Review Article

The mechanisms of intractable asthma

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ABSTRACT

Overwhelming evidence now points to asthma as a chronic inflammatory disease involving the airways. The T lymphocyte takes primacy in driving the inflammatory response through upregulation of cytokines, specifically those encoded in the IL-4 gene cluster: IL-4 and IL-13 (IgE isotype switching); IL-3, IL-5 and GM-CSF (eosinophil and basophil recruitment); and IL-9 (mast cell maturation). Additional cytokines of importance include TNFα and a range of related C-x-C and C-C cytokines. Although allergens are involved in initiating the Th-2 T-cell response, other factors are likely to operate that expand and maintain the inflammatory reaction. These include a potential role for superantigens and autoimmune mechanisms as well as the recruitment of accessory cytokine producing cells, especially mast cells and eosinophils. Leucocytes recruited from the microvasculature through interactions with specific adhesion molecules release an array of mediators, which in addition to causing bronchoconstriction also lead to damage to the epithelium and underlying structures. Neutral proteases from mast cells, metalloproteases from eosinophils and an array of mediators from the formed elements of the airway all contribute to the tissue destruction remodelling process. It was concluded that asthma is a dynamic disease process involving an interplay between inflammation and repair processes and that the differing proportions of these could account for the various disease phenotypes associated with severity and progression.

Key words: asthma, cytokines, eosinophils, inflammation, mast cells, repair, T cells.

THE HEALTH AND ECONOMIC BURDEN OF ASTHMA

Statistics worldwide indicate that the prevalence of asthma continues to increase, and the UK is no exception. However, the major health burden of asthma relates to severe, chronic and relapsing disease. The recent WHO/NHLBI technical report on A Global Strategy for Asthma describes severe persistent asthma as frequent or continuous daily symptoms despite treatment, frequent sleep disturbance on account of asthma, severe physical and other lifestyle limitations, peak expiratory flow (PEF) or forced expiratory volume in 1S (FEV1) <60% predicted, and within and between day variability of PEF>30%. Much of the mortality and some of the morbidity of asthma results from underestimation of the severity of the disease by both patient and doctor, inadequate action at the onset of deterioration and undertreatment. However, there remain many patients who receive anti-asthma drugs in large doses and yet remain symptomatic. The clinical phenotype of such patients is often complex and varied with no single pattern dominating.

The cost of illness for adult asthma, analysed by disease severity, reveals a disproportionate use of medical resources by patients with severe disease. In Canada, severe asthma comprised 10% of the asthma population and accounted for 51% of all direct medical care costs and 54% of total asthma costs. Patients with severe disease were three times more likely to consult an asthma specialist, 15 times more likely to use an accident and emergency department and 19 times more likely to require hospitalization. In Australia, severe asthma comprises 6% of the adult asthma population and consumes 47% of the total annual costs for this disease; an estimate similar to that for the UK. Thus, on the grounds of unsatisfactory treatment, quality of life and health economics, there is a strong case for focusing attention on this group of asthma patients.

CYTOKINE AND MEDIATOR NETWORKS AS THE BASIS OF ASTHMA

Asthma is a multifactorial disease that spans the full spectrum of activity from mild seasonal symptoms to being severe and intractable. When classifying asthma by severity, the majority falls into the mild–moderate range and can be managed well with currently available drugs. It is also at this end of the disease spectrum and in those with atopy that most of our understanding of inflammatory and mediator mechanisms of asthma has been based. Application of immunohistochemistry, in situ hybridization (ISH) and reverse transcriptase polymerase chain reaction (RT-PCR) to lavage cells, mucosal biopsies and T cells from the
airways of such patients reveals upregulation of the IL-4 gene cluster (IL-4, IL-13, IL-5, IL-3, IL-6, GM-CSF, IL-9) in T cells, mast cells and eosinophils. These cytokines are of crucial importance in the initiation and maintenance of the allergic inflammatory response through isotype switching of B cells to IgE synthesis (IL-4, IL-13), the selective maturation of Th2-like CD4⁺ T cells expressing the IL-4 gene cluster (IL-4), growth, maturation and activation of eosinophils and basophils (IL-3, IL-5, GM-CSF) and maturation of mast cells (SCF, IL-6, IL-9; Fig. 1). The expression of a counter regulatory signal provided by IFN-γ (from Th-1 cells) and macrophage/monocyte derived IL-12, which induces its synthesis, becomes reduced. This has led to the concept that cytokines from the Th-2 subtype of T cell dominate over those of the Th-1 subtype, leading to expression of the eosinophilic bronchitis characteristic of asthma. While far less is known of the cytokine networks in asthma, other than that associated with atopy, the therapeutic efficacy of disease-modifying drugs, especially corticosteroids, is likely to be the consequence of transcription factor-mediated downregulation of cytokine production and the pro-inflammatory pathways that these signalling molecules influence.

MECHANISMS OF ASTHMA SEVERITY AND CHRONICITY

There is overwhelming evidence to indicate that airway inflammation underlies the pathophysiology of asthma, but its relationship to disease severity is less clear. While there are eosinophils in the sputum, a persistent blood eosinophilia with increased circulating levels of eosinophil granule proteins broadly relates to disease severity. These measures are too variable to provide clinically useful markers to predict the level of airway inflammation.

The selective recruitment of cells from the microvasculature underlies the ongoing inflammation in severe and chronic disease. Increased mast cell, eosinophil and T cell survival through cytokine-mediated inhibition of apoptosis is also important. Lung transplantation has shown that alone, with its lymphoid tissue, the lung is able to sustain ongoing asthma also emphasizing the importance of local factors.³ From a therapeutic standpoint the wide variation observed in these biomarkers in relation to the varied clinical phenotypes suggest complex cellular and mediator mechanisms.

Corticosteroids are highly effective anti-asthma drugs acting to reduce the inflammatory response. However, there are many patients in whom only partial relief is achieved even with high doses. In a well-defined population of ‘corticosteroid-resistant’ asthmatics with preservation of β₂-agonist bronchodilatation, abnormalities of circulating monocyte and T-cell cytokine function have been described.⁴ However, such patients represent only a minority of the ‘difficult to control’ asthmatics.

The majority of asthma occurs in association with atopy, the predisposition to generate IgE in response to common environmental allergens through a Th-2-cell-dependent mechanism. Ongoing allergen-specific IgE production in severe disease provides the rationale for allergen avoidance and high altitude treatment. However, environmental interventions have no effect on non-allergic asthma and many patients with severe atopic disease only partially respond or fail to respond. Irrespective of atopy, powerful epidemiological studies have linked the total serum IgE to the presence of asthma and its level of disease progression.⁵ There have been very few long-term studies but what evidence does exist suggests that severe and poorly controlled asthma progresses because of an increasing, irreversible component.⁶ Severe and prolonged inflammation is almost always accompanied by tissue remodelling. The airways are no exception; however, the mechanism(s) involved and their contribution to the overall pathophysiology of severe and chronic asthma have not been evaluated.

It follows that the severity and chronicity of asthma results from the dysregulation of cytokine networks leading to persistent inflammation in structurally altered airways which become refractory to treatment. The responsibility for disease progression does not lie with any single cellular element but embraces T and B cells, mast cells, eosinophils, endothelial cells, epithelial cells and myofibroblasts acting co-operatively with each other and with formed elements of the airways, including smooth muscle and nerves, leading to the variable phenotype characteristic of severe disease. This integrated view of asthma as a chronic disease of ongoing inflammation and repair leads us to incriminate a number of effector cells.

Continuous T-cell activation

Severe disease increasingly engages airway T cells leading to their oligoclonal expansion, activation with a mixed pattern of...
cystokine expression and failure of normal counter regulatory mechanisms.

T-cell activation and expression of mRNA for cytokines is a common feature of all types of asthma. In mild disease, the level of T-cell involvement in the airways is low, but there is ample evidence for mast cell and eosinophil activation, which may explain why cromone-like drugs are more efficacious at this end of the asthma spectrum. By contrast our biopsy and lavage study of patients admitted to the Dutch Asthma Centre in Davos, together with observations of severely symptomatic asthmatic patients in the UK taking high doses of inhaled corticosteroids indicate that activated T cells drive the ongoing inflammatory process in severe disease by mechanisms in addition to eosinophil recruitment and activation. At 24 h after allergen challenge, we and others have shown increased IL-5 transcription in relation to T-cell recruitment and activation supporting the Th-2 hypothesis, however, our T-cell cloning studies have shown that laveage T cells from asthmatics of differing disease severity exhibit considerable heterogeneity of cytokine expression. At baseline, T cells from atopic asthmatic airways show strong expression of mRNA for IL-13, GM-CSF, IFN-γ and TNF-α whereas with allergen instillation there was a cytokine shift in favour of IL-3, IL-4, IL-5 and IL-13 and away from IFN-γ and TNF-α. These data support the recent T-cell cloning studies that recognize the existence of Th-2-like cells that are allergen responsive, while the majority of activated airway T cells in the airways serve other functions.

Using monensin to inhibit Golgi-mediated cytokine transport and flow cytometry applied to permeabilized cells, we have evaluated the cytokine protein production by airway T cells. Confirming our observations on airway T-cell clones, a high proportion of asthmatic BAL T cells produced IFN-γ and/or IL-2 and only a few accumulated IL-4 or IL-5. Even more surprising was the finding that in asthma cases, compared to normal controls, there is a significantly greater production of IFN-γ but not IL-2. During acute exacerbations of asthma there is evidence for T-cell upregulation in the peripheral circulation accompanied by enhanced IL-5 gene expression. However, during acute episodes in cases in the UK we have shown that proliferative T-cell responses to the house dust mite allergen, when compared with responses before or 8 weeks after the episode, decreased rather than increased indicating that factors influencing T cells other than allergens are important in directing the immune response during exacerbations. It is clear that little is known about T-cell cytokine responses influencing disease chronicity and severity in patients with asthma and how they may escape corticosteroid suppression.

Based on lung transplant experiments and bronchoscopy studies in mild–moderate disease, an important aspect of T-cell involvement is local expression. Studying the T-cell receptor (TCR) ζ V-gene usage will shed light on factors that shape the airway’s T-cell repertoire. In normal subjects, the peripheral TcR repertoire is diverse, resulting from the use of different VD and J segments in forming the α and β chain. As a consequence non-specific recruitment would result in a heterogeneous TcR repertoire at the inflammatory site, reflecting the populations in blood. The exciting observation that atopy (and asthma) is associated with linkage to specific polymorphisms of the Vα repertoire on chromosome 14 indicates that genetic determinants involving allergen recognition are an important component of T-cell selection. Specific stimulation of T cells would modify the Vα and Vβ repertoire so that allergen-driven responses would result in dominant oligoclonal T-cell interaction whereas superantigens would select a single dominant TcR Vα and Vβ family, but with random use of DJ segments. In preliminary experiments we have amplified the TcR repertoire in airways by polymerase chain reaction (PCR) of cDNA prepared from broncho-alveolar lavage (BAL) and blood lymphocytes of the same subjects and have found that TcR Vβ selection in favour of Vγ5 is a feature of airway T cells in atopic asthma. This supports the role of superantigens in driving the immune response. These promising initial experiments need to be expanded to embrace more exaggerated forms of the disease in the presence and absence of atopy.

**Mast cells and eosinophils**

The mast cell and eosinophil contribute to the maintenance of mucosal inflammation through their release of pro-inflammatory cytokines and mediators.

**Mast cell subtypes**

The mast cell has long been regarded as an important effector cell of asthma through its capacity to respond to IgE-dependent activation with release of both preformed and newly generated mediators. This function has found wide acceptance as the cause of acute allergen-induced and exercise-induced bronchoconstriction. We believe that as a constituent cell of the airway, the mast cell also plays a key role in maintaining chronicity of the inflammatory response. Of the two types of mast cell phenotyped through their granule neutral protease content, it is that containing only the unique four-chained neutral protease, tryptase, that dominates (MCs), although tryptase-containing and chymase-containing cells (MCrc) are found in relation to the submucous glands and microvasculature. Both MCs and MCrc require stem cell factor (SCF, c-kit ligand) for survival; MCs cells are under the influence of T-cell cytokines (IL-6, IL-9) while the MCrc phenotype is determined by fibroblast-derived growth factors. The relevance of mast cell products to asthma has been revealed in a study of 151 patients with different types of asthma in BAL fluid. The products of mast cell activation, tryptase, histamine and PGD2 were all present in higher concentrations than in normal patients with levels that correlated positively with albumin as a marker of microvascular permeability (P<0.01) and negatively with FEV1, suggesting an important link to disease activity.

**MECHANISMS OF INTRACTABLE ASTHMA**

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**Mast cell cytokines**

The importance of IL-4 and IL-5 in the pathogenesis of asthma is well established but, until recently, it was thought that their major source was the Th-2-like helper T cell. As an alternative source, we have shown that human mast cells are an important source of IL-4, IL-5, IL-6, IL-8 and TNFα. Both by immunohistochemistry and immunoelectronmicroscopy we are the first to show that human mast cells contain granule-associated IL-4, IL-5, IL-6 and TNF-α and that these are differentially expressed between the two mast cell subtypes. In purified lung mast cells mRNA for all of these cytokines is induced by IgE-dependent activation and is followed by protein secretion for up to 48 h. The inducing stimuli for secretion appears to be different, IL-4 and IL-5 being more dependent upon FcεRI receptor signalling, while SCF initiates TNF-α production. Both IL-6 and IL-8 mRNA is constitutively expressed at a high level, although IL-6 transcription could be further enhanced with SCF. Our recent finding that in severe atopic disease a proportion of MCT cells also contain preformed SCF provides an autocrine mechanism for local mast cell survival and activation. Since every mast cell is antigen specific, whereas this antigen specificity applies to only 1 in 300–5000 airway T cells, we hypothesize that cytokine release from airway mast cells initiated by allergens or other stimuli is pivotal in the induction and maintenance of the inflammatory response through upregulation of IgE (IL-4, IL-6), Th-2-like T cells (IL-4), mast cell (IL-6, SCF), eosinophil (IL-5, IL-8) and vascular adhesion molecule (TNF-α, IL-4) functions.

The use of monoclonal antibodies (mAb) directed to different epitopes of IL-4 in glycolmethacrylate sections of biopsies and free mast cells has allowed us to differentiate preformed (mAb 4D9) from the secreted (mAb 3H4) form of the cytokine. In both atopic and non-atopic asthma the proportion of bronchial mast cells demonstrating ring staining with 3H4 is considerably greater than in normal subjects, indicating ongoing cytokine release in the absence of specific challenge. Seasonal exposure to allergens increases, whereas treatment with inhaled corticosteroids decreases the number of 3H4+ mast cells without altering the overall number of cells staining with 4D9 for the preformed cytokine, indicating that IL-4 secretion is sensitive to changes in the micro-environment. Recent evidence shows that IL-4 binds strongly to the GAG side chains of heparin, a property it shares with IL-8 and TNF-α. Since heparin is a unique product of human mast cells, its secretion onto the mast cell surface is likely to provide a pericellular environment in which these cytokines are present in high concentrations to mediate local signalling effects pertinent to ongoing inflammation.

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**Fig. 2** Proposed mediator functions of mast cell tryptase in asthma.
Mast cell proteases

Tryptase, chymase, carboxypeptidase A and a cathepsin-G-like enzyme are also tightly bound to heparin which serves to preserve integrity and direct the specificity of the enzymes. Tryptase is the major secretory component of most cells and comprises in excess of 20% of the total granule content of protein, and elevated concentrations have been detected in BAL fluid collected from patients with chronic severe asthma as well as from milder cases of atopic asthma. Evidence is emerging that this serine protease may act as a key mediator of disease. Tryptase is capable of participating in tissue remodelling, being able to cleave several components of the extracellular matrix (e.g., collagen VI, fibronectin) in addition to activating matrix metalloproteases (stromelysin). The generation of kinins by tryptase may have important consequences, and this enzyme can also degrade certain neuropeptides postulated to have a regulatory role in asthma (VIP, CGRP). Interacting directly with the cell surface, we have shown that tryptase can also enhance proliferation of epithelial cells, fibroblasts and smooth muscle cells, upregulate expression of ICAM-1, stimulate IL-8 release and induce eosinophil chemotaxis and activation, all of which are dependent upon preservation of the enzyme’s catalytic site (Fig. 2).21-23

Chymase, a protease co-released with tryptase by MC\textsubscript{Tc}, can also cleave several structural proteins and activate other tissue-degrading proteases (stromelysin, gelatinase A) as well as degrade selective cytokines. In addition, chymase is one of the most potent secretagogues of mucus-secreting cells to have been described when injected into laboratory animals.24 We have shown that both tryptase and chymase, induce microvascular leakage and granulocyte accumulation (Fig. 3).

Despite their potential importance as mediators, relatively little is known of the expression of proteases in mast cells of the respiratory tract of asthmatic patients. Numbers of mast cells in bronchial tissue (as revealed by immunostaining for tryptase) are not greatly altered in asthma, although the elevated concentration of tryptase in BAL fluid indicates that the mast cells are in a more activated state. The process of protease synthesis and the kinetics of release from mast cells are not well understood. Multiple cDNA have been cloned for tryptase, but the significance of this variation is not known. Sequences have also been derived for cDNA for chymase, and for other proteases which have been detected in mast cells including carboxypeptidase and cathepsin G. These advances now permit new approaches to be applied to answer basic questions on the biology of mast-cell proteases and to better understand their roles in bronchial asthma.
The triggering role of IgE

Cytokine-induced IgE may lead to disease progression by shifting specificity from external to other antigens, including auto-antigens, and through the formation of auto-antibodies against IgE. Although the ability of IgE to bind to mast cells, and to mediate antigen-induced degranulation is clear, its role in maintaining chronic asthma is not fully understood. However, patients with chronic disease have raised levels of allergen-specific IgE in serum. IgE, particularly in its complex form, is capable of mediating release of a range of cytokines via Fc epsilon R1 and Fc epsilon R2 (CD23). Since both high and low affinity IgE receptors are upregulated on eosinophils and bronchial epithelial cells in asthma patients, the opportunities for IgE to contribute to local inflammatory processes are numerous.

The question is raised whether the IgE is specific for allergens, or whether the spectrum of recognition widens during disease progression to include viral antigens and auto-antigens, as for chronic urticaria. Past analysis of IgE specificities has been limited to serological investigation of mixed IgE but the new technology will allow investigation of individual IgE molecules. It will also be feasible to compare the molecular range of IgE found at local sites of inflammation with that in the blood. Methods for amplifying the variable region genes used to encode IgE have been developed and already reveal an unexpected asymmetric usage of immunoglobulin Vκ genes in patients with asthma. In the inflammatory environment, where there may be local release of cytokines possibly exacerbated by viral infection, it is conceivable that auto-antigens may be released. IgE antibodies could therefore be generated against allergens, viral antigens or auto-antigens. The high levels of IgE characteristic of chronic asthma could also induce auto-antibodies against IgE itself. These could have an important additional role in inflammation, either by cross-linking IgE on the mast cell surface, or by generating immune complexes which stimulate mononuclear phagocytes to release cytokines.

Endothelial-leucocyte recruitment

Cytokine driven activation of the microvasculature leads to increased expression of vascular adhesion molecules, mediators and chemokines to promote leucocyte activation and recruitment.

The recruitment of leucocytes to sites of inflammation involves a well co-ordinated and dynamic sequence of events in which several cell adhesion molecules (CAM) and chemotactic cytokines play an active role. In vitro lines of evidence predict a multi-step model involving: (i) initial low affinity selectin molecule-dependent ‘vascular rolling’ (margination); (ii) leucocyte activation by endothelial-derived chemo-attractants (e.g., IL-8, MCP-1); and (iii) a transition of β2-integrin-dependent high affinity leucocyte adherence cytokine-mediated upregulation of the Ig superfamily of adhesion proteins, ICAM and VCAM followed by (iv) transendothelial migration involving combinations of CAM.

In asthma the presence of eosinophils and mononuclear cells in the bronchial mucosa involves the initial recruitment from the microvasculature. We have shown that within 6 h of segmental allergen challenge of sensitized asthmatic airways there occurs marked endothelial upregulation of E-selectin and ICAM-1 accompanied by an influx of LFA-1+ leucocytes comprising neutrophils and eosinophils. By 24 h there was a marked increase in activated T cells and eosinophils present in BAL with variable expression of VCAM-1, an adhesion molecule not normally constitutively expressed. Because leucocyte recruitment occurs so rapidly we suggest that the first step involves upregulation of P-selectin (by histamine) and E-selectin (by TNF-α) released from activated mast cells which mediate rolling through a lectin interaction with the ligand sialyl Lewis x on the leucocyte surface. The IgE-dependent secretion of newly formed TNF-α would increase ICAM-1 expression while its interaction with mast-cell-derived IL-4 stabilizes TNF-α-induced VCAM-1 expression. These CAM interact with the integrins LFA-1 and VLA-4 to selectively recruit T cells and eosinophils.

Preliminary immunohistochemistry studies on biopsies from severe asthmatics from the National Dutch Asthma Hospital in Davos have revealed a marked upregulation of ICAM-1 and VCAM-1 in the absence of allergen exposure and while taking high doses of corticosteroids. We suggest that in severe asthma there is continued expression of endothelial CAM to promote ongoing leucocyte recruitment and activation. Such a mechanism would explain the finding of elevated circulating and BAL levels of soluble CAM in symptomatic asthma. The expression of E-selectin, ICAM-1 and VCAM-1 is controlled by the nuclear transcription factor NF-κB, a heterodimer p50/65 of which both subunits contain the 300 amino acid NF-κB/rel/dorsal (NRD) domain. The N-terminal end of NRD is involved in specific binding to DNA, while the C-terminal end...
contains the nuclear location signal (NLS), a cluster of positively charged amino acids necessary for translocation of NF-κB across the nuclear membrane. NF-κB binds to the decameric DNA sequence 5'-GGAGNN'TCC-3' found in the promoters of a number of genes that are upregulated in inflammation, especially the CAM and specific cytokines (IL-2, IL-6, IL-8, members of the IL-4 gene cluster and TNF-α; Fig. 4).

Within the E-selectin promoter there are three closely spaced binding sites for NF-κB clustered within a 40 bp segment and two additional regulatory elements, NF-ELAM-1 and NF-ELAM-2. The latter recognizes members of the cAMP-independent ATF-CREB family of transcription factors and the former is involved in the repression of the basal NF-κB enhancer in quiescent cells. Following cytokine exposure, all three NF-κB sites are essential for maximal promoter activity. The promoter for the human ICAM-1 gene contains binding sites for Sp-1, AP-1, AP-2, AP-3, NF-κB and a putative silencer, whereas NF-κB alone mediates VCAM-1 expression.

A range of factors have been shown to initiate activation of NF-κB, including TNF-α, IL-1, IL-2, LTβ, and viruses. Endothelial cells express a cytoplasmic inhibitor of NF-κB activity, IκBα, the over expression of which inhibits E-selectin and VCAM-1 transcription. IκBα binds selectively to NF-κB heterodimers and prevents its nuclear uptake by binding to the NLS. A pathway of NF-κB activation has been proposed that sequentially involves phosphorylation of IκBα, followed by its specific chymotryptic proteolysis which reveals the previously masked NLS site and nuclear translocation. The activation is only transient since NF-κB is also able to induce IκB mRNA transcription resulting in re-accumulation of IκBα and its functional inhibition of cytoplasmic NF-κB. Reactive oxygen intermediates (ROI) serve as secondary messengers of NF-κB activation and redox changes leading to activation of chymotryptic IκBα protease through modification of intracellular serpins. These intracellular events provide unique opportunities to investigate NF-κB activation in severe asthma, as it relates to increased CAM and cytokine expression, and to investigate pharmacological intervention with potential therapeutic significance.

**The epithelium as a source of pro-inflammatory products**

There is persistent epithelial activation with production and secretion of mediators and cytokines resulting in enhanced cell recruitment, bronchoconstriction and airway wall remodelling.

The bronchial epithelium has been viewed traditionally as a passive barrier which serves as a target for the inflammatory response, but it is also an important source of inflammatory products including arachidonic acid products, endothelin, nitric oxide (NO) and cytokines. In asthma, the increased expression of ICAM-1, HLA-DR and CD44 demonstrates the capacity of the epithelium to participate directly in inflammatory cell recruitment and activation.

**Arachidonic acid metabolism**

We and others have shown that the epithelium is a major source of 15-HETE and, although expression of immunoreactive 15-lipoxygenase is unaltered, Shannon et al. have shown increased enzyme activity in severe disease. Although 15-HETE and 15-dihydroxy acids exhibit some mediator functions, more active oxidate products of arachidonic acid are PGE₂ and PGF₂α, with their opposite actions in bronchial smooth muscle. We have recently shown that in mild–moderate asthma epithelial expression of the inducible form of cyclo-oxygenase (COX2) is enhanced over the constitutive form (COX1); a change that is suppressed by corticosteroid treatment in parallel with their clinical efficacy. In asthma poorly controlled with corticosteroids, COX2 upregulation might be expected to persist.

**Endothelin**

Human endothelin comprises three structurally distinct 21 amino acid peptides ET-1, ET-2 and ET-3 encoded on separate genes. In addition to its potent vasoconstrictor property, ET-1 is a potent contractant of airway smooth muscle mediated through the ETₐ receptor subtype, whereas ET-2 and ET-3 have lower binding affinities for ETₐ receptors. ET-1 is a mitogen for airway smooth muscle and in fibroblasts is chemo-attractant, mitogenic and provides an activating signal for collagen synthesis largely mediated through ETₐ receptors. ET-1 induces collagen production and is important in myofibroblast-mediated contraction of granulation tissue.

Human bronchial epithelial cells cultured from the airways of asthmatics secrete increased amounts of ET-1 which is sensitive to inhibition with corticosteroids. ET-1 immunoreactivity in vivo is also increased in the epithelium. In BAL, levels of ET-1 are increased in proportion to the resting level of airflow obstruction. The importance of ET-1 as a novel bronchconstrictor is revealed by its capacity to reduce FEV₁, by ≥ 20% of baseline at inhaled concentrations of 10⁻⁸-10⁻¹² mol/L. With effective corticosteroid treatment of asthma, both lavage ET-1 levels and ET-1 expression in the epithelium return to those found in normal subjects.

**Nitric oxide**

NO is a short lived, highly soluble, free radical which plays a major role in cell–cell communication. It is generated enzymatically from L-arginine by NO synthase which exists in both constitutive and inducible isoforms (iNOS). Both enzymes require NADPH as co-factor and are inhibited by L-arginine analogues such as N⁵-nitro-L-arginine (L-NNA) and N⁵-monomethyl-L-arginine (L-NMMA) while the inducible form is selectively inhibited by aminoguanidine. We have recently shown that iNOS immunolocalizes strongly to the bronchial epithelium in bronchial biopsies from asthmatics but only rarely in those from normal controls. Consistent with enhanced NO generation
airway mucosal inflammation is the increased NO detected in exhaled air of active asthma and rhinitis patients. In vitro iNOS is induced in response to IFN-γ, IL-1β and TNF-α and is inhibited by corticosteroids. We have evidence that in corticosteroid-responsive asthma iNOS in the epithelium is downregulated and associated with a reduction in exhaled NO. Whilst NO generated by iNOS also contributes to vasodilatation, when upregulated, this enzyme has a greater synthetic capacity than the constitutive enzymes in producing nanomolar concentrations of NO. These are cytotoxic through the formation of peroxynitrate and hydroxyl radicals and nitrosylation of key mitochondrial enzymes. In severe asthma, the epithelium is likely to be a major source of toxic levels of NO and as such may provide a novel surrogate marker of disease activity and response to treatment.

**Cytokines**

Human bronchial epithelial cells in vitro constitutively synthesize and release IL-1β, IL-6, IL-8 and GM-CSF with greatly enhanced production occurring on exposure to IL-1β or TNF-α. Enhanced release of these cytokines has also been reported in asthmatic epithelial cells in vitro. Application of IHC shows that the bronchial epithelium in asthma is a particularly rich source of IL-1β, IL-8 and GM-CSF.

IL-8, a member of the α(C-x-C) chemokine family, is particularly important in the expression of chronic and severe disease, although it is usually regarded as a neutrophil chemoattractant. We have shown that IL-8 can induce human eosinophils from asthmatic subjects to secrete IL-3, IL-5 and GM-CSF. In an explant model of nasal polyp tissue, repeated allergen exposure produces a supernatant exhibiting a markedly enhanced eosinophil survival property. The explant supernatant contains IL-2, IL-3 and IL-5 and particularly high amounts of GM-CSF and IL-8. Using a range of blocking mAb, the eosinophil survival properties imparted by the explant medium were shown to be IL-8 and GM-CSF. These findings help explain why in atopic but not in non-atopic subjects we were able to show that instillation of human recombinant IL-8 into the nasal cavity produced a marked eosinophil survival in addition to neutral influx.

IL-8 in bronchial biopsies from asthmatics and in the peripheral circulation binds strongly to IgA. Thus, despite clear immunostaining for IL-8 in the epithelium, no free IL-8 can be detected in detergent-extracted homogenates; however, IL-8-IgA complexes can be readily detected with significantly more being presented in the allergic asthmatics. In patients with chronic severe asthma, both free and complexed forms of IL-8 are present in mucosal tissue and in serum. Thus, although IL-8 can be formed by many cells in asthma, our studies point to the bronchial epithelium being the major site of production and concentration of this chemokine. It is of particular importance that IL-8 co-localizes with a secretory IgA in the epithelium, as secretory but not serum IgA is able to markedly upregulate the eosinophil chemotactic response to IL-8 reaching an optimum at 10⁻¹⁰ mol/L. Under the same conditions, the neutrophil response was inhibited. IL-8 is also known to complex with the glycosaminoglycan side chains of proteoglycans, an interaction that we have taken advantage of when purifying the cytokine from asthmatic BAL. The eosinophil-specific properties of IL-8 are greatly enhanced when it is complexed to a cell-bound matrix. These include the secretory piece of IgA containing up to 20% N-linked oligosaccharide and proteoglycans, such as the granule products of mast cells and eosinophils, and CD44 which is expressed in greater amounts in the asthmatic epithelium. We hypothesize that IL-8 binding regulates the activity of and changes the target cell specificity of this cytokine rendering it a potent attractant and activator of eosinophils. In contrast to ET-1 and iNOS, corticosteroids only partially inhibit IL-8 transcription by epithelial cells in vitro and have no effect on free or complexed IL-8 levels in BAL fluid. That this chemokine is regulated by NF-kB and is markedly increased in proportion to asthma severity, increases its importance among the chemokines as a prominent contributor to disease chronicity.

**Epithelial repair**

Epithelial damage results in persistent activation of repair mechanisms, abnormal epithelial-mesenchymal interactions and detrimental remodelling of the airway.

**Epithelial cell biology**

Epithelial damage is a key feature of asthma with the extent of damage being related to disease activity. A recent study has shown that impaired detection of bronchial obstruction, a characteristic feature of severe disease, closely relates to the level of eosinophil infiltration and the extent of epithelial damage when assessed in bronchial biopsies. We have provided evidence that the major structural site of damage is between the columnar and basal cells and between adjacent columnar cells, implicating disruption to the desmosomes. The cause of increased epithelial dysfunction in asthma is not understood although the arginine-rich basic proteins of the eosinophil and active radicals are considered to be important. The level of damage may reflect either increased fragility or increased insult to the epithelium. To understand the importance of epithelial disruption in severe and chronic asthma, it is important to understand the processes of damage, repair and regeneration of the epithelium, and which normal functions of the epithelium are compromised.

On the basis of our preliminary work and studies by others, we propose the following steps in epithelial damage and repair:

1. Immediate damage and effects; selective cell loss due to injury with loss of barrier function.
2. Immediate response; epithelial cells adjacent to areas of damage reduce cell–substrate adhesion, increase cell migration and form a temporary squamous barrier.
3. Proliferative response; division, differentiation and remodeling leads to reformation of fully functional differentiated epithelium.
(4) Ongoing damage; asthma occurring during the above processes may further compromise epithelial integrity. Lack of appropriate downregulation of the normal response may produce a similar effect.

Eosinophil-epithelial interactions

Cytokines

Eosinophils are a newly recognized source of cytokines including IL-3, IL-4, IL-5, GM-CSF, IL-6, IL-8, TNF-α, MP-1α, TGF-α and TGF-β. Important among these for eosinophil survival are IL-3, IL-5 and GM-CSF indicating an autocrine function. Eosinophils exposed to these cytokines have their life expectancy extended from days to weeks and are also more responsive to chemo-attractant and mediator-secreting stimuli. Additionally, since the three eosinophilopoitines antagonize the accelerating effect of corticosteroids on eosinophil apoptosis, their sustained production would serve to render the cells functionally ‘corticosteroid-resistant’.

The use of blocking antibodies in our organ culture model has clearly shown that IL-8 is important as an effector of eosinophil survival by stimulating the release of IL-3, IL-5 and GM-CSF. Within the limitations of available ELISA techniques, these cytokines were not released but remained cell associated. Eosinophils also synthesize and release IL-8 in response to PAF and other stimuli, indicating that within the inflammatory focus eosinophil survival and activation are supported by a cytokine network dependent upon paracrine and autocrine loops. We now wish to extend these observations in relation to asthma chronicity by investigating the expression, intracellular localization and mechanisms of release of IL-8, IL-5 and GM-CSF. Our hypothesis is that the expression of eosinophil survival cytokines is increased in bronchial tissue in proportion to disease severity and that the intracellular cytokines are presented as a complex with proteoglycans of the eosinophil granules similar to the role of mast cell heparin in presenting IL-4. We propose that cytokine release is dependent upon activation of cell-surface immunoglobulin receptors.

Secretory IgA has been suggested to be the principal immunoglobulin mediating eosinophil functions at mucosal surfaces. Eosinophil basic proteins are released in response to slgA via a mechanism which is enhanced by IL-3, IL-5 and GM-CSF. Functional effects of slgA are most probably mediated by binding to either FcαR, whose expression on eosinophils is increased in asthma, or to specific receptors for the secretory component. We have shown that in asthma peripheral blood eosinophils carry both IgA and the secretory component bound to the cell surface. Unexpectedly, the concentrations of the secretory component on asthmatic blood eosinophils was greater than in a cultured epithelial cell line known to generate the secretory component. We interpret this as a result of eosinophils from the mucosal site, a phenomenon previously suggested but never proven. We have also noted strong synergism between slgA and IL-8 induced eosinophil migration in vitro.

Although it is likely that enzymes of the 5-LO pathway are upregulated in eosinophils in asthma, this has not yet been investigated. If accompanied by a decrease in PGE₂ production then unimportant autocrine cAMP-mediated inhibitory effect of this mediator on a number of eosinophil responses is removed, including the slgA induced release of basic granule proteins and presumably cytokines. We have observed that eosinophils in culture secrete PGE₂ which is increased in the presence of PAF or IL-5. Over 90% of the PGE₂ is released into the fluid phase and available for mediating negative feedback. In NSAID-induced asthma, removal of PGE₂ inhibition of mast cell and eosinophil LTC₄ production has been suggested.

Eosinophils isolated from the airways are spontaneously cytotoxic towards alveolar epithelial cells. However, in the airways of asthmatics viable columnar cells that are shed from their basal cell attachments will remain firmly fixed to the membrane via hemidesmosome (α₅β₃) and fibronectin-integrin adhesion. Using a bovine branchial epithelial explant, we suggest that activated eosinophils mediate epithelial detachment via a cognate interaction involving ICAM-1 and the subsequent release of (i) metalloendoproteases, (ii) oxidants, and (iii) arginine-rich proteins.

Metalloendoproteases In BAL from patients with asthma we have shown increased concentrations of the 92 Kd gelatinase metalloproteinase along with a range of other metalloendoproteases. Eosinophils are an important source of the 92 Kd gelatinase which has a broad substrate specificity in being able to degrade both basement membrane collagen type IV and interstitial matrix molecules. Because of the extensive eosinophil infiltrate in severe asthma, this enzyme is likely to be important both in cell migration and tissue remodelling, but other than describing its existence little is known about its expression and regulation.

Oxidants The tissue damaging effect of the products of eosinophil peroxidase (EPO) are indicated by the effectiveness of antioxidants to inhibit eosinophil mediated injury to lung epithelial cells and interstitial matrix in vitro. EPO is released asynchronously with ECP or MBP and, as with many highly charged proteins, it largely remains cell associated. Thus, in line with our hypothesis that eosinophil-epithelial cell contact is required for effective epithelial disruption, we will investigate the hypothesis that EPO along with reactive oxygen increases epithelial fragility through pericellular proteoglycan and adhesion glycoprotein degradation. A close-coupled mechanism would serve to exclude naturally occurring antioxidants such as GSH, vitamin E or albumen, and concentrate the delivery of the oxidant injury.

Basic proteins Considerable evidence exists for the disruptive effects of eosinophil cationic proteins (MBP, ECP, EDN) on epithelial integrity in asthma. Their extreme cationicity renders matrix proteoglycans as susceptible targets. A number of proinflammatory cytokines, including IL-4, IL-8, IFN-γ and b-FGF are tightly bound to GAG-side chains of highly O-sulphated proteoglycans, an association which stabilizes the
cytokine, localizes its activity and determines its specificity. Using immunohistochemistry we suggest that IL-8 and IFN-γ localizes to interepithelial clefts on account of their association with the GAG of CD44 and N-sulphated carbohydrate moieties sLeA. Similarly, IL-4 binds to heparin on mast cells, to basic fibroblast growth factor (bFGF), heparin S04 in basement membranes and TGF-β to decorin. Levels of IL-8, TGF-β and b-FGF are elevated in BAL from asthmatics with further increases occurring with allergen challenge. We suggest the interaction of the eosinophil basic proteins with cytokine binding sites on matrix molecules results in the release of free cytokine so that a wider range of activity is achieved in severe disease. An additional possibility is that major basic protein and eosinophil cationic protein interact with the highly alkaline sensitive Ser-O-GAG linkage of proteoglycans to produce non-enzymatic hydrolysis to further affect tissue cytokine localization and activity.

Epithelial mast cell-epithelial interactions

In mucosal biopsies mast cells aggregate both within the epithelium and in relation to the connective tissue elements of the basement membrane and associated myofibroblasts. We have recently shown that, when assessed by flow cytometry on an H292 epithelial cell line, human mast cell tryptase is able to upregulate the cell surface expression of ICAM-1 to a similar extent as TNF-α. A small increase in P-selectin and an apparent downregulation of N-cadherin expression were also observed as the activity of tryptase was increased. Tryptase also stimulated DNA synthesis in epithelial cells measured by 3H-thymidine incorporation and produced a dose-related release of IL-8. Inhibition of tryptase with leupeptin or benzamidine HCl prevented these actions of tryptase indicating the obligatory requirement for an active catalytic site. These studies suggest an important role for mast cells in epithelial repair, in the recruitment of granulocytes and in rendering the lower respiratory tract vulnerable to human rhinovirus infection since the major type of human rhinovirus utilizes ICAM-1 to gain access to epithelial cells and upregulate cytokine production.

Epithelial cell-myofibroblast interactions

We have shown that the apparent thickening of the subepithelial basement membrane in asthma is due to the deposition of collagen types I, III and V and fibronectin produced by proliferating myofibroblasts. The presence of tenascin in the lamina reticulosa indicates that this is a site of high matrix turnover and cell migration towards which both epithelial cells and myofibroblasts contribute. In addition to their capacity to secrete matrix proteins, we have recently shown that cultures of human bronchial subepithelial myofibroblasts produce GM-CSF, IL-6, IL-8 and SCF constitutively, and that the supernatant from cultures could greatly extend eosinophil survival. GM-CSF transcription demonstrated by RNase protection assay, was greatly upregulated in the presence of TNFα and was accompanied by secretion of GM-CSF which accounted for the majority of the eosinophil survival capacity of the supernatants. The enhancement of GM-CSF production by TNFα was inhibited in a dose-dependent manner by prednisolone, but maximum inhibition was not achieved until a dose of 1 mmol/L was reached, a concentration far beyond that achieved therapeutically. These findings suggest that human myofibroblasts located beneath the bronchial epithelium establish close contact with eosinophils and mast cells and as such play a critical role in upregulating mucosal inflammation, especially in chronic disease.

An integrated model of asthma

The complexities of human asthma as an inflammatory disorder are only just being appreciated. We have passed through the era of believing that the disease is one of smooth muscle or mast cells or eosinophils or T cells to a picture where all these and other cells are involved in a co-operative fashion. Figure 5 attempts to demonstrate this by showing a close interrelationship between those factors responsible for inflammatory events and those involved in repair. Implicit in this model is interdependency between the classical cells of inflammation and the formed elements of the airway. Varying contributions from each of these processes provides a rational basis for the variable clinical phenotype and responses to therapeutic interventions.

REFERENCES


