.3.3.1, the sections rehydrated with 2% v/v normal rabbit serum in PBS, 50 ul per section, and left for 20 min., while the monoclonal antibodies dilutions were prepared, then drained without allowing them to dry, 50 ul of the appropriate dilutions of the monoclonal antibodies placed onto the sections and incubated for 90 min.

To minimize background staining, rabbit anti-mouse immunoglobulin conjugated to horseradish peroxidase (P161, Dakopatts a/s, Copenhagen, Denmark) was adsorbed with normal rat serum (1:5) in an incubator at 37^{0} C for 90 minutes. The solution was allowed to cool and diluted further in PBS to provided a final dilution of the P161 of 1:50.

After the incubation period, the sections were washed in PBS for 10 min. and 50 ul of the previously described solution of P161 put onto the sections, and incubated for 60 min. The sections were then washed in PBS for 10 min., and the specific antigen-antibody binding was revealed by diaminobenzidine development, as described in section 4.3.3.1. Normal rat spleen sections served as the positive control for the staining. At least one section per batch was left without the first layer monoclonal antibody, in order to provide a negative control for the presence of endogenous peroxidase activity.

5.2.8 - PANEL OF MONOCLONAL ANTIBODIES

- MRC OX-52 (Serotec, Oxford, UK) recognises a cell surface

structure with 2 chains of molecular weight 120000 and 95000 present on rat T lymphocytes (Robinson, Puklavec & Mason, 1986);

- MRC OX-19 (Serotec, Oxford, UK) recognises a determinant expressed on all rat thymocytes and peripheral T cells (Dallman, Thomas & Green, 1984). MRC OX-19 and MRC OX-52 were used together to enhance the staining (Holt & Schon-Hegrad, 1987) in a final dilution of 1:10;

- MRC OX-8 (Serotec, Oxford, UK) reacts against membrane glycoproteins present on rat CD8 positive cytotoxic/suppressor T cells (Barclay, 1981; Dallman, Mason & Webb, 1982), and some monocytes (Kaffenberger, Gruber & McVittie, 1987). It was used at a dilution of 1:10;

- W3/25 (Sera-Lab, Crawley, UK) recognises a determinant present in CD4 positive helper/inducer T cells and in many macrophages and dendritic cells (Mason, Arthur, Dallman et al, 1983), and was used at a dilution of 1:5;

- W3/13 (Sera-Lab, Crawley, UK) recognises a determinant present in most T-lymphocytes (Mason, Arthur, Dalmann et al, 1983) and also in some neutrophils and monocytes (Hedlund, Segren, Jansson et al, 1986);

- ED1 (kindly supplied by Dr. C. Dijkstra) recognises all rat macrophages and dendritic cells (Dijkstra, Dopp, Joling et al, 1985), and was used at a dilution of 1:500;

- ED2 (kindly supplied by Dr. C. Dijkstra) recognises a subset of rat macrophages and dendritic cells (Dijkstra, Dopp, Vogels et al, 1987), and was used at a dilution of 1:500;

- MRC OX-6 (Serotec, Oxford, UK) identifies a monomorphic determinant of the rat Ia antigen, present on B-lymphocytes, some

macrophages, activated T cells and certain epithelial cells (Barclay & Mayrhofer, 1981), used in a dilution of 1:20;

- MRC OX-39 (Serotec, Oxford, UK) recognises expression of the activation marker interleukin-2 receptor on the T cells and was used in a dilution of 1:2 (Patterson, Jefferies, Green et al, 1987);

- MRC OX-12 (Serotec, Oxford, UK) ia a monoclonal antibody against a determinant on rat kappa light chain (Hunt & Fowler, 1981), and was used in a dilution of 1:10.

5.2.9 - IMMUNOHISTOLOGY AND MORPHOMETRY

Slides from all three levels of each apical lobe were stained with each of the monoclonal antibodies. The sections from each group of animals stained with the same antibody were randomized and evaluated in "blind" manner. Three compartments of the lung were assessed:

- Bronchial epithelium: the area between the luminal border and the basement membrane;

- Bronchus-associated lymphoid tissue (BALT): the area between the lymphoepithelium and the surrounding stroma of the follicular aggregate;

- Peribronchial lamina propria: the area between the basement membrane and the surrounding lung parenchyma;

In a pilot study perivascular and alveolar areas were also qualitatively assessed but in most studies a quantitative method was employed, which excluded these two areas. The number of positively stained cells for each marker was calculated per unit area of each compartment, using the Solitaire Plus Image Analysis System (Seescan Imaging PLC, Cambridge, UK), as described in section 4.3.5.

5.2.10 - QUANTITATION OF MHC CLASS II EXPRESSION

The quantitation of the Major Histocompatibility Complex Class II Ia antigen was achieved by using the MRC OX-6 monoclonal antibody (see above). All slides were processed simultaneously with strictly the same amount of reagents and time of reaction in order to allow comparison of the optical density of the staining, and no counterstaining with haematoxylin was performed. Optic Densitometry was performed by using the same morphometric system provided with a dedicated black and white camera and appropriate software which measures the intensity of the average grey shade of a region defined by a frame. It can be directly related to the optical density of the brown precipitate formed during the staining. The relative expression of Ia staining was determined in each compartment and the results expressed as the mean ± SD of arbitrary units obtained in the readings. The results of all time points were accumulated.

5.2.11 - HISTOCHEMICAL METHOD

The presence of acid phosphatase in the macrophages was studied with a double staining technique in which the macrophage population was labelled with ED1 as previously described, followed by incubation at 37^{0} C during 90 minutes with a medium prepared with the hexazonium pararosaniline (see Appendix 2). The number of double labelled cells were expressed as a percentage of the total number of ED1 positive macrophages per unit area of each lung compartment examined. The results of all time points were accumulated.

5.2.12 - FOOTPAD TESTING

Pseudomonas aeruginosa antigens were prepared from frozen samples of P. aeruginosa strain P455. These were grown overnight at 37^{0} C in Brain-Heart Infusion Broth (Oxoid, Basinstoke, UK), harvested, washed twice in cold PBS and adjusted to 10^{7} CFU/ml in ice-cold PBS by nephelometry (Electrosellenium Ltd, London, UK). Five mililitre aliquots were prepared and submitted to sonication in a Dawe Soniprobe (Dawe Instruments Ltd., London, UK) until a translucent suspension was obtained. The aliquots were then pooled, a sample was plated onto Blood Agar Base No. 2 (Oxoid), incubated overnight at 37^{0} C, and examined for any sign of bacteriological growth.

At progressive times after surgery groups of Pa + LIG rats were taken and the thickness of both hind footpads measured with a dial-gauge precision caliper (Mitutoyo MFG Co. Ltd., Tokyo, Japan) calibrated in 0.001 mm; 0.2 ml of the antigenic preparation was then injected intradermally into the left footpad using an insulin-type syringe (Terumo Ltd., UK). An equivalent amount of sterile PBS was injected into the contralateral footpad. Footpad thickness was then re-measured 48 hours after the injections.

The animals were then killed and the hind feet were removed and fixed in formalin for 48 hours. Samples of tissue from all footpads were biopsied and embedded in paraffin wax for conventional haematoxylin-eosin staining. All subsequent procedures were performed in a blind manner. Samples of lung lavage fluid, lung and liver fragments were removed and cultured for Pseudomonas aeruginosa as described in section 5.2.

The differences in footpad thickness before and after the challenge were calculated and the means plus or minus standard

deviation established. Identical procedures were also performed concomitantly on the normal control animals.

5.2.13 - STATISTICAL ANALYSIS

The results for each parameter are presented as arithmetic means plus or minus standard deviation. Statistical analysis was performed with an Opus Technology PC V Turbo computer using the software package Minitab (Ryan, Joyner & Ryan,1985). Descriptive statistics are presented in Appendix 1. Significance testing of the means were calculated by Student's two-sample t-test between the different groups. Nonparametric tests were performed by Mann-Whitney two-sample rank test between the different groups. Kruskal-Wallis test were also performed and the results presented in Appendix 1.

5.3 - RESULTS

5.3.1 - IMMUNOPATHOLOGY OF ESTABLISHED EXPERIMENTAL BRONCHIECTASIS

5.3.1.1 - Post-operative Animal Status

No differences were seen in body weight at sacrifice between the five groups: Pa + LIG = 419.9±36.1 grams; Pa + NOLIG = 463.4±38.7 g; PBS + LIG = 410.0±105.3 g; S = 447.7±28.6 g; N = 449.7±28.4 g. No differences were seen in respiratory frequency, or condition of the surgical scar or fur.

5.3.1.2 - Bacteriological Studies

Pseudomonas aeruginosa was isolated from aliquots of bronchial lavage fluid and from samples of lung fragments obtained from all animals of the Pa + LIG group but none was isolated from samples obtained from animals in the other four groups. No Mycoplasma was isolated from the animals.

5.3.1.3 - Histopathology

The results for the histopathology of the animals are presented in Table 5.1. None of the apical lobes of the 11 rats in the Pa + NOLIG group, nor of those from the PBS + LIG or S groups exhibited bronchiectatic changes and the mononuclear cells were not significantly different in number or distribution from those in the normal (N) rats By contrast, all 13 rats in the Pa + LIG group exhibited histological changes very similar to that of human bronchiectasis, as defined by the presence of different degrees of bronchial dilatation and severe inflammatory changes. Three different histological types concerning the

TABLE 5.1

HISTOPATHOLOGY FINDINGS IN RATS FROM DIFFERENT EXPERIMENTAL GROUPS

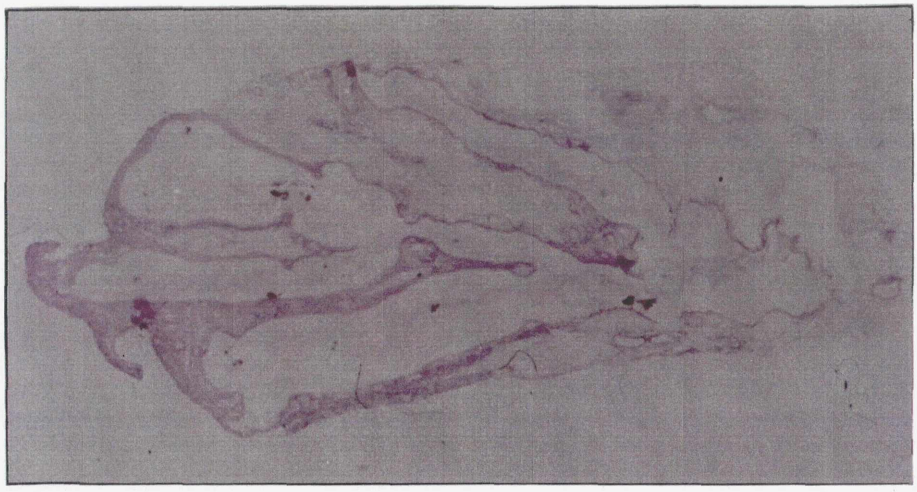
Change	Pa+LIG (n=13)	Pa+NOLIG (n=11)	PBS+LIG (n=5)	SHAM (n=5)	Normal (n=11)
Prox. dilatation	13	0	0	0	0
Distal dilatation	8	0	0	0	0
Luminal pus	11	0	0	0	0
EP metaplasia	5	0	0	0	0
EP ulceration	5	0	0	0	0
EP folding	4	0	0	0	0
Goblet cell hyp.	13	5	0	0	0
MNC in LP	13	0	0	0	0
Fibrosis in LP	13	2	0	0	0
Elastin loss	12	0	0	0	0
Neovasc. in LP	10	0	0	0	0
Incr. BALT no.	3	0	0	0	0
Other follicles	11	0	0	0	0
Perivasc. MNC	11	0	0	0	0
Vasc. hyperplasia	6	0	0	0	0
Alv. collapse	7	0	0	0	0
Alv. fibrosis	10	0	0	0	0
Pneumonitis	7	0	0	0	0

Data expressed as number of animals in each group displaying the change. group. Prox. = proximal; EP = epithelial; hyp. = hyperplasia; MNC = mononuclear cell; LP = lamina propria; neovasc. = neovascularization; incr. = increased; no. = numbers; vac. = vascular; alv. = alveolar. Pa + LIG = partial bronchial ligation followed by distal intrabronchial injection of live P. aeruginosa; Pa + NOLIG = intrabronchial injection of live P. aeruginosa without bronchial ligation; PBS + LIG = partial bronchial ligation with distal intrabronchial injection of sterile PBS; S = Sham operated animals; N = age-matched normal controls.

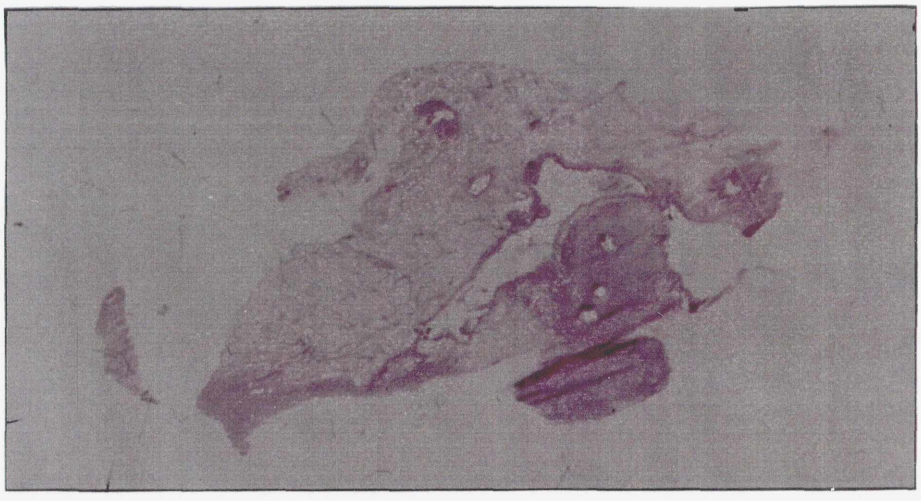
pattern of the bronchial dilatation could be distinguished: diffuse cylindrical bronchiectasis, affecting major airways and terminal bronchioles (6/13 animals); saccular bronchiectasis, as defined by gross saccular dilatation of bronchi and bronchioli (2/13); bronchiolectasis, defined as distortion and dilatation of the peripheral airways (5/13) (Plate 5.1). Other changes affected all lung compartments. In the epithelium hyperplasia of goblet cells, epithelial thickening and/or exfoliation, and mucosal folding were present. The bronchus-associated lymphoid tissue was severely affected, with extensive disorganization of structure and a great increase in size, reflecting a large increase in the number of mononuclear cells. Follicles were not only associated with the airways but were also found deep in the lung parenchyma. Peribronchial areas were also severely affected by the inflammatory process. All specimens showed increased numbers of mononuclear cells along the airways, isolated or forming clusters not associated with the BALT. In six animals with cylindrical and saccular bronchiectasis, huge collections of mononuclear cells extending from the basement membrane of the epithelium to the outer layers of peribronchial tissue were seen, sometimes in a continuous sheath. Hyperplasia of the muscle coat was also present. In most animals of the group, varying degrees of destruction of the elastic fibres and deposition of fibrotic tissue were also predominant features. Neovascularisation of the submucosal areas were also noted in most animals (Plate 5.2).

The perivascular areas, either periarteriolar or perivenular, showed increased accumulation of mononuclear cells, sometimes forming a continuous layer. Some vessels showed a marked hyperplasia

PLATE 5.1

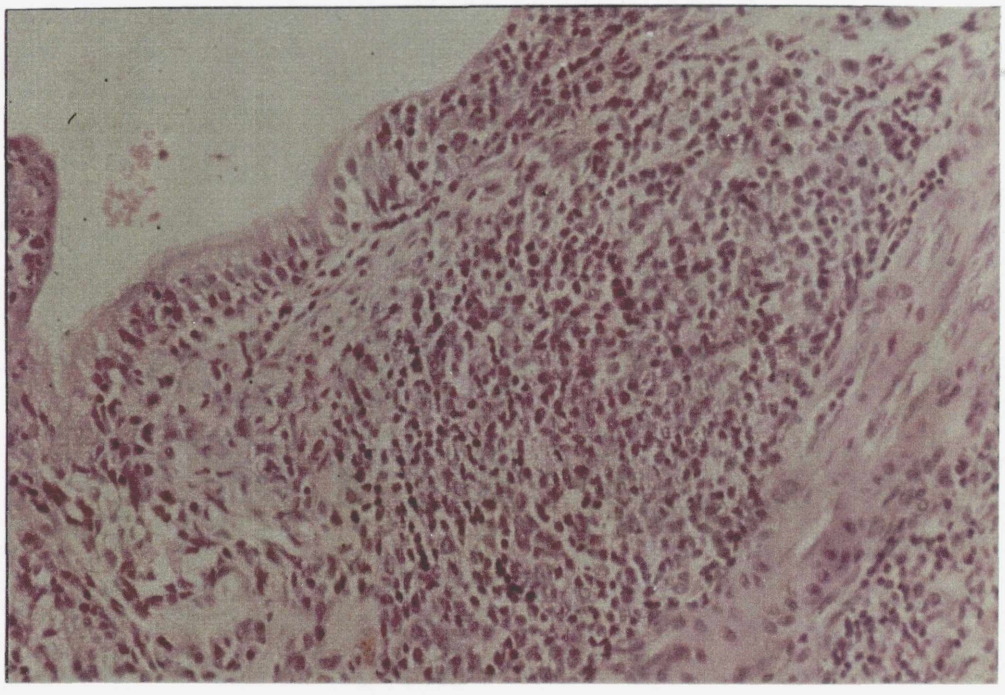


a: Saccular dilatation of the bronchial tree of a rat from the Pa + LIG group, 12 weeks after induction (haematoxylin-eosin, original magnification x10)

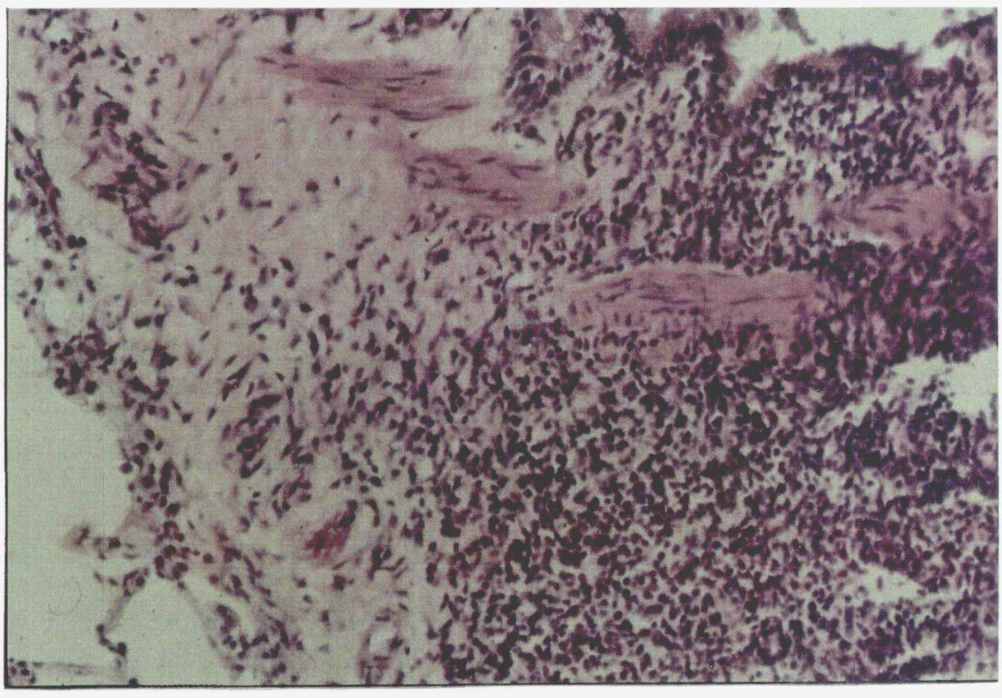


b: Cylindrical dilatation of the bronchial tree of a rat from the Pa + LIG group, 12 weeks after induction (haematoxylin-eosin, original magnification x10)

PLATE 5.2



a: Infiltration of mononuclear cells in the peribronchial area of a rat from the Pa + LIG group, 8 weeks after induction (haematoxylin-eosin, original magnification x250)



b: The bronchus-associated lymphoid tissue (BALT) of a rat from the Pa + LIG group, 4 weeks after induction (haematoxylin-eosin, original magnification x250)

of the intima, perhaps reflecting changes in the resistance to the circulation in the areas. The lung parenchyma was also severely affected, with large numbers of mononuclear cells detected both in the lumen and in the alveolar septal walls. In all three types of bronchiectasis there was partial replacement of the alveoli by fibrotic tissue, follicular aggregates of mononuclear cells or bronchus-related cysts - all effectively reducing the size of the entire lobe. Problems in inflating the lungs made this compartment unsuitable for detailed quantitative studies.

Four rats with cylindrical and saccular bronchiectasis also exhibited impressive hilar lymph node enlargement, sometimes occupying up to one third of the the whole diameter of the tissue section.

These results confirm that the histopathological features of the experimental model reflect many of the features seen in human bronchiectasis.

5.3.1.4 - Immunohistology of Normal Control Rat Lungs

The frequency and distribution of lymphocytes and macrophages observed in normal Wistar rats were very similar to that described in BN and WAG strain rats (Holt & Schon-Hegrad, 1987). In brief, immunocompetent cells, as defined by monoclonal antibodies are mainly concentrated in the BALT, whereas the other compartments show only scant numbers of those cells, mainly loosely distributed, without any evidence of cell clustering.

5.3.1.5 - Immunohistology of Lungs of the Experimental Control Groups

None of the lungs of the rats in the three control groups (Pa +

NOLIG, PBS + LIG, S) showed numbers of immunocompetent cells significantly different from those in the normal animals. Both histology and immunohistology of the lungs of the animals from these groups were also similar to those of the normal animals.

5.3.1.6 - Immunohistology of Lungs of Rats with Experimental Bronchiectasis

A qualitative assessment of the immunohistology of lungs from rats exhibiting experimental bronchiectasis was performed. Striking changes in the distribution and frequency of immunocompetent cells were seen in lungs of rats from the Pa + LIG group:

1. Class II MHC antigens

a) Epithelium: besides a slight increase in mononuclear inflammatory cells that expressed Ia antigens, epithelial cells were also positive for this marker. The large airways were entirely positive while the bronchioles were only positive in patches. In the two examples of saccular bronchiectasis strong Ia expression was seen throughout the epithelium. The bronchial epithelium of the normal and experimental control groups showed no positivity for Ia antigens;

b) BALT: although the absolute number of Ia positive cells in BALT was greatly increased, the proportion of Ia positive cells to the total number of cells in the BALT remained normal, because the large majority of cells in the BALT of normal rats constitutionally express these antigens. Also, large follicular aggregates of mononuclear cells distant from the bronchus expressed strong Ia positivity;

c) Peribronchial area: the expanded mononuclear cell population present in this compartment was strongly Ia positive with more than 50%

of cells expressing the antigen;

2. T-lymphocytes

a) Epithelium: a small increase was found in the number of T cells infiltrating the epithelium comparing with the control groups, and these were mainly OX-8 positively stained cells;

b) BALT: a large increase in size of BALT as compared with controls was found to be attributable to T cells. In some animals 80% or more of the cells in the huge lymphoid clusters that replaced the normal follicular structure were positively stained by at least one T cell marker. Forty to 80% of the cells expressed the phenotype of suppressor/cytotoxic T cells. The large non-bronchial follicular aggregates seen in the parenchyma were also composed mainly by T cells, mostly of suppressor/cytotoxic phenotype;

c) Peribronchial area: a large increase in T cells was found as compared with the controls, usually distributed densely beneath the basement membrane in a compact sheath, with isolated cells or small clusters of cells also present (Plate 5.3);

3. Macrophages

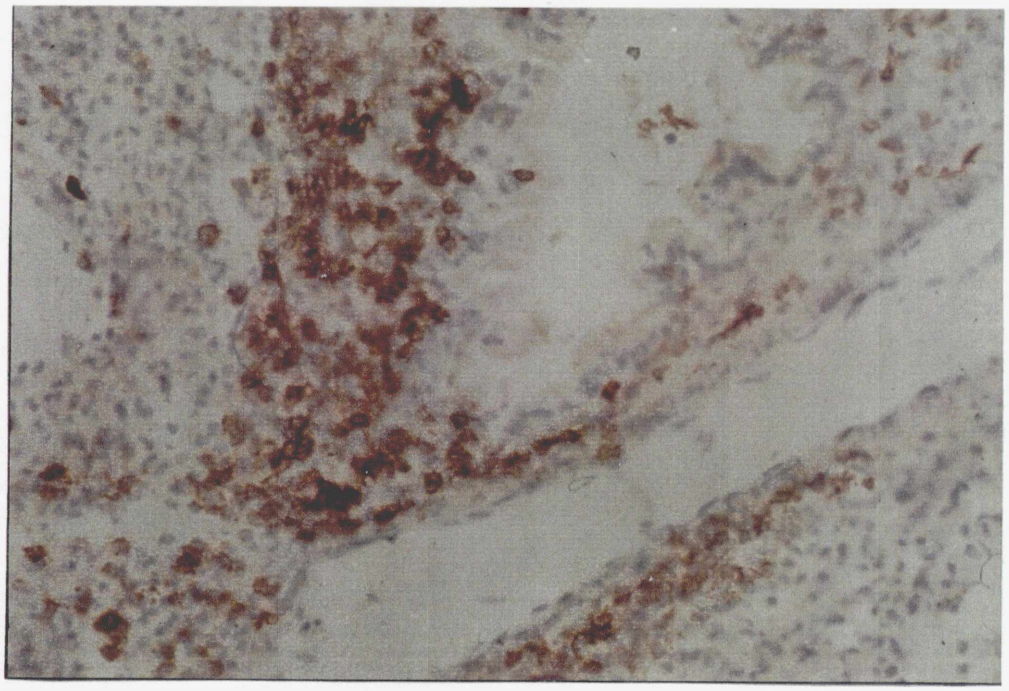
a) Epithelium: there was no increase in the number of ED1 or ED2 positive cells in this compartment;

b) BALT: many more ED1 positive cells were seen among the mononuclear cells in follicle-like aggregates than in the controls. The number staining with ED2 was similar to normal. Small numbers of ED1 and ED2 positive macrophages were also seen in the non-bronchial follicular aggregates seen in the lung parenchyma;

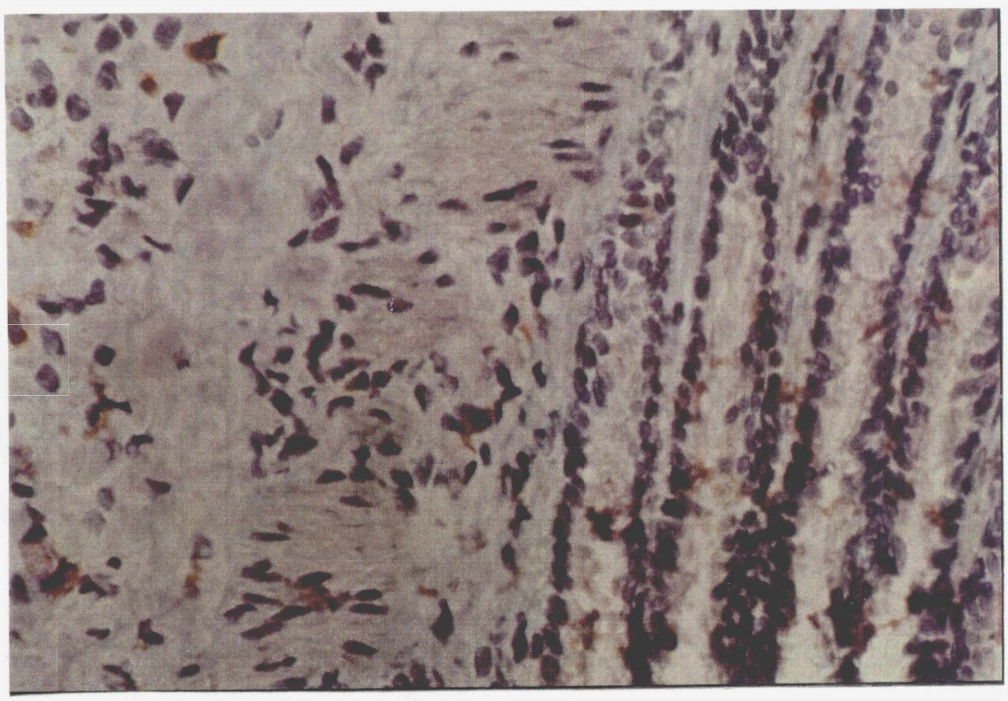
c) Peribronchial area: many more ED1 and ED2 positive cells were present in this compartment than in controls.

There appeared to be a relationship between the extent and pattern

PLATE 5.3



a: Infiltration of T-lymphocytes in peribronchial and perivascular areas of a rat from the Pa + LIG group, 8 weeks after induction (OX-19/OX-52, immunoperoxidase, original magnification x 250)



b: Distribution of T-lymphocytes in the normal bronchial epithelium and lamina propria of a rat from the N group (OX-19/OX-52, immunoperoxidase, original magnification x250)

of bronchiectasis and the expression of Ia antigens by the bronchial epithelium, as presented in Table 5.2. The subepithelial accumulation of mononuclear cells was increased in all cases and did not distinguish between the different morphological types of bronchiectasis although the presence of large follicular aggregates of mononuclear cells was a characteristic finding in rats with diffuse cylindrical bronchiectasis. The expression of Ia antigen by the epithelial cells was greatly increased in the two animals with saccular bronchiectasis but was somewhat less in those with diffuse cylindrical bronchiectasis.

In conclusion, this study revealed that the rat model using partial bronchial ligation followed by the injection of live P. aeruginosa caused the development of an inflammatory reaction in the bronchi and adjacent areas which reflected human bronchiectasis. It was therefore suitable for more detailed investigations concerning the development of the disease.

TABLE 5.2

RELATIONSHIP BETWEEN PATTERN OF BRONCHIECTASIS AND IMMUNOHISTOLOGY IN EXPERIMENTAL BRONCHIECTASIS

	PATTERN OF BRONCHIECTASIS		
Immunohistology	cylindrical (n=6)	saccular (n=2)	bronchiolectasis (n=5)
Large follicular aggregates of T-cells	5*	0	1
Ia expression by epithelial cells	3	2	0
Subepith. accumulation of macrophages/T-cells	4	1	3

* no. of animals in which relationship was seen.

5.3.2 - DEVELOPMENT OF HISTOPATHOLOGICAL AND IMMUNOPATHOLOGICAL LESIONS IN LUNGS OF RATS WITH EXPERIMENTAL BRONCHIECTASIS

Having established that the animal model generates a pathologic picture of bronchiectasis very similar to that seen in the human disease, specific questions regarding the development of the disease could be investigated in the model, particularly those of immunopathological nature.

5.3.2.1 - Post-operative Animal Status

The groups of rats did not appear grossly different in terms of weight and appearance during the development of the bronchiectasis. Table 5.3 shows mean weights (±SD) at sacrifice. Most of the rats survived the surgical, anaesthetic and infectious trauma and displayed similar growth to their normal littermates.

5.3.2.2 - Bacteriological Results

Pseudomonas aeruginosa was isolated from the lavage fluid or tissue fragments obtained from all animals of the Pa + LIG group up to 8 weeks, from 3/5 animals at 16 and 20 weeks, and from 1/3 animals at 24 weeks. The bacterium was isolated from lungs of 3/5 animals from the Pa + NOLIG group at week 2, 1/5 at week 4, but none was isolated subsequently: neither from the lavage fluid nor from tissue fragments obtained from the animals in the other control groups at any time point. No Mycoplasma was isolated.

5.3.2.3 - Histopathology

Bronchial dilatation was consistently observed in 29/35 animals of the Pa + LIG group (3/5 rats at week 2, 4/5 at week 4, 5/5 at weeks 8

TABLE 5.3

WEIGHT OF RATS IN EACH EXPERIMENTAL GROUP AT SACRIFICE AT THE DIFFERENT TIME POINTS

TIME POINT	Pa+LIG	Pa+NOLIG	PBS+LIG	SHAM	NORMAL
2 WEEKS	247.6	267.0	274.0	236.0	269.8
	(34.1)	(13.7)	(8.9)	(4.7)	(3.5)
4 WEEKS	249.2	366.6	329.8	297.0	315.2
	(27.6)	(21.1)	(18.5)	(51.4)	(21.8)
8 WEEKS	423.6	423.6	410.0	ND	432.6
	(15.6)	(45.8)	(105.3)	--	(16.1)
12 WEEKS	416.0	503.2	ND	447.7	466.8
	(56.6)	(31.6)	--	(28.6)	(40.7)
16 WEEKS	490.6	ND	ND	ND	482.2
	(17.6)	--	--	--	(39.2)
20 WEEKS	493.0	ND	ND	ND	465.0
	(36.9)	--	--	--	(33.2)
24 WEEKS	553.0	622.4	ND	513.0	543.0
	(89.9)	(55.6)	--	(51.3)	(30.1)

Data expressed as means (SD) of weight (in grams) of rats at sacrifice per experimental group at each time point. ND = not done.

and 12, 4/5 at weeks 16, 20, and 24). The apical lobes from two rats at 20 and 24 weeks were totally replaced by fibrous tissue, making it impossible to distinguish the presence of bronchial dilatation. This ranged from isolated distal dilatation and distortion of the bronchioli, to saccular dilatation compromising proximal and distal bronchi. More dilatation and lung destruction were observed at progressive time points. None of the 95 animals from the control groups developed any kind of bronchial dilatation.

Some form of damage to the bronchial epithelium affected all rats of the Pa + LIG group at all time points (Table 5.4). Exfoliation, ulceration, goblet cell hyperplasia, and metaplasia were noted. Mucosal foldings, similar to that seen in different forms of human bronchiectasis, were also present in some specimens. Epithelial damage was seen at week 2 in 5/5 rats of the Pa + NOLIG group, in 2/5 rats of the PBS + LIG group, and in 2/5 of the S group but no animals from either of these groups exhibited such damage from 4 weeks onwards, apart from mild goblet cell hyperplasia in a few specimens.

Bronchial wall infiltration by both acute and chronic inflammatory cells was present in all animals of the Pa + LIG group at all time points. Using staining of consecutive lung sections with haematoxylin-eosin and for endogenous peroxidase activity (see section 4.3.3.4), it was possible to distinguish the presence of some neutrophils infiltrating the bronchial wall but in tiny numbers as compared with those seen in the bronchial lumen or with the mononuclear cells infiltrating the bronchial wall. The number of neutrophils in the bronchial wall was particularly prominent at week 2, decreasing afterwards, despite the presence of large numbers of neutrophils in the

TABLE 5.4

LUNG HISTOPATHOLOGY OF THE RATS SUBSEQUENT TO PARTIAL BRONCHIAL LIGATION AND DISTAL INTRABRONCHIAL INJECTION OF P.aeruginosa

	TIME SINCE SURGERY (WEEKS)						
Change	2 n=5	4 n=5	8 n=5	12 n=5	16 n=5	20 n=5	24 n=5
Prox. dil.	3	4	3	5	4	4	4
Distal dil.	3	3	5	5	2	4	4
Luminal pus	5	5	5	5	3	4	4
EP metaplasia	2	3	3	2	1	2	1
EP ulceration	1	2	2	1	1	1	0
EP folding	0	0	1	1	1	1	2
Goblet cell hyp.	5	5	5	5	4	4	4
MNC in LP	5	5	5	5	5	4	4
Fibrosis in LP	2	4	5	5	5	4	4
Elastin loss	1	2	4	5	5	4	4
Neovasc. in LP	1	3	4	4	5	4	4
Incr. BALT no.	2	1	1	0	2	1	1
Other follicles	4	4	4	5	2	4	4
Perivasc. MNC	5	5	4	4	3	3	3
Vasc. Hyperpl.	1	1	2	2	2	1	1
Alv. collapse	4	1	2	4	4	4	4
Alv. fibrosis	0	1	3	5	4	4	4
Pneumonitis	4	2	1	5	2	1	1

Results expressed as number of animals exhibiting each histopathologic feature at each time point (see text for details of the other groups). prox. = proximal; dil. = dilatation; EP = epithelial; hyp. = hyperplasia; MNC = mononuclear cell; LP = lamina propria; neovasc. = neovascularisation; incr.= increased; alv. = alveolar.

bronchial lumen at all time points. In the other groups, a degree of inflammation (characterized by increased numbers of mononuclear cells and neutrophils infiltrating the peribronchial and perivascular areas) was only noted at week 2 (5/5 in the Pa + NOLIG group, 3/5 in the PBS + LIG group, and 1/5 in the S group), clearing completely by week 4. Peribronchial fibrosis was present in the Pa + LIG group in 2/5 animals at week 2, 4/5 at week 4, and in all animals at later time points. None of the 95 animals of the control groups developed fibrosis.

There was no significant difference in the number and distribution of lymphoid follicles between the groups at any time point but an expansion of the bronchus-associated lymphoid tissue (BALT) was seen in the rats of the Pa + LIG group. From 2 weeks onwards a number of lymphoid aggregates situated deeper in the lung parenchyma and apparently not associated with a bronchus, began to appear de novo in the Pa + LIG group. If BALT and follicular structures were considered together, statistical significance was reached when compared with the number of lymphoid aggregates seen in normals. Hilar and intrapulmonary lymph nodes, some of them much enlarged, also appeared in this group. The other groups failed to show these changes.

5.3.2.4 - Development of the Immunopathological Changes

The involvement of immunocompetent cells in the development of the histopathological changes in the animals undergoing the experimental procedures was examined in each of the lung compartments analysed. The p values presented here compare test or experimental control groups with normal controls by Student's t-test. Complete statistical analysis is presented in Appendix 1:

Intraepithelial infiltration: Significant intraepithelial

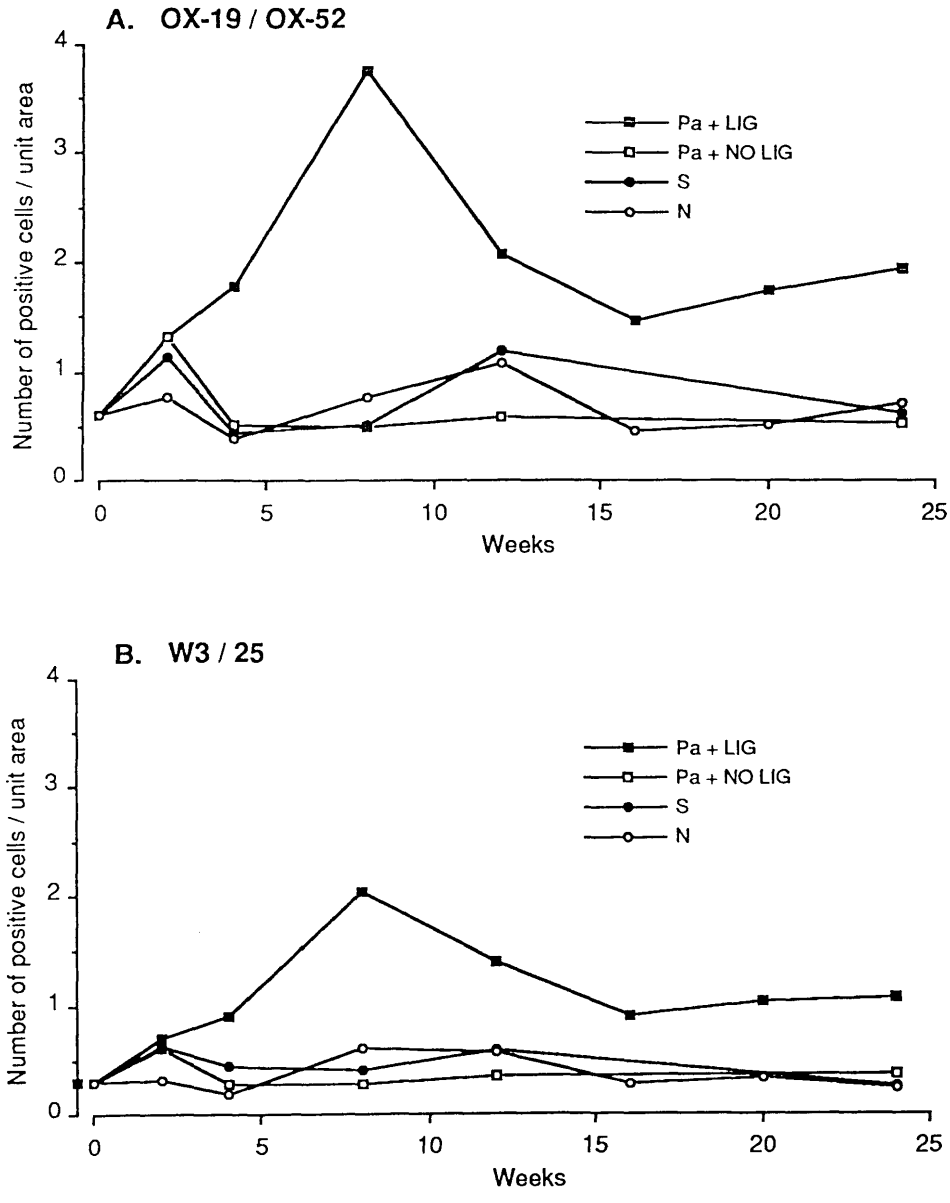
infiltration of T-lymphocytes in the Pa + LIG group was first noticed at week 2 (Fig. 5.1) when compared with the N group, mainly due to an increase of CD4 positive cells ($p = < 0.01$). Subsequently, this increase in the number of intraepithelial T cells was mainly due to an increment in the number of CD8 positive cells ($p < 0.003$ at week 4, $p = 0.0001$ at week 8, $p < 0.0001$ at week 20). Equivalent numbers of both subsets were seen at 24 weeks. No such increments were seen in the other experimental groups when compared with group N. Significant increase in ED1 positive macrophages was noted in the Pa + LIG group when compared with group N at weeks 2 ($p < 0.03$), 8 ($p < 0.005$), and 24 ($p = 0.02$) but no statistical significance was reached at the other time points. In the Pa + NOLIG group an increment in the number of intraepithelial macrophage-like cells was noted only at week 2 ($p < 0.01$) and none was seen in the PBS + LIG or S groups.

No OX-12 positive cells were ever seen infiltrating the bronchial epithelium of test or control groups of rats.

Immunocompetent cell proliferation in the BALT: A highly significant ($p < 0.0001$) increase in the number of T cells infiltrating the BALT of animals of the Pa + LIG group was first noticed at week 2, when compared with group N (Fig. 5.2). This increase was almost entirely due at this stage to increased numbers of CD4 positive helper T cells ($p = 0.001$). Similar results were obtained when the Pa + LIG group was compared with the Pa + NOLIG ($p < 0.0001$ for T cells, $p < 0.003$ for CD4 positive cells), PBS + LIG ($p < 0.0001$ for T cells, $P < 0.01$ for CD4 positive cells), and S groups ($p < 0.0004$ for T cells, $p = 0.003$ for CD4 positive cells). In none of the control animals in these three groups was such an increase in the cell numbers seen at any time

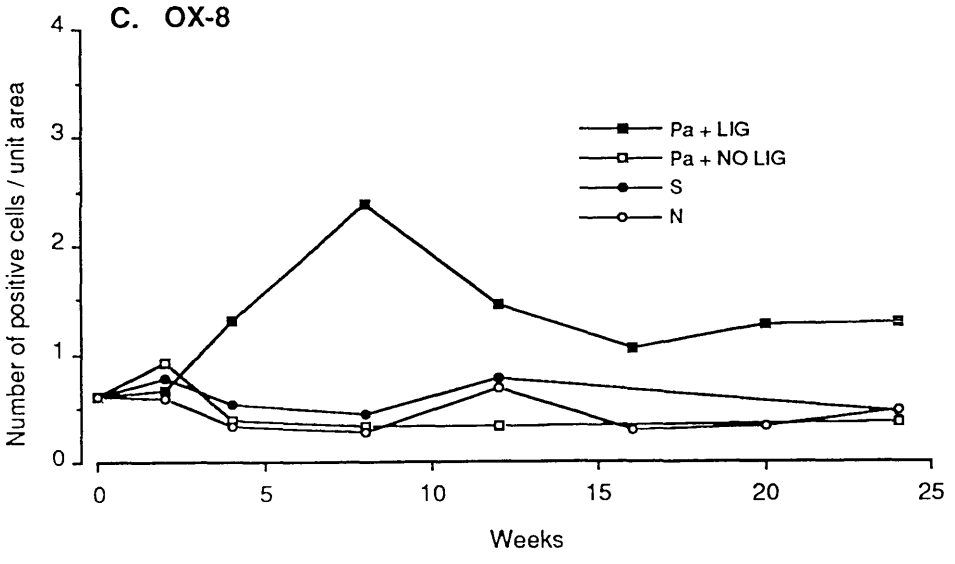
FIGURE 5.1

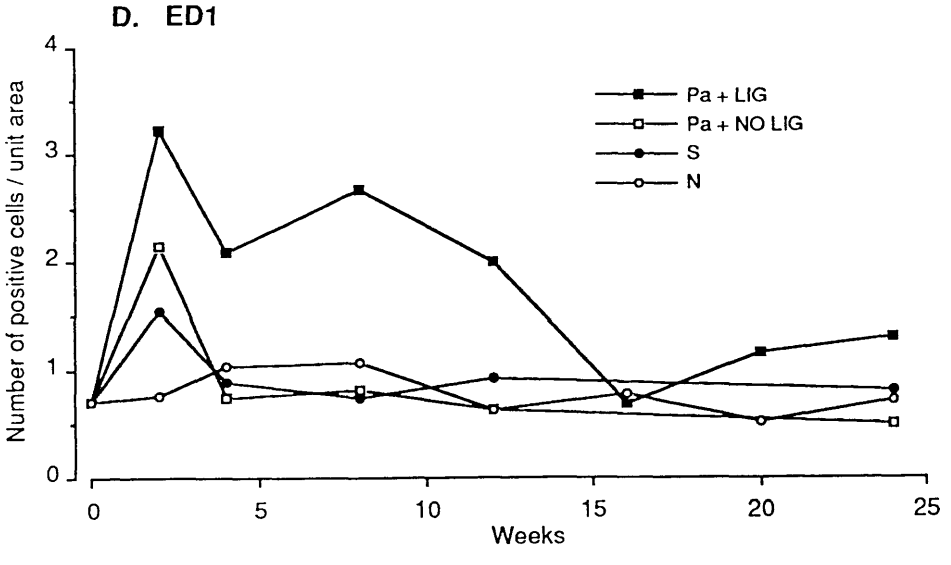
Changes in the composition and frequency of immunocompetent cells infiltrating the bronchial epithelium of animals with experimental bronchiectasis compared with controls.



(cont.)

FIGURE 5.1 (cont.)

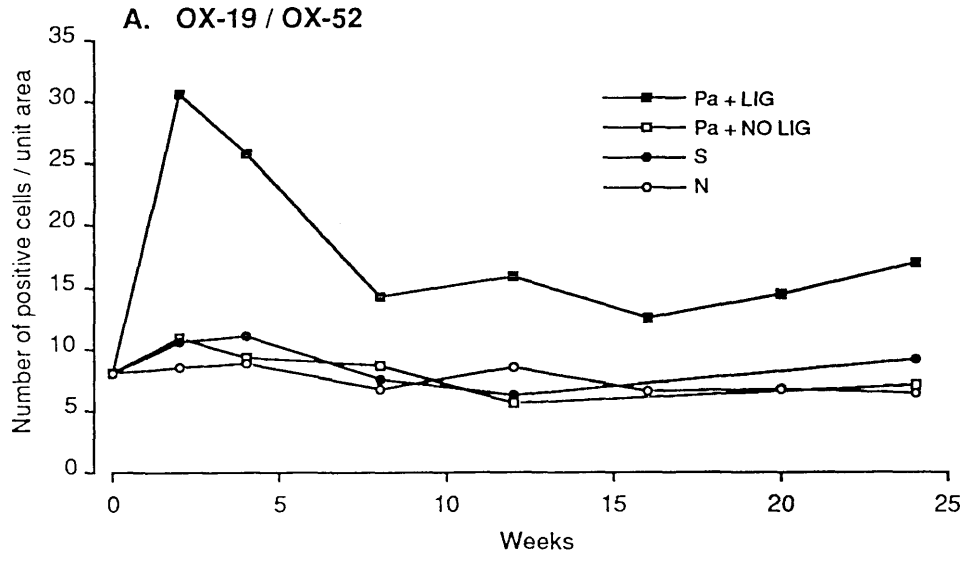


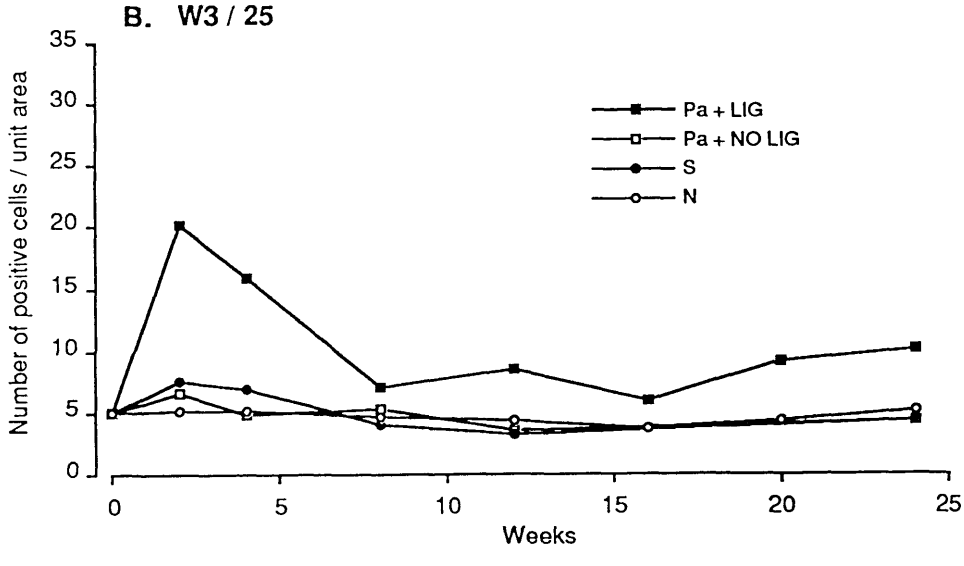


The graphs show changes in frequency of immunocompetent cells in the bronchial epithelium of test and control groups of rats. PBS + LIG and S groups are represented together. Standard deviations are omitted for clarity. Descriptive statistics in Appendix 1.

FIGURE 5.2

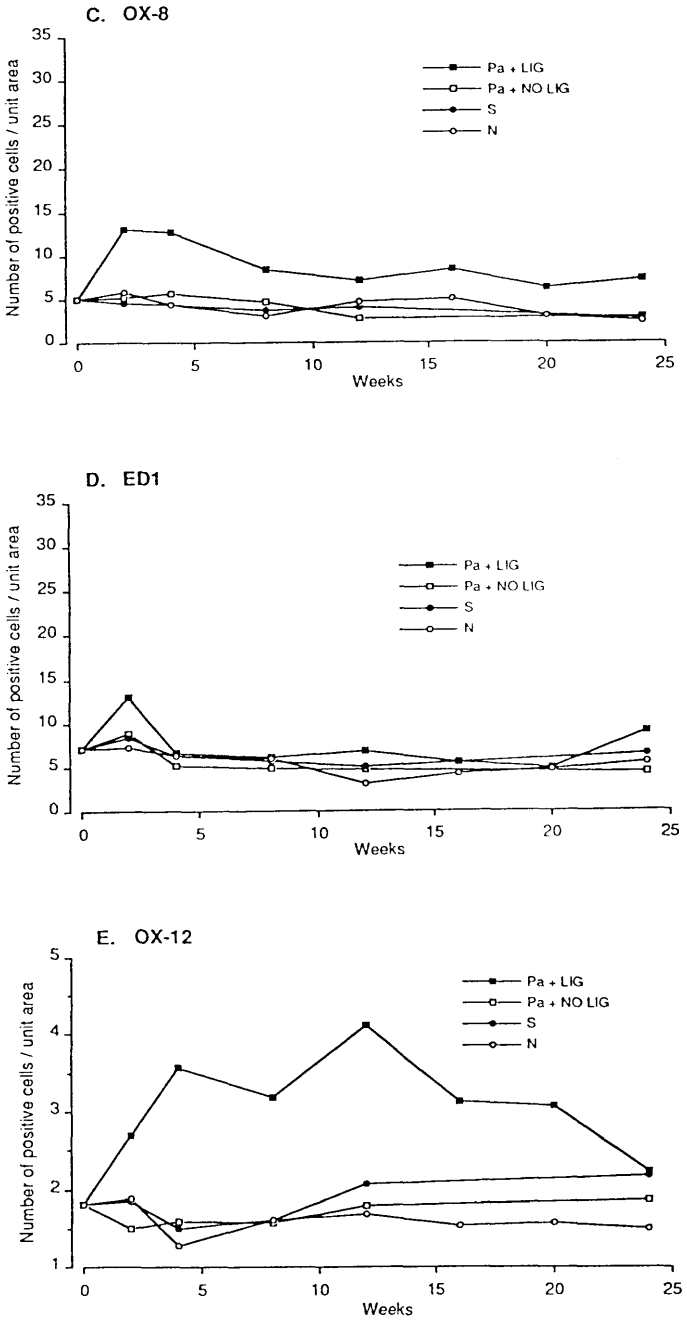
Changes in composition and frequency of immunocompetent cells infiltrating the bronchus-associated lymphoid tissue in animals with experimental bronchiectasis compared with controls.





(cont.)

FIGURE 5.2 (cont.)



The graphs show changes in frequency of immunocompetent cells in the BALT of test and control groups of rats. PBS + LIG and S groups are represented together. Standard deviations are omitted for clarity. Descriptive statistics in Appendix 1.

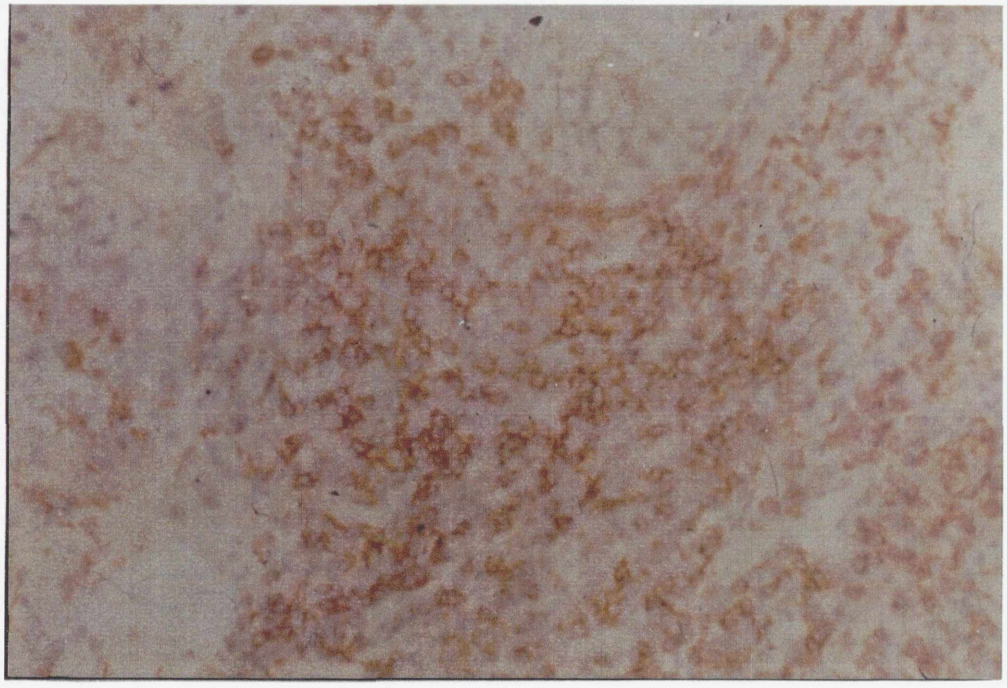
point. Subsequently, T cell numbers decreased but remained statistically different from N at all time points. An inversion of the CD4/CD8 ratios was seen at weeks 8 and 16, and at the other time points there were similar numbers of both subsets or slight predominance of CD4 positive cells (Plate 5.4).

The macrophage-like cell population in the BALT of animals from the Pa + LIG group did not demonstrate the same dramatic increase as that seen in the T cell population, when compared with group N, but was significantly increased at weeks 2 ($p = < 0.01$) and 12 ($p < 0.02$).

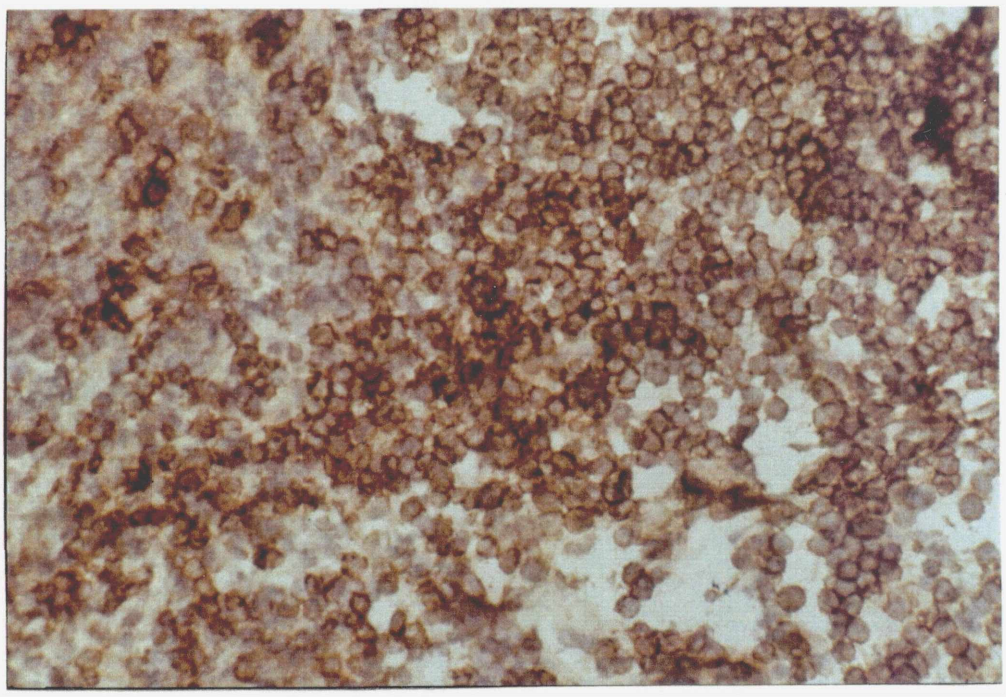
Cells of the B-lymphocyte lineage, as determined by the monoclonal antibody OX-12, showed a significant increase in the Pa + LIG group from week 4 onwards compared with the normal controls but no differences were seen between the experimental control groups and the normal animals at any time point.

Composition of the newly formed lymphoid aggregates: The T-lymphocyte proliferation seen in the BALT of animals from the Pa + LIG group was not exclusive to these structures but was also reflected in newly formed lymphoid aggregates seen in this group (Plate 5.4). Extensive aggregates of T-lymphocytes were present in some animals but in others this phenomenon was less marked, resulting in a large standard deviation (Table 5.5). No such aggregates were noted in the three control groups. As in the BALT, this T cell accumulation was most marked at week 2 but began to decline from week 8 onwards. At first, the T cells were almost exclusively CD4 positive helper cells but the numbers of CD8 positive cytotoxic/suppressor cells increased steadily up to week 8 and then began to decline. Many ED1 positive macrophage-like cells were also present in the aggregates, and numbers of OX12 positive cells equivalent to those seen in the BALT were noted.

PLATE 5.4



a: CD4+ T-lymphocytes infiltrating the bronchus-associated lymphoid tissue of a rat from the Pa + LIG group, 2 weeks after induction (W3/25, immunoperoxidase, original magnification x250)



b: T-lymphocytes infiltrating a parenchymal follicle in a rat from the Pa + LIG group, 4 weeks after induction (OX-19/OX-52, immunoperoxidase, original magnification x250)

TABLE 5.5

COMPOSITION OF THE NEWLY FORMED LYMPHOID AGGREGATES SEEN IN RATS DEVELOPING EXPERIMENTAL BRONCHIECTASIS FOLLOWING SURGERY

TIME FOLLOWING SURGERY (WEEKS)	Monoclonal antibodies 19/52	W3/25	OX8	ED1	OX12
2	20.24±13.69;	14.28±10.98;	8.99±4.02;	13.61±4.32;	2.5±1.1
4	40.38±24.81;	34.55±23.49;	7.72±3.92;	5.60±1.21;	3.5±0.8
8	37.76±33.03;	22.91±22.24;	17.99±11.5;	4.85±1.56;	3.4±1.2
12	18.36±10.53;	11.53±10.53;	9.33±3.22;	7.08±2.29;	4.1±1.0
16	9.64±2.85 ;	5.69±1.38 ;	6.19±4.99;	8.25±1.48;	3.1±0.8
20	14.56±1.41 ;	9.39±1.41 ;	6.21±1.87;	3.64±1.11;	2.9±1.6
24	9.90±4.74 ;	7.34±3.0 ;	4.87±2.39;	7.82±3.06;	2.2±0.8

Pa + LIG = intra-bronchial injection of Pseudomonas aeruginosa after partial bronchial ligation. Data expressed as mean ± SD of the numbers of positively stained cells per unit area (10^4 sq. u). No comparison possible with control groups since these lymphoid nodules are formed de novo in the test group; W = weeks.

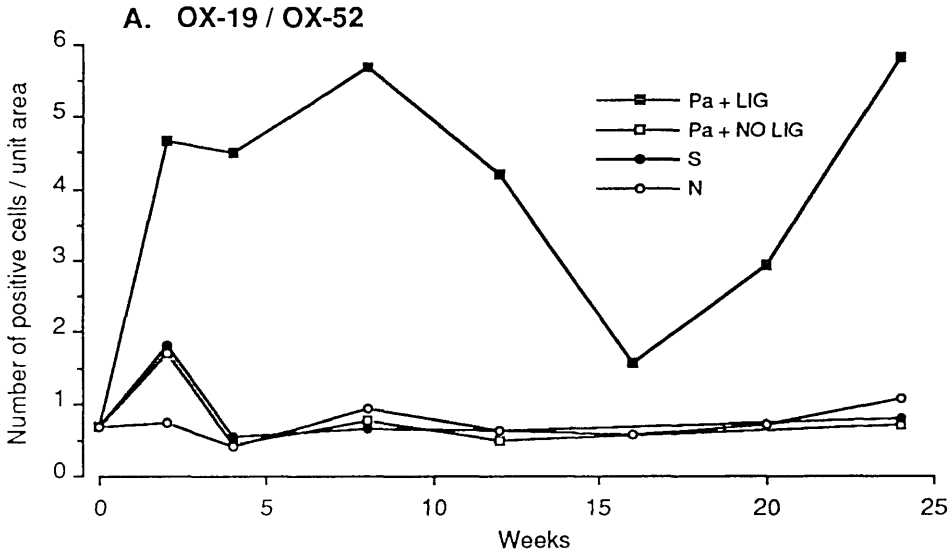
Peribronchial infiltration: A different pattern of infiltration to that in previously described compartments was noted in the bronchial lamina propria. A less marked but highly significant increase in the numbers of T cells present in the Pa + LIG group was seen at all time points, steadily increasing up to 8 weeks, declining at 16 weeks, then increasing again in a bi-phasic mode (Fig.5.3). In some animals, mainly at week 8, this infiltration was so massive as to form a continuous sheath (Plate 5.3). This T cell increment was initially due to both subsets, with a slight predominance of CD4 positive cells at the first time point but from 8 weeks onwards a predominance of CD8 positive cells over the CD4 positive cells or equivalent numbers of both phenotypes were noticed.

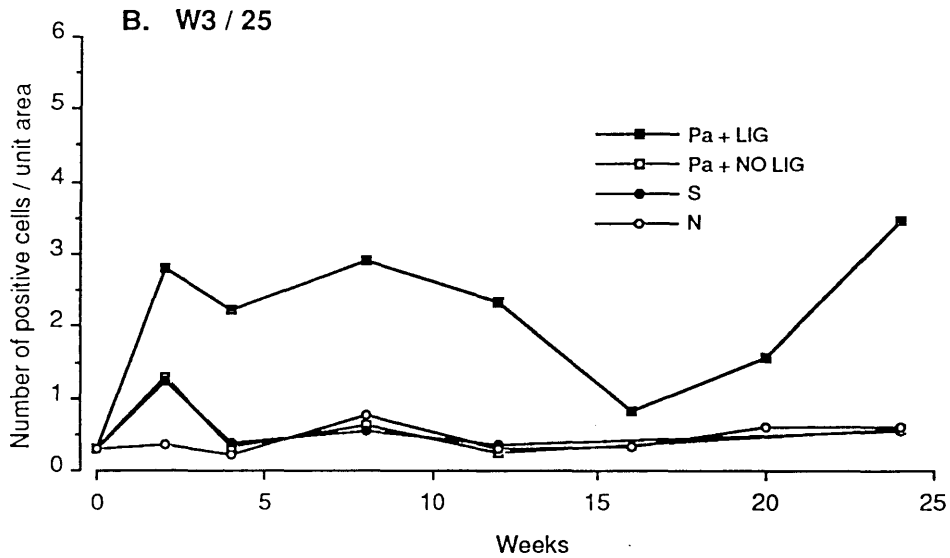
Significant increase in the macrophage population was also present in the Pa + LIG group compared with controls at all time points. In the Pa + NOLIG, PBS + LIG, and S groups, a small but significant increase in the T cell and macrophage populations was seen at week 2 but the numbers of positive cells were not different from group N after 4 weeks.

Significant increase in numbers of B-lymphocytes were noted at all time points when the Pa+ LIG group was compared with normal controls but no differences were seen between the experimental control groups and the normal animals at any time point.

FIGURE 5.3

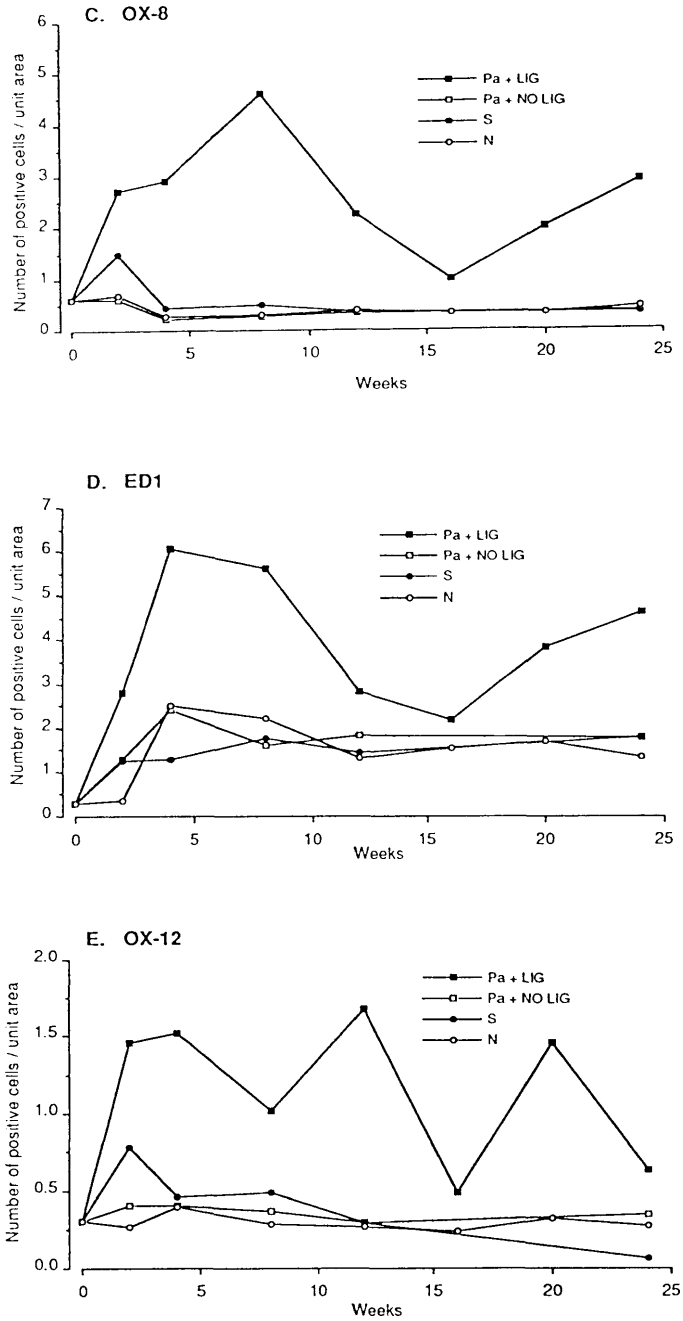
Changes in the composition and frequency of immunocompetent cells infiltrating the peribronchial areas of animals with experimental bronchiectasis compared with controls.





(cont.)

FIGURE 5.3 (cont.)



The graphs show changes in frequency of immunocompetent cells in the lamina propria of test and control groups of rats. PBS + LIG and S groups are represented together. Standard deviations are omitted for clarity. Descriptive statistics in Appendix 1.

5.3.3 - CHANGES IN LOCAL CELLULAR REACTIVITY: MARKERS OF CELL ACTIVATION IN EXPERIMENTAL BRONCHIECTASIS

5.3.3.1 - Expression of Interleukin-2 Receptor by T-lymphocytes

Expression of the interleukin-2 receptor by the T cells was studied. Virtually no staining of dendritic cells by the monoclonal antibody OX-39 was noticed in the lung compartments examined, which led to the conclusion that all OX-39 staining present was on T cells. In this analysis, readings at all time points were accumulated and the number of positively stained cells were counted in the lamina propria, BALT, and other follicular aggregates (Table 5.6).

Significantly higher proportions of T cells expressing OX-39 in animals of the Pa + LIG compared with the other three groups included in this study were seen in all compartments examined, principally in the lamina propria ($p < 0.0001$) (Plate 5.5). The number of OX-39 positive cells in rats from the Pa + LIG group was higher at the initial time points as compared with later points (data not shown).

5.3.3.2 - Increase in Acid Phosphatase Content in Macrophages

The presence of acid phosphatase in the macrophages was also studied (Table 5.7). Highly significant differences ($p < 0.0001$) were seen in all compartments between Pa + LIG and the other three groups. No differences were found in the earlier versus later time points, nor between the Pa + NOLIG, S, and N groups (Plate 5.5).

5.3.3.3 - Quantitation of Class II Ia Antigen Expression

Ia antigen was expressed by bronchial epithelial cells and by many

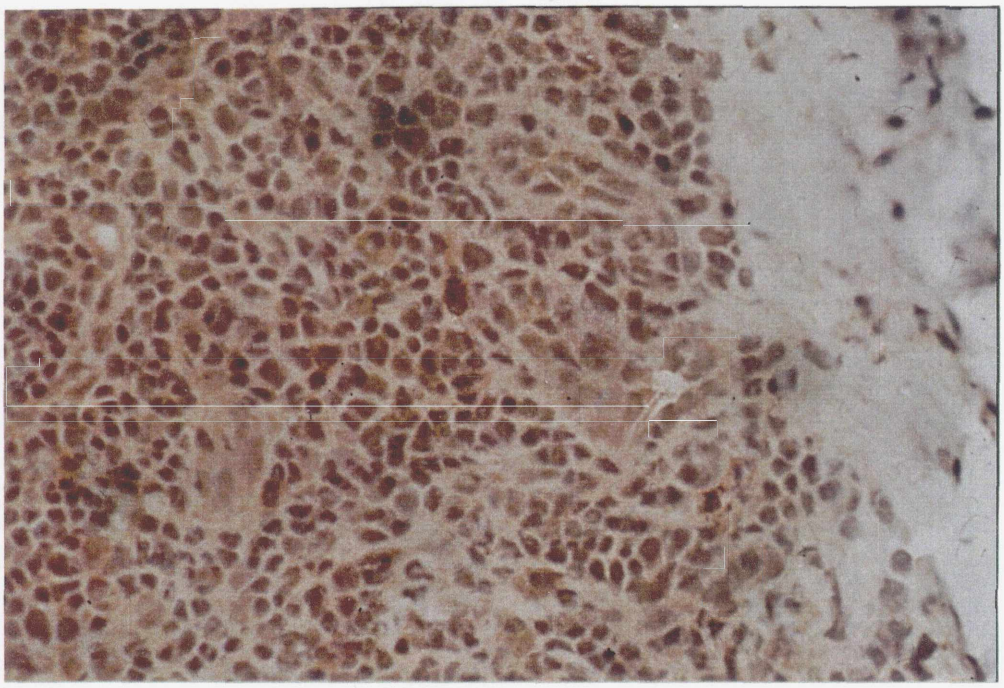
TABLE 5.6

DISTRIBUTION OF INTERLEUKIN-2 RECEPTOR POSITIVE CELLS IN THE BRONCHIAL WALL OF RATS WITH EXPERIMENTAL BRONCHIECTASIS AND CONTROLS

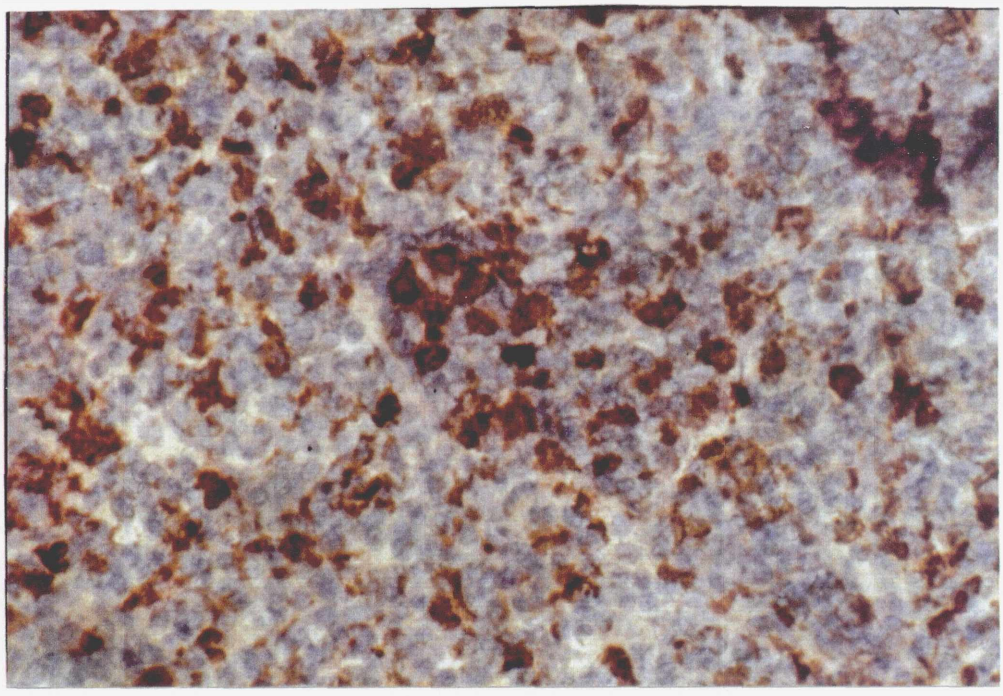
EXP'TAL GROUPS	LAMINA PROPRIA	BALT	FOLLICLES
Pa + LIG	0.54±0.41	2.68±2.35	1.94±1.59
	p <0.0001	p <0.0005	--
Pa + NOLIG	0.05±0.10	1.19±1.07	NP
	NS	NS	--
S	0	1.09±0.53	NP
	--	NS	--
N	0.04±0.08	0.84±0.49	NP

Data expressed as mean + SD of the numbers of OX-39+ cells/10^4 u^2 (readings at all time points were accumulated). NP = follicles not present; NS = statistically not significant; p values compare data from each experimental group with the age-matched normal control at each lung compartment analysed by Student's t-test. See Appendix 1 for descriptive statistics.

PLATE 5.5



a: Scant positive cells stained with the interleukin-2 receptor marker in the BALT of a rat from the Pa + LIG group, 2 weeks after induction (OX-39, immunoperoxidase, original magnification x250)



b: Macrophages stained in brown, also exhibiting cytoplasmic pink staining for acid phosphatase in the BALT of a rat from the Pa + Lig group, 12 weeks after induction (ED1, immunoperoxidase and pararosaniline staining, original magnification x250)

TABLE 5.7

DISTRIBUTION OF ACID PHOSPHATASE POSITIVE MACROPHAGES IN THE BRONCHIAL WALL OF RATS WITH EXPERIMENTAL BRONCHIECTASIS AND CONTROLS

GROUP	EPITHELIUM	LAMINA PROPRIA	BALT
Pa + LIG	56.6±5.5	56.7±11.1	54.8±10.4
	p <0.0001	p <0.0001	p <0.0001
Pa + NOLIG	26.5±13.1	32.9±5.5	35.4±9.5
	NS	NS	NS
S	29.4±11.9	30.3±8.6	32.0±7.9
	NS	NS	NS
N	33.5±11.9	32.3±10.2	36.5±8.56

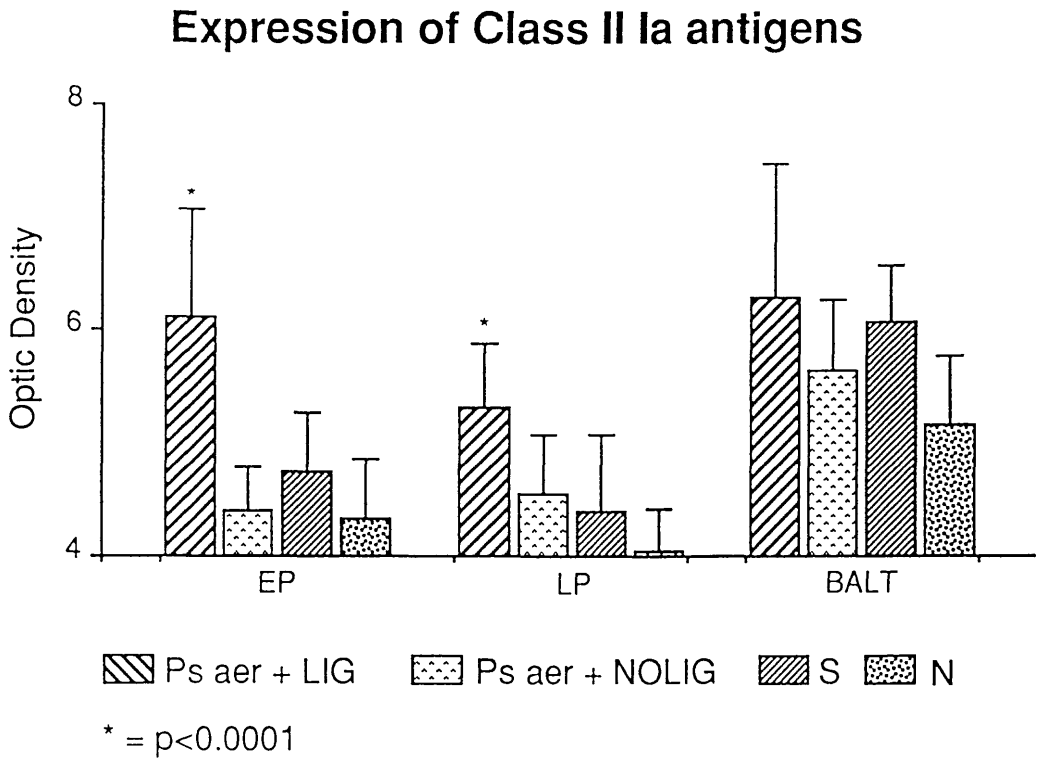
Data expressed as mean ± SD of the percent of ED1+/ACP+ cells in each compartment analysed (readings at all time points were accumulated). NS = statistically not significant; p values compare data from each experimental group with the age-matched normal controls at each lung compartment analysed by Student's t-test. See Appendix 1 for descriptive statistics.

mononuclear cells infiltrating the lung compartments of the Pa + LIG group but not by cells in the control groups. This Ia expression was quantitated in the different compartments of the lung. In order to determine the sensitivity of the system used for the quantitation, the readings obtained in the BALT of the rats from the N group (usually the only compartment of the normal rat lung to express the antigen uniformly) were compared with the readings obtained in the lamina propria and in the epithelium. The results were significantly different ($p = 0.005$ for the lamina propria, $p < 0.03$ for the epithelium), indicating that the quantitation system was sufficiently sensitive. The readings obtained in the different compartments of the lungs in all animals of the groups are presented in Fig. 5.4.

Highly significant differences were seen in the Ia readings obtained in the Pa + LIG group when compared with the other three groups, particularly in the epithelium ($p < 0.0001$ v. N; $p = 0.0001$ v. Pa + NOLIG; $p = 0.0003$ v. S) but also in the lamina propria ($p < 0.0001$ v. N; $p < 0.01$ v. Pa + NOLIG; $p < 0.03$ v. S). In the BALT the differences were less remarkable ($p = 0.006$ v. N; $p < 0.01$ v. Pa + NOLIG). No differences could be established between the readings from the Pa + NOLIG and S groups when compared with group N (Plate 5.6).

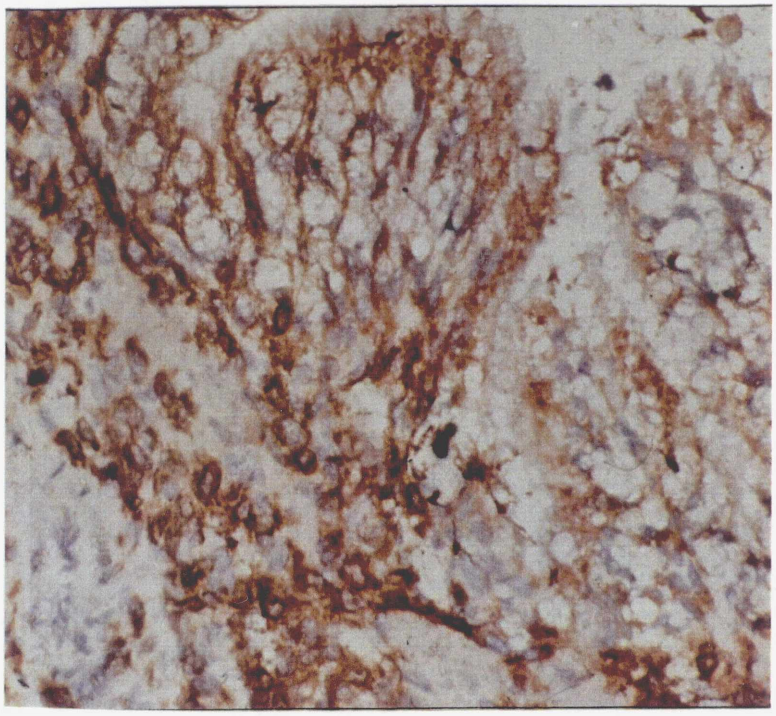
FIG. 5.4

EXPRESSION OF MHC CLASS II Ia ANTIGENS IN LUNG COMPARTMENTS OF TEST AND CONTROL GROUPS

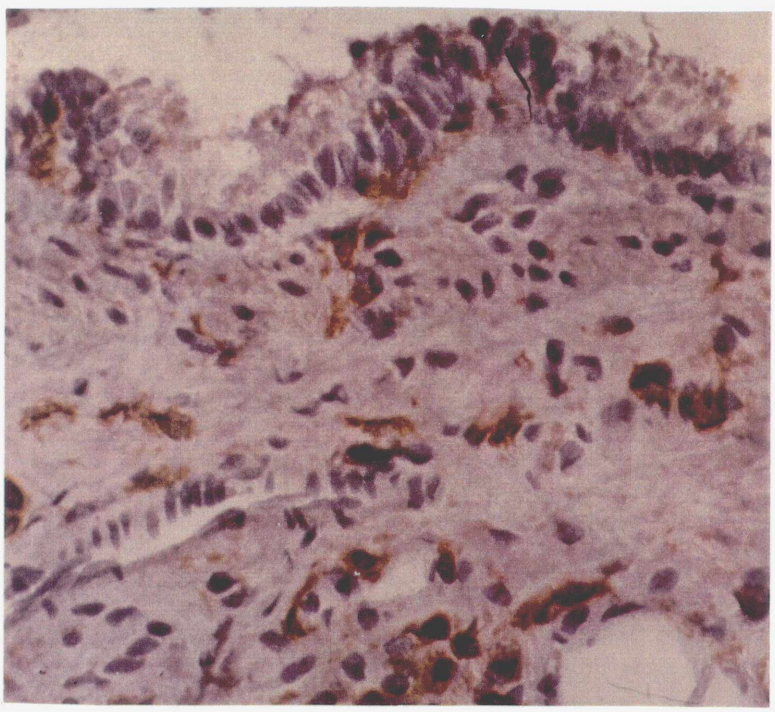


The bar graph shows the results of optic densitometry of lung compartments of test and control groups of rats. The results are expressed in arbitrary units. Descriptive statistics in Appendix 1.

PLATE 5.6



a: MHC Class II (Ia) positivity in the bronchial epithelium of a rat from the Pa + LIG group, 2 weeks after induction (OX-6, immunoperoxidase, original magnification x 250)



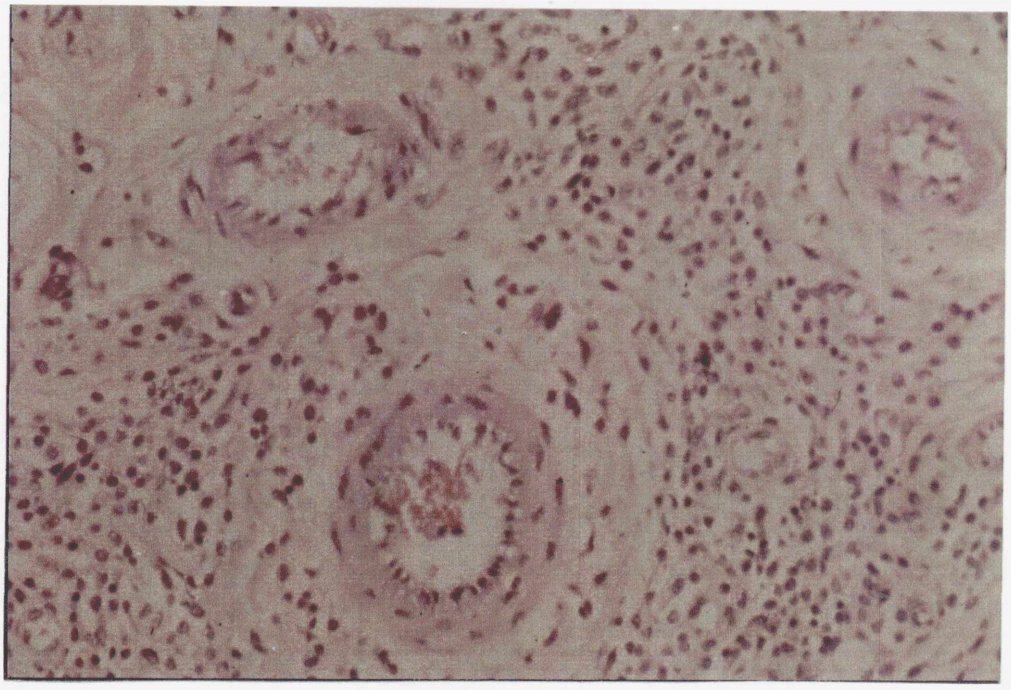
b: Bronchial epithelium negative for MHC Class II (Ia) staining in a rat from the N group (OX-6, immunoperoxidase, original magnification x250)

5.3.4 - DELAYED HYPERSENSITIVITY TO PSEUDOMONAS AERUGINOSA ANTIGENS IN RATS

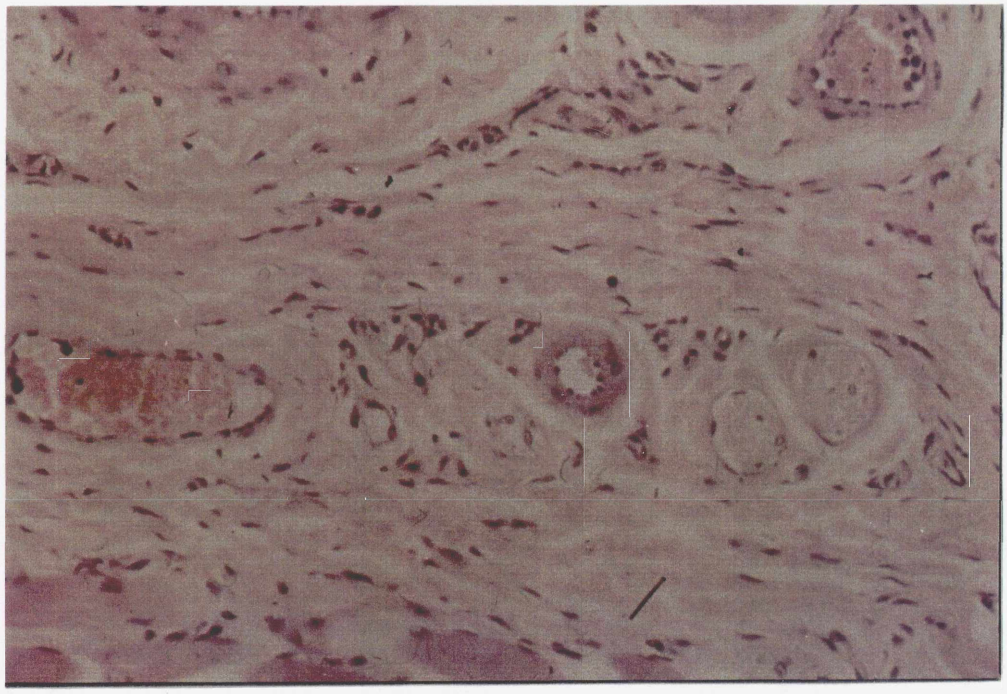
The Pa + LIG group showed a difference in thickness between the hind footpads: the right footpad, injected with sterile PBS, showed an increase of thickness of 0.045±0.068 mm (11 samples), while the left footpad, injected with sonicated pseudomonas, showed an increase of 0.177±0.15 mm ($p = < 0.02$). The normal controls did not show any difference between the footpads: right (PBS): 0.037±0.07 mm (8 samples); left (antigen): 0.025±0.046 mm. The comparisons between the increases in thickness of footpads injected with sonicated Pseudomonas aeruginosa between test and control groups revealed a statistically significant increase in the Pa + LIG group ($p = < 0.02$). The complete data, as well as descriptive statistics are presented in Appendix 1.

Histologically, no footpads from test or control groups injected with sterile PBS showed abnormal infiltration of mononuclear cells. The footpads of control animals injected with antigen showed a slight increase in the number of mononuclear and other inflammatory cells (Plate 5.7). In contrast, the footpads from all animals from Pa + LIG group injected with sonicated pseudomonas showed large increment in the numbers of mononuclear cells present in the dermis and other areas around the injection site, with histological appearance of small lymphocytes, macrophages, some neutrophils, and other inflammatory cells (Plate 5.7).

PLATE 5.7



a: Footpad of a rat from the Pa + LIg group (12 weeks), 48 hours after injection of pseudomonas antigen, showing infiltration of inflammatory cells (haematoxylin-eosin, original magnification x250)



b: Footpad of a rat from the N group, 48 hours after injection of pseudomonas antigens, with scant numbers of inflammatory cells (haematoxylin-eosin, original magnification x250)

5.5 - DISCUSSION

Guerreiro's model of experimental bronchiectasis in the rat closely mirrors human bronchiectasis, both histopathologically and immunopathologically. Introduction of Pseudomonas aeruginosa into a rat bronchus after its partial ligation produced a reliable model of bronchiectasis and chronic lung infection in which to study the pathogenesis of both conditions. It also provided evidence consistent with the "vicious circle" hypothesis for the pathogenesis of bronchiectasis, as proposed by Cole in 1984. Normally, insult to the respiratory tree by microorganisms or noxious agents is eliminated by mucociliary clearance with or without a self-limited acute inflammatory host response. This situation is illustrated in the experiments described here: the rats in control groups submitted to infection without bronchial ligation, bronchial ligation without infection, or sham operation developed a self-limited inflammatory response in the first two weeks after the insult - as seen by the presence of neutrophils, T-lymphocytes and macrophages in peribronchial and perivascular areas, accompanied by limited histologic damage. After this initial period, however, these lungs returned to normal. In the case of the rats submitted to partial bronchial ligation and injection of P. aeruginosa, failure to clear the bacterial load was probably associated with nonspecific and immunopathological reactions leading to bronchial dilatation, fibrosis, and in some cases, complete destruction of the normal bronchopulmonary architecture, in a "vicious circle" of events.

Such experiments confirm that the combination of partial bronchial ligation and intra-bronchial presence of viable Pseudomonas aeruginosa are necessary for the emergence of bronchiectasis. The mere presence of

the bacterium in a previously normal bronchus or the impairment of the mucociliary clearance without infection did not result in the emergence of the bronchial dilatation or chronic inflammation. Similar requirements were found by others (Tannenberg & Pinner, 1942; Cheng, 1952; Ventura & Domaradzki, 1967), whose findings also correlate with the human situation.

Damaging insults to the human bronchial tree especially in genetically predisposed individuals (or those with underlying disease) may compromise the first line bronchial defence mechanism of mucociliary clearance and predispose the individual to microbial colonisation of the bronchial tree. The colonising microorganisms are usually those which have been shown to release substances that impair the function of, or irreparably damage, ciliated epithelium (Wilson, Roberts & Cole, 1985; Wilson, Pitt, Taylor et al, 1987; Munro, Barker, Rutman et al, 1989; Steinfort, Wilson, Mitchell et al, 1989; Amitani, Wilson, Rutman et al, 1991). The host inflammatory response fails to eliminate these microbial colonists and becomes chronic, damaging adjacent lung tissue in the process. This chronic inflammation and tissue damage further reduce mucociliary clearance (Wilson & Cole, 1988) allowing increased microbial colonisation. The end result of such a chronic inflammatory process is bronchiectasis and progressive lung damage.

Bronchiectasis in the experimental model was characterised by the infiltration of large numbers of T cells, increased MHC Class II expression, emergence of follicles and other findings. The development of this cellular immune response during evolution of bronchiectasis and the demonstration that it was at its height at the time when the rats

developed histologically apparent bronchiectasis raise the possibility that this reaction might be causal. In established bronchiectasis, the continuing presence of large numbers of T-lymphocytes and macrophages in all lung compartments suggests an ongoing acquired immune response.

The histopathological findings of experimental bronchiectasis were also remarkably similar to those of the human condition described in the previous chapter. Most rats submitted to partial bronchial ligation and injection of Pseudomonas aeruginosa into the ligated bronchus developed bronchial dilatation, preceeded and accompanied by a severe inflammatory reaction. This bronchial dilatation was very heterogeneous, ranging from mild bronchiolectasis to severe saccular changes with almost complete disorganisation of the bronchopulmonary structure. The heterogeneous nature of the changes reflects the pattern seen in human disease (Dunnill, 1987).

Another interesting analogy was found between the pattern of the bronchial dilatation and that of the accumulation of inflammatory cells in peribronchial areas: in man, as pointed out by Whitwell (1952), follicular bronchiectasis tended to be associated with cylindrical dilatation; in experimental bronchiectasis in rats, large clusters of mononuclear and other inflammatory cells were also more commonly seen in peribronchial and parenchymal areas of rat lungs displaying cylindrical dilatation. In saccular bronchiectasis, however, inflammatory cells did not form follicles, either in human or in experimental diseases.

Apart from bronchial dilatation and disposition of inflammatory cells, several other histopathological aspects were shared by human and experimental bronchiectasis, such as the presence of intrabronchial secretion composed of mucus, neutrophils and cellular debris, loss of

bronchial elastin, neovascularisation of bronchiectatic areas, peribronchial fibrosis, and perivascular deposition of mononuclear cells. Particularly interesting were the changes exhibited by the bronchial epithelium. This compartment appeared sensitive to external factors, responding to them with goblet cell hyperplasia and other changes. Epithelial irregularities were found in all experimentally manipulated groups in early stages of the development of the model. In experimental control groups (PA + NOLIG, PBS + LIG, and S) but not in the normal group, a degree of goblet cell hyperplasia was present in animals at week two, perhaps as a nonspecific response to surgery or anaesthesia. This change tended to disappear from week four onwards, demonstrating the capacity of the bronchial epithelium to recover. However, in rats submitted to partial bronchial ligation and intrabronchial infection to induce bronchiectasis, these early changes tended to progress to widespread epithelial damage characterised by squamous metaplasia, epithelial shedding, patchy ulceration, epithelial and mucosal foldings, similar to that of the human disease.

The lymphoid tissue also showed important histological changes in animals with experimental bronchiectasis. The number of bronchus-associated lymphoid tissue (BALT) did not increase but its normal structure was seriously disrupted, reflecting the considerable recruitment of immunocompetent cells. The appearance of lymphoid aggregates in the lungs, apparently not related to an airway, probably represented massive perivascular accumulation of mononuclear cells. The fact that putative immunocompetent cells (represented by histologically defined lymphoid and non-lymphoid cells sometimes arranged in follicle-like structures) were much increased in the inflamed areas of lungs

with experimental bronchiectasis suggested that an ongoing immune reaction was part of the inflammatory process associated with the emergence of experimental bronchiectasis. These findings also correlated well with the human condition (Spencer, 1985).

Following bronchial infection, failure to clear the bacterial load due to partial bronchial obstruction is associated with a massive lymphocytic and macrophage response. The time studies used to compare development of the inflammatory lesions disclosed that during the development of bronchiectasis an early expansion of the T-lymphocyte population took place in the BALT, with an almost fourfold increase in the numbers of T cells as compared with the age-matched normal controls, together with the development of T cell follicles in the lung parenchyma. The major T cell subset both in the BALT and in the newly formed lymphoid aggregates during the first weeks of the inflammatory process was the CD4 positive helper subset. This situation was then superseded by a gradual increase in the numbers of CD8 positive cytotoxic/suppressor cells. Such changes would be compatible with recruitment of lymphocytes from the circulation in response to antigens in the bronchial lumen. It has been demonstrated that the high endothelium venules characteristic of the BALT are the place where the main lymphocytic traffic occurs in the bronchi (Otsuki, Ito & Magari, 1989). As the majority of these cells are CD4 positive helper cells, it could be postulated that this is similar to a Type IV hypersensitivity reaction. Potentially, the BALT acts as the draining lymphoid organ and the deeper follicles represent the outcome of perivascular accumulations of recruited T cells. This situation is reminiscent of the Mantoux reaction in man (Poulter, Seymour, Duke et al, 1982), particularly as a minor but significant population of non-lymphoid

macrophage cells is present in the locality possibly acting as antigen presenting cells (Unanue & Allen, 1987).

Also in the epithelium and peribronchial area, an early and highly significant accumulation of T-lymphocytes was apparent, and at the onset of the process a small predominance of CD4 positive over CD8 positive T cells was also observed. These findings could reflect the emergence of a Type IV hypersensitivity response. From week 8 onwards a predominance of CD8 positive cells, or an equilibrium between the two major subsets, was present in the bronchiectatic animals. Whether this predominance of the CD8 positive subset was an attempt to control the cell-mediated immune response, or whether it represented the emergence of a cytotoxic population itself involved in the tissue damaging events are questions yet to be answered. Similar responses have been described in viral infections (Maher, O'Toole, Wreghitt et al, 1985) and in allograft rejection (Hayry, 1989).

The time-dependent study showed clearly that severe inflammatory changes preceeded the development of bronchiectasis. The presence of a small but statistically significant population of OX-39 (interleukin-2 receptor) positive T cells in the lamina propria, BALT, and other lymphoid aggregates in rats with experimental bronchiectasis suggested that these T cells were activated. The increased expression of Class II MHC antigens by the epithelium and mononuclear cells infiltrating the peribronchial areas was further evidence of cellular activation. In the human condition, T-lymphocytes also exhibit signs of cell activation and immunological commitment, with increased expression of HLA-DR and CD45RO antigens. These data strongly suggest that T-lymphocytes are very important for the development of experimental bronchiectasis.

One of the important questions raised by these observations is whether the T cell response was driven by pseudomonas antigens. An example of T cell mediated inflammatory damage probably driven by bacterial antigens is provided by rheumatoid arthritis (Gaston, Life, Bailey et al, 1989; O'Brien, Happ, Dallas et al, 1989; Young & Elliott, 1989). Despite being an exclusive extracellular bacterium, it has been demonstrated that P. aeruginosa can elicit protective T-lymphocyte mediated immunity in mice (Pier & Markham, 1982; Markham, Goellner & Pier, 1984; Powderly, Pier & Markham, 1986a,b,c; Powderly, Pier & Markham, 1987; Markham, Pier & Powderly, 1988; Powderly, Schreiber, Pier et al, 1988). The T cell subset that mediates this bactericidal effect is of CD8 positive phenotype (Markham, Pier, Goellner et al, 1985; Markham & Powderly, 1988) and the accumulation of cells exhibiting this phenotype in the experimental lesions of the rat lung could be taken as evidence of the role of the bacterium in driving the immune response.

In this experimental model the live Pseudomonas aeruginosa comprises a large array of potentially immunogenic components (Appendix 3). To investigate possible systemic reactivity to them, sonicated bacteria was used as the source of pseudomonas antigens in classical skin tests for delayed hypersensitivity. Intradermal tests remain an useful tool for investigating a variety of cell-mediated reaction both in clinical and experimental conditions (Howard & Liew, 1984; Buckley, 1986; Dhaliwal & Liew, 1987; Czuprynski & Brown, 1990). The presence of statistically significant differences in thickness between the footpads receiving PBS as opposed to antigen suspension in rats with experimental bronchiectasis, and between test and control footpads receiving injection of the antigen preparation (confirmed by histology)

could be interpreted as an indirect evidence for specificity of the immune response seen in the lung against bacterial antigens. The relatively small differences found here could be explained by the fact that sonicated bacteria were employed, instead of purified potentially immunogenic components of the bacterium, which could "dilute" the effect of the relevant antigens involved in the immune reaction. Although there is no direct evidence from our data that the cell-mediated response observed in the bronchial wall is directed against antigens of the bacterium, the fact that the bacteria could be cultured from lung samples as far as 24 weeks after the induction of the model and that the footpad reaction depend upon the presence of the bacterium, is suggestive.

It has been shown that some exoproducts of P. aeruginosa, particularly the phenazine pigments, are strong inhibitors of T cell proliferation in vitro, by inhibiting the production of interleukin-2 and interleukin-2 receptor (Sorensen, Klinger, Cash et al, 1983; Muhlradt, Tsai & Conradt, 1986). This finding has been taken to suggest that the protracted course of P. aeruginosa infection could be due to to the suppressive effect of its exoproducts on T cell proliferation (Nutman, Bergman, Chase et al, 1987). However, there is contradictory evidence from the literature for marked effects of P. aeruginosa and its exoproducts and components on different aspects of the immune response (Petit & Daguet, 1983; Holt & Misfeldt, 1984; Issekutz & Stoltz, 1985; Daley, Pier, Liporace et al, 1985; Nicas & Iglewski, 1985; Blackwood, Lin, Rowe, 1987; Nutman, Berger, Chase et al, 1987; Horvat & Parmerly, 1988; Parmerly, Gale, Clabaugh et al, 1990). Most of these papers describe the effects of the bacterium or its specific

exoproducts on the inhibition of the immune response, particularly on T-lymphocyte activation. However, in one of the first reports on the effects of phenazine pigments from P. aeruginosa on T cell activation, Sorensen and colleagues (1983) pointed out that P. aeruginosa supernatants also contain antigens capable of inducing lymphocyte proliferation. These observations were confirmed by a recent paper (Ulmer, Pryjma, Tarnok et al, 1990) which showed that pyocyanine has dual dose-dependent stimulatory as well as inhibitory effects on immune responses in vitro. In general, stimulatory activity was found at low concentrations of the pigment, whereas high concentrations resulted in an inhibition of responses.

With such complex substances and interrelationships involved in P. aeruginosa infections, it is probable that the capability of this bacterium to persist is multifactorial, as pointed out by Nicas and Iglewski (1985). They suggested that the relative contribution of a given P. aeruginosa product may vary with the type of the infection. The in vivo data shown in this research, however, suggests that T cell proliferation and activation are important constituents of the severe inflammatory reaction accompanying the infection, and which parallel the development of bronchiectasis.

The induction of cell-mediated immune reactions in the lung is not itself a new observation. Using a model of classical delayed-type hypersensitivity reaction in the mouse lung described by Enander and colleagues (1984), Garssen and colleagues (1989) have shown that epicutaneous sensitization by picryl chloride followed by intranasal challenge seven days later result in a sudden increase in mononuclear cells, especially around vessels and bronchioli, and in interstitial tissues. The histological pattern they described closely resembles that

seen in the rat with experimental bronchiectasis described here. The authors showed that these responses were T cell dependent and antigen specific. They also found this reaction to be associated with airway hyperreactivity (Garssen, Van Loveren, Van Der Vliet et al, 1990).

The quantitation of MHC Class II antigen expression in tissue sections has been employed in studies of human diseases such as sarcoidosis (Campbell, Du Bois, Butcher et al, 1986). Here, an important finding was the significant increase in the expression of Class II MHC antigen by bronchial epithelial cell and mononuclear cells infiltrating the lung compartments of rats developing experimental bronchiectasis but not of controls. Normal rat epithelium is Ia negative (Holt & Schon-Hegrad, 1987) and the emergence of Ia could be taken to indicated the local release of lymphokines, such as interferon-gamma, which is known to promote Class II MHC expression, and could be taken as an indirect confirmation of T cell activation. Alternatively, the increased level of Class II MHC antigen might reflect an attempt by the immune system to down-regulate T cell activity (Gaspary, Jenkins & Katz, 1988; Markmann, Lo, Naji et al, 1988). A similar phenomenon has been described in the bronchial epithelium of rats acutely rejecting lung allografts soon after transplantation, a process known to be driven by classical cell-mediated immunity (Romaniuk, Prop, Petersen et al, 1987). In this process it is suggested that the mediator inducing the expression of Ia antigens is interferon-gamma, produced mainly by activated T cells and by natural killer cells (Kirby, Parfett, Reader et al, 1988). In experimental bronchiectasis large numbers of Ia positive, probably activated, T-lymphocytes, many of suppressor/cytotoxic phenotype (OX-8

positive), were present in the bronchial inflammatory response and it is possible that they could produce this lymphokine. Recently, bronchiolitis and bronchiectasis have been described as late complications of human heart-lung transplantation (Burke, Theodore, Dawkins et al, 1984) and the progression of these complications appears to be reduced by increasing the immunosuppression regimen (Glanville, Baldwin, Burke et al, 1987). Recent studies have emphasized the importance of expression of MHC Class I and II antigens by human bronchial epithelium, making it a possible target for allograft rejection mechanisms (Glanville, Tazellar, Theodore et al, 1989).

Since the preliminary reports of these changes (Lapa e Silva, Guerreiro, Noble et al, 1988; Lapa e Silva, Guerreiro, Munro et al, 1989a; Lapa e Silva, Guerreiro, Munro et al, 1989b; Lapa e Silva, Guerreiro, Noble et al, 1989; Lapa e Silva, Guerreiro, Munro et al, 1990), other groups, working with the Cash model of chronic lung infection by Pseudomonas aeruginosa, have shown similar changes in the composition and frequency of immunocompetent cells in the lungs (Iwata, Sato, Chida et al, 1990). Also, Tanaka and colleagues, using a modified Cash model in mice, in which they rechallenged the animals at 3, 6, and 9 weeks, measured immunocompetent cell populations and immunoglobulins in lung lavage fluid. They also found a large increment in all parameters observed, concluding that humoral and cellular immune responses occurred in the bronchial mucosae associated with chronic colonisation by Pseudomonas aeruginosa (Tanaka, Sato, Kato et al, 1990).

In conclusion, there is mounting evidence to support the concept that cell-mediated immunity to pseudomonas antigens is part of the

immune response against the bacterium and that this immune response may cause at least part of the tissue damage seen in the experimental condition described in this thesis. Confirmation of its causal nature, however, can only be achieved by other investigations.

C H A P T E R 6

IMMUNOPATHOGENESIS OF EXPERIMENTAL BRONCHIECTASIS: *IN VITRO* STUDIES

6.1 - INTRODUCTION

Evidence has been presented in the previous chapters to support the hypothesis that acquired immune mechanisms are associated with the development of experimental bronchiectasis. However, the finding of large numbers of T-lymphocytes and macrophages, some of them exhibiting signs of cellular activation suggestive of an ongoing immune response, does not answer the question as to whether this is a primary or secondary immune phenomenon. None of the methods employed revealed the function of the immunocompetent cells found to be present in large numbers in the lesions. It is undeniable that these cells are involved in the host response but whether they cause the observed response in part or in whole remains unresolved.

This question is addressed by determining the function of the immunocompetent cells from bronchiectatic sites in in vitro systems. For example, a direct role for T-lymphocytes in the pathogenesis of bronchiectasis would be supported if it were possible to demonstrate cytotoxic activity of these cells against intrinsic or extrinsic antigens. This approach has been used extensively in the past, especially in the investigation of immune mechanisms involved in interstitial lung diseases, where sampling of immunocompetent cells is possible by means of bronchoalveolar lavage. However, neither in human nor experimental bronchiectasis can bronchoalveolar lavage be used as a mean of obtaining immunocompetent cells from the lungs, despite previous attempts (Plusa & Wasek, 1987). In the case of human disease, poor fluid recovery due to loss of lung elasticity, gross bacterial and neutrophillic contamination, and ethical considerations rule out its use for clinical or research purposes. In the experimental model of bronchiectasis employed in this research, the partial bronchial

ligation used for induction of the process precludes lavage which even if attempted would not sample adequately the bronchoalveolar compartment of the involved lobe.

In recent years, a number of publications have proposed a reliable method for obtaining immunocompetent cells from the interstitial lung compartment by enzymatic digestion of lung fragments (Holt, Degebrodt, Venaille et al, 1985; Holt, Degebrodt, O'Leary et al, 1985; Holt, Robinson, Reid et al, 1986; Holt & Schon-Hegrad, 1987). The Holt method consists basically of incubation of lung slices in an enzymatic medium known to degrade lung matrix tissue in order to loosen immunocompetent and other cells from the parenchyma while keeping them alive and retaining them active functionally. After that, the cells can be separated into different subsets and treated in a similar way as cells recovered by bronchoalveolar lavage.

The Holt method, although laborious and time consuming, provides a way of sampling cells from the rat lung compartments shown in the previous chapter to be severely affected by the inflammatory process accompanying the development of experimental bronchiectasis. Thus with some adaptations enzymatic digestion of lung slices was employed to provide single mononuclear cell suspensions on which to perform investigations regarding the functional activities of the cellular components of the immune response.

Cell-mediated cytotoxic mechanisms are classically involved in immune responses generated to foreign transplantation antigens, or associated with auto-immunity and some infectious situations (Roitt, Brostoff & Male, 1989). This is a rather complex process involving recognition of the foreign antigen in the context of MHC Class I or II

antigens, T cell activation, and generation of T-lymphocytes with cytotoxic capabilities, usually among the CD8 positive T cell population but also, to a lesser extent, among the CD4 positive population. The cytotoxic T cell binds to the target and secretes enzymes, such as perforins, lymphokines and cytokines which damage the target cell membrane, modulate protein synthesis, and cause cytotoxic damage and cellular death (Roitt, Brostoff & Male, 1989).

This mechanism, one of the most effective processes generated by the immune system to protect the host, can also produce immunopathology, either in clinical or experimental conditions. For example, in the disease hypersensitivity pneumonitis a significant number of cells accounting for the alveolitis present in patients developing the condition display phenotypical and functional characteristics of active cytotoxic cells (Semenzato, Trentin, Zambello et al, 1988). In mice infected with respiratory syncytial virus (RSV) the injection of a RSV-specific cytotoxic T cell line clears the virus from the lungs but causes a lethal hemorrhagic pneumonitis within five days of the injection, characterized by increased peribronchiolar infiltration of lymphocytes, monocytes and neutrophils, together with areas of alveolar consolidation with haemorrhage (Cannon, Openshaw & Askonas, 1988).

Either in human bronchiectasis, as described in Chapter 4, or in experimental bronchiectasis, as described in Chapter 5, the presence of large numbers of T-lymphocytes in the bronchiectatic sites, many of them of CD8 positive phenotype, suggests that cell-mediated cytotoxic mechanisms, generated against opportunistic viruses or neo-antigens arising during the inflammatory process, could be partly responsible for the structural damage which is one of the main features of the

disease.

To address this question, a protocol was devised to investigate whether cytotoxic activity was present in cell suspensions obtained by collagenase digestion of rat apical lobes with experimentally induced bronchiectasis.

6.2 - METHODS

6.2.1 - ANIMALS AND EXPERIMENTAL DESIGN

Oubred, SPF Wistar Albino rats were purchased from Charles Rivers, Ltd., and experimental bronchiectasis induced by the method of Guerreiro of partial bronchial ligation and injection of live Pseudomonas aeruginosa bacteria, as described in section 5.2. Age-matched normal littermates, housed under the same conditions were used as controls.

6.2.2 - PREPARATION OF LUNGS

The animals were killed by intraperitoneal injection of 1 ml of Hypnorm (Janssen Pharmaceuticals, Marlow, UK) followed by cervical dislocation, after post-operative time intervals shown in previous experiments to be sufficient for the induction of experimental bronchiectasis. The fur was thoroughly wiped with 70% alcohol, the animal was exsanguinated by cardiac puncture, the blood being collected and the serum stored.

A midline incision was made from the cervical area to the abdomen, the sternum and part of the rib cage removed, and the abdominal aorta severed above the liver. A 4FG cannula (Portex) was introduced in the heart, and the vascular bed was flushed with sterile ice-cooled PBS until the lungs and abdominal organs glistened clear white. The contents of the thorax were removed "en bloc", the apical lobe and also the left lobe dissected out and placed in a sterile container wrapped in a moist gauze. This was kept on ice until use. No lung lavage was performed in this protocol. The trachea, remaining lung tissue, and part of the liver were aseptically removed and placed in sterile

plastic tubes.

The samples were quickly transported to the laboratory in ice. The serum was frozen and kept at -20^{0} C for future studies. The trachea, lung and liver were homogenised and plated onto Pseudomonas Isolation Agar (Oxoid, Basinstoke, UK), as explained previously (section 5.2.6). The apical and left lobes were submitted to the digestion protocol.

6.2.3 - COLLAGENASE DIGESTION

Once in the laboratory, all procedures were performed aseptically. The lobes were placed in separate sterile Petri dishes, weighed, and kept inside a laminar airflow cabinet used for cell culture. Working digestion solutions were freshly prepared with 54 ml RPMI 1640 (Gibco, Uxbridge, UK), 6 ml heat-inactivated fetal calf serum (FCS; Flow Laboratories Ltd, UK), 60 mg collagenase (Boehringer Ingelheim, Manheim, Germany), and 240 mg neutral protease (Dispase; Boehringer Ingelheim). A small volume of the solution (3 ml) was added to the Petri dishes containing the lobes, which were carefully sliced with scalpel. Macroscopic evidence of bronchiectasis and inflammatory changes were recorded. The fragments were transferred to plastic universal tubes and 20 ml of the digestion medium added to each universal, which was placed in a shaking water bath kept at 37^{0} C to allow optimum enzymatic activity. After 45 min, freshly prepared medium was added, containing the same amounts of the previously described components plus 240 ug of Deoxyribonuclease I (DNAase; Sigma Chemical Co., St. Louis, USA), and incubated for another 45 min under similar conditions. This was to prevent dead cell clumping and trapping of the viable cells being released from the lung matrix. The universals were

then removed from the bath and the solution aspirated several times with a wide bore Pasteur pipette to disperse mechanically the remaining tissue fragments.

The resulting suspension was quickly filtered through gauze to remove the non-digested fragments and diluted with medium containing 54 ml RPMI 1640, 6 ml FCS, and 240 ug DNAase (DNAase medium). The universals were centrifuged at 3000 rpm at 4^{0} C for 10 min, the supernatant discarded, and the cell pellet gently resuspended in DNAase medium. This process was repeated twice. Finally the cell pellet was resuspended in 3 ml DNAase medium, cells counted in a modified Neubauer haemocytometer chamber, and the cell viability assessed by Trypan Blue dye-exclusion assay.

6.2.4 - CELL FRACTIONATION

The cell suspension was fractionated in a 4-step Percoll discontinuous gradient. A stock solution was prepared in the following way: 90 ml Percoll (Pharmacia, Upsalla, Sweden), 9 ml 10x Hanks Balanced Salt Solution (HBSS; Sigma Chemical Co, St. Louis, USA), and 1.5 ml HEPES (Gibco, Uxbridge, UK). The stock solution was further diluted with sterile PBS and 10% FCS, and the following working dilutions prepared: 55%, 40%, 30%, and 20%. A discontinuous Percoll gradient was prepared by placing 5 ml of the 55% solution at the bottom of sterile centrifugation tubes, followed by careful layering of 5 ml of the 40% and then the 30% dilutions in the tube. Finally, the solution containing the cells, prepared by diluting 1 ml of the digest in 4 ml of the 20% Percoll solution was layered on top. A maximum of 1 x 10^{7} cells was placed in each gradient, which was then centrifuged at room temperature at 800 g for 30 min.

The top of the solution, containing mainly cellular debris and clumps, was aspirated and discarded. The interface cells were then carefully harvested, pooled (at this stage of the research), and RPMI supplemented with FCS added. This suspension was centrifuged and the cell pellet resuspended twice. Finally, the cell pellets were resuspended in 3 ml of complete medium (RPMI, FCS, glutamine, penicillin, streptomycin), counted in a Neubauer chamber, Trypan Blue dye-exclusion test performed, and the final solution adjusted to contain 2×10^6 viable cells/ml. This was kept at 4^0 C until use.

Aliquots from the cell suspension were obtained and cytospin preparations performed on glass slides using a Cytocentrifuge (Shandon, UK). The slides were allowed to dry under a fan and then stained with Pro-Diff (Braidwood Laboratories, Beckenham, UK), and differential counts performed with a light microscope.

6.2.5 - EFFECTOR CELLS

Crude suspensions of mononuclear and other inflammatory cells were used in all experiments described here. No attempt was made at this stage to separate the cell suspension into purified cell subsets, nor to induce cellular activation by engaging the T cell receptor with monoclonal antibodies or by the use of mitogens. The cell suspensions were adjusted to contain 2×10^6 per ml of complete medium (RPMI, 10% FCS, glutamine, penicilin, streptomycin) and kept on ice until use.

6.2.6 - TARGET CELLS

The murine mastocytoma cell line P815, known to be sensitive to T-cell mediated cytotoxicity, was cultured and when in log phase was

radiolabelled by incubation for 1 hour with 4.0 MBq Sodium Chromate (Cr-51; Amersham Int., Amersham, UK) at 37^{0} C, then washed twice and resuspended in complete medium to contain 5 X 10^{4} viable cells/ml.

6.2.7 - CYTOTOXIC ASSAYS

A lectin dependent cytotoxic assay was used to reveal total cytotoxic capacity of the effector cell suspension. The lectin phytohaemagglutinin (PHA; Gibco, Uxbridge, UK) in a final concentration of 1 ug/ml was used (this concentration was found capable of inducing ideal responses in proliferative assays). The presence of the lectin in the system provides a cross-linkage of glycoproteins on the surface of effector and target cells in such a way that the P815-PHA assay system can measure cytotoxicity of the effector cells regardless of specificity or MHC restriction.

The cytotoxic assays were performed in round-bottomed microtitre plates (Nunc; Gibco): 10 ul of the dilution of PHA was dispensed into the wells, followed by 100 ul of the concentration of target cells (5000 cells). Spontaneous chromium release was assessed by adding 100 ul of complete medium to the target cells, and maximum release obtained by adding 100 ul of the detergent Triton-X 100 (Sigma) at a concentration of 1% in complete medium. Any experiment with greater spontaneous release than 30% of the maximum release was discarded.

Three different concentrations of putative cytotoxic cells were added, in a volume of 100 ul in complete medium: 2 x 10^{6} (giving a 40:1 effector:target ratio); 1 x 10^{6} (20:1); 0.5 x 10^{6} (10:1). All experiments were performed in triplicate.

Once set up, the plates were covered and incubated for 4 hours at 37^{0} C in a humidified atmosphere of 5% CO2 in air. After this time 100

ul aliquots of the supernatant fluids were carefully harvested from each well, placed in plastic tubes, sealed and counted in a gamma counter (LKB Instruments Ltd., Croydon, UK). Before the supernatant fluids were harvested, the plates were carefully screened for signs of gross contamination, as determined by change of colour of the medium from pale pink to yellow. In this rare event, the plate was discarded.

The mean of three readings for each effector:target ratio was determined and the percent of specific cytotoxicity determined by the formula:

$$\% \text{ specific cytotoxicity} = \frac{T - SP}{Mx - SP} \times 100,$$

where T = test counts per minute, SP = spontaneous release, Mx = maximum release.

6.3 - RESULTS

A total of 26 rats prepared with the experimental procedures to induce bronchiectasis (Pa + LIG) and 15 age-matched normal littermates (N) were included in this study. They were killed at the following intervals after the surgery: 3 weeks (2/26); 12 weeks (14/26); 16 weeks (5/26); 20 weeks (5/26). No differences in body weight were seen: Pa + LIG = 486.5±82.4; N = 505.1±73.4. Macroscopic evidence of bronchial dilatation and inflammation involving the apical lobes were noticed during the slicing process in 23/26 animals from the Pa + LIG group. No evidence was found in left lobes of animals from this group, or in apical and left lobes of animals from the N group.

Bacteriological studies were performed in 24 tracheal and pulmonary fragments of animals from the Pa + LIG group: Pseudomonas aeruginosa was cultured from exactly half of the samples. Of these, the bacterium was isolated from five samples from animals operated 16 or more weeks before the sacrifice, once again showing the reliability of the model as far as chronic lung colonization was concerned. The microorganism was never isolated from lung fragments of animals from the N group, neither was it from any liver biopsies obtained from animals in both groups, suggesting that the observed chronic colonisation was largely confined to the lungs.

The cell yields were calculated after Percoll fractionation and presented as number of viable cells per gram of digested lobe. A large variation in cell yields was noticed, which could be partly attributed to the fact that in some samples large clumps of cells formed during the Percoll fractionation, despite the use of larger concentrations of DNAase than usually recommended, leading to variable amounts of cell loss. The range of cell yields per gram of tissue varied from 2.14×10^6

to 6.56 x 10^7 viable cells in lobes from normal animals, and from 1.78 x 10^6 to 1.08 x 10^8 in tissues from Pa + LIG group. The mean plus or minus standard deviation for the apical lobe of the test group was 1.69±0.98 x 10^8 cells/g, and of the N group 2.04±2.31 x 10^7. The results for the left lobe were: Pa + LIG = 1.45±2.58 x 10^7; N = 1.05±0.76 x 10^7. No statistically significant differences were seen.

Differential counts were performed after Pro-Diff staining of cytospins prepared from the cell suspensions. The numbers of lymphocytes, macrophages and other cell types were expressed as a percent of the total number of cells present in the preparation. Table 6.1 presents the results for the differential counts. In the apical lobe of the test group, a statistically significant increment in the relative proportion of lymphocytes was seen, in detriment to the number of macrophages, when compared with apical lobe cytospin preparations from the N group. Differences were also present when comparing the left lobe of the test ($p = < 0.0001$) and control ($p = 0.003$) groups. The proportions of the cell types were remarkably in the non-affected lobes (Plate 6.1).

A total of 12 separate cytotoxic assays were performed. In almost half of them the experiments were discarded due to inadequate target cell labelling or gross contamination. The results presented here are from seven successful experiments, involving 10 rat apical lobes in which bronchiectasis was induced and seven apical lobes obtained from age-matched normal littermates.

Table 6.2 displays the percentage of specific cytotoxicity obtained at different effector:target ratios for test and controls.

TABLE 6.1

DIFFERENTIAL COUNTS OF CYTOSPIN PREPARATIONS FROM CELL SUSPENSIONS OBTAINED BY COLLAGENASE DIGESTION OF RAT LUNGS

	Lymphocytes	Macrophages	Other cells
a) Apical lobe			
Pa+LIG	37.1±5.5(18)	56.5±4.8(18)	6.4±1.9(18)
N	25.9±5.4(8)	77.6±8.3(8)	6.5±3.6(8)
p	0.0003	<0.01	NS
b) Left lobe			
Pa+LIG	25.4±3.3(8)	66.8±8.3(8)	8.7±6.4(8)
N	28.4±4.4(6)	63.8±5.2(6)	7.8±3.5(6)
p	NS	NS	NS

Results presented as means ± SD of percents of each cell types (number of samples used in the calculation) Pa + LIG = partial bronchial ligation and intrabronchial injection of Pseudomonas aeruginosa; N = normal controls; p values compare means between test and control groups by Student's t-test; NS = statistically not significant.

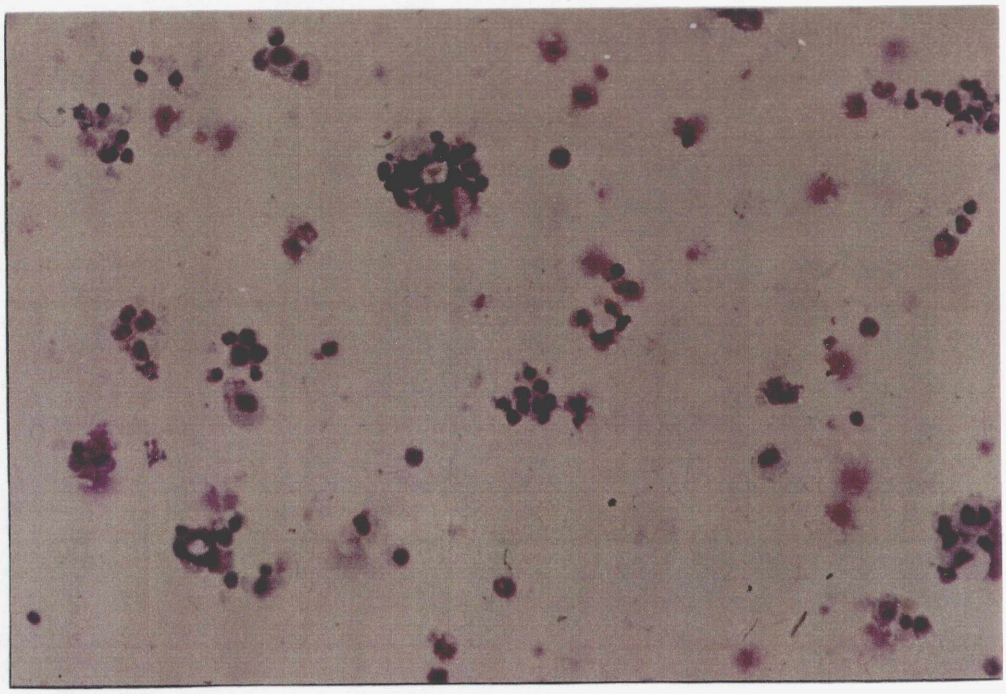
TABLE 6.2

SPECIFIC CYTOTOXIC ACTIVITY OF SINGLE CELL SUSPENSIONS OBTAINED BY COLLAGENASE DIGESTION OF RAT APICAL LOBES

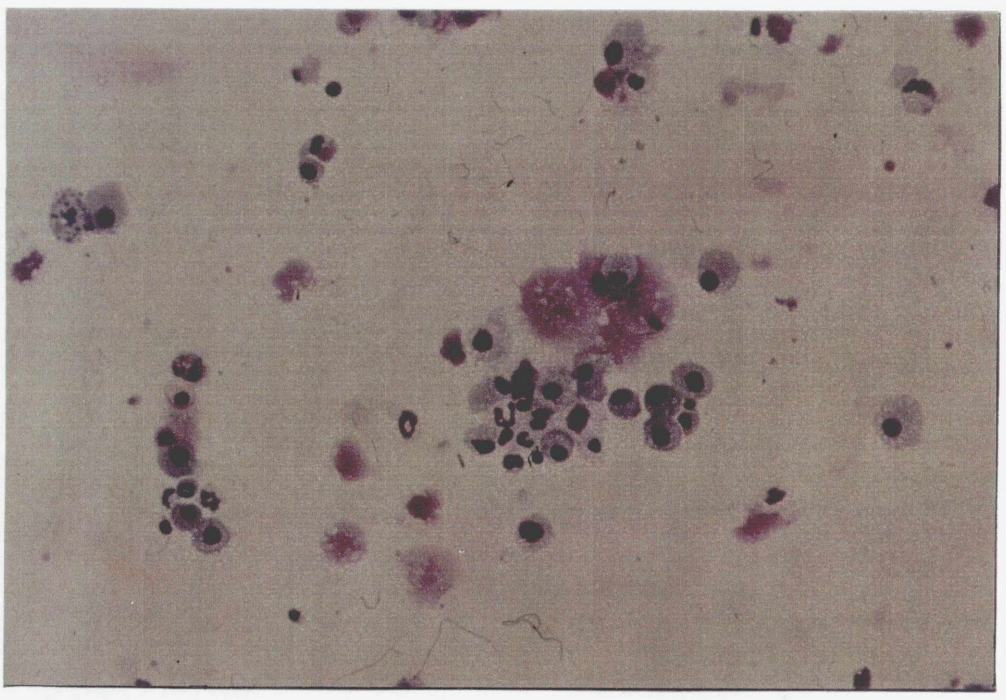
		Effector:target ratio		
		40:1 %	20:1 %	10:1 %
Experiment 1:	T1	15.2	8.1	8.75
	C1	2.6	-1.5	-0.4
Experiment 2:	T2	8.1	2.5	1.4
	C2	0	-0.2	-0.2
Experiment 3:	T3	2.5	-0.5	0.02
	C3	-0.2	-0.1	-0.2
Experiment 4:	T4	20.7	5.7	5.8
	C4	0.5	-2.2	-4.3
Experiment 5:	T5a	2.4	0.8	1.3
	T5b	3.3	2.7	2.0
	C5	1.3	1.6	-1.3
Experiment 6:	T6a	3.8	0.8	0.9
	T6b	1.9	0.8	0.6
	C6	1	0	-0.4
Experiment 7:	T7a	3.1	2.5	1.7
	T7b	3.9	-0.8	-0.5
	C7	1.1	0.7	0.6

Results presented as percent specific cytotoxicity of single cell suspensions obtained from apical lobes of test (T) and control (C) animals at different effector:target ratios.

PLATE 6.1



a: Cytospin preparation from a single cell suspension obtained by collagenase digestion of rat lung from the Pa+ LIG group, showing predominance of lymphocytes (Pro-Diff, original magnification x 250)



b: Cytospin preparation from a single cell suspension obtained by collagenase digestion of rat lung from the N group, showing predominance of macrophages (Pro-Diff, original magnification x 250)

6.4 - DISCUSSION

The Holt method of collagenase digestion of organ slices proved a reliable way of obtaining immunocompetent cells from the lung interstitium. Single cell suspensions derived from the digestion could be used for many investigational purposes, in the same way as lung cells sampled from the airways and airspaces by bronchoalveolar lavage. The method is ideal for the purposes of this research because it samples lung compartments shown in immunopathological studies to be infiltrated by immunocompetent cells that might be involved in the pathological process.

The results presented here were different from the original publications of the Holt method in two aspects: firstly, the cell yields obtained per gram of digested cells were lower. A possible explanation for this fact could be the relative inexperience in carrying out this laborious method. Secondly, the relative proportions of lymphocytes and macrophages were different. In Holt's original report (1985) there were, on average, 32% macrophages and 44% lymphocytes. In our results for normal tissues, 25% were lymphocytes and 65% macrophages. A possible explanation for these differences could be the fact that no attempt was made to remove the large numbers of alveolar macrophages by thorough lung lavage preceeding the digestion, as performed in Holt's protocol.

The results presented here suggested a trend towards the presence of enhanced cytotoxic activity in single cell suspensions obtained by collagenase digestion of rat apical lobes displaying experimentally induced bronchiectasis. Careful interpretation of these results is required. The fact that crude cell suspensions were employed makes it difficult to reach any conclusion as to whether a certain cell type,

particularly T-lymphocytes, displayed abnormal cytotoxic activity in the animal model of bronchiectasis. On the other hand, the putative cytotoxic cell in the crude suspension would be diluted in such a way that these results could underrepresent cytotoxicity of any single cell population.

The fact that no cytotoxic activity was seen in lung cells extracted from normal apical lobes is in accordance with observations made by others (Viney, Kinshaw & MacDonald, 1990) showing that non-stimulated lymphocytes extracted from normal mouse and rat intestine did not exhibit cytotoxicity. The presence of a degree of cytotoxicity, however small, in non-stimulated mononuclear cells obtained from lungs affected by severe inflammatory process could be interpreted as preliminary evidence for the presence of potentially damaging immune mechanisms.

Additional studies are necessary to draw definitive conclusions. The use of purified immunocompetent cell populations, as well as different target cells could shed light on the real dimension of this phenomenon.

C H A P T E R 7

GENERAL DISCUSSION AND PROSPECTS FOR FUTURE WORK

Bronchiectasis has been regarded for years as an infection, where adequate hygienic measures, vaccination and antibiotic therapy were sufficent to prevent or control its outcome. Although this may be true for a number of bronchiectasis patients, it is an oversimplistic view of the condition. The main postulate of this thesis is that an acquired immune response is one of the main contributors to the inflammatory process present in the affected areas of the lungs of patients suffering from bronchiectasis. All the main components of the immune response are represented in the inflamed sites, particularly the T-lymphocytes and macrophages. It has been postulated (Cole, 1984) that the development and progression of bronchiectasis depend on three principal factors: a defective bronchial mucociliary clearance mechanism, chronic bacterial colonisation of the airways, and the host's chronic inflammatory response. The studies presented here have characterised the inflammatory process as largely composed of activated and immunologically committed immunocompetent cells, which supported the idea that one of the driving forces leading to the development and progression of bronchiectasis is the host-mediated inflammatory process.

Up to now, most studies of the host's inflammatory component to bronchiectasis have concentrated on the role of nonspecific phagocytes, such as neutrophils and their products, in generating tissue damage. For example, investigation of the development of cystic fibrosis has concentrated almost exclusively on the role of neutrophils and their products, mainly neutrophil elastase, on the genesis and progression of the disease.

The idea that the immune system may be involved in the pathogenesis of bronchiectasis is not new. Hilton and Doyle (1978)

showed that the majority of patients presented with raised levels of serum immunoglobulins. Their finding was susequently confirmed by many authors, as reviewed in Section 1.6. They also pointed out the relationship between bronchiectasis and immune complexes, suggesting that local deposition of immune complexes in pulmonary tissues could perpetuate and increase tissue damage in the lungs (Hilton, Moore, Howat et al, 1979; Hilton, Hasleton, Bradlow et al, 1984). However, this idea was never fully proved, in fact only a handful of patients develop the stigmata of disease mediated by immune complex, such as vasculitis.

The involvement of other mechanisms of the immune system and study of the local milieu of bronchiectatic lesions has been neglected until recently. The present study reveals that T-lymphocytes rather than antibodies or other components of the immune response are the most relevant factors involved in the inflammatory reaction seen in affected areas. These findings mirror observations derived from work in chronically inflamed gut (MacDonald, 1990) and other bronchial diseases such as chronic bronchitis (Fournier, Lebargie, Ladurie et al, 1989) and asthma (Poulter, Powell & Burke, 1990).

Similar findings were also seen in the controlled circumstances of experimentally induced bronchiectasis. Guerreiro's model of experimental bronchiectasis, achieved by partially ligating the bronchus and introducing a bacterial inoculum into the ligated bronchus, also displays the elements of a cell-mediated immune response. The ligation probably operates as an adjuvant, "depoting" the bacterial antigen which then induces the severe host inflammatory response seen that is probably responsible for the development of

lesions in this model.

The presence of delayed-type hypersensitivity against Pseudomonas aeruginosa antigens in footpads of rats exhibiting histological bronchiectasis and chronic colonisation of the lung by the bacterium, indirectly suggested that the intense cellular infiltration seen in the affected lobe could be specifically directed against Pseudomonas aeruginosa. Preliminary results showing that enhanced in vitro cytotoxicity was also present in single cell suspensions obtained from affected lungs when compared with cells from normal lungs suggested a possible mechanism for the development of the lesions. By manipulating the conditions of the experimental model it was clear that bronchiectasis only developed when live bacteria were present in a bronchus whose clearance mechanisms was impaired. The immunocompetent cell infiltration that accompanyed the development of the experimental disease was also only present when these conditions were met.

This research did not set out to determine whether the immune response identified in the sites of bronchiectasis plays a primary or secondary role in the pathogenesis of the condition, and the methodology employed here was not appropriate to answer that question. Additional experiments are now required to deal with this and other relevant questions. The ideas proposed in this thesis about the role of acquired immune mechanisms could be tested in controlled and safe therapeutic trials first in the experimental model and then in man. Recent availability of drugs thought to have specificity for one of the main constituents of the immune response, the T-lymphocyte (particularly the activated T-lymphocyte), such as cyclosporin-A and its new generation of derivatives, could allow clarification of the relative contribution of immune mechanisms to the pathogenesis of the

condition. The recent availability of recombinant alpha-1-antitrypsin for use in replacement therapy by the aerosol route will allow the opportunity to ascertain the relative contribution of nonspecific mechanisms to the pathogenesis of the condition. However, it is hard to believe that one factor alone will be decisive in such a complex scenario. It is probable that several elements will contribute to the damage.

Guerreiro's model of experimental bronchiectasis has opened several possibilities of exploring the nature of the immune processes involved in the generation of the experimental condition. The technique of extraction of viable immunocompetent cells from the lung matrix by enzymatic digestion (discussed in chapter 6) provides a way of probing function of the local immunocompetent cell population as has been piloted in preliminary results of studies on cytotoxic activity of crude single-cell suspensions obtained from affected and non-affected lungs, described in chapter 6. A necessary follow-up study would sort the different cell types and test the purified cell populations against labelled target cells in cytotoxic studies. Initially, T-lymphocytes could be separated from the macrophage population by the technique of plastic adherence. T-lymphocyte subsets could also be separated by cell panning or by adherence to monoclonal antibody-coated magnetic beads. Possible problems with these techniques include the small numbers of each subset recovered by the sorting process. Possible ways of overcoming this problem would be the use of inbred animals, pooling the cells from different animals in order to achieve the necessary number of cells for the different experiments. Cytotoxicity could also be tested against different target cells.

A recently described *in vitro* model of infection in organ culture of respiratory epithelial explants fragments (Read, Wilson, Rutman et al, 1991) would allow the putative cytotoxic cell subset to be tested in the organ culture in order to determine whether ultrastructural changes develop in the organ explant. Cytotoxicity could also be tested in bronchial epithelium cell culture (B. B. Vargaftig, personal communication) in standard cytotoxic assays. These experiments might reflect the events taking place in the rat lungs during the development of experimental disease.

The extraction of viable cells from the lungs and their separation into different cell subsets also raises other possibilities. These subsets could be cultured and the cytokines in the culture supernatants could be assayed to determine the functional status of the cells. In order to determine the specificity of the putative memory cell population of T cells for *Pseudomonas aeruginosa* antigens, T cells could be co-cultured with relevant and irrelevant bacterial antigens and pulsed after four days with tritiated thymidine. The amount of thymidine incorporated by the cells would represent an index of the proliferation of the T cells in response to the antigens. To allow repeated experiments, T cells could be cloned and tested against many bacterial antigens. Once successful, the clones could also be used in adoptive transfer experiments, especially in partial reconstitution.

A crucial experiment in this research would be adoptive transfer. This possibility was opened by preliminary findings of the presence of delayed-type hypersensitivity against *Pseudomonas antigens* in footpads of rats exhibiting experimental bronchiectasis and chronic lung colonisation by the bacterium. T-lymphocytes could be sorted from the blood of bronchiectatic animals and transferred to previously

irradiated syngeneic animals. These rats would be examined for evidence of any inflammatory changes. The animals could then be tested against the same strain of bacterium previously used for induction of bronchiectasis in different ways: introduction of live bacteria into the bronchial tree without partial bronchial ligation; partial ligation of the bronchus followed by smaller inocula of the bacterium than those used for the induction of experimental bronchiectasis in the donor animals; introduction of heat-killed bacteria with and without bronchial ligation; introduction of different components and exoproducts of Pseudomonas aeruginosa into normal or partially ligated bronchi of reconstituted animals.

Finally, a relatively simple but crucial experiment would be to attempt to modulate the immune response in rats developing experimental bronchiectasis. The use of cyclosporin-A to modulate the activation of T-lymphocytes, particularly the CD4 positive T cell, may suggest whether the abnormal immune response identified in the bronchiectatic rat lungs is primary to the process or a secondary epiphenomenon. A more elegant way of testing the same question would be the in vivo use of monoclonal antibodies directed against those antigenic determinants shown in this research to be upregulated in the experimental process, in order to ablate components of the immune response. Particularly attractive would be the use of MRC OX-35, a CD4 marker equivalent to W3/25, or MRC OX-19, an anti pan-T cell antibody. Other possibilities would be the use of MRC OX-8 (suppressor/cytotoxic T-lymphocytes), MRC OX-6 (anti-Class II MHC molecules), and MRC-OX 39 (anti-interleukin-2 receptor).

REFERENCES

Abo T, Balch CM.
A differentiation antigen of human NK and K cells identified by a monoclonal antibody (HNK-1).
J. Immunol. 1981; 127:1024-1029.

Akbar AN, Amlot PL, Timms A, Lombardi G, Lechler R, Janossy G.
The development of primed/memory CD8+ lymphocytes in vitro and in rejecting kidneys after transplantation.
Clin. exp. Immunol. 1990; 81:225-231.

Akbar AN, Terry L, Timms A, Beverley PCL, Janossy G.
Loss of the CD45R and gain of UCHL1 reactivity is a feature of primed T cells.
J. Immunol. 1988; 140:2171-2178.

Akbar AN, Timms A, Janossy G.
Cellular events during memory T-cell activation in vitro: the UCHL1 (180000 MW) determinant is newly synthesized after mitosis.
Immunology 1989; 66:213-218.

Allison MC, Poulter LW, Dhillon AP, Pounder RE.
Immunohistological studies of surface antigen on colonic lymphoid cells in normal and inflamed mucosa.
Gastroenterology 1990; 99:421-430.

Amitani R, Wilson R, Rutman A, Read R, Ward C, Burnett D, Stockley RA, Cole PJ.
Effects of human neutrophil elastase and Pseudomonas aeruginosa proteinases on human respiratory epithelium.
Am. J. Respir. Cell Mol. Biol. 1991; 4:26-32.

Barclay AN.
The localization of populations of lymphocytes defined by monoclonal antibodies in rat lymphoid tissues.
Immunology 1981; 42:593-600.

Barclay AN, Mayhofer G.
Bone marrow origin of Ia-positive cells in the medulla of rat thymus.
J. Exp. Med. 1981; 153:1666-1671.

Barker AF, Bardana Jr. EJ.
Bronchiectasis: update of an orphan disease.
Am. Rev. Respir. Dis. 1988; 137:969-978.

Barrios R, Selman M, Franco R, Chapela R, Sullivan-Lopez J, Fortoul TI.
Subpopulations of T cells in lung biopsies from patients with pigeon breeder's disease.
Lung 1987; 165:181-187.

Bateson EM, Hayes JA, Woo-Ming M.
Endobronchial teratoma associated with bronchiectasis and bronchiolectasis.

Thorax 1968; 23:69-76.

Batori G, Bofill M, Petrayi G, Bollan SR, Janossy G.
Comparative analysis of monoclonal antibodies of workshop T series on suspensions and tissue sections.
In: Bernard A, ed. Leucocyte Typing. Paris, Springer Verlag, 1984, pp 469-475.

Becroft DMO.
Bronchiolitis obliterans, bronchiectasis, and other sequelae of adenovirus type 21 in young children.
J. Clin. Pathol. 1971; 24:72-82.

Berman JS.
Lymphocytes in the lung: Should we continue to exalt only BALT?
Am. J. Respir. Cell Mol. Biol. 1990; 3:101-102.

Beverley PCL.
Clinical and biological studies with monoclonal antibodies.
In: Goding JW, ed. Monoclonal antibodies: Principles and Practice-Production and Application of Monoclonal Antibodies in Cell Biology, Biochemistry and Immunology. London, Academic Press, 1983, pp 247-267.

Beverley PCL, Callard RE.
Distinctive functional characteristics of human T lymphocytes defined by E rosetting or a monoclonal anti-T-cell antibody.
Eur. J. Immunol. 1981; 11:329-334.

Bienenstock J.
Bronchus-associated lymphoid tissue.
In: Bienenstock J, ed. Immunology of the Lung and Upper Respiratory Tract. New York, McGraw Hill, 1984, pp 96-117.

Bienenstock J.
Mucosal immunological protection mechanisms in the airways.
Eur. J. Respir. Dis. 1986; 69 (suppl. 147):62-71.

Bienenstock J, Befus AD.
Mucosal immunology.
Immunology 1980; 41:249-270.

Bienenstock J, Befus D.
Gut- and bronchus-associated lymphoid tissue.
Am. J. Anat. 1984; 170:437-445.

Bienenstock J, Johnson N.
A morphologic study of rabbit bronchial lymphoid aggregates and lymphoepithelium.
Lab. Invest. 1976; 35:343-348.

Bienestock J, Johnson N, Perey DYE.
Bronchial lymphoid tissue. I. Morphologic characteristics.
Lab. Invest. 1973a; 28:686-692.

Bienenstock J, Johnson M, Perey DYE.

Bronchial lymphoid tissue. II. Functional characteristics.
Lab. Invest. 1973b; 28:693-698.

Bienenstock J, McDermott M, Befus AD.
A common mucosal immune system.
In: Ogra PL, ed., Immunology of Breast Milk. New York, Raven Press, 1979, pp 91-104.

Bienenstock J, McDermott, Befus AD.
The significance of bronchus-associated lymphoid tissue.
Bull. europ. Physiopath. resp. 1982; 18:153-177.

Blackwood LL, Lin T, Rowe JI.
Suppression of the delayed-type hypersensitivity and cell-mediated immune responses to Listeria monocytogenes induced by Pseudomonas aeruginosa.
Infect. Immun. 1987; 55:639-644.

Brenner MB, Mclean J, Dialynas DP, Strominger JL, Smith JA, Owen FL, Seidman JG, Ip S, Rosen F, Krangel MS.
Identification of a putative T-cell receptor.
Nature 1986; 322:145-149.

Brenner MB, Mclean J, Scheft H, Warnke RA, Jones N, Strominger JL.
Characterization and expression of the human alpha/beta T cell receptor by using a framework monoclonal antibody.
J. Immunol. 1987; 138:1502-1509.

Brogan TD, Davies BH, Ryley HC, Ross PJ, Neale L.
Composition of bronchopulmonary secretions from patients with bronchiectasis.
Thorax 1980; 35:624-627.

Bruce MC, Poncz L, Klinger KD,
Biochemical and pathological evidence for proteolytic destruction of lung connective tissue in cystic fibrosis.
Am. Rev. Respir. Dis. 1985; 132:529-535.

Buckley III CE.
Delayed hypersensitivity skin testing.
In: Rose NR, Friedman H, Fahey JL, eds. Manual of Clinical Laboratory Immunology, 3rd edition. Washington, American Society for Microbiology, 1986, pp 260-273.

Burke CM, Theodore J, Dawkins KD, Yousem SA, Blank N, Billingham ME, Van Kessel A, Jamieson SW, Oyer PE, Baldwin JC, Stinson EB, Shumway NE, Robin ED.
Post-transplant obliterative bronchiolitis and other late lung sequelae in human heart-lung transplantation.
Chest 1984; 86:824-829.

Burnett D, Crocker J, Stockley RA.
Cells containing IgA subclasses in bronchi of subjects with or without chronic obstructive lung disease.
J. Clin. Pathol. 1987; 40:1217-1220.

Butland RJA, Cole P, Citron KM, Turner-Warwick M.
Chronic bronchial suppuration and inflammatory bowel disease.
Quart. J. Med. 1981; 50:63-75.

Campana D.
The developmental stages of the human T cell receptors: a review.
Thymus 1989; 13:3-18.

Campana D, Coustan-Smith E, Janossy G.
Double and triple staining methods for studying the proliferative activity of human B and T lymphoid cells.
J. Immunol. Meth. 1988; 107:79-88.

Campana D, Janossy G, Coustan-Smith E, Amlot PL, Tian WT, Ip S, Wong L.
The expression of T cell receptor-associated proteins during T cell ontogeny in man.
J. Immunol. 1989; 142:57-66.

Campbell DA, Du Bois RM, Butcher RG, Poulter LW.
The density of HLA-DR antigen expression on alveolar macrophages is increased in pulmonary sarcoidosis.
Clin. exp. Immunol. 1986; 65:165-171.

Campbell DA, Poulter LW, Du Bois RM.
Immunocompetent cells in bronchoalveolar lavage reflect the cell populations in transbronchial biopsies in pulmonary sarcoidosis.
Am. Rev. Respir. Dis. 1985; 132:1300-1306.

Cannon MJ, Openshaw PJM, Askonas BA.
Cytotoxic T cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus.
J. Exp. Med. 1988; 168:1163-1168.

Cash HA, Straus DC, Bass JA.
Pseudomonas aeruginosa exoproducts as pulmonary virulence factors.
Can. J. Microbiol. 1983; 29:448-456.

Cash HA, Woods DE, McCollough B, Johanson WG Jr., Bass JA.
A rat model of chronic respiratory infection with Pseudomonas aeruginosa.
Am. Rev. Respir. Dis. 1979; 119:453-459.

Cheng KK.
The experimental production of bronchiectasis in rats.
J. Path. Bact. 1954; 67:89-98.

Chiu FT, Campbell AH.
Bronchogenic carcinoma causing non-terminal saccular bronchiectasis.
Aust. NZ J. Med. 1973; 3:200-203.

Clark M, Waldmann H.
Production of murine monoclonal antibodies.
In: Goding JW, ed. Monoclonal Antibodies: Principles and Practice-Production and Application of Monoclonal Antibodies in Cell Biology,

Biochemistry and Immunology. London, Academic Press, 1983, pp 1-25.

Clelland CA, Higenbottam TW, Stewart S, Scott JP, Wallwork J.
The histological changes in transbronchial biopsy after treatment of acute lung rejection in heart-lung transplantation.
J. Pathol. 1990; 161: 105-112.

Cole PJ.
Reccurrent bronchial infections and bronchiectasis.
In: Bellingham AJ. Advanced Medicine 16. Royal College of Physicians of London. London, Pitman Medical, 1980, pp 289-296.

Cole PJ.
A new look at the pathogenesis and management of persistent bronchial sepsis: a "vicious circle" hypothesis and its logical therapeutic connotations.
In: Davies RJ, ed. Strategies for the Management of Chronic Bronchial Sepsis. Oxford, The Medicine Publishing Foundation, 1984, pp 1-20.

Cole PJ.
Inflammation: a two-edged sword - the model of bronchiectasis.
Eur. J. Respir. Dis. 1986: 69 (suppl. 147):6-15.

Cole PJ.
Host - microbial interactions in chronic respiratory disease.
In: Reeves D, Geddes A, eds. Recent Advances in Infection 3. Edinburgh, Churchill Livingstone, 1989, pp 141-151.

Cole PJ.
Bronchiectasis.
In: Brewis RAL, Gibson GJ, Geddes DM, eds. Respiratory Medicine. London, Bailliere Tyndall, 1990, pp 726-759.

Cole P, Wilson R.
Host-microbial interrelationships in respiratory infection.
Chest 1989; 95 (suppl.):217S-221S.

Collings LA, Poulter LW, Janossy G.
The demonstration of cell surface antigens on T cells, B cells and accessory cells in paraffin-embedded human tissues.
J. Immunol. Meth. 1984; 75:227-239.

Cooke JC, Currie DC, Morgan AD, Kerr IH, Delany D, Strickland B, Cole PJ.
Role of computed tomography in diagnosis of bronchiectasis.
Thorax 1987; 42:272-277.

Crawford JM, Miller DA.
Lymphocyte subpopulations in rat lungs and Peyer's patches.
Am. Rev. Respir. Dis. 1984; 129:827-832.

Currie DC, Peters AM, Garbett ND, George P, Strickland B, Lavender JP, Cole PJ.
Indium-111 labelled granulocyte scanning to detect inflammation in the lungs of patients with chronic sputum expectoration.

Thorax 1990; 45:541-544.

Currie DC, Peters AM, George P, Lavender JP, Saverymuttu SH, Needham SG, Dhillon DP, Cole PJ.
Indium-111-labelled granulocyte accumulation in respiratory tract of patients with bronchiectasis.
Lancet 1987; i:1335-1339.

Czuprynski CJ, Brown JF.
Effects of purified anti-Lyt-2 mAb treatment on murine listeriosis: comparative roles of Lyt-2+ and L3T4+ cells in resistance to primary and secondary infection, delayed-type hypersensitivity and adoptive transfer of resistance.
Immunology 1990; 71:107-112.

Daley L, Pier GB, Liporace JD, Eardley DD.
Polyclonal B cell stimulation and interleukin 1 induction by the mucoid exopolysaccharide of Pseudomonas aeruginosa associated with cystic fibrosis.
J. Immunol. 1985; 134:3089-3093.

Dallman MJ, Mason DW, Webb M.
The roles of host and donor cells in the rejection of skin allografts by T cell-deprived rats injected with syngeneic T cells.
Eur. J. Immunol. 1982; 12:511-518.

Dallman MJ, Thomas ML, Green JR.
MRC OX-19: a monoclonal antibody that labels rat T lymphocytes and augments in vitro proliferative responses.
Eur. J. Immunol. 1984; 14:260-267.

Denney MK, Berkas EM, Snider TH, Nedwicki EG.
Foreign body bronchiectasis.
Dis. Chest 1968; 53:613-616.

Denny FW, Taylor-Robinson D, Allison AC.
The role of thymus-dependent immunity in Mycoplasma pulmonis infection in mice.
J. Med. Microbiol. 1972; 5:327-337.

Dhaliwal JS, Liew FY.
Induction of delayed-type hypersensitivity to Leishmania and the concomitant acceleration of disease development of progressive murine cutaneous leishmaniosis.
Infect. Immun. 1987; 55:645-651.

Dijkstra CD, Dopp EA, Joling P, Kraal G.
The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2, and ED3.
Immunology 1985; 54:589-599.

Dijkstra CD, Dopp EA, Vogels IMC, Van Noorden CJF.
Macrophages and dendritic cells in antigen-induced arthritis. An immunohistochemical study using cryostat sections of the whole knee

joint of rat.
Scand. J. Immunol. 1987; 26:513-523.

Dominique S, Bouchonnet F, Smiejan JM, Hance AJ.
Expression of surface antigens distinguishing "naive" and previously activated lymphocytes in bronchoalveolar lavage liquid.
Thorax 1990; 45:391-396.

Dunnill MS.
Bronchiectasis.
In: Dunnill MS, ed. Pulmonary Pathology, 2nd edition. Edinburgh, Churchill Livingstone, 1987, pp 81-95.

Ellis DA, Thornley PE, Wightman, AJ, Walker M, Chalmers J.
Present outlook in bronchiectasis: clinical and social history and review of factors influencing prognosis.
Thorax 1981; 36:659-664.

Enander I, Ahlstedt S, Nygren H.
Mononuclear cells, mast cells and mucous cells as part of the delayed hypersensitivity response to aerosolized antigen in mice.
Immunology 1984; 51:661-668.

Ernst PB, Befus AD, Bienenstock J.
Leukocytes in the intestinal epithelium: an unusual immunological compartment.
Immunol. Today 1985; 6:50-55.

Falini B, Flenghi L, Pileri S, Pelicci P, Fafioli M, Martelli MF, Moretta L, Ciccone E.
Distribution of T cells bearing different forms of the T cell receptor gamma/delta in normal and pathological human tissues.
J. Immunol. 1989; 143:2480-2488.

Field CE.
Bronchiectasis: third report on a follow-up study of medical and surgical cases from childhood.
Arch. Dis. Child. 1969; 44:551-561.

Fitch F, Lancki D, Havran W.
Koch's postulates and the molecular biology of T-cell function.
Immunol. Today 1988; 9:41-43.

Fournier M, Lebargy F, Ladurie FLR, Lenormand E, Pariente R.
Intra-epithelial T-lymphocyte subsets in the airways of normal subjects and of patients with chronic bronchitis.
Am. Rev. Respir. Dis. 1989; 140:737-742.

Freihorst J, Merrick JM, Ogra PL.
Effect of oral immunization with Pseudomonas aeruginosa on the development of specific antibacterial immunity in the lungs.
Infect. Immun. 1989; 57:235-238.

Frew AJ, Moqbel R, Azzawi M, Hartnell A, Barkans J, Jeffery PK, Kay AB, Scheper RJ, Varley J, Church MK, Holgate ST.

T lymphocytes and eosinophils in allergen-induced late-phase asthmatic reactions in the guinea pig.
Am. Rev. Respir. Dis. 1990; 141:407-413.

Garssen J, Nijkamp FP, Wagenaar SS, Zwart A, Askenase PW, Van Loveren H.
Regulation of delayed-type hypersensitivity-like responses in the mouse lung, determined with histological procedures: serotonin, T-cell suppressor-inducer factor and high antigen dose tolerance regulate the magnitude of T-cell dependent inflammatory reactions.
Immunology 1989; 68:51-58.

Garssen J, Van Loveren H, Van Der Vliet, Nijkamp FP.
A role for T cells in the induction of airway hyperreactivity.
Am. Rev. Respir. Dis. 1990; 141 (part 2):A115.

Gaspari AA, Jenkins MK, Katz SI.
Class II MHC-bearing keratynocytes induce antigen-specific unresponsiveness in hapten-specific TH1 clones.
J. Immunol. 1988; 141:2216-2220.

Gaston JSH, Life PF, Bailey LC, Bacon PA.
In vitro responses to a 65-kilodalton mycobacterial protein by synovial T cells from inflammatory arthritis patients.
J. Immunol. 1989; 143:2495-2500.

Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H.
Cell cycle analysis of a cell-proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67.
J. Immunol. 1984; 133: 1710-1715.

Glanville AR, Baldwin JC, Burke CM, Theodore J, Robin ED.
Obliterative bronchiolitis after heart-lung transplantation: apparent arrest by augmented immunosuppression.
Ann. Int. Med. 1987; 107:300-304.

Glanville AR,Tazellar HD, Theodore J, Imoto E, Pouse RV, Baldwin JC, Robin EO.
The distribution of MHC Class I and II antigens on bronchial epithelium.
Am. Rev. Respir. Dis. 1989; 139:330-334.

Gregson RL, Davey JJ, Prentice DE.
Postnatal development of bronchus-associated lymphoid tissue (BALT) in the rat. Rattus norvegicus.
Lab. Anim. 1979a; 13:231-238.

Gregson RL, Davey JJ, Prentice DE.
Bronchus-associated lymphoid tissue (BALT) in the laboratory-bred and wild rat. Rattus norvegicus.
Lab. Anim. 1979b; 13:239-243.

Griffith BP, Paradis IL, Zeevi A, Rabinowich H, Yousem SA, Duquesnoy RJ, Dauber JH, Hardesty RL.
Immunologically mediated disease of the airways after pulmonary

transplantation.
Ann. Surg. 1988; 208:371-378.

Guerreiro D, Heard B, Rohde J, Todd H, Sheppard M, Cole PJ.
Quantitation of experimental bronchiectasis.
Eur. Respir. J. 1990; 3:296s.

Guerreiro D, Rohde J, Todd H, Lapa e Silva JR, Sheppard M, Cole PJ.
Proliferation of bronchial epithelium in experimental bronchiectasis.
Thorax 1990a; 45:785.

Guerreiro D, Rohde J, Todd H, Lapa e Silva J, Sheppard M, Cole PJ.
Reduction of bronchial wall elastin in experimental bronchiectasis.
Respir. Med. 1990b; 84:429.

Halstensen TS, Scott H, Brandtzaeg P.
Intraepithelial T cells of the TcR gamma/delta+CD8- and V delta 1/J gamma 1+ phenotypes are increased in coeliac disease.
Scand. J. Immunol. 1989; 30:665-672.

Hanson LA.
Comparative immunological studies of the immune globulins of human milk and of blood serum.
Int. Arch. Allergy 1961; 18:241-267.

Harbeck RJ, Launder T, Staszak C.
Mononuclear cell pulmonary vasculitis in NZB/W mice.
Am. J. Pathol. 1986; 123:204-211.

Harvey J, Jones DB, Wright DH.
Leucocyte common antigen expression on T cells in normal and inflamed human gut.
Immunology 1989; 68:13-17.

Hata JS, Fick RB.
Pseudomonas aeruginosa and the airways disease of cystic fibrosis.
Clin. Chest Med. 1988; 9:679-689.

Hayry P.
Mechanisms of rejection.
Cur. Opin.Immunol. 1989; 1:1230-1235.

Hedlund G, Segren S, Jansson B, Sjogren HO.
Evidences for the existance of W3/13+ monocytes in the rat.
Scand. J. Immunol. 1986; 24:387-392.

Hill SL, Mitchell JL, Burnett D, Stockley RA..
IgG subclasses in sputum from patients with chronic bronchial sepsis.
Thorax 1990; 45:780.

Hilton AM, Doyle L.
Immunological abnormalities in bronchiectasis with chronic bronchial suppuration.
Br. J. Dis. Chest 1978; 72:207-216.

Hilton AM, Hasleton PS, Bradlow A, Leahy BC, Cooper KM, Moore M.
Cutaneous vasculitis and immune complexes in severe bronchiectasis.
Thorax 1984; 39:185-191.

Hilton AM, Moore M, Howat JMT, Kimber I.
Characterization of circulating immune complexes in bronchiectasis by gel filtration.
Clin. Allergy 1979; 9:65-74.

Hogg JC, Irving WL, Porter H, Evans, Dunnill MS, Fleming K.
In situ hybridization studies of adenoviral infections of the lung and their relationship to follicular bronchiectasis.
Am. Rev. Respir. Dis. 1989; 139:1531-1535.

Hogg N, MacDonald S, Slusarenko M, Beverley PCL.
Monoclonal antibodies specific for human monocytes, granulocytes, and endothelium.
Immunology 1984; 53:753-767.

Hoiby N.
Pseudomonas aeruginosa infection in cystic fobrosis. Diagnostic and prognostic significance of Pseudomonas aeruginosa precipitins determined by means of crossed immunoelectrophoresis. A survey.
Acta Pathol. Microbiol. Scand. 1977; 262 (suppl.):1-9.

Holland HK, Wingard JR, Beschorner WE, Saral R, Satos GW.
Bronchiolitis obliterans in bone marrow transplantation and its relationship to chronic graft-v-host disease and low serum IgG.
Blood 1988; 73:621-627.

Holland VA, Cagle PT, Windsor NT, Noon GP, Greenberg SD, Lawrence EC.
Lymphocyte subset populations in bronchiolitis obliterans after heart-lung transplantation.
Transplantation 1990; 50:955-959.

Holt PG, Degebrodt A, O'Leary C, Krska K, Plozza T.
T cell activation by antigen-presenting cells from lung tissue digests: suppression by endogenous macrophages.
Clin. exp. Immunol. 1985; 62:586-593.

Holt PG, Degebrodt A, Venaille T, O'Leary C, Krska K, Flexman J, Farrell H, Shellam G, Young P, Penhale J, Robertson T, Papadimitriou JM.
Preparation of interstitial lung cells by enzymatic digestion of tissue slices: preliminary characterization by morphology and performance in functional assays.
Immunology 1985; 54:139-147.

Holt PG, Robinson BWS, Reid M, Kees UR, Warton A, Dawson VH, Rose A, Schon-Hegrad M, Papadimitriou JM.
Extraction of immune and inflammatory cells from human lung parenchyma: evaluation of an enzymatic digestion procedure.
Clin. exp. Immunol. 1986; 66:188-200.

Holt PG, Schon-Hegrad MA.

Localization of T cells, macrophages and dendritic cells in rat respiratory tract tissue: implications for immune function studies.
Immunology 1987; 62:349-356.

Holt PG, Schon-Hegrad MA, Phillips MJ, McMenamin PG.
Ia-positive dendritic cells form a tighty meshed network within the human airway epithelium.
Clin. Exp. Allergy 1989; 19:597-601.

Holt PS, Misfeldt ML.
Alteration of murine immune response by Pseudomonas aeruginosa exotoxin-A.
Infect. Immun. 1984; 45:227-233.

Horan MA, Leahy BC, Fox RA, Stretton TB, Haeny H.
Immunological abnormalities in patients with chronic bronchial suppuration: a possible relationship with endotoxaemia.
Br. J. Dis. Chest 1984; 78:66-74.

Horvat RT, Parmely MJ.
Pseudomonas aeruginosa alkaline protease degrades human gamma interferon and inhibits its bioactivity.
Infect. Immun. 1988; 56:2925-2932.

Howard JG, Liew FY.
Mechanisms of acquired immunity in leishmaniasis.
Phil. Trans. R. Soc. Lond. 1984; B307:87-98.

Hunt SV, Fowler MH.
A repopulation assay for B and T lymphocyte stem cells employing radiation chimaeras.
Cell Tiss. Kin. 1981; 14:445-464.

Inghirami G, Zhu BY, Chess L, Knowles DM.
Flow cytometric and immunohistochemical characterization of the gamma/delta T-lymphocyte population in normal human lymphoid tissue and peripheral blood.
Am. J. Pathol. 1990; 136:357-367.

Issekutz TB, Stoltz JM.
Suppression of lymphocyte proliferation by Pseudomonas aeruginosa: mediation by Pseudomonas-activated suppressor monocytes.
Infect. Immun. 1985; 48:832-838.

Iwata M, Sato A, Chida K, Yasuda K, Shichi I, Okano A, Suda I.
Morphological and immunohistochemical studies of bronchus-associated lymphoid tissue (BALT) in chronic pulmonary infection model with Pseudomonas aeruginosa.
Am. Rev. Respir. Dis. 1990; 141(part 2):A621.

Janin-Mercier A, Mignot P, Delage J, Molina C.
Apport des methodes d'imunopathologie tissulaire au diagnostic des maladies broncho-pulmonaires.
Rev. Pneumol. Clin. 1985; 41:238-244.

Janossy G, Amlot P.
Immunofluorescence and immunohistochemistry.
In: Klaus GGB, ed. Lymphocytes. A Practical Approach. Oxford, IRL Press, 1987, pp 67-108.

Janossy G, Bofill M, Poulter LW.
Two-colour immunofluorescence: analysis of the lymphoid system with monoclonal antibodies.
In: Polak JM, Van Noorden S, eds. Immunocytochemistry: Modern Methods and Applications. Bristol, Wright, 1986, pp 439-455.

Janossy G, Bofill M, Poulter LW, Rawlings L, Buford GD, Navarrete C, Zyegler A, Keleman L.
Separate ontogeny of two macrophage-like accessory cell populations in the human fetus.
J. Immunol. 1986; 136:4354-4361.

Janossy G, Campana G, Akbar A.
Kinetics of T lymphocyte development.
In: Iversen OH, ed. Current Topics In Pathology, Vol. 79: Cell Kinetics of the Inflammatory Reaction. Berlin, Springer-Verlag, 1989, pp 59-99.

Janossy G, Campana D, Counstan-Smith E, Timms AM, Burns GF, Cawley JC, Erber WN, Mason DY.
Immunological markers.
In: Chanarin I, ed. Laboratory Haematology. An Account of Laboratory Techniques. Edinburgh, Churchill Livingstone, 1989, pp 181-216.

Janossy G, Duke O, Poulter LW, Panayi G, Bofill M, Goldstein G.
Rheumatoid arthritis: a disease of T-lymphocyte/macrophage immunoregulation?
Lancet 1981; II:839-842.

Janossy G, Prentice HG.
T cell subpopulations, monoclonal antibodies and their therapeutic applications.
Clin. Haematol. 1982; 11:631-661.

Janossy G, Tidman M, Papageorgiou ES, Kung PC, Goldstein G.
Distribution of T lymphocyte subsets in the human bone marrow and thymus - an analysis with monoclonal antibodies.
J. Immunol. 1981; 126:1608-1613.

Jennings AR.
Animal Pathology.
London, Bailliere Tindall, 1970.

Jose PJ, Forrester MJ, Williams TJ.
Detection of the complement fragment C5a in inflammatory exudates from the rabbit peritoneal cavity using radioimmunoassay.
J. Exp. Med. 1983; 158:2177-2182.

Kaffenberger K, Gruber DF, MacVittie TJ.
Rat monocytes in a model of combined injury express the OX8 antigen.
J. Leuk. Biol. 1987; 42:181-187.

Kaltreider HB.
Local immunity.
In: Bienenstock J, ed. Immunology of the Lung and Upper Respiratory Tract. New York, McGraw Hill, 1984, 191-215.

Kartagener M.
Zur pathogenese der bronchiektasien.
Beitr. Klin. Tuberk. 1933; 83:489.

Kirby LA, Parfett GJ, Reader JA, Pepper JR.
Lung transplantation in the rat: a model for study of the cellular mechanisms of allograft rejection.
Immunology 1988; 63:369-372.

Knapp W, Dorken B, Rieber P, Schmidt RE, Stein H, von Der Borne AEGKr.
Leukocyte Typing IV.
Oxford, Oxford University Press, 1989.

Kohler G, Milstein C.
Continuous cultures of fused cells secreting antibody of predetermined specificity.
Nature 1975; 256:495-497.

Konstan MW, Vargo KM, Davis PB.
Ibuprofen attenuates the inflammatory response to Pseudomonas aeruginosa in a rat model of chronic pulmonary infection.
Am. Rev. Respir. Dis. 1990; 141:186-192.

Kumar RK.
Quantitative immunohistologic assessment of lymphocyte populations in the pulmonary inflammatory response to intratracheal silica.
Am. J. Pathol. 1989; 135:605-614.

Kurklu EU, Williams MA, Le Roux BT.
Bronchiectasis consequent upon foreign body retention.
Thorax 1973; 28:601-602.

Laennec RTH.
De l'Auscultation Mediate ou Traite' du Diagnostic des Maladies des Poumon et du Coeur, Fonde', Principalement sur ce Nouveau Moyen d'Exploration.
Paris, JA Brosson et JS Chaude, 1819.

Lapa e Silva JR, Cole PJ, Jones A, Poulter LW.
Immunohistological investigation of bronchiectasis.
Thorax 1987; 42:709.

Lapa e Silva JR, Cole PJ, Poulter LW.
Phenotype of T-lymphocytes in the chronically inflamed bronchial wall in bronchiectasis.
Am. Rev. Respir. Dis. 1989; 139 (part 2):A62.

Lapa e Silva JR, Cole PJ, Poulter LW.
Characterisation of bronchial intraepithelial lymphocytes in

bronchiectasis.
Thorax 1990; 45:784.

Lapa e Silva JR, Guerreiro D, Munro NC, Cole PJ, Poulter LW.
Quantitation of Class II MHC antigen expression in experimental bronchiectasis.
Am. Rev. Respir. Dis. 1990; 141 (part 2): A680.

Lapa e Silva JR, Guerreiro D, Munro NC, Noble B, Cole PJ, Poulter LW.
Expression of activation markers by lung mononuclear cells in a rat model of bronchiectasis.
Thorax 1989; 44:893P.

Lapa e Silva JR, Guerreiro D, Munro NC, Noble B, Cole PJ, Poulter LW.
Cell-mediated immune kinetics in experimental bronchiectasis.
In: Challacombe SJ, Stokes CR, MacDonald TT, eds. Advances in Mucosal Immunology. Dordrecht, Kluwer Academic Publishers, 1990, pp 821-824

Lapa e Silva JR, Guerreiro D, Munro NC, Noble B, Poulter LW, Cole PJ.
Kinetics of cell mediated immune response in a rat model of bronchiectasis.
Thorax 1989a; 44:315P.

Lapa e Silva JR, Guerreiro D, Munro NC, Noble B, Poulter LW, Cole PJ.
Kinetics of cell-mediated immune response in experimental bronchiectasis.
Am. Rev. Respir. Dis. 1989b; 139 (part 2):A35.

Lapa e Silva JR, Guerreiro D, Munro NC, Poulter LW, Cole PJ.
T-lymphocyte and macrophage activation in a Pseudomonas-induced model of bronchiectasis.
Am. Rev. Respir. Dis. 1990; 141 (suppl.): A529.

Lapa e Silva, Guerreiro D, Noble B, Poulter LW, Cole PJ.
The immunopathology of experimental bronchiectasis in rats reflects human disease.
Thorax 1988; 43:861P.

Lapa e Silva JR, Guerreiro D, Noble B, Poulter LW, Cole PJ.
Immunopathology of experimental bronchiectasis.
Am. J. Respir. Cell Mol. Biol. 1989; 1:297-304.

Lapa e Silva JR, Jones JAH, Cole PJ, Poulter LW.
Functional capacity of macrophages and lymphocytes in the bronchial inflammation of bronchiectasis.
Thorax 1988a; 43:227P.

Lapa e Silva JR, Jones JAH, Cole PJ, Poulter LW.
Phenotype of CD8+ve suppressor/cytotoxic T lymphocyte in the bronchial inflammation of bronchiectasis.
Eur. Respir. J. 1988b; 1 (suppl. 2):249s.

Lapa e Silva JR, Jones JAH, Cole PJ, Poulter LW.
The immunological component of the cellular inflammatory infiltrate in bronchiectasis.

Thorax 1989; 44:668-73.

Lapa e Silva JR, Jones JAH, Noble B, Poulter LW, Cole PJ.
A cell mediated immune response is present in the chronic inflammation of bronchiectasis.
Am. Rev. Respir. Dis. 1988; 137(part 2):50.

Lapa e Silva JR, Munro NC, Guerreiro D, Poulter LW, Cole PJ.
Cytotoxic activity of lung cells obtained by collagenase digestion in a rat model of bronchiectasis.
Am. Rev. Respir. Dis. 1990; 141 (part 2): A680.

Lapa e Silva JR, Poulter LW, Cole PJ.
Phenotype of bronchial T-lymphocytes in the chronic inflammation of bronchiectasis.
In: Challacombe SJ, Stokes CR, MacDonald TT, eds. Advances in Mucosal Immunology. Dordrecht, Kluwer Academic Publishers, 1990, pp 636-637.

Le Roux BT, Mohlala ML, Odell JA, Whitton ID.
Suppurative disease of the lung and pleural space part II: Bronchiectasis.
In: Ravitch MM, ed. Current Problems in Surgery. Chicago, Year Book Medical Publishers, 1986, pp 94-159.

Lindskog GE.
Bronchiectasis revisited.
Yale J. Biol. Med. 1986; 59:41-53.

Ling NR, McLennan ICM, Mason DY.
B-cell and plasma cell antigens: new and previous defined clusters.
In: McMichael AJ, ed. Leukocyte Typing III. Oxford University Press, 1987, pp 302-336.

MacArthur CG, Cheung DLC, Spiro SG.
Endobronchial lipoma: a review of four cases.
Br. J. Dis. Chest 1977; 71:93-100.

MacDonald TT.
The role of activated T lymphocytes in gastrointestinal disease.
Clin. Exp. Allergy 1990, 20:247-252.

MacDonald TT, Spencer J.
Evidence that activated mucosal T cells play a role in the pathogenesis of enteropathy in human small intestine.
J. Exp. Med. 1988; 167:1341-1349.

Maher P, O'Toole CM, Wreghitt TG, Spiegelhalter DJ, English TAH.
Cytomegalovirus infection in cardiac transplant recipients associated with chronic T cell subset ratio inversion with expansion of a Leu-7+ Ts-c+ subset.
Clin. exp. Immunol. 1985; 62:515-524.

Markham RB, Goellner J, Pier GB.
<u>In vitro</u> T cell mediated killing of <u>Pseudomonas aeruginosa</u>. I. Evidence that a lymphokine mediates killing.

J. Immunol. 1984; 133:962-968.

Markham RB, Pier GB, Goellner JJ, Mizel SB.
In vitro T cell-mediated killing of Pseudomonas aeruginosa. II. The role of macrophages and T cell subsets in T cell killing.
J. Immunol. 1985; 134:4112-4117.

Markham RB, Pier GB, Powderly WG.
Suppressor T cells regulating the cell-mediated immune response to Pseudomonas aeruginosa can be generated by immunization with anti-bacterial T cells.
J. Immunol. 1988; 141:3975-3979.

Markham RB, Powderly WG.
Exposure of mice to live Pseudomonas aeruginosa generates protective cell-mediated immunity in the absence of an antibody response.
J. Immunol. 1988; 140:2039-2045.

Markmann J, Lo D, Naji A, Palmiter RD, Brinster RL, Heber-Katz E.
Antigen presenting function of Class II expressing pancreatic beta cells.
Nature 1988; 336:476-479.

Mason DW, Arthur RP, Dallman MJ, Green JR, Spickett GP, Thomas ML.
Functions of rat T-lymphocyte subsets isolated by means of monoclonal antibodies.
Immunol. Rev. 1983; 74:57-82.

Miller HRP, Nawa Y.
Nippostrongylus brasiliensis: intestinal goblet cell response in adoptively immunized rats.
Exp. Parasitol. 1979a; 47:81-90.

Miller HRP, Nawa Y.
Immune regulation of intestinal goblet cell differentiation. Specific induction of nonspecific protection against helminths?
Nouv. Rev. Fr. Haematol. 1979b; 21:31-45.

Muhlradt PF, Tsai H, Conradt P.
Effects of pyocyanine, a blue pigment from Pseudomonas aeruginosa, on separate steps of T cell activation: interkeukin 2 (IL2) production, IL2 receptor formation, proliferation and induction of cytolytic activity.
Eur. J. Immunol. 1986; 16:434-440.

Munro CS, Campbell DA, Collings LA, Poulter LW.
Monoclonal antibodies distinguish macrophages and epithelioid cells in sarcoidosis and leprosy.
Clin. exp. Immunol. 1987; 68:282-287.

Munro CS, Mitchell DN, Poulter LW, Cole PJ.
Early cellular responses to intradermal injection of Kveim suspension in normal subjects and those with sarcoidosis.
J. Clin. Pathol. 1986; 39:176-182.

Munro NC, Barker A, Rutman A, Tayler G, Watson D, McDonald-Gibson WJ, Towart R, Taylor WA, Wilson R, Cole PJ.
The effect of pyocyanin and 1-hydroxiphenazine on in vivo tracheal mucus velocity.
J. Appl. Physiol. 1989; 67:316-323.

Munro NC, Cooke JC, Currie DC, Strickland B, Cole PJ.
Comparison of thin section computed tomography with bronchography for identifying bronchiectatic segments in patients with chronic sputum production.
Thorax 1990; 45:135-139.

Murphy MB, Reen DJ, Fitzgerald MX.
Atopy, immunological changes, and respiratory function in bronchiectasis.
Thorax 1984; 39:179-184.

Murray JF.
Postnatal growth and development of the lung.
In: Murray JF, ed. The Normal Lung, 2nd edition. Philadelphia, W. B. Saunders Co., 1986, pp23-59.

Nicas TI, Iglewski BH.
The contribution of exoproducts to virulence of Pseudomonas aeruginosa.
Can. J. Microbiol. 1985; 31:387-392.

Noda Y, Yasuoka S, Tani K, Ogura T, Ogawara M, Kitatani F.
Secretory IgA (S-IgA) levels in sera from patients with diffuse panbronchiolitis.
Jap. J. Med. 1989; 28:189-195.

Nutman J, Berger M, Chase PA, Dearborn DG, Miller KM, Waller RL, Sorensen RU.
Studies on the mechanisms of T cell inhibition by the Pseudomonas aeruginosa phenazine pigment pyocyanine.
J. Immunol. 1987; 138:3481-3487.

O'Brien, Happ MP, Dallas A, Palmer E, Kubo R, Born WK.
Stimulation of a major subset of lymphocytes expressing T cell receptor gamma/delta by an antigen derived from Mycobacterium tuberculosis.
Cell 1989; 57:667-674.

Otsuki Y, Ito Y, Magari S.
Lymphocyte subpopulations in high endothelial venules and lymphatic capilaries of bronchus-associated lymphoid tissue (BALT) in the rat.
Am. J. Anat. 1989; 184:139-146.

Pabst R, Binns RM, Licence ST, Peter M.
Evidence of a selective major vascular pool of lymphocytes in the lung.
Am. Rev. Respir. Dis. 1987; 136:1213-1218.

Pabst R, Gehrke I.
Is the bronchus-associated lymphoid tissue (BALT) an integral structure of the lung in normal mammals, including humans?
Am. J. Respir. Cell Mol. Biol. 1990; 3:131-135.

Parmerly M, Gale A, Clabaugh M, Horvat R, Zhou WW.
Proteolytic inactivation of cytokines by Pseudomonas aeruginosa.
Infect. Immun. 1990; 58:3009-3014.

Paterson DJ, Jefferies WA, Green JR, Brandon MR, Corthesy P, Puklavec M, Williams MR.
Antigens of activated rat T lymphocytes including a molecule of 50000 M detected only on CD4 positive T blasts.
Mol. Immunol. 1987; 24:1281-1290.

Petit JC, Daguet GL.
Interference of Pseudomonas aeruginosa with immunospecific host defences.
Biomed. Pharmacoter. 1983; 37:422-428.

Pier GB.
Pulmonary disease associated with Pseudomonas aeruginosa in cystic fibrosis: current status of the host-bacterium interaction.
J. Infect. Dis. 1985; 151:575-580.

Pier GB, Markham RB.
Induction in mice of cell-mediated immunity to Pseudomonas aeruginosa by high molecular weight polysaccharide and vinblastine.
J. Immunol. 1982; 128:2121-2125.

Plesch BEC.
Histology and immunohistochemistry of bronchus-associated lymphoid tissue (BALT) in the rat.
In: Niewenhuis et al, eds. In Vivo Immunology. New York, Plenum Publishing Corp, 1982, pp 491-497.

Plesch BEC.
Development of bronchus-associated lymphoid tissue (BALT) in the rat, with special reference to T- and B-lymphocytes.
Dev. Comp. Immunol. 1983; 7:179-188.

Plusa T, Wasek Z.
Immunobiochemical evaluation of bronchoalveolar lavage (BAL) in atopic bronchial asthma, chronic bronchitis and bronchiectasis.
Allergol. Immunopathol. 1987; 15:209-213.

Poulter LW, Campbell DA, Munro C, Butcher RG.
The quantitation of HLA-DR expression on human cells using immunocytochemistry.
J. Immunol. Meth. 1987; 98:227-234.

Poulter LW, Campbell DA, Munro C, Janossy J.
Discrimination to macrophages and dendritic (ID) cells of man using monoclonal antibodies.
Scand. J. Immunol. 1986; 24:351-357.

Poulter LW, Chilosi M, Seymour GJ, Hobbs S, Janossy G.
Immunofluorescence membrane staining and cytochemistry, applied in combination for analysing cell interactions in situ.

In: Polak JM, Van Noorden S, eds. Immunocytochemistry, Practical Applications in Pathology and Biology. Bristol, Wright, 1983, pp 233-248.

Poulter LW, Powell C, Burke CB.
The relationship between bronchial immunopathology and hyperresponsiveness in asthma.
Eur. Respir. J. 1990; 3:792-799.

Poulter LW, Seymour GJ, Duke O, Janossy G, Panayi G.
Immunohistological analysis of delayed-type hypersensitivity in man.
Cell. Immunol. 1982; 74:358-369.

Powderly WG, Pier GB, Markham RB.
In vitro T cell-mediated killing of Pseudomonas aeruginosa. III. The role of suppressor T cells in nonresponder mice.
J. Immunol. 1986a; 137:299-303.

Powderly WG, Pier GB, Markham RB.
In vitro T cell killing of Pseudomonas aeruginosa. IV. Nonresponsiveness in polysaccharide-immunized BALB/c mice is attributable to vinblastine-sensitive suppressor T cells.
J. Immunol. 1986b; 137:2025-2030.

Powderly WG, Pier GB, Markham RB.
T lymphocyte-mediated protection against Pseudomonas aeruginosa infection in granulocytopenic mice.
J. Clin. Invest. 1986c; 78:375-380.

Powderly WG, Pier GB, Markham RB.
In vitro killing of Pseudomonas aeruginosa. V. Generation of bactericidal T cells in nonresponder mice.
J. Immunol. 1987; 138:2272-2277.

Powderly WG, Schreiber R, Pier GB, Markham RB.
T cells recognizing polysaccharide-specific B cells function as contrassupressor cells in the generation of T cell immunity to Pseudomonas aeruginosa.
J. Immunol. 1988; 140:2746-2752.

Raphael M, Puoletty P, Cavaille-Coll M, Rozenbaum W, Homond A, Nonnenmacher L, Delcourt A, Gluckman JC, Debre P.
Lymphadenopathy in patients at risk for acquired immunodeficiency syndrome. Histopathology and immunohistochemistry.
Arch. Pathol. Lab. Med. 1985; 109:128-132.

Ras G, Wilson R, Todd H, Taylor G, Cole P.
Effect of bacterial products on neutrophil migration in vitro.
Thorax 1990; 45:276-280.

Read RC, Wilson R, Rutman A, Lund V, Todd HC, Brain APR, Jeffery PK, Cole PJ.
Interaction of non typable Haemophilus influenzae with human respiratory mucosa in vitro.
J. Infect. Dis, 1991;163:549-558.

Reid LM.
Reduction of bronchial subdivisions in bronchiectasis.
Thorax 1950; 5:233-247.

Reinherz EL, Schlossman SF.
The differentiation and function of human T-lymphocytes.
Cell 1980; 19:821-827.

Rivasi P, Rivasi F, Botticelli A, Manenti A.
Le immunoglobuline della mucosa bronchiale nelle bronchiectasie acquisite i nei carcinomi bronchopolmonari.
Bol. Soc. It. Biol. Sper. 1975; 51:315-318.

Robinson AP, Puklavec M, Mason DW.
MRC OX-52: a rat T-cell antigen.
Immunology 1986; 57:527-531.

Roitt IM, Brostoff J, Male DK.
Immunology. 2nd edition.
London, Gower Medical Publishing, 1989.

Roles FC, Todd GS.
Bronchiectasis: diagnosis and treatment in relation to treatment.
Br. Med. J. 1933; 2:639-643.

Romaniuk A, Prop J, Petersen AH, Wildevuur CRH, Nieuwenhuis P.
Expression of Class II Major Histocompatibility Complex antigens by bronchial epithelium in rat lung allografts.
Transplantation 1987; 44:209-214.

Rommens JM, Ianuzzi MC, Kerem BS, Drumm ML, Melmer G, Dean M, Rozmahel R, Cole JL, Kennedy D, Hidara N, Zsiga M, Buchwald M, Riordam JR, Tsui LC, Collins FS.
Identification of the cystic fibrosis gene: chromosome walking and jumping.
Science 1989; 245:1059-1065.

Rosenzweig DY, Stead WW.
The role of tuberculosis and other forms of bronchopulmonary necrosis in the pathogenesis of bronchiectasis.
Am. Rev. Respir. Dis 1966; 93:769-785.

Rubin BK.
Immotile cilia syndrome (primary ciliary diskinesia) and inflammatory lung disease.
Clin. Chest Med.1988; 9:657-668.

Rutland J, Cole PJ.
Nasal mucociliary clearance and ciliary beat frequency in cystic fibrosis compared with sinusitis and bronchiectasis.
Thorax 1981; 36:654-658.

Ryan BF, Joiner BL, Ryan TA.
Minitab Handbook, 2nd Edition.

Boston, PWS-Kent Publishing Company, 1985.

Selby WS, Janossy G, Jewell DP.
Immunohistological characterization of intraepithelial lymphocytes of the human gastrointestinal tract.
Gut 1981; 22:169-176.

Semenzato G, Trentin L, Zambello R, Agostini G, Cipriani A, Marcer G.
Different types of cytotoxic lymphocytes recovered from the lungs of patients with hypersensitivity pneumonitis.
Am. Rev. Respir. Dis. 1988; 137:70-74.

Shanahan F, Brogan M, Targan S.
Human mucosal cytotoxic effector cells.
Gastroenterology 1987; 92:1951-1957.

Sibille Y, Reynolds HY.
Macrophages and polymorphonuclear neutrophils in lung defense and injury.
Am. Rev. Respir. Dis. 1990; 141:471-501.

Sicard FA, Forestier JA.
Methode generale d'exploration radiologique par l'huile iodee (Lipiodol).
Bull. Soc. Med. Hop. Paris 1922; 46:463.

Simecka JW. Davis JK, Cassel GH.
Distribution of Ia antigens and T lymphocyte subpopulations in rat lungs.
Immunology 1986; 57:93-98.

Smallman LA, Hill SL, Stockley RA.
Reduction in ciliary beat frequency in vitro by sputum from patients with bronchiectasis: a serine proteinase effect.
Thorax 1984; 39:663-667.

Smith SH, Brown MH, Rowe D, Callard RE, Beverley PCL.
Functional subsets of helper-inducer cells defined by a new monoclonal antibody, UCHL1.
Immunology 1986; 58:63-70.

Snider GL.
Interstitial pulmonary fibrosis.
Chest 1986; 89 (suppl.):115S-120S.

Snider GL, Lucey EC, Christensen TG, Stone PJ, Calore JD, Catanese A, Franzblau C.
Emphysema and bronchial secretory cell metaplasia induced in hammsters by human neutrophil elastase.
Am. Rev. Respir. Dis. 1984; 129:155-160.

Snider GL, Stone PJ, Lacey EC, Bruer R, Colore JD, Seshadri T, Catonese A, Maschler R, Schnebli HP.
Eglin-C, a polypeptide derived from the medicinal leech, prevents human neutrophil elastase-induced emphysema and bronchial secretory cell

metaplasia in the hamster.
Am. Rev. Respir. Dis. 1985; 132:1155-1161.

Somerville M, Rutman A, Wilson R, Cole PJ, Richardson PS.
Stimulation of secretion into human and feline airways by Pseudomonas aeruginosa proteases.
J. Appl. Physiol. 1991 (in press).

Sorensen RU, Klinger JD, Cash HA, Chase PA, Dearborn DG.
In vitro inhibition of lymphocyte proliferation by Pseudomonas aeruginosa phenazine pigments.
Infect. Immun. 1983; 41:321-330.

Spencer J, Isaacson PG, Diss TC, MacDonald TT.
Expression of disulfide-linked and non-disulfide-linked forms of the T cell receptor gamma/delta heterodimer in human intraepithelial lymphocytes.
Eur. J. Immunol. 1989; 19:1335-1338.

Spencer M.
Pathology of the Lung, 4th edition.
Oxford, Pergamon Press, 1985.

Steinfort C, Wilson R, Mitchell T, Feldman C, Rutman C, Todd H, Sykes D, Walker J, Saunders K, Andrew PW, Boulnois GJ, Cole PJ.
Effect of Streptoccocus pneumoniae on human respiratory epithelium in vitro.
Infect. Immun. 1989; 57:2006-2913.

Stockley RA.
The pathogenetic role of proteolytic enzymes in bronchiectasis.
In: Davies RJ, ed. Strategies for the Management of Chronic Bronchial Sepsis. Oxford, The Medicine Publishing Foundation, 1984, pp 35-43.

Stockley RA, Shaw J, Afford SC, Morrison HM, Burnett D.
Effect of alpha-1-proteinase inhibitor on neutrophil chemotaxis.
Am. J. Respir. Cell Mol. Biol. 1990; 2:163-170.

Stockley RA, Shaw J, Hill SL, Burnett D.
Neutrophil chemotaxis in bronchiectasis: a study of peripheral cells and lung secretions.
Clin. Sci. 1988; 74:645-650.

Strominger JL.
Developmental biology of T cell receptor.
Science 1989; 244:943-950.

Stutts KJ, Schwab JH, Chen MG, Knowles MR, Boucher RC.
Effects of Pseudomonas aeruginosa on bronchial epithelium ion transport.
Am. Rev. Respir. Dis. 1986; 134:17-21.

Sykes DA, Wilson R, Greenstone M, Currie DC, Steinfort C, Cole PL.
Deleterious effects of purulent sputum sol on human ciliary function in vitro: at least two factors identified.

Thorax 1987; 42:256-261.

Tanaka E, Sato A, Kato M, Kuze F.
Mucosal immune response of a murine model of chronic bronchial infection with Pseudomonas aeruginosa.
Am. Rev. Respir. Dis. 1990; 141 (part 2):A620.

Tannanberg J, Pinner M.
Atelectasis and bronchiectasis. An experimental study concerning their relationship.
J. Thor. Surg. 1942; 11:571-616.

Tegner H, Ohlsson K, Toremalm NG, Von Mecklenburg C.
Effect of human leukocyte enzymes on tracheal mucosa and its mucociliary activity.
Rhinology 1979; 17:199-206.

Tomasi TB, Tan EM, Solomon A, Prendergast RA.
Characteristics of an immune system common to certain external secretions.
J. Exp. Med. 1965; 121:101-124.

Trejdosiewicz LK, Badr-El-Din S, Smart CJ, Malizia G, Oakes DJ, Heatley RV, Losowsky MS.
Colonic mucosal T lymphocytes in ulcerative colitis: expression of CD7 antigen in relation to MHC Class II (HLA-D) antigens.
Dig. Dis. Sci. 1989; 34:1449-1456.

Uchiyama T, Broder S, Waldmann TA.
A monoclonal antibody (anti-TAC) reactive with activated and functionally mature human T cells. I. Production of anti-TAC monoclonal antibody and distribution of TAC positive cells.
J. Immunol. 1981; 126:1393-1397.

Ullrich R, Schieferdecker HL, Ziegler K, Riecken EO, Zeitz M.
Gamma/delta T cells in the human intestine express surface markers of activation and are preferentially located in the epithelium.
Cell. Immunol. 1990; 128:619-627.

Ulmer AJ, Pryjma J, Tarnok Z, Ernst M, Flad HD.
Inhibitory and stimulatory effects of Pseudomonas aeruginosa pyocyanine on human T and B lymphocytes and human monocytes.
Infect. Immun. 1990; 58:808-815.

Unanue ER, Allen PM.
The basis for the immunoregulatory role of macrophages and other dendritic cells.
Science 1987; 236:551-557.

Varpela E, Savilahti E, Lokki O.
Serum imunoglobulin A in chronic bronchial inflammation.
Scand. J. Respir. Dis. 1977; 58:33-40.

Ventura J, Domaradzki M.
Pathogenesis of experimental bronchiectasis in laboratory rats.

Arch. Path. 1967; 83:80-85.

Ventura J, Domaradzki M.
Role of Mycoplasma infection in the development of experimental bronchiectasis in the rat.
J. Path. Bact. 1967; 93:342-348.

Viney JL, Kilshaw PJ, MacDonald TT.
Cytotoxic alpha/beta+ and gamma/delta+ T cells in murine intestinal epithelium.
Eur. J. Immunol. 1990; 20:1623-1626.

Whitwell F.
A study of the pathology and pathogenesis of bronchiectasis.
Thorax 1952; 7:213-299.

Wilson R, Roberts D, Cole P.
Effects of bacterial products on human ciliary function in vitro.
Thorax 1985; 40:125-131.

Wilson R, Pitt T, Taylor G, Watson D, MacDermott J, Sykes D, Roberts D, Cole P.
Pyocyanin and 1-hydroxyphenazine inhibit the beating of human respiratory cilia in vitro.
J. Clin. Invest. 1987; 79:221-229.

Wilson R, Cole PJ.
The effect of bacterial products on ciliary function.
Am. Rev. Respir. Dis. 1988; 138:S49-S53.

Young RA, Elliott TJ.
Stress proteins, infection and immune surveillance
Cell 1989; 59: 5-8.

A P P E N D I X 1 - STATISTICAL TREATMENT OF DATA

DESCRIPTIVE STATISTICS FOR CHAPTER 4

DESCRIPTIVE STATISTICS FOR TABLE 4.4

MoAb: RFTmix Compartment: epithelium

Bronchiectasis	Controls
mean 7.64 (95% C.I. 5.64, 9.64)	mean 2.61 (95% C.I. 1.81, 3.40)
median 6.84 (95% C.I. 4.31, 9.33)	median 2.52 (95% C.I. 1.52, 3.50)
std 4.15 n 19	std 1.18 n 11
t-test 0.0001	Mann-Whitney 0.0003

MoAb: Leu7 Compartment: epithelium

Bronchiectasis	Controls
mean 0.96 (95% C.I. 0.13, 1.80)	mean 0.21 (95% C.I.-0.03, 0.46)
median 0.75 (95% C.I.-0.29, 1.80)	median 0 (95% C.I.-0.18, 0.80)
std 1.45 n 14	std 0.37 n 11
t-test 0.08	Mann-Whitney < 0.03

MoAb: RFD7 Compartment: epithelium

Bronchiectasis	Controls
mean 2.36 (95% C.I. 1.62, 3.09)	mean 0.66 (95% C.I. 0.36, 0.95)
median 1.76 (95% C.I. 0.83, 2.68)	median 0.52 (95% C.I. 0.15, 0.89)
std 1.43 n 17	std 0.44 n 11
t-test 0.0002	Mann-Whitney 0.0001

MoAb: UCHM1 Compartment: epithelium

Bronchiectasis	Controls
mean 1.51 (95% C.I. 1.08, 1.94)	mean 0.11 (95% C.I. 0.01, 0.46)
median 1.50 (95% C.I. 0.96, 2.04)	median 0 (95% C.I.-0.14, 0.14)
std 0.83 n 17	std 0.16 n 11
t-test 0.00005	Mann-Whitney 0.0002

MoAb: RFD1 Compartment: epithelium

Bronchiectasis	Controls
mean 1.99 (95% C.I. 1.44, 2.55)	mean 0.48 (95% C.I. 0.07, 0.85)
median 2.10 (95% C.I. 1.41, 2.70)	median 0.31 (95% C.I.-0.18, 0.80)
std 1.08 n 17	std 0.58 n 11
t-test 0.00005	Mann-Whitney 0.0001

DESCRIPTIVE STATISTICS FOR TABLE 4.5

MoAb: RFTmix Compartment: lamina propria

Bronchiectasis	Controls
mean 7.35 (95% C.I. 5.37, 9.33);	mean 1.67 (95% C.I. 1.21, 2.12)
median 7.56 (95% C.I. 5.08, 10.1);	median 1.66 (95% C.I. 1.08, 2.23)
std 4.47 n 22 ;	std 0.68 n 11
t-test 0.00005 ;	Mann-Whitney 0.00005

MoAb: Leu7 Compartment: lamina propria

Bronchiectasis	Controls
mean 0.50 (95% C.I. 0.15, 0.85);	mean 0.15 (95% C.I. 0.01, 0.29)
median 0.34 (95% C.I.-0.09, 0.78);	median 0 (95% C.I.-0.18, 0.18)
std 0.60 n 14 ;	std 0.22 n 11
t-test 0.056 ;	Mann-Whitney 0.04

MoAb: RFBmix Compartment: lamina propria

Bronchiectasis	Controls
mean 1.86 (95% C.I. 0.17, 3.56);	mean 0.01 (95% C.I.-0.01, 0.04)
median 0.15 (95% C.I.-1.97, 2.27);	median 0 (95% C.I.-0.03, 0.03)
std 3.72 n 21 ;	std 0.04 n 11
t-test 0.03 ;	Mann-Whitney 0.02

MoAb: RFD6 Compartment: lamina propria

Bronchiectasis	Controls
mean 3.56 (95% C.I. 1.56, 5.56);	mean 1.02 (95% C.I. 0.69, 1.35)
median 1.67 (95% C.I.-0.84, 4.18);	median 1.06 (95% C.I. 0.06, 1.48)
std 4.02 n 18 ;	std 0.46 n 11
t-test 0.02 ;	Mann-Whitney 0.03

MoAb: RFD7 Compartment: lamina propria

Bronchiectasis	Controls
mean 3.10 (95% C.I. 2.37, 3.83);	mean 0.79 (95% C.I. 0.39, 1.20)
median 2.92 (95% C.I. 2.01, 3.84);	median 0.71 (95% C.I. 0.20, 1.22)
std 1.61 n 21 ;	std 0.60 n 11
t-test 0.00005 ;	Mann-Whitney 0.00005

MoAb: UCHM1 Compartment: lamina propria

Bronchiectasis	Controls
mean 2.25 (95% C.I. 1.62, 2.87);	mean 0.22 (95% C.I. 0.09, 0.35)
median 2.51 (95% C.I. 1.73, 3.29);	median 0.22 (95% C.I. 0.06, 0.38)
std 1.33 n 20 ;	std 0.19 n 11
t-test 0.00005 ;	Mann-Whitney 0.0001

MoAb: RFD1 Compartment: lamina propria

Bronchiectasis	Controls
mean 1.63 (95% C.I. 1.19, 2.06);	mean 0.66 (95% C.I. 0.33, 0.99)
median 1.45 (95% C.I. 0.90, 1.99);	median 0.59 (95% C.I. 0.17, 1.01)
std 0.90 n 19 ;	std 0.49 n 11
t-test 0.0008 ;	Mann-Whitney 0.04

DESCRIPTIVE STATISTICS FOR TABLE 4.6

MoAb: RFTmix Compartment: submucosa

Bronchiectasis	Controls
mean 6.90 (95% C.I. 5.07, 8.73)	mean 1.77 (95% C.I. 1.34, 2.19)
median 6.01 (95% C.I. 3.72, 8.30)	median 1.74 (95% C.I. 1.20, 2.28)
std 4.02 n 21	std 0.60 n 10
t-test 0.00005	Mann-Whitney 0.00005

MoAb: Leu 7 Compartment: submucosa

Bronchiectasis	Controls
mean 0.56 (95% C.I. 0.19, 0.93)	mean 0.09 (95% C.I.-0.02, 0.20)
median 0.44 (95% C.I.-0.02, 0.90)	median 0 (95% C.I.-0.14, 0.14)
std 0.58 n 12	std 0.15 n 10
t-test 0.02	Mann-Whitney 0.02

MoAb: RFBmix Compartment: submucosa

Bronchiectasis	Controls
mean 2.25 (95% C.I. 0.25, 4.24)	mean 0.01 (95% C.I.-0.01, 0.02)
median 0 (95% C.I.-2.50, 2.50)	median 0 (95% C.I.-0.02, 0.02)
std 4.26 n 20	std 0.02 n 10
t-test 0.03	Mann-Whitney 0.07

MoAb: RFD6 Compartment: submucosa

Bronchiectasis	Controls
mean 4.09 (95% C.I. 1.99, 6.20)	mean 1.36 (95% C.I. 0.55, 2.17)
median 2.93 (95% C.I. 0.29, 5.57)	median 0.99 (95% C.I.-0.02, 2.01)
std 4.09 n 17	std 1.13 n 10
t-test 0.02	Mann-Whitney 0.02

MoAb: RFD7 Compartment: submucosa

Bronchiectasis	Controls
mean 3.19 (95% C.I. 2.52, 3.86)	mean 1.12 (95% C.I. 0.61, 1.63)
median 2.90 (95% C.I. 2.06, 3.74)	median 1.05 (95% C.I. 0.41, 1.69)
std 1.39 n 19	std 0.71 n 10
t-test 0.00005	Mann-Whitney 0.0002

MoAb: UCHM1 Compartment: submucosa

Bronchiectasis	Controls
mean 2.74 (95% C.I. 2.01, 3.47)	mean 0.20 (95% C.I. 0.02, 0.37)
median 2.70 (95% C.I. 1.79, 3.61)	median 0.20 (95% C.I.-0.11, 0.32)
std 1.51 n 19	std 0.24 n 10
t-test 0.00005	Mann-Whitney 0.0001

MoAb: RFD1 Compartment: submucosa

Bronchiectasis	Controls
mean 1.86 (95% C.I. 1.26, 2.46)	mean 0.77 (95% C.I. 0.33, 1.21)
median 1.62 (95% C.I. 0.87, 2.37)	median 0.69 (95% C.I. 0.14, 1.25)
std 1.21 n 18	std 0.61 n 10
t-test 0.05	Mann-Whitney 0.005

DESCRIPTIVE STATISTICS FOR TABLE 4.7

MoAb: RFTmix Compartment: lamina propria

Follicular		Non-follicular	
mean 10.91	(95% C.I. 8.33,13.47);	mean 4.63	(95% C.I. 3.15, 6.12)
median 10.16	(95% C.I. 6.93,13.39);	median 3.90	(95% C.I. 2.03, 5.76)
std 3.59	n 10 ;	std 2.34	n 12
t-test 0.0003	;	Mann-Whitney 0.0007	

MoAb: RFTmix Compartment: submucosa

Follicular		Non-follicular	
mean 9.98	(95% C.I. 7.38,12.59);	mean 4.09	(95% C.I. 3.15, 6.12)
median 10.16	(95% C.I. 6.95,13.49);	median 4.17	(95% C.I. 2.03, 5.40)
std 3.64	n 10 ;	std 1.46	n 11
t-test 0.0006	;	Mann-Whitney 0.0003	

MoAb: RFBmix Compartment: lamina propria

Follicular		Non-follicular	
mean 2.53	(95% C.I. 1.11, 3.95);	mean 0.01	(95% C.I.-0.15, 0.04)
median 2.04	(95% C.I. 0.26, 3.82);	median 0	(95% C.I.-0.03, 0.03)
std 1.85	n 9 ;	std 0.04	n 12
t-test 0.004	;	Mann-Whitney 0.00005	

MoAb: RFBmix Compartment: submucosa

Follicular		Non-follicular	
mean 3.08	(95% C.I. 0.83, 5.32);	mean 0.01	(95% C.I.-0.15, 0.04)
median 2.90	(95% C.I. 0.08, 6.59);	median 0	(95% C.I.-0.03, 0.03)
std 2.92	n 9 ;	std 0.06	n 11
t-test 0.02	;	Mann-Whitney 0.004	

MoAb: RFD6 Compartment: lamina propria

Follicular		Non-follicular	
mean 4.22	(95% C.I. 2.08, 6.35);	mean 1.22	(95% C.I. 0.68, 1.76)
median 3.92	(95% C.I. 1.24, 6.59);	median 1.03	(95% C.I. 0.36, 1.71)
std 2.78	n 9 ;	std 0.64	n 8
t-test 0.02	;	Mann-Whitney 0.02	

MoAb: RFD6 Compartment: subucosa

Follicular		Non-follicular	
mean 4.72	(95% C.I. 3.13, 6.32);	mean 1.39	(95% C.I. 0.98, 1.88)
median 5.10	(95% C.I. 3.10, 7.09);	median 1.26	(95% C.I. 0.64, 1.88)
std 2.07	n 9 ;	std 0.53	n 7
t-test 0.002	;	Mann-Whitney 0.003	

DESCRIPTIVE STATISTICS FOR TABLE 4.8

MoAb: CD8/CD25 Compartment: ALL

Bronchiectasis		Controls	
mean 4.59	(95% C.I. 4.47,4.71);	mean 0	(95% C.I. --)
median 3.00	(95% C.I. 2.85,3.15);	median 0	(95% C.I. --)
std 3.92	n 17	; std 0	n 10
t-test 0.001		; Mann-Whitney 0.001	

MoAb: CD8/CD38 Compartment: ALL

Bronchiectasis		Controls	
mean 12.31	(95% C.I. 12.21,12.40);	mean 0	(95% C.I. --)
median 10.00	(95% C.I. 9.88,10.12);	median 0	(95% C.I. --)
std 8.88	n 13	; std 0	n 9
t-test 0.0005		; Mann-Whitney 0.0005	

MoAb: CD8/CD7 Compartment: ALL

Bronchiectasis		Controls	
mean 78.16	(95% C.I. 78.01,78.31);	mean 23.5	(95% C.I. 23.23,23.76)
median 85.00	(95% C.I. 84.81,85.18);	median 17.5	(95% C.I. 17.17,17.83)
std 17.17	n 19	; std 21.74	n 10
t-test 0.00005		; Mann-Whitney 0.00005	

MoAb: CD8/HLA-DR Compartment: ALL

Bronchiectasis		Controls	
mean 19.21	(95% C.I. 19.15,19.27);	mean 2.8	(95% C.I. 2.77, 2.82)
median 20.00	(95% C.I. 19.92,20.09);	median 3.0	(95% C.I. 2.97, 3.03)
std 7.31	n 19	; std 2.2	n 10
t-test 0.00005		; Mann-Whitney 0.00005	

MoAb: CD8/CD45RO Compartment: ALL

Bronchiectasis		Controls	
mean 73.45	(95% C.I. 73.36,73.56);	mean 46.0	(95% C.I. 45.83,46.16)
median 80.00	(95% C.I. 79.87,80.13);	median 45.0	(95% C.I. 44.79,45.21)
std 9.87	n 13	; std 13.5	n 10
t-test 0.0001		; Mann-Whitney 0.0002	

MoAb: CD8/CD45RA Compartment: ALL

Bronchiectasis		Controls	
mean 18.51	(95% C.I. 18.43.18.62):	mean 20.5	(95% C.I. 20.44,20.55)
median 20.0	(95% C.I. 19.88,20.12);	median 20.0	(95% C.I. 19.93,20.67)
std 10.42	n 17	; std 4.37	n 10
t-test 0.5		; Mann-Whitney 0.18	

MoAb: CD8/Leu7 Compartment: ALL

Bronchiectasis		Controls	
mean 9.33	(95% C.I. 9.25, 9.41);	mean 0	(95% C.I. --)
median 5.0	(95% C.I. 4.89, 5.10);	median 0	(95% C.I. --)
std 8.42	n 15	; std 0	n 5
t-test 0.03		; Mann-Whitney 0.04	

DESCRIPTIVE STATISTICS FOR TABLE 4.9

MoAb: CD4/CD25 Compartment: ALL

Bronchiectasis	Controls
mean 1.23 (95% C.I. 1.22, 1.25)	mean 0 (95% C.I. --)
median 0 (95% C.I.-0.02, 0.02)	median 0 (95% C.I. --)
std 2.31 n 17	std 0 n 10
t-test NS	Mann-Whitney NS

MoAb: CD4/CD38 Compartment: ALL

Bronchiectasis	Controls
mean 6.11 (95% C.I. 6.05, 6.17)	mean 1.0 (95% C.I. 0.97, 1.02)
median 5.0 (95% C.I. 4.92, 5.07)	median 0 (95% C.I.-0.03, 0.03)
std 6.76 n 18	std 2.11 n 10
t-test 0.008	Mann-Whitney 0.04

MoAb: CD4/CD7 Compartment: ALL

Bronchiectasis	Controls
mean 27.6 (95% C.I. 27.4,27.88)	mean 15.5 (95% C.I. 15.35,15.65)
median 30.0 (95% C.I. 19.7,20.26)	median 12.5 (95% C.I. 12.31,12.69)
std 26.1 n 17	std 12.35 n 10
t-test NS	Mann-Whitney NS

MoAb: CD4/HLA-DR Compartment: ALL

Bronchiectasis	Controls
mean 21.05 (95% C.I. 20.84,21.3)	mean 3.0 (95% C.I. 2.95, 3.04)
median 20.0 (95% C.I. 19.73,20.3)	median 1.5 (95% C.I. 1.44, 1.55)
std 23.21 n 17	std 3.63 n 10
t-test 0.006	Mann-Whitney 0.02

MoAb: CD4/CD45RO Compartment: ALL

Bronchiectasis	Controls
mean 63.12 (95% C.I. 62.94,63.3)	mean 46.1 (95% C.I. 45.9, 46.3)
median 60.0 (95% C.I. 59.77,60.2)	median 45.0 (95% C.I. 44.7, 45.2)
std 18.87 n 16	std 13.87 n 9
t-test 0.02	Mann-Whitney 0.02

MoAb: CD4/CD45RA Compartment: ALL

Bronchiectasis	Controls
mean 46.15 (95% C.I. 45.8,46.46)	mean 28.33 (95% C.I. 28.2, 28.47)
median 40.0 (95% C.I. 39.6,40.39)	median 30.0 (95% C.I. 29.8, 30.17)
std 29.59 n 13	std 10.61 n 9
t-test NS	Mann-Whitney NS

DESCRIPTIVE STATISTICS FOR TABLE 4.10

MoAb: CD4/CD7 Compartment: ALL

Follicular		Non-follicular	
mean 45.55	(95% C.I. 36.6, 56.5);	mean 7.5	(95% C.I.-10.2, 25.2)
median 50.0	(95% C.I. 36.3, 63.7);	median 0	(95% C.I.-22.2, 22.2)
std 14.24 n 9	;	std 21.2 n 8	
t-test 0.002	;	Mann-Whitney 0.005	

MoAb: CD4/CD25 Compartment: ALL

Follicular		Non-follicular	
mean 2.62	(95% C.I. 1.74, 3.51);	mean 0.01	(95% C.I.-0.01, 0.01)
median 3.0	(95% C.I. 1.89, 4.11);	median 0	(95% C.I.-0.01, 0.01)
std 1.06 n 8	;	std 0.01 n 9	
t-test 0.0002	;	Mann-Whitney 0.001	

MoAb: CD4/CD38 Compartment: ALL

Follicular		Non-folicular	
mean 10.0	(95% C.I. 6.67, 13.3);	mean 2.22	(95% C.I.-2.90, 7.34)
median 10.0	(95% C.I. 5.83, 14.2);	median 0	(95% C.I.-6.42, 6.42)
std 4.33 n 9	;	std 6.66 n 9	
t-test 0.02	;	Mann-Whitney 0.003	

MoAb: CD4/HLA-DR Compartment: ALL

Follicular		Non-follicular	
mean 33.5	(95% C.I. 11.41,55.6);	mean 10.0	(95% C.I.-0.17, 20.17)
median 20.0	(95% C.I. -7.68,47.7);	median 0	(95% C.I.-12.7, 12.74)
std 26.41 n 8	;	std 13.22 n 9	
t-test 0.05	;	Mann-Whitney 0.05	

DESCRIPTIVE STATISTICS FOR TABLE 4.11

MoAb: IgA Compartment: lamina propria

Bronchiectasis	Controls
mean 1.08 (95% C.I. 0.03, 2.13)	mean 0.48 (95% C.I. 0.27, 0.68)
median 0.55 (95% C.I.-0.76, 1.86)	median 0.46 (95% C.I. 0.21, 0.74)
std 0.99 n 6	std 0.24 n 8
t-test NS	Mann-Whitney NS

MoAb: IgA Compartment: submucosa

Bronchiectasis	Controls
mean 1.63 (95% C.I. 0.75, 2.52)	mean 0.60 (95% C.I. 0.18, 1.02)
median 1.55 (95% C.I. 0.44, 2.66)	median 0.44 (95% C.I.-0.08, 0.96)
std 0.84 n 6	std 0.45 n 7
t-test 0.04	Mann-Whitney 0.03

MoAb: IgM Compartment: lamina propria

Bronchiectasis	Controls
mean 0.51 (95% C.I. 0.08, 0.32)	mean 0.33 (95% C.I. 0.02, 0.65)
median 0.61 (95% C.I. 0.37, 0.85)	median 0.31 (95% C.I.-0.08, 0.71)
std 0.20 n 7	std 0.34 n 7
t-test NS	Mann-Whitney NS

MoAb: IgM Compartment: submucosa

Bronchiectasis	Controls
mean 1.16 (95% C.I. 0.42, 1.89)	mean 0.34 (95% C.I. 0.11, 0.58)
median 1.0 (95% C.I. 0.07, 1.93)	median 0.4 (95% C.I. 0.10, 0.69)
std 0.79 n 7	std 0.25 n 7
t-test 0.04	Mann-Whitney 0.055

MoAb: IgG Compartment: lamina propria

Bronchiectasis	Controls
mean 0.75 (95% C.I. 0.30, 1.21)	mean 0.40 (95% C.I. 0.15, 0.65)
median 0.57 (95% C.I.-0.01, 1.14)	median 0.43 (95% C.I. 0.12, 0.75)
std 0.49 n 7	std 0.23 n 6
t-test NS	Mann-Whitney NS

MoAb: IgG Compartment: submucosa

Bronchiectasis	Controls
mean 1.12 (95% C.I. 0.34, 1.88)	mean 0.48 (95% C.I. 0.04, 0.93)
median 0.62 (95% C.I.-0.34, 1.58)	median 0.62 (95% C.I. 0.06, 1.18)
std 0.83 n 7	std 0.48 n 7
t-test NS	Mann-Whitney NS

DESCRIPTIVE STATISTICS FOR TABLE 4.12A

MoAb: CD3 Compartment: epithelium

Bronchiectasis	Controls
mean 6.71 (95% C.I. 6.68, 6.74);	mean 2.74 (95% C.I. 2.72, 2.76)
median 6.83 (95% C.I. 6.79, 6.86);	median 2.72 (95% C.I. 2.70, 2.74)
std 2.85 n 15 ;	std 1.05 n 7
t-test 0.0001 ;	Mann-Whitney 0.002

MoAb: TCR delta1 Compartment: epithelium

Bronchiectasis	Controls
mean 1.14 (95% C.I. 1.13, 1.14);	mean 0.28 (95% C.I. 0.11, 0.44)
median 1.10 (95% C.I. 1.09, 1.11);	median 0.22 (95% C.I. 0.01, 0.43)
std 0.54 n 15 ;	std 0.18 n 7
t-test < 0.00005 ;	Mann-Whitney 0.0004

MoAb: Beta F1 Compartment: epithelium

Bronchiectasis	Controls
mean 4.36 (95% C.I. 4.33, 4.38);	mean 2.05 (95% C.I. 1.53, 2.57)
median 4.56 (95% C.I. 4.53, 4.59);	median 2.27 (95% C.I. 1.61, 2.92
std 2.24 n 15 ;	std 0.55 n 7
t-test < 0.002 ;	Mann-Whitney 0.02

MoAb: TCR delta1/CD3 Compartment: epithelium

Bronchiectasis	Controls
mean 19.6 (95% C.I. 19.5, 19.7);	mean 10.7 (95% C.I. 5.9, 15.4)
median 15.0 (95% C.I. 14.8, 15.1);	median 12.2 (95% C.I. 6.3, 18.1)
std 11.6 n 15 ;	std 5.1 n 7
t-test < 0.03 ;	Mann-Whitney < 0.04

MoAb: Beta F1/CD3 Compartment: epithelium

Bronchiectasis	Controls
mean 66.4 (95% C.I. 66.2, 66.7);	mean 78.6 (95% C.I. 62.4, 94.9)
median 76.3 (95% C.I. 75.9, 76.6);	median 86.4 (95% C.I. 66.0, 106.8)
std 24.6 n 15 ;	std 17.6 n 7
t-test NS ;	Mann-Whitney NS

DESCRIPTIVE STATISTICS FOR TABLE 4.12B

MoAb: CD3/CD8 Compartment: epithelium

Bronchiectasis	Controls
mean 84.9 (95% C.I. 84.8, 85.0)	mean 78.8 (95% C.I. 70.7, 86.9)
median 89.0 (95% C.I. 88.9 89.3)	median 80.0 (95% C.I. 69.8, 90.2)
std 10.8 n 14	std 8.8 n 7
t-test NS	Mann-Whitney NS

MoAb: CD3/CD4 Compartment: epithelium

Bronchiectasis	Controls
mean 15.3 (95% C.I. 15.0, 15.2)	mean 10.7 (95% C.I. 4.9, 16.5)
median 13.0 (95% C.I. 12.9, 13.1)	median 10.0 (95% C.I. 2.7, 17.3)
std 10.6 n 14	std 6.32 n 7
t-test NS	Mann-Whitney NS

MoAb: CD3+/CD4-CD8- Compartment: epithelium

Bronchiectasis	Controls
mean 6.6 (95% C.I. 6.5, 6.6)	mean 3.4 (95% C.I. 1.7, 5.2)
median 5.5 (95% C.I. 5.4, 5.5)	median 4.0 (95% C.I. 1.8, 6.2)
std 3.8 n 12	std 1.9 n 7
t-test 0.03	Mann-Whitney 0.063

MoAb: CD8/CD5 Compartment: epithelium

Bronchiectasis	Controls
mean 9.5 (95% C.I. 9.4, 9.53)	mean 13.6 (95% C.I. 7.6, 19.5)
median 10.0 (95% C.I. 9.6, 10.04)	median 10.0 (95% C.I. 2.6, 17.4)
std 3.4 n 14	std 6.4 n 7
t-test NS	Mann-Whitney NS

MoAb: CD8/CD45RO Compartment: epithelium

Bronchiectasis	Controls
mean 75.5 (95% C.I. 75.4, 75.6)	mean 52.7 (95% C.I. 42.4, 62.9)
median 76.5 (95% C.I. 76.4, 76.6)	median 54.0 (95% C.I. 41.1, 66.8)
std 8.84 n 10	std 11.1 n 7
t-test 0.001	Mann-Whitney 0.002

MoAb: CD8/HLA-DR Compartment: epithelium

Bronchiectasis	Controls
mean 15.9 (95% C.I. 15.8, 16.0)	mean 3.8 (95% C.I. 1.97, 5.74)
median 15.0 (95% C.I. 14.9, 15.1)	median 3.0 (95% C.I. 0.64, 5.36)
std 7.3 n 13	std 2.0 n 7
t-test 0.0001	Mann-Whitney 0.0007

- DESCRIPTIVE STATISTICS FOR CHAPTER 5

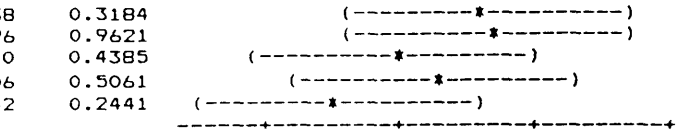
FIGURE 5.1 A - INFILTRATION OF OX-19/52+ CELLS IN BRONCHIAL EPITHELIUM

TIME POINT - 2 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	1.3138	0.3184
2	5	1.3296	0.9621
3	5	0.9910	0.4385
4	5	1.1306	0.5061
5	5	0.7642	0.2441

POOLED STDEV = 0.5541



MTB > krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	1.3790	17.6	1.56
2	5	1.0200	13.2	0.07
3	5	1.0500	12.3	-0.24
4	5	1.0500	14.1	0.37
5	5	0.7240	7.8	-1.77
OVERALL	25		13.0	

H = 4.61 d.f. = 4 p = 0.330
H = 4.61 d.f. = 4 p = 0.330 (adj. for ties)

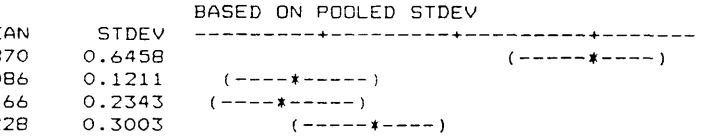
Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 4 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	1.7870	0.6458
2	5	0.5086	0.1211
3	5	0.4466	0.2343
4	5	0.8228	0.3003
5	5	0.3868	0.0456

POOLED STDEV = 0.3403



MTB > krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	1.9460	22.6	3.26
2	5	0.4280	11.3	-0.58
3	5	0.3830	8.0	-1.70
4	5	0.9820	15.6	0.88
5	5	0.3630	7.5	-1.87
OVERALL	25		13.0	

H = 14.50 d.f. = 4 p = 0.006
H = 14.50 d.f. = 4 p = 0.006 (adj. for ties)

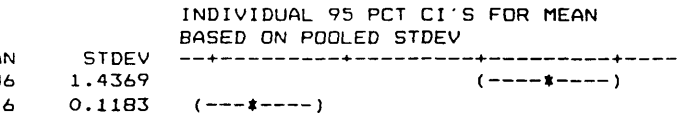
Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 8 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	3.7686	1.4369
2	5	0.5016	0.1183
3	5	0.5126	0.1573
4	5	0.7736	0.3974

POOLED STDEV = 0.7519



MTB > krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	3.7500	18.0	3.27
2	5	0.4760	7.0	-1.53
3	5	0.5270	7.2	-1.44
4	5	0.7840	9.8	-0.31
OVERALL	20		10.5	

H = 11.41 d.f. = 3 p = 0.010

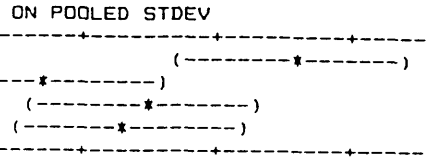
Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=N

TIME POINT: 12 WEEKS

LEVEL	N	MEAN	STDEV
1	5	2.0654	1.3544
2	5	0.5946	0.1707
3	5	1.1880	0.3273
4	5	1.0760	0.1868

POOLED STDEV = 0.7081

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV




```
MTB > krus c6 c7
```

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	1.4000	16.2	2.49
2	5	0.5000	3.0	-3.27
3	5	1.1200	11.9	0.61
4	5	1.1200	10.9	0.17
OVERALL	20		10.5	

H = 12.98 d.f. = 3 p = 0.005
H = 12.99 d.f. = 3 p = 0.005 (adj. for ties)

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

TIME POINT: 16 WEEKS

TWOSAMPLE T FOR C1 VS C2

	N	MEAN	STDEV	SE MEAN
C1	4	1.469	0.993	0.50
C2	5	0.459	0.167	0.075

95 PCT CI FOR MU C1 - MU C2: (-0.59, 2.607)

TTEST MU C1 = MU C2 (VS NE): T= 2.01 P=0.14 DF= 3

```
MTB > mann c1 c2

Mann-Whitney Confidence Interval and Test

C1          N =   4     Median =       1.204
C2          N =   5     Median =       0.384
Point estimate for ETA1-ETA2 is        0.678
96.3 pct c.i. for ETA1-ETA2 is (0.137,2.473)
W = 29.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0373
The test is significant at 0.0365 (adjusted for ties)
```

Level 1=Pa+LIG; Level 2=N

TIME POINT: 20 WEEKS

TWOSAMPLE T FOR C1 VS C2

	N	MEAN	STDEV	SE MEAN
C1	4	1.738	0.327	0.16
C2	5	0.511	0.160	0.072

95 PCT CI FOR MU C1 - MU C2: (0.73, 1.722)

TTEST MU C1 = MU C2 (VS NE): T= 6.88 P=0.0023 DF= 4

```
MTB > mann c1 c2

Mann-Whitney Confidence Interval and Test

C1          N =   4     Median =       1.7580
C2          N =   5     Median =       0.4800
Point estimate for ETA1-ETA2 is        1.2770
96.3 pct c.i. for ETA1-ETA2 is (0.6980,1.6940)
W = 30.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0200
```

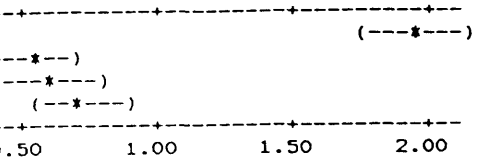
Level 1=Pa+LIG; Level 2=N

TIME POINT: 24 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	1.9522	0.2836
2	5	0.5298	0.2005
3	5	0.6160	0.1545
4	5	0.7196	0.1401

POOLED STDEV = 0.2025



DATA> .krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	2.0200	18.0	3.27
2	5	0.5200	5.8	-2.05
3	5	0.6250	7.6	-1.27
4	5	0.7220	10.6	0.04
OVERALL	20		10.5	

H = 12.39 d.f. = 3 p = 0.006
H = 12.41 d.f. = 3 p = 0.006 (adj. for ties)

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

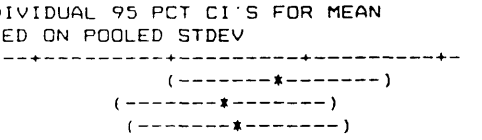
FIGURE 5.1 B - INFILTRATION OF W3/25+ CELLS IN BRONCHIAL EPITHELIUM

TIME POINT - 2 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	0.7028	0.1617
2	5	0.6050	0.3415
3	5	0.6222	0.1749
4	5	0.6428	0.1989
5	5	0.3200	0.0508

POOLED STDEV = 0.2076



MTB > krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	0.7280	17.6	1.56
2	5	0.5270	12.6	-0.14
3	5	0.5920	15.0	0.68
4	5	0.6280	16.4	1.15
5	5	0.3410	3.4	-3.26
OVERALL	25		13.0	

H = 11.91 d.f. = 4 p = 0.018

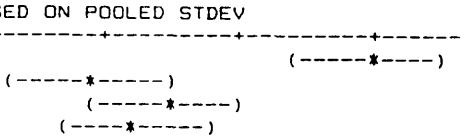
Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 4 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	0.8932	0.3232
2	5	0.2700	0.0835
3	5	0.4430	0.1975
4	5	0.3696	0.0787
5	5	0.1798	0.0662

POOLED STDEV = 0.1794



MTB > set c6krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	0.9200	22.6	3.26
2	5	0.2720	8.9	-1.39
3	5	0.4500	15.2	0.75
4	5	0.3480	14.2	0.41
5	5	0.1810	4.1	-3.02
OVERALL	25		13.0	

H = 17.95 d.f. = 4 p = 0.001
H = 17.96 d.f. = 4 p = 0.001 (adj. for ties)

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 8 WEEKS

```
                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
LEVEL      N      MEAN     STDEV   --+---------+---------+---------+----
    1      5    2.0324    0.7150                                 (-----*-----)
    2      5    0.2784    0.0523   (-----*-----)
    3      5    0.3960    0.1658     (-----*-----)
    4      5    0.6106    0.4157        (---  -*----)
                                    --+---------+---------+---------+----
POOLED STDEV =    0.4226            0.00      0.70      1.40      2.10
```

```
DATA> krus c6 c7

LEVEL     NOBS     MEDIAN  AVE. RANK    Z VALUE
    1        5     1.8700        18.0       3.27
    2        5     0.2840         5.2      -2.31
    3        5     0.3500         8.8      -0.74
    4        5     0.3280        10.0      -0.22
OVERALL     20                   10.5

H = 12.50  d.f. = 3  p = 0.006
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=N

TIME POINT: 12 WEEKS

```
                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
LEVEL      N      MEAN     STDEV   --+---------+---------+---------+----
    1      5    1.3880    0.7890                           (-------*-------)
    2      5    0.3432    0.0868  (-------*-------)
    3      5    0.5840    0.2659        (-------*-------)
    4      5    0.5720    0.1404       (-------*-------)
                                    --+---------+---------+---------+----
POOLED STDEV =    0.4244            0.00      0.50      1.00      1.50
```

```
MTB > krus c6 c7

LEVEL     NOBS     MEDIAN  AVE. RANK    Z VALUE
    1        5     1.2200        16.8       2.75
    2        5     0.3240         4.3      -2.71
    3        5     0.4800         9.7      -0.35
    4        5     0.5200        11.2       0.31
OVERALL     20                   10.5

H = 11.32  d.f. = 3  p = 0.010
H = 11.43  d.f. = 3  p = 0.010 (adj. for ties)
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

TIME POINT: 16 WEEKS

```
TWOSAMPLE T FOR C1 VS C2
    N      MEAN     STDEV   SE MEAN
C1  4     0.892     0.576      0.29
C2  4     0.275     0.143     0.072

95 PCT CI FOR MU C1 - MU C2: (-0.33, 1.561)

TTEST MU C1 = MU C2 (VS NE): T= 2.08  P=0.13  DF=  3

MTB > mann c1 c2

Mann-Whitney Confidence Interval and Test

C1          N =   4     Median =       0.720
C2          N =   4     Median =       0.214
Point estimate for ETA1-ETA2 is        0.422
97.0 pct c.i. for ETA1-ETA2 is (-0.077,1.536)
W = 25.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0606

Cannot reject at alpha = 0.05
```

Level 1=Pa+LIG; Level 2=N

TIME POINT: 20 WEEKS

```
TWOSAMPLE T FOR C1 VS C2
    N      MEAN     STDEV   SE MEAN
C1  4     1.033     0.217      0.11
C2  4     0.325     0.106     0.053

95 PCT CI FOR MU C1 - MU C2: (0.37, 1.042)

TTEST MU C1 = MU C2 (VS NE): T= 5.87  P=0.0042  DF=  4

MTB > mann c1 c2

Mann-Whitney Confidence Interval and Test

C1          N =    4      Median =       1.0040
C2          N =    4      Median =       0.3470
Point estimate for ETA1-ETA2 is       0.6750
97.0 pct c.i. for ETA1-ETA2 is (0.3879,1.1281)
W = 26.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0304
```

Level 1=Pa+LIG; Level 2=N

TIME POINT: 24 WEEKS

```
                                  INDIVIDUAL 95 PCT CI'S FOR MEAN
                                  BASED ON POOLED STDEV
LEVEL       N      MEAN     STDEV ---------+---------+---------+---------
    1       5    1.0640    0.1479                                (---*----)
    2       5    0.3592    0.2680       (---*----)
    3       5    0.2584    0.1209    (---*----)
    4       5    0.2440    0.1124    (----*----)
                                     ---------+---------+---------+---------
POOLED STDEV =   0.1739               0.35      0.70      1.05

MTB > krus c6 c7

LEVEL     NOBS     MEDIAN  AVE. RANK   Z VALUE
    1        5     1.0800       18.0      3.27
    2        5     0.2500        9.3     -0.52
    3        5     0.2160        7.8     -1.18
    4        5     0.2500        6.9     -1.57
OVERALL     20                   10.5

H = 11.13  d.f. = 3  p = 0.011
H = 11.15  d.f. = 3  p = 0.011 (adj. for ties)
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

FIGURE 5.1 C - INFILTRATION OF OX-8+ CELLS IN BRONCHIAL EPITHELIUM

TIME POINT - 2 WEEKS

```
                                  INDIVIDUAL 95 PCT CI'S FOR MEAN
                                  BASED ON POOLED STDEV
LEVEL       N      MEAN     STDEV --+---------+---------+---------+----
    1       5    0.6680    0.2246    (----------*----------)
    2       5    0.9156    0.6582              (----------*----------)
    3       5    0.7760    0.2324         (----------*----------)
    4       5    0.7686    0.2703         (----------*----------)
    5       5    0.5928    0.1992    (----------*----------)
                                     --+---------+---------+---------+----
POOLED STDEV =   0.3607              0.30      0.60      0.90      1.20
MTB > krus c6 c7

LEVEL     NOBS     MEDIAN  AVE. RANK   Z VALUE
    1        5     0.6720       11.8     -0.41
    2        5     0.7480       13.4      0.14
    3        5     0.8500       15.7      0.92
    4        5     0.8400       14.5      0.51
    5        5     0.5720        9.6     -1.15
OVERALL     25                   13.0

H = 2.10  d.f. = 4  p = 0.718
H = 2.10  d.f. = 4  p = 0.717 (adj. for ties)
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 4 WEEKS

```
                                        INDIVIDUAL 95 PCT CI'S FOR MEAN
                                        BASED ON POOLED STDEV
LEVEL      N      MEAN      STDEV      ---------+---------+---------+-------
    1      5    1.3052     0.4999                          (----*----)
    2      5    0.3884     0.0914       (----*----)
    3      5    0.4482     0.2345        (----*----)
    4      5    0.5390     0.2040          (----*----)
    5      5    0.3236     0.0503     (----*----)
                                       ---------+---------+---------+-------
POOLED STDEV =    0.2673                     0.50      1.00      1.50
```

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	1.4200	22.6	3.26
2	5	0.3720	9.4	-1.22
3	5	0.5200	11.8	-0.41
4	5	0.6400	14.0	0.34
5	5	0.3410	7.2	-1.97
OVERALL	25		13.0	

H = 13.03 d.f. = 4 p = 0.011

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 8 WEEKS

```
                                        INDIVIDUAL 95 PCT CI'S FOR MEAN
                                        BASED ON POOLED STDEV
LEVEL      N      MEAN      STDEV      ---+---------+---------+---------+----
    1      5    2.3768     0.7637                                  (----*----)
    2      5    0.3312     0.1157     (----*----)
    3      5    0.4314     0.2575      (----*----)
    4      5    0.2708     0.1526     (----*----)
                                       ---+---------+---------+---------+----
POOLED STDEV =    0.4142               0.00      0.80      1.60      2.40
```

DATA> krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	2.7420	18.0	3.27
2	5	0.2780	7.6	-1.27
3	5	0.3350	9.6	-0.39
4	5	0.2800	6.8	-1.61
OVERALL	20		10.5	

H = 11.31 d.f. = 3 p = 0.010
H = 11.32 d.f. = 3 p = 0.010 (adj. for ties)

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=N

TIME POINT: 12 WEEKS

```
                                        INDIVIDUAL 95 PCT CI'S FOR MEAN
                                        BASED ON POOLED STDEV
LEVEL      N      MEAN      STDEV      ----+---------+---------+---------+--
    1      5    1.4580     0.9718                            (--------*--------)
    2      5    0.3260     0.1658     (--------*--------)
    3      5    0.7680     0.4129              (--------*--------)
    4      5    0.6880     0.1404            (--------*--------)
                                       ----+---------+---------+---------+--
POOLED STDEV =    0.5390               0.00      0.60      1.20      1.80
```

MTB > krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	0.9000	16.1	2.44
2	5	0.3200	4.2	-2.75
3	5	0.8200	11.3	0.35
4	5	0.7200	10.4	-0.04
OVERALL	20		10.5	

H = 10.24 d.f. = 3 p = 0.017
H = 10.26 d.f. = 3 p = 0.017 (adj. for ties)

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

TIME POINT: 16 WEEKS

```
TWOSAMPLE T FOR C1 VS C2
    N      MEAN      STDEV    SE MEAN
C1  4     1.046      0.569       0.28
C2  5     0.301      0.147      0.066

95 PCT CI FOR MU C1 - MU C2: (-0.18, 1.673)

TTEST MU C1 = MU C2 (VS NE):    2.55  P=0.084  DF=  3

MTB > mann c1 c2

Mann-Whitney Confidence Interval and Test

C1          N =    4     Median =       0.927
C2          N =    5     Median =       0.308
Point estimate for ETA1-ETA2 is        0.612
96.3 pct c.i. for ETA1-ETA2 is (0.196,1.566)
W = 29.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0373
```

Level 1=Pa+LIG; Level 2=N

TIME POINT: 20 WEEKS

```
TWOSAMPLE T FOR C1 VS C2
    N      MEAN      STDEV    SE MEAN
C1  4     1.266      0.173      0.087
C2  5     0.329      0.125      0.056

95 PCT CI FOR MU C1 - MU C2: (0.672, 1.202)

TTEST MU C1 = MU C2 (VS NE): T= 9.09  P=0.0003  DF=  5

MTB > mann c1 c2

Mann-Whitney Confidence Interval and Test

C1          N =    4     Median =       1.2660
C2          N =    5     Median =       0.3720
Point estimate for ETA1-ETA2 is        0.9955
96.3 pct c.i. for ETA1-ETA2 is (0.6970,1.2941)
W = 30.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0200
```

Level 1=Pa+LIG; Level 2=N

TIME POINT: 24 WEEKS

```
                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
LEVEL      N      MEAN     STDEV   ----+---------+---------+---------+--
    1      5    1.2840    0.1780                                (----*---)
    2      5    0.3640    0.0891  (---*----)
    3      5    0.4596    0.1512     (---*---)
    4      5    0.4784    0.1878     (---*---)
                                    ----+---------+---------+---------+--
POOLED STDEV =  0.1563              0.35      0.70      1.05      1.40

MTB > krus c8 c7

LEVEL     NOBS     MEDIAN  AVE. RANK   Z VALUE
    1        5     1.3200       18.0      3.27
    2        5     0.3800        6.1     -1.92
    3        5     0.4280        9.0     -0.65
    4        5     0.4220        8.9     -0.70
OVERALL     20                  10.5

H = 11.49  d.f. = 3  p = 0.010
H = 11.50  d.f. = 3  p = 0.010 (adj. for ties)
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

FIGURE 5.1 D - INFILTRATION OF ED1+ CELLS IN BRONCHIAL EPITHELIUM

TIME POINT - 2 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	3.231	2.001
2	5	2.141	0.816
3	5	1.535	0.749
4	5	1.937	1.561
5	5	0.744	0.239

POOLED STDEV = 1.243

0.0 1.5 3.0 4.5

DATA> krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	2.8400	18.8	1.97
2	5	1.8130	16.5	1.19
3	5	1.3500	12.2	-0.27
4	5	1.5380	12.9	-0.03
5	5	0.7240	4.6	-2.85
OVERALL	25		13.0	

H = 10.81 d.f. = 4 p = 0.029
H = 10.81 d.f. = 4 p = 0.029 (adj. for ties)

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 4 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	2.0860	1.6880
2	5	0.7294	0.1775
3	5	0.7990	0.2877
4	5	0.8748	0.3352
5	5	1.0318	0.3608

POOLED STDEV = 0.8008

0.00 0.80 1.60 2.40

MTB krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	1.0060	18.2	1.77
2	5	0.6780	8.0	-1.70
3	5	0.8200	9.8	-1.09
4	5	0.9920	12.4	-0.20
5	5	1.0900	16.6	1.22
OVERALL	25		13.0	

H = 6.98 d.f. = 4 p = 0.138

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 8 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	2.6784	1.6598
2	5	0.8116	0.3134
3	5	0.7404	0.3108
4	5	1.0586	0.5169

POOLED STDEV = 0.8968

0.0 1.0 2.0 3.0

MTB > krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	1.9480	17.8	3.19
2	5	0.7140	7.5	-1.31
3	5	0.8500	6.3	-1.83
4	5	1.0800	10.4	-0.04
OVERALL	20		10.5	

H = 11.42 d.f. = 3 p = 0.010
H = 11.43 d.f. = 3 p = 0.010 (adj. for ties)

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=N

TIME POINT: 12 WEEKS

```
                                        INDIVIDUAL 95 PCT CI'S FOR MEAN
                                        BASED ON POOLED STDEV
LEVEL      N      MEAN      STDEV  -+---------+---------+---------+-----
    1      5    2.0024     1.3186                      (-------*-------)
    2      5    0.6180     0.2067  (-------*-------)
    3      5    0.9200     0.2112     (--------*-------)
    4      5    0.6204     0.2170  (-------*-------)
                                   -+---------+---------+---------+-----
POOLED STDEV =   0.6843          0.00      0.80      1.60      2.40

 MTB > sekrus c6 c7

 LEVEL     NOBS     MEDIAN  AVE. RANK    Z VALUE
     1        5     1.8000        17.2       2.92
     2        5     0.5880         6.2      -1.88
     3        5     0.8400        12.2       0.74
     4        5     0.5760         6.4      -1.79
 OVERALL     20                   10.5

 H = 11.87  d.f. = 3  p = 0.008
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

TIME POINT: 16 WEEKS

```
 TWOSAMPLE T FOR C1 VS C2
     N      MEAN     STDEV   SE MEAN
C1   4     0.688     0.351      0.18
C2   4     0.774     0.174     0.087

 95 PCT CI FOR MU C1 - MU C2: (-0.63, 0.459)

 TTEST MU C1 = MU C2 (VS NE): T= -0.44  P=0.69  DF=  4

 MTB > mann c1 c2

 Mann-Whitney Confidence Interval and Test

 C1          N =    4     Median =      0.5330
 C2          N =    4     Median =      0.7595
 Point estimate for ETA1-ETA2 is      -0.1935
 97.0 pct c.i. for ETA1-ETA2 is (-0.5243,0.6359)
 W = 14.0
 Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.3123

 Cannot reject at alpha = 0.05
```

Level 1=Pa+LIG; Level 2=N

TIME POINT: 20 WEEKS

```
 TWOSAMPLE T FOR C1 VS C2
     N      MEAN     STDEV   SE MEAN
C1   4     1.149     0.396      0.20
C2   5     0.517     0.205     0.092

 95 PCT CI FOR MU C1 - MU C2: (0.03, 1.239)

 TTEST MU C1 = MU C2 (VS NE): T= 2.90  P=0.044  DF=  4

 MTB > mann c1 c2

 Mann-Whitney Confidence Interval and Test

 C1          N =    4     Median =      1.0225
 C2          N =    5     Median =      0.5700
 Point estimate for ETA1-ETA2 is      0.5235
 96.3 pct c.i. for ETA1-ETA2 is (0.2049,1.2959)
 W = 30.0
 Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0200
```

Level 1=Pa+LIG; Level 2=N

TIME POINT: 24 WEEKS

```
                                        INDIVIDUAL 95 PCT CI'S FOR MEAN
                                        BASED ON POOLED STDEV
LEVEL      N      MEAN     STDEV  ------+---------+---------+---------+
    1      5    1.3114    0.3653                          (-------*------)
    2      5    0.5010    0.2832   (-------*------)
    3      5    0.8104    0.3519           (------*-------)
    4      5    0.7244    0.2661         (------*-------)
                                    ------+---------+---------+---------+
POOLED STDEV =    0.3195             0.40      0.80      1.20      1.60
```

```
DATA> kaus c6 c7

LEVEL     NOBS     MEDIAN  AVE. RANK    Z VALUE
    1        5     1.2000       17.0        2.84
    2        5     0.5800        6.0       -1.96
    3        5     0.7000       10.0       -0.22
    4        5     0.7180        9.0       -0.65
OVERALL     20                   10.5

H = 9.29  d.f. = 3  p = 0.026
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

FIGURE 5.2 A - INFILTRATION OF OX-19/52+ CELLS IN LAMINA PROPRIA

TIME POINT - 2 WEEKS

```
                                        INDIVIDUAL 95 PCT CI'S FOR MEAN
                                        BASED ON POOLED STDEV
LEVEL      N      MEAN     STDEV  --+---------+---------+---------+----
    1      5     4.673     1.786                                (-----*-----)
    2      5     1.704     0.769          (-----*-----)
    3      5     1.809     0.776          (-----*-----)
    4      5     2.114     0.893            (-----*-----)
    5      5     0.732     0.247   (-----*-----)
                                    --+---------+---------+---------+----
POOLED STDEV =    1.024            0.0       1.6       3.2       4.8
```

```
DATA> krus c6 c7

LEVEL     NOBS     MEDIAN  AVE. RANK    Z VALUE
    1        5     4.3650       22.0        3.06
    2        5     2.0330       11.8       -0.41
    3        5     2.0000       12.4       -0.20
    4        5     2.0600       15.2        0.75
    5        5     0.6750        3.6       -3.19
OVERALL     25                   13.0

H = 16.25  d.f. = 4  p = 0.003
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 4 WEEKS

```
                                        INDIVIDUAL 95 PCT CI'S FOR MEAN
                                        BASED ON POOLED STDEV
LEVEL      N      MEAN     STDEV  -+---------+---------+---------+-----
    1      5    4.5138    1.2462                                (---*---)
    2      5    0.4420    0.1937  (---*---)
    3      5    0.6814    0.2275    (---*--)
    4      5    0.5510    0.2867   (---*--)
    5      5    0.4162    0.2633  (---*---)
                                   -+---------+---------+---------+-----
POOLED STDEV =    0.5990          0.0       1.5       3.0       4.5
```

```
MTB > krus c6 c7

LEVEL     NOBS     MEDIAN  AVE. RANK    Z VALUE
    1        5     4.4120       23.0        3.40
    2        5     0.4200        9.0       -1.36
    3        5     0.7850       14.7        0.58
    4        5     0.4500       11.5       -0.51
    5        5     0.3140        6.8       -2.11
OVERALL     25                   13.0

H = 14.73  d.f. = 4  p = 0.006
H = 14.74  d.f. = 4  p = 0.006 (adj. for ties)
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 8 WEEKS

```
                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
LEVEL      N      MEAN     STDEV   --+---------+---------+---------+----
    1      5     5.695     1.995                                (----*-----)
    2      5     0.777     0.596    (----*----)
    3      5     0.649     0.232   (----*----)
    4      5     0.937     0.462     (----*----)
                                    --+---------+---------+---------+----
POOLED STDEV =    1.073             0.0       2.0       4.0       6.0
```

```
DATA> krus c6 c7

LEVEL     NOBS     MEDIAN  AVE. RANK   Z VALUE
    1        5     4.8790        18.0      3.27
    2        5     0.5080         7.4     -1.35
    3        5     0.6900         6.8     -1.61
    4        5     0.7520         9.8     -0.31
OVERALL     20                   10.5

H = 11.43  d.f. = 3  p = 0.010
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=N

TIME POINT: 12 WEEKS

```
                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
LEVEL      N      MEAN     STDEV   ---+---------+---------+---------+---
    1      5    4.2150    1.7510                               (-----*-----)
    2      5    0.5070    0.2111   (----*-----)
    3      5    0.6372    0.2327    (----*-----)
    4      5    0.6320    0.2326    (----*-----)
                                    ---+---------+---------+---------+---
POOLED STDEV =   0.8971             0.0       1.5       3.0       4.5
```

```
DATA> krus c6 c7

LEVEL     NOBS     MEDIAN  AVE. RANK   Z VALUE
    1        5     3.8400        18.0      3.27
    2        5     0.4800         6.4     -1.79
    3        5     0.6250         8.9     -0.70
    4        5     0.5800         8.7     -0.79
OVERALL     20                   10.5

H = 11.27  d.f. = 3  p = 0.011
H = 11.27  d.f. = 3  p = 0.011 (adj. for ties)
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

TIME POINT: 16 WEEKS

```
TWOSAMPLE T FOR C1 VS C2
    N      MEAN     STDEV   SE MEAN
C1  5     0.577     0.118     0.053
C2  5     1.562     0.337      0.15

95 PCT CI FOR MU C1 - MU C2: (-1.428, -0.54)

TTEST MU C1 = MU C2 (VS NE): T= -6.17  P=0.0035  DF=  4

MTB > mann c1 c2

Mann-Whitney Confidence Interval and Test

C1          N =   5     Median =      0.6180
C2          N =   5     Median =      1.4000
Point estimate for ETA1-ETA2 is     -0.8720
96.3 pct c.i. for ETA1-ETA2 is (-1.5421,-0.6779)
W = 15.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0122
The test is significant at 0.0119 (adjusted for ties)
```

Level 2=Pa+LIG; Level 1=N

TIME POINT: 20 WEEKS

```
TWOSAMPLE T FOR C1 VS C2
     N      MEAN     STDEV   SE MEAN
C1   5     2.950     0.372      0.17
C2   5     0.715     0.234      0.10

95 PCT CI FOR MU C1 - MU C2: (1.75, 2.72)

TTEST MU C1 = MU C2 (VS NE): T= 11.37  P=0.0000  DF=  6

MTB > mann c1 c2

Mann-Whitney Confidence Interval and Test

C1          N =    5     Median =      3.0000
C2          N =    5     Median =      0.7180
Point estimate for ETA1-ETA2 is       2.2820
96.3 pct c.i. for ETA1-ETA2 is (1.6979,2.7403)
W = 40.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0122
The test is significant at 0.0119 (adjusted for ties)
```

Level 1=Pa+LIG; Level 2=N

TIME POINT: 24 WEEKS

```
                                  INDIVIDUAL 95 PCT CI'S FOR MEAN
                                  BASED ON POOLED STDEV
LEVEL      N      MEAN     STDEV  --+---------+---------+---------+----
    1      5     5.824     2.010                                 (----*----)
    2      5     0.726     0.207   (----*----)
    3      5     0.799     0.561   (----*----)
    4      5     1.062     0.251     (----*----)
                                  --+---------+---------+---------+----
POOLED STDEV =    1.056           0.0       2.0       4.0       6.0

  DATA> krus c6 c7

  LEVEL     NOBS     MEDIAN  AVE. RANK   Z VALUE
      1        5     6.4160        18.0      3.27
      2        5     0.7800         6.3     -1.83
      3        5     0.7140         6.8     -1.61
      4        5     1.0200        10.9      0.17
  OVERALL     20                   10.5

  H = 12.53  d.f. = 3  p = 0.006
  H = 12.54  d.f. = 3  p = 0.006 (adj. for ties)
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

FIGURE 5.2 B - INFILTRATION OF W3/25+ CELLS IN LAMINA PROPRIA

TIME POINT - 2 WEEKS

```
                                  INDIVIDUAL 95 PCT CI'S FOR MEAN
                                  BASED ON POOLED STDEV
  LEVEL      N      MEAN     STDEV  ----+---------+---------+---------+--
      1      5    2.8074    1.2323                       (-----*------)
      2      5    1.2804    0.8113            (------*-----)
      3      5    1.2440    0.6103            (-----*------)
      4      5    1.3848    1.0211             (------*-----)
      5      5    0.3588    0.1056  (------*------)
                                  ----+---------+---------+---------+--
  POOLED STDEV =    0.8489          0.0       1.2       2.4       3.6
  MTB > krus c6 c7

  LEVEL     NOBS     MEDIAN  AVE. RANK   Z VALUE
      1        5     2.3280        21.2      2.79
      2        5     1.7420        13.0      0.00
      3        5     1.2000        13.6      0.20
      4        5     1.4240        13.3      0.10
      5        5     0.3280         3.9     -3.09
  OVERALL     25                   13.0

  H = 13.89  d.f. = 4  p = 0.008
  H = 13.91  d.f. = 4  p = 0.008 (adj. for ties)
```

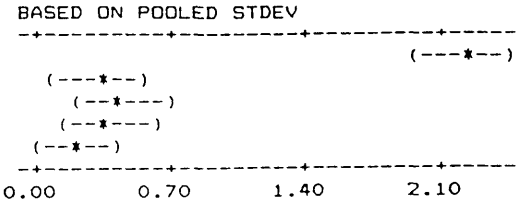
Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 4 WEEKS

LEVEL	N	MEAN	STDEV
1	5	2.2328	0.4689
2	5	0.3344	0.1327
3	5	0.4300	0.2162
4	5	0.3788	0.1729
5	5	0.2084	0.1543

POOLED STDEV = 0.2600

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV



MTB > krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	2.4200	23.0	3.40
2	5	0.3720	10.5	-0.85
3	5	0.4400	13.6	0.20
4	5	0.3480	11.6	-0.48
5	5	0.1480	6.3	-2.28
OVERALL	25		13.0	

H = 14.17 d.f. = 4 p = 0.007
H = 14.18 d.f. = 4 p = 0.007 (adj. for ties)

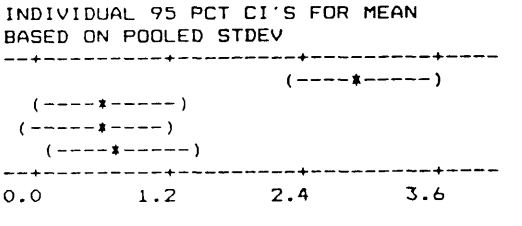
Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 8 WEEKS

LEVEL	N	MEAN	STDEV
1	5	2.9040	1.2730
2	5	0.6284	0.4935
3	5	0.5552	0.2950
4	5	0.7688	0.3322

POOLED STDEV = 0.7179

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV



MTB > krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	2.3400	18.0	3.27
2	5	0.4200	7.2	-1.44
3	5	0.4400	7.3	-1.40
4	5	0.7180	9.5	-0.44
OVERALL	20		10.5	

H = 11.20 d.f. = 3 p = 0.011
H = 11.21 d.f. = 3 p = 0.011 (adj. for ties)

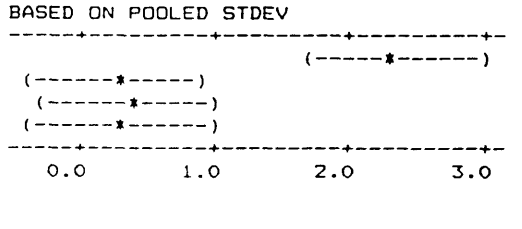
Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=N

TIME POINT: 12 WEEKS

LEVEL	N	MEAN	STDEV
1	5	2.3440	1.3879
2	5	0.2560	0.1851
3	5	0.3692	0.2568
4	5	0.3146	0.1236

POOLED STDEV = 0.7145

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV



DATA> krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	1.8400	18.0	3.27
2	5	0.3600	6.9	-1.57
3	5	0.4480	9.3	-0.52
4	5	0.2480	7.8	-1.18
OVERALL	20		10.5	

H = 11.13 d.f. = 3 p = 0.011
H = 11.18 d.f. = 3 p = 0.011 (adj. for ties)

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

TIME POINT: 16 WEEKS

```
TWOSAMPLE T FOR C1 VS C2
    N      MEAN      STDEV   SE MEAN
C1  5     0.332      0.120     0.054
C2  5     0.818      0.189     0.085

95 PCT CI FOR MU C1 - MU C2: (-0.731, -0.241)

TTEST MU C1 = MU C2 (VS NE): T= -4.85  P=0.0028  DF=  6

MTB > mann c1 c2

Mann-Whitney Confidence Interval and Test

C1          N =    5     Median =      0.3180
C2          N =    5     Median =      0.8000
Point estimate for ETA1-ETA2 is      -0.4820
96.3 pct c.i. for ETA1-ETA2 is (-0.7999,-0.2300)
W = 15.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0122
```

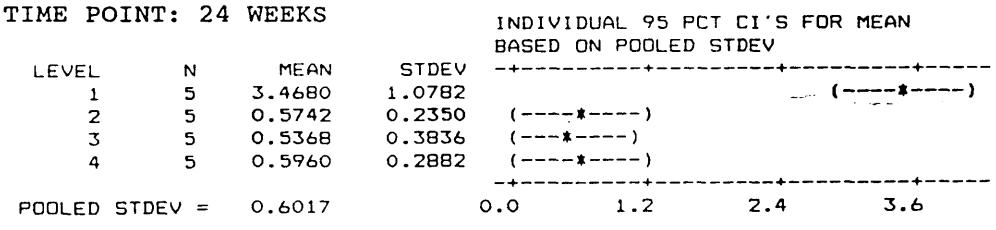
Level 2=Pa+LIG; Level 1=N

TIME POINT: 20 WEEKS

```
TWOSAMPLE T FOR C1 VS C2
    N      MEAN      STDEV   SE MEAN
C1  5     1.567      0.463     0.21
C2  5     0.595      0.252     0.11

95 PCT CI FOR MU C1 - MU C2: (0.40, 1.55)

TTEST MU C1 = MU C2 (VS NE): T= 4.13  P=0.0062  DF=  6

MTB > mann c1 c2

Mann-Whitney Confidence Interval and Test

C1          N =    5     Median =      1.5180
C2          N =    5     Median =      0.4840
Point estimate for ETA1-ETA2 is      1.0180
96.3 pct c.i. for ETA1-ETA2 is (0.4063,1.6218)
W = 40.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0122
The test is significant at 0.0119 (adjusted for ties)
```

Level 1=Pa+LIG; Level 2=N

TIME POINT: 24 WEEKS




```
DATA> krus c6 c7

LEVEL     NOBS     MEDIAN  AVE. RANK   Z VALUE
    1        5     3.7400       18.0      3.27
    2        5     0.4500        8.6     -0.83
    3        5     0.4120        7.0     -1.53
    4        5     0.5000        8.4     -0.92
OVERALL     20                  10.5

H = 10.93  d.f. = 3  p = 0.012
H = 10.94  d.f. = 3  p = 0.012 (adj. for ties)
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

FIGURE 5.2 C - INFILTRATION OF OX-8+ CELLS IN LAMINA PROPRIA

TIME POINT - 2 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	2.7312	1.2254
2	5	0.5938	0.1416
3	5	1.4740	0.4479
4	5	1.5462	0.6728
5	5	0.6768	0.1652

POOLED STDEV = 0.6637

0.0 1.0 2.0 3.0

MTB > krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	3.1250	21.0	2.72
2	5	0.6250	4.3	-2.96
3	5	1.5600	16.2	1.09
4	5	1.5000	16.4	1.15
5	5	0.7280	7.1	-2.00
OVERALL	25		13.0	

H = 18.12 d.f. = 4 p = 0.001
H = 18.13 d.f. = 4 p = 0.001 (adj. for ties)

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 4 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	2.9066	1.6460
2	5	0.2374	0.1529
3	5	0.5240	0.2332
4	5	0.4428	0.2364
5	5	0.2664	0.1542

POOLED STDEV = 0.7572

0.0 1.2 2.4 3.6

MTB > krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	2.2900	23.0	3.40
2	5	0.1660	6.4	-2.24
3	5	0.5500	14.4	0.48
4	5	0.4200	12.2	-0.27
5	5	0.2080	9.0	-1.36
OVERALL	25		13.0	

H = 14.97 d.f. = 4 p = 0.005
H = 15.00 d.f. = 4 p = 0.005 (adj. for ties)

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 8 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	4.5876	1.4568
2	5	0.2664	0.1984
3	5	0.4986	0.2150
4	5	0.3020	0.2577

POOLED STDEV = 0.7540

0.0 1.6 3.2 4.8

DATA> krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	4.8200	18.0	3.27
2	5	0.1800	6.3	-1.83
3	5	0.5640	10.8	0.13
4	5	0.1800	6.9	-1.57
OVERALL	20		10.5	

H = 12.42 d.f. = 3 p = 0.006
H = 12.45 d.f. = 3 p = 0.006 (adj. for ties)

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=N

TIME POINT: 12 WEEKS

```
                                        INDIVIDUAL 95 PCT CI'S FOR MEAN
                                        BASED ON POOLED STDEV
  LEVEL      N      MEAN      STDEV    ----------+---------+---------+------
      1      5    2.2580     0.5958                                  (---*----)
      2      5    0.3328     0.1936  (----*---)
      3      5    0.3488     0.0766   (---*---)
      4      5    0.3990     0.1475   (----*---)
                                      ----------+---------+---------+------
POOLED STDEV =    0.3241              0.70      1.40      2.10

DATA> krus c6 c7

LEVEL     NOBS     MEDIAN  AVE. RANK   Z VALUE
    1        5     2.1400       18.0      3.27
    2        5     0.3400        6.8     -1.61
    3        5     0.3400        8.1     -1.05
    4        5     0.4000        9.1     -0.61
OVERALL     20                  10.5

H = 11.04  d.f. = 3  p = 0.012
H = 11.13  d.f. = 3  p = 0.011 (adj. for ties)
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

TIME POINT: 16 WEEKS

```
TWOSAMPLE T FOR C1 VS C2
     N      MEAN     STDEV   SE MEAN
C1   5     0.341     0.103     0.046
C2   5     0.993     0.211     0.094

95 PCT CI FOR MU C1 - MU C2: (-0.922, -0.382)

TTEST MU C1 = MU C2 (VS NE): T= -6.22  P=0.0016  DF=  5

MTB > mann c1 c2

Mann-Whitney Confidence Interval and Test

C1          N =    5     Median =      0.3180
C2          N =    5     Median =      1.1000
Point estimate for ETA1-ETA2 is      -0.7090
96.3 pct c.i. for ETA1-ETA2 is (-0.8901,-0.3459)
W = 15.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0122
```

Level 2=Pa+LIG; Level 1=N

TIME POINT: 20 WEEKS

```
TWOSAMPLE T FOR C1 VS C2
     N      MEAN     STDEV   SE MEAN
C1   5     2.015     0.366     0.16
C2   5     0.333     0.149     0.067

95 PCT CI FOR MU C1 - MU C2: (1.23, 2.136)

TTEST MU C1 = MU C2 (VS NE): T= 9.53  P=0.0002  DF=  5

MTB > mann c1 c2

Mann-Whitney Confidence Interval and Test

C1          N =    5     Median =      2.0400
C2          N =    5     Median =      0.2420
Point estimate for ETA1-ETA2 is      1.7860
96.3 pct c.i. for ETA1-ETA2 is (1.1739,2.0879)
W = 40.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0122
```

Level 1=Pa+LIG; Level 2=N

TIME POINT: 24 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	2.9120	1.0939
2	5	0.3558	0.1174
3	5	0.3400	0.3408
4	5	0.4340	0.2403

POOLED STDEV = 0.5883

MTB > krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	2.6600	18.0	3.27
2	5	0.3250	8.6	-0.83
3	5	0.2100	6.2	-1.88
4	5	0.3300	9.2	-0.57
OVERALL	20		10.5	

H = 11.43 d.f. = 3 p = 0.010
H = 11.48 d.f. = 3 p = 0.010 (adj. for ties)

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

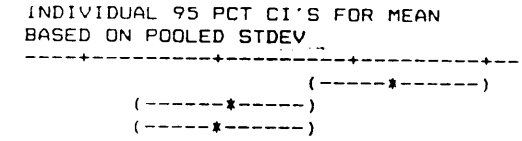
FIGURE 5.2 D - INFILTRATION OF ED1+ CELLS IN LAMINA PROPRIA

TIME POINT - 2 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	2.8074	1.2323
2	5	1.2804	0.8113
3	5	1.2440	0.6103
4	5	1.3848	1.0211
5	5	0.3588	0.1056

POOLED STDEV = 0.8489



MTB > krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	2.3280	21.2	2.79
2	5	1.7420	13.0	0.00
3	5	1.2000	13.6	0.20
4	5	1.4240	13.3	0.10
5	5	0.3280	3.9	-3.09
OVERALL	25		13.0	

H = 13.89 d.f. = 4 p = 0.008
H = 13.91 d.f. = 4 p = 0.008 (adj. for ties)

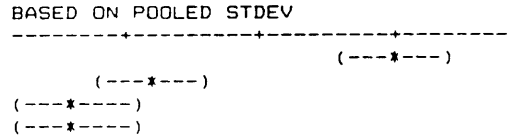
Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 4 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	6.0600	1.3074
2	5	2.3950	1.1965
3	5	1.2730	0.5973
4	5	1.2966	0.6887
5	5	2.5108	0.2795

POOLED STDEV = 0.9000



LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	6.607	23.0	3.40
2	5	2.085	13.0	0.00
3	5	1.255	6.2	-2.31
4	5	1.270	7.4	-1.90
5	5	2.346	15.4	0.82
OVERALL	25		13.0	

H = 16.93 d.f. = 4 p = 0.002

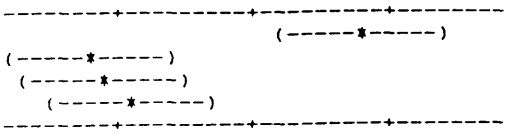
Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 8 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	5.618	1.876
2	5	1.617	0.482
3	5	1.774	1.052
4	5	2.225	1.043

POOLED STDEV = 1.219



DATA> krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	6.560	17.6	3.10
2	5	1.754	7.4	-1.35
3	5	1.250	7.0	-1.53
4	5	1.780	10.0	-0.22
OVERALL	20		10.5	

H = 10.36 d.f. = 3 p = 0.016

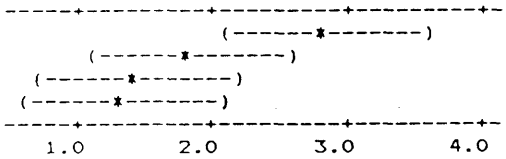
Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=N

TIME POINT: 12 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	2.8248	1.0939
2	5	1.8200	1.0606
3	5	1.4320	0.1842
4	5	1.3154	0.3611

POOLED STDEV = 0.7883



DATA> krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	3.160	16.1	2.44
2	5	1.380	9.9	-0.26
3	5	1.370	9.3	-0.52
4	5	1.210	6.7	-1.66
OVERALL	20		10.5	

H = 6.80 d.f. = 3 p = 0.079
H = 6.81 d.f. = 3 p = 0.079 (adj. for ties)

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

TIME POINT: 16 WEEKS

TWOSAMPLE T FOR C1 VS C2

	N	MEAN	STDEV	SE MEAN
C1	5	1.547	0.378	0.17
C2	5	2.184	0.193	0.086

95 PCT CI FOR MU C1 - MU C2: (-1.13, -0.149)

TTEST MU C1 = MU C2 (VS NE): T= -3.36 P=0.020 DF= 5

MTB > mann c1 c2

Mann-Whitney Confidence Interval and Test

C1 N = 5 Median = 1.6720
C2 N = 5 Median = 2.2000
Point estimate for ETA1-ETA2 is -0.5280
96.3 pct c.i. for ETA1-ETA2 is (-1.1793,-0.2082)
W = 15.0
Test of ETA1 = ETA2 vs. ETA1 n.e. ETA2 is significant at 0.0122

Level 2=Pa+LIG; Level 1=N

TIME POINT: 20 WEEKS

```
TWOSAMPLE T FOR C1 VS C2
    N      MEAN      STDEV   SE MEAN
C1  5     3.849      0.578      0.26
C2  5     1.690      0.501      0.22

95 PCT CI FOR MU C1 - MU C2: (1.35, 2.97)

TTEST MU C1 = MU C2 (VS NE): T= 6.31  P=0.0004  DF=  7

MTB > mann c1 c2

Mann-Whitney Confidence Interval and Test

C1          N =   5     Median =       4.000
C2          N =   5     Median =       1.722
Point estimate for ETA1-ETA2 is        2.252
96.3 pct c.i. for ETA1-ETA2 is (1.337,3.088)
W = 40.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0122
```

Level 1=Pa+LIG; Level 2=N

TIME POINT: 24 WEEKS

```
                                  INDIVIDUAL 95 PCT CI'S FOR MEAN
                                  BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  -------+---------+---------+---------
     1      5    4.6650    1.1359                            (----*----)
     2      5    1.8100    0.8589       (----*----)
     3      5    1.7994    0.2619       (----*----)
     4      5    1.3548    0.6912    (----*----)
                                     -------+---------+---------+---------
POOLED STDEV =    0.8022               1.5       3.0       4.5
```

```
DATA> krus c6 c7

LEVEL     NOBS     MEDIAN  AVE. RANK   Z VALUE
    1        5      4.500       18.0      3.27
    2        5      1.700        8.0     -1.09
    3        5      1.800        9.7     -0.35
    4        5      1.420        6.3     -1.83
OVERALL     20                  10.5

H = 11.54  d.f. = 3  p = 0.009
H = 11.55  d.f. = 3  p = 0.009 (adj. for ties)
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

FIGURE 5.2 E - INFILTRATION OF OX-12+ CELLS IN LAMINA PROPRIA

TIME POINT - 2 WEEKS

```
                                  INDIVIDUAL 95 PCT CI'S FOR MEAN
                                  BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV  ---+---------+---------+---------+---
     1      5    1.4600    0.2527                      (------*------)
     2      5    0.4000    0.3391     (------*------)
     3      5    0.7780    0.5678           (------*------)
     4      5    0.7380    0.6813         (------*------)
     5      5    0.2660    0.1244   (------*------)
                                    ---+---------+---------+---------+---
POOLED STDEV =    0.4429             0.00      0.60      1.20      1.80
MTB > krus c6 c7
```

```
LEVEL     NOBS     MEDIAN  AVE. RANK   Z VALUE
    1        5     1.6000       21.2      2.79
    2        5     0.3000        9.0     -1.36
    3        5     0.5200       14.1      0.37
    4        5     0.4800       13.5      0.17
    5        5     0.3000        7.2     -1.97
OVERALL     25                  13.0

H = 10.92  d.f. = 4  p = 0.028
H = 10.97  d.f. = 4  p = 0.028 (adj. for ties)
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 4 WEEKS

LEVEL	N	MEAN	STDEV
1	5	1.5260	0.4477
2	5	0.4000	0.3391
3	5	0.5210	0.2473
4	5	0.4600	0.2558
5	5	0.3940	0.2486

POOLED STDEV = 0.3175

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

```
---------+---------+---------+-------
                                (-----*-----)
 (-----*-----)
   (-----*-----)
  (-----*-----)
 (-----*-----)
---------+---------+---------+-------
       0.50      1.00      1.50
```

DATA> krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	1.7400	23.0	3.40
2	5	0.3000	9.4	-1.22
3	5	0.5200	12.0	-0.34
4	5	0.4500	11.1	-0.65
5	5	0.3300	9.5	-1.19
OVERALL	25		13.0	

H = 11.98 d.f. = 4 p = 0.018
H = 11.99 d.f. = 4 p = 0.018 (adj. for ties)

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 8 WEEKS

LEVEL	N	MEAN	STDEV
1	5	1.0200	0.2168
2	5	0.3660	0.2701
3	5	0.4844	0.2497
4	5	0.2860	0.1897

POOLED STDEV = 0,2336

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

```
---------+---------+---------+-------
                          (-----*-----)
   (-----*------)
       (-----*-----)
 (-----*------)
---------+---------+---------+-------
      0.35      0.70      1.05
```

DATA> krus c66 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	0.9000	17.8	3.19
2	5	0.3200	7.8	-1.18
3	5	0.4500	9.8	-0.31
4	5	0.3000	6.6	-1.70
OVERALL	20		10.5	

H = 10.90 d.f. = 3 p = 0.013
H = 10.95 d.f. = 3 p = 0.012 (adj. for ties)

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=N

TIME POINT: 12 WEEKS

LEVEL	N	MEAN	STDEV
1	5	1.6780	0.3467
2	5	0.2960	0.1053
3	5	0.2960	0.1205
4	5	0.2660	0.1652

POOLED STDEV = 0.2080

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

```
---------+---------+---------+-------
                                (---*---)
 (---*---)
 (---*---)
(---*---)
---------+---------+---------+-------
       0.50      1.00      1.50
```

DATA> krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	1.7000	18.0	3.27
2	5	0.3000	7.9	-1.13
3	5	0.3300	8.3	-0.96
4	5	0.3000	7.8	-1.18
OVERALL	20		10.5	

H = 10.73 d.f. = 3 p = 0.014
H = 11.05 d.f. = 3 p = 0.012 (adj. for ties)

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

TIME POINT: 16 WEEKS

```
TWOSAMPLE T FOR C1 VS C2
    N      MEAN      STDEV   SE MEAN
C1  5     0.240      0.124     0.056
C2  5     0.488      0.206     0.092

95 PCT CI FOR MU C1 - MU C2: (-0.512, 0.016)

TTEST MU C1 = MU C2 (VS NE): T= -2.30  P=0.061  DF=  6

MTB > mann c1 c2

Mann-Whitney Confidence Interval and Test

C1          N =    5     Median =      0.2500
C2          N =    5     Median =      0.4600
Point estimate for ETA1-ETA2 is     -0.2200
96.3 pct c.i. for ETA1-ETA2 is (-0.5499,0.0099)
W = 18.5
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0758
The test is significant at 0.0749 (adjusted for ties)
```

Level 2=Pa+LIG; Level 1=N

TIME POINT: 20 WEEKS

```
TWOSAMPLE T FOR C1 VS C2
    N      MEAN      STDEV   SE MEAN
C1  5     1.460      0.261      0.12
C2  5     0.318      0.164     0.074

95 PCT CI FOR MU C1 - MU C2: (0.80, 1.479)

TTEST MU C1 = MU C2 (VS NE): T= 8.28  P=0.0002  DF=  6

MTB > mann c1 c2

Mann-Whitney Confidence Interval and Test

C1          N =    5     Median =      1.5000
C2          N =    5     Median =      0.3800
Point estimate for ETA1-ETA2 is      1.1200
96.3 pct c.i. for ETA1-ETA2 is (0.8000,1.5199)
W = 40.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0122
The test is significant at 0.0119 (adjusted for ties)
```

Level 1=Pa+LIG; Level 2=N

TIME POINT: 24 WEEKS

```
                                  INDIVIDUAL 95 PCT CI'S FOR MEAN
                                  BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV ----+---------+---------+---------+--
     1      5    0.6340    0.2598                          (-----*------)
     2      5    0.3460    0.1268               (------*-----)
     3      5    0.0640    0.0921  (-------*-----)
     4      5    0.2740    0.1486            (-----*-----)
                                  ----+---------+---------+---------+--
POOLED STDEV =    0.1689           0.00      0.25      0.50      0.75

DATA> krus c6 c7

LEVEL     NOBS     MEDIAN  AVE. RANK   Z VALUE
    1        5  7.20E-01        16.5      2.62
    2        5  4.00E-01        12.1      0.70
    3        5  0.00E+00         3.5     -3.06
    4        5  2.30E-01         9.9     -0.26
OVERALL     20                  10.5

H = 12.56  d.f. = 3  p = 0.006
H = 12.67  d.f. = 3  p = 0.006 (adj. for ties)
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

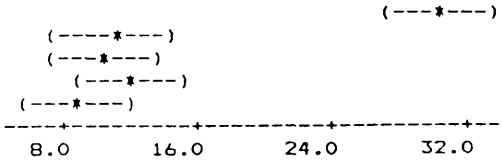
FIGURE 5.3 A - INFILTRATION OF OX-19/OX-52+ CELLS IN BALT

TIME POINT - 2 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	30.605	3.054
2	5	10.893	2.163
3	5	10.584	2.738
4	5	11.901	5.303
5	5	8.589	3.625

POOLED STDEV = 3.543



DATA> krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	30.03	23.0	3.40
2	5	10.35	11.2	-0.61
3	5	10.25	10.2	-0.95
4	5	12.60	12.0	-0.34
5	5	10.12	8.6	-1.49
OVERALL	25		13.0	

H = 12.13 d.f. = 4 p = 0.017

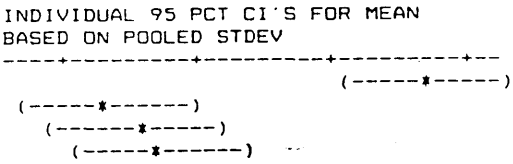
Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 4 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	25.774	6.071
2	5	9.294	2.477
3	5	11.006	3.313
4	5	12.131	6.833
5	5	8.843	3.219

POOLED STDEV = 4.712



DATA> krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	24.540	22.6	3.26
2	5	9.420	9.4	-1.22
3	5	10.650	12.6	-0.14
4	5	10.210	12.2	-0.27
5	5	8.121	8.2	-1.63
OVERALL	25		13.0	

H = 11.90 d.f. = 4 p = 0.019

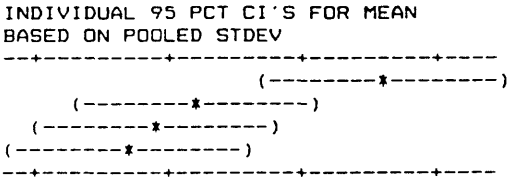
Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 8 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	14.274	4.806
2	5	8.688	4.498
3	5	7.548	2.770
4	5	6.795	2.160

POOLED STDEV = 3.730



MTB > krustc6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	14.720	16.2	2.49
2	5	8.500	10.0	-0.22
3	5	8.870	9.0	-0.65
4	5	6.280	6.8	-1.61
OVERALL	20		10.5	

H = 6.95 d.f. = 3 p = 0.074

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=N

TIME POINT: 12 WEEKS

```
                                          INDIVIDUAL 95 PCT CI'S FOR MEAN
                                          BASED ON POOLED STDEV
LEVEL      N      MEAN     STDEV  ------+---------+---------+---------+-
    1      5    15.874     5.515                          (-----*-----)
    2      5     5.628     1.138  (-----*-----)
    3      5     6.274     2.364    (------*-----)
    4      5     8.444     2.415          (-----*-----)
                                   ------+---------+---------+---------+-
POOLED STDEV =    3.284              5.0      10.0      15.0      20.0

MTB > krus c6 c7

LEVEL     NOBS     MEDIAN  AVE. RANK  Z VALUE
    1        5     15.060       17.6      3.10
    2        5      5.620        5.0     -2.40
    3           6.540        7.4     -1.35
    4        5     7.900       12.0      0.65
OVERALL     20                 10.5

H = 13.22  d.f. = 3  p = 0.004
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

TIME POINT: 16 WEEKS

```
TWOSAMPLE T FOR C1 VS C2
    N      MEAN     STDEV   SE MEAN
C1  5      6.56      2.26       1.0
C2  5     12.56      2.76       1.2

95 PCT CI FOR MU C1 - MU C2: (-9.8, -2.2)

TTEST MU C1 = MU C2 (VS NE): T= -3.76  P=0.0071  DF=  7

MTB > mann c1 c2]

Mann-Whitney Confidence Interval and Test

C1          N =   5     Median =       5.413
C2          N =   5     Median =      13.000
Point estimate for ETA1-ETA2 is      -6.411
96.3 pct c.i. for ETA1-ETA2 is (-9.788,-1.580)
W = 16.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0216
```

Level 2=Pa+LIG; Level 1=N

TIME POINT: 20 WEEKS

```
TWOSAMPLE T FOR C1 VS C2
    N      MEAN     STDEV   SE MEAN
C1  4     14.38      2.50       1.3
C2  5      6.71      1.25      0.56

95 PCT CI FOR MU C1 - MU C2: (3.9, 11.48)

TTEST MU C1 = MU C2 (VS NE): T= 5.60  P=0.0050  DF=  4

MTB > mann c1 c2

Mann-Whitney Confidence Interval and Test

C1          N =   4     Median =      14.208
C2          N =   5     Median =       6.180
Point estimate for ETA1-ETA2 is       7.456
96.3 pct c.i. for ETA1-ETA2 is (3.679,11.761)
W = 30.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0200
```

Level 1=Pa+LIG; Level 2=N

TIME POINT: 24 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	17.027	5.797
2	5	7.006	0.920
3	5	9.096	3.188
4	5	6.461	1.420
POOLED STDEV =		3.414	

```
                                  (-----*-----)
                    (-----*-----)
                         (-----*------)
               (------*-----)
               ----+---------+---------+---------+--
                  5.0       10.0      15.0      20.0
```

```
MTB > set c6krus c6 c7
```

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	14.660	18.0	3.27
2	5	7.230	7.6	-1.27
3	5	8.420	10.2	-0.13
4	5	6.490	6.2	-1.88
OVERALL	20		10.5	

H = 11.89 d.f. = 3 p = 0.008

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

FIGURE 5.3 B - INFILTRATION OF W3/25+ CELLS IN BALT

TIME POINT - 2 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	20.289	5.161
2	5	6.612	2.358
3	5	7.622	2.918
4	5	7.856	2.742
5	5	5.158	2.501
POOLED STDEV =		3.301	

```
                                                (----*----)
                (----*----)
                  (----*----)
                  (----*----)
          (-----*----)
          -------+---------+---------+---------
               6.0      12.0      18.0
```

```
DATA> krus c6 c7
```

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	19.360	23.0	3.40
2	5	7.260	9.7	-1.12
3	5	8.100	12.3	-0.24
4	5	8.430	12.8	-0.07
5	5	5.728	7.2	-1.97
OVERALL	25		13.0	

H = 13.39 d.f. = 4 p = 0.010
H = 13.40 d.f. = 4 p = 0.010 (adj. for ties)

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 4 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	15.818	7.990
2	5	4.816	1.654
3	5	6.920	4.537
4	5	8.928	6.411
5	5	5.136	2.433
POOLED STDEV =		5.180	

```
                                  (-------*-------)
                (-------*-------)
                   (--------*-------)
                       (-------*-------)
                 (-------*-------)
                -+---------+---------+---------+-----
                0.0       6.0      12.0      18.0
```

```
MTB > krus c6 c7
```

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	13.420	20.9	2.68
2	5	4.260	9.0	-1.36
3	5	5.000	11.4	-0.54
4	5	5.800	14.5	0.51
5	5	4.280	9.2	-1.29
OVERALL	25		13.0	

H = 9.01 d.f. = 4 p = 0.062
H = 9.02 d.f. = 4 p = 0.061 (adj. for ties)

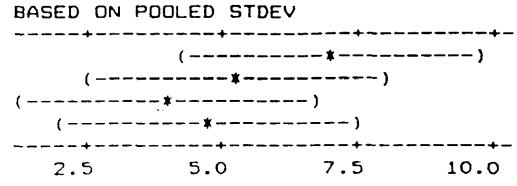
Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 8 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	6.994	2.812
2	5	5.294	3.913
3	5	4.010	2.729
4	5	4.712	1.476

POOLED STDEV = 2.866



DATA> krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	7.420	14.2	1.61
2	5	3.420	9.3	-0.52
3	5	3.530	7.8	-1.18
4	5	3.840	10.7	0.09
OVERALL	20		10.5	

H = 3.21 d.f. = 3 p = 0.361
H = 3.21 d.f. = 3 p = 0.361 (adj. for ties)

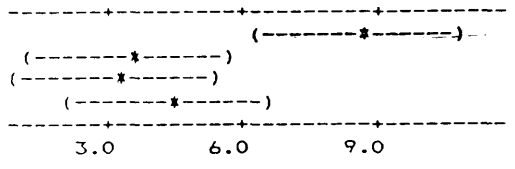
Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=N

TIME POINT: 12 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	8.572	3.915
2	5	3.556	1.587
3	5	3.182	1.473
4	5	4.392	1.327

POOLED STDEV = 2.333



MTB > krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	7.120	16.6	2.66
2	5	2.780	7.2	-1.44
3	5	2.900	7.2	-1.44
4	5	4.240	11.0	0.22
OVERALL	20		10.5	

H = 8.46 d.f. = 3 p = 0.038

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

TIME POINT: 16 WEEKS

TWOSAMPLE T FOR C1 VS C2

	N	MEAN	STDEV	SE MEAN
C1	5	3.72	1.26	0.56
C2	5	5.918	0.608	0.27

95 PCT CI FOR MU C1 - MU C2: (-3.81, -0.59)

TTEST MU C1 = MU C2 (VS NE): T= -3.51 P=0.017 DF= 5

MTB > mann c1 c2

Mann-Whitney Confidence Interval and Test

C1 N = 5 Median = 3.118
C2 N = 5 Median = 6.110
Point estimate for ETA1-ETA2 is -2.569
96.3 pct c.i. for ETA1-ETA2 is (-3.362,-0.269)
W = 16.0
Test of ETA1 = ETA2 vs. ETA1 n.e. ETA2 is significant at 0.0216

Level 2=Pa+LIG; Level 1=N

TIME POINT: 20 WEEKS

```
TWOSAMPLE T FOR C1 VS C2
     N      MEAN      STDEV   SE MEAN
C1   4      9.10       2.68       1.3
C2   5     4.314      0.527      0.24

95 PCT CI FOR MU C1 - MU C2: (0.4, 9.12)

TTEST MU C1 = MU C2 (VS NE): T= 3.51  P=0.039  DF=  3

MTB > maan c1 c2

Mann-Whitney Confidence Interval and Test

C1          N =    4     Median =        9.010
C2          N =    5     Median =        4.180
Point estimate for ETA1-ETA2 is         4.584
96.3 pct c.i. for ETA1-ETA2 is (2.002,7.760)
W = 30.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0200
```

Level 1=Pa+LIG; Level 2=N

TIME POINT: 24 WEEKS

```
                                  INDIVIDUAL 95 PCT CI'S FOR MEAN
                                  BASED ON POOLED STDEV
  LEVEL       N       MEAN      STDEV  ----+---------+---------+---------+--
      1       5     10.192      4.675                      (-------*-------)
      2       5      4.268      0.405  (-------*-------)
      3       5      4.402      0.956   (-------*-------)
      4       5      5.114      1.132     (-------*-------)
                                        ----+---------+---------+---------+--
POOLED STDEV =      2.460                 3.0       6.0       9.0      12.0
MTB > krus c6 c7

LEVEL     NOBS     MEDIAN  AVE. RANK   Z VALUE
    1        5      8.420       17.9      3.23
    2        5      4.200        6.4     -1.79
    3        5      4.210        7.8     -1.18
    4        5      4.820        9.9     -0.26
OVERALL     20                   10.5

H = 11.32  d.f. = 3  p = 0.010
H = 11.33  d.f. = 3  p = 0.010 (adj. for ties)
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

FIGURE 5.3 C - INFILTRATION OF OX-8+ CELLS IN BALT

TIME POINT - 2 WEEKS

```
                                  INDIVIDUAL 95 PCT CI'S FOR MEAN
                                  BASED ON POOLED STDEV
  LEVEL       N       MEAN      STDEV  ----------+---------+---------+------
      1       5     13.042      7.719                      (------*------)
      2       5      5.098      1.940    (------*------)
      3       5      4.470      1.528   (------*------)
      4       5      3.789      1.486  (------*------)
      5       5      5.746      1.582     (------*------)
                                        ----------+---------+---------+------
POOLED STDEV =      3.752                       5.0       10.0      15.0
MTB > krus c6 c7

LEVEL     NOBS     MEDIAN  AVE. RANK   Z VALUE
    1        5     18.250       18.8      1.97
    2        5      4.790       12.2     -0.27
    3        5      5.132       10.5     -0.85
    4        5      4.040        7.6     -1.83
    5        5      6.000       15.9      0.99
OVERALL     25                   13.0

H = 7.21  d.f. = 4  p = 0.126
H = 7.21  d.f. = 4  p = 0.126 (adj. for ties)
```

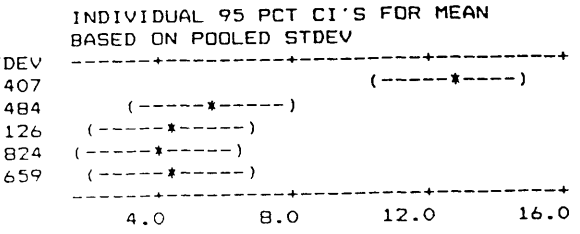
Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 4 WEEKS

LEVEL	N	MEAN	STDEV
1	5	12.671	4.407
2	5	5.648	1.484
3	5	4.354	1.126
4	5	4.084	0.824
5	5	4.294	2.659

POOLED STDEV = 2.475

INDIVIDUAL 95 PCT CI'S FOR MEAN BASED ON POOLED STDEV



DATA> krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	13.000	22.0	3.06
2	5	5.620	15.0	0.68
3	5	4.220	9.5	-1.19
4	5	3.850	8.7	-1.46
5	5	3.540	9.8	-1.09
OVERALL	25		13.0	

H = 11.63 d.f. = 4 p = 0.021
H = 11.68 d.f. = 4 p = 0.020 (adj. for ties)

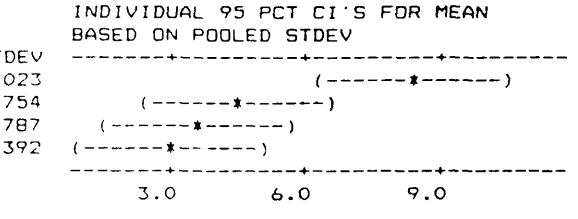
Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 8 WEEKS

LEVEL	N	MEAN	STDEV
1	5	8.352	3.023
2	5	4.590	2.754
3	5	3.722	0.787
4	5	3.016	1.392

POOLED STDEV = 2.195

INDIVIDUAL 95 PCT CI'S FOR MEAN BASED ON POOLED STDEV



MTB > krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	8.540	16.4	2.57
2	5	4.250	10.1	-0.17
3	5	4.120	8.8	-0.74
4	5	2.480	6.7	-1.66
OVERALL	20		10.5	

H = 7.47 d.f. = 3 p = 0.059
H = 7.48 d.f. = 3 p = 0.059 (adj. for ties)

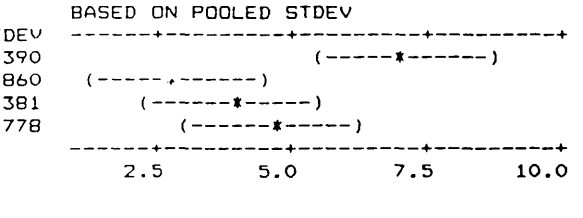
Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=N

TIME POINT: 12 WEEKS

LEVEL	N	MEAN	STDEV
1	5	7.082	1.390
2	5	2.792	0.860
3	5	3.952	1.381
4	5	4.640	2.778

POOLED STDEV = 1.753

INDIVIDUAL 95 PCT CI'S FOR MEAN BASED ON POOLED STDEV



DATA> krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	7.500	16.4	2.57
2	5	2.480	4.8	-2.49
3	5	3.290	9.6	-0.39
4	5	4.800	11.2	0.31
OVERALL	20		10.5	

H = 9.80 d.f. = 3 p = 0.021
H = 9.81 d.f. = 3 p = 0.021 (adj. for ties)

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

TIME POINT: 16 WEEKS

```
TWOSAMPLE T FOR C1 VS C2
    N      MEAN      STDEV   SE MEAN
C1  5      4.97       1.46      0.65
C2  5      8.29       3.01       1.3

95 PCT CI FOR MU C1 - MU C2: (-7.17, 0.5)

TTEST MU C1 = MU C2 (VS NE): T= -2.22  P=0.077  DF=  5

MTB > mann c1 c2

Mann-Whitney Confidence Interval and Test

C1          N =    5     Median =       4.500
C2          N =    5     Median =       8.000
Point estimate for ETA1-ETA2 is      -2.882
96.3 pct c.i. for ETA1-ETA2 is (-8.299,0.079)
W = 18.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0601

Cannot reject at alpha = 0.05
```

Level 2=Pa+LIG; Level 1=N

TIME POINT: 20 WEEKS

```
TWOSAMPLE T FOR C1 VS C2
    N      MEAN      STDEV   SE MEAN
C1  4      6.26       1.76      0.88
C2  5      2.99       1.07      0.48

95 PCT CI FOR MU C1 - MU C2: (0.50, 6.06)

TTEST MU C1 = MU C2 (VS NE): T= 3.28  P=0.031  DF=  4

MTB > mann c1 c2

Mann-Whitney Confidence Interval and Test

C1          N =    4     Median =       6.890
C2          N =    5     Median =       2.712
Point estimate for ETA1-ETA2 is       3.650
96.3 pct c.i. for ETA1-ETA2 is (-0.040,5.439)
W = 28.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0662

Cannot reject at alpha = 0.05
```

Level 1=Pa+LIG; Level 2=N

TIME POINT: 24 WEEKS

```
                                  INDIVIDUAL 95 PCT CI'S FOR MEAN
                                  BASED ON POOLED STDEV
  LEVEL      N      MEAN     STDEV  ------+---------+---------+---------+
      1      5     7.270     2.266                        (----*----)
      2      5     2.904     0.957     (----*----)
      3      5     2.700     0.667    (----*----)
      4      5     2.372     0.849  (----*-----)
                                  ------+---------+---------+---------+
POOLED STDEV =     1.343              2.5       5.0       7.5      10.0
MTB > krus c6 c7

LEVEL     NOBS     MEDIAN  AVE. RANK    Z VALUE
    1        5      6.330       18.0       3.27
    2        5      2.800        9.1      -0.61
    3        5      2.500        8.5      -0.87
    4        5      2.320        6.4      -1.79
OVERALL     20                  10.5

H = 11.29  d.f. = 3  p = 0.011
H = 11.31  d.f. = 3  p = 0.010 (adj. for ties)
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

FIGURE 5.3 D - INFILTRATION OF ED1+ CELLS IN BALT

TIME POINT - 2 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	13.008	2.866
2	5	8.775	1.918
3	5	8.410	3.075
4	5	9.135	2.136
5	5	7.228	2.488

POOLED STDEV = 2.534

6.0 9.0 12.0 15.0

MTB >

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	11.694	21.6	2.92
2	5	8.923	11.2	-0.61
3	5	9.580	11.8	-0.41
4	5	10.140	13.0	0.00
5	5	7.080	7.4	-1.90
OVERALL	25		13.0	

H = 10.15 d.f. = 4 p = 0.039

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 4 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	6.643	1.153
2	5	5.100	1.710
3	5	6.266	2.486
4	5	6.406	3.425
5	5	6.188	2.225

POOLED STDEV = 2.329

3.2 4.8 6.4 8.0

MTB > kruskal c6 krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	6.480	16.4	1.15
2	5	5.400	11.0	-0.68
3	5	5.310	11.8	-0.41
4	5	5.370	11.2	-0.61
5	5	5.980	14.6	0.54
OVERALL	25		13.0	

H = 2.10 d.f. = 4 p = 0.717

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 8 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	6.098	1.007
2	5	4.836	1.685
3	5	5.544	2.335
4	5	5.972	2.490

POOLED STDEV = 1.969

3.0 4.5 6.0 7.5

MTB > krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	5.600	12.4	0.83
2	5	4.670	7.8	-1.18
3	5	5.200	9.9	-0.26
4	5	5.600	11.9	0.61
OVERALL	20		10.5	

H = 1.89 d.f. = 3 p = 0.596
H = 1.89 d.f. = 3 p = 0.595 (adj. for ties)

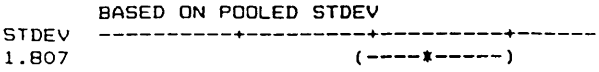
Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=N

TIME POINT: 12 WEEKS

LEVEL	N	MEAN	STDEV
1	5	6.820	1.807
2	5	4.706	0.426
3	5	4.962	0.931
4	5	3.112	1.068

POOLED STDEV = 1.168

INDIVIDUAL 95 PCT CI'S FOR MEAN BASED ON POOLED STDEV



MTB >

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	6.950	16.0	2.40
2	5	4.750	10.2	-0.13
3	5	5.040	11.6	0.48
4	5	2.660	4.2	-2.75
OVERALL	20		10.5	

H = 10.18 d.f. = 3 p = 0.017

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

TIME POINT: 16 WEEKS

TWOSAMPLE T FOR C1 VS C2

	N	MEAN	STDEV	SE MEAN
C1	5	4.106	0.946	0.42
C2	5	5.38	1.25	0.56

95 PCT CI FOR MU C1 - MU C2: (-2.93, 0.38)

TTEST MU C1 = MU C2 (VS NE): T= -1.82 P=0.11 DF= 7

MTB > mann c1 c2

Mann-Whitney Confidence Interval and Test

C1 N = 5 Median = 3.922
C2 N = 5 Median = 4.930
Point estimate for ETA1-ETA2 is -1.110
96.3 pct c.i. for ETA1-ETA2 is (-3.407,0.399)
W = 19.0
Test of ETA1 = ETA2 vs. ETA1 n.e. ETA2 is significant at 0.0947
The test is significant at 0.0937 (adjusted for ties)

Cannot reject at alpha = 0.05

Level 2=Pa+LIG; Level 1=N

TIME POINT: 20 WEEKS

TWOSAMPLE T FOR C1 VS C2

	N	MEAN	STDEV	SE MEAN
C1	4	4.86	1.25	0.62
C2	5	4.632	0.751	0.34

95 PCT CI FOR MU C1 - MU C2: (-1.74, 2.20)

TTEST MU C1 = MU C2 (VS NE): T= 0.33 P=0.76 DF= 4

MTB > mann c1 c2

Mann-Whitney Confidence Interval and Test

C1 N = 4 Median = 4.875
C2 N = 5 Median = 4.840
Point estimate for ETA1-ETA2 is 0.295
96.3 pct c.i. for ETA1-ETA2 is (-1.722,2.269)
W = 22.0
Test of ETA1 = ETA2 vs. ETA1 n.e. ETA2 is significant at 0.7133

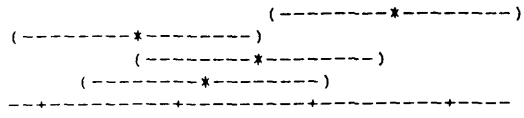
Cannot reject at alpha = 0.05

Level 1=Pa+LIG; Level 2=N

TIME POINT: 24 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	8.930	3.473
2	5	4.300	0.738
3	5	6.498	2.715
4	5	5.458	1.614



POOLED STDEV = 2.376

MTB > krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	7.830	15.8	2.31
2	5	4.100	5.4	-2.23
3	5	5.000	11.9	0.61
4	5	4.980	8.9	-0.70
OVERALL	20		10.5	

H = 8.37 d.f. = 3 p = 0.039
H = 8.38 d.f. = 3 p = 0.039 (adj. for ties)

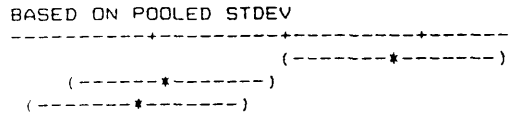
Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

FIGURE 5.3 E - INFILTRATION OF OX-12+ CELLS IN BALT

TIME POINT - 2 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	2.6860	1.0607
2	5	1.5000	0.2236
3	5	1.3164	0.3619
4	5	1.8520	0.6226
5	5	1.8760	0.2736



POOLED STDEV = 0.5947

MTB > krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	3.230	18.8	1.97
2	5	1.500	9.3	-1.26
3	5	1.500	7.5	-1.87
4	5	1.530	13.0	0.00
5	5	1.800	16.4	1.15
OVERALL	25		13.0	

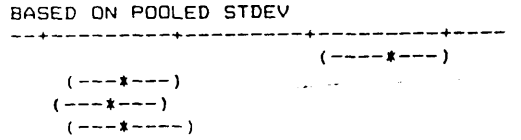
H = 8.23 d.f. = 4 p = 0.084
H = 8.32 d.f. = 4 p = 0.081 (adj. for ties)

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 4 WEEKS

INDIVIDUAL 95 PCT CI'S FOR MEAN
BASED ON POOLED STDEV

LEVEL	N	MEAN	STDEV
1	5	3.5640	0.8559
2	5	1.5860	0.2024
3	5	1.5000	0.2236
4	5	1.6400	0.2074
5	5	1.2840	0.4422



POOLED STDEV = 0.4609

DATA> krus c6 c7

LEVEL	NOBS	MEDIAN	AVE. RANK	Z VALUE
1	5	3.200	23.0	3.40
2	5	1.530	11.6	-0.48
3	5	1.500	9.8	-1.09
4	5	1.600	13.2	0.07
5	5	1.400	7.4	-1.90
OVERALL	25		13.0	

H = 13.26 d.f. = 4 p = 0.010
H = 13.44 d.f. = 4 p = 0.010 (adj. for ties)

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=S; Level 5=N

TIME POINT: 8 WEEKS

```
                                        INDIVIDUAL 95 PCT CI'S FOR MEAN
                                        BASED ON POOLED STDEV
  LEVEL      N      MEAN      STDEV    ---------+---------+---------+---------
      1      5    3.1800     0.9121                          (------*------)
      2      5    1.5680     0.2176    (------*-----)
      3      5    1.6100     0.4321    (------*------)
      4      5    1.6000     0.5148    (------*------)
                                        ---------+---------+---------+---------
  POOLED STDEV =    0.5769                    1.60      2.40      3.20
  MTB > krus c6 c7

  LEVEL     NOBS     MEDIAN  AVE. RANK   Z VALUE
      1        5      3.400       17.0       2.84
      2        5      1.500        8.4      -0.92
      3        5      1.650        9.6      -0.39
      4        5      1.400        7.0      -1.53
  OVERALL     20                   10.5

  H = 8.53  d.f. = 3  p = 0.037
  H = 8.57  d.f. = 3  p = 0.036 (adj. for ties)
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=PBS+LIG; Level 4=N

TIME POINT: 12 WEEKS

```
                                        INDIVIDUAL 95 PCT CI'S FOR MEAN
                                        BASED ON POOLED STDEV
  LEVEL      N      MEAN      STDEV    ---+---------+---------+---------+----
      1      5    4.1200     0.9985                         (----*-----)
      2      5    1.7800     0.2588     (----*----)
      3      5    2.0600     0.6007       (----*----)
      4      5    1.6720     0.6284     (----*----)
                                        ---+---------+---------+---------+----
  POOLED STDEV =    0.6745              1.2       2.4       3.6       4.8

  DATA> brwstc6 c7

  LEVEL     NOBS     MEDIAN  AVE. RANK   Z VALUE
      1        5      3.800       18.0       3.27
      2        5      1.700        6.7      -1.66
      3        5      2.360       10.3      -0.09
      4        5      1.800        7.0      -1.53
  OVERALL     20                   10.5

  H = 11.85  d.f. = 3  p = 0.008
  H = 11.93  d.f. = 3  p = 0.008 (adj. for ties)
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

TIME POINT: 16 WEEKS

```
  TWOSAMPLE T FOR C1 VS C2
     N      MEAN      STDEV   SE MEAN
C1   5     1.540      0.344      0.15
C2   5     3.128      0.774      0.35

  95 PCT CI FOR MU C1 - MU C2: (-2.56, -0.61)

  TTEST MU C1 = MU C2 (VS NE): T= -4.19  P=0.0086  DF=  5

  MTB > mann c1 c2]

  Mann-Whitney Confidence Interval and Test

  C1          N =    5     Median =       1.700
  C2          N =    5     Median =       2.760
  Point estimate for ETA1-ETA2 is      -1.360
  96.3 pct c.i. for ETA1-ETA2 is (-2.690,-0.590)
  W = 15.0
  Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0122
  The test is significant at 0.0119 (adjusted for ties)
```

Level 2=Pa+LIG; Level 1=N

TIME POINT: 20 WEEKS

```
TWOSAMPLE T FOR C1 VS C2
    N      MEAN     STDEV   SE MEAN
C1  3      3.07      1.21      0.70
C2  5     1.560     0.305      0.14

95 PCT CI FOR MU C1 - MU C2: (-1.55, 4.56)

TTEST MU C1 = MU C2 (VS NE): T= 2.12  P=0.17  DF=  2

MTB > mann c1 c2

Mann-Whitney Confidence Interval and Test

C1          N =    3     Median =       3.200
C2          N =    5     Median =       1.700
Point estimate for ETA1-ETA2 is        1.500
96.3 pct c.i. for ETA1-ETA2 is (0.000,3.099)
W = 20.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0736
The test is significant at 0.0668 (adjusted for ties)

Cannot reject at alpha = 0.05
```

Level 1=Pa+LIG; Level 2=N

TIME POINT: 24 WEEKS

```
                                  INDIVIDUAL 95 PCT CI'S FOR MEAN
                                  BASED ON POOLED STDEV
 LEVEL      N      MEAN     STDEV ----------+---------+---------+------
     1      5    2.2280    0.7067                (---------*---------)
     2      5    1.8580    0.3047          (---------*---------)
     3      5    2.1660    0.5940                (---------*---------)
     4      5    1.5000    0.2236   (---------*---------)
                                  ----------+---------+---------+------
POOLED STDEV =    0.4988                   1.50      2.00      2.50
MTB > krus c6 c7

LEVEL     NOBS     MEDIAN  AVE. RANK   Z VALUE
    1        5      2.200       13.4      1.27
    2        5      1.890       10.4     -0.04
    3        5      1.800       13.5      1.31
    4        5      1.500        4.7     -2.53
OVERALL     20                  10.5

H = 7.29  d.f. = 3  p = 0.064
H = 7.34  d.f. = 3  p = 0.062 (adj. for ties)
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 3=S; Level 4=N

TABLE 5.7 - INTERLEUKIN-2+ CELLS - LAMINA PROPRIA

```
TWOSAMPLE T FOR C1 VS C5
     N      MEAN     STDEV   SE MEAN
C1  33     0.531     0.402     0.070
C5  24    0.0250    0.0442    0.0090

95 PCT CI FOR MU C1 - MU C5: (0.362, 0.6494)

TTEST MU C1 = MU C5 (VS NE): T= 7.18  P=0.0000  DF=  33

MTB > mann c1 c5

Mann-Whitney Confidence Interval and Test

C1          N =  33     Median =      0.4160
C5          N =  24     Median =      0.0000
Point estimate for ETA1-ETA2 is       0.4120
95.0 pct c.i. for ETA1-ETA2 is (0.2470,0.5970)
W = 1353.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0000
The test is significant at 0.0000 (adjusted for ties)

TWOSAMPLE T FOR C2 VS C5
     N      MEAN     STDEV   SE MEAN
C2  17    0.0297    0.0673     0.016
C5  24    0.0250    0.0442    0.0090

95 PCT CI FOR MU C2 - MU C5: (-0.034, 0.0431)

TTEST MU C2 = MU C5 (VS NE): T= 0.25  P=0.80  DF=  25

MTB > mann c2 c5

Mann-Whitney Confidence Interval and Test

C2          N =  17     Median =     0.00000
C5          N =  24     Median =     0.00000
Point estimate for ETA1-ETA2 is      0.00000
95.1 pct c.i. for ETA1-ETA2 is (0.00000,-0.00001)
W = 351.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.8843
The test is significant at 0.8403 (adjusted for ties)

Cannot reject at alpha = 0.05
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 5=N

BRONCHUS-ASSOCIATED LYMPHOID TISSUE

```
TWOSAMPLE T FOR C1 VS C5
     N      MEAN     STDEV   SE MEAN
C1  28      2.51      1.31      0.25
C5  21     1.187     0.992      0.22

95 PCT CI FOR MU C1 - MU C5: (0.66, 1.98)

TTEST MU C1 = MU C5 (VS NE): T= 4.01  P=0.0002  DF=  46

MTB > mann c1 c5

Mann-Whitney Confidence Interval and Test

C1          N =  28     Median =       2.395
C5          N =  21     Median =       0.625
Point estimate for ETA1-ETA2 is        1.247
95.1 pct c.i. for ETA1-ETA2 is (0.634,2.012)
W = 872.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0005
The test is significant at 0.0005 (adjusted for ties)
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 5=N

BRONCHUS-ASSOCIATED LYMPHOID TISSUE

```
TWOSAMPLE T FOR C2 VS C5
     N      MEAN     STDEV   SE MEAN
C2  17     1.358     0.818      0.20
C5  21     1.187     0.992      0.22

95 PCT CI FOR MU C2 - MU C5: (-0.43, 0.77)

TTEST MU C2 = MU C5 (VS NE): T= 0.58  P=0.56  DF=  35

MTB > mann c2 c5

Mann-Whitney Confidence Interval and Test

C2          N =  17     Median =       1.240
C5          N =  21     Median =       0.625
Point estimate for ETA1-ETA2 is        0.399
95.1 pct c.i. for ETA1-ETA2 is (-0.460,0.811)
W = 361.5
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.3865
The test is significant at 0.3864 (adjusted for ties)

Cannot reject at alpha = 0.05
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 5=N

TABLE 5.8 - ACID PHOSPHATASE+ MACROPHAGES - EPITHELIUM

```
TWOSAMPLE T FOR C1 VS C5
     N      MEAN     STDEV   SE MEAN
C1  33      55.6      15.7       2.7
C5  25     31.94      9.72       1.9

95 PCT CI FOR MU C1 - MU C5: (16.9, 30.4)

TTEST MU C1 = MU C5 (VS NE): T= 7.06  P=0.0000  DF=  54

MTB > mann c1 c5

Mann-Whitney Confidence Interval and Test

C1          N =  33     Median =       53.50
C5          N =  25     Median =       33.30
Point estimate for ETA1-ETA2 is        25.00
95.0 pct c.i. for ETA1-ETA2 is (16.70,33.30)
W = 1290.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0000
The test is significant at 0.0000 (adjusted for ties)


TWOSAMPLE T FOR C2 VS C5
     N      MEAN     STDEV   SE MEAN
C2  19     31.12      6.20       1.4
C5  25     31.94      9.72       1.9

95 PCT CI FOR MU C2 - MU C5: (-5.7, 4.0)

TTEST MU C2 = MU C5 (VS NE): T= -0.34  P=0.73  DF=  40

MTB > mann c2 c5

Mann-Whitney Confidence Interval and Test

C2          N =  19     Median =      33.300
C5          N =  25     Median =      33.300
Point estimate for ETA1-ETA2 is        0.000
95.1 pct c.i. for ETA1-ETA2 is (-4.199,4.198)
W = 418.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.8311
The test is significant at 0.8223 (adjusted for ties)

Cannot reject at alpha = 0.05
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level4=S; Level 5=N

LAMINA PROPRIA

```
TWOSAMPLE T FOR C1 VS C5
     N       MEAN      STDEV   SE MEAN
C1  32       56.0       10.8       1.9
C5  24      31.16       9.37       1.9

95 PCT CI FOR MU C1 - MU C5: (19.4, 30.3)

TTEST MU C1 = MU C5 (VS NE): T= 9.17  P=0.0000  DF=  52

MTB > mann c1 c5

Mann-Whitney Confidence Interval and Test

C1          N =  32      Median =       56.050
C5          N =  24      Median =       33.150
Point estimate for ETA1-ETA2 is        24.635
95.0 pct c.i. for ETA1-ETA2 is (19.101,30.397)
W = 1266.5
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0000
The test is significant at 0.0000 (adjusted for ties)

TWOSAMPLE T FOR C2 VS C5
     N       MEAN      STDEV   SE MEAN
C2  25      33.94       8.05       1.6
C5  24      31.16       9.37       1.9

95 PCT CI FOR MU C2 - MU C5: (-2.3, 7.8)

TTEST MU C2 = MU C5 (VS NE): T= 1.11  P=0.27  DF=  45

MTB > mann c2 c5

Mann-Whitney Confidence Interval and Test

C2          N =  25      Median =       33.300
C5          N =  24      Median =       33.150
Point estimate for ETA1-ETA2 is         2.300
95.1 pct c.i. for ETA1-ETA2 is (-1.401,7.098)
W = 688.5
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.2077
The test is significant at 0.2051 (adjusted for ties)
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 4=S; Level 5=N

BRONCHUS-ASSOCIATED LYMPHOID TISSUE

```
TWOSAMPLE T FOR C1 VS C5
     N       MEAN      STDEV   SE MEAN
C1  32       57.0       11.9       2.1
C5  19      32.12       8.65       2.0

95 PCT CI FOR MU C1 - MU C5: (19.0, 30.7)

TTEST MU C1 = MU C5 (VS NE): T= 8.59  P=0.0000  DF=  46

MTB > mann c1 c5

Mann-Whitney Confidence Interval and Test

C1          N =  32      Median =       55.57
C5          N =  19      Median =       32.40
Point estimate for ETA1-ETA2 is        24.30
95.2 pct c.i. for ETA1-ETA2 is (18.70,30.90)
W = 1112.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0000
The test is significant at 0.0000 (adjusted for ties)
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 5=N

BRONCHUS-ASSOCIATED LYMPHOID TISSUE

```
TWOSAMPLE T FOR C2 VS C5
     N      MEAN     STDEV   SE MEAN
C2  20     35.26      9.01       2.0
C5  19     32.12      8.65       2.0

95 PCT CI FOR MU C2 - MU C5: (-2.6, 8.9)

TTEST MU C2 = MU C5 (VS NE): T= 1.11  P=0.27  DF=  36

MTB > mann c2 c5

Mann-Whitney Confidence Interval and Test

C2          N =  20     Median =      34.450
C5          N =  19     Median =      32.400
Point estimate for ETA1-ETA2 is        2.800
95.2 pct c.i. for ETA1-ETA2 is (-1.197,8.000)
W = 448.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.1820
The test is significant at 0.1815 (adjusted for ties)

Cannot reject at alpha = 0.05
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 5=N

FIG. 5.6 - EXPRESSION OF CLASS II IA ANTIGENS - EPITHELIUM

```
TWOSAMPLE T FOR C1 VS C5
     N      MEAN     STDEV   SE MEAN
C1  24     6.105     0.969      0.20
C5   8     4.320     0.539      0.19

95 PCT CI FOR MU C1 - MU C5: (1.21, 2.36)

TTEST MU C1 = MU C5 (VS NE): T= 6.50  P=0.0000  DF=  22

MTB > mann c1 c5

Mann-Whitney Confidence Interval and Test

C1          N =  24     Median =      5.783
C5          N =   8     Median =      4.286
Point estimate for ETA1-ETA2 is       1.631
95.2 pct c.i. for ETA1-ETA2 is (0.989,2.583)
W = 488.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0001


TWOSAMPLE T FOR C2 VS C5
     N      MEAN     STDEV   SE MEAN
C2   7     4.390     0.370      0.14
C5   8     4.320     0.539      0.19

95 PCT CI FOR MU C2 - MU C5: (-0.45, 0.59)

TTEST MU C2 = MU C5 (VS NE): T= 0.29  P=0.77  DF=  12

MTB > mann c2 c5

Mann-Whitney Confidence Interval and Test

C2          N =   7     Median =      4.4960
C5          N =   8     Median =      4.2865
Point estimate for ETA1-ETA2 is       0.0600
95.7 pct c.i. for ETA1-ETA2 is (-0.5219,0.5402)
W = 60.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.6854

Cannot reject at alpha = 0.05
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 4=S; Level 5=N

LAMINA PROPRIA

```
TWOSAMPLE T FOR C1 VS C5
     N      MEAN     STDEV   SE MEAN
C1  23     5.309     0.567      0.12
C5   8     4.048     0.373      0.13

95 PCT CI FOR MU C1 - MU C5: (0.89, 1.63)

TTEST MU C1 = MU C5 (VS NE): T= 7.12  P=0.0000  DF=  18

MTB > mann c1 c5

Mann-Whitney Confidence Interval and Test

C1          N =  23     Median =      5.3270
C5          N =   8     Median =      4.1205
Point estimate for ETA1-ETA2 is       1.2585
95.0 pct c.i. for ETA1-ETA2 is (0.8121,1.7080)
W = 454.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0001

TWOSAMPLE T FOR C2 VS C5
     N      MEAN     STDEV   SE MEAN
C2   7     4.550     0.531      0.20
C5   8     4.048     0.373      0.13

95 PCT CI FOR MU C2 - MU C5: (-0.03, 1.04)

TTEST MU C2 = MU C5 (VS NE): T= 2.09  P=0.063  DF=  10

MTB > mann c2 c5

Mann-Whitney Confidence Interval and Test

C2          N =   7     Median =      4.5940
C5          N =   8     Median =      4.1205
Point estimate for ETA1-ETA2 is       0.5115
95.7 pct c.i. for ETA1-ETA2 is (-0.0617,1.0579)
W = 70.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.1182

Cannot reject at alpha = 0.05
```

Level 1=Pa+LIG; Level 2=Pa+NOLIG; Level 5=N

SECTION 5.3.4 - DELAYED-TYPE HYPERSENSITIVITY TO PSEUDOMONAS ANTIGENS

```
TWOSAMPLE T FOR C1 VS C2
     N      MEAN     STDEV   SE MEAN
C1  11    0.0455    0.0688     0.021
C2   8    0.0375    0.0744     0.026

95 PCT CI FOR MU C1 - MU C2: (-0.064, 0.080)

TTEST MU C1 = MU C2 (VS NE): T= 0.24  P=0.82  DF=  14

MTB > mann c1 c2

Mann-Whitney Confidence Interval and Test

C1          N =  11     Median =      0.0000
C2          N =   8     Median =      0.0000
Point estimate for ETA1-ETA2 is      -0.0000
95.7 pct c.i. for ETA1-ETA2 is (0.0000,0.1000)
W = 114.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.7726
The test is significant at 0.7242 (adjusted for ties)

Cannot reject at alpha = 0.05
```

C1=RF TEST; C2=RF CONTROL; C3= LF TEST; C4=LF CONTROL

DELAYED-TYPE HYPERSENSITIVITY TO PSEUDOMONAS ANTIGENS

```
TWOSAMPLE T FOR C3 VS C4
     N       MEAN      STDEV   SE MEAN
C3  11      0.177      0.151     0.045
C4   8     0.0250     0.0463     0.016

95 PCT CI FOR MU C3 - MU C4: (0.047, 0.257)

TTEST MU C3 = MU C4 (VS NE): T= 3.15  P=0.0083  DF=  12

MTB > mann c3 c4

Mann-Whitney Confidence Interval and Test

C3          N =   11      Median =      0.2000
C4          N =    8      Median =      0.0000
Point estimate for ETA1-ETA2 is       0.1250
95.7 pct c.i. for ETA1-ETA2 is (-0.0000,0.3000)
W = 137.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0287
The test is significant at 0.0200 (adjusted for ties)

MTB > twos c1 c3

TWOSAMPLE T FOR C1 VS C3
     N       MEAN      STDEV   SE MEAN
C1  11     0.0455     0.0688     0.021
C3  11      0.177      0.151     0.045

95 PCT CI FOR MU C1 - MU C3: (-0.240, -0.024)

TTEST MU C1 = MU C3 (VS NE): T= -2.64  P=0.020  DF=  13

MTB > mann c1 c3

Mann-Whitney Confidence Interval and Test

C1          N =   11      Median =      0.0000
C3          N =   11      Median =      0.2000
Point estimate for ETA1-ETA2 is      -0.1000
95.1 pct c.i. for ETA1-ETA2 is (-0.2500,-0.0000)
W = 94.5
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.0386
The test is significant at 0.0286 (adjusted for ties)

MTB > twos c2 c4

TWOSAMPLE T FOR C2 VS C4
     N       MEAN      STDEV   SE MEAN
C2   8     0.0375     0.0744     0.026
C4   8     0.0250     0.0463     0.016

95 PCT CI FOR MU C2 - MU C4: (-0.056, 0.081)

TTEST MU C2 = MU C4 (VS NE): T= 0.40  P=0.69  DF=  11

MTB > mann c2 c4

Mann-Whitney Confidence Interval and Test

C2          N =    8      Median =      0.0000
C4          N =    8      Median =      0.0000
Point estimate for ETA1-ETA2 is       0.0000
95.9 pct c.i. for ETA1-ETA2 is (-0.0000,0.1000)
W = 69.0
Test of ETA1 = ETA2  vs.  ETA1 n.e. ETA2 is significant at 0.9581
The test is significant at 0.9447 (adjusted for ties)

Cannot reject at alpha = 0.05
```

C1=RF TEST; C2=RF CONTROL; C3= LF TEST; C4=LF CONTROL

A P P E N D I X 2 : S O L U T I O N S

1) Phosphate-buffered saline (PBS): sodium chloride 80 g, potassium chloride 2 g, sodium hydrogen phosphate 11.5 g, potassium dihydrogen phosphate 2 g in 1 l; pH 7.2.

2) Poly-l-lysine: 5 mg poly-l-lysine (Sigma Chemical Co, St. Louis, USA) in 500 ml sterile water.

3) Toluidine Blue (Sigma): for metachromatic staining prepare 0.5% aqueous solution.

4) Haematoxylin-Eosin:

- 10% formalin (BDH) in saline
- Harris haematoxylin (Accustain-Sigma)
- Acid/Alcohol - 1% concentrated HCl (BDH) in 70% alcohol (BDH)
- Alkaline Water: 5% Na2C)3 (Sigma) in destilled water
- Eosin: 1% Eosin Y (Sigma) in destilled water.

5) Miller's Elastic Van Gieson (modified Weigert Elastin Technique): commercially available Miller's solution (BDH). Van Gieson solution: 1% acid fuchsin (BDH) aqueous solution (18 ml), saturated aqueous picric acid (BDH - 100 ml), distilled water 100 ml.

6) D.P.X.: a mounting medium produced by the mixture of distrene (a polystyrene), a plasticizer (dibutylphthalate) and xylene, commercially available from DBH.

7) Polysiloxane: water-repellent prepared by the mixture of 84% neat propanol (BDH), 1% concentrated H2SO4 (BDH) and 15% di-methyl-polysiloxane (Sigma).

8) Tris-HCl: stock solution prepared as 1 M Tris (hydroxymethyl) aminomethane (BDH - 121.1 g/l); working solution for DAB development: dilute to 0.05 M and add 0.1 N HCl dropwise to pH 7.6.

9) DAB development: freshly prepared solution of 16.6 ml 0.05 M Tris-HCl, 10 mg 3.3'-diaminobenzidine tetrahydrochloride (Sigma), 166 ul H2O2 (Sigma).

10) Acid Phosphatase development medium:

a) Barbital buffer: 9.7 g sodium acetate (Sigma), 14.7 g barbital sodium (Sigma), 500 ml distilled water;

b) substrate solution: 10 mg naphtyl ASBI phosphate (Sigma), 1 ml NN-dimethylformamide (Sigma);

c) pararosaniline solution: 2 g pararosanilin-HCl (Sigma), 50 ml 2N HCl (BDH);

d) sodium nitrite solution: 4 g sodium nitrite (Sigma) in 100 ml distilled water;

Solution A: 5 ml barbital buffer (a), 12 ml distilled water, 1 ml substrate solution (b);

Solution B: 0.8 ml pararosaniline (c), 0.8 ml sodium nitrite solution (d);

Prepare solution A, then add solution B. Use NaOH or HCl to bring the pH to 5.0.

A P P E N D I X 3

BACTERIOLOGICAL PROFILE OF PSEUDOMONAS AERUGINOSA STRAIN P455

Clinical isolate from bronchiectasis sputum.

Blue green - strong pigment production on Kings agar.

Antibiotic sensitivity: sensitive to carbenicillin, azlocillin, gentamicin, tobramycin, piperacillin, ceftazidine, ciprofloxacin.

ENZYME	ACTIVITY
Oxidase	Positive
NO3 - NO2	Positive
Indole production	Negative
Urease	Negative
Beta Galactosidase	Negative
DNAse	Negative
Haemolysin	Negative
Glucose oxidation	Positive
Glucose Fermentation	Positive
N-acetyl glucosamine	Assimilated
Arginine dihydrolase	Positive
Lecithinase	Weak positive
Elastase	Positive
Gelatin hydrolysis	Positive
Casein hydrolysis	Positive
Alkaline protease	Positive
Phospholipase	Positive
Serotype	1
Phage type + reactions	7.F8.109.1214

(Prepared by Mr. Howard Todd)

A P P E N D I X 4: PUBLICATIONS DERIVED FROM THIS THESIS

1) Lapa e Silva JR, Jones JAH, Cole PJ, Poulter LW. The immunological component of the cellular inflammatory infiltrate in bronchiectasis. Thorax 1989; 44:668-73.

2) Lapa e Silva JR, Guerreiro D, Noble B, Poulter LW, Cole PJ. Immunopathology of experimental bronchiectasis. Am. J. Respir. Cell Mol. Biol. 1989;1:297-304.

3) Lapa e Silva JR, Poulter LW, Cole PJ. Phenotype of bronchial T-lymphocytes in the chronic inflammation of bronchiectasis. In: MacDonald TT, Challacombe SJ, Bland PW, Stokes CR, Heatley RV, Mowat AM, eds. Advances in Mucosal Immunology. Kluwer Academic Publishers B.V., 1990, pp. 636-637, London.

4) Lapa e Silva JR, Guerreiro D, Munro NC, Noble B, Cole PJ, Poulter LW. Cell-mediated immune kinetics in experimental bronchiectasis. In: MacDonald TT, Challacombe SJ, Bland PW, Stokes CR, Heatley RV, Mowat AM, eds. Advances in Mucosal Immunology. Kluwer Academic Publishers B. V., 1990, pp. 821-824, London.

5) Lapa e Silva JR, Pouter LW, Cole PJ. T-lymphocyte populations in the chronic inflammation of bronchiectasis. Submitted.

6) Lapa e Silva JR, Guerreiro D, Munro NC, Noble B, Cole PJ, Poulter LW. Pseudomonas-induced bronchiectasis in the rat: development of lymphocyte and macrophage responses. Submitted.

7) Lapa e Silva JR, Cole PJ, Poulter LW. Characterization of bronchial intraepithelial lymphocytes in bronchiectasis. In preparation.

8) Lapa e Silva JR, Poulter PJ, Cole PJ. Immunopathology of follicular bronchiectasis in man. In preparation.

9) Lapa e Silva JR, Guerreiro D, Munro NC, Roberts PME, Poulter LW,

Cole PJ. Cytotoxic mechanisms in the immunopathogenesis of experimental bronchiectasis in rat. In preparation.

The results of this research were presented in part in 35 communications to scientific meetings or seminars between July 1987 and October 1990, of which 21 were published in abstract form.

Immunopathology of Experimental Bronchiectasis

Jose R. Lapa e Silva, Diamantino Guerreiro, Bernice Noble, Leonard W. Poulter, and Peter J. Cole

Host Defence Unit, Department of Thoracic Medicine, National Heart and Lung Institute, Brompton Hospital, and Academic Department of Immunology, Royal Free Hospital School of Medicine, London, United Kingdom

In human bronchiectasis, the bronchial wall is the seat of abnormal mononuclear cell infiltration, which suggests the presence of a cell-mediated immune reaction. The histopathology of a recently devised animal model of experimental bronchiectasis resembles that of the human disease. We have investigated its immunohistology to validate the similarity to that of human bronchiectasis in order to provide a model for the study of cellular immune aspects of the pathogenesis of bronchiectasis. The immunohistology of the bronchial wall mononuclear cell population in experimental rat bronchiectasis was compared with that in control and normal rats. The control rats did not develop bronchiectasis, and the composition and distribution of mononuclear cells in the bronchial wall were similar to those of normal animals. In the rats developing bronchiectasis, there was infiltration of T lymphocytes, macrophages, and dendritic cells (as defined by monoclonal antibodies) in all compartments of the lung, particularly in the bronchial wall and around vessels. The bronchus-associated lymphoid tissue was disrupted by heavy infiltration of T cells, and follicular aggregates of T lymphocytes were seen deeper in the lung parenchyma. Expression of Ia antigen increased in the bronchial epithelium and in large numbers of mononuclear cells throughout the lung. These findings suggest that a cell-mediated immune response appears during the development of experimental bronchiectasis in this rat model. This cellular immune response is similar to that described in human bronchiectasis and may enable this animal model to be used in defining the role of cellular immunity in the pathogenesis of bronchiectasis.

Bronchiectasis is a chronic lung disease in which there is irreversible dilatation of one or more bronchi, usually associated with chronic purulent sputum production (1). The affected bronchi are the seat of persistent inflammation (2), which may lead to scarring and shrinkage of the lung and ultimately, in some cases, to cor pulmonale and death or heart-lung transplantation.

Although the pathology of bronchiectasis has been well described (2, 3), its pathogenesis remains poorly understood and little work has been attempted in this area since that of Whitwell (2) in 1952. Recently, a "vicious circle" hypothesis for pathogenesis has been proposed by Cole (4, 5). According to this, damaging insults to the bronchial tree (or underlying disease) may compromise the first line bronchial defense mechanism of mucociliary clearance and predispose the individual to microbial colonization of the bronchial tree. These microorganisms appear to be those that are able to release substances that impair the function of and damage ciliated epithelium (6). The host's inflammatory response fails to eliminate these microbial colonists and therefore becomes chronic (7), damaging "innocent bystander" host lung tissue in the process. This damage is likely to reduce mucociliary clearance further and lead to increasing microbial colonization and progressive lung damage. There is immunohistologic evidence that a significant cell-mediated immune response develops in inflamed areas of the bronchiectatic lung (8, 9) and this could be responsible, at least in part, for progressive lung damage—either by the emergence of a cytotoxic T lymphocyte population or by the activation of macrophages. This mechanism has been identified in several chronic inflammatory diseases such as rheumatoid arthritis (10), psoriasis (11), and ulcerative colitis (12).

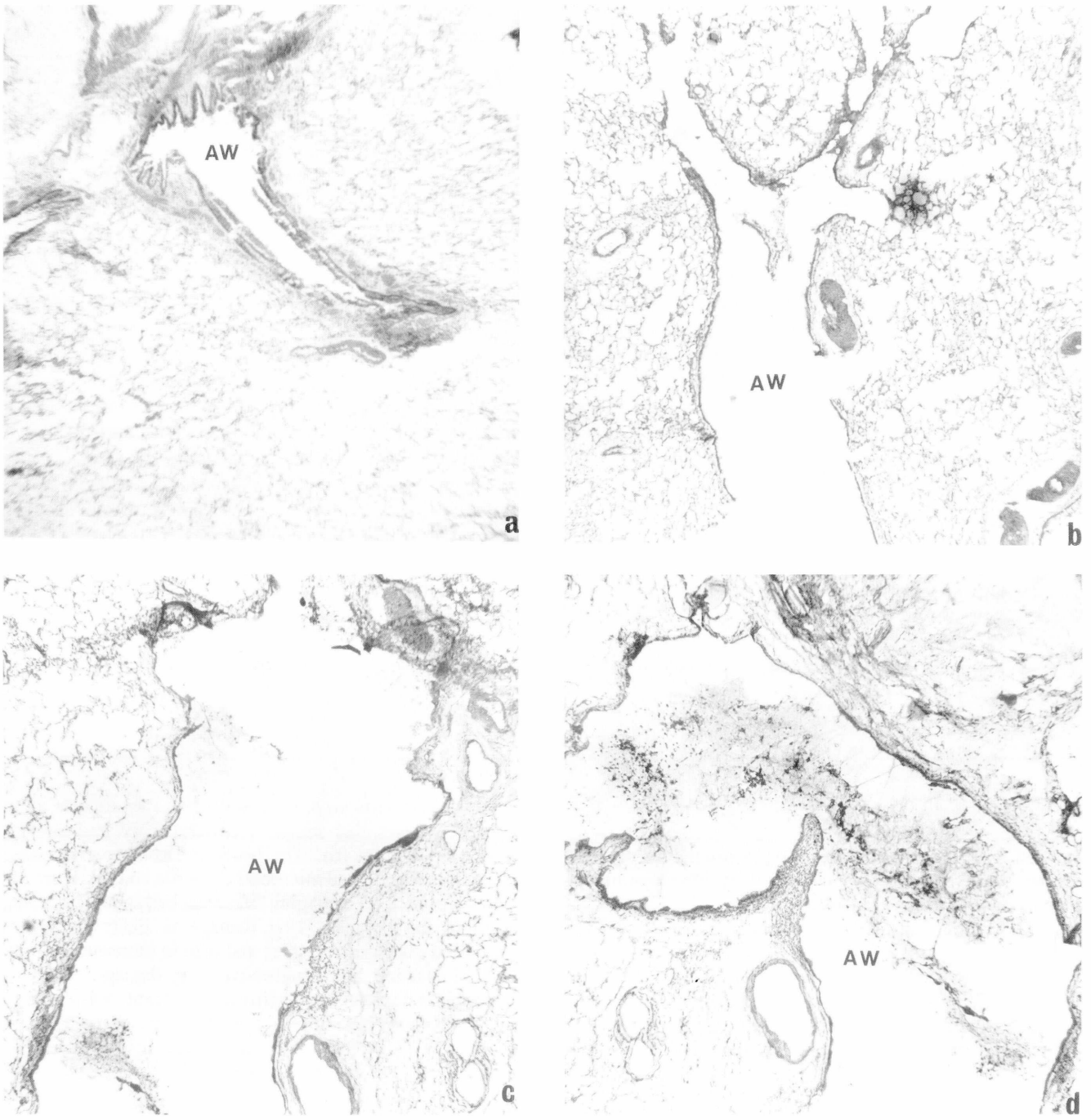
Diagnostic bronchoalveolar lavage and open lung biopsy, which have proved invaluable in the investigation of lung diseases such as sarcoidosis and cryptogenic fibrosing alveolitis, cannot usually be undertaken in bronchiectasis as the diagnosis is established by less invasive radiologic means. Therefore, to facilitate investigation of the pathogenesis of bronchiectasis, particularly the role of nonspecific and immunologically specific inflammatory processes, a rat model of the disease has been developed (D. Guerreiro, B. E. Heard, H. Todd *et al*. Similarities of a new animal model to human *Pseudomonas*-associated bronchiectasis. Unpublished work). We describe the immunohistology of the

Key Words: bronchiectasis, immunopathology, cell-mediated immunity

(Received in original form October 31, 1988 and in revised form February 22, 1989)

Address correspondence to: Peter J. Cole, Host Defence Unit, Department of Thoracic Medicine, National Heart and Lung Institute, Brompton Hospital, Dovehouse Street, London, SW3 6LY United Kingdom.

Abbreviations: partial ligation of the apical lobe bronchus followed by injection of *Pseudomonas aeruginosa*, Pa + LIG; *P. aeruginosa* injected without prior ligation of the bronchus (Pa + NOLIG); normal, N; bronchus-associated lymphoid tissue, BALT.



inflammatory lesions that develop in this model and discuss its similarities to that of resection specimens of human bronchiectasis.

Materials and Methods

Animal Model

Bronchiectasis was induced in SPF Wistar outbred rats (Charles Rivers Ltd., Margate, UK) by a technique of apical lobe ligation. In brief, 160-g animals were anesthetized with an i.m. injection of 0.02 ml of Hypnorm (Janssen Pharmaceutical Ltd., Marlow, UK), intubated with a 4FG cannula (Portex Ltd., Hythe, UK), ventilated with a small animal ventilator (Harvard Apparatus Ltd., Edenbridge, UK), and placed on their left side. Right thoracotomy through the fifth intercostal space was performed aseptically. The apical lobe was then retracted towards the diaphragm so as to expose the apical bronchus, which was partially ligated as near as possible to the bifurcation of the main bronchus, using surgical suture 7/0 prolene (Ethicon Ltd., Edinburgh, UK). A volume of 0.02 ml of bacterial suspension was then injected into the ligated bronchus, the lungs were inflated

Figure 1. Histopathology of experimental bronchiectasis produced in the rat. (Hematoxylin-eosin stain; original magnification: ×40; aw = airways.) (a) Normal control rat; (b) bronchiolectasis; (c) diffuse cylindrical bronchiectasis; (d) saccular bronchiectasis; (e) human follicular bronchiectasis.

manually with a 5-ml syringe, and the chest wall was closed in layers. Once spontaneous breathing was resumed, the animals were extubated and allowed to recover.

The bacterial suspension contained a clinical isolate of *Pseudomonas aeruginosa* (P455) from a bronchiectatic patient, which had been cultured in Oxoid Nutrient Broth No. 2 for 18 h at 37° C and centrifuged at 3,000 rpm for 20 min at 4° C; the cell pellet was washed twice and finally resuspended in sterile PBS. Viable counts were undertaken, and the suspension was adjusted by nephelometry to contain 1×10^9 viable CFU/ml.

Experimental Design and Animals

Three groups of rats were included in the study. In one group ($n = 13$), partial ligation of the apical lobe bronchus was followed by injection of *P. aeruginosa* (Pa + LIG). In the second experimental control group ($n = 11$), *P. aeruginosa* was injected without prior ligation of the bronchus (Pa + NOLIG). The third group consisted of 10 normal, age-matched control rats (N). The mean weight (± SD) of the rats at surgery was 163 ± 13 g. The rats were killed between 8 and 12 wk after the surgery.

Lung Lavage and Tissue Preparation

The animals were anesthetized with 0.5 ml of Hypnorm (Janssen Pharmaceutical) and exsanguinated by cardiac puncture. Lung lavage was performed by instilling 5 ml of sterile PBS through a 4FG cannula (Portex) inserted into the pulmonary tree through a small incision in the trachea. The liquid recovered was cultured for bacteria and showed the presence of large numbers of viable *P. aeruginosa* in all animals of the Pa + LIG group but not in the other groups. After the lungs were excised, the apical lobe of the right lung was dissected, a 4FG cannula was inserted into the lobar bronchus, and the lobe was inflated gently with 1 ml of Histocon (Polysciences, Inc., Warrington, PA). Immediately after inflation, the lung was mounted on cork with the hilar aspect upwards, covered with OCT compound (Miles Laboratories Inc., Naperville, IL), and snap-frozen in isopentane cooled by liquid nitrogen. The block was stored in liquid nitrogen until use. Cryostat sections of 6 μm were cut from three different levels of the specimen (central, intermediate, and peripheral), mounted on poly-L-lysine coated glass slides, air-dried at room temperature for 1 h, fixed in chloroform-acetone for 10 min, and stored at −20° C until use. Serial sections were used to evaluate coexpression of different antigens by a given cell type. Conventional histology stains, including hematoxylin-eosin and toluidine blue, and a stain for elastin (Miller's–Van Gieson), were performed.

Immunohistochemical Procedures

An indirect immunoperoxidase method was used for the immunohistochemical studies (13). The specific antigen-antibody binding was revealed by diaminobenzidine (Sigma Chemical Co., St. Louis, MO) development. Normal rat spleen served as the positive control. The panel of monoclonal antibodies used is shown in Table 1.

Immunohistology

Slides from all three levels of each tissue sample were stained with each of the monoclonal antibodies. The sections from each group of animals stained with the same antibody were randomized and independently evaluated in "blind" manner by two investigators. Five compartments of the lung were assessed: epithelium, bronchus-associated lymphoid tissue (BALT), peribronchial area, perivascular area, and alveolus.

Because significant numbers of mononuclear cells are found in the normal rat lung (17), a scoring system was devised to compare the number of positively stained cells in the

TABLE 1
Panel of monoclonal antibodies

Designation	Specificity	Dilution	Source	References
OX-6	Class II MHC (Ia) antigens	1:20	Sera-Lab	14
OX-8	Suppressor T cells, natural killer cells	1:10	Sera-Lab	14
W3/13	T cells, neutrophils, monocytes	1:10	Sera-Lab	14
W3/25	Helper T cells, macrophages, dendritic cells	1:10	Sera-Lab	14
OX-19*	T cells	1:5	Serotec	14
OX-52*	T cells	1:5	Serotec	15
ED1	Macrophages, dendritic cells	1:500	Dr. Dijkstra	16
ED2	Macrophages, some dendritic cells	1:500	Dr. Dijkstra	16

* A mixture of the two antibodies was employed to enhance staining (17). The final dilution of each of these two antibodies was 1:10.

lungs of rats with experimental bronchiectasis with the number in the normal control animals: zero = same number of positively stained cells; + = 2 to 5 times more positively stained cells than in controls; ++ = 5 to 20 times more positively stained cells than in controls; +++ = >20 times more positively stained cells than in controls.

The individual score for each rat was a mean of the scores obtained from observations of at least three slides taken from each of the levels sampled. The data are presented as the mean for each group. The reproductibility of the scores attributed by the two independent investigators was 82%.

Results

Histopathology

None of the apical lobes of the 11 rats in the Pa + NOLIG group exhibited bronchiectatic changes, and the mononuclear cells were not significantly different in number or distribution from those in the N rats. By contrast, all 13 rats in the Pa + LIG group exhibited histologic changes typical of human bronchiectasis. Three different histologic types could be distinguished in this group of rats (Figure 1): diffuse cylindrical bronchiectasis (6 of 13 rats) affecting major airways and terminal bronchioles; saccular bronchiectasis (2 of 13 rats); bronchiolectasis (5 of 13 rats). In each tissue compartment, the following major changes were seen. (*1*) Epithelium: hyperplasia of goblet cells, epithelial thickening, and/or exfoliation were present. (*2*) BALT: the BALT was severely affected, with extensive disorganization of structure and a great increase in size, reflecting a large increase in the number of mononuclear cells. Follicles were not only associated with the airways but were also found deep in the lung parenchyma. (*3*) Peribronchial area: all specimens showed increased numbers of mononuclear cells along the airways, isolated or forming clusters not associated with the BALT. In 6 animals with cylindrical and saccular bronchiectasis, huge collections of mononuclear cells extending from the basement membrane of the epithelium to the outer layers of peribronchial tissue were seen, sometimes in a continuous sheath. Hyperplasia of the muscularis mucosae was also present. (*4*) Perivascular area: increased numbers of mononuclear cells accumulated around vessels, sometimes forming a continuous layer. (*5*) Alveolus: large numbers of mononuclear cells were detected both in the lumen and in the alveolar septal walls. In all three types of bronchiectasis, there was partial replacement of the alveoli by fibrotic tissue, follicular aggregates of mononuclear cells, or bronchus-related cysts – all reducing the size of the entire lobe.

Four rats with cylindrical and saccular bronchiectasis also exhibited impressive hilar lymph node enlargement, sometimes occupying up to one third of the whole diameter of the tissue section.

Immunohistology of Normal Rat Lungs

The frequency and distribution of lymphocytes and macrophages observed in normal Wistar rats were very similar to that described in BN and WAG strain rats (17), as shown in Table 2.

TABLE 2

Distribution and relative frequency of mononuclear cells in the lungs of normal rats, expressed as numbers of positively stained cells per hpf

Monoclonal Antibody	Epithelium	BALT*	Peri-bronchial	Peri-vascular	Alveolar Septa/lumen
OX-6	<1	80–90	10	3	5/0
OX-8	<1	5–10	3	<1	2/0
W3/13	<1	10	3	<1	2/0
W3/25	<1	10–20	10	5	2/0
OX-19/OX-52	<1	10–20	8	3	2/0
ED1	<1	5–10	5	1	5/5
ED2	0	0	3	3	1/1

* Percentage of positively stained mononuclear cells.

Immunohistology of Pa + LIG Rats with Experimental Bronchiectasis

Striking changes in the distribution and frequency of immunocompetent cells were seen in rats of the Pa + LIG group (Figure 2). In contrast, the frequency and distribution of cells in the Pa + NOLIG group were similar to that seen in the normal control group (data not shown).

1. Class II Major Histocompatibility Complex (MHC) antigens (Figure 3). (*1*) Epithelium: besides a slight increase in mononuclear inflammatory cells that expressed Ia antigens, epithelial cells were also positive. The large airways were entirely positive, while the bronchioles were only positive in patches. In the two examples of saccular bronchiectasis, strong Ia expression was seen throughout the epithelium (Table 3). (*2*) BALT: although the absolute number of Ia-positive cells in BALT was greatly increased, the proportion of Ia-positive cells to the total number of cells in the BALT remained normal. (*3*) Peribronchial area: the expanded mononuclear cell population present in this compartment was strongly Ia positive, with more than 50% of cells expressing the antigen. (*4*) Perivascular area: significant perivascular accumulation of mononuclear cells was found, more than 50% of the cells being Ia positive. (*5*) Alveolus: a large increment in Ia-positive cells was seen in the alveolar septal wall, but it was difficult to assess Ia expression in the alveolar lumen due to unsatisfactory inflation.

2. T lymphocytes (Figure 4). (*1*) Epithelium: a small increase was found in the number of T cells infiltrating the epithelium, and these were mainly OX-8 positively stained cells. (*2*) BALT: a large increase in size of BALT was found to be attributable to T cells. In some animals, 80% or more of the cells in the huge lymphoid clusters that replaced the normal follicular structure were positively stained by at least one T cell marker. Forty to eighty percent of the cells expressed the phenotype of suppressor/cytotoxic T cells. (*3*) Peribronchial area: a large increase in T cells was found, usually distributed densely beneath the basement membrane in a compact sheath, with isolated cells or small clusters of cells also present. (*4*) Perivascular area: T cells in perivascular sites were usually isolated but sometimes formed compact perivascular sheaths. (*5*) Alveolus: the slight increase

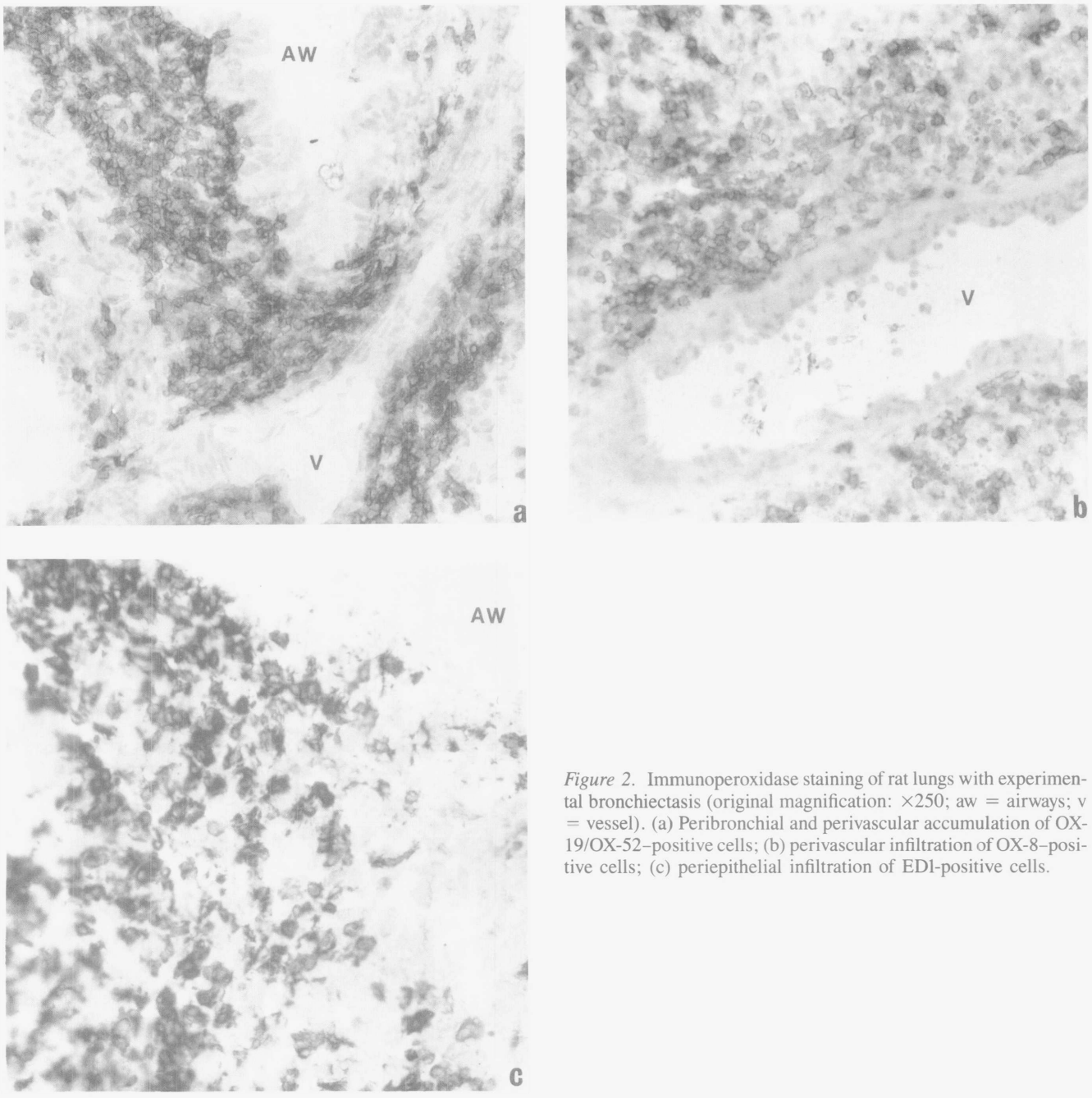


Figure 2. Immunoperoxidase staining of rat lungs with experimental bronchiectasis (original magnification: ×250; aw = airways; v = vessel). (a) Peribronchial and perivascular accumulation of OX-19/OX-52–positive cells; (b) perivascular infiltration of OX-8–positive cells; (c) periepithelial infiltration of ED1-positive cells.

TABLE 3

Relationship between pattern of bronchiectasis and immunohistopathology in experimental bronchiectasis

Immunohistology	Diffuse Cylindrical (n = 6)	Saccular (n = 2)	Bronchiolectasis (n = 5)
Large follicular aggregates of T cells	5*	0	1
Ia expression by epithelial cells	3	2	0
Subepithelial accumulation of macrophages/T cells	4	1	3

* Number of animals in which relationship was seen.

in number of alveolar T cells was almost completely confined to the septal walls.

3. Macrophages (Figure 5). (*1*) Epithelium: there was no increase in the number of macrophage-like cells in this compartment. (*2*) BALT: many more ED1-positive cells were seen among the mononuclear cells in follicle-like aggregates than in the controls. The number staining with ED2 was similar to normal. (*3*) Peribronchial area: many more ED1- and ED2-positive cells were present in this compartment than in the controls. (*4*) Perivascular area: a slight increase of ED1-positive cells was seen in perivascular sites, mainly in the connective tissue. (*5*) Alveolus: large numbers of ED1-

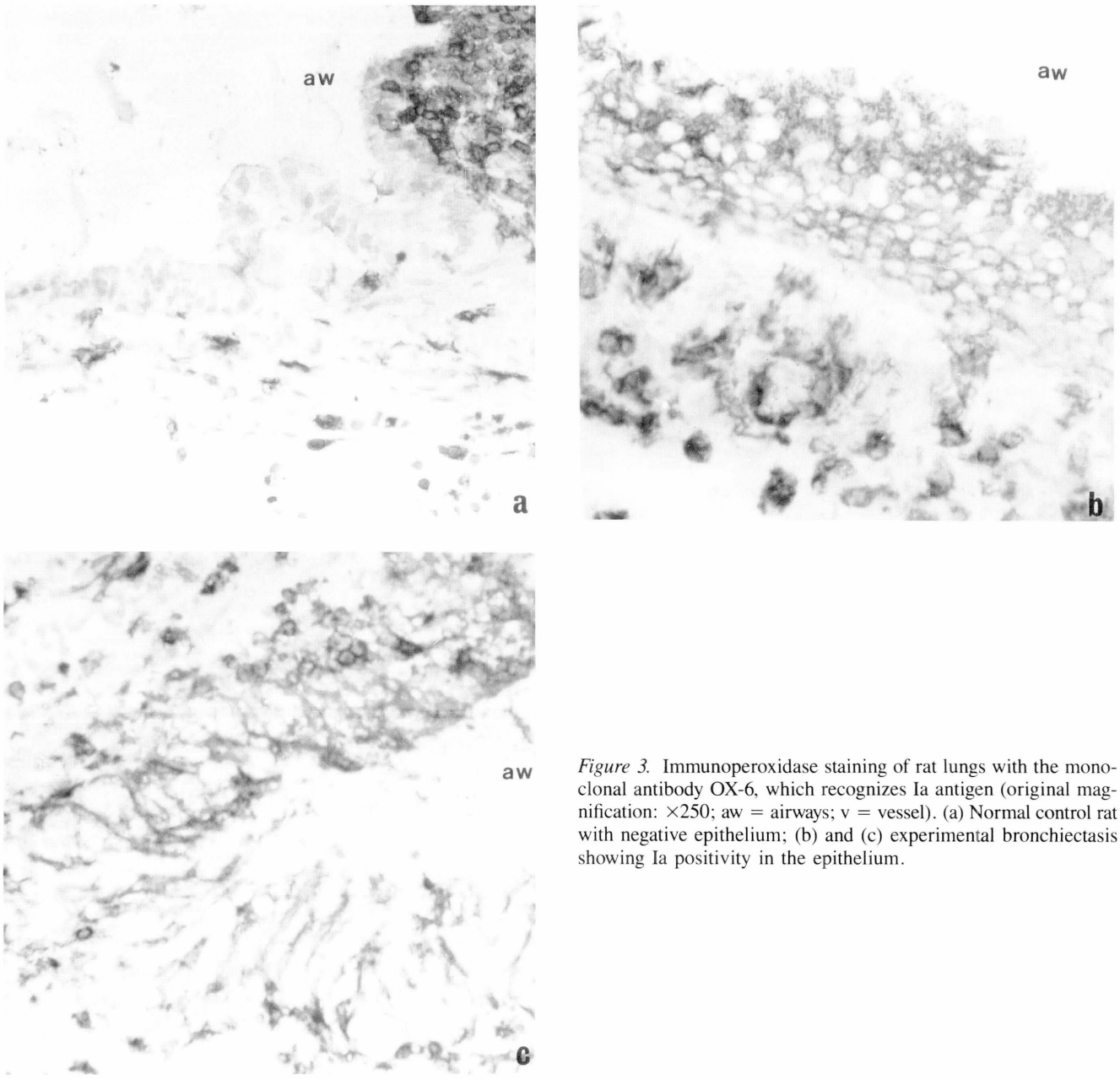


Figure 3. Immunoperoxidase staining of rat lungs with the monoclonal antibody OX-6, which recognizes Ia antigen (original magnification: ×250; aw = airways; v = vessel). (a) Normal control rat with negative epithelium; (b) and (c) experimental bronchiectasis showing Ia positivity in the epithelium.

positive cells were present in the alveolar compartment, and many free alveolar (putative) macrophages could be identified with this marker. Increased ED1 stain was also noticed in the alveolar septal walls, but ED2 staining was similar to normal.

There appeared to be a relationship between the extent and pattern of bronchiectasis and the expression of Ia antigens by the bronchial epithelium (Table 3). The subepithelial accumulation of mononuclear cells was increased in all cases and did not distinguish between the different morphologic types of bronchiectasis, but the presence of large follicular aggregates of mononuclear cells was a characteristic finding in rats with diffuse cylindrical bronchiectasis. The expression of Ia antigen by the epithelial cells was greatly increased in the two animals with saccular bronchiectasis but was somewhat less in those with diffuse cylindrical bronchiectasis.

Discussion

Experimental bronchiectasis in the rat closely mirrors human disease. The presence of large numbers of activated T lymphocytes and macrophages in the bronchial wall infiltrates in human bronchiectasis suggests that an ongoing cell-mediated immune response contributes to the inflammatory process (8, 9). In the present study, T lymphocytes were also found to predominate in the infiltrates of experimental bronchiectasis in the rat. They were evenly distributed throughout the tissue, constituting more than 80% of the mononuclear cell infiltration around the airways, and organized as "folli-

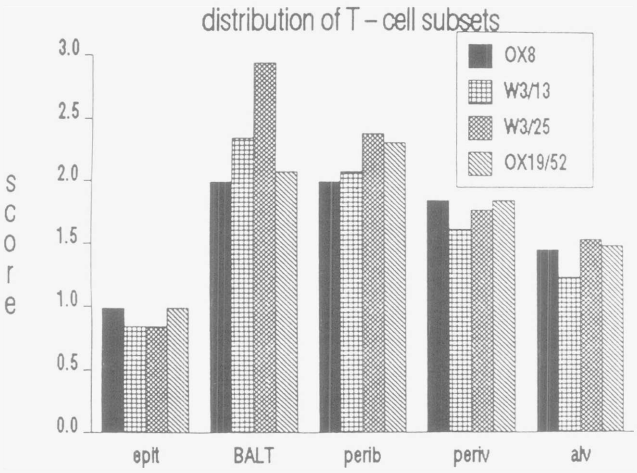


Figure 4. Cells positively stained with the monoclonal antibodies OX-8, W3/13, W3/25, and OX-19/OX-52 in the compartments of the apical lobe in rats with experimental bronchiectasis. The scoring system compares the number of positively stained cells in the lungs of rats with experimental bronchiectasis with the number in the normal control animals: zero = same number of positively stained cells; + = 2 to 5 times more positively stained cells than in controls; ++ = 5 to 20 times more positively stained cells than in controls; +++ = >20 times more positively stained cells than in controls.

The numbers of cells positively stained by each monoclonal antibody in the normal control rats are presented in Table 2.

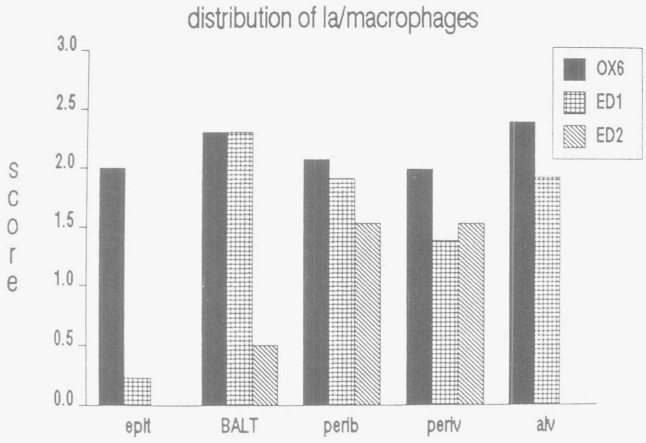


Figure 5. Cells positively stained with the monoclonal antibodies OX-6, ED1, and ED2 in the compartment of the apical lobe in rats with experimental bronchiectasis. The scoring system compares the number of positively stained cells in the lungs of rats with experimental bronchiectasis with the number in the normal control animals: zero = same number of positively stained cells; + = 2 to 5 times more positively stained cells than in controls; ++ = 5 to 20 times more positively stained cells than in controls; +++ = >20 times more positively stained cells than in controls.

The numbers of cells positively stained by each monoclonal antibody in normal control rats are presented in Table 2.

cles" or as a continuous sheath of cells around vessels and airways. The disorganization of BALT was prominent, and the usually small number of T lymphocytes arranged in the outer edge was substituted by a massive infiltration of T cells as defined by T lymphocyte markers (OX-19, OX-52, W3/13, W3/25). Many suppressor-cytotoxic T cells were present (OX-8 positive). Large numbers of macrophages and dendritic cells stained by the monoclonal antibodies ED1 and ED2 were seen in the areas of lymphocytic infiltration, many expressing Class II MHC antigens (OX-6 positive) as seen in stained serial sections.

In normal rat lungs, a compartmentalization of subsets of macrophage-like cells (distinguished by the monoclonal antibodies ED1 and ED2) exists, as expressed by the predominance of ED1-positive cells in the epithelium, BALT and alveolar area, and similar numbers of ED1- and ED2-positive cells in the peribronchial and perivascular areas. In experimental bronchiectasis, a striking increase in the numbers of cells stained by both markers was seen, but the distribution remained the same. This may suggest that local cell populations are predominantly involved in the inflammatory reaction.

There was correlation between the emergence of Class II MHC (Ia) antigen in the epithelial cells and the histologic severity of bronchiectatic changes. In cases of diffuse cylindrical and saccular bronchiectasis, strong expression of epithelial Ia was seen, whereas less severely affected animals showed only limited Ia expression. A similar phenomenon has been described in the bronchial epithelium of rats acutely rejecting lung allografts soon after transplantation (18). In this process, it is suggested that the mediator inducing the expression of Ia antigens is gamma-interferon, produced mainly by activated T cells and by natural killer cells (19). In experimental bronchiectasis, large numbers of Ia-positive, activated T lymphocytes, many of suppressor/cytotoxic phenotype (OX-8 positive), are present in the bronchial inflammatory response and it is possible that they produce this lymphokine. More recently, bronchiolitis and bronchiectasis have been described as late complications of human heart-lung transplantation (20) and the progression of these complications appears to be reduced by increasing the immunosuppression regimen (21).

The presence of T lymphocytes, macrophages, and dendritic cells expressing large amounts of Ia, at the same sites, in the bronchiectatic lesions of these experimental rats constitutes the necessary conditions for the promotion of a type IV delayed hypersensitivity reaction (22). These findings are consistent with the "vicious circle" hypothesis (4, 5), for the pathogenesis of bronchiectasis in which an uncontrolled host inflammatory reaction to a colonizing microbial load constitutes the basis for the emergence and progression of the condition. The demonstrated cell-mediated immune response forms one component of this inflammatory response.

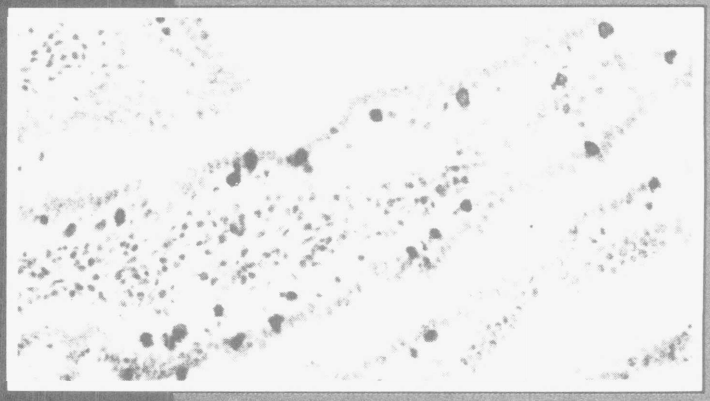
This animal model of bronchiectasis is therefore similar to human bronchiectasis in respect to its immunopathology and offers the opportunity of clarifying a number of aspects of pathogenesis of the disease. Among these are the relative contributions of the microbe and host to the inflammatory lesions, the kinetics of expression of delayed hypersensitivity mechanisms in chronic lung inflammation, and determination of the functional capacities of the immunocompetent cells in the lesions.

Acknowledgments: The writers thank Mr. H. Todd, Mrs. J. A. L. Rohde, Mr. J. A. H. Jones for technical support, Dr. N. Munro for invaluable advice, and Dr. C. D. Dijkstra for kindly supplying the monoclonal antibodies ED1 and ED2. This work was supported by grants from the British Lung Foundation and Clinical Research Committee of Brompton Hospital. Dr. Lapa e Silva is a recipient of a scholarship from the Brazilian Research Council (CNPq).

References

1. Barker, A. L., and E. J. Bardana, Jr. 1988. Bronchiectasis: update of an orphan disease. *Am. Rev. Respir. Dis.* 137:969–978.
2. Whitwell, F. 1952. A study of the pathology and pathogenesis of bronchiectasis. *Thorax* 7:213–239.
3. Reid, L. M. 1950. Reduction in bronchial subdivisions in bronchiectasis. *Thorax* 5:233–247.
4. Cole, P. J. 1984. A new look at the pathogenesis and management of persistent bronchial sepsis: a "vicious circle" hypothesis and its logical therapeutical connotations. *In* Strategies for the Management of Chronic Bronchial Sepsis. R. J. Davies, editor. The Medicine Publishing Foundation, Oxford. 1–20.
5. Cole, P. J., and R. Wilson. 1989. Host-microbial interrelationship in respiratory infection. *Chest* 95:217S–221S.
6. Wilson, R., T. Pitt, G. Taylor, *et al.* 1987. Pyocyanin and 1-hydroxyphenazine produced by *Pseudomonas aeruginosa* inhibit beating of human respiratory cilia *in vitro*. *J. Clin. Invest.* 79:221–229.
7. Currie, D. C., S. H. Saverymuttu, A. M. Peters, *et al.* 1987. Indium-labelled granulocyte accumulation in the respiratory tract of patients with bronchiectasis. *Lancet* 1:1335–1339.
8. Lapa e Silva, J. R., J. A. H. Jones, B. Noble, L. W. Poulter, and P. J. Cole. 1988. A cell-mediated immune response is present in the chronic inflammation of bronchiectasis (abstract). *Am. Rev. Respir. Dis.* 137(Suppl:A50).
9. Lapa e Silva, J. R., J. A. H. Jones, P. J. Cole, and L. W. Poulter. 1989. The immunological component of the cellular inflammatory infiltrate in bronchiectasis. *Thorax* (In press).
10. Poulter, L. W., O. Duke, G. S. Panayi, S. Hobbs, M. J. Raftery, and G. Janossy. 1985. Activated T-lymphocytes of the synovial membrane in rheumatoid arthritis and other arthropathies. *Scand. J. Immunol.* 22:683–689.
11. Poulter, L. W., R. Russel-Jones, and S. Hobbs. 1984. The significance of antigen presenting cells in psoriasis. *In* Immunodermatology. D. C. MacDonald, editor. Butterworth, London. 185–188.
12. Poulter, L. W., M. Allison, A. T. Richardson, and G. Janossy. 1987. The distribution of lymphocyte subsets in inflammatory bowel disease and reactive synovitis: a comparison. *In* Spondyloarthropathies: Involvement of the Gut. H. Mielants, and E. M. Veys, editors. Elsevier, Amsterdam. 213–220.
13. Munro, C. S., D. N. Mitchell, L. W. Poulter, and P. J. Cole. 1986. Early cellular responses to intradermal injection of Kveim suspensions in normal subjects and those with sarcoidosis. *J. Clin. Pathol.* 39:176–182.
14. Mason, D. W., R. P. Arthur, M. J. Dallman, J. R. Green, G. P. Spickett, and M. L. Thomas. 1983. Functions of rat T-lymphocyte subsets isolated by means of monoclonal antibodies. *Immunol. Rev.* 74:57–63.
15. Robinson, A. P., M. Puklavec, and D. W. Mason. 1986. MRC OX-52: a rat T-cell antigen. *Immunology* 57:527–531.
16. Dijkstra, C. D., E. A. Dopp, P. Joling, and G. Krall. 1985. The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2, and ED3. *Immunology* 54:589–599.
17. Holt, P. G., and M. A. Schon-Hegrad. 1987. Localization of T cells, macrophages and dendritic cells in rat respiratory tract tissue: implications for immune function studies. *Immunology* 62:349–356.
18. Romaniuk, A., J. Prop, A. H. Petersen, C. R. H. Wildevuir, and P. Nieuwenhuis. 1988. Expression of Class II major histocompatibility complex antigens by bronchial epithelium in rat lung allografts. *Transplantation* 44: 209–213.
19. Kirby, L. A., G. J. Parfett, J. A. Reader, and J. R. Pepper. 1988. Lung transplantation in the rat: a model for study of the cellular mechanisms of allograft rejection. *Immunology* 63:369–372.
20. Burke, C. M., J. Theodore, K. D. Dawkins *et al.* 1984. Post-transplant obliterative bronchiolitis and other late lung sequelae in human heart-lung transplantation. *Chest* 86:824–829.
21. Glanville, A. R., J. C. Baldwin, C. M. Burke, J. Theodore, and E. D. Robin. 1987. Obliterative bronchiolitis after heart-lung transplantation: apparent arrest by augmented immunosuppression. *Ann. Intern. Med.* 107:300–304.
22. Unanue, E. R., and P. M. Allen. 1987. The basis for the immunoregulatory role of macrophages and other dendritic cells. *Science* 236:551–557.

Advances in Mucosal Immunology



Edited by
Thomas T. MacDonald
Stephen J. Challacombe
Paul W. Bland
Christopher R. Stokes
Richard V. Heatley
Allan McI Mowat

258
Cell-mediated immune kinetics in experimental bronchiectasis

JR Lapa e Silva, D Guerreirro, NC Munro, B Noble, PJ Cole and LW Poulter

Host Defence Unit, Department Thoracic Medicine, National Heart and Lung Institute, Brompton Hospital and Academic Department of Immunology, Royal Free Hospital School of Medicine, London, UK

ABSTRACT. In a recently developed rat model of bronchiectasis (BX) generated by partial bronchial ligation and injection of Pseudomonas aeruginosa (Pa), the composition and distribution of immunocompetent cells were analysed at progressive timeintervals by immunohistochemical methods. Four groups of 25 animals were prepared: 1) Partial ligation and injection of Pa (Pa+LIG); 2) Injection of Pa without bronchial ligation (Pa+NOLIG); 3) Sham operated (S); 4) Age-matched normal controls (N). Bronchiectasis developed in 22/25 animals of the Pa+LIG group but in none of the 75 controls of the other 3 groups. This bronchial dilation began within 2 weeks and was accompanied by a T-lymphocyte proliferation beginning in the bronchus-associated lymphoid tissue (BALT). Up to week 4 this was composed mainly of CD4+ve cells. Large de novo lymphoid aggregates appeared in the lung parenchyma, with an initial (2-4 wks.) predominance of CD4+ cells. From week 8 onwards, a predominance of CD8+ cells was noted in both areas. The bronchial lamina propria of rats from the Pa+LIG group was infiltrated by significantly larger numbers of T-lymphocytes (mainly of CD8+ phenotype) and macrophages when compared with the other 3 groups, at all time points. The bronchial epithelium of 17/22 animals showing bronchial dilation in the Pa+LIG group expressed Ia antigen but that of the 75 animals in the other groups did not. These findings support the hypothesis that chronic inflammation in bronchiectasis is associated with a cell-mediated immune response developing as a consequence of bacterial infection in an airway where the clearance mechanisms have been impaired.

1. INTRODUCTION.

Bronchiectasis is a chronic lung disorder characterised by irreversible dilation of parts of the bronchial tree, usually associated with persistent inflammation which may lead to scarring and shrinkage of the lung and ultimately, in some cases, to cor pulmonale and death (1). The pathogenesis of bronchiectasis remains poorly understood and little work has been attempted in this area since that of Whitwell (2) in 1952. Recently, the "vicious circle" hypothesis for its pathogenesis proposed

by Cole (3) postulated that microbial colonisation of the bronchial tree subverts the host's normally protective inflammatory response into a tissue-damaging one resulting in progressive lung damage. One of the microorganisms frequently associated with chronic bronchial infection in bronchiectasis is Pseudomonas aeruginosa (1). The immunohistology of the inflammatory lesions which develop in a recently devised rat model of Pseudomonas-associated bronchiectasis has been shown (4) to resemble that previously found in resection specimens of human bronchiectasis (5). We have now examined the kinetics of development of this cell-mediated immune response using the animal model.

2. MATERIAL AND METHODS

2.1. Animal model. Bronchiectasis was induced in SPF Wistar outbred rats (Charles Rivers Ltd., Margate, UK) by a technique of apical lobe ligation. In brief, 160g animals were anaesthetised with an intramuscular injection of 0.02 ml of Hypnorm (Janssen Pharmaceutical Ltd., Marlow, U.K.), intubated with a 4FG cannula (Portex Ltd., Hythe, U.K.), ventilated with a small animal ventilator (Harvard Apparatus Ltd., Edenbridge, U.K.), and placed on their left side. Right thoracotomy through the 5th intercostal space was performed aseptically. The apical lobe was then retracted towards the diaphragm so as to expose the apical bronchus, which was partially ligated as near as possible to its orginin from the main bronchus, using surgical suture 7/0 prolene (Ethicon Ltd., Edinburgh, U.K.). A volume of 0.02 ml of bacterial suspension composed of 1×10^9 of per ml a clinical isolate of Pseudomonas aeruginosa (P455) was then injected into the ligated bronchus, the lungs inflated manually with a 5 ml syringe, and the chest wall closed in layers. Once spontaneous breathing was resumed, the animals were extubated and allowed to recover.

2.2. Experimental Design and Animals. Four groups of rats were included in the study, each containing 25 animals: Group 1 (Pa + LIG) - partial ligation of the apical lobe bronchus was followed by injection of P. aeruginosa as described above; Group 2 (Pa + NOLIG)- a similar amount of P.aeruginosa was injected without prior ligation of the bronchus; Group 3 (S) - sham operated animals; Group 4 (N) - age-matched normal controls. Five rats from each group were sacrificed after 2,4,8,12, and 16 weeks.

2.3. Immunohistochemical Procedures. Cryostat sections of 6 μm were obtained from the inflated apical lobes. An indirect immunoperoxidase method was used for the immunohistochemical studies using diaminobenzidine (Sigma Chemical Company, Inc., St. Louis, USA) development. The panel of monoclonal antibodies used was the following: MRC OX-52 (Serotec, Oxford, U.K.), present on rat T lymphocytes; MRC OX-19 (Serotec), rat T cells. MRC OX-19 and MRC OX-52 were used together to enhance the staining; MRC OX-8 (Serotec), rat cytotoxic/suppressor T cells; MRC OX-6 (Serotec), rat Ia antigen; ED1 (supplied by Dr. C. Dijkstra), rat macrophages and dendritic cells.

Three compartments of the lung tissue were assessed: epithelium, bronchus-associated lymphoid tissue (BALT) and other lymphoid aggregates, and peribronchial area. The positively stained cells were quantified using a graticule, which corresponded to a total

area of 0.024 mm at 400 x magnification. The results were expressed as the mean number of positive cells per unit area (0.024 mm^2) + standard deviation (SD). Student's t-test for non-paired data was used and the data considered statistically significant when $p =< 0.05$.

3. RESULTS

3.1 T-Lymphocyte proliferation in BALT: Significant T-lymphocyte proliferation in BALT of animals of the Pa + LIG group was first observed at week 2 (p = 0.002), when compared with the N group. None of the animals in the Pa + NOLIG or S groups expressed such proliferation and a statistically significant difference was also seen when both control groups were compared with the Pa + LIG group (p =0.002, and p =0.003, respectively). Subsequently, T cell numbers decreased but were still significantly different from those in normal controls at weeks 4 (p = 008), 8 (p = 0.02), 12 (p = 0.01), and 16 (p = 0.04). Similar findings were seen in comparison with the Pa + NOLIG and S groups. This proliferation was due initially to increase in helper T-cells. A shift to proliferation of cytotoxic/ suppressor T-cells was seen from week 4 onwards. The macrophage-like cell populations in BALT did not change significantly in any group at any time point.

3.2. Composition of the newly formed lymphoid aggregates: T-lymphocyte proliferation was also seen in newly formed lymphoid aggregates in all animals of the Pa + LIG group. Extensive aggregates of T-lymphocytes were present in some animals but in others this phenomenon was less marked. No such aggregates were noticed however in the controls. This T cell accumulation was most marked at week 2 but began to decline from 8 weeks onwards. At first, the T-cells were almost exclusively putative helper cells (OX-8 negative) but the numbers of cytotoxic/ suppressor cells increased steadily up to week 8 and then began to decline. At week 12 almost all T cells were OX-8 positive. Many ED1 positive macrophages were also present in the aggregates.

3.3. Peribronchial infiltration: A less marked but statistically significant increase in the T-cell population was seen from week 2 in the Pa + LIG group when compared with the Pa + NOLIG, S, and N groups and this population remained significantly increased at all time points. The two control groups failed to show any difference when compared with normal rats. This T-cell increment was almost entirely due to the OX-8 positive subset at all time points. A statistically significant increase in the macrophage-like cells population at week 2 was seen in both Pa + LIG (p = 0.001), Pa + NOLIG (p = 0.01), and S groups (p = 0.0001) when compared with normal controls. This increase was not evident in the Pa + NOLIG and S groups from week 4 onwards, but in the Pa + LIG group macrophage infiltration remained significant compared with that in normal rats at all time points.

3.4. Expression of Ia by the bronchial epithelium: Ia antigen was expressed by the bronchial epithelium of 17/25 animals in the Pa + LIG group but not by the epithelium of the 75 rats of the three control groups. An association between the presence of bronchial dilatation and the epithelial expression of Ia was noticed: of the 22 rats of this group showing bronchiectasis, 17 expressed Ia antigen in the bronchial epithelium.

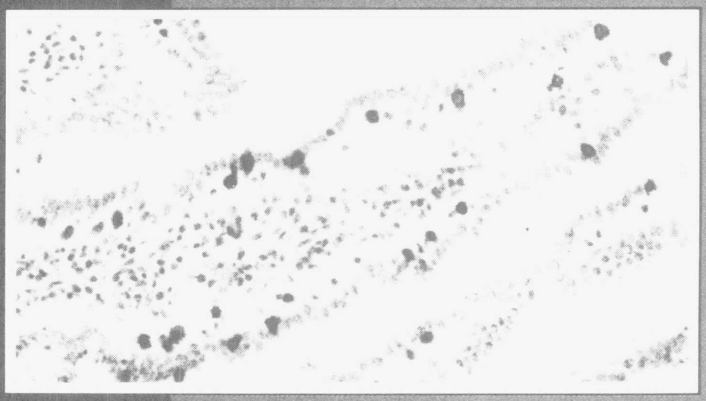
4. DISCUSSION

Introduction of Pseudomonas aeruginosa into the rat bronchus after partial ligation produces a model of bronchiectasis in which to test the "vicious circle" hypothesis of the pathogenesis of the condition. As a first step we have examined the kinetics of the cellular immunological inflammatory reactions associated with bronchiectasis and the results confirm the previous suggestion that this inflammation is associated with a cell-mediated immune response. Here we have shown that, following bronchial infection, failure to clear the bacterial load due to partial bronchial obstruction is associated with a massive cell-mediated immune response and imbalance between benefit and damage. The data produced here suggest that Pseudomonas aeruginosa may induce a local cell-mediated immune response in situations in which the mucociliary mechanisms of the bronchi are previously impaired. However, no direct evidence exists to confirm that the cell-mediated response observed in the bronchial wall is directed against antigens of the Pseudomonas. Other possibilities are that neo-antigens are released by the experimental procedure and that these promote auto-immune phenomena locally or, alternatively, that opportunistic viruses may take advantage of the bronchial damage and promote an immunological response. These possibilities could all explain the predominance of the CD8 positive subset in the bronchial wall.

5. REFERENCES.

1. Cole, P.J. (1989) "Bronchiectasis", in A. Brewis, J. Gibson, and D. M. Geddes (eds.), Textbook of Respiratory Medicine, Bailliere Tyndall, London, (in press).
2. Whitwell, F. (1952) "A study of the pathology and pthogenesis of bronchiectasis", Thorax 7, 213-239.
3. Cole, P.J. (1984) "A new look at the pathogenesis and management of persistent bronchial sepsis: a "vicious circle" hypothesis and its logical therapeutic connotations", in R. J. Davies (ed.), Strategies for the Management of Chronic Bronchial Sepsis, The Medicine Publishing Foundation, Oxford, pp. 1-20.
4. Lapa e Silva, J.R., Guerreiro, D. , Noble, B., Poulter, L.W. and Cole P.J. (1989) "Immunopathology of experimental bronchiectasis", Am. J. Respir. Cell Mol. Biol., (in press).
5. Lapa e Silva, J. R., Jones, J.A.H., Cole, P.J. and Poulter, L. W. (1989) "The immunological component of the cellular inflammatory infiltrate in bronchiectasis", Thorax, (in press).

Advances in Mucosal Immunology



Edited by
Thomas T. MacDonald
Stephen J. Challacombe
Paul W. Bland
Christopher R. Stokes
Richard V. Heatley
Allan McI Mowat

198
Phenotype of bronchial T-lymphocyte population in the chronic inflammation of bronchiectasis

JR Lapa e Silva, LW Poulter and PJ Cole

Host Defence Unit, Department of Thoracic Medicine, National Heart and Lung Institute, Brompton Hospital and Academic Department of Immunology, Royal Free Hospital School of Medicine, London, UK

1. INTRODUCTION

Bronchiectasis is a chronic disease characterised by irreversible dilation of one or more bronchi. The affected bronchi are seat of persistent inflammation which may lead to scarring and shrinkage of the lung, and in some cases to cor pulmonale and death. The understanding of the pathogenesis bronchiectasis is still poor. Recently a "vicious circle" hypothesis for the pathogenesis of the disease has been proposed by Cole. According to this, damaging insults to the bronchial tree or underlying disease may compromise the first line bronchial defence mechanisms of mucociliary clearance and predispose the individual to microbial colonization of the bronchial tree. The host's inflammatory response fails to eliminate these microbial colonists and becomes chronic, damaging adjacent lung disease in the process. There is immunohistological evidence that a significant cell-mediated immune response develops in inflamed areas of bronchiectatic lungs. This inflammation could be responsible, at least in part, for the progressive lung damage seen in the disease, either by the emergence of a cytotoxic T-lymphocyte population or by the activation of macrophages. Such mechanisms have been identified in several chronic inflammatory diseases such as rheumatoid arthritis, psoriasis, and inflammatory bowel disease. We present here the phenotype of the T-lymphocyte population present in the bronchial wall of resection specimens of 23 bronchiectatic patients in comparison to non-bronchiectatic controls.

2. MATERIALS AND METHODS

Patients: Tissues from 23 patients with radiographically proved bronchiectasis were obtained at lobar resection or by bronchial biopsy during clinically indicated fibreoptic bronchoscopy. Controls: Tissues from 7 patients without bronchiectasis (four carcinomas, three pneumonias) were used as controls, being obtained by bronchial biopsy during clinically indicated fibreoptic bronchoscopy (three cases), or at resection for bronchial carcinoma, from non-compromised areas (four

cases). All samples were snap frozen in liquid nitrogen and cryostat sections of 6 microns preopared and some were stained for conventional histology. Indirect immunoperoxidase using a pan T-cell marker was employed to determine the distribution of T-lymphocytes in the bronchial mucosa. Double immunofluorescence was used to investigate the CD4/CD8 ratios in the bronchial wall and the co-expression of other molecules by the T cells using of a combination of specific anti-mouse antisera used in this study: CD7 (blast T cells); RFDR (Class II MHC); CD25 (Interleukin-2 receptor); CD38 (activated T-cells); UCHL1 (LCA-memory cells); CD45R (LCA-virgin cells);

3. RESULTS

TABLE1- Distribution of T-lymphocytes in the bronchial mucosa

	Epithelium	Lamina Propria	Submucosa
BX	26.9+10.4 (20)	29.7+21 (22)	28.7+20.8 (21)
C	7.1+1.6 (6)	5.4+4.1(7)	6.5+3.4 (5)
P	0.0001	0.0001	0.0001

BX= bronchiectatic samples C= controls p= p value (x) = number of samples. Both CD4+ and CD8+ subsets were observed but there was a consistent overall predominance of CD8+ cells. Further analysis of the membrane antigens by both subsets was carried out and is expressed here as the percent of doubly-labelled cells (SD ommited for clarity):

TABLE2 - Phenotype of CD4 and CD8 positive T-lymphocytes

	CD7		CD25		CD38		HLA-DR		UCHL 1		SN130	
	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
BX	27.6	83.0	2.0	4.3	6.1	13.6	27.3	19.3	61.8	75.5	43.1	18.6
C	15.0	30.0	0	0	1.2	0	3.7	3.5	35.0	35.0	25.0	17.5
P	NS	.05	NS	.05	.05	.01	.01	0001	.0001	.0001	NS	NS

4. DISCUSSION

This paper showed that T-lymphocytes constitutes a major component of the inflammatory infiltrates present in the bronchial wall of bronchiectasis. The differences between bronchiectatic and control suggested that these cells may play a role in the pathogenesis of this condition. Also important was the predominance of the CD8+ cells when compared with the CD4+ subset. The CD8+ population expressed significant amounts of activation markers, as IL-2 receptor, CD7, CD38, and HLA-DR. The only activation marker expressed in significant amounts by the CD4+ cells was the HLA-DR. Differences between BX and C were noted in the proportions of CD8+UCHL1+ and CD4+UCHL1+, whereas no differences could be found in the proportions of CD8+CD45R+ or CD4+CD45R+. It has been demonstrated that after T cell activation, a switch of leucoyte common antigen expression occurs which is detected by the disappearance of CD45R and the appearance of UCHL1 positivity. The damage created by this inflammation in the bronchi and surrounding tissues in bronchiectasis indicates a cytotoxic role for the CD8 positive T cells, the overall characteristic of which reflect those seen in cell-mediated immune reactions.

Reprinted from Thorax 1989;**44**:668–673

The immunological component of the cellular inflammatory infiltrate in bronchiectasis

J R LAPA E SILVA, J A H JONES, P J COLE, L W POULTER

British Medical Association, Tavistock Square, London WC1H 9JR

Thorax 1989;**44**:668–673

The immunological component of the cellular inflammatory infiltrate in bronchiectasis

J R LAPA E SILVA, J A H JONES, P J COLE, L W POULTER

From the Host Defence Unit, Department of Thoracic Medicine, National Heart and Lung Institute, Brompton Hospital, and the Academic Department of Immunology, Royal Free Hospital and School of Medicine, London

ABSTRACT Immunohistological analysis of bronchial biopsy specimens from nine patients with bronchiectasis and four control subjects was performed with a panel of monoclonal antibodies selected to show lymphocyte and macrophage subsets and signs of cellular activation. The cells taking part in the inflammatory response in the bronchial wall of patients with bronchiectasis were almost exclusively mononuclear cells, most of them T lymphocytes. B lymphocytes were observed in biopsy specimens from only two out of nine patients. $CD8^+$ T cells outnumbered $CD4^+$ cells in all patients in a ratio ranging from 2:1 to 10:1. Most T lymphocytes also strongly expressed CD7 antigen and a proportion of them expressed HLA-DR. Most of the lymphocytic infiltration occurred just beneath the basement membrane of the epithelium, though intraepithelial and submucosal infiltration was also seen. Non-lymphoid mononuclear cells expressing the phenotype of dendritic cells and macrophages were found dispersed throughout the infiltrate, most of them expressing HLA-DR. These observations support the hypothesis that cell mediated immunological reactions contribute to the inflammation associated with bronchiectasis.

Introduction

Bronchiectasis is a chronic disease characterised by irreversible dilatation of the bronchi and, in most cases, by persistent production of purulent sputum. Since the advent of antibiotics the most florid saccular manifestations of the disease have been less frequent, but a progressive form of disease in young adults is now well recognised.[1]

Understanding of the pathogenesis of the disease has increased in recent years,[2] but many aspects remain obscure. Data are accruing to support the hypothesis of Cole and coworkers[1] that the chronic inflammatory host response to microbial colonisation in bronchiectasis contributes substantially to progressive bronchial damage. Although this persistent host inflammatory response in the bronchial wall and surrounding lung tissue has potential benefits (for example, in the resolution of acute infection), these may be outweighed by the damage it causes, leading ultimately to scarring and shrinkage of the lung and cardiorespiratory failure. The histological pattern in bronchiectasis is seen principally as an infiltration of mononuclear cells into the affected area of the bronchial wall. The intensity of infiltration varies from rather dispersed mononuclear cells in milder cases to striking collections of lymphoid follicles and nodules in the most advanced cases, sometimes completely obstructing the most peripheral airways.[3]

The cellular components of the inflammatory host response have been investigated in certain lung diseases—for example, sarcoidosis,[4,5] cryptogenic fibrosing alveolitis[6]—and notably in rheumatoid arthritis,[7] but not so far in bronchiectasis. We have used immunohistochemical methods to ascertain whether immunocompetent cells are present in affected bronchial tissues of patients with bronchiectasis.

Methods

Tissue from nine patients with radiographically proved bronchiectasis were obtained at lobar resection or by bronchial biopsy during fibreoptic bronchoscopy. Six of the nine patients had postinfective bronchiectasis and the other three patients had bronchiectasis associated with other conditions (table 1). Samples of affected airways were dissected from surgical specimens. Endoscopic biopsy specimens were taken from segmental or subsegmental bronchi of radiologically affected lobes. The biopsies were intended to obtain samples of bronchial mucosa and submucosa. The average size of the specimens was 1–2 mm^3. Control tissue was obtained from four patients

Address for reprint requests: Professor P J Cole, Host Defence Unit, Department of Thoracic Medicine, National Heart and Lung Institute, Brompton Hospital, Dovehouse Street, London SW3 6LY.

Accepted 24 April 1989

Table 1 *Characteristics of patients with bronchiectasis and controls*

Patient No	*Age, sex*	*Main diagnosis (duration (y) of symptoms)*	*Tissue obtained*	
			at	*from*
Bronchiectasis				
1	20, F	Cystic fibrosis (20)	Necropsy	LLL
2	40, M	Young's syndrome (39)	Bronchoscopic* biopsy	LLL
3	37, M	Postinfective bronchiectasis (30)	Lobectomy	ML
4	28, F	Postinfective bronchiectasis (6)	Lobectomy	LLL
5	43, F	Postinfective bronchiectasis (42)	Bronchoscopic biopsy	LLL
6	39, M	Ulcerative colitis (18)	Lobectomy	LLL
7	60, M	Postinfective bronchiectasis (55)	Lobectomy	LLL
8	66, F	Postinfective bronchiectasis (9)	Lobectomy	LLL
9	43, F	Postinfective bronchiectasis (38)	Lobectomy	LLL
Controls				
A	73, M	Pneumonia (1/12)	Bronchoscopic biopsy	RUL
B	43, M	Carcinoma (1½)	Lobectomy	LLL
C	67, M	Pneumonia (1/12)	Bronchoscopic biopsy	RUL
D	29, F	Pneumonia (1/12)	Bronchoscopic biopsy	LLL

*Fibreoptic bronchoscopy.
LLL—left lower lobe; ML—middle lobe; RUL—right upper lobe.

without bronchiectasis (three pneumonia, one carcinoma) (table 1) by bronchial biopsy at clinically indicated fibreoptic bronchoscopy (three cases) or at resection for bronchial carcinoma (one case). No other disease was present in the control cases. All patients had previously given written consent for the procedures and the study had the approval of the ethics committee of the Brompton Hospital.

All tissue samples were covered in Tissue-Tek Optimal Cutting Temperature Compound (Miles Laboratories, Kankakee, Illinois), orientated on cork discs, snap frozen in isopentane cooled by liquid nitrogen, and stored at −70°C. Cryostat sections of 6 μm were placed on microscope slides coated with poly-L-lysine, air dried for two hours, fixed in chloroform-acetone (1:1) for 10 minutes, wrapped in cling film (Handywrap, Payne Scientific, Berks), and stored at −20°C until use.

Some sections were stained with conventional histological stains—haematoxylin and eosin, toluidine blue, and Miller's van Gieson stain (for elastin). Other sections were examined with a panel of murine monoclonal antibodies for specific surface antigens on lymphocytes and macrophage like cells (table 2). The reaction of the antibodies RFTmix, RFT8, RFBmix, RFD1, RFD7, and RFDR was revealed by immunoperoxidase staining for mouse immunoglobulin with the use of 3'3'-diaminobenzidine (Sigma Chemical Company, St Louis, Missouri) as the disclosing agent. The immunofluorescence studies used heavy chain specific anti-mouse antisera conjugated to fluorescein isothiocyanate and tetramethyl rhodamine isothiocyanate (Southern Biotechnology Associates, Alabama).[8 9] The use of a combination of two monoclonal antibodies with different heavy chain specificities allowed study of the relative proportions of cells in the section expressing either or both of the surface markers concerned. At least three sections of each specimen of bronchiectatic and control tissues were stained with any one of the monoclonal antibodies and techniques described.

Double immunofluorescence was used to investigate the CD4:CD8 (Leu3a:RFT8) ratios in the bronchial wall and the expression of CD7 on T lymphocytes (RFTmix:RFT2). Three sections of each sample were stained with both monoclonal antibodies

Table 2 *Panel of monoclonal antibodies*

Cluster designation	*Name*	*Specificity in normal tissues*	*Source*	*Reference*
CD4	Leu3a	T helper cells	Becton Dickinson	17
CD7	RFT2	Pan T cells (strongly expressed by blasts)	RFHSM	18
CD5, 8, 2	RFTmix	All T cells	RFHSM	19
CD8	RFT8	T suppressor-cytotoxic cells	RFHSM	19
CD23, 24	RFBmix	Panperipheral B cells	RFHSM	20
—	RFD1(*)	Dendritic cells	RFHSM	21
—	RFD7	Mature macrophages, small proportion of B lymphocytes	RFHSM	22
—	RFDR	MHC class II antigens	RFHSM	23

*RFD1 precipitated a 28–33 Kd molecule from appropriate cell lines but its expression is restricted in immunohistological stains to the cells shown above. It is, for example, not expressed on Langerhans cells. It therefore reacts with an epitope on a class II major histocompatibility (MHC) molecule that is only "visible" on a restricted population of cells or when a specific function is being expressed.
RFHSM—Royal Free Hospital School of Medicine.

and the numbers of positive cells counted in all areas. At least 100 cells were counted in each compartment and the results (in the case of RFTmix/CD7) expressed as the percentages of double labelled cells. Proportions of CD4:CD8 cells were expressed as ratios. Sections of tonsil resection specimens were used as positive controls and consecutive sections of the bronchial tissue under study as negative controls. Immunofluorescent staining was read with a Zeiss fluorescence microscope.

Immunoperoxidase stained sections were read separately by two observers and scored semiquantitatively in five categories: no positive cells = 0; very few positive cells = 1; few positive cells = 2; a moderate number of positive cells = 3; many positive cells = 4. At least three different stained sections from the same block were assessed independently by the two observers; the reproducibility of the scores was 84%. The results are presented in terms of the mean (SD) score assigned after observation of sections from all specimens within each group. Student's *t* test for non-paired data was used for the comparison of the frequency of positive cells in the two groups of patients.

The frequency of positive cells was analysed in three compartments of the bronchial wall: epithelium, lamina propria, and submucosa.

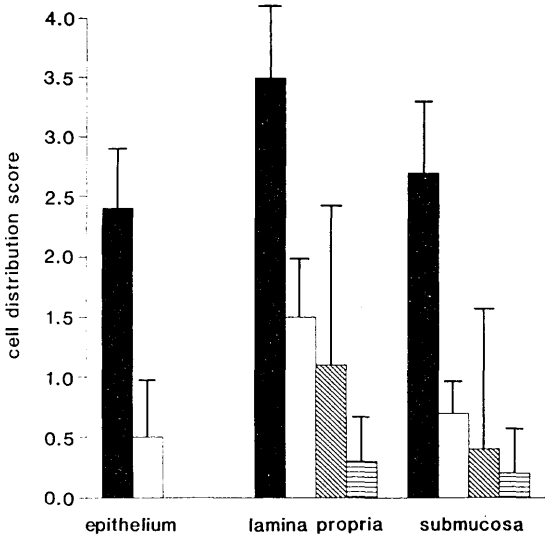


Fig 1 *Relative frequency of T lymphocytes in bronchiectatic (■) and control (□) samples and of B lymphocytes in bronchiectatic (▨) and control (▤) samples in epithelium, lamina propria, and submucosa. For scoring see under "Methods."*

Results

CONVENTIONAL HISTOLOGY

The histological appearances of the bronchiectatic tissue was classified according to the method of Whitwell[3] as showing mild or moderate follicular bronchiectasis. No specimens showed severe follicular bronchiectasis or any of the other forms (saccular and atelectatic bronchiectasis) described by Whitwell. The epithelium was relatively well preserved, with few areas of disruption. Mononuclear cells were seen to have infiltrated the cells of the pseudostratified epithelium. The smooth muscle was intact, but the elastic layer showed considerable damage in the more advanced cases. The submucosal glands were enlarged, but there was none of the destruction seen in severe cases.[3] Some specimens showed increased vascularity, mainly in the submucosa. The cartilage was well preserved. In one tissue nests of giant epithelioid cells were present in the deeper stroma of the bronchial wall.

Infiltration by mononuclear cells was the most striking and constant feature, varying from a mild dispersed infiltrate to massive numbers of cells sometimes arranged in a follicular pattern and mainly in the lamina propria. There were relatively few polymorphonuclear neutrophils in the bronchial wall, though many were present in the bronchial lumen along with cellular debris and mucus.

The histological appearance of one control tissue was not entirely normal, showing some cell infiltration and gland hyperplasia, compatible with very mild chronic inflammation (this was from a patient with carcinoma).

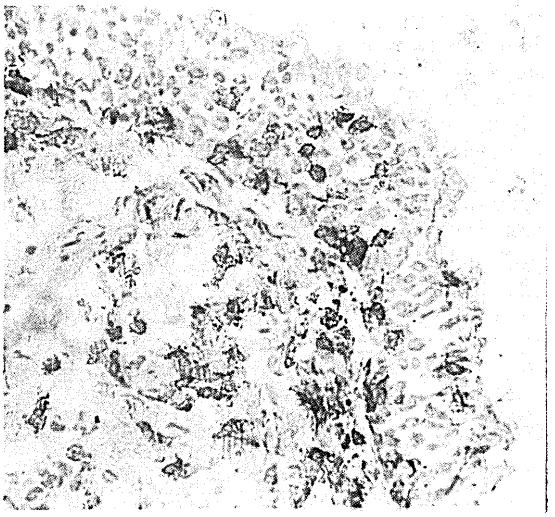


Fig 2 *Accumulation of T lymphocytes under the bronchial basement membrane and infiltrating the bronchial epithelium in a specimen from a patient with bronchiectasis. (Immunoperoxidase stain.)*

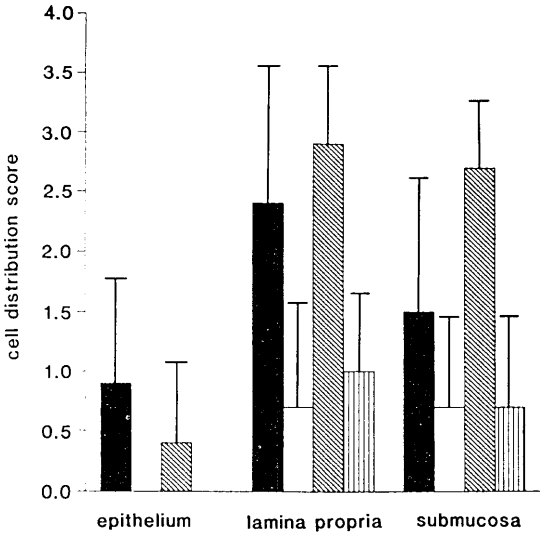


Fig 3 *Relative frequency of RFD1$^+$ cells in bronchiectatic (■) and control (□) samples and of RFD7$^+$ cells in bronchiectatic (▨) and control (▥) samples in the epithelium, lamina propria, and submucosa. For scoring see under "Methods."*

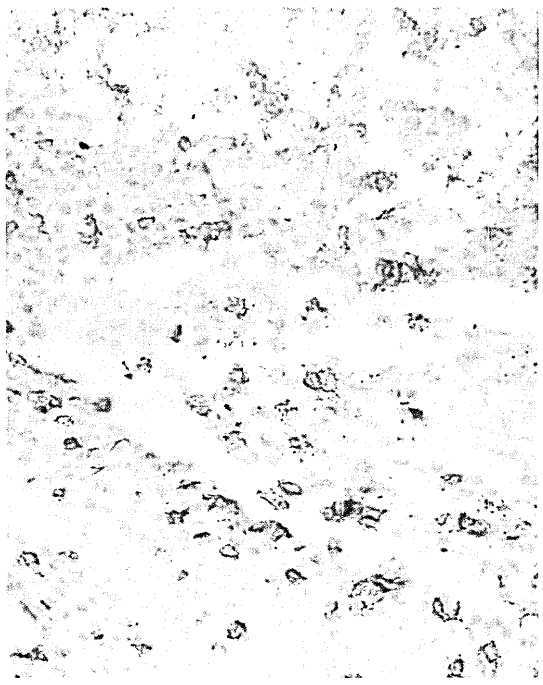


Fig 4 *Diffusely distributed CD8$^+$ T cells adjacent to and within the epithelium of the bronchial wall of a specimen from a patient with bronchiectasis. (Immunoperoxidase stain.)*

IMMUNOHISTOLOGY

Lymphocytes (fig 1)

The most striking feature was the large number of T lymphocytes stained with the RFTmix monoclonal antibody infiltrating all compartments (epithelium, lamina propria, and submucosa) of the bronchiectatic tissue. This accounted for most of the mononuclear cells present in the chronic inflammatory reaction. This marker was the only one to show a significant difference between bronchiectatic and control groups ($p < 0{\cdot}01$) in the three areas of the bronchial wall. Differences in the distribution of other cell types were apparent in some but not all areas of the tissue (see below).

T lymphocytes had infiltrated the epithelium of all patients, but the predominant distribution was in the lamina propria (fig 2). These T cells were often arranged just below the basement membrane of the epithelium, sometimes isolated but usually packed in clusters of cells. In the submucosa they were mainly around glands and vessels. In contrast, B lymphocytes were seen in only two specimens and no significant differences were found between control subjects and patients with bronchiectasis. The bronchial wall of one patient showed clusters of B lymphocytes staining for RFB mix packed in huge follicles in the lamina propria and submucosa. The bronchial wall of a second subject showed a group of B cells (mixed with T cells) just beneath the basal layer of the epithelium, possibly representing bronchus associated lymphoid tissue.

The control tissues differed mainly in the number of T cells staining for RFTmix. There were far fewer such positively stained cells than in the bronchiectatic tissues and they were dispersed mainly in the epithelium and lamina propria. There were a few B lymphocytes in one section only.

Macrophage like cells (fig 3).

Dendritic cells staining for RFD1 were present in all tissues except those from one patient. The predominant distribution was in the lamina propria, where they were sometimes found in groups but were usually isolated. The tissue from one patient showed many cells staining for RFD1 in the alveolar area contiguous with the bronchial wall. Only the numbers of RFD1$^+$ cells present in the lamina propria were significantly different when the bronchiectatic and control groups were compared ($p < 0{\cdot}01$).

Mature macrophages staining for RFD7 were present in tissue from all patients. These cells were not confined to any particular area and were usually isolated, but in two patients there were groups of positively stained cells in the deeper stroma of the bronchus. The frequency of positively stained cells was greater in tissue from the patients than from the control subjects in the lamina propria and submucosa ($p < 0{\cdot}01$) but not in the epithelium ($p = 0{\cdot}29$). The control tissues either were negative for both RFD1

and RFD7 markers or showed very few positively stained cells.

SUBSETS AND MARKERS OF ACTIVATION OF T LYMPHOCYTES

Most T lymphocytes were of the CD8 subset (fig 4). The number of T cells expressing the CD8 (RFT8) marker nearly always (in 7/9 cases) exceeded the number of cells expressing CD4 (Leu3a) in all compartments of the bronchial wall in patients with bronchiectasis. The mean (SD) ratio of CD4 to CD8 was 1:6·7 (3·2) in bronchiectatic tissue and 1:2·5 (0·6) in control tissue ($p < 0·01$). In control tissues most T cells were also of the CD8 phenotype but the number of such positive cells was very small.

Most of the T cells in bronchiectatic tissue stained positively for the lymphoblast marker CD7 (RFT2) (85%(7%)) whereas T cells in control tissue were usually negative for this marker (27·5%(20%); $p < 0·01$).

EXPRESSION OF HLA-DR ANTIGEN

The HLA-DR antigen was strongly expressed by the epithelium and mononuclear infiltrates in tissue from all patients with bronchiectasis. There was some positive staining in the control tissues, but this was irregularly distributed in the epithelium, with very low expression in cells of the lamina propria and submucosa.

Discussion

Whitwell's study of the pathology of bronchiectasis emphasised chronic infiltration of mononuclear cells as the common histological pattern in all types of this disease. Immunohistochemical methods have been used to identify the different kinds of cells present in this chronic inflammation, their degree of activation, some of their functions and their interrelationship. Although our knowledge of mucosal immunity has increased in the last few years, many mechanisms are still poorly understood.[10] In healthy tissues of the tracheobronchial tree lymphocytes are present in the intraepithelial compartment and lamina propria, but their phenotype has been little studied. Bronchus associated lymphoid tissue, a follicular collection containing mainly B lymphocytes overlaid by specialised epithelium, is distinct and usually present in areas of airflow turbulence, probably functioning mainly in the sampling of antigen.[11 12]

This study shows that the predominant cells present in the inflammatory infiltrates of bronchiectasis are immunologically active T lymphocytes. Few B lymphocytes were present and these were mainly restricted to the areas of follicle formation. This may be partly due to the fact that our study was restricted to more localised forms of bronchiectasis. In the severe follicular forms of the disease the proportions may be different.

The presence in bronchiectatic tissue of cells with a dendritic appearance and the phenotype of antigen presenting cells and mature macrophages together with T lymphocytes suggests a cell mediated immune response. This is further supported by the strong expression of the major histocompatibility complex class II molecules (HLA-DR) by bronchial epithelial cells and by most infiltrating cells, and by the strong expression of CD7 antigen by most of the T cells. All these phenomena are found in delayed hypersensitivity reactions.[13]

The predominance of suppressor-cytotoxic T cells over helper T cells has also been observed in cryptogenic fibrosing alveolitis,[14] whereas in other chronic inflammatory diseases, such as rheumatoid arthritis[15] and ulcerative colitis,[16] the common pattern is the predominance of helper T cells.

The "vicious circle" hypothesis of the pathogenesis of bronchiectasis proposed by Cole[1] suggests that initial damage to or underlying disease of the respiratory tract allows microbial colonisation as a result of reduced mucociliary clearance. The colonising organisms incite an inflammatory response that becomes chronic and causes tissue damage, which impairs bronchial mucociliary clearance still further. The results of our study are consistent with this hypothesis—the presence of a cell mediated immune response being one component of the bronchial inflammation. The immune response we have identified within the lung in bronchiectasis may persist either because of constant antigenic stimulation by intrabronchial microrganisms or because of an underlying defect of the local immune response. To examine this possibility functional studies of the cells taking part in the immune response are required.

We are grateful to Drs C Steinfort, D Currie, E Sheffield, M Spiteri, and M Johnson and Mr P Goldstraw for the supply of the specimens; to Dr B Noble for valuable advice, and to Mrs M Whitney and Miss A Condez for technical support. J R L e S is funded by the Brazilian Research Council (CNPq).

References

1 Cole PJ. A new look at the pathogenesis and management of persistent bronchial sepsis: a "vicious circle" hypothesis and its logical therapeutical connotations. In: Davies RJ, ed. *Strategies for the management of chronic bronchial sepsis*. Oxford: Medicine Publishing Foundation, 1984:1–20.

2 Cole PJ, Wilson R. Host-microbial interrelationships in

respiratory infection. *Chest* 1989;**95**:217–21S.
3 Whitwell FA. A study of the pathology and pathogenesis of bronchiectasis. *Thorax* 1952;**7**:213–39.
4 Campbell DA, DuBois RM, Butcher RG, Poulter LW. Density of HLA-DR antigen expression on alveolar macrophages is increased in pulmonary sarcoidosis. *Clin Exp Immunol* 1986;**65**:165–71.
5 Munro CS, Mitchell DA, Poulter LW, Cole PJ. Early cellular responses to intradermal injection of Kveim suspension in normal subjects and those with sarcoidosis. *J Clin Pathol* 1986;**39**:176–82.
6 Crystal RG, Bitterman PB, Rennard SI, Hance AJ, Keogh BA. Interstitial lung disease of unknown cause. *N Engl J Med* 1984;**310**:235–44.
7 Janossy G, Panayi, Duke O, Bofill M, Poulter LW, Goldstein G. Rheumatoid arthritis: a disease of T-lymphocyte immune regulation. *Lancet* 1981;ii:839–42.
8 Poulter LW, Chilosi M, Seymour GL, Hobbs S, Janossy G. Immunofluorescence membrane staining and cytochemistry, applied in combination for analysing cell interaction "in situ". In: Polak JM, Noorden S, eds. *Immunocytochemistry: practical applications in pathology and biology*. Bristol: Wright, 1983:233–48.
9 Janossy G, Bofill M, Poulter LW. Two-colour immunofluorescence: analysis of the lymphoid system with monoclonal antibodies. In: Polak JM, Noorden S, eds. *Immunocytochemistry. Modern methods and applications*. Bristol: Wright, 1986:438–55.
10 Bienenstock J. Mucosal immunological protection mechanisms in the airways. *Eur J Respir Dis* 1986; **69**(suppl 147):62–71.
11 Bienenstock J. Bronchus-associated lymphoid tissue. In: Bienenstock J, ed *Immunology of the lung and upper respiratory tract*. New York: McGraw Hill 1984: 96–117.
12 Bienenstock J, Befus D. Gut- and Bronchus-associated lymphoid tissue. *Am J Anat* 1984;**170**:437–45.
13 Unanue ER, Allen PM. The basis of the immunoregulatory role of macrophages and other accessory cells. *Science* 1987;**236**:551–7.
14 Kallenberg CGM, Schilizzi BM, Beaumont F, Poppema S, De Leij J, The TH. Expression of class II MHC antigens on alveolar epithelium in fibrosing alveolitis. *Clin exp Immunol* 1987;**67**:182–90.
15 Duke O, Panayi GS, Janossy G, Poulter LW. An immunohistological analysis of lymphocyte population and their microenvironment in the synovial membranes of patients with rheumatoid arthritis using monoclonal antibodies. *Clin exp Immunol* 1982;**49**: 22–30.
16 Poulter LW, Allison M, Richardson AT, Janossy G. The distribution of lymphocyte subset in inflammatory disease and reactive synovitis: a comparison. In: Mielants H, Veys EM, eds. *Spondyloarthropaties: involvement of the gut*. Amsterdam: Elsevier 1987: 213–20.
17 Evans RL, Wall DW, Platsourcas CD, *et al.* Thymus-dependent membrane antigens in man: inhibition of cell-mediated lympholysis by monoclonal antibodies to the TH2 antigen. *Proc Natl Acad Sci* 1981;**78**:544–7.
18 Poulter LW, Duke O, Hobbs S, Raftery MJ, Janossy G. Activated T-lymphocytes of the synovial membrane in rheumatoid arthrytis and other arthropathies. *Scand J Immunol* 1985;**22**:683–90.
19 Janossy G, Prentice HG. T cell subpopulations, monoclonal antibodies and their therapeutic applications. *Clin Haematol* 1982;**11**:631–60.
20 Janossy G. Human Lymphocyte subsets: their tissue distribution. *Progr Immunol* 1984;**5**:1085–101.
21 Poulter LW, Collings LA, Tung KS, Waters MFA. Parasitism of antigen presenting cells in hyperbacillary leprosy. *Clin Exp Immunol* 1984;**55**:611–7.
22 Munro CS, Campbell DA, Collins LA, Poulter LW. Mononuclear antibodies distinguish macrophages and epithelioid cells in sarcoidosis and leprosy. *Clin Exp Immunol* 1987;**68**:282–7.
23 Collings LA, Tidman N, Poulter LW. Quantitation of HLA-DR expression by cells involved in the skin lesions of tuberculoid and lepromatous leprosy. *Clin Exp Immunol* 1985;**61**:58–60.