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Anti-Inflammatory Drugs in Asthma

Anthony P. Sampson
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Editors

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Anti-inflammatory drugs in asthma: The pathophysiology of asthma

James L. Lordan and Ratko Djukanović

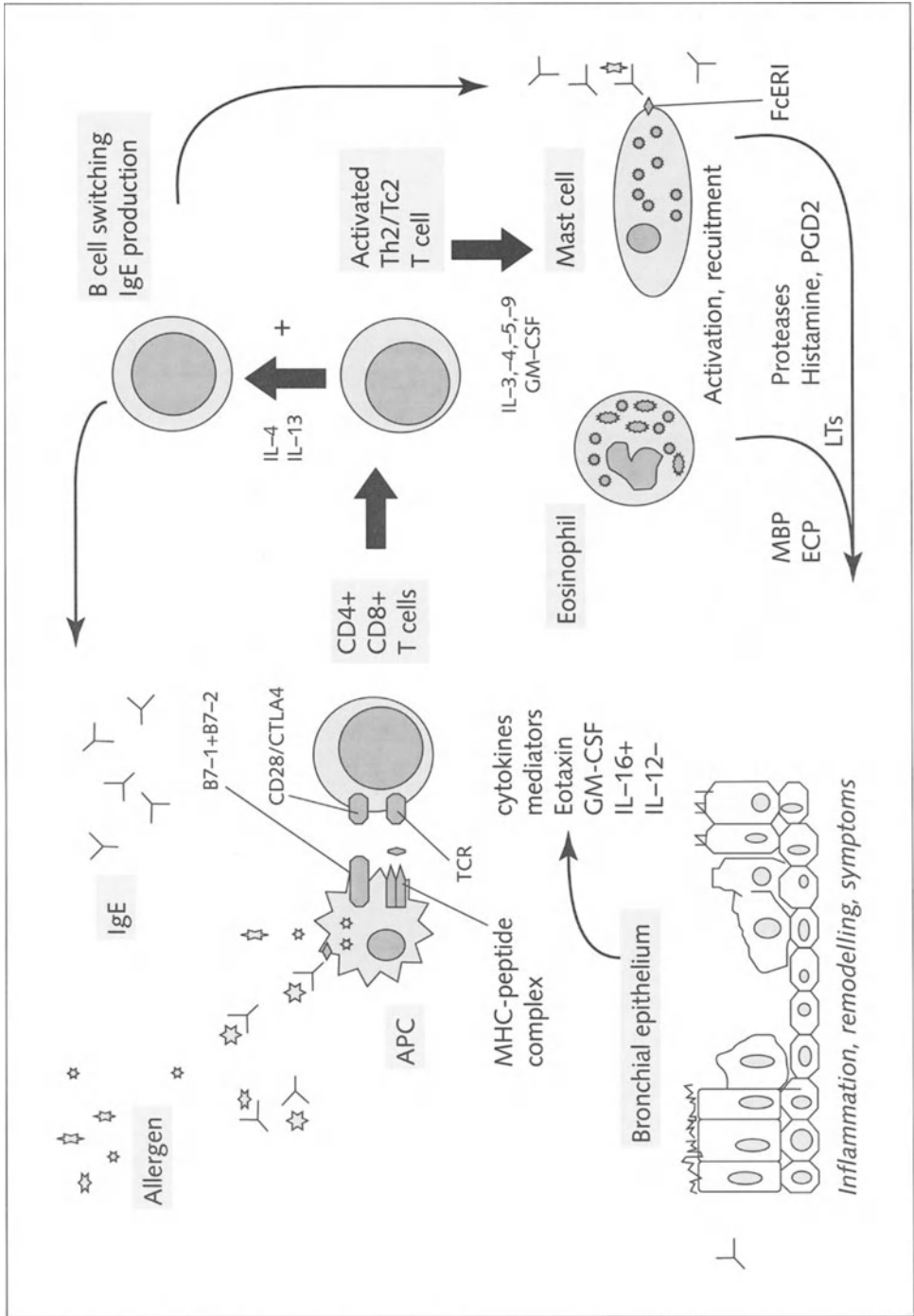
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Introduction

Asthma is a chronic condition characterised by widespread, variable and reversible airflow obstruction which is either spontaneous or pharmacologically induced. The underlying pathophysiological feature of asthma is increased airway responsiveness which develops on a basis of diffuse bronchial inflammation. The prevalence of asthma is increasing worldwide despite improved treatment which has resulted from a more comprehensive understanding of its pathogenesis [1]. In most countries asthma affects between 4 and 8% of the population, with a trend towards an increase in morbidity as judged by increased hospital admissions [2]. The reasons for this are unclear, but environmental factors such as indoor and outdoor air pollution and changes in lifestyle are considered to be amongst the contributing factors.

Asthma is clearly a complex process mediated by inflammatory cells and the formed and structural elements of the airway, involving numerous cytokines, chemokines, and costimulatory and adhesion molecules that interact in a complex network (Fig. 1). There is accumulating evidence establishing a link between chronic inflammation and remodelling of the airways in asthma. The present evidence of relatively permanent, irreversible structural changes found at an early stage in asthma supports the recommendations of current asthma management guidelines that effective anti-inflammatory treatment be introduced at an early stage in the management of asthma.

Asthma has been closely associated with atopy, the ability to generate an IgE response to environmental allergens which is detected by positive skin tests to one or more allergens, although it is interesting to note that only one-fifth of atopic individuals actually develop asthma. Whilst the presence of allergen specific IgE has been considered to be central to allergic responses in human allergic disease, studies using IgE-deficient mice have demonstrated that bronchial eosinophilic inflammation and airway hyperresponsiveness (AHR) can occur in the absence of IgE [3]. To what extent these observations are relevant to human disease remains to be elucidated. Clinical studies using a humanised monoclonal antibody to IgE show that



despite a dramatic depletion of IgE from the circulation with the prevention of the early and late phase response to allergen challenge, the clinical response in allergic asthmatics is quite poor [4, 5]. These studies suggest that while IgE is likely to be contributing to the inflammatory process in asthma, other immune mechanisms must be operating in parallel [6].

The genetic basis of asthma

There is evidence from family aggregation studies to support an important role for genetic contribution factors into the development of atopy and asthma. The prevalence of asthma in first-order relatives of asthmatics is 20–25% compared with 4–5% in the general population [1]. The relative contribution of genetic factors to the onset of allergy and asthma is estimated at 40–60% [7]. Twin studies, including a large study of 7000 same-sex twins by Edfors-Lubs, provide evidence for the genetic contribution to the onset of asthma, with concordance for asthma in monozygotic twins being 19%, and only 4.8% for dizygotic twins, who share less common genetic material [8]. Asthma is a complex genetic disorder, with studies suggesting a polygenic inheritance pattern which interacts with environmental factors to express the full clinical and pathophysiological phenotype [1].

Genetic studies are being used to characterise and map the genes responsible for the asthmatic phenotype. Studies in British families by Cookson and colleagues were the first to provide evidence of linkage of atopy to a marker (D11S97) on chromosome 11q13 in the vicinity of genes for CD20 (a B cell marker) and FcεRI-β, the β component of the high affinity IgE receptor [9–12]. An iso-leucine to leucine polymorphism at position 181 was identified in the FcεRI-β gene and was found in 15% of individuals, being associated with the phenotype of high total serum IgE [13]. A polymorphism (Glu 237-Gly) was found in 5.3% of the population in a study by Hill and colleagues and this was associated with AHR and atopy [5014]. There is

Figure 1

The inflammatory process in asthma involves the activation of Th2 type T cells by antigen presenting cells (APCs) resident in the airway wall (i.e. dendritic cells). T cell derived cytokines, including Interleukin-4 and IL-13 promote the switching of B cells to the synthesis of IgE. Cytokines are involved in the activation and differentiation of eosinophils and mast cells with release of pro-inflammatory mediators, leading to epithelial damage, bronchoconstriction and symptoms of asthma. The bronchial epithelium plays an active role by the release of mediators and cytokines. MHC, major histocompatibility complex class II; TCR, T cell receptor; FcεRI, high affinity IgE receptor; MBP, major basic protein; ECP, eosinophil cationic protein; LT, leukotrienes; PGD₂, Prostaglandin D₂.

also evidence for linkage of atopy and AHR to chromosome 5q31–33, which contains the genes for many cytokines considered to play an important role in asthma (interleukins- (IL-) 3, 4, 5, 9, and 13 and GM-CSF) [15]. In a study of Amish families involving 349 affected sib pairs, Marsh and colleagues provided evidence of linkage of total serum IgE with markers located at 5q31 [16]. A study in Dutch families, using affected sib pair analysis and linkage analysis, has also provided evidence of linkage of total serum IgE and AHR to markers on chromosome 5q [17, 18]. Asthma and elevated serum IgE levels have further been linked to markers on chromosome 12q which contains candidate asthma genes including IFN γ , mast cell growth factor (MGF), insulin-like growth factor-1 (IGF-1), and the constitutive form of nitric oxide synthase (NOS-1) [19, 20].

In agreement with the complex nature of asthma pathogenesis, there is evidence that the human leucocyte antigen (HLA) and T cell receptor (TCR) genes may genetically also contribute to determine antigen-specific IgE responses. Studies have shown associations between HLA alleles (HLA DRB1) and specific IgE responses [21]. HLA-DQw2 is more prevalent in Chinese children with asthma and house dust mite (HDM) allergy [22]. An association is found with certain alleles (HLA-DQB1*0503 and HLA-DQB1*0201/0301) and the onset of isocyanate-induced asthma in exposed individuals, while other alleles (HLA-DQB1*0501 and DQA1*0101-DQB1*0501-DR1) appeared to be protective (HLA-DQB1*0501 and DQA1*0101-DQB1*0501-DR1) [23]. The alpha chain of the T cell receptor (TCR), located on chromosome 11q, has been linked to specific IgE responses in a population of atopic asthmatics [24]. On chromosome 5q31–33 the β 2 adrenoreceptor gene has been linked with a phenotype based on elevated IgE and bronchial hyper-reactivity. Of significant relevance to treatment of asthma are the genes which might determine response to anti-inflammatory drugs, and one such plausible candidate for corticosteroid resistance is the glucocorticoid receptor gene [2, 25].

Prenatal origin of asthma and allergy

A familial tendency is seen in allergic patients, with 50% of children with one parent and 80% of those with two allergic parents becoming atopic [26]. Significantly more infants of an atopic mother develop allergy than those with an allergic father [27]. It is hypothesized that factors which determine allergic responses may cross the placenta and activate the process of initial sensitization of the fetal immune system. Thus, IL-10, a cytokine known believed to deviate the immune response to a Th2 type immune response, thus favouring the development of atopy in the foetus of an atopic mother [28], is detectable in amniotic fluid with at higher levels seen in atopic mothers than non-atopic mothers [29]. The maternal antigen presenting cells (APC) of the mother may pre-process antigen to peptides capable of crossing the placenta, allowing the foetal APCs to present this specific peptide to foetal T cells [76]. There

is also evidence to suggest that house dust mite antigens are present in cord blood, enabling foetal exposure to allergen to occur *in utero*. Infants who subsequently develop allergy during later childhood demonstrate an altered immune response at birth, with raised proliferative responses of T cells from cord blood and defective IFN γ production of IFN γ (a cytokine which downregulates allergic responses) to specific allergic stimulation [30]. Circulating allergen-specific T cells can be demonstrated as early as 15 weeks of gestation, and increased proliferative responses to antigen can be seen from 22 weeks, suggesting that the second trimester may be vital for initial sensitisation [30]. Circulating allergen-specific T cells are detectable at birth and these cells are thought to migrate over the following 6 to 12 months to tissue sites where disease will be expressed [31–33]. The early origin of atopy therefore appears to be central to a strategy aimed at early intervention. In keeping with this notion, studies are under way to assess whether allergen avoidance during the second trimester of pregnancy and the first year of life is effective in preventing the sensitization and subsequent development of atopy and allergy.

Pathological features of asthma

Asthma is characterised by variable damage to the epithelium with and increased numbers of mucosal and submucosal inflammatory cells, including eosinophils, mast cells and lymphocytes [34, 35]. Other prominent features of asthma are hypertrophy of airway smooth muscle, thickening of the basement membrane, mucosal oedema and excessive secretion of mucus, all of which contribute to airways narrowing. In severe exacerbations both large and small airways show gross damage and shedding of the epithelium, and the airways may become occluded by inspissated mucus and cellular debris which form tenacious plugs [34, 35]. Studies suggest differences in the type of inflammation depending on the time-course of asthma exacerbation, with infiltration of eosinophils being noted in slow-onset fatal asthma and an excess of neutrophils being seen the prominent feature of sudden acute asthma deaths [36].

Cellular mechanisms in asthma

The ability to obtain representative samples of the lower respiratory tract and technological improvements in analysing samples have revolutionised our understanding of the cellular and molecular basis of asthma. The advent of flexible bronchoscopy as a research tool, and the ability to safely obtain and analyse bronchial biopsies, bronchial brushings and bronchoalveolar lavage (BAL) fluid in asthmatic individuals, together with the recent validation of induced sputum techniques, have provided essential samples for research purposes.

Traditionally asthma has been viewed as a condition characterised by airway infiltration with activated mast cells and eosinophils, orchestrated by specific Th2 polarised T lymphocytes [37]. It is increasingly evident that other structures, including the bronchial epithelium, endothelium, fibroblasts and the extracellular matrix are involved in the recruitment of inflammatory cells, tissue damage and the subsequent repair and remodelling process that is characteristic of asthma.

Antigen presentation and co-stimulation

Environmental allergens and antigen presentation play an important role in the initiation and maintenance of airway inflammation. As with any immune response, antigen presentation of allergens to the immune system is central to subsequent responses. On exposure to antigen, T cells require the assistance of antigen-presenting cells (APC), such as dendritic cells, tissue macrophages, or in the case of secondary presentation, B cells, for optimal activation [38]. Antigen presentation involves the intracellular processing of allergen to peptides which are presented to the T cell receptor (TCR) in the context of major histocompatibility complex (MHC) class II molecules on the surface of APCs. For this process to be effective at eliciting a T cell response, ligation of costimulatory molecules on the surface of T cells and APC is required, resulting in optimal T cell clonal expansion and activation (Fig. 2).

The dendritic cells (DC), which form an intricate network within the bronchial epithelium, are believed to be the main APC in the airways. They develop from monocytes in the presence of IL-3, stem cell factor (SCF) and GM-CSF, having a unique capacity to stimulate naïve T cells. In rats, dendritic cells can be shown to migrate to regional (peribronchial, and mediastinal) lymph nodes for presentation of antigen to the immune system [39]. Of relevance to antigen presentation in asthma is the finding that dendritic cell numbers are elevated in the mucosa of asthmatic individuals and are reduced by treatment with inhaled corticosteroids in association with improved disease control [40, 41]. Although the mechanisms responsible for this increase in DC numbers are largely unknown, local T cell-mediated inflammation is enhanced by increased production of granulocyte macrophage-colony stimulating factor (GM-CSF) which enhances the ability of DC to differentiate and present antigen effectively [39]. Other cells, such as macrophages or B cells, may be involved in antigen presentation, but their role in asthma is poorly understood. Macrophages recovered by BAL are generally poor antigen presenting cells and have predominantly an immuno-suppressive role in the lungs, despite the fact that tissue macrophages promote inflammation through the production of pro-inflammatory mediators, such as PGE₂, hydrogen peroxide, leukotrienes, and cytokines such as IL-1, GM-CSF and MCP-1 [42–46]. Alveolar macrophages have a reduced capacity to bind with T cells, which may be due to reduced density of LFA-1 expression on their

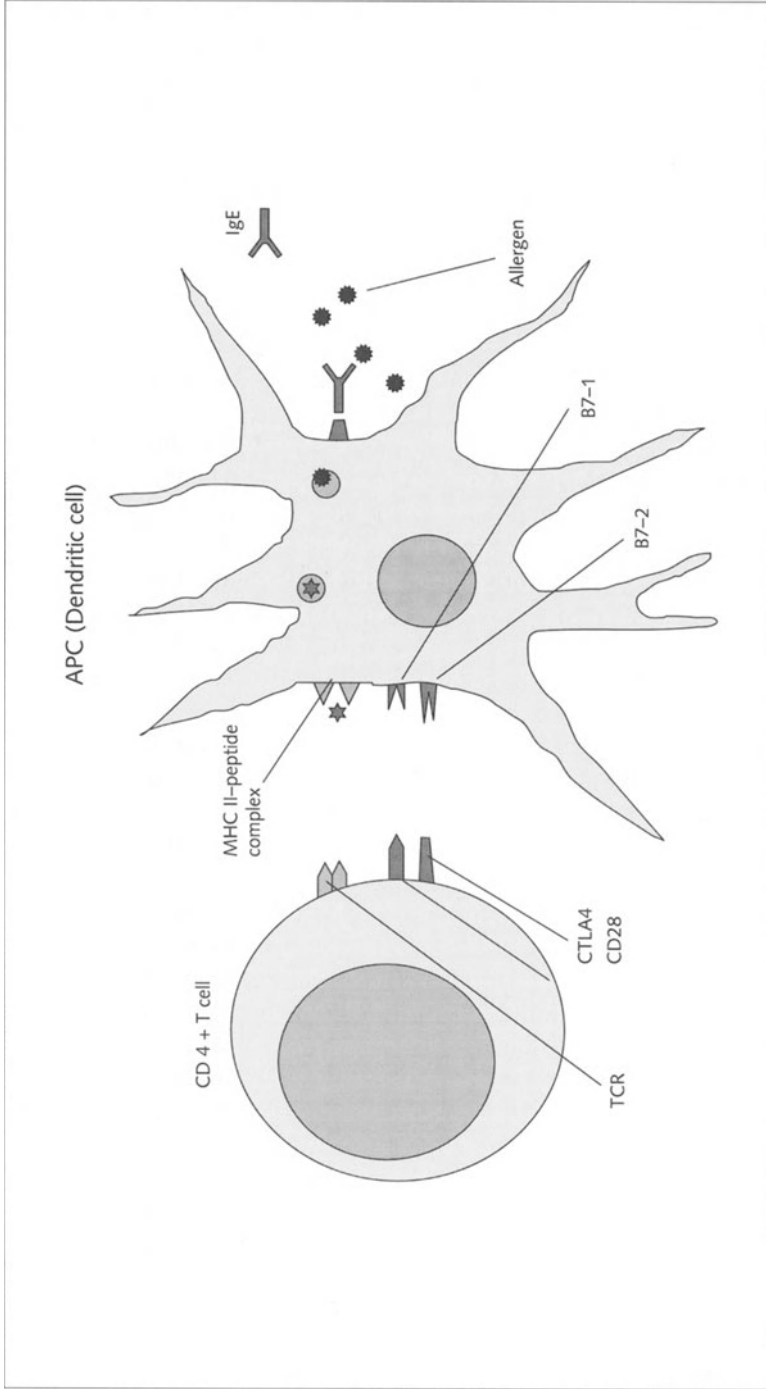


Figure 2
Activation of T cells by antigen presenting cells (APCs) involves the processing of allergen by APCs and presentation of peptide to the T cell receptor via the major histocompatibility complex (MHC) class II molecules. Optimum T cell activation requires a costimulatory signal delivered via the CD28/CTLA4-CD80 (B7-1)/CD86 (B7-2) pathway. Antigen uptake by APCs is potentiated by IgE-facilitated mechanisms involving the high affinity IgE receptor (FcεR1).

cell surface AM [47, 48]. Defective expression of B7-1 and B7-2 expression has previously been shown on AMs which would reduce the ability to present antigen efficiently [49], although a recent study by Agea and coworkers has shown elevated expression of B7-2 on AM in BAL fluid from atopic asthmatics compared with normal control subjects [50].

Costimulation via the CD28:B7 pathway

There is considerable evidence to support an important role for costimulatory molecules in regulating the proliferation and activation of T cells in the immune response. Of particular relevance are the interaction between CD28 and B7, and the CD40 binding with CD40 ligand (CD40L) [51, 52].

Following interaction between the T cell receptor (TCR) on the T cell membrane and the major histocompatibility (MHC) antigen-peptide complex on the APC, CD28 molecules expressed on the surface of T cells interact with B7-1 (CD80) and B7-2 (CD86) ligands expressed on the surface of APCs to provide a powerful augmenting stimulus to T cell activation. Studies have suggested that binding of another molecule present on activated T cells, CTLA-4, to B7-1 and B7-2 delivers a negative signal and functions to downregulate T cell activity [53, 54]. However, the role of CTLA4 remains uncertain, with recent studies suggesting that CTLA-4 might also deliver a positive signal to T cell activation [55, 56]. The development of CTLA4-Ig, a fusion protein consisting of the extracellular domain of CTLA4 and the Fc portion of a human immunoglobulin G₁ (IgG₁), has provided a useful tool to study the role of CD28/B7 interactions in immune responses. A number of studies have shown that interference with interaction by CTLA4-Ig is able to switch off T cell activation [57]. Recent studies using an ovalbumin sensitive murine model of asthma showed that systemic or intra-nasal CTLA4-Ig treatment suppresses the response to inhaled allergen (increased AHR, IgE production, recruitment of eosinophils into the lungs, production of IL-4, IL-5, and IL-10 and increased IFN γ production from CD3-TCR-activated T cells) [31, 54, 58–60]. Anti B7-2 treatment has been shown to have effects similar in magnitude on eosinophil infiltration, cytokine production, IgE production and to reduce bronchial hyperresponsiveness, suggesting that interaction of B7-2 with CD28 is important in the development of a Th-2 type inflammatory response in mice.

This observation has recently been shown to be of relevance to human allergic disease. *In vitro* studies have shown that exposure of naïve cord blood mononuclear cells to Der P allergen or Th-2 type cytokines, IL-4 or GM-CSF, results in increased expression of B7-2 and to a lesser extent B7-1. Blockade of CD28-B7 signalling by CTLA4-Ig or anti-B7-2 antibody has been shown to inhibit allergen-induced proliferation and cytokine mRNA production by PBMC from both atopic and non-atopic subjects [61]. A recent study by Spinozzi and colleagues has also shown increased

expression of B7-2 on BAL fluid alveolar macrophages from atopic asthmatics compared to normal controls, and to patients with pulmonary sarcoidosis or extrinsic allergic alveolitis [50].

The role of co-stimulation was recently studied in a human bronchial explant model of asthma. Bronchial biopsies obtained at bronchoscopy from house dust mite (HDM) sensitive atopic asthmatics were cultured for 24 h in the presence of house dust mite allergen extract. In this bronchial explant model, allergen was shown to stimulate the production of the cytokines IL-5 and IL-13 [62]. Addition of CTLA4-Ig fusion protein to the culture medium effectively blocked production of both IL-5 and IL-13 found on stimulation *ex vivo* with house dust mite antigen [62]. These studies confirm the requirement for interaction between co-stimulatory molecules in cytokine production and allergic inflammation, and point to the CD28-B7 pathway as being important to the allergen-induced AHR in asthma. Studies of organ transplantation in primates suggest that CTLA4-Ig is extremely effective in preventing organ rejection. While Phase I clinical trials have shown CTLA-4-Ig treatment of patients with psoriasis vulgaris to be well tolerated and to result in clinical improvement, its role in asthma management merits further investigation [63].

The role of CD40-CD40L interaction

There is considerable recent interest in the role of CD40 and its ligand CD40L in inflammation. CD40 is expressed on a number of cells, including B cells, T cells, monocytes, dendritic cells, eosinophils, and endothelial cells [64]. Ligation of CD40 on monocytes and dendritic cells results in the secretion of cytokines IL-1, -6, -8, -10, -12, TNF α , MIP1 α and up-regulation of costimulatory molecule expression such as CD54 (ICAM-1), CD58 (LFA-3), B7-1 and B7-2 on the cell surface. In co-cultures of dendritic cells and T cells, the interruption of CD40 signalling with an anti-CD40L monoclonal antibody results in reduced T cell proliferation. Ligation of CD40L on human activated T cells by specific monoclonal antibodies or with CD40 transfected T cells considerably enhances their cytokine production [65]. The interruption of CD40-CD40L interactions appears to improve the disease state in murine models of many conditions (e.g. collagen arthritis, lupus nephritis, experimental allergic encephalomyelitis and *Leishmania* infection) [64]. However, while its effects on B cell function and IgE production in allergy have been well studied, the role in T cell function is less clear.

Facilitated antigen presentation

Emerging evidence suggests that IgE may potentiate allergen-specific responses by binding to high- and low- affinity IgE receptors (Fc ϵ RI and Fc ϵ RII) on the surface

of antigen presenting cells and facilitating the capture and internalization of antigen which can subsequently be processed and presented to T cells. It has recently been demonstrated that IgE/FcεRI-mediated antigen uptake results in 100–1000-fold increased effectiveness in antigen presentation by APCs [66]. A recent study of bronchial biopsies has shown that pulmonary DCs and mast cells stain positively for the α sub-unit of FcεRI [67]. This suggests a possible role for IgE and facilitated antigen presentation in the induction and maintenance of chronic inflammation in asthma.

Transcription factors

There is increasing research interest in the role of transcription factors in the expression of cytokine genes involved in asthma. Transcription factors are intracellular proteins that bind to regulatory sequences of target genes, activating gene transcription, and subsequent cytokine mRNA and protein production. It is also increasingly recognised that therapies for asthma, such as corticosteroids and cyclosporin A, may function through interaction with transcription factors. Many stimuli including cytokines, activators of protein kinase C, viruses, and oxidants activate transcription factors including NFκB [68]. Cytokines act by binding to receptors on target cells with increased intracellular production of various protein kinases and phosphorylation of nuclear factor kappa B (NFκB) and activator protein (AP)-1. NFκB is present in the cytoplasm in an inactive form complexed to an inhibitory protein IκB. Various protein kinase molecules phosphorylate IκB to release free NFκB which then localises to the nucleus and binds to a specific κB recognition element on the promoter region of several genes. NFκB is an important regulator of several inducible genes, such as inducible nitric oxide synthase (iNOS), TNFα, GM-CSF, MIP-1α, MCP-1, eotaxin, ICAM-1 and VCAM-1 [68, 69]. TNFα stimulates the activation of NFκB and AP-1 in the lung, and treatment with corticosteroids inhibits activation of these transcription factors via a direct interaction between activated glucocorticosteroid receptor (GR), and the p65 subunit of NFκB [70].

The cytokine mediators of asthma

A number of cytokines have been implicated in controlling and perpetuating the chronic inflammation in asthma [71]. While studies of BAL and bronchial biopsies using *in situ* hybridisation show T cells to be a major source of cytokines in asthma, it is clear that other cells including eosinophils, mast cells, bronchial epithelial cells and the structural elements of the airways contribute to the production of cytokines (Table 1).

Table 1 - The cellular origin of cytokines and chemokines in asthma

Cell type	Cytokines and chemokines produced
T lymphocytes	IL-2, -3, -4, -5, -10, -12, -13, -16 GM-CSF, TNF α , IFN γ , RANTES, MIP-1 α , MCP-3
Eosinophil	IL-1 α , -2, -3, -4, -6, -8, -10, -16, GM-CSF, TNF α , IFN γ , TGF α , TGF β 1, MIP-1 α , IGF-II
Mast cell	IL-3, -4, -5, -6, -8, -10, -13, -16, GM-CSF, SCF, TNF α
Bronchial epithelium	IL-5 [72], -6, -8, -16, GM-CSF, TNF α , PDGF, IL-18, IDGF, MIP-1 α , MCP-3, RANTES, Eotaxin
Alveolar macrophages	IL-1, -10, -12, -13, -18, GM-CSF, TNF α , MCP-1

Cytokines derived from T cells and mast cells, such as IL-4, IL-5, and IL-10 play central roles in the pathogenesis of asthma by their ability to promote recruitment, activation, terminal differentiation, and prolonged survival of eosinophils in the airways, and, in the case of IL-4, to promote B cell switching to IgE production [73]. IL-4 enhances eosinophil recruitment to the airways by increasing VCAM-1 expression on endothelial cells to promote adherence of eosinophils bearing VLA-4 [74, 75, 121]. IL-3 and GM-CSF also contribute to eosinophilic inflammation by promoting eosinophil survival and mast cell and basophil development. GM-CSF is also involved in priming neutrophils and eosinophils [7Barnes PJ 941]. Although T cells and mast cells are likely to initiate the production of eosinophilotactic cytokines, eosinophils are also capable of enhancing eosinophilic inflammation by producing IL-4, IL-5, GM-CSF and TNF α [76]. A number of studies have confirmed increased expression of the Th2-type cytokines which promote the allergic response (IL-4 and IL-5), but not the Th1-type cytokine IFN γ , in BAL and bronchial biopsies from asthmatics [77, 78].

While there is a considerable body of evidence to support the role of cytokines such as IL-3, IL-4, IL-5, IL-6 and GM-CSF in asthma, a number of studies have recently focussed on IL-10, IL-12, IL-13, IL-16 and IL-18. IL-10 inhibits T cell proliferation and cytokine production [79, 80]. *In vivo* studies in murine models of allergic disease show that IL-10 treatment can reduce the airway eosinophilic response to allergen inhalation by preventing the release of pro-inflammatory cytokines IL-3, IL-5, GM-CSF and TNF α [76]. It has been suggested that IL-10 is produced as a late event after T cell activation in asthma [Robinson DS 9680], with IL-10 mRNA expression being increased after allergen challenge and localised predominantly to CD3⁺ T cells, but also to CD68⁺ alveolar macrophages. It has thus been suggested This suggests that IL-10 may be useful as a potential therapeutic

agent in asthma by controlling Th2-mediated inflammatory processes and preventing eosinophil accumulation [76, 80].

In vitro studies show that IL-12 promotes IFN γ production, suppresses IgE production, and promotes development of a Th1 phenotype [81, 82, 77]. For In sensitised mice, IL-12 treatment has been shown to abrogate airway hyperresponsiveness, eosinophilia and the production of Th-2 type cytokines after allergen inhalation [Gavett 9583]. Studies using anti-IFN γ antibodies show that the action of IL-12, at least in mice, is partially mediated by IFN γ production. The expression of IL-12 in bronchial biopsies is lower in asthmatics than normal controls, with mRNA for IL-12 being localised to alveolar macrophages and T cells [77]. A recent observation that treatment with prednisolone results in a significant increase in IL-12 mRNA positive cells in bronchial biopsies from steroid sensitive (SS) asthmatics but not steroid resistant (SR) asthmatic patients [77]. IL-12 provides further insight into the mechanisms of severe asthma that does not respond readily to corticosteroids. In addition, it suggests that treatment with IL-12 may prove useful in the management of asthma.

IL-13 is produced mainly by activated T cells and has a potentially important role in regulating allergic inflammation in asthma by promoting B cell proliferation, differentiation and immunoglobulin secretion via enhancement of class switching from IgG to IgE. This cytokine upregulates MHC class II and Fc ϵ R2 (low affinity IgE receptor) molecules and can promote dendritic cell development [84, 85]. IL-13 also upregulates VCAM-1 expression on vascular endothelial cells, which together with IL-4 and TNF α promotes eosinophil accumulation [86, 87]. Segmental allergen challenge in asthmatics has conclusively shown elevated expression of cells expressing BAL IL-13 mRNA and IL-13 levels in BAL [85, 88]. As noted for IL-2 and IL-4, treatment with prednisolone results in improvement in lung function which is associated with a significant downregulation in IL-13 mRNA cellular expression in glucocorticosteroid sensitive (GS) asthmatics, but not in glucocorticosteroid resistant (GR) asthmatics who showed little clinical improvement after treatment [77, 86]. These observations support the hypothesis that corticosteroid action is mediated partly by influencing IL-12 and IL-13 expression [77].

IL-16 is a newly characterised cytokine which has been shown to be produced by CD4⁺ and CD8⁺ T cells, epithelial cells, mast cells and eosinophils [89, 92, 113]. It uses the surface molecule CD4 as its ligand and thus activates cells that are CD4⁺, namely monocytes and CD4⁺ T cells [90]. Bronchial biopsy studies using ISH show expression of IL-16 mRNA on epithelial cells from asthmatics but not normal or atopic controls. The epithelial and subepithelial IL-16 mRNA expression was significantly associated with airways hyperresponsiveness and CD4⁺ T cell infiltration in the bronchial mucosa [91]. IL-16 has been detected early in BAL post endobronchial allergen challenge in asthmatics but not in control subjects [92]. This suggests a role for early IL-16 release in the selective recruitment of CD4⁺ T cells and eosinophils to the inflamed bronchial mucosa in asthma.

IL-18 is a recently characterised cytokine synthesised by activated macrophages and bronchial epithelial cells [93]. In association with IL-12, IL-18 acts on Th1 type T cells to produce IFN γ , and *in vivo* and *in vitro* studies in mice show that IL-12 and IL-18 strongly activate B cells to produce IFN γ [94]. Further studies are necessary to characterise the function of IL-18 in asthma.

TNF α is produced by many cells including mast cells, T cells, neutrophils, epithelial cells and monocytes [95–97]. Bronchial biopsy studies confirm mast cells to be a major source of TNF α in the human airways, with increased expression seen in asthmatics [96]. TNF γ levels in BAL are also raised in asthmatics in comparison to controls with higher levels seen in more symptomatic subjects [98]. TNF α may cause increased AHR via induction of pro-inflammatory mediators or by acting directly on bronchial smooth muscle [Kips JC 17,9299]. It is a chemoattractant for neutrophils and monocytes, and enhances eosinophil and mast cell cytotoxicity. Furthermore, TNF α increases vascular permeability [100–102]. TNF α also acts indirectly on eosinophils by stimulating myofibroblasts to produce GM-CSF which prolongs their survival. TNF α upregulates adhesion molecules, including VCAM-1, E-selectin and ICAM-1 involved in the recruitment of eosinophils, T cells and neutrophils to the site of inflammation [103–105]. Finally, TNF α appears to play an important role in airways remodelling by promoting fibroblast activity [106].

The orchestrating role of T lymphocytes in the inflammation of asthma

There is considerable evidence to support the view that the fine balance between Th1 and Th2 cytokines that exists in disease is dysfunctional, leading to a predominant Th2 type pattern in asthma. The asthmatic process is driven by the persistence of chronically activated memory T cells sensitised to specific allergens which localise to the airways after appropriate antigen exposure, or as some evidence suggests, after viral infection. This hypothesis is supported by observations in studies using BAL and bronchial biopsies from asthmatic subjects, including those studying treatment effects. Elevated numbers of activated CD4⁺, CD25⁺ cells are seen in blood in acute severe asthma, with a direct correlation being evident between activated peripheral blood T cells and airflow obstruction [107, 202]. Using immunohistochemistry, an increased number of activated (CD25⁺, CD3⁺) T cells can be demonstrated in the lamina propria and submucosa of bronchial biopsies from symptomatic asthmatics [108, 109]. Flow cytometry of BAL cells from asthmatics shows a correlation between CD4⁺, CD25⁺ lymphocytes and eosinophils, and the majority of CD4⁺ T cells are of the memory (CD45RO⁺) phenotype [110].

Using techniques such as double ISH, immunohistochemistry and semi-quantitative PCR, T cells have been shown to be the major source of mRNA encoding IL-4 and IL-5 in bronchial biopsies from both atopic and non-atopic asthmatics [111,

112]. Furthermore, CD4⁺ lymphocytes expressing mRNA for IL-4, IL-5 and GM-CSF, but not IL-2, IL-3 or IFN γ , are increased in the peripheral blood of asthmatics. The percentage of CD4⁺ T lymphocytes expressing IL-5 mRNA correlates with disease severity and the number of eosinophils in bronchial biopsy specimens. Glucocorticoid treatment improves lung function and reduces the percentage of CD4⁺ T cells expressing mRNA encoding IL-3, IL-5 and GM-CSF but not IL-2, IL-4 or IFN γ [115]. An elevated number of IL-5 mRNA expressing cells are seen in mucosal biopsies of asthmatics in comparison to controls, and these correlate with the number of CD25⁺ and EG2⁺ cells and total eosinophil counts suggesting that T cell products regulate eosinophil accumulation and function [116]. BAL from atopic asthmatics shows an increased number of cells expressing mRNA for IL-3, IL-4, IL-5 and GM-CSF, with IL-4 and IL-5 being localised to T cells, suggesting activation of, and cytokine production by, Th2 type T cells [78]. Prednisolone treatment of asthmatics results in clinical improvement and reduced AHR, accompanied by reduced eosinophil counts and reduced expression of mRNA for IL-4 and IL-5 and increased expression of IFN γ by cells in BAL [117, 118].

In situ hybridisation (ISH) techniques demonstrate significant associations between IL-4, IL-5 and GM-CSF expression by BAL cells and airflow obstruction, bronchial hyper-responsiveness and asthma symptom scores [119]. IL-5 is significantly higher in asthmatics than atopic non-asthmatic controls while IL-4 is elevated in both atopic groups compared to normal non-atopic controls [111, 112]. This suggests that IL-5 may be more closely related to the asthmatic phenotype while IL-4 may be associated with the overproduction of IgE [120]. The combined use of immunohistochemistry and *in situ* hybridisation localises mRNA for IL-4 and IL-5 predominantly to CD4⁺, but also to CD8⁺ T cells [121].

T cells – determinants of asthma severity

The notion that asthma is not a single disease entity but a spectrum of what is best referred to as the asthma syndrome is well accepted. Since the natural history of asthma has not been a subject of scrutiny in large studies, it is unclear what proportion of asthmatics are predisposed to develop severe disease early in their life as opposed to progressing gradually from mild, through moderate, to severe disease. Severe asthma is characterised by decreased responsiveness, both in clinical and pathological terms, to corticosteroid treatment. A minority of these patients can be shown to be truly resistant irrespective of the dose of corticosteroids used.

Peripheral blood mononuclear cells (PBMCs) from patients with glucocorticosteroid resistant (GR) asthma are seen to proliferate in response to the mitogen, phytohaemagglutinin (PHA), despite the presence of high doses of dexamethasone

[120]. A clear relationship can be shown between clinical responsiveness to steroids and *in vitro* effects of steroids on PHA induced T cell proliferation [120]. After treatment with oral prednisolone, glucocorticosteroid sensitive (GS) asthmatics experience a significant decrease in the number of cells in BAL expressing mRNA for IL-4 and IL-5 with an increase in IFN γ expression. GR asthmatics show no change in the number of cells expressing IL-4 or IL-5, but display a significant reduction in the number of IFN γ positive cells [122]. These findings are compatible with reports of a defect in glucocorticosteroid receptor binding affinity in GR asthmatics which can be maintained *in vitro* by the addition of IL-2 and IL-4 together, but not individually [123, 124]. It has been suggested that steroid resistance may be due to altered expression of genes regulating T cell cytokine production [125]. Studies suggest that corticosteroid resistance results from an altered ability of the cellular glucocorticosteroid receptor (GCR) to bind to glucocorticoid response elements (GREs) in the promoter region of the glucocorticoid responsive genes in GR patients [125]. Another hypothesis is that a dysregulation of transcription factors, with increased expression of the pro-inflammatory molecule AP-1 in GR asthmatics, may be involved in steroid resistance by interfering with steroid action by binding to the glucocorticosteroid receptor preventing GCR DNA binding [125, 126]. Leung et al. have identified GRC abnormalities using a [³H]-dexamethasone radio ligand-binding assay of PBMCs, categorising GR asthmatics as Type 1 when a decrease in GCR binding affinity restricted to T cells was present, and Type 2 when the GCR binding affinity was normal but the numbers of GCR binding sites per cell were found to be abnormally low in both T cells and non-T cells.

Unfortunately, effective and safe agents enabling suppression of corticosteroid-resistant components of airway inflammation are not available. A number of drugs used to inhibit organ rejection in transplanted patients (cyclosporin) and so-called corticosteroid sparing agents (methotrexate, azathioprine) have been used in severe asthma. Cyclosporin inhibits T cell activation by blocking calcium dependent transcription of mRNA encoding a number of cytokines. In a group of GR asthmatics it is seen to improve lung function, reduce the number of exacerbations and have a cortico-steroid-sparing effect [126–128, 201]. Cyclosporin reduces the magnitude of the late allergic response (LAR) to allergen challenge, suggesting cyclosporin action through inhibition of T cell function in this model of asthma [129, 201]. However, the clinical efficacy of cyclosporin remains to be fully established. For this reason, a number of novel therapeutic agents are being considered for the treatment of severe asthma, including :immuno-suppressive agents (e.g. FK506, mycophenolate mofetil and rapamycin), ;anti-CD4 antibodies, ;cytokine receptor antagonists (e.g. IL-1 receptor antagonist), anti-cytokine antibodies (e.g. anti-IL-4 and anti-IL-5 antibodies), IL-12 modulation of the Th2 response, and agents that may block the co-stimulatory pathways required for T cell activation (e.g. CTLA4Ig fusion protein and anti-B7-2 antibody) [130].

The effector role of eosinophils

Eosinophils are considered to be responsible for much of the mucosal inflammation and resulting epithelial damage in asthma. A number of proteins are released by eosinophils on activation, including major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin and eosinophil peroxidase, which have been shown to damage epithelial integrity by disruption of epithelial desmosomes and tight junctions [6]. Eosinophil counts are consistently shown to be increased in the lumen and in sub-mucosa and epithelium of bronchial biopsies both in unchallenged airways and after segmental allergen challenge [131, 132].

Whilst a correlation between disease severity and eosinophils can be demonstrated using cell counts in the lumen and mucosa, the strength of correlation is best when luminal eosinophils, counted in sputum, are investigated [133]. In addition to producing vaso- and bronchoactive mediators, eosinophils have been shown to synthesise a number of pro-inflammatory cytokines including IL-3, IL-4, IL-5, IL-6, TNF α , GM-CSF and transforming growth factors (TGF α and TGF β) [134]. In addition, using recombinant polymerase chain reaction (RT-PCR), mRNA for IL-4 has been detected in blood eosinophils of asthmatic patients [135]. IL-3, IL-5 and GM-CSF are thought to prime eosinophil mediator release and prolong eosinophil survival by inhibiting apoptosis [118].

Mast cells

Mast cells have long been viewed as pivotal to the development of anaphylactic or immediate type reactions. They produce a number of inflammatory mediators including histamine, heparin, tryptase, prostaglandins (PGD₂), leukotriene C₄ (LTC₄) and thromboxane A₂ (TXA₂) which possess potent broncho- and vasoactive properties [6]. Mast cells containing tryptase alone (MC_T) are found in increased numbers in the airway biopsies of asthma patients and correlated inversely with PC₂₀ (i.e. the cumulative dose of spasmogen, such as histamine, required to cause a 20% fall in FEV₁ from baseline) [37]. Mast cell activation markers have also been shown to correlate positively with markers of vascular permeability and negatively with FEV₁, suggesting an association with disease activity. Studies have shown an increased release of mast cell mediators in the BAL fluid of asthmatics [6].

In the presence of specific allergen, cross linkage of IgE molecules bound to high affinity IgE receptors (Fc ϵ R1) on the mast cell surface causes cellular activation and release of mediators resulting in acute bronchoconstriction. Mast cells can be activated by stimuli such as airway fluid osmolar changes as in exercise-induced asthma, and by drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) in susceptible individuals [136].

It is now fully appreciated that mast cells have the capacity to produce a number of cytokines which have previously been attributed to T cells, including IL-4, IL-5, IL-6, IL-8, GM-CSF and TNF α [137]. IL-4 has been shown to bind to glycosaminoglycan (GAG) side-chains of heparin produced by mast cells and stored in their granules, which is likely to mediate local cytokine signalling. The production of cytokines such as TNF α and IL-5 and the release of the neutral protease tryptase by mast cells, in association with the later recruitment and activation of Th-2 type T cells, are likely to contribute significantly to the LAR [138, 139]. To what extent the cytokines secreted by mast cells contribute to allergic inflammation is unclear. Using a combination of immunohistochemistry and *in situ* hybridisation, studies have shown that the greatest proportion of cells producing IL-3, IL-4 and IL-5 are T cells [121]. However, in view of the relatively low frequency of allergen specific T cells in the airways of asthmatics [115] it is uncertain what the trigger factors for the synthesis of these cytokines may be. The relative contribution of mast cells and T cells is further complicated by the fact that probably all of the mast cells in the airways possess specific IgE which enables them to respond in an allergen specific manner.

The bronchial epithelium as a source of pro-inflammatory cytokines

The bronchial epithelium was previously thought to be a passive barrier that was damaged by inflammation in asthma. It is now known to be an important source of mediators including arachidonic acid products, nitric oxide, endothelins, cytokines and molecules involved in repair, demonstrating an active role for the epithelium in regulating the inflammation, repair and remodelling processes of asthma. The epithelium can be activated by mediators such as histamine, cytokines, leukotrienes, by IgE dependent mechanisms or by stimuli including, occupational chemicals, pollutants, viruses, or the shifts in epithelial lining fluid osmolarity that may occur in exercise induced asthma [140–144]. The epithelium is a major source of 15-HETE, PGE₂, nitric oxide (NO) and endothelin-1 [6]. The arachidonic acid metabolite, 15-HETE induces chemotaxis of inflammatory cells, increases mucus production and enhances the EAR. PGE₂ has vasodilator and bronchodilator properties. Increased PGE₂ production by the epithelium inhibits IL-12 production by dendritic cells promoting Th2 differentiation by T cells [97, 145].

The bronchial epithelium is capable of storing and releasing cytokines (IL-1 β , IL-6, IL-11, GM-CSF, IL-16 and IL-18) and chemokines (IL-8, MIP-1 α , MCP-3, RANTES and eotaxin) [146–149]. Cytokine release is controlled by inducible genes activated by pro-inflammatory cytokines (TNF α , IL-1 α and IFN γ), by growth factors (TGF α , HB-EGF and amphiregulin) and by epithelial detachment and deformation [68, 150, 151]. The transcription factors, NF- κ B and STATS, control inducible genes that regulate mediator release. Immuno-histochemical studies show

increased expression of NF- κ B and STATs in asthmatic bronchial epithelium [152]. Asthmatic bronchial epithelium expresses the adhesion molecules ICAM-1, α 3 β 1 and α v β 6 [153]. ICAM-1 is the binding receptor for rhinovirus and increased ICAM-1 expression may contribute to virus-induced exacerbations in asthma [154]. α 3 β 1 is a ligand for fibronectin, laminins and collagens, the extracellular matrix proteins involved in repair and remodelling [155]. α v β 6 is a ligand for fibronectin and tenascin, the structural proteins deposited in increased amounts beneath the epithelial basement membrane.

Nitric oxide (NO) is a highly soluble free radical with a short half-life, involved in cellular signalling and is produced by constitutive and inducible forms of NO synthase (iNOS). Increased levels of NO are found in the exhaled air of patients with active asthma or rhinitis. In severe asthma, the epithelium is likely to be a major source of NO [156–158]. Bronchial biopsy studies show that iNOS immune activity localises to the bronchial epithelium in asthmatics but is rarely seen in airway biopsies of normal controls [6]. Bronchial epithelial iNOS activity is downregulated in corticosteroid sensitive asthma and is associated with a reduction in exhaled NO levels. It may be a useful marker of disease activity in asthma [159, 160].

Human endothelins include three polypeptides (ET-1, ET-2 and ET-3). ET-1 is a potent vasoconstrictor, contractor of smooth muscle, mitogen for airway smooth muscle and fibroblasts and is capable of activating collagen synthesis [161]. ET-1 is produced by bronchial epithelial cells [162], vascular endothelial cells [163], mast cells and macrophages and ET-1 expression is increased in bronchial epithelial biopsies of asthmatics [164]. Studies show that ET-1 levels in BALF of asthmatics are increased in proportion to the level of resting airflow obstruction in asthma [165]. ET-1 levels in BALF and bronchial biopsy epithelial expression of ET-1 have been shown to fall to normal levels in asthmatics treated with effective doses of corticosteroids [165]. This suggests a role for endothelins in asthma, particularly in the late allergic reaction (LAR) but also possibly in the remodelling process.

Inhaled anti-inflammatory drugs such as corticosteroids have a dramatic effect in improving asthma symptoms, lung function and airway reactivity. These agents have powerful inhibitory effects on mediator release and cytokine production by the asthmatic epithelium [166, 167]. Inhaled corticosteroids promote a reduction in bronchial eosinophilic infiltration and downregulate the expression of transcription factors such as NF- κ B known to be important in inflammation [168].

The process of airway remodelling in asthma

A number of studies indicate that irreversible airway obstruction can occur in asthma, which suggests that structural changes consistent with a remodelling process occurs in the airways of asthmatics [169]. In patients who died from severe asthma, prominent airway wall thickening is noted. The bronchi of wall diameter greater

than 2 mm in thickness demonstrate increased thickness of the total wall, sub-epithelial layer and muscle layer with associated hypertrophy and hyperplasia of the airway smooth muscle layer [170, 171]. A recent three-dimensional morphometric study of asthmatic airways suggests that smooth muscle hyperplasia is more prominent in large airways whereas hypertrophy is more prominent in the affected smaller airways [171]. Studies have demonstrated prominent hyperplasia of mucus glands in the airway wall in cases of fatal asthma [172]. Increased angiogenesis and airway smooth muscle hypertrophy is also a feature of the remodelling process of asthma [173, 174]. Computerised tomography studies of asthmatics demonstrated abnormalities in airway wall thickness in 90% of the cases studied; these were related to both the duration and severity of asthma in cases with an irreversible component of airways disease [175, 176]. Thickened basement membrane zone abnormalities noted at postmortem of asthmatics have been confirmed in bronchial biopsy studies using fibreoptic bronchoscopy. Electron microscopy studies localise the increased basement membrane thickness to the lamina reticularis layer. Immunohistochemistry studies confirm that this thickened layer is composed of collagen types III, V, I and fibronectin [177]. Myofibroblast numbers are increased in the airway walls of asthmatics and are considered to be responsible for this collagen deposition [178]. A recent study has shown that the submucosa, as well as the reticular basement membrane, contains significantly more collagen than normal controls, suggesting that airway scar formation may have more significant effects than were previously considered [179]. *In vitro* studies suggest that this remodelling process involves a number of growth factors and cytokines including TGF β ₁, platelet-derived growth factor, basic fibroblast growth factor, TNF α , IL-4, endothelin and other molecules including tryptase and histamine suggesting a role for mast cells in tissue remodelling in asthma [180, 181]. A number of extra-cellular matrix proteins contribute to the inflammation and remodelling of asthma. Eosinophils express integrins such as VLA-4, VLA-6 and LFA-1 which are known to adhere to matrix proteins such as laminin, fibronectin and fibrinogen [182, 183]. This is thought to encourage the accumulation, increase the activation and prolong the survival of eosinophils locally in the bronchial tissue [184]. Studies of asthmatic bronchial biopsies, using *in situ* hybridisation and immunohistochemistry techniques have shown activated eosinophils in the reticular lamina to be a major source of TGF β ₁ mRNA and immunoreactivity which is associated with the degree of airway fibrosis, linking chronic allergic inflammation with the structural remodelling and decline in FEV₁ seen in asthma [185]. Mast cells also express integrins and *in vitro* studies have shown that fibronectin adherence encourages both IgE mediated mast cell degranulation, chemotaxis and proliferation. In asthmatics, matrix protein interaction is capable of altering IgE mediated cytokine release by basophils [184]. Elevated levels of glycosaminoglycan (GAG) side-chains, particularly chondroitin and dermatan sulphate have been noted in the urine of asthmatics during a severe asthmatic attack [186]. It is considered that these are produced by the breakdown of extracellular

matrix proteoglycan in the bronchi of asthmatics. Tryptase is known to cleave certain components of the extracellular matrix (e.g. fibronectin, collagen VI) and activate matrix metalloproteases (stromolysin). Tryptase can also enhance proliferation of fibroblasts, smooth muscle and epithelial cells. Asthmatic airways have an increased expression of these mediators which are thought to promote mitogenesis of myofibroblasts and airway smooth muscle and synthesis of collagen [187].

Studies in children and occupational asthma indicate that airway changes of remodelling occur at an early stage of the disease [188]. The majority of studies in asthmatics treated with inhaled corticosteroids show no significant reversibility of airway collagen deposition despite relieving symptoms, improving lung function and modifying bronchial hyperreactivity [189, 190]. This supports the recommendation of early introduction of effective anti-inflammatory treatment in an attempt to prevent the development of irreversible structural changes.

Mechanisms of asthma severity and chronicity

Despite the considerable improvement in the understanding of asthma pathogenesis, the mechanisms which determine disease severity remain poorly understood. The degree of sputum, BALF and blood eosinophilia and, to a lesser extent, levels of ECP detected in clotted blood have been found to broadly relate to disease severity. T cell activation and expression of Th2 type cytokines are found in all degrees of asthma but, as discussed above, it is most prominent in severe disease, which is poorly controlled by corticosteroids [191]. Physiological indices of asthma severity such as methacholine responsiveness are related to the number and activity of eosinophils and activated CD4⁺ T cells in BALF from atopic asthmatics [192, 193]. AHR but not symptom scores have been shown to be inversely related to the number of activated eosinophils and mast cells in bronchial biopsies from asthmatics not treated with corticosteroids [194]. In asthmatics treated with inhaled corticosteroids, AHR, but not symptom score or lung function, is inversely related to the number of infiltrating mast cells, activated eosinophils, CD8⁺, and CD45RO⁺ T cells in bronchial biopsies from these patients [193].

Studies involving *post mortem* examination of patients with sudden asthma related deaths, and sputum analysis of patients in *status asthmaticus* support the role of neutrophils in severe asthma [195, 196]. A recent bronchoscopy study showed that whilst eosinophil numbers may or may not be raised in the mucosa, a significantly higher neutrophil count is seen in bronchial and transbronchial biopsies of severe, high-dose oral corticosteroid dependent asthmatics compared with moderate asthmatics and normal controls. Lipid-derived and mast cell derived mediators remained elevated in the severe patients despite treatment with high dose corticosteroids [197]. Interestingly, a lower percentage of macrophages was seen in the severe asthmatics compared to moderate asthmatics. Some studies have shown that

corticosteroids can enhance neutrophil function through increased leukotriene and superoxide production and inhibition of apoptosis [198]. It is possible that corticosteroids could reduce the lymphocyte and eosinophil mediated inflammation but exacerbate neutrophil mediated inflammation in severe asthmatics [199].

Concluding remarks

The last few years have seen a dramatic improvement in our understanding of asthma which has probably surpassed that of other respiratory diseases. We have learned about the orchestrating role of Th2-type cytokines which dominate inflammatory responses in asthma and are beginning to understand the regulation of their production at the gene level. Detailed morphological studies have led to the concept of airways remodelling as a consequence of chronic inflammation which is most likely fixed. This has contributed to better management of asthma. However, many issues remain unresolved, the most important being the inability to completely abrogate the inflammatory response in more severe disease, which is not only becoming more prevalent but is also draining a major proportion of funds dedicated to asthma management [200]. The identification of several cytokines as participating in airways inflammation has meant that targeting individual cytokines is unlikely to lead to major improvements in disease management. Future efforts will have to be directed at elucidating the relative contribution of individual cell types and mediators in the hope that the wide syndrome of asthma may eventually be broken down into subtypes, hopefully with distinct patterns of mediators that are involved in their pathogenesis.

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Corticosteroids

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Introduction

Corticosteroids are the most effective therapy currently available for asthma and improvement with corticosteroids is one of the hallmarks of asthma. Inhaled glucocorticoids have revolutionised asthma treatment and have now become the mainstay of therapy for patients with chronic disease [1]. There has recently been an enormous increase in our understanding of the molecular mechanisms whereby glucocorticoids suppress inflammation in asthma and this has led to changes in the way corticosteroids are used and may point the way to the development of more specific therapies in the future [2, 3].

Molecular mechanisms

Glucocorticoids are very effective antiinflammatory therapy in asthma and the molecular mechanisms involved in suppression of inflammation in asthma have recently become clarified. It is evident that corticosteroids are so effective because they block many of the inflammatory pathways that are abnormally activated in asthma.

Glucocorticoid receptors

Glucocorticoids exert their effects by binding to glucocorticoid receptors (GR) which are localised to the cytoplasm of target cells. The affinity of cortisol binding to GR is approximately 30 nM, which falls within the normal range for plasma concentrations of free hormone. There is a single class of GR that binds glucocorticoids, with no evidence for subtypes of differing affinity in different tissues. Recently a splice variant of GR, termed GR- β , has been identified that does not bind glucocorticoids, but binds to DNA and may therefore interfere with the action of corticosteroids [6]. However, it is unlikely that there is sufficient GR- β present in cells to

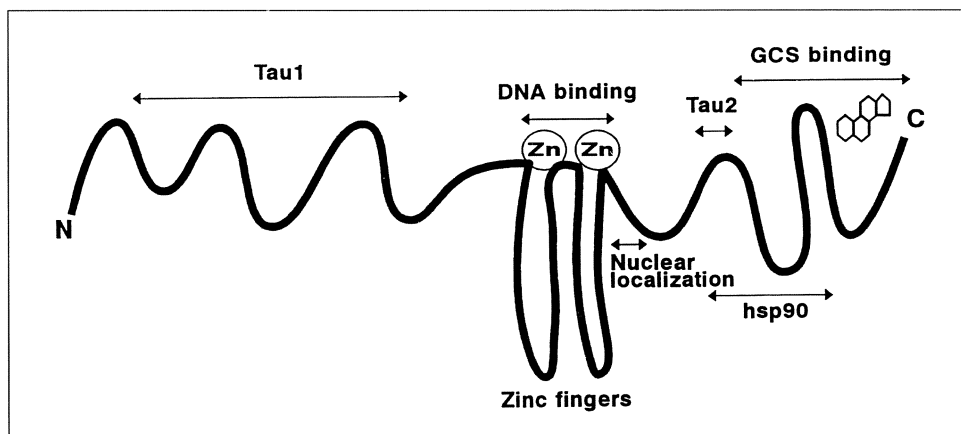


Figure 1
Domains of the glucocorticoid receptor.

functionally inhibit glucocorticoid action. The structure of GR has been elucidated using site-directed mutagenesis, which has revealed distinct domains [7, 8]. The glucocorticoid binding domain is at the C-terminal end of the molecule and in the middle of the molecule are two finger-like projections that interact with DNA. Each of these “zinc fingers” is formed by a zinc atom bound to four cysteine residues (Fig. 1). An N-terminal domain ($\tau 1$) that is involved in transcriptional trans-activation of genes once binding to DNA has occurred and this region may also be involved in binding to other transcription factors [9]. Another trans-activating domain ($\tau 2$) is adjacent to the steroid-binding domain and is also important for the nuclear translocation of the receptor. The inactivated GR is bound to a protein complex (~300 kDa) that includes two molecules of 90 kDa heat shock protein (hsp90) and various other inhibitory proteins. The hsp 90 molecules act as a “molecular chaperone” preventing the unoccupied GR localising to the nuclear compartment. Once the glucocorticoid binds to GR hsp90 dissociates, thus exposing two nuclear localisation signals and allowing the nuclear localisation of the activated GR-corticosteroid complex and its binding to DNA (Fig. 2).

Effects on gene transcription

Glucocorticoids produce their effect on responsive cells by activating GR to directly or indirectly regulate the transcription of certain target genes [10, 11]. The number of genes per cell directly regulated by corticosteroids is estimated to be between 10 and 100, but many genes are indirectly regulated through an interaction with

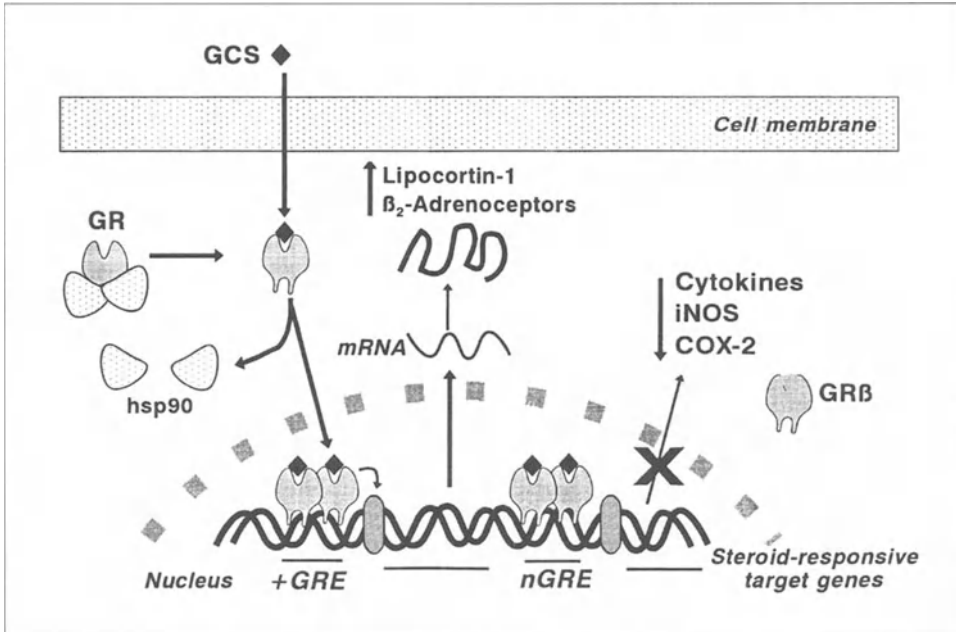


Figure 2

Classical model of glucocorticoid action. The glucocorticoid enters the cell and binds to a cytoplasmic glucocorticoid receptor (GR) that is complexed with two molecules of a 90 kDa heat shock protein (hsp90). GR translocates to the nucleus where, as a dimer, it binds to a glucocorticoid recognition sequence (GRE) on the 5'-upstream promoter sequence of corticosteroid-responsive genes. GREs may increase transcription and nGREs may decrease transcription, resulting in increased or decreased messenger RNA (mRNA) and protein synthesis.

other transcription factors, as discussed below. Upon activation GR forms a homodimer which binds to DNA at consensus sites termed glucocorticoid response elements (GREs) in the 5'-upstream promoter region of corticosteroid-responsive genes. This interaction changes the rate of transcription, resulting in either induction or repression of the gene. The consensus sequence for GRE binding is the palindromic 15-base pair sequence $GGTACAnnnTGTTCT$ (where n is any nucleotide), although for repression of transcription the putative negative GRE (nGRE) has a more variable sequence ($ATYACnnTnTGATCn$). Crystallographic studies indicate that the zinc finger binding to DNA occurs within the major groove of DNA with one finger of each receptor in the homodimer interacting with one-half of the DNA palindrome. Interaction with other transcription factors may also be important in determining differential corticosteroid responsiveness in different cell types. Other transcription factors binding in the vicinity of GRE may influence the transactivat-

ing efficiency of GRE binding and the relative abundance of different transcription factors may contribute to the corticosteroid responsiveness of a particular cell type. GR may also inhibit protein synthesis by reducing the stability of mRNA via enhanced transcription of specific ribonucleases that break down mRNA containing constitutive AU-rich sequences in the untranslated 3'-region, thus shortening the turnover time of mRNA.

Interaction with transcription factors

Activated GR may bind directly with other activated transcription factors as a protein-protein interaction. This could be an important determinant of corticosteroid responsiveness and is a key mechanism whereby glucocorticoids exert their anti-inflammatory actions [4]. This interaction was first demonstrated for the collagenase gene which is induced by the transcription factor activator protein-1 (AP-1), which is a heterodimer of Fos and Jun oncoproteins. AP-1, activated by phorbol esters or tumour necrosis factor- α (TNF α), forms a protein-protein complex with activated GR, and this prevents GR interacting with DNA and thereby reduces corticosteroid responsiveness [12]. In human lung TNF α and phorbol esters increase AP-1 binding to DNA and this is inhibited by glucocorticoids [13, 14]. GR also interacts with other transcription factors that are activated by inflammatory signals, including nuclear factor- κ B (NF- κ B) in a similar manner [13–17] (Fig. 3). There is also evidence that β_2 -agonists, via cyclic AMP formation and activation of protein kinase A, result in the activation of the transcription factor CREB that binds to a cyclic AMP responsive element (CRE) on genes. A direct interaction between CREB and GR has been demonstrated [18]. These interactions between activated GR and transcription factors occur within the nucleus, but recent observations suggest that these protein-protein interactions may also occur in the cytoplasm [19].

Effects on chromatin structure

There has recently been increasing evidence that glucocorticoids may have effects on the chromatin structure. DNA in chromosomes is wound around histone molecules in the form of nucleosomes. Several transcription factors interact with large co-activator molecules, such as CREB binding protein (CBP) and the related p300, which bind to the basal transcription factor apparatus [20]. Several transcription factors have now been shown to bind directly to CBP, including AP-1, NF- κ B and STATs [21]. Since binding sites on this molecule may be limited, this may result in competition between transcription factors for the limited binding sites available, so that there is an indirect rather than a direct protein-protein interaction (Fig. 4). CBP also interacts with nuclear hormone receptors, such as GR. These nuclear hormone

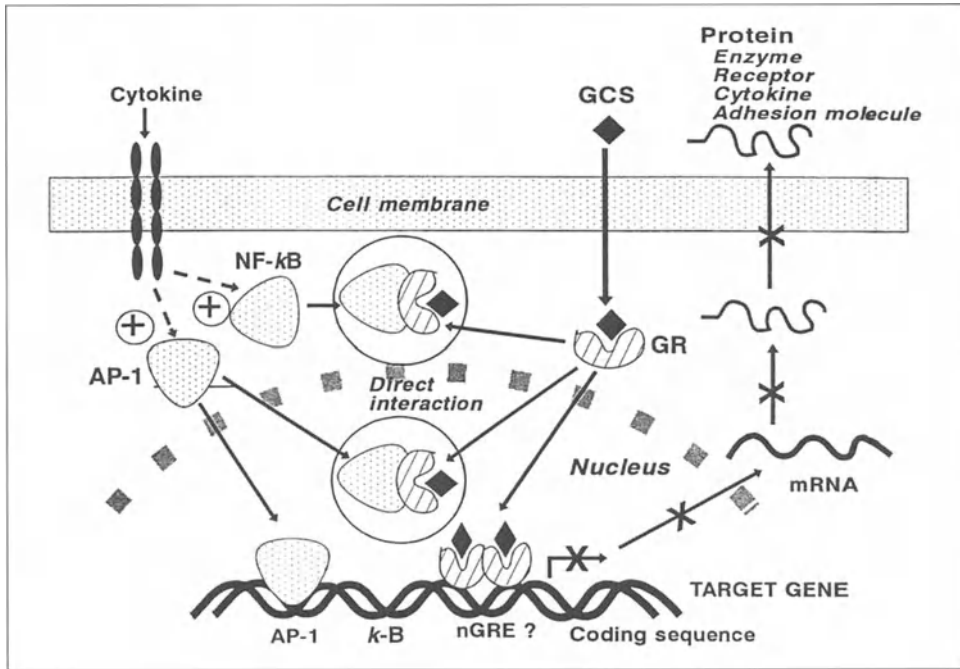


Figure 3

Direct interaction between the transcription factors activator protein-1 (AP-1) and nuclear factor-kappa B (NF-κB) and the glucocorticoid receptor (GR) may result in mutual repression. In this way corticosteroids may counteract the chronic inflammatory effects of cytokines that activate these transcription factors.

receptors may interact with CBP and the basal transcriptional apparatus through binding to other nuclear coactivator proteins, including corticosteroid receptor coactivator-1 (SRC-1) [22, 23], transcription factor intermediary factor-2 (TIF2) or glucocorticoid receptor interacting protein-1 [24]. DNA is wound around histone proteins to form nucleosomes and the chromatin fibre in chromosomes. At a microscopic level that chromatin may become dense or opaque due to the winding or unwinding of DNA around the histone core. CBP and p300 have histone acetylation activity which is activated by the binding of transcription factors, such as AP-1 and NF-κB [25]. Acetylation of histone residues results in unwinding of DNA coiled around the histone core, thus opening up the chromatin structure, which allows transcription factors to bind more readily, thereby increasing transcription (Fig. 4). Repression of genes reverses this process by histone deacetylation [26]. The process of deacetylation involves the binding of hormone or vitamin receptors to co-repressor molecules, such as nuclear receptor co-repressor (N-CoR) which forms a

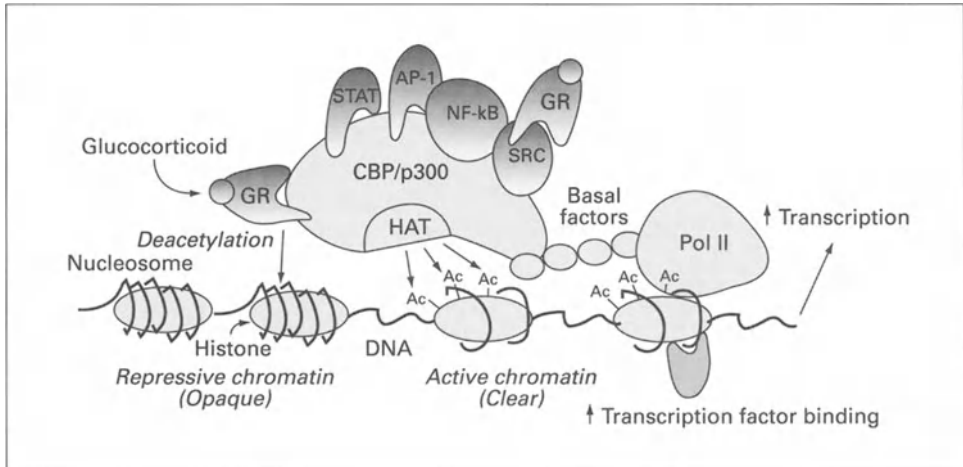


Figure 4

Effect of glucocorticoids on chromatin structure. Transcription factors, such as STATs, AP-1 and NF- κ B bind to co-activator molecules, such as CREB binding protein (CBP) or p300, which have intrinsic histone acetyltransferase (HAT) activity, resulting in acetylation (-Ac) of histone residues. This leads to unwinding of DNA and this allows increased binding of transcription factors resulting in increased gene transcription. Glucocorticoid receptors (GR) after activation by glucocorticoids bind to a glucocorticoid receptor co-activator which is bound to CBP. This results in deacetylation of histone, with increased coiling of DNA around histone, thus preventing transcription factor binding leading to gene repression.

complex with another repressor molecule Sin3 and a histone deacetylase [27, 28]. Deacetylation of histone increases the winding of DNA round histone residues, resulting in dense chromatin structure and reduced access of transcription factors to their binding sites, thereby leading to repressed transcription of inflammatory genes. Activated GR may bind to several transcription co-repressor molecules that associate with proteins that have histone deacetylase activity, resulting in deacetylation of histone, increased winding of DNA round histone residues and thus reduced access of transcription factors to their binding sites and therefore repression of inflammatory genes [26] (Fig. 4).

Target genes in inflammation control

Glucocorticoids may control inflammation by inhibiting many aspects of the inflammatory process through increasing the transcription of anti-inflammatory genes and decreasing the transcription of inflammatory genes [2, 4] (Tab. 1).

Table 1 - Effect of corticosteroids on gene transcription

Increased transcription

Lipocortin-1 (phospholipase A₂ inhibitor)
 β_2 -Adrenoceptor
 Secretory leukocyte inhibitory protein
 Clara cell protein (CC10)
 IL-1 receptor antagonist
 IL-1R2 (decoy receptor)
 I κ B- α (inhibitor of NF- κ B)

Decreased transcription

Cytokines (IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, IL-12, IL-13, TNF α , GM-CSF, SCF)
 Chemokines (IL-8, RANTES, MIP-1 α , MCP-1, MCP-3, MCP-4, eotaxin)
 Inducible nitric oxide synthase (iNOS)
 Inducible cyclooxygenase (COX-2)
 Cytoplasmic phospholipase A₂ (cPLA₂)
 Endothelin-1
 NK₁-receptors, NK₂-receptors
 Adhesion molecules (ICAM-1, E-selectin)

Antiinflammatory proteins

Glucocorticoids may suppress inflammation by increasing the synthesis of anti-inflammatory proteins. Corticosteroids increase the synthesis of lipocortin-1, a 37 kDa protein that has an inhibitory effect on phospholipase A₂ (PLA₂), and therefore may inhibit the production of lipid mediators. Corticosteroids induce the formation of lipocortin-1 in several cells and recombinant lipocortin-1 has acute anti-inflammatory properties [29]. However, glucocorticoids do not induce lipocortin-1 expression in all cells and this may be only one of many genes regulated by glucocorticoids. Glucocorticoids also increase the synthesis of secretory leukocyte protease inhibitor (SLPI) in human airway epithelial cells by increasing gene transcription [30]. SLPI is the predominant antiprotease in conducting airways and may be important in reducing airway inflammation by counteracting inflammatory enzymes, such as tryptase.

Interleukin (IL)-1 receptor antagonist (IL-1ra) is a cytokine that blocks the binding of IL-1 to its receptors. Its synthesis is increased by glucocorticoids, thus counteracting the effect of the proinflammatory cytokine IL-1. Thus, treatment of asth-

matic patients with inhaled glucocorticoids results in an increased expression of IL-1ra in airway epithelial cells *in vitro* and *in vivo* [31, 32]. IL-1 interacts with two types of surface receptor, designated IL-1R1 and IL-1R2. The inflammatory effects of IL-1 β are mediated exclusively via IL-1R1, whereas IL-1R2 has no signalling activity, but binds IL-1 and therefore acts as a “molecular decoy” that interferes with the actions of IL-1. Glucocorticoids are potent inducers of this decoy IL-1 receptor and result in release of a soluble form of the receptor, thus reducing the functional activity of IL-1 [33].

IL-10 is another antiinflammatory cytokine secreted predominantly by macrophages in the lung which inhibits the transcription of many proinflammatory cytokines and chemokines and this appears to be mediated via an inhibitory effect on NF- κ B [34]. IL-10 secretion by alveolar macrophages may be impaired in asthmatic patients, resulting in increased macrophage cytokine secretion [35, 36]. Glucocorticoid treatment in asthmatic patients increases IL-10 secretion by these cells, although this appears to be an indirect effect, since treatment of alveolar macrophages *in vitro* with glucocorticoids tends to decrease IL-10 secretion [36].

NF- κ B is regulated by the inhibitory protein I κ B to which it is bound in the cytoplasm [37]. There is some evidence that glucocorticoids increase the synthesis and transcription of the predominant form of I κ B, I κ B- α , in mononuclear cells and T lymphocytes, thus terminating the activation of NF- κ B [38, 39], but this has not been seen in other cell types [40–42]. The I κ B- α gene does not appear to have any GRE consensus sequence, so any effect of glucocorticoids is probably mediated via other transcription factors.

In epithelial cells glucocorticoids also increase the expression of the enzyme neutral endopeptidase (NEP), which degrades inflammatory peptides such as substance P, bradykinin and endothelin-1 [43]. Asthmatic patients treated with inhaled glucocorticoids have a higher level of NEP expression than untreated patients [44].

β_2 -Adrenoceptors

Corticosteroids increase the expression of β_2 -adrenoceptors by increasing the rate of transcription and the human β_2 -receptor gene has three potential GREs [45]. Corticosteroids double the rate of β_2 -receptor gene transcription in human lung *in vitro*, resulting in increased expression of β_2 -receptors [46]. Using autoradiographic mapping and *in situ* hybridisation in animals to localise the increase in β_2 -receptor expression, there appears to be an increase in all cell types, including airway epithelial cells and airway smooth muscle, after chronic glucocorticoid treatment [47]. This may be relevant in asthma as it may prevent down-regulation in response to prolonged treatment with β_2 -agonists. In rats glucocorticoids prevent the down-regulation and reduced transcription of β_2 -receptors in response to chronic β -agonist exposure [47].

Cytokines

Although it is not yet possible to be certain of the most critical aspects of corticosteroid action in asthma, it is likely that their inhibitory effects on cytokine synthesis are of particular relevance. Corticosteroids inhibit the transcription of several cytokines that are relevant in asthma, including IL-1 β , TNF α , granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2, IL-3, IL-4, IL-5, IL-6, IL-11 and the chemokines IL-8, RANTES, macrophage chemotactic protein (MCP)-1, MCP-3, MCP-4, macrophage inflammatory protein (MIP)-1 α and eotaxin. These inhibitory effects were at one time thought to be mediated directly via interaction of GR with a nGRE in the upstream promoter sequence of the cytokine gene, resulting in reduced gene transcription. Surprisingly, there is no apparent nGRE consensus sequence in the upstream promoter region of these cytokines, suggesting that glucocorticoids inhibit transcription indirectly. Thus, the 5'-promoter sequence of the human IL-2 gene has no GRE consensus sequences, yet glucocorticoids are potent inhibitors of IL-2 gene transcription in T-lymphocytes. Transcription of the IL-2 gene is predominantly regulated by a cell-specific transcription factor nuclear factor of activated T-cells (NF-AT), which is activated in the cytoplasm on T-cell receptor stimulation via calcineurin. A nuclear factor is also necessary for increased activation and this factor appears to be AP-1, which binds directly to NF-AT to form a transcriptional complex [48]. Glucocorticoids therefore inhibit IL-2 gene transcription indirectly by binding to AP-1, thus preventing increased transcription due to NF-AT [49]. There may be marked differences in the response of different cells and of different cytokines to the inhibitory action of glucocorticoids and this may be dependent on the relative abundance of transcription factors. Thus in alveolar macrophages and peripheral blood monocytes GM-CSF secretion is more potently inhibited by glucocorticoids than IL-1 β or IL-6 secretion [50].

Inflammatory enzymes

Nitric oxide (NO) synthase may be induced by proinflammatory cytokines, resulting in increased NO production. NO may amplify asthmatic inflammation and contribute to epithelial shedding and airway hyperresponsiveness through the formation of peroxynitrite. The induction of the inducible form of NOS (iNOS) is potently inhibited by glucocorticoids. In cultured human pulmonary epithelial cells pro-inflammatory cytokines result in increased expression of iNOS and increased NO formation, due to increased transcription of the iNOS gene, and this is inhibited by glucocorticoids [51]. There is no nGRE in the promoter sequence of the iNOS gene, but NF- κ B appears to be an important transcription factor in regulating iNOS gene transcription [52]. Glucocorticoids may therefore prevent induction of iNOS by inactivating NF- κ B, thereby inhibiting transcription.

Glucocorticoids inhibit the synthesis of several inflammatory mediators implicated in asthma through an inhibitory effect on enzyme induction. Glucocorticoids

inhibit the induction of the gene coding for inducible cyclooxygenase (COX-2) in monocytes and epithelial cells and this also appears to be via NF- κ B activation [53-55]. Glucocorticoids also inhibit the gene transcription of a form of PLA₂ (cPLA₂) induced by cytokines [56].

Corticosteroids also inhibit the synthesis of endothelin-1 in lung and airway epithelial cells and this effect may also be via inhibition of transcription factors that regulate its expression [57].

Inflammatory receptors

Glucocorticoids also decrease the transcription of genes coding for certain receptors. Thus the NK1-receptor which mediates the inflammatory effects of substance P in the airways may show increased gene expression in asthma [58]. This may be inhibited by corticosteroids through an interaction with AP-1 as the NK₁ receptor gene promoter region has no GRE, but has an AP-1 response element [59]. Glucocorticoids also inhibit the transcription of the NK₂-receptor which mediates the bronchoconstrictor effects of tachykinins [60].

Apoptosis

Corticosteroids markedly reduce the survival of certain inflammatory cells, such as eosinophils. Eosinophil survival is dependent on the presence of certain cytokines, such as IL-5 and GM-CSF. Exposure to corticosteroids blocks the effects of these cytokines and leads to programmed cell death or apoptosis [61].

Adhesion molecules

Adhesion molecules play a key role in the trafficking of inflammatory cells to sites of inflammation. The expression of many adhesion molecules on endothelial cells is induced by cytokines and corticosteroids may lead indirectly to a reduced expression via their inhibitory effects on cytokines, such as IL-1 β and TNF α . Corticosteroids may also have a direct inhibitory effect on the expression of adhesion molecules, such as ICAM-1 and E-selectin at the level of gene transcription [62]. ICAM-1 expression in bronchial epithelial cell lines and monocytes is inhibited by glucocorticoids [63].

Effects on cell function

Corticosteroids may have direct inhibitory actions on several inflammatory cells implicated in pulmonary and airway diseases (Fig. 5).

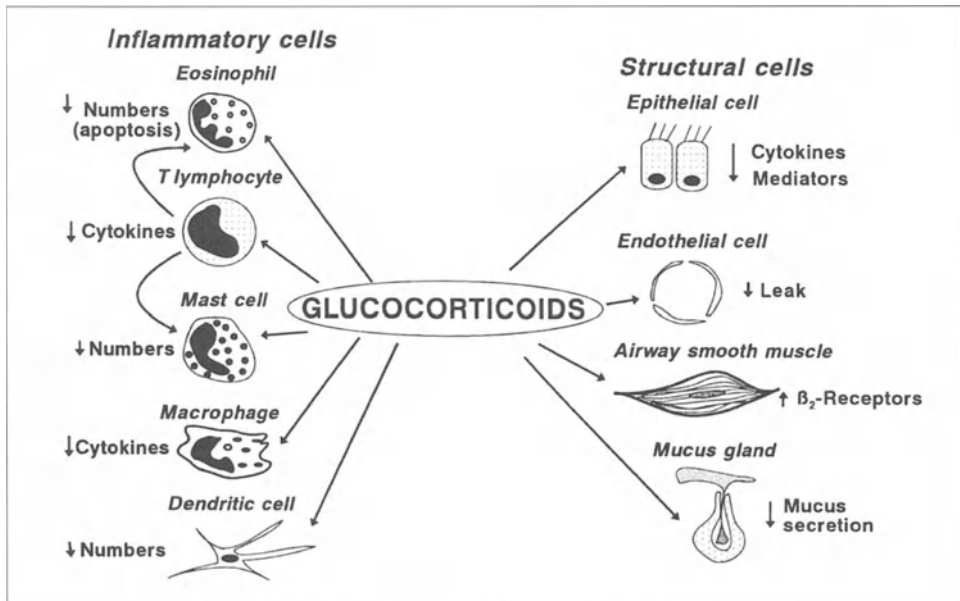


Figure 5
Cellular effect of glucocorticoids.

Macrophages

Corticosteroids inhibit the release of inflammatory mediators and cytokines from alveolar macrophages *in vitro* [50], although their effect after inhalation *in vivo* is modest [64]. Corticosteroids may be more effective in inhibiting cytokine release from alveolar macrophages than in inhibition of lipid mediators and reactive oxygen species *in vitro* [65]. Inhaled corticosteroids reduce the secretion of chemokines and proinflammatory cytokines from alveolar macrophages from asthmatic patients, whereas the secretion of IL-10 is increased [36]. Oral prednisone inhibits the increased gene expression of IL-1 β in alveolar macrophages obtained by bronchoalveolar lavage from asthmatic patients [66].

Eosinophils

Corticosteroids have a direct inhibitory effect on mediator release from eosinophils, although they are only weakly effective in inhibiting secretion of reactive oxygen species and eosinophil basic proteins [67]. Corticosteroids inhibit the permissive action of cytokines such as GM-CSF and IL-5 on eosinophil survival [68, 69] and this contributes to the reduction in airway eosinophils seen with corticosteroid ther-

apy. One of the best described actions of corticosteroids in asthma is a reduction in circulating eosinophils, which may reflect an action on eosinophil production in the bone marrow. Inhaled corticosteroids inhibit the increase in circulating eosinophil count at night in patients with nocturnal asthma and also reduce plasma concentrations of eosinophil cationic protein [70]. After inhaled corticosteroids (budesonide 800 µg b.i.d.) there is a marked reduction in the number of low-density eosinophils, presumably reflecting inhibition of cytokine production in the airways [71].

T Lymphocytes

An important target cell in asthma may be the T lymphocyte, since corticosteroids are very effective in inhibition of activation of these cells and in blocking the release of cytokines which are likely to play an important role in the recruitment and survival of inflammatory cells involved in asthmatic inflammation. Thus glucocorticoids potentially inhibit the secretion of IL-5 from T lymphocytes [72].

Mast cells

While corticosteroids do not appear to have a direct inhibitory effect on mediator release from lung mast cells [73], chronic corticosteroid treatment is associated with a marked reduction in mucosal mast cell number [74, 75]. This may be linked to a reduction in IL-5 and stem cell factor (SCF) production, which are necessary for mast cell expression at mucosal surfaces. Mast cells also secrete various cytokines (TNF α , IL-4, IL-5, IL-6, IL-8), but whether this is inhibited by corticosteroids has not yet been reported.

Dendritic cells

Dendritic cells in the epithelium of the respiratory tract appear to play a critical role in antigen presentation in the lung as they have the capacity to take up allergen, process it into peptides and present it via MHC molecules on the cell surface for presentation to uncommitted T lymphocytes [76]. In experimental animals the number of dendritic cells is markedly reduced by systemic and inhaled corticosteroids, thus dampening the immune response in the airways [77]. Topical corticosteroids markedly reduce the numbers of dendritic cell in the nasal mucosa [78], and it is likely that a similar effect would be seen in airways.

Neutrophils

Neutrophils, which are not prominent in the bronchial biopsies of asthmatic patients, are not very sensitive to the effects of corticosteroids. Indeed, systemic cor-

corticosteroids increase peripheral neutrophil counts which may reflect an increased survival time due to an inhibitory action of neutrophil apoptosis (in complete contrast to the increased apoptosis seen in eosinophils) [79].

Endothelial cells

GR gene expression in the airways is most prominent in endothelial cells of the bronchial circulation and airway epithelial cells. Corticosteroids do not appear to directly inhibit the expression of adhesion molecules, although they may inhibit cell adhesion indirectly by suppression of cytokines involved in the regulation of adhesion molecule expression. Corticosteroids may have an inhibitory action on airway microvascular leak induced by inflammatory mediators [80, 81]. This appears to be a direct effect on postcapillary venular epithelial cells. The mechanism for this antipermeability effect has not been fully elucidated, but there is evidence that synthesis of a 100 kDa protein distinct from lipocortin-1 termed vasocortin may be involved [82]. Although there have been no direct measurements of the effects of corticosteroids on airway microvascular leakage in asthmatic airways, regular treatment with inhaled corticosteroids decreases the elevated plasma proteins found in bronchoalveolar lavage fluid of patients with stable asthma [83].

Epithelial cells

Epithelial cells may be an important source of inflammatory mediators in asthmatic airways and may drive and amplify the inflammatory response in the airways [84, 85]. Airway epithelium may be one of the most important targets for inhaled glucocorticoids in asthma [3, 86]. Corticosteroids inhibit the increased transcription of the IL-8 gene induced by TNF α in cultured human airway epithelial cells *in vitro* [87, 88] and the transcription of the RANTES gene in an epithelial cell line [89]. Inhaled corticosteroids inhibit the increased expression of GM-CSF and RANTES in the epithelium of asthmatic patients [84, 90, 91]. There is increased expression of iNOS in the airway epithelium of patients with asthma [92] and this may account for the increase in NO in the exhaled air of patients with asthma compared with normal subjects [93]. Asthmatic patients who are taking regular inhaled corticosteroid therapy, however do not show such an increase in exhaled NO [93], suggesting that glucocorticoids have suppressed epithelial iNOS expression. Furthermore double-blind randomised studies show that oral and inhaled glucocorticoids reduce the elevated exhaled NO in asthmatic patients to normal values [94, 95]. Glucocorticoids also decrease the transcription of other inflammatory proteins in airway epithelial cells, including COX-2, cPLA₂ and endothelin-1 [53, 56, 57]. Airway epithelial cells may be the key cellular target of inhaled corticosteroids; by inhibiting the transcription of several inflammatory genes inhaled corticosteroids may reduce inflammation in the airway wall (Fig. 6).

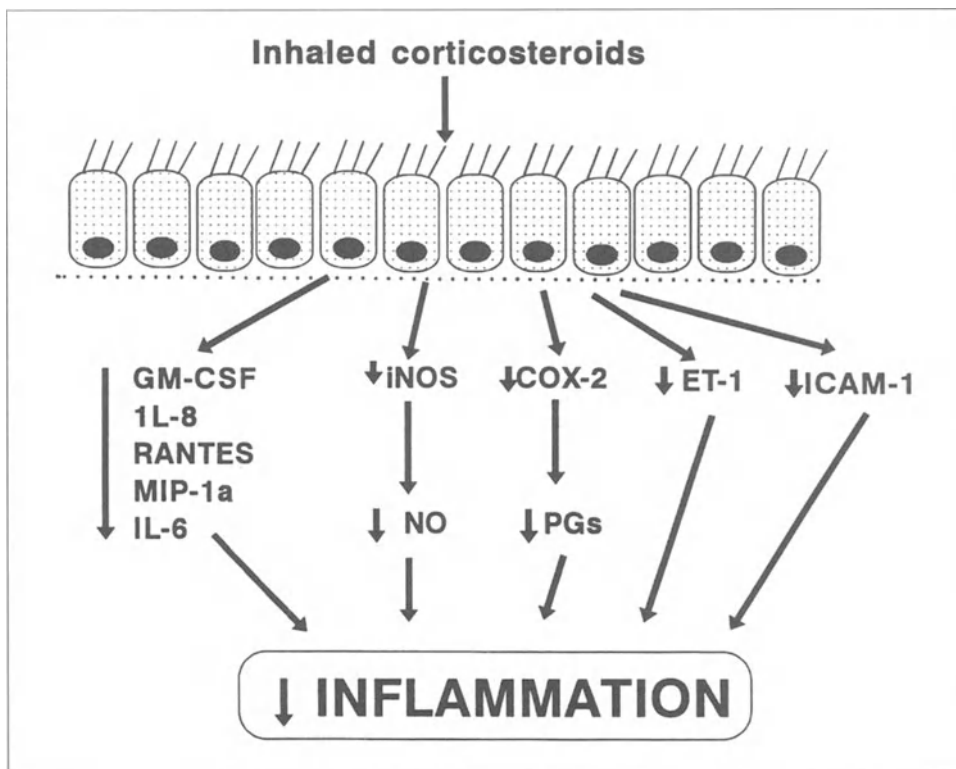


Figure 6
Inhaled corticosteroids may inhibit the transcription of several "inflammatory" genes in airway epithelial cells and thus reduce inflammation in the airway wall.

Mucus secretion

Corticosteroids inhibit mucus secretion in airways and this may be a direct action of corticosteroids on submucosal gland cells [96]. Recent studies suggest that corticosteroids may also inhibit the expression of mucin genes, such as MUC2 and MUC5AC [97]. In addition, there are indirect inhibitory effects due to the reduction in inflammatory mediators that stimulate increased mucus secretion.

Effects on asthmatic inflammation

Glucocorticoids are remarkably effective in controlling the inflammation in asthmatic airways and it is likely that they have multiple cellular effects. Biopsy studies in patients with asthma have now confirmed that inhaled corticosteroids reduce the

number and activation of inflammatory cells in the airway [74, 75, 91, 98, 99]. Similar results have been reported in bronchoalveolar lavage of asthmatic patients, with a reduction in both eosinophil number and eosinophil cationic protein concentrations, a marker of eosinophil degranulation, after inhaled budesonide [100]. These effects may be due to inhibition of cytokine synthesis in inflammatory and structural cells. There is also a reduction in activated CD4⁺ T-cells (CD4⁺/CD25⁺) in bronchoalveolar lavage fluid after inhaled glucocorticoids [101]. The disrupted epithelium is restored and the ciliated to goblet cell ratio is normalised after three months of therapy with inhaled corticosteroids [74]. There is also some evidence for a reduction in the thickness of the basement membrane [91], although in asthmatic patients taking inhaled corticosteroids for over 10 years the characteristic thickening of the basement membrane was still present [102].

Effects on airway hyperresponsiveness

By reducing airway inflammation inhaled corticosteroids consistently reduce airway hyperresponsiveness (AHR) in asthmatic adults and children [103]. Chronic treatment with inhaled corticosteroids reduces responsiveness to histamine, cholinergic agonists, allergen (early and late responses), exercise, fog, cold air, bradykinin, adenosine and irritants (such as sulphur dioxide and metabisulphite). The reduction in AHR takes place over several weeks and may not be maximal until after several months of therapy. The magnitude of reduction is variable between patients and is in the order of one to two doubling dilutions for most challenges and often fails to return to the normal range. This may reflect suppression of the inflammation but persistence of structural changes which cannot be reversed by corticosteroids. Inhaled corticosteroids not only make the airways less sensitive to spasmogens, but they also limit the maximal airway narrowing in response to spasmogens [104].

Clinical efficacy of inhaled corticosteroids

Inhaled corticosteroids are very effective in controlling asthma symptoms in asthmatic patients of all ages and severity [1, 105].

Studies in adults

Inhaled corticosteroids were first introduced to reduce the requirement for oral corticosteroids in patients with severe asthma and many studies have confirmed that the majority of patients can be weaned off oral corticosteroids [106]. As experience has been gained with inhaled corticosteroids they have been introduced in patients

with milder asthma, with the recognition that inflammation is present even in patients with mild asthma [107]. Inhaled anti-inflammatory drugs have now become first-line therapy in any patient who needs to use a β_2 -agonist inhaler more than once a day, and this is reflected in national and international guidelines for the management of chronic asthma [108–110]. In patients with newly-diagnosed asthma inhaled corticosteroids (budesonide 600 μg twice daily) reduced symptoms and β_2 -agonist inhaler usage and improved peak expiratory flows. These effects persisted over the two years of the study, whereas in a parallel group treated with inhaled β_2 -agonists alone there was no significant change in symptoms or lung function [111]. In another study patients with mild asthma treated with a low dose of inhaled corticosteroid (budesonide 200 μg b.d.) showed fewer symptoms and a progressive improvement in lung function over several months and many patients became completely asymptomatic [112]. Similarly inhaled beclomethasone dipropionate (BDP, 400 μg b.d.) improved asthma symptoms and lung function and this was maintained over the 2.5 years of the study [113]. There was also a significant reduction in the number of exacerbations. Although the effects of inhaled corticosteroids on AHR may take several months to reach a plateau, the reduction in asthma symptoms occurs more rapidly [114].

High dose inhaled corticosteroids have now been introduced in many countries for the control of more severe asthma. This markedly reduces the need for maintenance oral corticosteroids and has revolutionised the management of more severe and unstable asthma [115–117]. Inhaled corticosteroids are the treatment of choice in nocturnal asthma, which is a manifestation of inflamed airways, reducing night time awakening and reducing the diurnal variation in airway function [118, 119].

Inhaled corticosteroids effectively control asthmatic inflammation but must be taken regularly. When inhaled corticosteroids are discontinued there is usually a gradual increase in symptoms and airway responsiveness back to pretreatment values [114], although in patients with mild asthma who have been treated with inhaled corticosteroids for a long time symptoms may not recur in some patients [120].

Studies in children

Inhaled corticosteroids are equally effective in children. In an extensive study of children aged 7–17 years there was a significant improvement in symptoms, peak flow variability and lung function compared to a regular inhaled β_2 -agonist which was maintained over the 22 months of the study [121], but asthma deteriorated when the inhaled corticosteroids were withdrawn [122]. There was a high proportion of drop-outs (45%) in the group treated with inhaled β_2 -agonist alone. Inhaled corticosteroids are also effective in younger children. Nebulised budesonide reduced the need for oral corticosteroids and also improved lung function in children under the

age of three [123]. Inhaled corticosteroids given via a large volume spacer improved asthma symptoms and reduced the number of exacerbations in preschool children and in infants [124, 125].

Prevention of irreversible changes

Some patients with asthma develop an element of irreversible airflow obstruction, but the pathophysiological basis of this is not yet understood. It is likely that it is the result of chronic airway inflammation and that it may be prevented by treatment with inhaled corticosteroids. There is some evidence that the annual decline in lung function may be slowed by the introduction of inhaled corticosteroids [126]. Delay in starting inhaled corticosteroids may result in less overall improvement in lung function in both adults and children [127–129].

Pharmacokinetics

The pharmacokinetics of inhaled corticosteroids are important in determining the concentration of drug reaching target cells in the airways and in the fraction of drug reaching the systemic circulation and therefore causing side-effects [1, 130, 131]. Beneficial properties in an inhaled corticosteroid are a high topical potency, a low systemic bioavailability of the swallowed portion of the dose and rapid metabolic clearance of any corticosteroid reaching the systemic circulation. After inhalation a large proportion of the inhaled dose (80–90%) is deposited on the oropharynx and is then swallowed and therefore available for absorption via the liver into the systemic circulation (Fig. 7). This fraction is markedly reduced by using a large volume spacer device with a metered dose inhaler (MDI) or by mouth washing and discarding the washing with dry powder inhalers. Between 10 and 20% of inhaled drug enters the respiratory tract, where it is deposited in the airways and this fraction is available for absorption into the systemic circulation. Most of the early studies on the distribution of inhaled corticosteroids were conducted in healthy volunteers, and it is not certain what effect inflammatory disease, airway obstruction, age of the patient or concomitant medication may have on the disposition of the inhaled dose. There may be important differences in the metabolism of different inhaled corticosteroids. BDP is metabolised to its more active metabolite beclomethasone monopropionate in many tissues including lung, but there is no information about the absorption or metabolism of this metabolite in humans. Flunisolide and budesonide are subject to extensive first-pass metabolism in the liver so that less reaches the systemic circulation [132, 133]. Little is known about the distribution of triamcinolone [134]. FP is almost completely metabolised by first-pass metabolism, which reduces systemic effects [135].

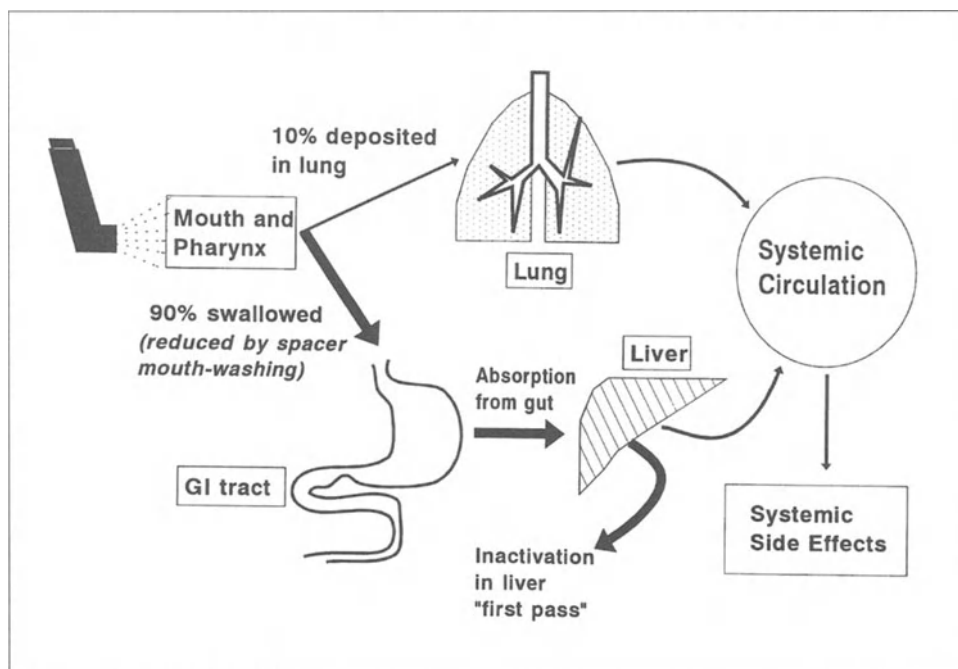


Figure 7
Pharmacokinetics of inhaled corticosteroids.

When inhaled corticosteroids were first introduced it was recommended that they should be given four times daily, but several studies have now demonstrated that twice daily administration gives comparable control [136, 137], although four times daily administration may be preferable in patients with more severe asthma [138]. However, patients may find it difficult to comply with such frequent administration unless they have troublesome symptoms. For patients with mild asthma who require $\leq 400 \mu\text{g}$ daily, once daily therapy may be sufficient [139].

Side-effects of inhaled corticosteroids

The efficacy of inhaled corticosteroids is now established in short- and long-term studies in adults and children, but there are still concerns about side-effects, particularly in children and when high inhaled doses are needed. Several side-effects have been recognised.

Local side-effects

Side effects due to the local deposition of the inhaled corticosteroid in the oropharynx may occur with inhaled corticosteroids, but the frequency of complaints depends on the dose and frequency of administration and on the delivery system used.

Dysphonia

The commonest complaint is of hoarseness of the voice (dysphonia) and may occur in over 50% of patients using MDI [140, 141]. Dysphonia is not appreciably reduced by using spacers, but may be less with dry powder devices [142]. Dysphonia may be due to myopathy of laryngeal muscles and is reversible when treatment is withdrawn [141]. For most patients it is not troublesome but may be disabling in singers and lecturers.

Oropharyngeal candidiasis

Oropharyngeal candidiasis (thrush) may be a problem in some patients, particularly in the elderly, with concomitant oral corticosteroids and more than twice daily administration [140]. Large volume spacer devices protect against this local side-effect by reducing the dose of inhaled corticosteroid that deposits in the oropharynx.

Other local complications

There is no evidence that inhaled corticosteroids, even in high doses, increase the frequency of infections, including tuberculosis, in the lower respiratory tract [143, 144]. There is no evidence for atrophy of the airway epithelium and even after 10 years of treatment with inhaled corticosteroids there is no evidence for any structural changes in the epithelium [102]. Cough and throat irritation, sometimes accompanied by reflex bronchoconstriction, may occur when inhaled corticosteroids are given via a metered dose inhaler. These symptoms are likely to be due to surfactants in pressurised aerosols as they disappear after switching to a dry powder corticosteroid inhaler device [145].

Systemic side-effects

The efficacy of inhaled corticosteroids in the control of asthma is undisputed, but there are concerns about systemic effects of inhaled corticosteroids, particularly as they are likely to be used over long periods and in children of all ages [105]. The

safety of inhaled corticosteroids has been extensively investigated since their introduction 30 years ago [146]. One of the major problems is to decide whether a measurable systemic effect has any significant clinical consequence, and this necessitates careful long-term follow-up studies. As biochemical markers of systemic corticosteroid effects become more sensitive, then systemic effects may be seen more often, but this does not mean that these effects are clinically relevant. There are several case reports of adverse systemic effects of inhaled corticosteroids, and these are often idiosyncratic reactions, which may be due to abnormal pharmacokinetic handling of the inhaled corticosteroid. The systemic effect of an inhaled corticosteroid will depend on several factors, including the dose delivered to the patient, the site of delivery (gastrointestinal tract and lung), the delivery system used and individual differences in the patient's response to the corticosteroid.

Effect of delivery systems

The systemic effect of an inhaled corticosteroid is dependent on the amount of drug absorbed into the systemic circulation. As noted above, approximately 90% of the inhaled dose from an MDI deposits in the oropharynx and is swallowed and subsequently absorbed from the gastrointestinal tract. Use of a large volume spacer device markedly reduces the oropharyngeal deposition, and therefore the systemic effects of inhaled corticosteroids [147]. For dry powder inhalers similar reductions in systemic effects may be achieved with mouth-washing and discarding the fluid. All patients using a daily dose of $\geq 800 \mu\text{g}$ of an inhaled corticosteroid should therefore use either a spacer or mouth washing to reduce systemic absorption. Approximately 10% of an MDI enters the lung and this fraction (which presumably exerts the therapeutic effect) may be absorbed into the systemic circulation. As the fraction of inhaled corticosteroid deposited in the oropharynx is reduced, the proportion of the inhaled dose entering the lungs is increased. More efficient delivery to the lungs is therefore accompanied by increased systemic absorption, but this is offset by a reduction in the dose needed for optimal control of airway inflammation. For example, a multiple dry powder delivery system, the Turbohaler, delivers approximately twice as much corticosteroid to the lungs as other devices, and therefore has increased systemic effects. However this is compensated for by the fact that only half the dose is required [148].

Hypothalamic-pituitary-adrenal axis

Glucocorticoids may cause hypothalamic-pituitary-adrenal (HPA) axis suppression by reducing corticotrophin (ACTH) production, which reduces cortisol secretion by the adrenal gland. The degree of HPA suppression is dependent on dose, duration, frequency and timing of corticosteroid administration. The clinical significance of HPA axis suppression is two-fold. Firstly, prolonged adrenal suppression may lead

to reduced adrenal response to stress. There is no evidence that cortisol responses to the stress of an asthma exacerbation or insulin-induced hypoglycaemia are impaired, even with high doses of inhaled corticosteroids [149]. Secondly, measurement of HPA axis function provides evidence for systemic effects of an inhaled corticosteroid. Basal adrenal cortisol secretion may be measured by a morning plasma cortisol, 24 h urinary cortisol or by plasma cortisol profile over 24 h [150]. Other tests measure the HPA response following stimulation with tetracosactrin (which measures adrenal reserve) or stimulation with metyrapone and insulin (which measure the response to stress).

There are many studies of HPA axis function in asthmatic patients with inhaled corticosteroids, but the results are inconsistent as they have often been uncontrolled and patients have also been taking courses of oral corticosteroids (which may affect the HPA axis for weeks) [151]. BDP, budesonide and FP at high doses by conventional MDI (> 1600 µg daily) give a dose-related decrease in morning serum cortisol levels and 24 h urinary cortisol, although values still lie well within the normal range [152–154]. However, when a large volume spacer is used doses of 2000 µg daily of BDP or budesonide have little effect on 24 h urinary cortisol excretion [155]. Studies with inhaled flunisolide and triamcinolone in children show no effect on 24 h cortisol excretion at doses of up to 1000 µg daily [156, 157]. Stimulation tests of HPA axis function similarly show no consistent effects of doses of 1500 µg or less of inhaled corticosteroid. At high doses (> 1500 µg daily) budesonide and FP have less effect than BDP on HPA axis function [153, 158]. In children no suppression of urinary cortisol is seen with doses of BDP of 800 µg or less [159–161]. In studies where plasma cortisol has been measured at frequent intervals there was a significant reduction in cortisol peaks with doses of inhaled BDP as low as 400 µg daily [162], although this does not appear to be dose-related in the range 400–1000 µg [163, 164]. The clinical significance of these effects is not certain, however.

Overall, the studies which are not confounded by concomitant treatment with oral corticosteroids, have consistently shown that there are no significant suppressive effects on HPA axis function at doses of ≤ 1500 µg in adults and ≤ 400 µg in children.

Effects on bone metabolism

Corticosteroids lead to a reduction in bone mass by direct effects on bone formation and resorption and indirectly by suppression of the pituitary-gonadal and HPA axes, effects on intestinal calcium absorption, renal tubular calcium reabsorption and secondary hyperparathyroidism [165]. The effects of oral corticosteroids on osteoporosis and increased risk of vertebral and rib fractures are well known, but there are no reports suggesting that long-term treatment with inhaled corticosteroids is associated with an increased risk of fractures. Bone densitometry has been used to

assess the effect of inhaled corticosteroids on bone mass. Although there is evidence that bone density is less in patients taking high-dose inhaled corticosteroids, interpretation is confounded by the fact that these patients are also taking intermittent courses of oral corticosteroids [166].

Changes in bone mass occur very slowly and several biochemical indices have been used to assess the short-term effects of inhaled corticosteroids on bone metabolism. Bone formation has been measured by plasma concentrations of bone-specific alkaline phosphatase, serum osteocalcin, a non-collagenous 49-amino acid peptide secreted by osteoblasts, or by procollagen peptides. Bone resorption may be assessed by urinary hydroxyproline after a 12-h fast, urinary calcium excretion and pyridinium cross-link excretion. It is important to consider the age, diet, time of day and physical activity of the patient in interpreting any abnormalities. It is also necessary to choose appropriate control groups as asthma itself may have an effect on some of the measurements, such as osteocalcin [167]. Inhaled corticosteroids, even at doses up to 2000 µg daily, have no significant effect on calcium excretion, but acute and reversible dose-related suppression of serum osteocalcin has been reported with BDP and budesonide when given by conventional MDI in several studies [151]. Budesonide consistently has less effect than BDP at equivalent doses and only BDP increases urinary hydroxyproline at high doses [168]. With a large volume spacer even doses of 2000 µg daily of either BDP or budesonide are without effect on plasma osteocalcin concentrations, however [155]. Urinary pyridinium and deoxypyridinoline cross-links, which are a more accurate and stable measurement of bone and collagen degradation, are not increased with inhaled corticosteroids (BDP > 1000 µg daily), even with intermittent courses of oral corticosteroids [166]. It is important to monitor changes in markers of bone formation as well as bone degradation, as the net effect on bone turnover is important.

There has been particular concern about the effect of inhaled corticosteroids on bone metabolism in growing children. A very low dose of oral corticosteroids (prednisolone 2.5 mg) causes significant changes in serum osteocalcin and urinary hydroxyproline excretion, whereas daily BDP and budesonide at doses up to 800 µg daily have no effect [167, 169]. It is important to recognise that the changes in biochemical indices of bone metabolism are less than those seen with even low doses of oral corticosteroids. This suggests that even high doses of inhaled corticosteroids, particularly when used with a spacer device, are unlikely to have any long-term effect on bone structure. Careful long-term follow-up studies in patients with asthma are needed.

There is no evidence that inhaled corticosteroids increase the frequency of fractures. Long-term treatment with high dose inhaled corticosteroids has not been associated with any consistent change in bone density [170, 171]. Indeed, in elderly patients there may be an increase in bone density due to increased mobility [171].

Effects on connective tissue

Oral and topical corticosteroids cause thinning of the skin, telangiectasiae and easy bruising, probably as a result of loss of extracellular ground substance within the dermis, due to an inhibitory effect on dermal fibroblasts. There are reports of increased skin bruising and purpura in patients using high doses of inhaled BDP, but the amount of intermittent oral corticosteroids in these patients is not known [172, 173]. Easy bruising in association with inhaled corticosteroids is more frequent in elderly patients [174] and there are no reports of this problem in children. Long-term prospective studies with objective measurements of skin thickness are needed with different inhaled corticosteroids.

Ocular effects

Long-term treatment with oral corticosteroids increases the risk of posterior subcapsular cataracts and there are several case reports describing cataracts in individual patients taking inhaled corticosteroids [146]. In a study of 48 patients who were exposed to oral and/or high dose inhaled corticosteroids the prevalence of posterior subcapsular cataracts (27%) correlated with the daily dose and duration of oral corticosteroids, but not with the dose and duration of inhaled corticosteroids [175]. In a recent cross-sectional study in patients aged 5–25 years taking either inhaled BDP or budesonide, no cataracts were found on slit-lamp examination, even in patients taking 2000 µg daily for over 10 years [176]. Recently there has been a report of a slight increase in the risk of glaucoma in patients taking very high doses of inhaled corticosteroids [177].

Growth

There has been particular concern that inhaled corticosteroids may cause stunting of growth and several studies have addressed this issue. Asthma itself (as with other chronic diseases) may have an effect on the growth pattern and has been associated with delayed onset of puberty and deceleration of growth velocity that is more pronounced with more severe disease [178]. However, asthmatic children appear to grow for longer, so that their final height is normal. The effect of asthma on growth makes it difficult to assess the effects of inhaled corticosteroids on growth in cross-sectional studies, particularly as courses of oral corticosteroids are a confounding factor. Longitudinal studies have demonstrated that there is no significant effect of inhaled corticosteroids on statural growth in doses of up to 800 µg daily and for up to 5 years of treatment [121, 151, 179, 180]. A prospective study of inhaled BDP (400 µg daily) versus theophylline in children with mild to moderate asthma showed no effect on height, although there was some reduction in growth velocity compared to children treated with theophylline [181]. However it is not possible to relate changes in growth velocity to final height as other

studies have demonstrated that there is a “catch up” period. In a longitudinal study in children aged 2–7 years with severe asthma, budesonide 200 µg daily had no effect on growth over 3–5 years [161]. In children with virally-induced wheezing BDP 400 µg daily has been reported to reduce growth compared with a placebo [182]. A meta-analysis of 21 studies, including over 800 children, showed no effect of inhaled BDP on statural height, even with higher doses and long duration of therapy [183].

Short-term growth measurements (knemometry) have demonstrated that even a low dose of an oral corticosteroid (prednisolone 2.5 mg) is sufficient to give complete suppression of lower leg growth. However inhaled budesonide up to 400 µg is without effect, although some suppression is seen with 800 µg and with 400 µg BDP [184, 185]. The relationship between knemometry measurements and final height are uncertain since low doses of oral corticosteroid that have no effect on final height cause profound suppression.

Metabolic effects

Several metabolic effects have been reported after inhaled corticosteroids, but there is no evidence that these are clinically relevant at therapeutic doses. In adults fasting glucose and insulin are unchanged after doses of BDP up to 2000 µg daily [186] and in children with inhaled budesonide up to 800 µg daily [187]. In normal individuals high dose inhaled BDP may slightly increase resistance to insulin [188]. However, in patients with poorly controlled asthma high doses of BDP and budesonide paradoxically decrease insulin resistance and improve glucose tolerance, suggesting that the disease itself may lead to abnormalities in carbohydrate metabolism [189]. Neither BDP 2000 µg daily in adults nor budesonide 800 µg daily in children have any effect on plasma cholesterol or triglycerides [186, 187].

Haematological effects

Inhaled corticosteroids may reduce the numbers of circulating eosinophils in asthmatic patients [71], possibly due to an effect on local cytokine generation in the airways. Inhaled corticosteroids may cause a small increase in circulating neutrophil counts [155, 190].

Central nervous system effects

There are various reports of psychiatric disturbance, including emotional lability, euphoria, depression, aggressiveness and insomnia, after inhaled corticosteroids. Only eight such patients have so far been reported, suggesting that this is very infrequent and a causal link with inhaled corticosteroids has usually not been established [151].

Table 2 - Side-effects of inhaled corticosteroids

Local side-effects	Systemic side-effects
Dysphonia	Adrenal suppression
Oropharyngeal candidiasis	Growth suppression
Cough	Bruising
	Osteoporosis
	Cataracts
	Glaucoma
	Metabolic abnormalities (glucose, insulin, triglycerides)
	Psychiatric disturbances

Safety in pregnancy

Based on extensive clinical experience, inhaled corticosteroids appear to be safe in pregnancy, although no controlled studies have been performed. There is no evidence for any adverse effects of inhaled corticosteroids on the pregnancy, the delivery or on the foetus [146]. It is important to recognise that poorly controlled asthma may increase the incidence of perinatal mortality and retard intra-uterine growth, so that more effective control of asthma with inhaled corticosteroids may reduce these problems.

Clinical use of inhaled corticosteroids

Inhaled corticosteroids are now recommended as first-line therapy for all but the mildest of asthmatic patients [1]. Inhaled corticosteroids should be started in any patient who needs to use a β -agonist inhaler for symptom control more than once daily (or possibly three times weekly). It is conventional to start with a low dose of inhaled corticosteroid and to increase the dose until asthma control is achieved. However, this may take time and a preferable approach is to start with a dose of corticosteroids in the middle of the dose range (400 μ g twice daily) to establish control of asthma more rapidly [191]. Once control is achieved (defined as normal or best possible lung function and infrequent need to use an inhaled μ_2 -agonist) the dose of inhaled corticosteroid should be reduced in a step-wise manner to the lowest dose needed for optimal control. It may take as long as three months to reach a plateau in response and any changes in dose should be made at intervals of three months or more. This strategy (“start high – go low”) is emphasised in the revised BTS Guidelines for Asthma Management [110]. When doses of ≥ 800 μ g daily are

needed a large volume spacer device should be used with an MDI and mouth washing with a dry powder inhaler in order to reduce local and systemic side-effects. Inhaled corticosteroids are usually given as a twice daily dose in order to increase compliance. When asthma is more unstable four times daily dosage is preferable [138]. For patients who require ≤ 400 μg daily, once daily dosing appears to be as effective as twice daily dosing, at least for budesonide [139].

The dose of inhaled corticosteroid should be increased to 2000 μg daily if necessary, but higher doses may result in systemic effects and it may be preferable to add a low dose of oral corticosteroid, since higher doses of inhaled corticosteroids are expensive and have a high incidence of local side-effects. Nebulised budesonide has been advocated in order to give an increased dose of inhaled corticosteroid and to reduce the requirement for oral corticosteroids [192], but this treatment is expensive and may achieve its effects largely via systemic absorption.

Most of the guidelines for asthma treatment suggest that additional bronchodilators (slow-release theophylline preparations, inhaled and oral long-acting β_2 -agonists and inhaled anticholinergics) should be introduced after increasing the dose of inhaled corticosteroid to 1600–2000 μg daily. However an alternative approach is to introduce these treatments when patients are taking 400–800 μg inhaled corticosteroid daily. Addition of the long-acting inhaled β_2 -agonist salmeterol provides better control of asthma symptoms than doubling the dose of inhaled corticosteroids [193, 194]. Similarly, addition of low dose oral theophylline gives better control than doubling the dose of inhaled corticosteroid in patients not controlled on budesonide 800 μg daily [195].

Inhaled corticosteroids may be the most cost-effective way of controlling asthma, since reducing the frequency of asthma attacks will save on total costs [196, 197]. Inhaled corticosteroids improve the quality of life of patients with asthma and allow many patients a normal lifestyle [198].

Systemic corticosteroids

Oral or intravenous corticosteroids may be indicated in several situations. Prednisolone, rather than prednisone, is the preferred oral corticosteroid as prednisone has to be converted in the liver to the active prednisolone. In pregnant patients prednisone may be preferable as it is not converted to prednisolone in the foetal liver, thus diminishing the exposure of the foetus to glucocorticoids. Enteric-coated preparations of prednisolone are used to reduce side-effects (particularly gastric side-effects) and give delayed and reduced peak plasma concentrations, although the bioavailability and therapeutic efficacy of these preparations are similar to uncoated tablets. Prednisolone and prednisone are preferable to dexamethasone, betamethasone or triamcinolone, which have longer plasma half-lives and therefore an increased frequency of adverse effects.

Short courses of oral corticosteroids (30–40 mg prednisolone daily for 1–2 weeks or until the peak flow values return to best attainable) are indicated for exacerbations of asthma, and the dose may be tailed off over 1 week once the exacerbation is resolved. The tail-off period is not strictly necessary [199], but some patients find it reassuring.

Maintenance oral corticosteroids are only needed in a small proportion of asthmatic patients with the most severe asthma that cannot be controlled with maximal doses of inhaled corticosteroids (2000 µg daily) and additional bronchodilators. The minimal dose of oral corticosteroid needed for control should be used and reductions in the dose should be made slowly in patients who have been on oral corticosteroids for long periods (e.g. by 2.5 mg per month for doses down to 10 mg daily and thereafter by 1 mg per month). Oral corticosteroids are usually given as a single morning dose as this reduces the risk of adverse effects since it coincides with the peak diurnal concentrations. There is some evidence that administration in the afternoon may be optimal for some patients who have severe nocturnal asthma [200]. Alternate day administration may also reduce adverse effects, but control of asthma may not be as good on the day when the oral dose is omitted in some patients.

Intramuscular triamcinolone acetonide (80 mg monthly) has been advocated in patients with severe asthma as an alternative to oral corticosteroids [201, 202]. This may be considered in patients in whom compliance is a particular problem, but the major concern is the high frequency of proximal myopathy associated with this fluorinated corticosteroid. Some patients who do not respond well to prednisolone are reported to respond to oral betamethasone, presumably because of pharmacokinetic handling problems with prednisolone [203].

Corticosteroid-sparing therapy

In patients who have serious side-effects with maintenance corticosteroid therapy there are several treatments which have been shown to reduce the requirement for oral corticosteroids [204]. These treatments are commonly termed corticosteroid-sparing, although this is a misleading description that could be applied to any additional asthma therapy (including bronchodilators). The amount of corticosteroid sparing with these therapies is not impressive.

Several immunosuppressive agents have been shown to have corticosteroid effects, including methotrexate [205, 206], oral gold [207] and cyclosporin A [208, 209]. These therapies all have side-effects that may be more troublesome than those of oral corticosteroids and are therefore only indicated as an additional therapy to reduce the requirement of oral corticosteroids. None of these treatments is very effective, but there are occasional patients who appear to show a particularly good response. Because of side-effects these treatments cannot be considered as a way to

reduce the requirement for inhaled corticosteroids. Side-effects are a problem with these immunosuppressive drugs and include nausea, vomiting, hepatic dysfunction, hepatic fibrosis, pulmonary fibrosis and increased infections for methotrexate and renal dysfunction for cyclosporin and oral gold. Several other therapies, including azathioprine, dapsone and hydroxychloroquine have not been found to be beneficial. The macrolide antibiotic troleandomycin is also reported to have corticosteroid-sparing effects, but this is only seen with methylprednisolone and is due to reduced metabolism of this corticosteroid, so that there is little therapeutic gain [210].

Acute severe asthma

Intravenous hydrocortisone is given in acute severe asthma. The recommended dose is 200 mg i.v. [211]. While the value of corticosteroids in acute severe asthma has been questioned, others have found that they speed the resolution of attacks [212]. There is no apparent advantage in giving very high doses of intravenous corticosteroids (such as methylprednisolone 1 g). Indeed, intravenous corticosteroids have occasionally been associated with an acute severe myopathy [213]. In a recent study no difference in recovery from acute severe asthma was seen whether i.v. hydrocortisone in doses of 50, 200 or 500 mg every 6 h were used [214] and another placebo controlled study showed no beneficial effect of i.v. corticosteroids [215]. Intravenous corticosteroids are indicated in acute asthma if lung function is < 30% predicted and in it there is no significant improvement with nebulised β_2 -agonist. Intravenous therapy is usually given until a satisfactory response is obtained and then oral prednisolone may be substituted. Oral prednisolone (40–60 mg) has a similar effect to intravenous hydrocortisone and is easier to administer [212, 216]. Oral prednisolone is the preferred treatment for acute severe asthma, providing there are no contraindications to oral therapy [110].

Corticosteroid-resistant asthma

Although corticosteroids are highly effective in the control of asthma and other chronic inflammatory or immune diseases, a small proportion of patients with asthma fail to respond even to high doses of oral corticosteroids [217–219]. Resistance to the therapeutic effects of glucocorticoids is also recognised in other inflammatory and immune diseases, including rheumatoid arthritis and inflammatory bowel disease. Corticosteroid-resistant patients, although uncommon, present considerable management problems. Recently, new insights into the mechanisms whereby glucocorticoids suppress chronic inflammation have shed new light on the molecular basis of glucocorticoid resistance in asthma [217].

Clinical features

Corticosteroid resistance (CR) in asthma was first described by Schwartz et al. in 1968, in six asthmatic patients who did not respond clinically to high doses of systemic corticosteroids and in whom there was also a reduced eosinopaenic response [220]. Carmichael and colleagues reported a larger group of patients with chronic asthma who were corticosteroid-resistant [221]. These patients failed to improve their mean peak expiratory flow (PEF) by > 15% after taking prednisolone 20 mg daily for at least 7 days. They differed clinically from corticosteroid-sensitive patients only in having a longer duration of symptoms, lower morning PEF values and a more frequent family history of asthma. These patients are not Addisonian and they do not suffer from the abnormalities in sex hormones described in familial glucocorticoid resistance. Plasma cortisol and adrenal suppression in response to exogenous cortisol are normal in these patients [222].

Absolute corticosteroid resistance in asthma is rare, but there are no population studies giving an estimate of the proportion of patients who are resistant. It is likely that most specialists would only have a few such patients in their clinic and the prevalence is probably < 1:1000 asthmatic patients. Much more common is a reduced responsiveness to corticosteroids, so that large inhaled or oral doses are needed to control asthma adequately. It is important to establish that the patient has asthma, rather than chronic obstructive pulmonary disease (COPD), "pseudoasthma" (a hysterical conversion syndrome involving vocal cord dysfunction), left ventricular failure or cystic fibrosis that do not respond to corticosteroids [223]. Asthmatic patients are characterised by a variability in PEF and, in particular, a diurnal variability of > 15% and episodic symptoms. It is also important to identify provoking factors (allergens, drugs, psychological problems) that may increase the severity of asthma and its resistance to therapy.

Distinction between corticosteroid-sensitive (CS) and CR asthmatics depends on the response to a high dose of oral corticosteroids given for a reasonable period. In research studies prednisolone is usually given in a dose of 40 mg daily for 2 weeks with twice daily monitoring of PEF. In SR asthma patients fail to improve the morning PEF or FEV₁ by > 15%. Patients with CR asthma show the typical diurnal variability in PEF and bronchodilate in response to inhaled β_2 -agonists. Bronchial biopsy shows the typical inflammatory infiltrate of eosinophils in CR patients [224]. It is clearly important to establish that the patient is taking the oral corticosteroid by measurement of plasma cortisol, which is suppressed after high dose oral corticosteroids in both CS and CR patients [222] or by measurement of plasma prednisolone concentrations. Patients with COPD fail to improve lung function after a course of oral corticosteroids, but are distinguished from CR asthmatic patients by their lack of acute bronchodilator response and absence of diurnal variability in PEF.

Another group of patients with asthma is responsive to corticosteroids, but only in relatively high oral doses. These patients are best described as steroid-dependent

(i.e. dependent on oral corticosteroids as opposed to inhaled corticosteroids). These patients deteriorate when the dose of oral corticosteroids is reduced. Rarely a maintenance dose of > 40 mg prednisolone daily may be required and such patients may mistakenly be classified as CR. Steroid-dependent asthmatic patients usually have severe disease and are presumed to have a high level of inflammation in their airways.

Mechanisms of corticosteroid resistance

There may be several mechanisms for resistance to the effects of glucocorticoids [217–219]. Although a family history of asthma is more common in patients with SR than CS asthma, little is known of the inheritance of CR asthma. Resistance to the inflammatory and immune effects of glucocorticoids should be distinguished from the very rare familial glucocorticoid resistance, where there is an abnormality of glucocorticoid binding to GR.

Familial glucocorticoid resistance

The rare inherited syndrome familial glucocorticoid resistance (FGR) is characterised by high circulating levels of cortisol without signs of symptoms of Cushing's syndrome [225]. Clinical manifestations, which may be absent, are due to an excess of non-glucocorticoid adrenal corticosteroids, stimulated by high ACTH levels, resulting in hypertension with hypokalaemia and/or signs of androgen excess (usually hirsutism and menstrual abnormalities in females). Only about 12 cases have so far been reported. Several abnormalities in GR function have been described in peripheral blood leukocytes or fibroblasts from these patients. These include a decreased affinity of GR for cortisol, a reduced number of GRs, GR thermolability and an abnormality in the binding of the GR complex to DNA. The molecular basis of the disease in four patients with a reduction in GR appears to be a point mutation in the corticosteroid-binding domain of GR.

Resistance to anti-inflammatory actions of corticosteroids

Resistance to the anti-inflammatory and immunomodulatory effects of glucocorticoids differs from the familial glucocorticoid resistance described above, as it is not associated with high circulating concentrations of cortisol or ACTH, and is not accompanied by hypertension, hypokalaemia or androgen excess. Furthermore, these patients are not Addisonian and show normal adrenal suppression. This suggests that any abnormality is unlikely to be due to the same abnormalities in the corticosteroid-binding domain of GR, as described in FGR. Analysis of GR has failed to demonstrate any major abnormality in predicted structure in CR compared with

CS asthma [226]. Corticosteroid resistance may be primary (inherited or acquired of unknown cause) or secondary to some factor known to reduce glucocorticoid responsiveness (glucocorticoids themselves, cytokines, β -adrenergic agonists). There are several possible sites where abnormalities in the anti-inflammatory response to glucocorticoids in asthma may arise.

Pharmacokinetic abnormalities

The initial suggestion of Schwartz et al. was that defective responses to corticosteroids were due to increased clearance of the glucocorticoid, resulting in reduced clinical and eosinopenic response [220]. There is no evidence for altered bioavailability or plasma clearance of prednisolone or methylprednisolone in patients with corticosteroid-resistant asthma [227, 228]. Metabolism of glucocorticoids may be increased by induction of P-450 enzymes in response to certain drugs (e.g. rifampicin, carbamazepine), which may thus lead to a secondary corticosteroid resistance [130].

Antibodies to lipocortin-1

Some anti-inflammatory effects of glucocorticoids may be due to induction of lipocortin-1 [29]. In some patients with corticosteroid-resistant rheumatoid arthritis autoantibodies to lipocortin-1 have been described [229]. However, two independent studies have failed to demonstrate the presence of IgG or IgM lipocortin-1 antibodies in either SR or corticosteroid-dependent asthma [230, 231].

Cellular abnormalities

Glucocorticoid resistance has been documented *in vitro* in monocytes and T-lymphocytes from CR asthmatic patients, with a reduction in the inhibitory effect of corticosteroids on cytokine production. These studies in circulating leukocytes suggest that the defect in glucocorticoid responsiveness extends outside the respiratory tract and is therefore unlikely to be secondary to inflammatory changes in the airways. In patients with SR asthma the reduced blanching response to topical glucocorticoids applied to the skin further indicates that there is a generalised abnormality that is unlikely to be secondary to local cytokine production [232].

Abnormalities in GR function

In FGR there is an abnormality in GR structure that results in reduced glucocorticoid binding affinity. GR binding in monocytes and T lymphocytes of SR asthma shows either no difference in GR affinity and receptor density or a relative reduction in GR affinity [227, 233–235]. Corrigan et al. found some reduction of GR

affinity in T cells from SR asthmatic patients but this could not account for the resistance to PHA-induced proliferative responses in cells from the same patients [227]. Sher et al. described two types of glucocorticoid resistance: a reduced affinity of GR binding confined to T lymphocytes which reverted to normal after 48 h in culture, and a much less common reduction in GR density (in only 2/17 SR patients) which did not normalise with prolonged incubation [235]. This suggests that there may be different types of corticosteroid resistance in asthma. The small reduction in GR affinity is unlikely to be of functional significance and is not associated with elevated plasma cortisol concentrations, as observed in patients with FGR. The small reduction in GR affinity may be secondary to cytokine exposure, since the normalisation of GR affinity *in vitro* is prevented by a combination of IL-2 and IL-4 [235] and this combination of cytokines reduces the binding affinity in nuclear GR in T lymphocytes, although either cytokine alone has no effect [236]. This suggests that corticosteroid resistance may occur in the airways of patients with asthma as a secondary phenomenon due to the local production of cytokines. In SR asthmatic patients there is a significant increase in the numbers of BAL cells expressing IL-2 and IL-4 mRNA compared to SS asthmatics, but no difference in IFN γ mRNA positive cells. After oral prednisone for 1 week there is a reduction in IL-4 expressing cells and a rise in IFN γ positive cells in SS asthma, whereas in SR asthma there was no fall in IL-4 positive cells and a fall in IFN γ positive cells [224]. This may indicate that there are different patterns of cytokine release that may contribute to corticosteroid resistance. Although this may account for the increased requirement for glucocorticoids in more severe asthma, it is unlikely to account for the reduced corticosteroid response seen in circulating mononuclear cells and in the skin of patients with no response to oral glucocorticoids.

There is, however, a marked reduction in GR-GRE binding in mononuclear cells of patients with CR asthma and Scatchard analysis has demonstrated a marked reduction in GR available for DNA binding compared with cells from patients with CS asthma [237].

Interaction between GR and transcription factors

In mononuclear cells of CS patients and normal control subjects the phorbol ester PMA, which activates AP-1, results in reduced GRE binding. This inhibitory effect is significantly abrogated in the PBMC of patients with SR asthma, indicating a likely abnormality in the interaction between GR and AP-1 [238]. This defect does not appear to apply to the other transcription factors, NF- κ B and CREB, that also interact with GR [238]. The abnormality in the interaction between GR and AP-1 is unlikely to be due to a defect in GR, since the protein sequence of GR in patients with SR asthma is normal [226]. It is more likely to be due to a defect in AP-1 or its activation. Indeed, activation of c-Fos by phorbol esters is potentiated in the cells of patients with SR compared to SS asthma [239] and one of the key enzymes

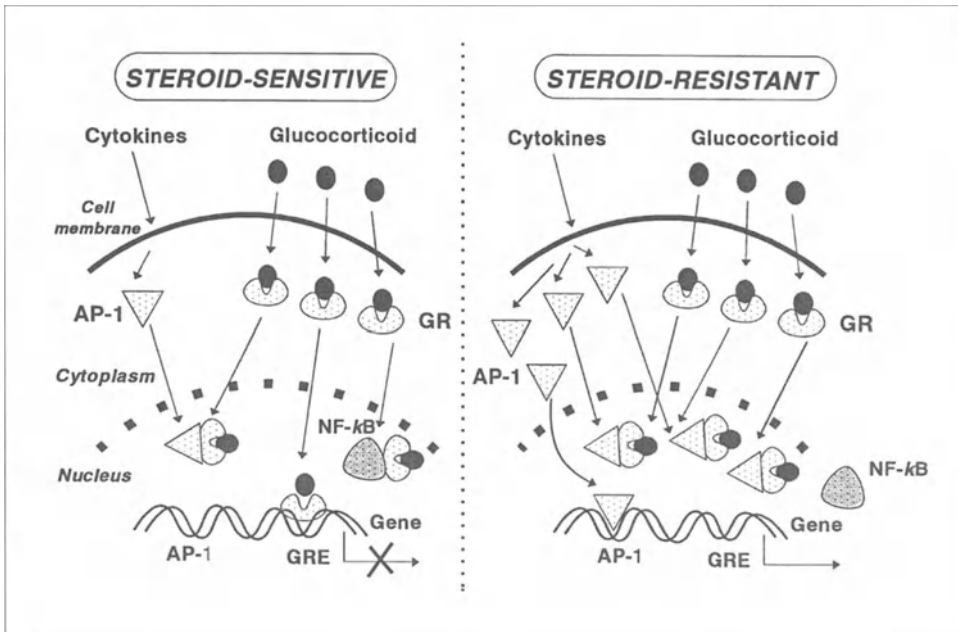


Figure 8

Proposed mechanism of primary corticosteroid-resistance in asthma. Increased activation of activator protein-1 (AP-1) results in the consumption of glucocorticoid receptors (GR), thus preventing the anti-inflammatory action of corticosteroids, either through binding to GREs or through inhibition of NF-κB.

involved in activation of AP-1, namely Jun N-terminal (JNK) kinase, is abnormally activated in these patients [240]. The increased basal and cytokine-induced AP-1 activity may lead to consumption of GR, so that corticosteroids are not able to suppress the inflammatory response, either through interacting with GRE or with other transcription factors, such as NF-κB (Fig. 8).

An abnormality in AP-1 may also account for the selective resistance to the effects of corticosteroid in SR asthma, since AP-1 is more likely to be important in the regulation of some genes than in others. It would also explain why resistance is seen to the antiinflammatory effects of corticosteroids, since such resistance can only arise when AP-1 is activated at the inflammatory site, whereas the hormonal effects of corticosteroids at uninflamed sites will not be impaired. Furthermore, there may also be differences in the corticosteroid resistance of different target cells, depending upon the relative balance of transcription factors.

Secondary corticosteroid resistance

Although complete corticosteroid resistance is uncommon, there may be a spectrum of corticosteroid responsiveness in asthma. This may reflect several mechanisms that are secondary either to disease activity itself or to the effects of therapy.

Down-regulation of GR

Down-regulation of GR in circulating lymphocytes after oral prednisolone has been demonstrated in normal individuals [241]. Whether high local concentrations of inhaled glucocorticoids reduce GR expression in surface cells of the airway, such as epithelial cells, is not yet certain. It is possible that certain individuals may be more susceptible to the effects of down-regulation. If effective GR density is reduced by direct interaction with other transcription factors, such as AP-1 and NF- κ B, then the down-regulating effect of glucocorticoids on GR would be expected to have a greater functional consequence.

Effects of cytokines

Several proinflammatory cytokines, including IL-1 β , IL-6 and TNF α , activate AP-1 and NF- κ B in human lung [13, 242]. As all these cytokines are known to be secreted in asthmatic inflammation, this suggests that these transcription factors will be activated in the cells of asthmatic airways. These activated transcription factors may then form protein-protein complexes with activated GR, both in the cytoplasm and within the nucleus, thus reducing the number of effective GR and thereby decreasing corticosteroid responsiveness [4] (Fig. 9). In a model *in vitro* system increased expression of c-Fos or c-Jun oncoproteins prevents the activation of mouse mammary tumour virus promoter by GR, thus creating a model of corticosteroid resistance [12]. Addition of recombinant c-Jun or c-Fos proteins to partially purified GR results in inhibition of DNA binding [12]. Phorbol esters, which activate AP-1, result in attenuation of glucocorticoid-mediated gene activation [243]. Any reduction in glucocorticoid responsiveness would be greater as the intensity of asthmatic inflammation increased and may contribute, for example, to the failure of oral or intravenous glucocorticoids to control acute exacerbations of asthma. Once the inflammation is brought under control with large doses of oral glucocorticoids, corticosteroid responsiveness increases again so that lower doses of inhaled or oral glucocorticoids are needed to control asthmatic inflammation. Increased resistance may also be due to the effects of cytokines on GR receptor function, since high concentrations of IL-2 and IL-4 have been shown to reduce GR affinity in T lymphocytes *in vitro* [236]. This effect would only be seen in mucosal T cells of patients with severe asthma and it is therefore difficult to obtain evidence to support this possibility.

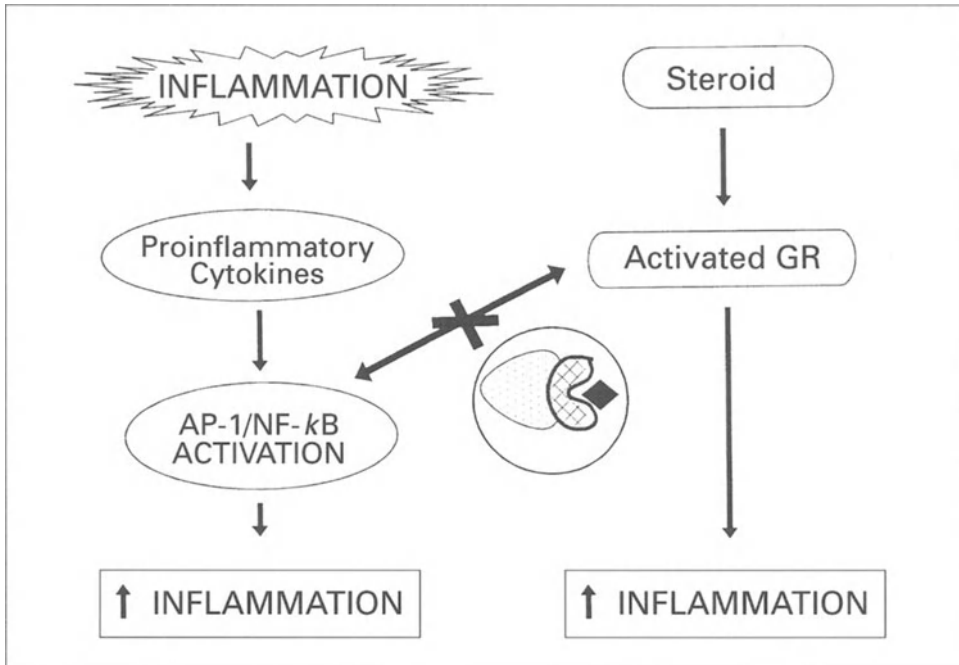


Figure 9

Secondary corticosteroid resistance may arise in the presence of cytokine-mediated inflammation through an interaction between the cytokine-activated transcription factors, such as activator protein-1 (AP-1) and nuclear factor- κ B (NF- κ B), and the glucocorticoid receptor (GR), resulting in a reduced availability of GR for control of the inflammatory response. This can only be overcome by increasing the dose of glucocorticoid administered.

Effect of β_2 -agonists

High concentrations of β_2 -agonists activate CREB in rat and human lung and in inflammatory cells via an increase in cyclic AMP concentration [244, 245]. This results in reduced GRE binding due to the formation of GR-CREB complexes [246]. This predicts that high concentrations of β_2 -agonists would induce corticosteroid resistance. In asthmatic patients, while 3 weeks of treatment with an inhaled corticosteroid blocked the airway response to inhaled allergen, concomitant treatment with inhaled corticosteroid and a relatively large dose of inhaled β -agonist appeared to provide no significant protection against allergen challenge [247]. This suggests that high doses of an inhaled β_2 -agonists might interfere with the anti-asthma effect of inhaled glucocorticoids. It is possible that some patients who use very high doses of inhaled β_2 -agonists (over two canisters per month of metered dose inhalers or

regular nebulised doses), may develop a degree of corticosteroid resistance that is overcome by increasing the dose of inhaled or oral glucocorticoid. Corticosteroid responsiveness might be restored by reducing the dose of inhaled β_2 -agonists. In an uncontrolled study in corticosteroid-dependent patients with severe asthma, gradual withdrawal of nebulised β_2 -agonists resulted in a reduced requirement for oral prednisolone [248].

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Phosphodiesterases in asthma

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Introduction

Airway obstruction and hyperreactivity in asthma are mainly caused by accumulation of inflammatory cells and their mediators promoting bronchoconstriction, airway oedema and airway remodeling. Cyclic AMP counteracts a huge variety of inflammatory cell functions involved in the development and maintenance of asthma. In addition, cyclic AMP has been shown to reverse bronchial constriction, airway oedema and smooth muscle proliferation and may therefore protect against airway remodeling in asthma. cAMP is generated from ATP by adenylate cyclases that are activated by G-protein coupled receptors such as the β_2 -receptor. In fact, attenuating cAMP generation may aggravate clinical asthma as reported with β_2 -antagonists. On the other hand, there is overwhelming evidence that β_2 -agonists improve asthma. However, long-term administration of β_2 -agonists may be hampered by the phenomenon of tachyphylaxis, e.g. receptor desensitization and post-receptor events. In particular, it was repeatedly demonstrated that continuous use of inhaled β_2 -agonists is associated with an impairment of their acute protective effects against bronchoconstrictive stimuli [1–4]. Continuous inhalation of β_2 -agonists impaired their bronchoprotective effect against AMP-induced hyperreactivity to a greater extent compared to hyperreactivity triggered by methacholine. These results may imply that β_2 -agonists tend to desensitize mast cell responses more strongly than direct smooth muscle responses [4]. Apart from enhanced cAMP generation another option to increase cAMP is to inhibit its decay. Cyclic nucleotide hydrolysing phosphodiesterases (PDE) represent a superfamily of enzymes which break down cAMP and cGMP. Thus PDE inhibitors should increase cAMP in bronchial smooth muscle and inflammatory cells and have anti-asthmatic effects.

Seven PDE families have been characterized mainly based on substrate, activator or inhibitor sensitivities (Tab. 1) (families 8 and 9 have been identified recently). Several of these PDE families comprise subtypes encoded by different genes. As an example, four subtypes (PDE4A–D) have been identified for PDE4. mRNA-transcription of subtypes involves alternative splicing resulting in the expression of PDE

Table 1 - Classification of PDE isoenzyme families. Seven PDE families based on substrate preference and sensitivities to activators or inhibitors have been characterised.

PDE family	Preferred substrate	Activator	Inhibitor
PDE1	cAMP, cGMP	Ca ²⁺ -Calmodulin	
PDE2	cAMP	cGMP	EHNA
PDE3	cAMP		cGMP, Milrinone
PDE4	cAMP		Rolipram
PDE5	cGMP		Sildenafil
PDE6	cGMP		
PDE7	cAMP		

species with distinct N-terminal ends. This is exemplified by the five N-terminal splice variants so far described for PDE4D. As will be discussed below, N-terminal splicing may serve to direct intracellular localisation of the enzyme, post-translational modification or formation of distinct conformers. Phosphodiesterases share homologies in their amino acid sequence. A catalytic domain (~270 amino acids) is flanked by a hydrophilic C-terminus and an N-terminus that comprises regulatory sites. The catalytic core has a higher degree of homology within phosphodiesterases of one family (~80%) than with other families (~25%–40%). The catalytic core contains the PDE-specific sequence HD(X)₂H(X₄)N and two Zn²⁺-binding domains. The N-terminal domain contains the calmodulin-binding site (PDE1), the cGMP-binding site (PDE2 or 5), hydrophobic membrane association domains (PDE3) or membrane-targeting domains (PDE4) or other functionally relevant regions.

In humans, there is cell-specific expression of PDE families, subtypes or splice variants. Peripheral blood eosinophils, neutrophils, and monocytes contain almost exclusively PDE4. T lymphocytes, lung mast cells, and bronchial and vascular smooth muscle cells express PDE4 and 3. Human alveolar macrophages additionally exhibit PDE1 whereas human endothelial cells express PDE2, 3 and 4. Finally, PDE1 and 4 were detected in human bronchial epithelial cells. Regarding subtypes, PDE3A has been located in platelets, vascular smooth muscle and myocardium, whereas PDE3B was found in adipocytes, liver, pancreatic β -cells, the neuronal system and T lymphocytes. PDE4C was not found in peripheral blood cells, but it was abundant in the CNS. Studies examining whether expression of phosphodiesterase variants is up- or down-regulated in disease states such as asthma are at an early stage. Earlier studies implied that there may be an up-regulation of PDE in peripheral blood cells of patients with atopic dermatitis [5, 6], but others could not confirm these findings [7]. Results from studies investigating the pattern of PDE variants in eosinophils, T lymphocytes or neutrophils infiltrating the bronchial mucosa in asthmatics are eagerly awaited.

Recently, the interesting observation was made that distinct subtypes of PDE1 were increasingly expressed in maturing or proliferating cells. Firstly, it was shown that monocytes cultured with human AB serum for several days assume the phenotype of macrophages and this was associated with an enhanced expression of PDE1 that was absent in monocytes but detected in human alveolar macrophages [8, 9]. Secondly, PDE1B1 was expressed in proliferating T lymphocytes following incubation with PHA, but was absent in resting T cells. In fact, PDE1B1 antisense promotes apoptosis of lymphocytic cells, implying a functional role of PDE1B1 up-regulation in these cells [10]. Thirdly, proliferating human vascular smooth muscle cells express PDE1C but this was undetectable in quiescent cells [11]. From these latter findings the hypothesis may be raised that PDE1C up-regulation facilitates smooth muscle remodeling in vascular (pulmonary hypertension, atherosclerosis) disease and asthma and that PDE1C antisense or selective PDE1C inhibitors might reverse vascular or bronchial smooth muscle remodeling.

From the pattern of PDE isoenzymes described above it was concluded that particularly PDE4 inhibitors or dual-selective PDE3 and 4 inhibitors might provide benefit as efficient anti-asthma drugs since these families are found in almost all inflammatory cells. The development of new PDE4 or PDE3 and 4 inhibitors for asthma is greatly driven by the encouraging clinical experience with theophylline – a non-selective PDE inhibitor. In fact, although theophylline has been considered for decades as a pure bronchodilator, recent intensive research revealed and unequivocally confirmed that theophylline triggers a multitude of anti-inflammatory effects that certainly contribute to its effects in clinical asthma. *In vitro*, theophylline inhibits lipopolysaccharide (LPS)-induced TNF α formation in human monocytes and alveolar macrophages; human T lymphocyte proliferation, chemotaxis and cytokine generation; production of reactive oxygen species (ROS), degranulation, LTC₄ synthesis, and chemotaxis in human eosinophils; anti-IgE induced histamine release from human lung mast cells; degranulation and ROS formation in human neutrophils. Moreover, theophylline promotes human eosinophil apoptosis. Theophylline inhibited inflammatory cell accumulation in diverse allergen challenge models and attenuated airway oedema formation in experimental animals. Controlled clinical trials revealed that theophylline acts to reduce T lymphocyte infiltrates, to reduce IL-4 and IL-5 expression and to attenuate the number of activated eosinophils in bronchial biopsies obtained from asthmatics. There is certainty from a diversity of clinical trials that theophylline inhibits late and early responses to allergen challenge, again adding strong evidence to its anti-inflammatory profile [12–22]. Finally, two recent trials have shown that theophylline has steroid-sparing effects. Adding theophylline to budesonide 800 $\mu\text{g}/\text{day}$ or beclomethasone 400 $\mu\text{g}/\text{day}$ was at least equivalent and in some clinical parameters even significantly superior to a doubled steroid dose (i.e. budesonide 1600 $\mu\text{g}/\text{day}$ or beclomethasone 800 $\mu\text{g}/\text{day}$) [23, 24]. In other trials it was shown that benefit from theophylline is still gained in those patients who do not further improve despite increas-

ing steroid dose. It may be hypothesized that theophylline synergises with steroids also by inhibiting some of the inflammatory responses resistant to steroids.

New phosphodiesterase inhibitors for asthma are required in particular to improve the benefit-risk ratio. Originally, attention was paid to the hypothesis that family-selective PDE inhibitors i.e. PDE4 and PDE4 and 3 inhibitors might provide a solution. However, both disappointing clinical results with first generation selective inhibitors as well as considerable recent progress in PDE research fueled a slightly different concept, and the current paradigm has changed. Through post-translational regulation, association to other proteins or membranes PDEs are present in diverse functional states (conformations) and the second generation inhibitor should inhibit the state associated with inflammatory cells and processes but not the state assumed in non-inflammatory tissues (e.g. CNS, myocardium, vasculature).

This review will focus on PDE4 and PDE3 and recent progress with the development of selective (PDE4) or dual-selective (PDE3 and 4) inhibitors for asthma. Chemical structures of PDE4 inhibitors and dual-selective PDE3 and 4 inhibitors are illustrated in Figure 1 (see pages 110/111). Other PDE families which have so far not been in the focus of drug discovery for asthma have recently been extensively reviewed by others.

Phosphodiesterase 4 family

PDE4 is a specific cyclic AMP hydrolysing phosphodiesterase activity that is potently inhibited by rolipram but not affected by cGMP. In early chromatographical investigations of heart and smooth muscle extracts, this phosphodiesterase activity eluted as the fourth activity from anion exchange columns [25]. Subsequent investigations revealed the ubiquitous presence of PDE4. In human peripheral blood eosinophils, monocytes, neutrophils, B cells but also neuronal cells, PDE4 represents the predominant isoenzyme. In other systems such as human alveolar macrophages, T cells, dendritic cells, endothelial cells, smooth muscle cells and bronchial epithelial cells, PDE4 was coexpressed with other PDE isoenzymes, in particular PDE3. This abundance of PDE4 in cells that orchestrate inflammation in bronchial asthma or rheumatoid arthritis was paralleled by evidence that PDE4 inhibitors such as rolipram potently suppressed a multitude of inflammatory cell functions e.g. ROS generation, cytokine synthesis, cysteinyl leukotriene-production, chemotaxis, and T lymphocyte proliferation. Hence, PDE4 or PDE3/4 inhibitors are thought to be capable of resolving inflammation in bronchial asthma or other inflammatory diseases.

However, the presence of PDE4 in neuronal cells implies that inhibitors of this isoenzyme family may induce CNS effects. In fact, the original intention with the classical PDE4 inhibitor rolipram was to develop an antidepressant drug. Moreover, clinical development of first generation PDE4 inhibitors failed due to CNS-induced adverse effects such as nausea or vomiting that occurred at therapeutic plasma con-

centrations. Therefore, it was necessary to elaborate strategies that allow to the dissociation of the anti-inflammatory actions of PDE4 inhibitors from their CNS-driven adverse effects based on the specific interaction of these second generation PDE4 inhibitors with PDE4 molecules. An intensive research campaign over the past decade revealed that PDE4 is encoded by four distinct genes (HSPDE4A–D). Transcription of mRNA from these genes is associated with alternate splicing of 5'-situated exons and finally PDE4 protein is expressed as a certain N-terminal splice variant of one of the four subtypes PDE4A–D. For human PDE4, thirteen splice variants have been characterised. Certainly, cellular systems may exhibit a different expression of these splice variants and it was reasoned that splice-variant selective PDE4 inhibitors may improve the ratio between anti-inflammatory potential and adverse effects.

The important breakthrough in PDE4 research is however, the discovery that PDE4 proteins may exist in two different conformational states. These conformations can be distinguished by their different affinity for the classical PDE4 inhibitor rolipram [26]. This discovery was the result of fundamental investigations to explain the early enigmatic finding that rolipram inhibits PDE4 catalytic activity with $K_i \approx 1 \mu\text{M}$, whereas rolipram binding affinity to PDE4 is in the range of $K_i \approx 1 \text{ nM}$. Recent pharmacological studies suggest that the protein conformation with low affinity for rolipram (LAR-conformation) may predominate in inflammatory cells. On the other hand, in neuronal cells the PDE conformation with a high affinity for rolipram (HAR-conformation) may predominate. Indeed, there is an excellent correlation between interaction of PDE4 inhibitors with the LAR conformation and inhibition of $\text{TNF}\alpha$ -generation from human monocytes [27, 28], IL-2 from murine splenocytes [29], superoxide from guinea pig eosinophils [30], degranulation of guinea pig mast cells [31], human T lymphocyte proliferation induced by antigen [32] and antigen-induced bronchoconstriction [33, 34]. In contrast, the correlation between association with HAR conformation and these functional responses is substantially weaker. On the other hand, interaction of PDE4 inhibitors with the HAR conformation strongly correlates with emesis [35, 36] and acid secretion from rabbit isolated gastric glands [37] (Tab. 2). In comparison, the correlation between association of PDE4 inhibitors with the LAR conformation and these functional responses is dramatically reduced. In conclusion, second generation PDE4 inhibitors designed to exhibit a more potent interaction with the LAR-conformer than with the HAR-conformer of the PDE4 molecule are suggested to dissociate therapeutic efficacy from adverse effects resulting in an improved ratio between anti-inflammatory potential and emesis threshold. Taking into account these encouraging conclusions it should not escape our attention that the PDE4-inhibitor induced suppression of certain inflammatory functions such as neutrophil degranulation [28] but also PDE4-inhibitor induced relaxation of methacholine-precontracted guinea pig trachea [38] may more closely correlate with HAR-conformer association than with LAR-conformer interaction.

Table 2 - Correlation of inhibition of HAR or LAR conformation with functional effects. IC₅₀ values obtained for either inhibition of PDE4 catalysis (reflecting inhibition of the LAR conformer) or [³H]rolipram binding to PDE4 (reflecting inhibition of the HAR conformer) were correlated to corresponding data for the inhibition or activation of functional responses. Higher degree of correlation between HAR or LAR and the functional response is depicted. Notably for some functional effects their correlation to one of the conformers depends on the stimulus. For example, reduction of human T-lymphocyte proliferation induced by antigen correlates to LAR-inhibition whereas suppression of PHA-stimulated proliferation appears to be more closely associated with HAR-inhibition.

Cell/Tissue	Correlation with		Reference
	HAR	LAR	
Human monocytes		LPS-induced TNF α release	[27, 28]
Mice splenocytes		<i>Staph. aur.</i> -induced IL2 release	[29]
Human T lymphocytes		Antigen-induced proliferation	[32]
Guinea pig mast cells		PGD ₂ -release	[31]
Guinea pig eosinophils		ROS generation	[30]
Guinea pig antigen-induced bronchoconstriction		Relaxation	[33, 34]
Behavior, CNS	Emesis		[35, 36]
Human T lymphocytes	PHA-induced proliferation		[98]
Rabbit gastric glands	Acid secretion		[37]
Human neutrophil	Degranulation		[28]
Guinea pig peritoneal macrophages	cAMP-accumulation		[45]
Guinea pig histamine/metacholine- induced tracheal constriction	Relaxation		[38]

In view of the pivotal role of the LAR and HAR conformers for the design of PDE4 inhibitors an intriguing aspect is to investigate for each of the PDE4 splice variants the LAR/HAR ratio or the interconvertibility of these conformers. In fact, currently available data indicate that the percentage of LAR conformer for human recombinant PDE4A5, PDE4B2 and PDE4D3 is 58% [39], 66% [40] and 47% [41], respectively. However, the extent to which a PDE4 variant assumes LAR or HAR conformation depends on the experimental conditions and may be different in intact cells.

Protein kinase A-triggered phosphorylation of PDE4D3 [42, 43] or membrane association of PDE4A5 [44, 45] may assist to augment the proportion of the corresponding HAR conformer. In addition, the hypothesis was raised that *in vitro* treatment of cellular extracts containing PDE4 with orthovanadate in combination with glutathione may result in an augmented amount of the HAR conformer reflected by an increased affinity of PDE4 to rolipram [45–47]. Orthovanadate in combination with glutathione also enhanced PDE4 activity in eosinophil membranes [47, 48]. In view of the fact that orthovanadate serves as a phosphotyrosine-phosphatase inhibitor it may be reasoned that orthovanadate acts by maintaining a tyrosine kinase-induced activated conformer of PDE4. Recent investigations give rise to the view that tyrosine kinases may stimulate PDE4 activity. The tyrosine kinase inhibitor genistein suppressed Ro20-1724-sensitive PDE4 activity in a neuronal cell line by 85% [49]. Genistein was shown to amplify GHRH-stimulated cAMP increase in rat anterior pituitary cells or rat pinealocytes. These effects were reversed by orthovanadate, occurred independently of the cAMP stimulus used and were not observed in the presence of phosphodiesterase inhibitors [50, 51]. Interestingly, strong suppression of PAF-induced human eosinophil LTC₄ and superoxide generation by tyrosine kinase inhibitors was demonstrated [52] which may also be explained by the proposed tyrosine kinase-PDE4 crosstalk. These data are corroborated by evidence that src tyrosine kinase activates PDE4 in rat thecal interstitial and mouse Leydig cells [53]. The tight association of SH3-binding domains located in the N-terminal end of PDE4A with SH3-domains in src-tyrosyl kinases [54], suggesting an *in situ* co-localization of certain PDE4 splice variants with tyrosine kinases, further supports the significance of this putative crosstalk between two major signalling cascades. In summary, based on available data the hypothesis arises that tyrosine kinases may activate PDE4 variants and augment the proportion of HAR conformers. In this respect, tyrosine kinases and protein kinase A comparably activate PDE4 by enhancing their phosphorylated form and the HAR fraction. Orthovanadate, by suppressing phosphotyrosine phosphatase imitates tyrosine kinase-mediated PDE4 modification.

The concept of optimising PDE4 inhibitors by screening for structures showing a high ratio of affinity for the LAR conformer compared to the HAR conformer has now been incorporated in the drug discovery strategies of leading pharmaceutical companies [34, 41, 55, 56]. A very promising candidate is SB207499 (Fig. 1) which associates 33-fold more potently with the LAR conformer versus the HAR conformer of PDE4A5, when compared to rolipram [41]. This compound is currently undergoing clinical investigations as an oral antiasthmatic.

Gene and protein structure of PDE4 variants

Four PDE4 genes (PDE4A, B, C and D) have been distinguished. PDE4A and PDE4C genes are located on chromosome 19, whereas PDE4B and PDE4D genes

are on chromosome 1 and chromosome 5, respectively. These genes are composed of a multitude of exons and introns. Hence, transcription from each of the four genes may result in several mRNA-products emerging from alternative splicing of exons situated near the 5'-end of the DNA open reading frame (ORF). cDNAs encoding different splice variants from rat and human PDE4 genes have been generated based on screening of cDNA libraries with oligonucleotides conservative for a specific PDE4 gene. cDNA was then transfected in cellular systems and corresponding PDE4 proteins were expressed. The amino acid sequences of expressed PDE4 protein variants have been analyzed. In addition, subcellular localisation and catalytic activity of PDE4 splice variants was characterized.

In general, PDE4 protein structures comprise three domains of amino acid sequences which are highly conserved within this enzyme family. A catalytic domain is flanked at its N-terminal end by two upstream conserved regions (UCR1 and UCR2) formed of about 60–100 amino acids. In human and rat PDE4 genes, two splice junctions have been identified. Alternative splicing at the junction next to the 5' end of the ORF results in protein structures that express both the UCR1 and UCR2 encoding regions (long form splice variants). On the other hand, PDE4 variants expressing the UCR2 but not the UCR1 region (short form splice variants) are elaborated by alternate splicing at the splice junction next to the 5' end of the region encoding the catalytic domain. The amino acid sequence at the N-terminal end may (i) serve to confer association to membranes and cytoskeletal proteins and hence, subcellular compartmentation, (ii) represent a site for phosphorylation, (iii) function as an endogenous inhibitor of catalytic activity, (iv) support interaction with other enzymes, and (v) affect sensitivity to exogenous PDE4 inhibitors. Hence, the diversity of PDE4 variants and their unique expression pattern in different cellular systems assists in cell-specific regulation of intracellular signalling and modulates the crosstalk of major signalling pathways.

Table 3 details the classification of currently characterized human PDE4 variants and their rat homologues.

Phosphodiesterase 4A variants

In humans, a single PDE4A variant (PDE4A5) has been detected so far [44, 57–60]. The PDE4A5 protein consists of 886 amino acids and covers both N-terminal UCR1 and UCR2 conserved regions and the catalytic domain (long-form). cDNA for PDE4A5 was isolated from a human monocyte cDNA library [57]. PCR amplification with PDE4A5-specific oligonucleotide primers and Western blotting using antibodies specific for PDE4A5 located PDE4A5 mRNA-transcripts and protein in diverse systems e.g. human monocytes, T cells, B-cells, eosinophils and human brain [7, 61]. Recently, Zn²⁺-binding domains have been identified in the PDE4A5 protein structure and a strong association of PDE4A5 with Zn²⁺ was described

Table 3 - Characteristics of PDE4 subtypes and splice variants.

Four different PDE4 genes are distinguished. Alternative splicing from at least two splice junctions results in short forms and long forms of PDE4.

Human PDE4	Rat homologue	Structure	Comments
	4A1 (RD1)	short-form	membrane-bound
4A5 (PDE46)	4A5 (RNPDE6)	long-form	cAMP → mRNA ↑, protein ↑
	4A8 (RNPDE39)	short-form	exclusively in rat testis
4B1	RPDE4B1	long-form	
4B2	RPDE4B2	short-form	cAMP → mRNA ↑, protein ↑
4B3	4B3 (pRPDE74)	long-form	
4C-791		long-form	fetal human lung
4C-426			fetal human lung
4C-D54			human testis
4C-D109			human testis, inactive
4D1	RPDE4D1	short-form	cAMP → mRNA ↑, protein ↑
4D2	RPDE4D2	short-form	cAMP → mRNA ↑, protein ↑
4D3	RPDE4D3	long-form	cAMP → phosphorylation
4D4		long-form	
4D5		long-form	

(K_d = 0.4 μM). In fact, Zn²⁺ enhanced PDE4A activity, reduced the ability of inhibitors to suppress PDE4A activity by a factor of 10 and attenuated the association of rolipram with the enzyme by a factor of 30 [62].

Human recombinant PDE4A5 was engineered in COS-7 cells and PDE4 activity was detected in both cytosolic (88%) and particulate (12%) fractions [44]. Moreover, immunofluorescence studies were suggestive of an association with membrane structures such as cytoskeletal or cytoskeletal-associated proteins. From these studies it was speculated that the amount of PDE 4A5 associated to membrane or cytoskeletal structures *in situ* was higher than estimated by simple activity measurements in cytosolic and particulate fractions of cell lysates. In fact, studies with the rat homologue RNPDE6 (rat PDE4A5) of human PDE4A5 revealed that certain regions of the N-terminal end constitute SH3-binding domains [54]. These SH3-binding domains confer tight association with SH3-domains. Proteins with SH3-domains are tyrosyl kinases of the Src family but also structural proteins such as fodrin [63, 64]. It has been shown that complete src-kinases and isolated SH3-domains excised from these src-kinases bind to RNPDE6. The src-kinases lyn and fyn exhibit the strongest association to RNPDE6 reflected by a seven-fold higher binding affinity compared to v-src. Binding of these tyrosyl kinases does not affect

PDE activity. In contrast, src-kinases csk and crk inhibited PDE4 activity although their binding affinity was reduced compared to v-src [54].

Whether association with RNPDE6 modulates functions of src tyrosyl kinases has not yet been comprehensively explored. However, it merits attention that PDE association to src kinases directs capacity for cAMP hydrolysis to the immediate vicinity of these tyrosyl kinases. As an example, the tyrosyl kinase csk is phosphorylated and subsequently inactivated by cAMP-activated protein kinase [65]. Csk phosphorylates and inhibits other tyrosine kinases e.g. v-src resulting in suppression of expression of inflammation proteins such as TNF α , IL-1 or iNOS [66]. In this case, PDE association to csk may prevent cAMP-induced inactivation of this protein kinase and ultimately support the function of csk to counteract inflammatory processes. Apart from this indirect crosstalk mediated by catalytic PDE activity, it is unknown at present whether RPDE6 through protein-protein interaction directly modifies src kinases. Certainly, an additional function of enzymes distinct from their well-characterized catalytic activity is not uncommon which is illustrated by the immunophilins. In particular, the FK506-binding protein exhibits catalytic activity as peptidyl-prolyl *cis-trans* isomerase and associates to calcineurin phosphatase resulting in inhibition of the phosphatase activity [67, 68]. Moreover, the interaction of the synaptic vesicle phosphoprotein synapsin I with src SH3-domains attenuates tyrosine phosphorylating capacity [69]. These findings show that direct interaction of proteins with SH3 domains of src may modify tyrosine kinase activity. In view of cAMP-dependent up-regulation of PDE4A mRNA transcripts and protein [70] and considering an orchestrating role of src-kinases in cellular functions as diverse as T lymphocyte proliferation [71], monocyte TNF α generation [72], apoptosis [73, 74], and eosinophil degranulation [75] it would be attractive to hypothesize that PDE4A protein may regulate src tyrosyl kinase activity independently from cAMP breakdown.

PDE4A5 engineered in COS-7 cells that is bound to the particulate fraction of cell lysates exhibits only about half the maximum enzyme activity determined for PDE4A5 found in the cytosolic fraction. Moreover, the potency of rolipram in inhibiting particulate PDE4A5 activity ($K_i = 37$ nM) was about 43-fold higher than its potency in suppressing cytosolic enzyme activity ($K_i = 1.6$ μ M). Whereas inhibition by rolipram of cytosolic PDE4 activity was purely competitive, inhibition data obtained with the particulate enzyme were best fitted by a partial competitive model [44]. Although the authors did not measure rolipram binding, they formulated the hypothesis that the cytosolic and particulate enzyme fractions have a different proportion of PDE4A5 conformers. Expression of PDE4A5 deletion mutants in COS cells revealed that UCR1 and UCR2 was required for high affinity rolipram binding whereas inhibition of catalytic activity was still found in an enzyme truncated to the catalytic core. Rolipram inhibition of the full-length PDE4A5 followed a shallow log dose-inhibition curve ($N = 0.69$) and was about five-fold more potent compared to suppression of the truncated core enzyme that followed competitive kinetics with

a Hill coefficient close to unity [60]. These results are consistent with the hypothesis that full-length recombinant PDE4A5 coexists in two distinct conformers e.g. HAR and LAR. In contrast the core enzyme may only adopt the LAR conformation. The amino acid sequence located N-terminal to the catalytic domain is required for constitution of the HAR conformer.

Analysis of rolipram and RP73401 binding to PDE4A5 deletion mutants further extended these results [59]. RP73401 represents a benzamide originally designed as an anti-asthmatic drug [76]. In contrast to rolipram, this compound associated to the PDE4-high affinity rolipram binding site at concentrations comparable to those required for inhibition of catalysis ($IC_{50} \approx 0.6$ nM) [59, 77]. In consequence the hypothesis was raised that RP73401 exhibits similar capacity to label LAR and HAR [46]. This hypothesis is supported by the finding that deletion of 331 amino acids at the N-terminal end of PDE4A5 retains high affinity binding of RP73401 whereas rolipram high affinity binding is lost [59]. In parallel, RP73401-induced inhibition of catalysis is not affected by N-terminal deletion of 331 amino acids whereas such a truncation impairs rolipram inhibition of cAMP-hydrolysis by a factor of ~ 6 [59]. In addition, these authors detected high ($K_d \approx 1$ nM) and low affinity ($K_d \approx 100$ nM) rolipram binding to the full length human recombinant PDE4A5 (Met1-886) [78] as well as to a fully active truncated form of PDE4A5 (Met265-886) using an equilibrium filter binding technique. Inhibition of cAMP hydrolysis by rolipram was found to be in a range comparable to low-affinity rolipram binding. This indicates that standard measurement of inhibition of PDE4-induced cAMP hydrolysis by rolipram may predominantly reflect catalytic inhibition of the LAR conformer. A strong argument for the concept of two PDE4A5 conformers with different affinities for rolipram but similar affinities for RP73401 is provided by the finding that the B_{max} value for [3H]RP73401 binding is two to three-fold higher than that for [3H]rolipram binding [39]. These data based on standard filtration technique are consistent with the view that RP73401 labels HAR and LAR whereas with rolipram only binding to HAR is detectable under these experimental conditions. Taken together, there is considerable evidence that for human PDE4A5 two conformers can be distinguished. Rolipram discriminates between these conformers whereas RP73401 binds and inhibits catalytic activity of both conformers with identical affinity. Rolipram and RP73401 bind to the catalytic site of the conformers since cAMP is a competitive inhibitor of association of these compounds with PDE4A5 [59].

However, the structural requirements within the central conserved region for inhibitor association compared to cAMP binding to PDE4A5 are different. PDE4A5 point mutants where either His505 or His506 were replaced by Asn using site directed mutagenesis displayed an increase in cAMP- K_M . In parallel, the IC_{50} for cAMP competition of [3H]rolipram and [3H]RP73401 binding reflected a decrease in cAMP affinity for the enzyme. On the other hand, binding and inhibition curves for rolipram and RP73401 were not affected [39].

From the PDE4 variants expressed in rat tissue the short form RNPDE4A1 (RD1) is of interest since a 24 amino acid residue confers complete membrane association in its native state in rat cerebellum [79] and when engineered in COS cells [80, 81] or a human carcinoma thyroid cell line [82]. Deletion of the 24 amino acid residue flanking the N-terminal end of the core region results in an enzyme which is completely located in the cytosol [80]. On the other hand, a chimeric construct of the N-terminal domain of RD1 with chloramphenicol-acetyltransferase, which is cytosolic in its native state, was exclusively expressed in a membrane associated form [83]. In addition, deletion of the N-terminal end results in a substantial increase in V_{MAX} suggesting that this unique 24 amino acid chain acts as an endogenous inhibitor of PDE4 activity [79, 80]. Notably, neither K_M nor rolipram inhibition was affected by deletion of the N-terminal 24 amino acids [80]. Immunofluorescent studies revealed that RD1 primarily associated to the Golgi apparatus [79, 82]. Interestingly, RD1 engineered in human thyroid carcinoma FTC cell lines was accompanied by a down-regulation of endogenous PDE1C mRNA transcript and enzyme activity [82]. It remains an open question whether transcription of PDE1C mRNA is regulated by cAMP.

Phosphodiesterase 4B variants

Three PDE4B variants can be distinguished in humans. PDE4B1 and PDE4B3 are long forms, whereas PDE4B2 represents a short form [58, 84–86]. The rat homologue of PDE4B2 was completely associated to membranes, whereas rat PDE4B1 was cytosolic [87]. In contrast, each of the human PDE4B1-3 splice variants engineered in COS7 cells was expressed in both cytosolic and particulate fractions [86]. However, PDE4B1 and B2 but not PDE4B3 were released from the microsomal particulate fraction following exposure to Triton X-100 suggesting a different mechanism of enzyme association to membrane fractions for PDE4B1 and 4B2 in comparison to PDE4B3. This hypothesis is corroborated by the finding that V_{MAX} values determined for particulate PDE4B1 and PDE4B2 were substantially reduced compared to the cytosolic forms, whereas particulate PDE4B3 showed similar maximum enzyme activity to the cytosolic enzyme [86]. Comprehensive kinetic analysis of cytosolic PDE4B1-3 expressed in COS-7 cells revealed a higher V_{MAX} of the short form PDE4B2 compared to the long forms PDE4B1 and PDE4B3 [86]. These data support the view that the N-terminal end and also membrane association may depress enzyme activity. In contrast to the long forms where rolipram-induced inhibition of PDE4 activity was roughly identical for the cytosolic and particulate species, the selective PDE4 inhibitor attenuated PDE4B2 more potently when expressed in the cytosolic ($IC_{50} \approx 20$ nM) than in the particulate fraction ($IC_{50} \approx 200$ nM) [86]. Although the shallow log dose inhibition curves were suggestive of co-existence of the HAR and LAR conformers for both membrane-associated and cyto-

lic PDE4 forms, it may be hypothesized that the cytosolic PDE4B2 species is dominated by the HAR conformer whereas the LAR conformer represents the predominant folding of PDE4B2 in its membrane-bound state.

That PDE4B2 protein forms HAR and LAR conformations has recently been unequivocally demonstrated [40]. These authors separated high-affinity rolipram binding ($K_d \approx 7$ nM) paralleled by high-affinity enzyme inhibition ($K_i \approx 5\text{--}10$ nM) from low-affinity rolipram binding ($K_d \approx 210$ nM) paralleled by low-affinity enzyme inhibition ($K_i \approx 200\text{--}400$ nM). Rolipram bound with stoichiometries of 0.3 and 0.6 molecules rolipram per molecule of PDE4B2 to the HAR conformer and LAR conformer, respectively. In contrast, an N-terminal and C-terminal deletion mutant of PDE4B2 encompassing amino acids 152–528 of the 564 amino acid sequence exclusively assumed the LAR conformation. Rolipram association to this deletion mutant fit to a low-affinity, single site ($K_d \approx 1$ μ M). In parallel, rolipram-induced inhibition of PDE4B2 (152–528) activity gave a single low-affinity inhibition constant ($K_i \approx 400$ nM). Stoichiometry of rolipram binding to the PDE4B2 (152–528) deletion mutant was 0.9 molecules rolipram per PDE4B2 molecule. Stoichiometry of rolipram binding strongly supports the concept that full-length PDE4B2 protein assumes two conformations that are discriminated by their different affinity for rolipram whereas the PDE4B2 (152–528) deletion mutant exclusively exists in the LAR conformer. Similar to these data are findings with PDE4A5 discussed in the previous section, where deletion of the N-terminal end was associated with a loss of high affinity rolipram binding [59, 78]. Hence, domains in the N-terminal end of PDE4 variants appear indispensable to form the HAR conformer.

The distinct characterisation of HAR and LAR conformers of highly purified human recombinant PDE4B2 in this study [40] is based on techniques for the measurement of enzyme activity and rolipram binding different from those used in previous investigations. To assess cAMP hydrolysing capacity PDE4B2 preparations were preincubated with rolipram for 1 h before the activity assay was performed. Notably, this protocol imitates conditions in the rolipram binding experiments. This technique gave two distinct inhibition constants whereas PDE activity measurement immediately after addition of rolipram i.e. according to the standard common protocol gave a single (low affinity) inhibition constant only. Isothermal titration calorimetry served to assess rolipram binding to PDE4B2. This technique described the binding of rolipram to PDE4B2 protein by evaluating measurements of the energy generated through association of rolipram to the enzyme. Following this technique two distinct binding constants were obtained whereas standard filter binding experiments exclusively detected high affinity rolipram binding.

In conclusion, these experiments add further evidence that rolipram binding is associated with inhibition of catalytic activity. The apparent discrepancy between high affinity rolipram binding and low affinity rolipram inhibition of enzyme catalysis observed previously derives from the limitations of standard protocols for assessment of rolipram inhibition of PDE4 catalysis and rolipram binding to PDE4.

Point mutations within the catalytic core region of rat PDE4B1 resulted in phosphodiesterase species that were resistant to rolipram inhibition. Moreover, these point mutations did not show high affinity rolipram binding. In contrast, the modified enzymes demonstrated K_M and V_{MAX} values comparable to native rat PDE4B1 [88]. These findings reiterate the view that structural elements required for rolipram interaction with the phosphodiesterase 4 protein may be at least in part different from those required for cyclic AMP association [39].

Phosphodiesterase 4C variants

cDNA encoding human PDE4C was first isolated from a substantia nigra cDNA library and transfection of PDE4C cDNA resulted in expression of corresponding PDE4 activity [89]. Northern blotting with PDE4C specific riboprobes and RT PCR with PDE4C specific oligonucleotide primers revealed that PDE4C is located in diverse human systems e.g. brain, liver, lung, heart. However, PDE4C was completely absent in peripheral blood leukocytes e.g. neutrophils, eosinophils, T cells, B-lymphocytes [89, 90]. Recently, cDNA from two different human PDE4C splice variants encompassing 791 and 426 amino acids have been isolated from human fetal lung cDNA library. Screening of human testis cDNA library revealed the presence of another splice variant. Proteins encoded by these three splice variants displayed PDE activity sensitive to phosphodiesterase 4 inhibitors. A fourth splice variant was identified in human testis mRNA. This variant lacked 55 amino acids in the catalytic domain and was inactive [91]. The significance of PDE4C splice variants remains to be investigated.

Phosphodiesterase 4D variants

There are at least five splice variants of PDE4D. The variants PDE4D1 and PDE4D2 are short forms whereas PDE4D3-5 are long forms. PDE4D species were first detected in rat [92–94] and have now also been found in human tissue [58, 95]. PDE4D1-3 transcripts and corresponding PDE4D1 and PDE4D2 proteins were identified in human mononuclear cells [96, 97]. Other sources of human PDE4D variants are monocytes [7, 27], CD4⁺- and CD8⁺-T cells [7, 98], TH2-T lymphocytes [99], Jurkat T cells (PDE4D1 and 2) [100], B lymphocytes and eosinophils [7]. cDNA encoding human PDE4D1–5 has recently been isolated from HeLa cell cDNA libraries using riboprobes specific for PDE4D [101]. Transfection of cDNA in COS-7 cells resulted in the expression of five enzymatically active PDE4D species. The PDE4D variants obtained differed based on their subcellular distribution and inhibition by rolipram. Whereas the short forms PDE4D1 and PDE4D2 were exclusively cytosolic, the additional N-terminal extension in the long forms PDE4D3-5

conferred partial association to the particulate fraction (30–60% of total PDE4 protein). Rolipram inhibited all cytosolic PDE4D activities except PDE4D3 with comparable high potency ($IC_{50} \approx 50\text{--}80\text{ nM}$) accompanied by a shallow concentration-inhibition curve (Hill coefficient $n \approx 0.5$). In contrast, the log concentration inhibition curve for rolipram-induced inhibition of PDE4D3 activity was substantially more steep reflected by a Hill coefficient close to unity. Moreover, rolipram less potently depressed PDE4D3 activity ($IC_{50} \approx 140\text{ nM}$) compared to the other PDE4D species. Notably, particulate PDE4D5 activity is less sensitive to rolipram by a factor of ~ 7 than its cytosolic homologue. This rightward shift of the log concentration inhibition curve is accompanied by an increased curve steepness. These results may indicate that in this study PDE4D3 and particulate PDE4D5 predominantly form the LAR conformations, whereas other species are present in both LAR and HAR conformers. Recent work raised the hypothesis that protein kinase A - induced phosphorylation of PDE4D3 directs transition of the LAR into the HAR conformer [43]. If phosphorylation is the pivotal event that converts LAR into HAR it is conceivable that PDE4D3 is completely dephosphorylated in the ATP-deprived state of cell-free extracts and therefore, the LAR conformer predominates. In consequence the conclusion may be drawn that for PDE4D3 the proportion of HAR / LAR conformers is different in cell-free extracts compared to *in situ* conditions.

Deletion of an N-terminal end encompassing 121 amino acids from PDE4D1 resulted in a six-fold increase in V_{MAX} . These findings complement data obtained with PDE4A species where an inhibitory N-terminal end has also been described [102].

In summary, four different genes encode PDE4. mRNA-transcription from these genes results in a diversity of N-terminal splice variants. N-terminal ends are versatile tools that direct PDE4-localisation or association with other proteins or regulate PDE4 activity. Ultimately the diversity of N-terminal ends serves to translocate and regulate PDE4 activity in defined intracellular compartments where the enzyme affords precise regulation of cAMP and consequently, protein kinase A activity.

There is now ample evidence that PDE4A, 4B and 4D species are present in at least two different conformers. Rolipram binds and inhibits the PDE conformers with different potency. In contrast, RP73401 does not discriminate between the conformers. Rolipram and RP73401 act at the (catalytic) cAMP-binding site. These claims are supported by experimental results.

- (i) In standard assays rolipram shows high affinity binding to PDE4 but low affinity catalytic PDE4 inhibition. In most systems, log-dose inhibition curves for rolipram are shallow.
- (ii) High affinity rolipram binding sites have been localized on highly purified, recombinant PDE4 [40, 84, 103]
- (iii) cAMP competitively inhibits [^3H]rolipram and [^3H]RP73401 binding to PDE4A [59, 103]

- (iv) Rolipram binding to PDE4A reveals high and low affinity binding sites, whereas RP73401 binding kinetics would fit to a homogenous population of binding sites.
- (v) B_{MAX} for high affinity rolipram binding to PDE4A is about half the B_{MAX} for RP73401 binding.
- (iv) Convincing data suggest that PDE4B2 forms two conformers. One conformer shows high affinity rolipram binding sites and hence, is inhibited by low concentrations of rolipram. The other conformer is characterized by low affinity rolipram binding sites and is inhibited by higher concentrations of rolipram.
- (v) Compared to other PDE4D variants rolipram exhibits less potency to suppress PDE4D3. On the other hand, log concentration inhibition curves for rolipram-inhibition of PDE4D3 are substantially steeper (Hill coefficient close to unity) compared to the other PDE4D species. This implies that under cell free conditions LAR represents the predominant conformation of PDE4D3.

Regarding standard assays of rolipram binding and catalytic inhibition it appears that rolipram binding detects HAR whereas catalytic inhibition visualizes predominantly the LAR conformation. However, both assays are conducted in cell free systems and the ultimate relevance of rolipram binding and catalytic inhibition *in situ* remains largely unknown.

Interaction of PDE4 inhibitors with LAR or HAR conformers correlates with different cellular responses. Whereas HAR correlates with behavioral effects, LAR may be more strongly associated with the gating of inflammatory responses.

PDE4 regulation

Expression and activity of the diverse PDE4 variants is selectively regulated by a sophisticated network of cellular messengers. cAMP has been found to enhance mRNA transcription of certain PDE4 splice variants. In addition, some PDE4 variants may be subject to posttranslational modification such as protein kinase A dependent phosphorylation resulting in increased PDE4 activity. Phosphatidic acid has also been described to up-regulate enzyme activity of PDE4 species. PDE4 variants interact with tyrosine kinases or other SH3-domain comprising proteins which may modulate their catalytic activity (see previous section for discussion). In this chapter, regulation by enhanced mRNA transcription, phosphorylation and phosphatidic acid will be reviewed.

cAMP-dependent transcriptional regulation of PDE4 subtypes

Up-regulation of PDE4 activity by long-term incubation with cAMP-increasing agents was detected almost two decades ago (Tab. 4). Incubation of rat Sertoli cells

Table 4 - Cell-specific cAMP-induced transcriptional up-regulation of PDE4 variants. Enhanced mRNA transcription for PDE4 variants by cAMP appears to depend on the cell-type indicating a role of cell-specific cofactors. In the case of PDE4D, the short forms PDE4D1 and PDE4D2 are transcriptionally up-regulated by cAMP. In contrast, the long-form PDE4D3 is resistant to this type of up-regulation. Instead, PDE4D3 activity is increased as a consequence of protein kinase A-dependent phosphorylation.

Tissue	PDE isoenzyme	Reference
Rat brain	4A	[124]
Rat Sertoli cells	4D, 4B	[104], [113]
Rat aortic smooth muscle cells	3/4	[107]
U937 cells, human monocytes	4A, 4B	[105], [61]
Mono Mac 6 cells	4A, 4B, 4D	[70]
Guinea pig macrophages	4	[108]
HaCaT cells	4	[109]
Myoblasts	4	[110]
Rat PC12 cells	4	[111]
Jurkat cells	3, 4D1, 4D2	[100]
Myocardium	1C, 4B	[123]

with FSH, dibutyryl cAMP or 3-isobutyl-1-methylxanthine for several hours resulted in an approximate 10-fold increase in PDE4 activity [104]. More recently it was found that 1 μ M salbutamol in combination with 30 μ M rolipram added to human monocytic U937 cells over 4 h induced an up to four-fold up-regulation of PDE4 [105]. These data were extended by findings that dibutyryl cAMP over 18-24 h induced a two to three-fold increase in PDE4 activity from human peripheral blood monocytes and human monocytic MonoMac6 cells [70]. Long-term cAMP-dependent up-regulation of PDE4 was also shown in other systems (summarized in Tab. 4), e.g. rat aortic vascular smooth muscle cells [106, 107], guinea pig macrophages [108], human keratinocyte HACAT cells [109], myoblast cell lines [110] and rat pheochromocytoma (PC12) cells [111]. Protein kinase A inhibitors reversed long-term PDE4 upregulation which suggests that this process is mediated by protein kinase A [109]. This hypothesis is further corroborated by data showing that L6 myoblasts expressing an inhibitory mutant of the regulatory subunit of protein kinase A were not capable of causing cAMP-dependent PDE4 up-regulation [110]. Regarding the mechanism of long-term cAMP-induced PDE4 up-regulation, it was found that incubation with cycloheximide or actinomycin D inhibited this up-regulation [104, 105, 109]. These results indicated that mRNA transcription

and *de novo* protein synthesis were involved. In fact, following incubation of rat sertoli cells with FSH there was a time and concentration dependent increase in PDE4 mRNA [112] followed by augmented PDE4 protein and activity [113]. Later it was shown under identical experimental conditions that the amount of PDE4D mRNA transcripts increased, whereas the amount of PDE4B mRNA transcripts was less affected [113]. Identification of the amazing number of splice variants of PDE4A-D subtypes was paralleled by findings that some but not all splice variants were transcriptionally regulated by cAMP. Among the PDE4D splice variants, cAMP increased mRNA transcripts of the short forms PDE4D1 and PDE4D2. In contrast, mRNA transcription of the long form PDE4D3 was not affected by cAMP [114]. In human monocytic MonoMac-6 cells exposed to dibutyryl cAMP mRNA transcripts for PDE4A5, PDE4B2 and PDE4D1 increased [70]. Notably, although PDE4A5 represents a long form splice variant its mRNA transcription appears to be regulated by cAMP. These findings are corroborated by data obtained in human monocytic U-937 cells [90, 115] and human monocytes [61] where increases in PDE4A and PDE4B mRNA transcripts and protein were observed following long-term combined exposure to rolipram and salbutamol although in these cells PDE4D was not affected. On the other hand, incubation of Jurkat-T cells with forskolin augmented the amount of mRNA transcripts for PDE4D1 and PDE4D2 but also PDE3, whereas PDE4D3, PDE4D4, PDE4B and PDE4C were not affected and mRNA transcription of a PDE4A variant decreased [100]. The mechanism of cAMP-dependent up-regulation of PDE4D1-mRNA transcripts has recently been investigated [116]. An intronic, cAMP-responsive promoter region has been identified in the PDE4D gene. Activation of this 5' upstream intronic promoter region directs transcription of truncated mRNA products from the PDE4D gene – the short forms PDE4D1 and PDE4D2. A TATA-box was not detected in this promoter sequence but instead GC-rich regions were found which may also serve to signal the transcription start site. RNase protection assays revealed at least two cap sites for transcription initiation. DNA-sequence encompassing 1500 bp 5' upstream of the translation start site coupled to a luciferase reporter gene resulted in ~five-fold activation of baseline luciferase expression. cAMP further increased luciferase expression in this construct by a factor of ~4.5. These results demonstrated that the intron 5' upstream of the PDE4D1 open reading frame acts as promoter and confers cAMP responsiveness. A cAMP response element (CRE) was identified in the 5' untranslated region of the transcribed PDE4D1 mRNA. No further CRE were detected up to 1500 bp 5' upstream of the translation initiation site. However, deletion of a region comprising the CRE within the 1500 bp 5' upstream of the translation initiation site resulted in a fragmented promoter that still conferred cAMP-inducibility of a luciferase reporter gene. Hence, cAMP may act to increase PDE4D1 mRNA transcription by mechanisms independent from CREB-induced CRE activation. AP-consensus sites have been identified upstream of the proximal cap site and there is evidence that cAMP may be capable of amplifying AP-mediat-

ed transcription. In fact, *c-fos* expression, AP-binding to DNA and AP-directed transcription of reporter genes may be enhanced by cAMP [117–119]. In view of these findings the observation may be of interest that TPA – a potent stimulus of AP-induced transcription – synergistically potentiated cAMP-induced luciferase expression directed by the PDE4D1 promoter [116]. In parallel to PDE4D1, mRNA transcription of PDE4B2 is also regulated by a cAMP-responsive intronic promoter. However, this promoter includes a TATA-box that confers efficient baseline mRNA transcription [116]. A more rapid cAMP-responsive up-regulation of PDE4B2 mRNA compared to PDE4D1 mRNA was observed in human monocytic MonoMac 6 cells [70].

Heterologous desensitization by PDE4 up-regulation

cAMP-dependent transcriptional regulation of PDE4 may cause heterologous desensitization of cellular responses induced by β_2 -agonists or other cAMP-generating drugs. In human monocytic U-937 cells exposed to rolipram in combination with salbutamol over 4 h, PGE₂-induced cAMP accumulation was dramatically reduced compared to control cells. The additional presence of rolipram restored this impaired cAMP accumulation indicating that it was operated by PDE4 up-regulation. In parallel, the capacity of PGE₂ to suppress monocytic LTD₄-induced Ca²⁺-mobilisation was substantially reduced in U937 cells exposed to salbutamol and rolipram for 4 h compared to control cells. Again, rolipram reversed this functional desensitization [115]. An impaired capacity for cellular cAMP accumulation as a consequence of protracted cAMP elevation has also been shown in Jurkat-T lymphocytes [120], rat aortic smooth muscle cells [107] and the human keratinocyte HaCaT cells [109]. In these systems, cAMP-dependent PDE4 up-regulation based on *de novo* protein synthesis has been shown and PDE inhibitors could reverse the concomitant desensitization of cAMP accumulation.

Heterologous desensitisation following prolonged administration of β_2 -agonists may have therapeutic implications in asthma [121]. In asthmatics, regular treatment with β_2 -agonists resulted in tachyphylaxis to their protective effects against methacholine- or AMP-induced bronchoconstriction [1–4]. Although part of these effects may originate in β -receptor desensitisation, the hypothesis was raised that PDE4 up-regulation may also be involved [121]. Future clinical trials may investigate whether PDE inhibitors e.g. theophylline restore the impaired bronchoprotective effects of β_2 -agonists caused by their regular administration.

cAMP-dependent transcriptional regulation of PDE4 may drive the cardioprotective effects of prostacyclin in the ischaemic myocardium. In an experimental approach, hearts removed from rats pretreated intramuscularly with 7-oxo PGI₂ for 48 h exhibited a substantially impaired inotropic response to isoproterenol associated with reduced isoproterenol-induced cAMP accumulation compared to sham-

treated animals. Whereas the capacity of the cAMP-generating system was found to be unaffected, exposure of intact rats to 7-oxo PGI₂ induced a marked alteration in the pattern of myocardial PDE subtypes. Strong increases in PDE1C and PDE4B3 transcripts were described accompanied by reduced expression of PDE4D1–3 species. These alterations in PDE transcripts were paralleled by changes in protein levels and by increased PDE4 and PDE1 activities [122, 123]. The increased PDE1C expression induced by 7-oxo prostacyclin may limit Ca²⁺-induced myocardial damage by enhancing Ca²⁺-dependent down-modulation of myocardial cAMP. Other experiments demonstrated that pharmacologically induced impaired or activated noradrenergic signalling in the intact rat is associated with reduced or enhanced PDE4A protein, respectively in cerebral cortex [124]. These latter studies with intact animals provide convincing evidence for the physiological relevance of cAMP-operated transcriptional PDE4 up-regulation.

Protein kinase A-induced phosphorylation and up-regulation of PDE4

A substantial but transient increase in cAMP content of U937 cells is induced by salbutamol or PGE₂. In fact, cAMP levels returned to baseline after 15 min exposure to salbutamol or PGE₂. However, the combination of salbutamol with rolipram exerts a profound cAMP increase lasting over >4 h [42, 105]. Desensitization of the cAMP-generating system may provide an explanation for these findings. Alternatively, a short-term up-regulation of PDE4 may be involved. This hypothesis is particularly supported by the marked protracted cAMP increase induced by the addition of rolipram. Indeed, Torphy and colleagues [105] observed a significant increase of PDE4 activity after only 15 min incubation of U937 cells with PGE₂ (10 μM) or salbutamol (10 μM). Corroborating these data, PDE4 in human keratinocyte HaCaT cells was significantly up-regulated after 30 min incubation with rolipram (10 μM) and salbutamol (1 μM) and PDE4 up-regulation was only partly inhibited by cycloheximide [109]. In addition, the PDE4 activity increase following incubation of rat thyroid FRTL-5 cells with TSH, dibutyryl cAMP or forskolin over 10–15 min was resistant to cycloheximide, supporting the concept that short-term PDE4 up-regulation was independent from *de novo* protein synthesis [125]. On the other hand, the phosphatase inhibitor okadaic acid enhanced PDE4 up-regulation in FRTL-5 cells and in consequence the concept arose that short-term cAMP-induced increase in PDE4 activity may be driven by PDE4 phosphorylation. Indeed, protein kinase A phosphorylates PDE4 which was paralleled by an increase in PDE4 activity in rat thyroid FRTL-5 cells [43, 125] and human monocytic U937 cells [42]. Protein kinase A-induced short-term up-regulation of PDE4 activity has also been described in rat parotid tissue [126], UMR-106 osteoblast like cells [127], LRM 55 astroglial cells [128] or bovine vascular smooth muscle cells [129] indicating that PDE4 activity regulation by cAMP-

mediated phosphorylation represents an ubiquitous event. However, protein kinase A-triggered PDE4 phosphorylation is restricted to particular splice variants. As an example, within the PDE4D subfamily PKA phosphorylates the long form PDE4D3 but not the short forms PDE4D1 and PDE4D2 [42, 43, 125]. Meanwhile, based on elegant experiments using site directed mutagenesis, Sette and Conti [43] discovered that protein-kinase A mediated PDE4D3 activity increase is associated with phosphorylation of serine 54 at the N-terminal end of the enzyme. In fact, substitution of serine 54 by alanine completely abolished phosphorylation and PDE-activity increase. Furthermore, as a consequence of protein kinase A-triggered phosphorylation of PDE4D3, altered sensitivity of the enzyme to PDE4 inhibitors occurred. RS-25344, a highly specific PDE4 inhibitor blocked activity of the phosphorylated PDE4D3 form with ~100-fold higher potency than the non-phosphorylated enzyme [42]. From experiments with rolipram the hypothesis was raised that PDE4D3 phosphorylation tips the balance of HAR to LAR conformers in favour of the HAR conformer [43]. Careful analysis of log concentration inhibition curves revealed that rolipram displayed high ($IC_{50} \sim 1$ nM) and low ($IC_{50} \sim 1$ μ M) affinity inhibition of basal PDE4D3 catalytic activity. These IC_{50} values are close to those obtained for rolipram binding to HAR and LAR. Phosphorylation-induced PDE4D3 activity increment was almost entirely based on an increase in the PDE activity that was inhibited by low nanomolar rolipram concentrations. In contrast, activity of the PDE4D3 conformer inhibited by micromolar rolipram concentrations was not affected. These findings imply an increase in HAR conformation induced by PDE4D3 phosphorylation. Rolipram log concentration-inhibition curves suggest that 72% of basal PDE4D3 assumes the LAR conformer with the remaining 28% in the HAR state. In contrast, regarding the phosphorylated PDE4D3 62% of the enzyme forms the HAR state with only 38% in the LAR conformation [43]. In view of the concept that anti-inflammatory effects of PDE4 inhibitors are related to LAR whereas unwanted CNS effects are based on their interaction with HAR those PDE4 inhibitors which lock PDE4D3 in the non-phosphorylated state should provide anti-inflammatory drugs with reduced side-effects.

Regulation of PDE4 activity by phosphatidic acid

Phosphatidic acid (PA) was found to up-regulate PDE4 activity of some but not all variants, most probably based on a direct interaction. In particular, incubation of the long forms PDE4A5, PDE4B2 and PDE4D3 with PA resulted in a 1.5–2-fold up-regulation of PDE4 activity. On the other hand, activities of the short forms PDE4B2, PDE4D1 and PDE4D2 were not affected. PA-induced PDE4 up-regulation may facilitate Con-A stimulated proliferation of rat thymocytes [130, 131].

Candidate PDE4 inhibitors as antiasthmatics

Based on the fact that PDE4 was localized in almost all inflammatory cells and also in bronchial smooth muscle, and that PDE4 inhibitors have the capacity to suppress diverse inflammatory cell functions, it was anticipated that PDE4 inhibitors would be potent anti-asthmatic drugs. As discussed before, although an abundance of PDE4 inhibiting structures were synthesized and exhibited anti-inflammatory potency *in vitro* and in experimental animals none of them has been licensed or reached phase III clinical trials mainly due to intolerable CNS side-effects. In fact, definite proof of antiasthmatic potency of PDE4 inhibitors in large-scale controlled clinical studies is still awaited. However, based on the concept that a beneficial ratio of LAR/HAR inhibition should improve the benefit-risk ratio there are some promising PDE4 inhibitors which recently reached phase I or II clinical trials. These compounds designed in consideration of the LAR/HAR-concept have been denominated as "second generation PDE4 inhibitors". Chemical structures of the compounds are depicted in Figure 1. Affinity ratios to HAR and LAR of these compounds related to rolipram are given in Table 5.

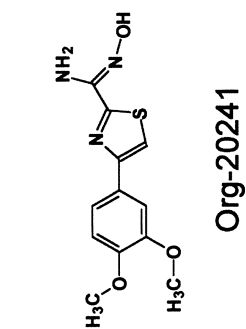
SB207499 (Ariflo®)

SB207499 (Ariflo®) represents a second generation PDE4 inhibitor. The potency ratios for inhibition of the HAR vs LAR conformers of PDE4A, PDE4B2 and PDE4D3 are at least four-fold higher than for rolipram implying that SB207499 in comparison to rolipram preferentially inhibits PDE4 in its LAR conformer (see also Tab. 5). Whereas rolipram inhibits the HAR conformer of all PDE4 variants tested with at least 20-fold higher potency than the LAR conformers, SB207499 inhibits the LAR conformer of PDE4A with two-fold higher potency than its HAR conformer and for PDE4B2 and PDE4D3 inhibition of HAR was only about two-fold and five-fold more potent than for LAR. In fact, binding experiments revealed that SB207499 adhered to a two-fold higher number of binding sites on PDE4 than rolipram. This finding reiterates that SB207499 has the capacity to bind to both HAR and LAR [41].

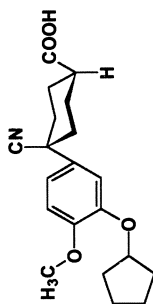
This distinct profile of SB207499 affinity to the PDE4 conformers is reflected by its *in vitro* effects. SB207499 inhibits T cell proliferation and monocyte TNF α generation with comparable potency as rolipram. This is in accordance with earlier findings that inhibition of these inflammatory functions is mediated by LAR. In contrast, SB207499 proves to be ~160-fold less potent than rolipram in attenuating gastric gland acid secretion [132]. On the other hand, anti-IgE induced basophil degranulation was clearly more potently inhibited by rolipram than by SB207499 and this implies that similar to neutrophil degranulation, inhibition of basophil degranulation requires blocking of the HAR conformer. The compound is currently being clinically tested as an oral anti-asthmatic drug.

Table 5 - Inhibition of HAR and LAR by PDE4 inhibitors. PDE4 inhibitors may be distinguished by their affinities to the two PDE4 conformers. IC₅₀ [μM] values for inhibition of catalysis (IC₅₀) reflecting affinity to LAR as well as for inhibition of [³H]rolipram binding (RB) reflecting affinity to HAR are given. Results from different authors (references in brackets) are documented. The relative ratios were calculated from the ratios IC₅₀/RB for each of the compounds related to 100% which was defined as the ratio IC₅₀/RB for rolipram. It becomes evident that particularly SB207499, CDP840, RP73401 and benafentrine exhibit preferential affinity to LAR compared to rolipram, Ro-201724, denbutylline or RS25344.

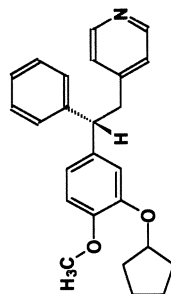
	[27]		[34]		[133]		[37]		relative ratio			
	IC ₅₀	RB	IC ₅₀	RB	IC ₅₀	RB	IC ₅₀	RB	[27]	[34]	[133]	[37]
Rolipram	0.31	0.0017	0.103	0.005	2.2	0.006	0.63	0.007	100	100	100	100
Ro 20-1724	2.4	0.0170					9.34	0.1	77			104
Denbutylline	0.2	0.0041	0.123	0.019			0.54	0.021	27	31		28.6
CDP840			0.007	0.06	0.143	0.008				0.6	4.8	
SB207499			0.05	0.077	0.149	0.077				3.2	0.5	
RP 73401	0.0012	0.0004	0.0005	0.005	0.008	0.001			1.6	0.5	2.1	
CP80366					0.5	0.008					16.7	
RS25344			0.011	0.001						53		
Zardaverine							1.11	0.45				2.7
Benafentrine							2.19	7.5				0.3



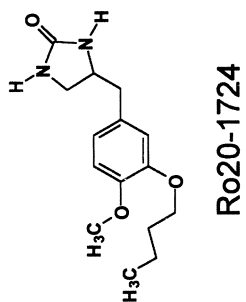
Org-20241



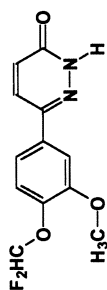
Ariflo



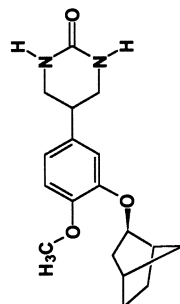
CDP840



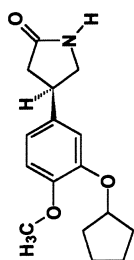
Ro20-1724



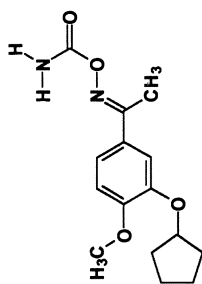
Zardaverine



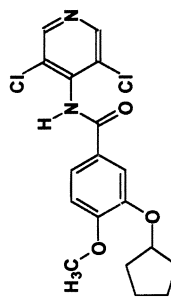
CP-80633



Rolipram



WAY-PDA-641



Piclamilast

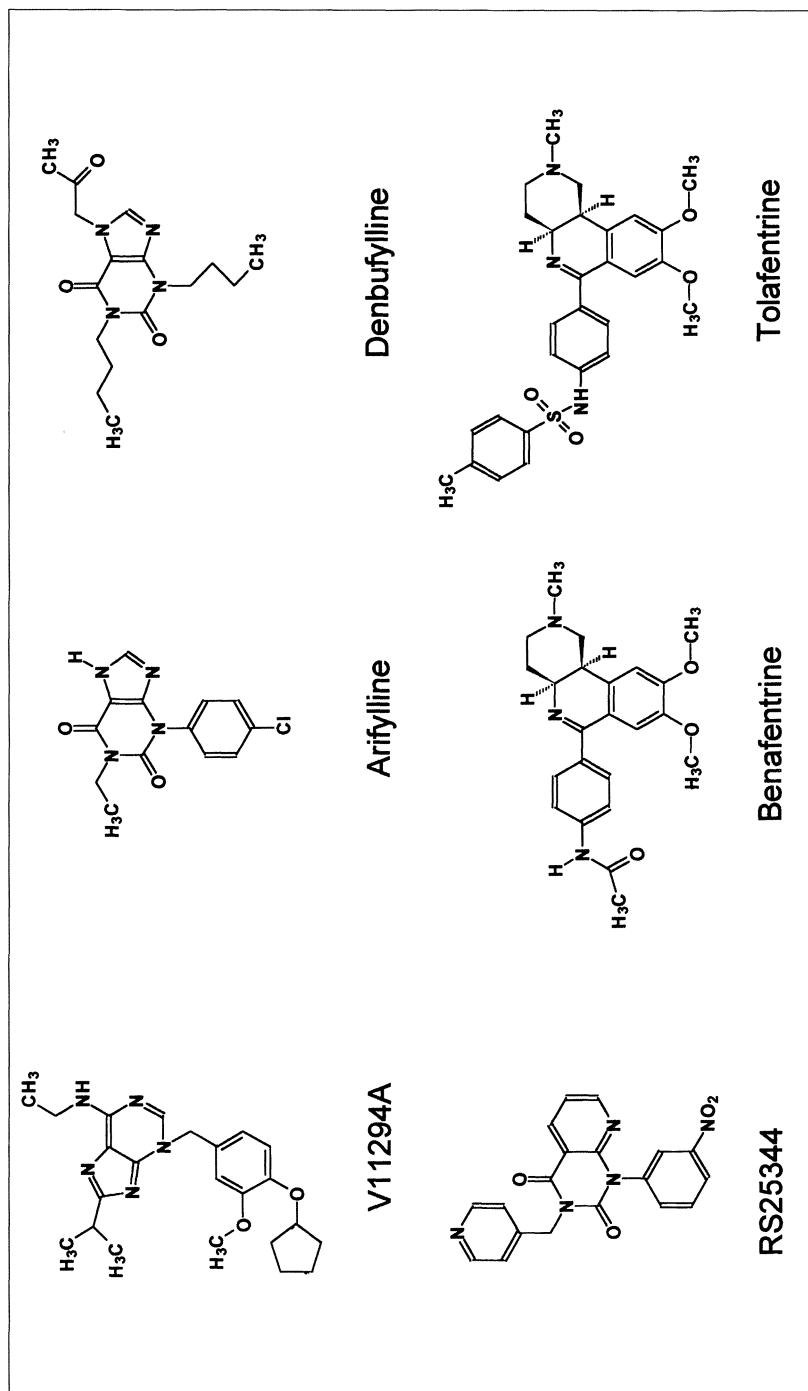


Figure 1
 Chemical structures of PDE4 inhibitors. Zardaverine, tolafentrine, benafentrine and Org-20241 are dual-selective PDE3&4 inhibitors. The other compounds are selective inhibitors of PDE4. Most structures are related to rolipram e.g. piclamilast (RP73401), ariflo (SB207499), CP-80633, WAY-PDA-641, CDP840 or V11284A. Tolafentrine and benafentrine are benzozaphthyridines.

CDP840

CDP840 was shown to suppress PDE4 hydrolysing capacity of subtypes A, B and D with comparable potency ($IC_{50}=2.9\text{--}4.3$ nM) whereas the compound inhibited PDE4C ~25-fold less potently. In addition, CDP840 was less efficient in competing for [3H]rolipram binding to guinea pig brain membranes ($IC_{50}=60$ nM) than in inhibiting PDE4 activity (Tab. 5). CDP840 had a fairly high oral bioavailability. These findings should predict a favorable profile of CDP840 [34]. However, other recent investigations came to different conclusions based on their findings that CDP840 inhibited catalytic PDE4 activity (reflecting LAR) less potently than the compound competed with [3H]rolipram binding to brain membranes [133]. In the above studies, competition of [3H]rolipram binding has not been measured with the recombinant human PDE4 variants that served for determination of PDE4 catalysis but with guinea pig and mice brain membranes. The discrepancy between the two studies emphasizes that PDE4 catalysis and [3H]rolipram-binding should be assessed with identical human recombinant PDE4 variants.

In animal models CDP840 mostly given by the intraperitoneal route has been shown to reduce antigen-induced pulmonary eosinophil accumulation and bronchoconstriction and to attenuate ozone-triggered airway hyperreactivity [34, 134–136]. Finally, in mild-moderate asthmatic humans CDP840 given orally at a daily dose of 30 mg over 10 days significantly suppressed late airway obstruction to allergen challenge. An oral dose of 30 mg/day was well tolerated as reflected by the absence of adverse events [137]. However, development of CDP840 was discontinued.

CP80366

CP80633 inhibits PDE4A, B and D with comparable IC_{50} values (0.3–0.5 μM), whereas [3H]rolipram binding to brain membranes is competed at substantially lower concentrations (8 nM) (Tab. 5) [133]. Therefore, CP80633 does not represent a second generation PDE4 inhibitor. The compound stimulated PGE_1 -facilitated cAMP increase in human monocytes, inhibited LPS-triggered human monocytic $TNF\alpha$ -generation and zymosan-stimulated human eosinophil ROS release with IC_{50} values close to those required for suppression of catalysis [133]. The capacity of CP-80633 to counteract allergen-challenge induced bronchoconstriction and bronchoalveolar cellular infiltration in animal models further adds to the anti-inflammatory profile of the compound. In another system it was found that CP-80633 given by the oral route (10 mg/kg) to mice elevated plasma cAMP by a factor of 6 and inhibited LPS-induced systemic $TNF\alpha$ production by 95% [138]. The compound has been tested in phase II trials in patients with atopic dermatitis. It was found that topical application of CP-80633 over 28 days significantly reduced

skin inflammation in these patients. Oral administration of CP80633 to humans resulted in emesis at plasma concentrations above 0.16 $\mu\text{g/ml}$ [139].

RP73401

The benzamide RP73401 (Piclamilast) was the first described second generation PDE4 inhibitor. In fact, in the catalytic assay (reflecting LAR) RP73401 suppressed human recombinant PDE4D3 with IC_{50} of 0.1 nM and the benzamide exhibited a comparable affinity to PDE4D3 ($K_d = 0.08$ nM) as determined in the [^3H]RP73401 binding assay (reflecting HAR). RP73401 attenuated catalytic activity of PDE4A and PDE4B with comparable efficacy as PDE4D and competed for [^3H]rolipram binding to brain membranes with $\text{IC}_{50} = 1$ nM i.e. in the range of PDE4 catalysis inhibition [34, 41, 133]. In conclusion, RP73401 does not discriminate between the HAR and LAR conformers whereas in contrast rolipram exhibits clear preference for the HAR conformer (Table 5).

In vitro, RP73401 enhanced β_2 -agonist facilitated eosinophil and monocyte cAMP increase and consequently inhibited eosinophil LTC_4 and ROS generation, eosinophil degranulation [20, 77, 140], LPS-induced $\text{TNF}\alpha$ expression in human monocytes [27], and IL-2 release from *Staph. aureus* enterotoxin stimulated CD3-differentiated splenocytes [29] in the lower nanomolar range. Rolipram was substantially less potent than RP73401 in attenuating monocyte $\text{TNF}\alpha$ generation and splenocyte differentiation, reiterating that these functional responses are inhibited by interaction with the LAR (where rolipram is less potent than RP73401) rather than the HAR conformer (where rolipram shows comparable potency to RP73401) of PDE4 (Tab. 5). On the other hand RP73401 counteracted methacholine-induced contraction of guinea pig trachea which is considered as an HAR-correlated response [38] (Tab. 2) in a dose range comparable to rolipram [46]. In animal models of airway inflammation RP73401 given by the intratracheal route dose-dependently inhibited antigen-induced bronchoconstriction and bronchoalveolar inflammatory cell influx. In addition, RP73401 counteracted histamine-induced microvascular leakage into the guinea pig lung [141]. The compound is currently being developed for rheumatoid arthritis [139].

Phosphodiesterase 3

PDE3 is also described as the cGMP-inhibited PDE activity. In fact, although PDE3 exhibits similar affinity for cAMP and cGMP as substrates ($K_M = 0.1\text{--}0.8$ μM), the V_{MAX} for cAMP is about 4–10 times higher compared to cGMP. Abundant PDE3 activity is found in the myocardium where it may be tightly associated with the sarcoplasmic reticulum. Therefore, there was considerable interest

from the pharmaceutical industry in developing selective PDE3 inhibitors as inotropics in chronic heart failure (CHF) culminating in the clinical development of amrinone, milrinone and enoximone. Whilst these compounds have meanwhile been licensed in some regions for acute treatment of otherwise refractory CHF where they result in some improvement of systemic haemodynamics, its chronic administration has been discouraged based on clinical trials demonstrating a variety of haemodynamic adverse events and finally, increased mortality [142–144]. Indeed, in the severely damaged ischemic myocardium cAMP increase may facilitate the generation of arrhythmias.

So far two different PDE3 subtypes encoded by different genes have been identified. PDE3A is located on chromosome 12 and PDE3B on chromosome 11 [145]. PDE3A and PDE3B have a different cellular expression pattern. PDE3A is predominantly found in myocardium, vasculature, bronchial smooth muscle, platelets. In contrast, human lymphocytes [146] but also adipocytes, hepatocytes, pancreatic B cells and renal epithelial cells [145] contain PDE3B. Hence, the hypothesis arises that selective inhibition of PDE3B should support anti-inflammatory effects without cardiovascular adverse effects.

Subtypes are distinguished by a 44 amino acid insert in the N-terminal end of the catalytic core which is highly homologous for one subtype among different species but shows substantially less homology if the two subtypes are compared. This 44 amino acid insert is unique for PDE3 species. Binding of PDE3 selective inhibitors is exclusively mediated by the catalytic domain since it is not affected by either N-terminal mutants or site directed mutants in the 44 amino acid insert [147]. The N-terminal region contains a hydrophobic site that directs membrane association. Expression of full-length human recombinant PDE3A and PDE3B in SF9 cells results in membrane associated PDE3 activity. On the other hand, expression of PDE3 forms truncated at their N-terminal end results in predominant cytosolic expression [148]. It is not known so far whether the platelet PDE3A species which is cytosolic represents a splice variant that omits the hydrophobic membrane association region. The occurrence of multiple splice products from PDE3A has been proposed based on the use of different initiation sites in the PDE3A gene [149]. The N-terminal region also comprises the protein kinase A phosphorylation site. In rat adipocytes PDE3B serine 302 has been identified as a target for PKA-induced phosphorylation [150]. PKA-induced phosphorylation is associated with up-regulation of PDE3 activity. In addition to phosphorylation, transcriptional regulation of PDE3 has been observed. In Jurkat cells, chronic cAMP-exposure resulted in a biphasic PDE3 up-regulation [100]. The initial PDE3 increase based on phosphorylation whereas the later PDE3 up-regulation was reversed by prior incubation with actinomycin D indicating that enhanced mRNA transcription was involved. Exposure of vascular smooth muscle cells in culture to forskolin or 8-bromo cAMP also enhanced PDE3 activity based on *de novo* protein synthesis [107]. In an animal model of pulmonary hypertension induced by chronic hypoxia there was an up-reg-

ulation of PDE3A paralleled by an elevation of the corresponding mRNA detected by Northern blot technique [151].

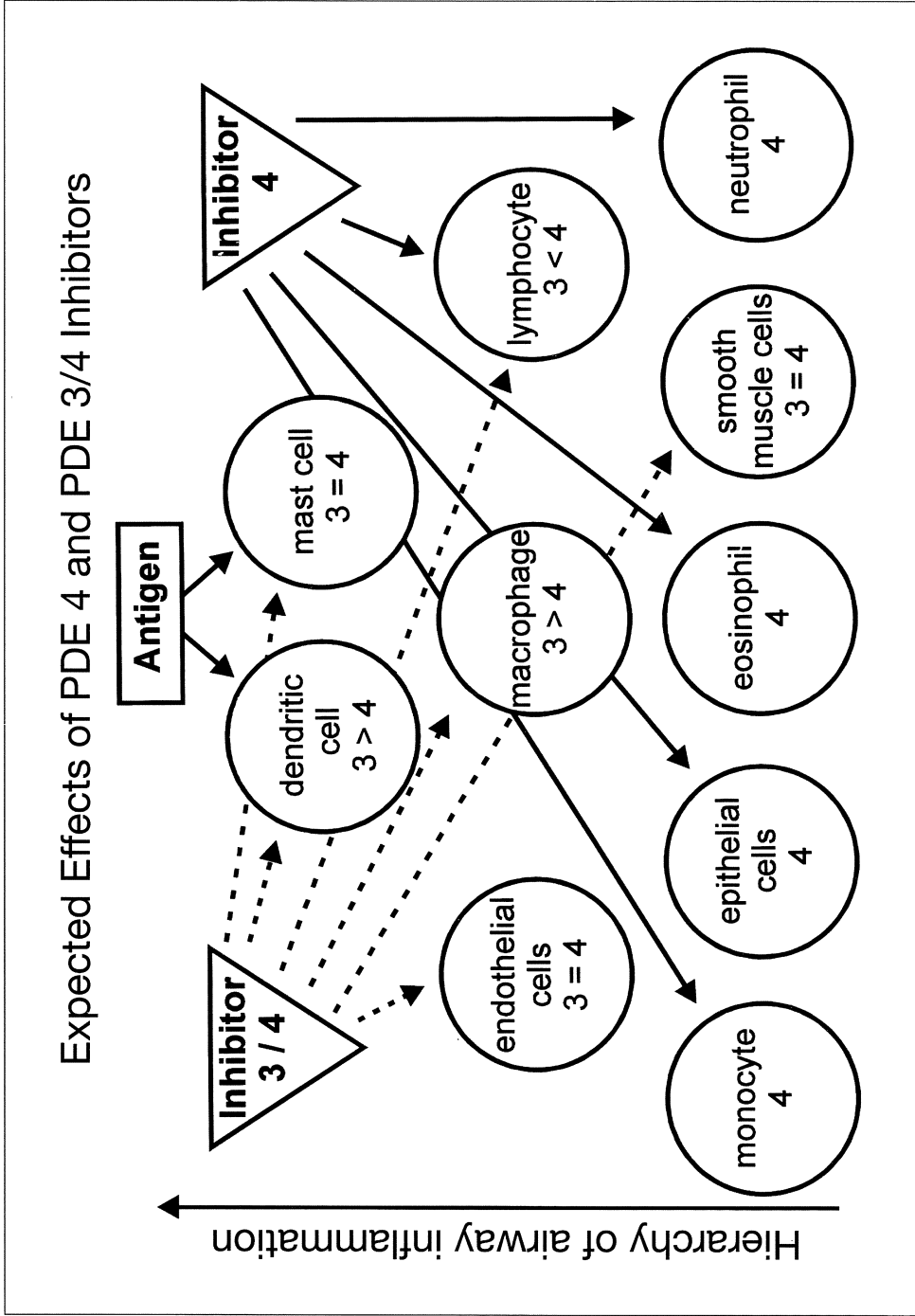
PDE3 occurs in macrophages and T lymphocytes, endothelial cells, mast cells, bronchial and vascular smooth muscle cells. Selective PDE3 inhibitors may have some functional effects in animal models relevant to asthma. In particular, selective PDE3 inhibitors may cause bronchodilation as a consequence of the occurrence of PDE3 in human bronchi. In fact, it was found that the selective PDE3 inhibitor SKF94120 reduced the basal tone of human bronchial rings [152]. In a clinical setting, the PDE3 selective inhibitor cilostazol attenuated methacholine-induced bronchial hyperresponsiveness [153].

Whilst exclusive inhibition of PDE3 does not represent a promising strategy for drug development in asthma the fact that additional inhibition of PDE3 frequently amplifies the effects of PDE4 inhibitors implies that dual selective type PDE3 and 4 inhibitors could be an attractive concept in asthma-related drug discovery. This may be of particular interest since compounds such as benafentrine interact predominantly with LAR (Tab. 5). In the last section of this chapter, synergisms between PDE3 and PDE4 inhibition will be briefly discussed.

Synergistic and additive effects of PDE3 and 4 inhibition

PDE3 and PDE4 inhibitors may act synergistically to enhance intracellular cAMP and consequently, to modulate functional responses in cells orchestrating the inflammatory response (Fig. 2). Quiescent human peripheral blood CD4⁺ and CD8⁺-T lymphocytes expressed PDE3B [145], PDE4A, PDE4B and PDE4D and PDE7. On the other hand, PDE1, PDE2 and PDE5 were absent in these cells [98, 154, 155]. Following PHA stimulation for several hours however, there was an induction of PDE1B [10]. PDE3 inhibitors, whilst almost inactive by themselves, synergistically enhanced reduction of T lymphocyte proliferation and IL-2 generation by inhibition of PDE4. 100 nM rolipram inhibited PHA-induced [³H]thymidine incorporation in human CD4⁺-T lymphocytes by about 20%. The selective PDE3 inhibitor SKF95654 (10 μM) did not affect [³H]thymidine incorporation. Adding 10 μM SKF95654 to 100 nM rolipram resulted in about 50% reduction of PHA-induced [³H]thymidine incorporation. Similarly, addition of 10 μM SKF95654 which by itself did not affect IL-2 generation to 10 nM rolipram that induced 25% inhibition of IL-2 synthesis resulted in 60% reduction of PHA-induced IL-2-generation in CD4⁺-T lymphocytes [98].

In human alveolar macrophages there is coexpression of PDE1, PDE3 and PDE4 [9]. Rolipram or the PDE3-selective inhibitor motapizone suppressed LPS-stimulated TNFα generation dose-dependently following biphasic log concentration inhibition curves. These biphasic curves reflect functional effects of selective PDE3 or PDE4 inhibition at lower inhibitor concentrations, whereas at higher con-



centrations rolipram or motapizone lost their selectivity and additionally inhibited other isoenzymes. At concentrations $< 1 \mu\text{M}$ motapizone synergistically amplified the reduction of $\text{TNF}\alpha$ release induced by rolipram [156]. These data were corroborated by findings that in human monocyte-derived macrophages LPS-stimulated $\text{TNF}\alpha$ generation was synergistically reduced by exposure to a combination of PDE3 and 4 inhibitors (40–50% reduction) when compared to each of the selective inhibitors alone ($< 15\%$ reduction) [8]. Monocyte-derived macrophages showed a PDE isoenzyme profile comparable to human alveolar macrophages; PDE1, 3 and 4 were expressed [8]. In contrast, LPS-induced $\text{TNF}\alpha$ synthesis in human monocytes (i.e. the precursor cells of monocyte derived macrophages) was almost completely inhibited by rolipram reflecting the predominant expression of PDE4 in these cells [8].

Rolipram synergistically enhanced inhibition of serum-stimulated [^3H]thymidin incorporation into vascular smooth muscle cells by the PDE3 inhibitor cilostamide [157]. With $1 \mu\text{M}$ cilostamide and $10 \mu\text{M}$ rolipram, [^3H]thymidine incorporation was attenuated by about 25% and 20%, respectively. Combined administration resulted in 80% reduction. Rat aortic rings precontracted with phenylephrine showed less than 20% relaxation to either $3 \mu\text{M}$ milrinone (PDE3 selective) or $10 \mu\text{M}$ rolipram. However, the combined inhibition of PDE3 and PDE4 by these compounds resulted in $> 90\%$ relaxation. Human pulmonary artery precontracted with $\text{PGF}_{2\alpha}$ did not respond to $1 \mu\text{M}$ rolipram. Exposure to $1 \mu\text{M}$ motapizone (selective PDE3 inhibitor) induced about 30% relaxation. However, $1 \mu\text{M}$ rolipram was synergistic to $1 \mu\text{M}$ motapizone resulting in 75% relaxation [158]. Similarly, in human bronchi, combined exposure to rolipram and the PDE3-selective inhibitor SKF94120 showed a trend for overadditive reduction of inherent airway tone [152].

Figure 2

Targets of PDE4 and PDE3/4 inhibitors. A multitude of cells are involved in airway inflammation. Dendritic cells function as antigen presenting cells whereas antigen directly activates mast cells. Lymphocytes orchestrate airway inflammation. Eosinophils or neutrophils predominantly constitute terminal effector cells i.e. cytotoxic proteins (ECP, EDN) are released as a consequence of eosinophil activation. Smooth muscle cells may not only exhibit a contractile phenotype but they may also assume proliferative, motile or synthetic (cytokine producing) phenotypes. Epithelial and endothelial cells generate a multitude of cytokines and other mediators apart from their barrier function. Terminal effector cells e.g. eosinophils exclusively express PDE4. On the other hand in cells orchestrating inflammation e.g. T-lymphocytes or in antigen-presenting cells e.g. dendritic cells PDE3 and PDE4 are detected. In these cells selective PDE4 inhibitors partially but not completely inhibit functional responses. However, complete inhibition is attained by dual-selective PDE3&4 inhibitors. Dual-selective PDE3&4 inhibitors are therefore an attractive approach in asthma management.

cAMP-dependent transcriptional and post-translational up-regulation (PKA-dependent phosphorylation) of PDE3 and PDE4 may be one explanation for the synergistic functional effects of PDE3 and PDE4 inhibitors. One may postulate that exclusive selective inhibition of PDE3 may provide a cAMP-signal that phosphorylates PDE4 or stimulates transcription of PDE4 mRNA. Increased PDE4 activity masks functional effects of PDE3 inhibitors by down-regulating cAMP. Simultaneous PDE4 inhibition would then result in synergistic cAMP response and functional effect.

Synergisms between PDE3 and PDE4 inhibitors and the occurrence of both PDE3 and PDE4 in diverse cellular systems involved in asthma were decisive for the design of dual-defective PDE3 and 4 inhibitors. Preclinical and some clinical experience with the dual-selective inhibitors zardaverine, AH21-132 and tolafentrine will be briefly reviewed.

Zardaverine

The pyridazinone derivative zardaverine inhibited PDE3 from human platelets and PDE4 from human neutrophils with IC_{50} values of 0.6 and 0.2 μ M, respectively [159]. Other isoenzymes were hardly affected up to 100 μ M. Zardaverine inhibited human monocyte PDE4 with IC_{50} 1.1 μ M. For competition of zardaverine with [3 H]rolipram binding to rat brain membranes an IC_{50} of 0.45 μ M was calculated (Tab. 5) [37]. However, zardaverine more strongly inhibited [3 H]rolipram binding to PDE4 of human origin. Zardaverine counteracted the contraction of guinea pig trachea in response to a variety of spasmogens (e.g. histamine, ovalbumin, U46619, and LTC_4) [160]. In addition, zardaverine potently reduced endogenous tone of isolated human bronchi [152]. In the anaesthetized guinea pig zardaverine given intravenously (1–60 μ mol/kg) inhibited histamine induced bronchoconstriction, and following oral application (1–100 μ mol/kg) attenuated acetylcholine- or ovalbumin-induced dyspnoea [160]. In the rat, oral zardaverine (3–30 μ mol/kg) shows bronchodilator activity [161].

Zardaverine protects against a multitude of inflammatory responses (for review refer to [162]). In particular, zardaverine inhibited LPS-stimulated $TNF\alpha$ release from human alveolar macrophages and human T lymphocyte proliferation with monophasic log concentration-inhibition curves, corresponding to IC_{50} values of 1.85 and 1.7 μ M, respectively [156]. Zardaverine inhibited the increase in endothelial permeability of HUVEC (human umbilical vein endothelial cell) monolayers induced by thrombin or *E. coli* haemolysin and this effect was further accentuated in combination with adenylate cyclase activators [163]. In cultured porcine pulmonary artery endothelial cells, zardaverine at concentrations up to 10 μ M had no effect on H_2O_2 -induced endothelial hyperpermeability. Endothelial cells from porcine pulmonary artery expressed PDE1, 2, 3 and 4, and PDE3 and 4 should have

been strongly inhibited by 10 μM zardaverine. However, in the presence of 1 μM PGE_1 that was inactive on its own, 10 nM zardaverine completely inhibited H_2O_2 -induced increase in endothelial permeability. Hence, PGE_1 synergistically potentiated the efficacy of zardaverine by a factor of > 10,000. In addition, in the presence of PGE_1 , synergistic suppression of endothelial hyperpermeability by combined PDE3 and PDE4 inhibition may be deduced from the experimental findings. 10 nM rolipram or 10 nM motapizone did not reduce the H_2O_2 -induced increase in endothelial permeability in the presence of 1 μM PGE_1 . However, 10 nM zardaverine completely inhibited H_2O_2 -induced increased endothelial permeability. 10 nM zardaverine afforded only ~10% inhibition of PDE3 and 4 which is less than the extent of PDE3 and 4 inhibition obtained by combining 10 nM rolipram and 10 nM motapizone (25% inhibition) [164].

Provided salbutamol (1 μM) was present, zardaverine inhibited C5a-stimulated human eosinophil degranulation and chemoluminescence with IC_{50} values (0.4–0.9 μM) in the range of those obtained for PDE3 and PDE4 inhibition. In contrast, zardaverine up to 10 μM was inactive when salbutamol was absent [20]. On the other hand zardaverine attenuated fMLP-stimulated human eosinophil LTC_4 synthesis in the absence of salbutamol (IC_{50} = 0.3 μM) [140].

The finding that cAMP-triggered inhibition of some functional responses (e.g. eosinophil LTC_4 generation) is operated by PDE inhibition alone whereas reduction of other responses (e.g. eosinophil degranulation, endothelial hyperpermeability) requires the additional presence of an adenylate cyclase enhancing stimulus merits attention. The following explanations may be provided. Firstly, cAMP-triggered modulation of different functional responses may require different thresholds of protein kinase A activation. Secondly, cells and membrane compartments may differ regarding their expression of “high cycling” and “low cycling” adenylate cyclase subtypes. “High cycling” adenylate cyclase offers high baseline cAMP generation whereas “low cycling” adenylate cyclase elaborates less cAMP under baseline conditions. It appears evident that coincidence of “low cycling” conditions with a high threshold of PKA-activation requires both inhibition of PDE activity and stimulation of cAMP generation to modulate a functional response [165]. In fact, in an elegant experimental setting, Underwood and colleagues recently demonstrated [166] that endogenous catecholamines were required for rolipram-induced inhibition of antigen-induced bronchial obstruction in the anaesthetized guinea pig to be apparent. Both β_2 -antagonists and adrenalectomy dramatically reduced the anti-obstructive potency of intravenously given rolipram [166]. On the other hand, PDE inhibition alone may suppress functional responses in the case of “high cycling” conditions accompanied by a low “functional” PKA activation threshold [165]. Thirdly, cells may release endogenously formed stimulants of adenylate cyclase activity, in particular following stimulation. This is illustrated by data showing adenosine release from human neutrophils incubated with fMLP [167] and PAF-induced PGE_2 generation in human eosinophils [18]. In this context, it is of interest that fMLP

stimulation of neutrophils resulted in a transient cAMP increase that was potentiated by the PDE4-selective inhibitor Ro 20-1724 and abolished by adenosine deaminase [167]. Extending these data, Schudt and colleagues [159] reported that 1 μM zardaverine, that only slightly and insignificantly increased the cAMP content of quiescent neutrophils, dramatically potentiated cAMP increase following fMLP stimulation by about three-fold. Taken together, these data suggest that adenosine released by stimulated human neutrophils supports modulation of functional responses by PDE inhibitors such as zardaverine in these cells.

In the isolated perfused rat lung, zardaverine inhibited LPS-induced bronchoconstriction ($\text{IC}_{50} = 1.8 \mu\text{M}$) and in parallel, reduced TXA_2 release in the perfusate [168]. 10 μM zardaverine protected against increased endothelial cell permeability and oedema formation in the isolated perfused rabbit lung challenged with H_2O_2 [169]. Furthermore, it was demonstrated that zardaverine inhibits antigen-induced accumulation of eosinophils in guinea pig airways [170–172]. Zardaverine given by the oral route attenuated LPS-induced liver injury in mice [173].

In clinical trials, single doses of zardaverine (1.5–6 mg) given by the inhaled route (MDI) afforded a modest increase in FEV_1 in mild-moderate asthmatics. About 55% of the asthmatics responded to zardaverine with an FEV_1 increase of at least 10% [162, 174–176]. Zardaverine protected against the early airway reaction to allergen challenge but only slightly reduced the extent of the late airway reaction [162]. Administration of zardaverine was associated with the occasional occurrence of nausea and vomiting typically associated with first generation PDE4 inhibitors. Clinical development of zardaverine was discontinued.

AH21-132 (Benafentrine)

The benzonaphthyridine benafentrine (AH21-132) inhibited PDE3 (human platelets) and PDE4 (human neutrophils) with IC_{50} values of 1.74 and 1.76 μM , respectively. Bronchodilatory, anti-inflammatory and clinical effects of this compound have been recently extensively reviewed [162]. Briefly, AH21-132 reversed human bronchus contractility induced by histamine, carbachol or endogenous spasmogens with IC_{50} values of 4.0, 8.0, and 4.7 μM , respectively. In the anaesthetized, ventilated guinea pig the benzonaphthyridine protected against bombesin-induced airway obstruction. The anti-inflammatory potential of benafentrine is reflected by the finding that the compound inhibited PAF- or antigen-induced airway eosinophil accumulation [162]. AH21-132 showed some clinical efficacy in asthmatics where the benzonaphthyridine induced transient bronchodilation and protected against allergen-challenge induced late airway obstruction [162]. Interestingly, single daily doses of 800 mg or repeated daily doses of 200 mg over 14 days given orally were well tolerated. The high oral tolerability of benafentrine may originate in the LAR-specificity of this compound (Tab. 5).

Table 6 - Summary of expected adverse effects with classical PDE3 or PDE4 inhibitors and strategies for design of PDE inhibitors avoiding these unwanted effects.

PDE3 and PDE4 are expressed in diverse cellular systems apart from those relevant in airway inflammation. PDE3 is abundant in the cardiovascular system and cAMP increase followed by inhibition of PDE3 results in enhanced myocardial contractility, vascular smooth muscle relaxation and inhibition of platelet aggregation. Nausea and vomiting may originate in cAMP increase in the area postrema as a consequence of PDE4 inhibition. On the other hand, gastric acid hypersecretion may represent a peripheral effect of PDE4 inhibition. Hence, non-selective PDE3 and 4 inhibition would result in a variety of unwanted effects. However, implementing recent findings regarding cell-specific prevalence of conformers or subtypes of PDE3 or PDE4 in drug discovery strategies would minimize the occurrence of these unwanted effects.

	PDE3	PDE4
Adverse events	Tachycardia Arrhythmia SVRI-reduction CI-increase Platelet inhibition	Nausea Vomiting Gastric acid secretion Headache
Avoidance strategy	PDE3B inhibition	PDE4-LAR inhibition

SVRI, systemic vascular resistance index; CI, cardiac index

Tolafentrine

Tolafentrine is another benzonaphthyridine. The compound inhibits PDE1–5 with IC₅₀ values of 18, 0.8, 0.09, 0.06 and 2 µM, respectively. Tolafentrine relaxes isolated human bronchial smooth muscle *in vitro* [177] and inhibits histamine-induced bronchoconstriction in the anaesthetized guinea pig *in vivo* [178]. Tolafentrine inhibits LPS-stimulated TNFα-generation in human alveolar macrophages, proliferation of human lymphocytes [156], degranulation and chemoluminescence of human eosinophils (when combined with salbutamol) [20], and fMLP-stimulated eosinophil LTC₄ synthesis [140]. These *in vitro* findings might predict an anti-inflammatory potential of tolafentrine *in vivo*.

Although dual-selective PDE3/4 inhibitors are considered as promising new drugs in asthma, there are some concerns regarding cardiovascular side-effects due to the PDE3 inhibiting component (Tab. 6). However, in clinical trials with dual selective inhibitors, haemodynamic effects were not observed. Regarding the potential of PDE3 inhibitors to induce cardiovascular effects such as those in milrinone

trials [142, 143] it should be taken into account that patients included in those trials had severe cardiac failure i.e. these studies targeted a completely different population from asthma trials. However, since PDE3B is abundant in human lymphocytes but not in the cardiovascular system the safety profile may be increased by selectively inhibiting PDE3B. In fact, an ideal second generation dual selective PDE3/4 inhibitor for asthma should selectively inhibit PDE3B but not PDE3A and show selectivity for the LAR conformer of PDE4 subtypes. A dual selective PDE3B/4-LAR inhibitor with high oral bioavailability would represent a cutting edge strategy for future drug development in asthma.

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β_2 -Agonists

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Introduction

β_2 -Adrenoceptor agonists are widely used as bronchodilators and have been used to treat acute attacks of asthma for decades. They can be divided into the very short acting which last 1 to 2 h (e.g. rimiterol), the short-acting such as salbutamol which produce an effect for 4 to 6 h and the newer longer acting β -agonists such as salmeterol and formoterol which maintain bronchodilatation for at least 12 h [1, 2]. β -Agonists antagonise the effects of a wide variety of bronchoconstrictor agents on airway smooth muscle *in vitro* and *in vivo* and are thus functional antagonists. The main mechanism by which they cause smooth muscle relaxation involves the cyclic 3'5' adenosine monophosphate (cAMP) second messenger system which is linked to the β_2 -adrenoceptor by a coupling G-protein and adenylate cyclase, the effector enzyme [3] (Fig. 1). Cyclic AMP is able to modulate a number of processes which are important in governing the contractile state of the cell [4].

The reason for a long duration of action may differ between salmeterol and formoterol. Salmeterol has a large non-polar N-substituent which is thought to interact with specific sites in the receptor to prolong its action and this may explain why the duration of action appears not to be concentration dependent [5]. Formoterol has a long duration of action when inhaled but not when given orally and its duration of action is concentration dependent. It has been proposed that because it is very lipophilic it enters the membrane depot rapidly and only re-equilibrates gradually with the aqueous phase where it interacts with β_2 adrenoceptors [6].

In addition to being bronchodilators β -agonists may also have anti-inflammatory activity. This article discusses the evidence for this and its clinical relevance. Since short and long acting β -agonists have different roles and functions they will be discussed separately.

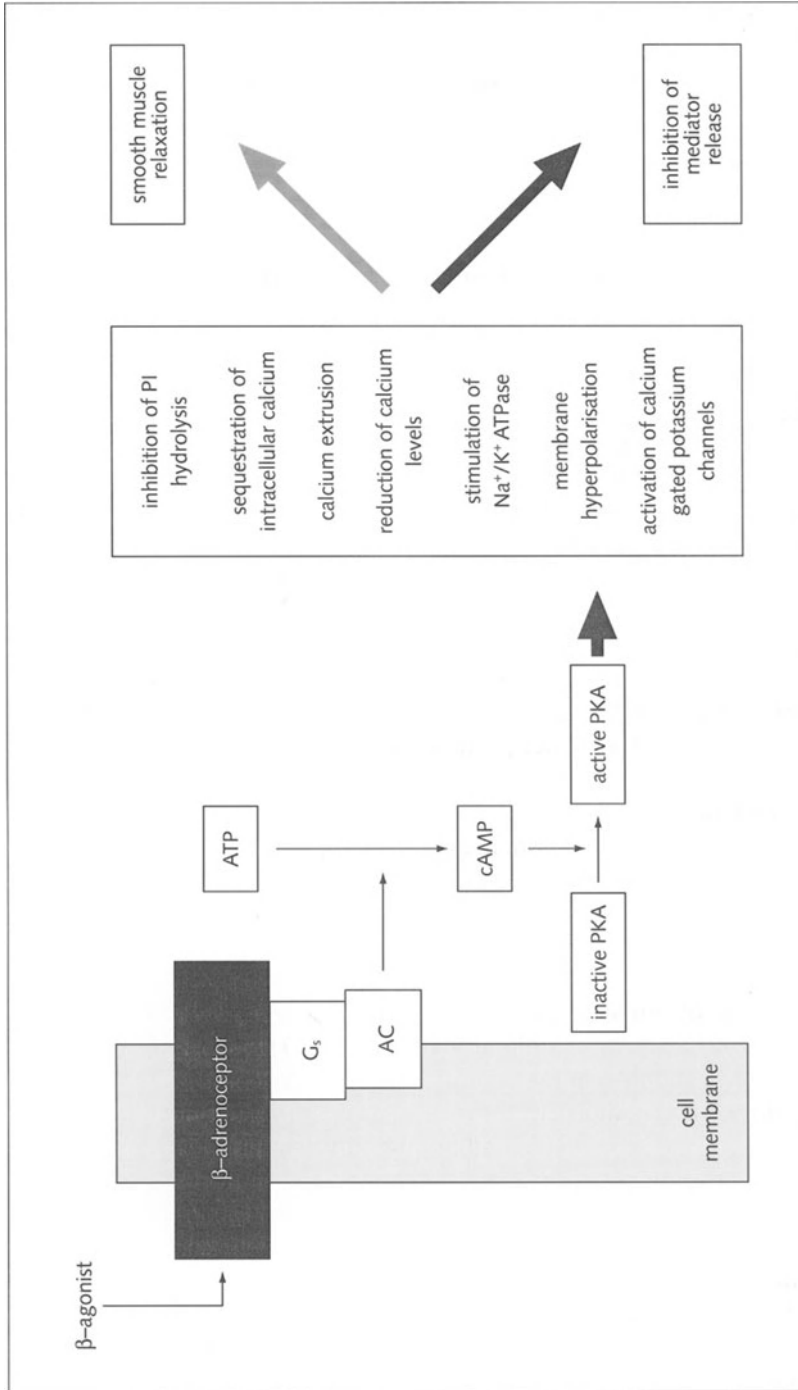


Figure 1
 β_2 -Receptors are coupled to adenylyl cyclase (AC) via a stimulatory guanine nucleotide regulatory protein (G_s). AC catalyses the conversion of ATP to cyclic AMP which in turn activates protein kinase A (PKA) which phosphorylates several proteins to produce intracellular effects that lead to smooth muscle relaxation and inhibition of mediator release.

Do β -agonists have anti-inflammatory activity?

The anti-inflammatory effects of β -agonists have been studied *in vitro*, in animal studies, in bronchial biopsies in man and in clinical studies.

Evidence from studies *in vitro* and in animals

The effect of β -agonists on two aspects of inflammation, plasma exudation and activation and mediator release from inflammatory cells have been studied.

Plasma exudation

Plasma exudation is an important part of airway inflammation and may contribute to the pathophysiology of asthma by increasing airway oedema and thus airflow obstruction and bronchial hyperresponsiveness, and by allowing pro-inflammatory substances into the airways [7].

Short-acting β -agonists

The short acting β -agonists terbutaline and salbutamol have been shown to inhibit plasma exudation induced by bradykinin and histamine in several animal preparations including hamster cheek pouches [8] and guinea pig airways *in vivo* [9, 10]. The dose of β -agonist required to inhibit the increase in microvascular permeability is generally greater than that required to reverse the increase in airway resistance caused by the same mediators when both are given by the intravenous route but not when given by inhalation [10, 11].

Long-acting β -agonists

Bronchodilator doses of inhaled salmeterol and formoterol inhibit histamine induced plasma exudation in guinea pig airway [12, 13]. The effect of salmeterol was maintained for 6 h, formoterol for 5 h and salbutamol for 2 to 4 h in one study [14]. The mechanism for the anti-permeability effects of β -agonists is unclear but they are β_2 -adrenoceptor mediated [15] and are probably due to a direct action on the endothelial cells of leaky post-capillary venules [16, 17].

Effects on inflammatory cells

β_2 -Adrenoceptors coupled to adenylate cyclase are present on several human inflammatory cells including lung mast cells [18], eosinophils [19], alveolar macrophages [20], B and T lymphocytes [21–23] and neutrophils [24]. Many studies have demon-

strated effects from both short and long acting β -agonists on these cells (Tab. 1) although tachyphylaxis tends to develop very quickly.

Short-acting β -agonists

Salbutamol and isoprenaline are potent inhibitors of IgE dependent release of histamine from chopped human lung [25, 26] and of histamine and prostaglandin D_2 from dispersed human lung mast cells [27]. Salbutamol inhibited the release of superoxide anions and leukotriene B_4 induced hydrogen peroxide release from eosinophils in one study [28], though this has not been seen in all studies possibly due to methodological differences. It appears that longer incubation of eosinophils with a β -agonist results in a loss of its inhibitory effect, presumably due to tachyphylaxis [19]. Salbutamol inhibited the accumulation of neutrophils in guinea pig lung [13] whilst isoprenaline has been shown to inhibit various responses of isolated human neutrophils that are capable of causing tissue inflammation including cell activation, generation of superoxides and release of beta glucuronidase [29]. Although not demonstrated with β -agonists, an increase in intracellular cAMP inhibits interleukin-2 production which in turn has caused a decrease in T lymphocyte proliferation [30–32]. The functional significance of the β_2 adrenoceptors found on macrophages is unclear since short-acting β -agonists appear not to inhibit mediator release *in vitro* [33–34].

Long acting β -agonists

Salmeterol and formoterol are also potent inhibitors of IgE dependent histamine release from human lung fragments [6, 35] and dispersed lung mast cells [36]. Salmeterol and formoterol inhibit antigen and platelet activating factor induced eosinophil and neutrophil accumulation in guinea pig airways [13, 37] and the leukotriene B_4 induced release of hydrogen peroxide from guinea pig eosinophils was inhibited by formoterol but not salmeterol [28]. The lack of effect of salmeterol in this respect may be due to the low density of β_2 -adrenoceptors on eosinophils, since salmeterol is a partial agonist [28]. Salmeterol has also been shown to reduce *Pseudomonas aeruginosa* damage to both nasal epithelium *in vitro* and cultured nasal epithelial cells, an effect which is attributed to a rise in intracellular cAMP which inhibits various toxins produced by the bacteria [38]. Most of the effects described above are β_2 -adrenoceptor mediated and inhibited by β_2 -adrenoceptor antagonists; salmeterol may however have effects which are not β_2 -adrenoceptor mediated, possibly due to its membrane stabilising properties. Examples include a potent inhibitory effect of salmeterol on $TNF\alpha$ production in a human monocytic cell line (THP-1) [39], inhibition of T lymphocyte activation in mice [39] and modification of thromboxane B_2 release from human alveolar macrophages [34, 40].

Table 1 - Examples of the inhibitory effects of β -agonists on inflammatory cellsa) short acting β -agonists

Cell type	Drug	Inhibition of	Ref.
Chopped lung	salbutamol	IgE dependent histamine release	[25]
	isoprenaline		[26]
Dispersed lung mast cells	salbutamol	IgE dependent histamine and PGD ₂ release	[27]
Guinea pig eosinophils	salbutamol	release of superoxide anions and LTB ₄ induced H ₂ O ₂ release	[28]
Neutrophils	isoprenaline	release of human neutrophil beta glucuronidase generation of superoxides cell activation	[29]
Guinea pig neutrophils	salbutamol	accumulation	[13]
Macrophages	isoprenaline	no effect on mediator release	[33]
	salbutamol		[34]
Lymphocytes	intracellular cAMP	increase in T lymphocyte proliferation	[32]
	raising agents		[30, 31]

b) long acting β -agonists

Cell type	Drug	Inhibition of	Ref.
Chopped lung	salmeterol	IgE dependent histamine, LTC ₄ and PGD ₂ release	[6]
	formoterol		[35]
Dispersed lung mast cells	salmeterol	IgE dependent histamine release	[36]
	formoterol		
Eosinophils	salmeterol	H ₂ O ₂ release and eosinophil accumulation	[28]
	formoterol		[37]
Neutrophils	salmeterol	antigen and PAF induced neutrophil accumulation	[13]
	formoterol		
Alveolar macrophages	salmeterol	modification of TXB ₂ release	[34, 40]
Cell lines	salmeterol	TNF α production	[39]
Murine lymphocytes	salmeterol	cell activation	[39]

IgE, immunoglobulin E; PGD₂, prostaglandin D₂; LTB₄, leukotriene B₄; H₂O₂, hydrogen peroxide; LTC₄, leukotriene C₄; PAF, platelet activating factor; TXB₂, thromboxane B₂; TNF α , tumour necrosis factor alpha.

Evidence from bronchial biopsy, bronchoalveolar lavage, induced sputum and peripheral blood studies in man

Bronchial biopsy and bronchoalveolar lavage have been used to establish the nature of the inflammatory response within asthmatic airways and to investigate the effects of various challenges and treatment regimens on the inflammatory response. More recently sputum induced by hypertonic saline has been developed as a less-invasive method of studying airway inflammation, with sputum being analysed for various constituents including total and differential cell counts and inflammatory mediators [41]. Eosinophils and their activation markers in peripheral blood have also been measured.

Short-acting β -agonists

Several studies have looked at bronchial biopsies or bronchoalveolar lavage before and after a period of regular treatment with short-acting β -agonists. Study details are given in Table 2 and the main findings in Table 3. In the first study, a small, 3-month, double-blind parallel group study by Laitinen et al. [42], bronchial biopsies were performed in seven patients with mild asthma before and after regular terbutaline treatment. They found a decrease in lymphocytes in the epithelium, a decrease in lymphocytes, mast cells and plasma cells in the lamina propria but no change in eosinophils, neutrophils, macrophages or monocytes. However two patients received a 6-day course of prednisolone during the treatment period which may have influenced the results. Manolitsas et al. [43] using bronchial biopsies and bronchoalveolar lavage found an increase in both total and activated eosinophils compared with baseline following 16 weeks treatment with regular salbutamol in 13 patients with mild asthma, but no change in mast cell or lymphocyte numbers. When comparisons were made with placebo there were no statistically significant differences in cell number or activation. Two shorter studies found no changes in inflammatory cell number or activation. Evans et al. [44] found no changes in eosinophil numbers, eosinophil cationic protein (ECP) levels or mast cell numbers in induced sputum from 30 rhinitic non-asthmatic subjects before and after 4 weeks' therapy with salbutamol, whilst Di Lorenzo et al. [45] found no change in eosinophil counts in blood or in serum ECP levels after 1-weeks' therapy with salbutamol. An alternative to looking at inflammatory markers in the basal state is to study the inflammatory response to an antigen challenge. Twentyman et al. found a reduction in the antigen induced increase in neutrophils at 8 h post challenge and in neutrophils and basophils at 24 h post challenge following a single dose of salbutamol in nine subjects with mild asthma [46], whilst Howarth et al. [47] showed that pre-treatment with a single dose of salbutamol reduced the antigen induced rise in plasma histamine and neutrophil chemotactic factor levels for up to 30 minutes in eight subjects with mild asthma.

Table 2 - Bronchial biopsy, BAL, induced sputum and peripheral blood studies

a) short acting β -agonists

Method (Ref.)	Sample size	Study type and duration	Treatment
Biopsy [42]	14	R, DB, PG, 12 weeks	375 μ g bd terb
Biopsy and BAL [43]	48	R, DB, PG, 16 weeks	200 μ g qds salb
Peripheral blood [45]	20	R, O, CO, 1 week	200 μ g qds salb
Induced sputum [44]	30	R, DB, PG, PC, 4 weeks	200 μ g qds salb
▲ peripheral blood [46]	9	R, DB, PC, CO, SD	200 μ g salb
▲ peripheral blood [47]	8	PC, CO, SD	200 μ g salb

b) long acting β -agonists

Method (ref)	Sample size	Study type and duration	Treatment
BAL [48]	12	R, DB, CO, PC, 4 weeks	50 μ g bd salm
BAL [49]	23	R, DB, PG, PC, 6 weeks	50 μ g bd salm
biopsy [50]	23	R, DB, PG, PC, 6 weeks	50 μ g bd salm
BAL [51]	23	R, DB, PG, PC, 6 weeks	50 μ g bd salm
BAL [52]	10	R, DB, CO, PC, 8 weeks	50 μ g bd salm
peripheral blood [45]	20	R, O, CO, 1 week	50 μ g bd salm
▲ induced sputum and peripheral blood [56]	8	R, DB, CO, PC, SD	100 μ g salm
▲ induced sputum and peripheral blood [53]	6	SB, CO, PC, SD	24 μ g form
▲ peripheral blood [54]	12	R, DB, CO, PC, SD	50 μ g salm
▲ peripheral blood [55]	19	R, DB, PG, PC, SD	50 μ g salm

BAL, bronchoalveolar lavage; R, randomised; DB, double blind; SB, single blind; CO, cross-over; PG, parallel group; PC, placebo controlled; O, open; SD, single dose; terb, terbutaline; salb, salbutamol; salm, salmeterol; form, formoterol.

▲, studies carried out before and after antigen challenge.

Long-acting β -agonists

The effect of regular long acting β -agonists on bronchial biopsies in subjects with asthma has also been studied (Tabs. 2 and 3). In 1991, Dahl et al. [46] reported that 4 weeks' treatment with salmeterol produced an improvement in the appearance of

Table 3 - Effects of β -agonists on inflammatory markers seen in bronchial biopsy, BAL, induced sputum and peripheral blood studies

a) short acting β -agonists

Study type (ref)	β -Agonist	Eosino- phils	Neutro- phils	Lympho- cytes	Macro- phages	Mast cells	Baso- phils
B [42]	terb (375 μ g bd)	x	x	↓	x	↓	-
B & BAL [43]	salb (200 μ g qds)	↑	-	x	-	x	-
PB [45]	salb (200 μ g qds)	x	-	-	-	-	-
IS [44]	salb (200 μ g qds)	x	x	x	x	x	-
▲ PB [46]	salb (200 μ g)	x	↓ at 8 h	x	-	-	↓ at 8 & 24 h
▲ PB [47]	salb (200 μ g)	-	↓ in NCF	-	-	↓ in histamine	-

b) long acting β -agonists

Study type (ref)	β -Agonist	Eosino- phils	Neutro- phils	Lympho- cytes	Macro- phages	Mast cells	Baso- phils
BAL [48]	salm (50 μ g bd)	↓ in ECP	-	-	↓ in O ₂ radical generation	-	-
BAL [49]	salm (50 μ g bd)	x	-	-	-	x	-
B [50]	salm (50 μ g bd)	x	-	x	-	x	-
BAL [51]	salm (50 μ g bd)	-	-	x	-	-	-
BAL [52]	salm (50 μ g bd)	x	x	x	x	x	-
PB [45]	salm (50 μ g bd)	↓ in ECP	-	-	-	-	-
▲ IS & PB [53]	form (24 μ g)	↓ in ECP	-	x	-	-	-
▲ PB [54]	salm (50 μ g)	↓ in ECP	-	-	-	-	-
▲ PB [55]	salm (50 μ g)	x	-	-	-	-	-
▲ IS & PB [56]	salm (100 μ g)	x	-	-	-	-	-

BAL, bronchoalveolar lavage; B, biopsy; IS, induced sputum; PB, peripheral blood; terb, terbutaline; salb, salbutamol; salm, salmeterol; form, formoterol; ECP, eosinophil cationic protein; x, no effect; -, not measured; ↓, decrease; ↑, increase.

▲, studies carried out before and after antigen challenge.

the mucosa compared with baseline and a reduction in the oxidative metabolism of macrophages, but the differential cell count following bronchoalveolar lavage was unaltered. The authors also reported a statistically significant decrease in ECP levels in lavage. More recently another group found a fall in mean serum ECP concentration with no change in blood eosinophil count following salmeterol treatment for 1 week in 20 patients with mild atopic asthma, suggesting that salmeterol may modify and reduce the release of granular proteins from eosinophils but not total cell numbers [45]. Conversely, several studies have not been able to demonstrate any anti-inflammatory effects of salmeterol. Roberts et al. [49–51] performed detailed analyses of a wide range of inflammatory markers in bronchial biopsies and bronchoalveolar lavage in 23 subjects with mild asthma after six weeks therapy with salmeterol and placebo. Despite improvements in symptoms and morning peak flow after salmeterol there was no difference in differential cell counts, histamine, albumin, prostaglandin D₂ or tryptase levels in bronchoalveolar lavage, no difference in T lymphocyte numbers or activation status assessed by flow cytometry and no difference in numbers of mast cells, eosinophils or T lymphocytes on bronchial biopsy. Similarly, a crossover study in nine subjects with mild asthma showed no significant change in BAL cell profile, or in percentages of CD4 and CD8 lymphocytes following 8 weeks' salmeterol treatment compared with placebo [50].

All these studies have looked at patients with stable asthma. Four further studies have looked at the effects of long acting β -agonists on the inflammatory response to an antigen challenge [53–56]. In all four the long acting β -agonist inhibited the early and late response to antigen and provided protection against the subsequent increase in bronchial responsiveness. The effect on inflammatory markers was less clear cut. Formoterol had no effect on the increase in sputum and blood eosinophils and lymphocytes that occurred following antigen challenge in six subjects with mild, atopic asthma studied by Wong et al. [53] although it did inhibit the rise in serum ECP 24 h after the challenge. Similarly, a single dose of salmeterol administered prior to antigen challenge in 12 patients with mild asthma had no effect on the antigen induced rise in blood eosinophils although it again prevented the rise in serum ECP [52]. In two further studies however a single dose of salmeterol administered prior to antigen challenge did not affect the numbers or activation markers of eosinophils in peripheral blood or induced sputum [55, 56].

Clinical studies

Determining the extent to which the effects of β -agonists in clinical practice can be attributed to an anti-inflammatory effect rather than bronchodilatation is very difficult. Antigen challenge has been used as a model but interpretation of the effects of β -agonists on the response to antigen challenge is not straightforward. β -Agonists

are functional antagonists and would be expected to counter the bronchoconstrictive effect of antigen even if they were acting solely on airway smooth muscle.

Short acting β -agonists

The short acting β -agonists inhibit the early response to antigen [55] and when nebulised salbutamol was given in higher doses (2.5 mg) the late response to antigen and post antigen increase in bronchial responsiveness were also inhibited [57]. High doses of salbutamol have a longer duration of action than smaller doses but the inhibition of the late response lasted for a longer time than the bronchodilatation seen with salbutamol alone on the control day, suggesting that salbutamol might be having effects other than functional antagonism [56].

Long acting β -agonists

Salmeterol and formoterol have been shown to inhibit both the early and the late response to antigen [53–56, 58]. The extent of the inhibitory effect of salmeterol on the early and late response to antigen in the study by Twentyman et al. [58] was broadly similar to the bronchodilator effect seen when salmeterol was given alone on the control day, in keeping with functional antagonism. Measurements made 32 h after the antigen challenge when any direct effects of salmeterol had worn off, showed a slightly greater FEV₁ and reduced bronchial reactivity following salmeterol than following placebo, suggesting that salmeterol might have had a more specific anti-inflammatory effect during the antigen challenge. However, when inflammatory indices have been compared in blood and sputum following antigen challenges preceded by long acting β -agonists or placebo the differences have been small. The rise in ECP levels following antigen challenge was inhibited by salmeterol in two of the studies [53, 54] but not in the other two [55, 56].

Conclusion

Both short and long acting β -agonists have actions which can be described as anti-inflammatory. Both reduce plasma exudation *in vitro* and in animal models and both have inhibitory effects on several of the inflammatory cells found in the lung including inhibition of mediator release and cell accumulation. Although these actions would be expected to reduce inflammation in the airways, studies of bronchial biopsies and bronchoalveolar lavage following regular treatment with both short and long acting β -agonists have been largely negative with no consistent increase or decrease in inflammatory cell numbers in the biopsies or bronchoalveolar lavage. Although most of these studies have been small (see Tab. 2), with insufficient power to determine small effects of β -agonists, it is clear that any changes

that occur with β -agonists are considerably less than those seen with corticosteroids. It is also important to note that the studies are less able to comment on the functional activity of inflammatory cells.

Determining the extent to which the beneficial effects of β -agonists in patients are due to their anti-inflammatory effect is difficult. It seems likely that the rapid action of short acting bronchodilators in relieving acute asthma is due predominantly to a relaxant effect on smooth muscle. When taken on a regular basis the long acting β -agonists have been shown to provide clinical benefit compared to placebo whereas the effects of regular short acting β -agonists have differed little from placebo [59–62]. The reason for the difference between short and long acting β -agonists in this respect is not clear but it is now apparent that the bronchodilator effect of short acting β -agonists wanes considerably between doses. The same is probably true for their actions on inflammatory cells and this may allow intermittent release of mediators. The more sustained action of a long acting β -agonist would ensure a more continuous inhibitory effect on inflammatory cells and this may explain their greater clinical efficacy.

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Mast cell stabilizing drugs

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Introduction

Currently available medication for treating airway inflammation is limited to only a few proven antiinflammatory agents. Cromolyn sodium (sodium cromoglycate, SCG) and nedocromil sodium, traditionally referred to as mast cell stabilizing agents, comprise an important group of mild to moderate anti-inflammatory drugs [1].

Sodium cromoglycate (Cromolyn sodium)

Sodium cromoglycate (SCG) has been developed from derivatives of khellin and found to inhibit allergen-induced bronchoconstriction in man [2]. It is believed to prevent degranulation of mast cells via a membrane stabilizing effect brought about by the phosphorylation of a protein that results in blocking ionic calcium transport across the cell membrane.

Cellular effects of sodium cromoglycate

Prevention of the release of mast cell products such as histamine, PGD₂ and LTC₄ accounts for the action of this drug on the early asthmatic reaction (EAR) which is IgE and mast cell-dependent. Cox demonstrated that SCG inhibits the release of mediators from mast cells triggered by the interaction between antigen and IgE [3]. Sodium cromoglycate inhibited histamine release from passively sensitized human lung fragments in a concentration range of 0.2–200 μM, producing a maximum inhibition of 33% (compared to a maximum inhibition of 72% produced by the β₂-agonist salbutamol) [4]. Sodium cromoglycate was more effective in inhibiting release of PGD₂ (compared to histamine) from human dispersed lung mast cells [5].

The origin of the mast cells is important for the inhibitory activity of cromoglycate. Flint and colleagues demonstrated that mast cells recovered by bronchoalveo-

lar lavage in man were more sensitive to the inhibitory activity of SCG than mast cells obtained by digesting human lung fragments [6]. SCG also inhibits the activation of mast cells by other stimuli [7].

Besides mast cells, other inflammatory cells are sensitive to the inhibitory activity of SCG. Human neutrophils, eosinophils, platelets and macrophages have been used to demonstrate the broad inhibitory activity of SCG on the *in vitro* activation of inflammatory cells [8]. This wide variety of activity of SCG may account for the suppressive activity of this drug on the late asthmatic reaction (LAR). Finally, it was demonstrated that SCG inhibits, in a dose-dependent manner, IL-4-driven T cell-dependent IgE synthesis by PBMC from the majority (65%) of normal human subjects as well as IgE synthesis by B cells stimulated with anti-CD40 plus IL-4 or with hydrocortisone plus IL-4. SCG acts directly on B cells and the inhibition resides at the level of deletional switch recombination. SCG has a minimal effect on B cells that have already undergone isotype switching [9]. These new findings suggest a novel potential mechanism for the prevention of allergic disease.

Cromoglycate may also interfere with neural mechanisms. Dixon et al. demonstrated that SCG inhibited the activation of autonomic C fibre nerves stimulated by capsaicin in dogs *in vivo*, resulting in an inhibition of reflex bronchoconstriction [10]. These and other experiments suggest that SCG also inhibits the activation of certain types of nerves [11].

Clinical pharmacology: protective effect of SCG in bronchial challenges

Effect of SCG on airway reactions following allergen challenge

Altounyan and others have demonstrated that SCG, inhaled before allergen challenge, significantly inhibited the immediate bronchoconstriction that developed following the inhalation of allergen [2]. In addition, SCG given before allergen challenge also inhibits the development of the late asthmatic reaction [12] and prevents the increase in airway responsiveness observed following the last asthmatic reaction [13].

Effect of SCG on airway reactions following challenge with non-allergic stimuli

Immediate pretreatment with SCG has no protective effect on the bronchoconstriction induced by agonists that act directly on airway smooth muscle such as histamine, methacholine, prostaglandins or leukotrienes. In contrast, therapeutic doses of SCG significantly inhibit the bronchoconstriction induced by exercise, cold air, adenosine, substance P, bradykinin, sulphur dioxide and distilled water [14].

Silverman and Andrea demonstrated that SCG only inhibits exercise-induced asthma (EIA) when inhaled before exercise. SCG given after exercise but before the

bronchoconstriction developed was not effective [15]. Deal et al. [16] showed that SCG inhibits the bronchoconstriction induced by isocapnic hyperventilation. The airway narrowing induced by hyperventilation of cold air was also inhibited by cromoglycate [17]. The role of mast cells in EIA and in the airway reaction to cold air is still a matter of debate and therefore no uniformly accepted hypothesis on the mechanism responsible for the protective activity of SCG in these clinical pharmacology models is available.

The airway reaction to inhaled adenosine is at least partially due to the activation of mast cells, since H₁-antagonists significantly inhibit the bronchoconstriction following the inhalation of adenosine [18]. SCG has a significant protective activity on the adenosine-induced bronchoconstriction. The protective activity of SCG in this model can be explained at least partially by a mast cell stabilizing activity.

Dixon and Barnes observed a significant protective effect of SCG on the bradykinin-induced bronchoconstriction in mild asthmatics [19]. SCG also inhibits the bronchoconstriction in asthmatics induced by the inhalation of sulphur dioxide [20] or metabisulphite [21]. The airway reaction induced by sulphur dioxide or metabisulphite is significantly inhibited by anticholinergics. The bronchoconstriction induced by these agonists is however not inhibited by high doses of the presently available H₁-antagonists and the role of mast cells in these types of airway reactions is therefore less likely. The inhibitory effect of SCG on the activation of some autonomic nerve fibres may play a role [14].

Effect of chronic treatment with SCG on long-term changes in non-allergic airway responsiveness

Although the administration of SCG immediately before challenge does not inhibit the bronchial response to methacholine or histamine, Löwhagen and Rak observed that treatment with SCG prevented the seasonal increase in bronchial responsiveness to histamine in pollen-allergic asthmatics [22]. The effect of SCG on airway responsiveness in non-allergic asthmatics is rather controversial. Löwhagen and Rak did not observe a significant change in histamine responsiveness in non-allergic asthma following treatment with SCG during 8 weeks [23]. In contrast, in the study of Petty et al. the histamine responsiveness decreased significantly over 12 weeks in the SCG-treated group, although the final degree of bronchial responsiveness did not differ significantly from the placebo-treated group [24].

Clinical use

The drug is particularly active in prevention of seasonal allergic asthma in children and young adults. SCG seems less effective in older patients or in patients whose

asthma is not allergic. However, other studies have demonstrated the clinical efficacy of SCG in chronic asthmatics, with a decrease of concomitant medication used, an increase in pulmonary function and significantly fewer exacerbations (reviewed in [25]). On the other hand, long-term prophylactic inhalation therapy with inhaled SCG with a spacer device in 1–4 year old children with moderate asthma was not more effective than placebo [26].

Inhaled sodium cromoglycate may be effective in cough induced by treatment with an ACE-inhibitor. In a double-blind crossover study, 9 of 10 patients reported a reduction in cough after treatment with sodium cromoglycate. Moreover, the cough sensitivity to capsaicin was significantly reduced by treatment with cromoglycate [27].

SCG has to be taken by inhalation and has a relatively short duration of action, requiring administration every 3 to 4 h. SCG is a safe drug. Long-term follow-up studies have shown no serious side-effects [28]. Local irritation of the throat or an occasional mild bronchospasm are more often seen with the dry powder preparation than with the nebulizer and the metered dose inhaler.

Nedocromil sodium

Nedocromil sodium is a pyranoquinoline dicarboxylic acid that has been developed for the treatment of bronchial asthma. Several clinical trials have demonstrated the therapeutic efficacy of this relatively new prophylactic agent in the treatment of asthma.

Cellular effects of nedocromil sodium

In vitro studies of human eosinophils, neutrophils, mast cells, and bronchoalveolar cells demonstrate that nedocromil sodium exerts its anti-inflammatory effects through regulation of mediator production and release from these cells as well as modulation of inflammatory cell migration. Nedocromil sodium inhibits chemotaxis of human eosinophils and neutrophils induced by platelet activating factor (PAF), zymosan activated serum (ZAS), leukotriene B₄ (LTB₄), or N-formyl-methionyl-leucylphenylalanine (fMLP) in healthy adults. Both nedocromil and cromoglycate are similarly effective in inhibiting neutrophil chemotaxis induced by PAF, ZAS, LTB₄, and fMLP. *In vitro* neutrophil migration was inhibited by 35% after incubation with nedocromil sodium 10⁻⁶ mol/l. Nedocromil sodium also affects the release of a wide variety of inflammatory mediators from various cells (reviewed in [29]). Nedocromil sodium inhibits IgE and IgG4 production in human B cells stimulated with IL-4, presumably at the level of switching, as does SCG. The

same authors also demonstrated that nedocromil sodium inhibits the production of IgM, IgG1, IgG2, IgG3, IgG4, IgA, and IgA2 by tonsillar B cells stimulated with *S. aureus* Cowan strain I and IL-6 without affecting proliferation, suggesting that nedocromil sodium may act as a B cell regulatory reagent, thus modulating allergic inflammation [9].

Interleukin (IL)-6 production by alveolar macrophages obtained from patients with asthma, and stimulated by allergen and anti-IgE [30] and IL-1 induced production of IL-8 by cultured human bronchial epithelial cells from normal bronchi [31] was inhibited *in vitro* by nedocromil sodium. However, no effect was observed on the ozone-induced synthesis of IL-8 by bronchial epithelial cells *in vitro*, whereas nedocromil sodium clearly inhibited release of GM-CSF, TNF α and sICAM by these cells [32]. The increased release of GM-CSF induced by IL-1 in bronchial epithelium obtained from patients undergoing surgery was inhibited by nedocromil sodium 10^{-6} mol/l, although the constitutive production was not affected [33]. The effect of nedocromil sodium on leakage of plasma proteins into the airway lumen – a feature of chronic inflammation – was assessed by Schoonbrood et al. [34]. There was a very limited effect of treatment on PEF and FEV $_1$, and no effect on symptom score. However, a decrease was noted for the plasma protein leakage as measured in the sputum sol phase. The maximal effect occurred after 4 weeks of treatment. This reduction of protein exudation suggests that nedocromil sodium has anti-inflammatory properties, the mechanism of which is not known [34].

It appears that nedocromil sodium may not be able to alter the resident airway inflammatory cells in mild to moderate asthmatics [35]. Recently, the effect of nedocromil sodium was compared with regular albuterol and albuterol as required, on inflammatory changes in bronchial mucosal biopsies and BAL, obtained before and 4 months after treatment. No significant alteration in the number of inflammatory cells was observed, but there was a decrease in the number of eosinophils with nedocromil sodium and an increase with albuterol [36]. Contradictory results have been reported with regard to inhibitory effects of nedocromil sodium on the release of LTC $_4$ from stimulated eosinophils and on the release of superoxide anion and LTB $_4$ by stimulated neutrophils. Histamine release from mast cells obtained by bronchoalveolar lavage from healthy volunteers and asthmatic patients was inhibited more readily by nedocromil sodium than by cromoglycate [37]. Nedocromil sodium also inhibits histamine release within asthmatic airways following hyperosmolar saline challenge [38].

The neurogenic component of asthmatic inflammation is also inhibited by nedocromil sodium. In guinea pigs, noncholinergic, tachykinin-mediated airway contraction was found to be inhibited by pretreatment with nedocromil [39].

It is apparent from these studies that the mechanism of action of nedocromil sodium in the treatment of asthma is broad. The precise mechanism by which this drug exerts its antiinflammatory effect in man remains unclear.

Clinical pharmacology: protective effect of nedocromil sodium in bronchial challenges

Effect of nedocromil on airway reactions following allergen challenge

Pretreatment with nedocromil significantly inhibits the immediate reaction and the late reaction following allergen challenge. The inhalation of nedocromil following the immediate reaction partially but significantly reduced the magnitude of the late asthmatic reaction [40]. Inhalation of nedocromil sodium 15 min before house dust mite challenge inhibited the early and late reactions, with a greater protective effect against the late reaction, in 11 of 14 patients with asthma and positive skin tests for *Dermatophagoides pteronyssinus* allergen. There was also a significant improvement in bronchial hyperresponsiveness 3 and 24 h after challenge [41]. However, when given after the occurrence of the early-phase asthmatic reaction, nedocromil sodium was not able to prevent the development of the late reaction: it delayed the onset by 1.5 h. The allergen-induced increase of bronchial responsiveness was not prevented [42].

Effect of nedocromil on airway reactions following challenge with non-allergic stimuli

Pretreatment with nedocromil immediately before challenge does not modify the airway responsiveness to histamine or methacholine [14]. However, the inhalation of 2 or 4 mg of nedocromil significantly inhibits the airway response to almost all indirect stimuli. Nedocromil shares this protective activity with cromoglycate but is more potent in protecting against non-allergic stimuli involving neuronal activation such as metabisulphite and bradykinin.

Crimi et al. have compared the protective effect of 4 mg of nedocromil, 10 mg of cromoglycate and placebo against adenosine challenge [43]. Both agents had a significant protective effect and nedocromil was significantly better than cromoglycate at the doses used. This study confirmed an investigation by Altounyan et al. [44].

Joos et al. investigated the protective effect of nedocromil against neurokinin A-induced bronchoconstriction: 4 mg of nedocromil almost completely prevented the dose-dependent bronchoconstriction induced in asthmatics by the inhalation of neurokinin A [45]. Crimi et al. confirmed the protective activity of nedocromil against neuropeptide-induced bronchoconstriction. The inhalation of 4 mg of nedocromil 30 min before inhalation challenge with substance P significantly increased the dose of substance P necessary to decrease the FEV₁ by 20% [46].

Dixon et al. studied the effect of nedocromil on the bronchospasm induced by sulphur dioxide in a group of six asthmatics [20]. Both 2 and 4 mg of nedocromil significantly inhibited the bronchoconstriction and dyspnoea, but using metabisulphite instead of sulphur dioxide, Dixon and Ind investigated in two double-blind, placebo-controlled studies the protective effect of 4 mg of nedocromil, 200 µg of

ipratropium bromide or 180 mg of terfenadine [21]. Both ipratropium and nedocromil were protective and nedocromil almost completely prevented any bronchoconstrictive reaction to metabisulphite. The fact that terfenadine had no protective activity attests to the negligible role of the mast cell in this airway reaction. Wright et al. compared the protective effect of 8 mg of nedocromil with that of 4 mg of cromoglycate against metabisulphite challenge in a larger group of asthmatics. Nedocromil blocked the response to metabisulphite in 15 out of 20 asthmatics, whilst this dose of cromoglycate was only protective in three out of 20 subjects [47].

Dixon and Barnes compared the protective effect of 4 mg of nedocromil, 10 mg of cromoglycate and placebo against bradykinin challenge in eight asthmatics. Both drugs had significant protective activity, nedocromil being slightly better than cromoglycate [19].

Robuschi et al. compared 4 mg of nedocromil to placebo in a study using challenge with distilled water. Nedocromil significantly inhibited the fog-induced bronchoconstriction [48]. Along the same line, Roberts and Thomson demonstrated that 2 and 4 mg of nedocromil, inhaled 25 min before exercise, significantly protected against EIA [49].

Juniper et al. studied the effect of 1, 2 and 4 mg of nedocromil on the bronchospasm induced by the hyperventilation of cold air in a placebo-controlled study in 12 asthmatics. The response to cold air was measured at 20, 90 and 300 min after inhalation of the drug. All three doses has a significant protective effect at 20 min, but later on the protective effect of nedocromil was no longer significant [50].

Effect of prolonged treatment with nedocromil on airway responsiveness

Dorward et al. compared nedocromil to placebo in a double-blind cross-over study in pollen-sensitive asthmatics. The active treatment period had a duration of 2 weeks and nedocromil was inhaled at a dose of 4 mg bid. Nedocromil significantly prevented the seasonal increase in airway responsiveness to histamine [51].

Orefice et al. investigated the effect of chronic treatment with nedocromil on methacholine responsiveness in asthmatics [52]. Patients were treated with 4 mg of nedocromil q.d.s. or placebo, and this was added to their usual antiasthma treatment. A significant decrease in the airway responsiveness to methacholine was observed at 8 and 12 weeks of nedocromil treatment.

In a parallel-group study design, Bel et al. compared the effect of 4 mg of nedocromil, 100 µg of beclomethasone dipropionate and placebo, given four times per day for 4 months, on methacholine responsiveness in non-steroid-dependent non-atopic asthmatics. Both nedocromil and beclomethasone dipropionate significantly decreased the airway responsiveness to methacholine after 8 weeks of treatment [53]. In contrast, beclomethasone 200 µg twice or 4 times daily for 6 to 8 weeks was more effective than nedocromil sodium 4 mg twice or 4 times daily for the same period in reducing bronchial hyperreactivity to histamine and distilled water [54].

Nedocromil-sodium: therapeutic efficacy in asthma

Two double-blind, double-placebo, randomized cross-over studies compared the effects of nedocromil sodium with continuous β_2 -agonist treatment [55, 56]. Effects of nedocromil sodium and salbutamol (sal) treatment were investigated in allergic, mild to moderately severe asthmatics. Both studies showed a significant decrease in symptom scores and diurnal PEF variation during nedocromil sodium compared with sal treatment. In addition, de Jong et al. also showed a significant increase in PC₂₀ propranolol (0.5 doubling concentrations) and morning PEF (16 L/Min) and a decrease in day-to-day PEF variation and rescue ipratropium bromide use (0.6 puffs/day) [56]. Wasserman et al. showed that regular treatment with nedocromil sodium provided a greater level of asthma symptom control than regular treatment with albuterol with a significantly greater improvement in daytime and nighttime asthma and morning chest tightness [57]. Quality of life was evaluated in a year long, double-blind, placebo-controlled comparative study with nedocromil sodium. The impacts component of the St. George's Respiratory Questionnaire was significantly improved in patients receiving nedocromil sodium (4 mg), as were night-time asthma, asthma severity at clinic and daytime inhaled bronchodilator use [58].

The effects of nedocromil sodium added to treatment with bronchodilators were investigated in seven placebo-controlled, group comparative studies in adults with mild to moderate, mostly allergic asthma (reviewed in [29]). Patients were maintained on oral and/or inhaled bronchodilator therapy and received nedocromil sodium or placebo after a baseline period of 2 to 4 weeks. Most of the studies showed a significant increase in FEV₁ (0.2–0.5 L) and PEF (10–75 L/min), a decrease in β_2 -agonist use (1–3 puffs/day), and an improvement of total symptom scores during nedocromil sodium compared with placebo treatment. The morning PEF values during nedocromil sodium therapy continued to increase over the period of treatment [59].

Nedocromil sodium versus corticosteroids

Inhaled corticosteroids are very effective anti-inflammatory agents in the treatment of asthma. Investigations into the effects of nedocromil sodium compared with corticosteroids can be classified into three categories: (1) Nedocromil sodium compared with inhaled corticosteroids; (2) the addition of nedocromil sodium and the ability of patients to reduce their concomitant corticosteroid therapy without further deterioration of their asthma; and (3) the effects of nedocromil sodium added to corticosteroid inhalation.

The effects of nedocromil sodium compared with inhaled corticosteroid therapy

In two placebo-controlled studies both nedocromil sodium (16 mg/d) and beclomethasone (400 µg/d) significantly improved symptom scores when compared with placebo. FEV₁ also improved during both treatments, but significantly more with beclomethasone than with nedocromil sodium treatment. Bel et al. demonstrated a significant improvement in airway hyperresponsiveness, as measured by the PC₂₀ methacholine, both during nedocromil sodium and beclomethasone (1.5 and 1.7 doubling concentrations, respectively) compared with placebo treatment, in nonatopic patients [53]. In three crossover studies in mostly allergic asthmatics both beclomethasone and nedocromil sodium were significantly better at improving PC₂₀ histamine (1 to 3 doubling concentrations) but beclomethasone improved the PC₂₀ values significantly more compared with nedocromil sodium (reviewed in [29]).

Addition of nedocromil sodium to corticosteroid therapy: steroid sparing effect?

In six placebo-controlled studies (reviewed in [29]) attempts were made to reduce the doses of inhaled or oral corticosteroids. About half of the patients were allergic, with mild to severe asthma. Paananen et al. stopped inhaled corticosteroids after 3 weeks of active treatment. The results showed a general impairment in PEF values and symptom scores, an increase in β₂-agonist use and a high number of dropouts during both nedocromil sodium and placebo treatment. Ruffin et al. and Bone et al. halved the amount of inhaled corticosteroids during the baseline period, to make asthma unstable. During nedocromil sodium treatment, PEF values (20 L/min) and symptom scores and β₂-agonist use improved significantly compared to placebo.

In a group of steroid-dependent chronic asthmatic patients, with a mean oral prednisolone use of > 8 mg/day, it was not possible to reduce the amount of prednisolone significantly more during nedocromil sodium compared with placebo treatment. None of the clinical outcome parameters (FEV₁, PEF values, β₂-agonist use and symptoms scores) changed in either treatment group [60]. In another study [61] a significantly greater reduction of the oral steroid dose during nedocromil sodium treatment was achieved, compared with placebo treatment. However, there was no change in FEV₁, PEF or symptom scores. During nedocromil sodium treatment the β₂-agonist use was increased, as was the use in the placebo group.

In a placebo-controlled study, Orefice et al. compared the effects of treatment with nedocromil sodium (16 mg/day) with cromoglycate (40 mg/day) and beclomethasone (1500 µg/day) [62]. Compared with placebo, all patients managed to improve FEV₁ (0.2 l) and PC₂₀ methacholine (1.2 doubling doses). β₂-agonist use and symptom scores decreased significantly during beclomethasone, compared with beclomethasone and placebo treatment, but not in comparison with nedocromil sodium treatment. However the four treatment groups are difficult to compare

because all patients, with the exception of the beclomethasone group, received the oral corticosteroid daflazacort.

The effects of nedocromil sodium when added to the treatment of patients already inhaling corticosteroids

The effects of nedocromil sodium (8–16 mg/day), added to the therapy of subjects who were mostly steroid-dependent with stable, moderate to severe asthma were investigated in five placebo-controlled studies (reviewed in [29]). Most of the studies showed significant improvements in PEF values (14–30 L/min) and symptom scores, and decreases in β_2 -agonist use (about 1 puff/day) in patients treated with nedocromil sodium, compared with placebo. In contrast to patients not receiving corticosteroids, the addition of nedocromil sodium to corticosteroids leads only to an improvement in PEF during the first 4 to 8 weeks, reaching a plateau at that time without further improvement.

In spite of these confounding factors, many studies have demonstrated the non-steroidal anti-inflammatory drug nedocromil sodium to be effective. To summarize:

- (i) Nedocromil sodium treatment is clearly superior to placebo and in some studies it provides better clinical improvement compared to continuous bronchodilator therapy.
- (ii) Several studies demonstrate that the effects of nedocromil sodium (16 mg/day) are equal to these of beclomethasone (400 μ g/day) for some outcome parameters. For changes in symptom score and β_2 -agonist use beclomethasone gives significantly better results.
- (iii) No conclusive data can be drawn from studies replacing corticosteroid treatment with nedocromil sodium, because the withdrawal of corticosteroids was too rapid and the amounts used too great.
- (iv) Nedocromil sodium seems to be effective especially in mild to moderate allergic and nonallergic asthma.
- (v) There may be an important role for nedocromil sodium as an additional therapy in patients already using high doses of inhaled corticosteroids.
- (iv) Compared with inhaled cromoglycate (4×10 mg/day), nedocromil sodium (4×4 mg/day) is more effective in reducing asthma symptoms and extra bronchodilator use. In patients more than 50 years old, there seems to be no difference in the efficacy of the two drugs.
- (vii) It is still not clear whether nedocromil sodium can be used as a steroid sparing agent.

Nedocromil sodium and steroid-resistant asthma

Some patients with asthma respond poorly to corticosteroids and have persistent airway inflammation and daily clinical symptoms. Marin et al. demonstrated that

inhaled nedocromil sodium improves pulmonary function and decreased asthma severity in steroid-resistant asthma [63].

Nedocromil sodium and COPD

The effect of 10 weeks of treatment with nedocromil sodium in non-allergic subjects with COPD was reported. Nedocromil sodium failed to improve clinical parameters (airway responsiveness to histamine, methacholine, AMP, pulmonary function and symptom scores). The only difference with placebo was seen in the lower number of dropouts (because of exacerbations). Also both patients and clinicians favoured treatment with nedocromil sodium [64].

Safety

No major side-effects have been noted during the therapeutic evaluation with nedocromil. The drug has an excellent safety profile up to the present. The most frequent side-effect is unpleasant taste and occasionally local irritation of the throat and the airways. Some patients treated with nedocromil may also experience headache.

Summary and conclusion

Cromoglycate and nedocromil work through a number of different mechanisms. Their original designation as “mast cell stabilizing agents” is an oversimplification of their multiple pharmacological activities. According to the international guidelines [1] cromolyn sodium and nedocromil are considered to be long-term-control medications. They have a mild to moderate anti-inflammatory effect and can be the initial choice for long-term-control therapy in children. They can also be used as preventive treatment prior to exercise or unavoidable exposure to known allergens. Safety is one of the primary advantages of these agents. A therapeutic response often occurs within 2 weeks, but a 4-to-6 week trial may be needed to determine the maximum benefit.

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Anti-leukotriene therapy for asthma

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Introduction

Human bronchial asthma is a chronic disorder of the airways, associated in most cases with atopy (i.e. specific immunoglobulin E responses to allergens), and characterised by a range of abnormalities on various levels. Early definitions of asthma stressed the aspects of variable lung function, reversible spontaneously or with treatment, but more recent definitions also place emphasis on the underlying airway inflammation that is thought to promote bronchial hyperresponsiveness [1]. The asthma syndrome comprises firstly, clinical symptoms, such as chest tightness, dyspnoea, coughing, and/or wheeze, secondly, pathophysiological features, such as variable airway obstruction and bronchial hyperresponsiveness (BHR) to a variety of stimuli which may be physical (e.g. cold dry air, hyper/hypotonic saline), pharmacological (e.g. histamine, methacholine), chemical (e.g. SO₂), or physiological (e.g. exercise), and thirdly, pathohistological parameters, particularly eosinophilic airway inflammation, resulting in structural changes within the airways (“airway remodelling”) [2, 3]. Asthma may occur in varying degrees of severity ranging from intermittent to severe persistent forms [1].

Airway inflammation plays a central role in the pathogenesis of asthma [2]. Hence, in recent asthma management guidelines, anti-inflammatory therapy (“controller” medication, mostly inhaled corticosteroids) is advocated as first-line treatment of persistent asthma, in combination with “reliever” medication (specific β_2 -bronchodilators) as required for rescue medication [1]. Despite the well-documented effectiveness and relative safety of these drugs, too many asthmatic patients are still being treated suboptimally, resulting in a poor quality of life [4]. The reasons for suboptimal treatment include poor compliance with treatment, sometimes due to misunderstanding by patients of the different roles of anti-inflammatory prophylaxis and bronchodilator “rescue” drugs, and sometimes due to largely misplaced anxiety about adverse drug effects, particularly with corticosteroids. In addition, a world-wide trend of increasing prevalence, morbidity, and mortality of asthma has been observed in the last decade [1]. Therefore, many research groups have sought

alternative anti-inflammatory drugs, preferably to be combined with bronchodilator activity. The anti-leukotriene (anti-LT) drugs seem to comply with this profile.

Experimental models of asthma

In preclinical studies, exacerbations of asthma can be mimicked in appropriate patients by a number of well-documented models including exercise and aspirin challenge, inhalation of allergen or ozone, experimental virus infections, or even withdrawal of regular anti-asthma controller treatment [5].

The characteristics of the allergen challenge model make it one of the most suitable systems for intervention studies with experimental anti-asthma drugs. Inhalation of a specific allergen by a sensitive asthmatic patient induces acute airway narrowing, the so-called early asthmatic response (EAR), usually defined as a fall in forced expiratory volume in 1 s (FEV_1) of at least 20% from baseline [6]. The EAR involves IgE-triggered mast cell mediator release, predominantly causing acute airway smooth muscle contraction, occurring within 10 min after allergen inhalation and mostly recovering within 3 h [6, 7]. In approximately 50% of cases, the EAR is followed by a late asthmatic response (LAR) at 3–7 h, defined as a fall in FEV_1 of at least 15% from baseline [6]. The LAR is characterised by inflammatory events within the airways in which activated eosinophils are thought to play a key role. In particular, airway oedema and mucus hypersecretion, as well as airway smooth muscle contraction, may make significant contributions to airflow obstruction in the LAR. Eosinophil release of pro-inflammatory mediators and basic proteins may induce the subsequent BHR which may last for several days or weeks following the LAR [7–9], perhaps due to damage to the airway epithelium which allows greater access of airborne irritants to the bronchial submucosa. The LAR is thus thought to mimic the underlying pathophysiological changes in the asthmatic airway.

Despite many advantages for the investigator, experimental models of asthma can only reflect the acute inflammatory processes occurring within the airways during exacerbations of asthma. Inflammatory changes in clinical asthma may occur in response to persistent exposure to low levels of airborne allergens or sensitising chemicals, rather than the large bolus doses of allergens and stimuli administered in the laboratory. Long-term follow-up studies in patients with persistent asthma are therefore essential to evaluate the possible effects of novel anti-asthma therapies on the chronic, structural changes which determine clinical outcome. Both types of intervention have been applied with anti-LT drugs [10]. Although airway inflammation and remodelling involve complex networks of inflammatory cells and mediators, the central role of the leukotriene family of mediators in these processes has been amply demonstrated by the surprising effectiveness of anti-LT drugs.

Leukotrienes and human lung cells

The leukotriene synthetic pathway

The eicosanoids are a heterogeneous group of biologically-active mediators derived from the 20-carbon fatty acid arachidonic acid, and include leukotrienes (LTs), prostaglandins (PGs), thromboxane (TX), lipoxins (LX), and hydroxylated derivatives (HETEs)[11]. Leukotrienes are derived from arachidonic acid via the 5-lipoxygenase (5-LO) pathway (Fig. 1) [11, 12]. 5-LO is a 78 kDa haem protein found in the cytosol of a limited number of resting cells of myeloid origin [13, 14]. 5-LO activating protein (FLAP), an 18 kDa nuclear membrane-bound protein, is essential for translation and activation of 5-LO, together with Ca^{2+} and ATP [15, 16]. First, free arachidonic acid cleaved from membrane phospholipids by cytosolic phospholipase A_2 or other phospholipases, is donated by FLAP to 5-LO, which converts it to 5-

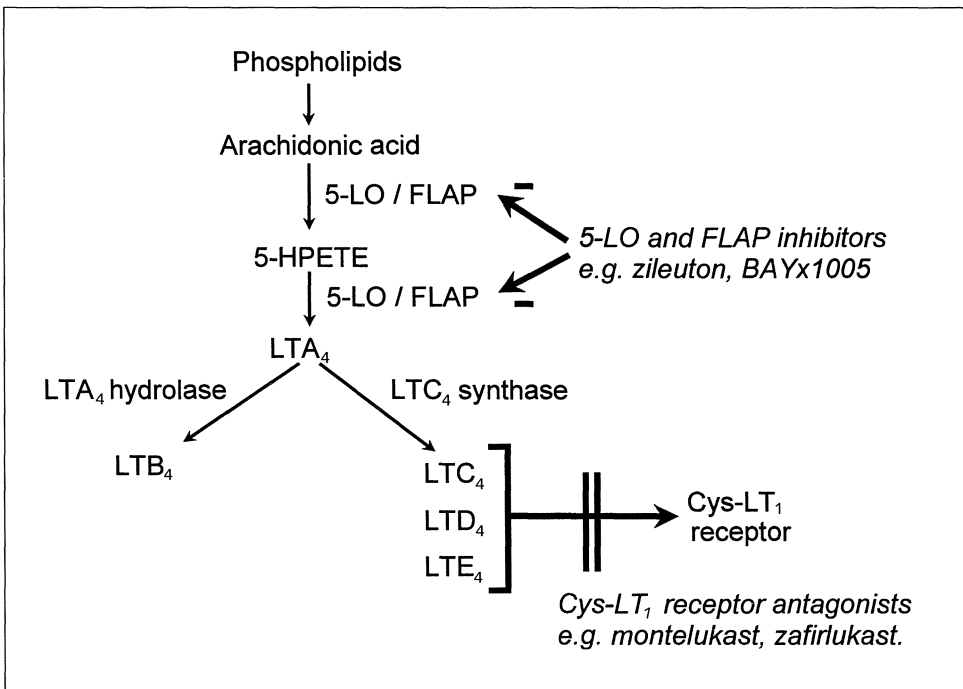


Figure 1

Arachidonic acid metabolism: 5-lipoxygenase pathway with sites of action of leukotriene receptor antagonists and leukotriene biosynthesis inhibitors.

Abbreviations: 5-LO, 5-lipoxygenase; FLAP, 5-lipoxygenase activating protein; (cys)-LT, (cysteinyloxy)- leukotriene; 5-HPETE, 5-hydroperoxy-eicosatetraenoic acid

hydroperoxy-eicosatetraenoic acid (5-HPETE) and thence to the unstable epoxide LTA₄. Subsequently, depending on cell-type, LTA₄ is converted either to LTB₄ by LTA₄ hydrolase [17], or to the first of the cysteinyl-leukotrienes (cys-LTs), LTC₄, by LTC₄ synthase [18]. LTC₄ synthase, a homodimer of 16.6 kDa subunits, is an integral membrane protein with striking sequence homology to FLAP [19]. Following carrier-mediated export from the cell [20], LTC₄ is metabolized via LTD₄ to the relatively stable LTE₄ by extracellular enzymes found in lung tissue and plasma [21, 22]. Both LTE₄ and LTB₄ are then degraded by ω - and β -oxidation in the liver [23]. LTB₄ may also be rapidly degraded by ω -oxidation within activated neutrophils at the inflammatory site [24]. A small proportion (about 5%) of total systemic LTC₄ is excreted unchanged as LTE₄ in the urine [25].

Actions of leukotrienes on human lung cells

While LTB₄ is predominantly synthesized by monocytes, macrophages, and neutrophils, cys-LTs are mainly produced by mast cells, basophils, and eosinophils [11, 12, 26–31]. Monocyte-macrophages may also contribute to cys-LT formation. Both subfamilies of leukotrienes possess distinct biologic properties which may account for many of the features of asthma, as well as of other inflammatory diseases [11, 12, 32, 33]. LTB₄ is a potent chemoattractant and activator of neutrophils, monocytes, and eosinophils in several species *in vitro* and *in vivo* [11, 12, 34, 35], but does not contract human airway smooth muscle [36]. In contrast, cys-LTs are exquisitely potent in contracting airway smooth muscle in normals and asthmatics [37–40]. They are also potent enhancers of airway microvascular permeability, resulting in oedema, and they stimulate mucus secretion [41, 42]. Furthermore, recent evidence shows that cys-LTs also possess potent and specific chemoattractant activity for eosinophils in animals and humans both *in vitro* and *in vivo* [35, 43–45]. This is in contrast to other chemoattractant lipids such as platelet-activating factor (PAF) and LTB₄ which also attract neutrophils.

Leukotriene receptors

The pro-inflammatory effects of leukotrienes are mediated by stimulation of specific receptors [46]. Although bronchoconstriction of isolated human airways by cys-LTs is mediated by a direct effect at specific receptors on airway smooth muscle [36], such effects may be supplemented or modulated in whole lung *in vivo* by secondary release of other pro-inflammatory mediators, such as neuropeptides [47]. While LTB₄ is likely to occupy a distinct receptor, the BLT receptor [11], many details of cys-LT receptor pharmacology remain to be clarified. In guinea-pig airways, distinct receptor subtypes have been isolated for LTC₄ and LTD₄, termed LTR_c and LTR_d

respectively [48, 49]. The latter has been characterised as a 45 kDa membrane protein [50]. Conflicting data exist on possible receptor heterogeneity in human airways. Investigations with an early leukotriene receptor antagonist (FPL-55712) in isolated human airways suggested that all cys-LTs interact with a single receptor [51], whereas binding studies with [³H]-leukotriene radioligands pointed to the existence of two distinct cys-LT receptors: one for LTD₄ and LTE₄, and a second for LTC₄ [46]. Such experiments may be confounded by non-specific binding of ³H-LTC₄ to cell-associated enzymes including LTC₄ synthase, or to the γ -glutamyl transpeptidase which converts LTC₄ to LTD₄. Recent evidence however suggests heterogeneity of cys-LT receptors in different tissues; LTC₄ and LTD₄ are regarded as full agonists, and LTE₄ as a partial agonist at cys-LT₁ receptors located on airway smooth muscle, which mediate airway smooth muscle contraction, while cys-LT₂ receptors, located in the smooth muscle of pulmonary arteries and veins, mediate relaxation [46, 52]. Among the cys-LT receptor antagonists, all of which block the cys-LT₁ receptor, only Bay u9773 also blocks the cys-LT₂ receptor [52]. In addition, evidence has been provided for similar (auxiliary?) receptors located on human pulmonary vein endothelial cells, which are likely to bear both cys-LT₁ receptors, associated with contraction, and cys-LT₂ receptors, which mediate relaxation [46]. The further development of potent, selective antagonists for each receptor subtype should clarify the role and mechanism of action of these receptors subsets in the pathophysiology of asthma.

Detection of endogenous leukotriene production

A number of studies have detected the release of cys-LTs into relevant body compartments in clinical asthma and in various challenge models of asthma. Inhaled allergen challenge of atopic asthmatics causes significant rises in BAL fluid LTC₄ levels 5 min after challenge [53] and in urinary LTE₄ levels within 4 h [54]. The former is accompanied by rises in BAL fluid histamine, tryptase, and PGD₂, suggesting that all four mediators are released by degranulating mast cells. In symptomatic patients with atopic asthma, increased levels of LTB₄ and/or cys-LTs have been demonstrated in the exhaled breath condensate, and in biological fluids including blood, urine, sputum, and bronchoalveolar lavage fluid [54–64]. This generation of leukotrienes may either result from direct activation of inflammatory cells [7, 28], or from secondary release by other mediators, such as platelet activating factor (PAF) [65], or bradykinin [66]. Despite conflicting data on cys-LT production following exercise or isocapnic hyperventilation of cold and/or dry air [67–72], several intervention studies using anti-LT drugs have attested that leukotrienes are likely to be involved in the bronchoconstriction induced by these challenges [10, 73].

In aspirin-sensitive asthmatic patients, who are mostly non-atopic [74], basal production of cys-LTs is particularly exaggerated, as reflected by increased urinary

LTE₄ levels as compared to aspirin-tolerant asthmatics or normals [75]. Challenge with oral aspirin or inhaled lysine-aspirin produces significant increases in BAL fluid and urinary cys-LTs only in aspirin-intolerant asthmatics [76, 77], probably because NSAIDs remove the chronic suppression of cys-LT synthesis by endogenous PGE₂ [78]. The increased basal cys-LT synthesis in aspirin-intolerant asthmatics, and the BHR to inhaled NSAIDs, are closely related to markedly higher expression of LTC₄ synthase, the terminal enzyme for cys-LT synthesis, in the bronchial wall of these patients [79]. This is due partly to chronically increased numbers of bronchial mucosal eosinophils in aspirin-intolerant asthmatics [79, 80], and partly to the higher proportion of eosinophils expressing the enzyme, compared to aspirin-tolerant asthmatics [79]. In addition, evidence exists for a selective target organ hypersensitivity to LTE₄ in patients with aspirin-induced asthma [81].

Effects of inhaled leukotrienes in man

Upon inhalation, cys-LTs induce clinical symptoms of asthma, including bronchoconstriction [82, 83]. The cys-LTs are several orders of magnitude more potent than other bronchoconstrictors such as histamine and PGD₂, and the bronchoconstriction is longer-lived, lasting 30–40 min compared to the 5–10 min response to histamine. Inhaled cys-LTs also cause increased bronchial responsiveness both in normal and asthmatic individuals [82, 85–87], although late phase responses to inhaled cys-LTs are not reported [88]. In addition, recent studies in asthmatic patients have shown selective influx of eosinophils into the airway in bronchial biopsies [44], and in hypertonic saline-induced sputum [45], 4 h after inhalation of LTE₄ and LTD₄, respectively. Furthermore, strong evidence exists from animal studies that cys-LTs may contribute to the development of structural changes within the airways, particularly by increasing the airway smooth muscle mass [89–91]. Apart from airway smooth muscle hypertrophy/hyperplasia, this airway remodelling is characterised by loss of airway epithelium and thickening of the airway wall through deposition of fibronectin and collagen [2, 92], and, from the pathophysiological point of view, it is a major determinant of excessive airway narrowing which may cause serious clinical symptoms [93]. In contrast to the cys-LTs, inhalation of LTB₄ by normal or atopic asthmatic subjects does not cause acute bronchoconstriction and does not change bronchial responsiveness to histamine [94].

Anti-leukotriene drugs

Despite the possible involvement of other pro-inflammatory mediators such as histamine, prostanoids, and platelet activating factor in asthma [56–58], therapy with

experimental drugs directed against single mediators have mostly shown disappointing results in the treatment of asthma, especially in the case of PAF antagonists [95]. The anti-LT drugs are exceptions to this rule. Two categories of anti-LT agents have been developed. Firstly, the leukotriene receptor antagonists (LTRA) block either cys-LT receptors (cys-LT₁) or LTB₄ receptors (BLT), and secondly, the leukotriene synthesis inhibitors (LTSIs) which block either 5-LO activity or FLAP activity (Fig. 1), and hence prevent the synthesis both of cys-LTs and of LTB₄ [10]. Little evidence exists of any meaningful differences in the therapeutic benefits of LTSIs compared to cysLTRAs .

Leukotriene B₄ receptor (BLT) antagonists

LTB₄ has potent chemotactic activity on neutrophils and eosinophils both *in vitro* [33] and *in vivo* [34], and increased LTB₄ levels have been reported in body fluids of symptomatic asthmatics [60, 61, 63]. Both neutrophils and eosinophils recruited to the airways of asthmatics by segmental allergen challenge show decreased migratory responses to LTB₄ *in vitro* [96, 97], suggesting desensitisation following exposure to endogenous LTB₄ *in vivo*. Similarly, in normals, inhalation of LTB₄ causes a rapid blood neutropaenia due to margination of circulating neutrophils within the lung vasculature, but patients with stable mild persistent asthma show an attenuated response [98], again suggesting neutrophil desensitisation due to prior exposure to excess endogenous LTB₄. Potent (but non-selective) chemoattractants such as LTB₄ may cause specific eosinophil migration in atopic subjects, whose eosinophils may be primed by specific (but weak) chemokines such as interleukin-5 [99].

Specific BLT receptor antagonists have been developed. So far, few such compounds have been applied in human studies of asthma. In sensitized guinea-pigs, the BLT antagonist U-75302 considerably reduced peribronchial eosinophil infiltration following antigen challenge, without affecting neutrophil migration [100]. However, this was not confirmed in humans *in vivo* with the BLT receptor antagonist LY-293111 [101]. In the latter study, LY-293111 failed to protect against allergen-induced bronchoconstriction, and had no effect on bronchial responsiveness to histamine, despite significant decreases in neutrophil counts and in myeloperoxidase levels in BAL fluid 24 h after challenge [101]. The role of neutrophils and of LTB₄ in allergen-induced airway responses is still under debate [7, 8, 102, 103]. Hence, BLT receptor antagonists may have greater effects in models in which neutrophils are likely to play a more prominent role, such as ozone inhalation, occupational asthma, or experimental virus infections [103]. Evidence for the involvement of neutrophils in sudden-onset fatal asthma [104] and in nocturnal asthma [105] reinforces the need to examine anti-LT drugs in clinically-relevant models other than allergen challenge.

Cysteinyl-leukotriene receptor (cys-LT₁) antagonists (cysLTRAs)

The cys-LT receptor antagonists (cysLTRAs) antagonize the effects of cys-LTs at the cys-LT₁ receptor [51]. The first drugs in this category to be evaluated in humans *in vivo* included FPL-55712, L-648051, L-649923, LY-171883 (tomelukast), LY-170680 (sulukast), RG-12525 and SKF-104353 (pobilukast) [106–112]. These compounds had relatively low potency, producing a rightward shift in the LTD₄ dose-response curve of only three to 12-fold. Lack of potency, and in some cases undesirable side effects, led to the development of most of these compounds being suspended. In the past 6–7 years, more potent and/or selective cysLTRAs have been developed, among which pranlukast (SB-205312; Ultair or Onon), zafirlukast (ICI 204219; Accolate) and montelukast (MK-0476; Singulair) have been registered, or are close to registration, in a number of countries including the USA, Japan, and several countries in Europe [10]. These compounds are administered orally at a recommended rate of once or twice daily.

The cysLTRAs have a dual mechanism of action. Firstly, these agents have bronchodilator activity, which is additive to β_2 -agonists in patients with mild to moderate persistent asthma [113, 114]. This suggests that part of their efficacy relies on blockade of LT-induced airway oedema and/or mucus hypersecretion, as well as on blocking LT-induced airway smooth muscle contraction. In the inhaled allergen challenge model, potent cysLTRAs inhibit 75–88% of the early response (EAR) and 54–63% of the late response (LAR) [10]. Furthermore, cysLTRAs provide clinically significant protection against bronchoconstriction induced by inhaled lysine-aspirin [115] or exercise challenge [116] in susceptible patients (Tab. 1). Secondly, strong evidence exists for anti-inflammatory activity of cysLTRA [117–122], in that they reduce airway inflammatory cell counts and mediator levels, reduce bronchial hyperresponsiveness, and allow reductions in steroid dosage, suggesting modulation of the underlying disease processes (Tab. 2).

Clinical effects of cysLTRAs

An increasing number of long-term follow-up studies investigating cysLTRAs in clinical asthma are currently being performed. Consequently, many results are not yet published in full. Several studies in patients with different gradations of asthma have been reported, showing that pranlukast, zafirlukast and montelukast, alone or in combination with anti-inflammatory anti-asthma therapy (mostly theophylline or corticosteroids), are capable of improving both clinical criteria and lung function parameters (Tab. 2). In these studies, more or less comparable increases in lung function parameters (FEV₁ and peak flow) have been measured, which are dose- and duration-dependent, and often accompanied by clinically sig-

nificant decreases in daytime and nighttime symptom scores, and in β_2 -agonist use in patients with mild to moderate, or with moderate to severe persistent asthma (Tab. 2) [122–125].

Apart from bronchodilation, cysLTRAs possess anti-inflammatory properties, also dependent on the dose and duration of treatment [117–122, 126–128]. In studies in patients with moderate to severe persistent asthma, 6 to 12 weeks of treatment with various cysLTRAs had a steroid-sparing effect [120–122]. Moreover, oral pranlukast produced small but significant decreases in baseline bronchial responsiveness to methacholine after only 1 week of treatment in patients with stable asthma [121], and this was sustained for up to 24 weeks [128]. Two- to five-fold reductions in baseline bronchial responsiveness to methacholine are obtained with 2–12 weeks of treatment with oral zafirlukast in mild to moderate atopic asthmatics [129]. One week of treatment with oral zafirlukast also produces a significant decrease in basophils and eosinophils in the BAL fluid of patients with mild to moderate atopic asthma 48 h following segmental allergen challenge [117]. Similarly, 4 weeks of treatment with oral montelukast provided a significant reduction in sputum eosinophils in patients with mild to moderate persistent asthma [127]. These studies clearly indicate that cysLTRAs are capable of reducing airway eosinophilia and the associated BHR in clinical asthma.

Leukotriene synthesis inhibitors

Since LTB_4 , as well as cys-LTs, may contribute to chronic airway inflammation in asthma, pharmacological agents have been developed (LTSIs) which block the synthesis of both leukotriene subfamilies, by inhibiting 5-lipoxygenase activity (5-LO inhibitors) or 5-LO activating protein activity (FLAP antagonists) (Fig. 1). Fewer long-term clinical studies of LTSIs have been published compared to cysLTRAs trials, and direct comparison is complicated by the trend for LTSI in slightly more severe asthma patient groups than those investigated in cysLTRAs trials. However, LTSIs appear to provide improvements in clinical and lung function parameters in asthma comparable to those obtained with the cysLTRAs (Tabs. 1 and 2).

Clinical effects of LTSIs

In repeated-dose studies in asthma, most experience has been accumulated with the first generation 5-LO inhibitor zileuton, mostly at oral doses of either 400 or 600 mg q.i.d. [130] (Tab. 2). Zileuton (formally Leutrol™, Zyflo) has recently been registered for treatment of asthma in the United States. Besides, the FLAP antagonists MK-0591 and BAY-x1005 have also shown improvements in clinical and lung function parameters after 4 to 6 weeks of treatment [131, 132].

Table 1 - Effects of cys-LT₁ receptor antagonists and leukotriene biosynthesis inhibitors on various bronchoprovocation tests in asthma

Compound	Type	Stimulus	Dose, route	Effect (% mean inhibition)	Ref.
ICI-204, 219 (zafirlukast*; Accolate#)	cysLTR	allergen	40 mg, p.o.	EAR (max) ↓88%	[151]
		exercise	0.4 mg, I	LAR (max) ↓54%	[152]
		eosinophils ^{\$}	20 mg, p.o.	↓52%	[153]
		basophils ^{\$\$}	7 (2 × 160 mg), p.o.	↓40%	[117]
MK-571	cysLTR	allergen	450 mg, i.v.	↓45%	[154]
		exercise	160 mg, i.v.	↓57%	[155]
MK-0476 (montelukast*; Singulair#)	cysLTR	allergen	2 × 10 mg, p.o.	EAR (AUC ₀₋₃) ↓88%	[156]
		exercise	160 mg, i.v.	LAR (AUC ₃₋₁₀) ↓63%	[156]
SK&F104, 353 (pobilukast*)	cysLTR	exercise	10 mg, p.o.	↓68%	[157]
		aspirin	893 µg, I	EAR (max) ↓54%	[158]
		exercise	800 µg, I	LAR (AUC ₃₋₈) ↓57%	[148]
		aspirin	750 mg, p.o.	LAR (max) ↓36%	[115]
ONO-1078 = SB205, 312 (pranlukast*; Ultair/Onon#)	cysLTR	dipyrrone	225 mg, p.o.	↓41%	[159]
		exercise	14 (1 × 450 mg), p.o.	↑14-fold dipyrrone dose ↓45%	[160]

Table 1 (continued)

Compound	Type	Stimulus	Dose, route	Effect (% mean inhibition)	Ref.
A-64077 (zileuton* ; Leutrol = Zylflo#)	5-LO inhibitor	cold, dry air	800 mg, p.o.	↑47% cold, dry air extra for ↓10% FEV ₁	[161]
		exercise	4 × 600 mg, p.o.	↓41%	[162]
		aspirin	6-8 (4 × 600 mg), p.o.	↓76%	[146]
		eosinophils	8 (4 × 600 mg), p.o.	↓66%	[138]
ZD2138	5-LO inhibitor	aspirin	2(1 × 350 mg), p.o.	↓76%	[163]
MK-886	FLAP-antagonist	allergen	500+250 mg, p.o.	EAR (AUC ₀₋₃) ↓58% LAR (AUC ₃₋₇) ↓44%	[164]
MK-0591	FLAP-antagonist	allergen	3 × 250 mg, p.o.	EAR (AUC ₀₋₃) ↓79% LAR (AUC ₃₋₈) ↓39%	[165]
BAY-x1005	FLAP-antagonist	allergen	3.5 (2 × 500 mg), p.o.	EAR (AUC ₀₋₃) ↓87% EAR (max) ↓57% LAR (AUC ₃₋₇) ↓60% LAR (max) ↓46%	[166]

cysLTR_A, cysteinyl leukotriene receptor antagonist; EAR, early asthmatic response; LAR, late asthmatic response; AUC, area under the time-response curve over an indicated time-interval; 5-LO, 5-lipoxygenase; FLAP, 5-lipoxygenase activating protein; p.o., per os; i.v., intravenous; I, inhaled; *, generic name; #, trade name; \$/\$\$, airway eosinophils and basophils, respectively, after a segmental allergen provocation test; dipyrone, a pyrazolone derivative.

Table 2 - Effect of Cys-LT₁ receptor antagonists and leukotriene synthesis inhibitors in clinical asthma

Compound	Asthma (n = no. of patients)	Dose, route	Effect	Ref.
MK-571	mild-moderate (n = 43)	2 wk (3 × 75 mg), p.o.+ 4 wk (3 × 150 mg), p.o.	↑8–14% FEV ₁ ↓30% symptom scores ↓30% β ₂ -agonist use	[167]
pranlukast	moderate-severe (n = 42)	8 wk (2 × 450 mg)	↑16% morning/evening PEF ↓40% daytime symptom scores ↓60% nighttime symptom scores	[168]
pranlukast	moderate-severe (n = 586)	12 wk (2 × 150/300/450 mg)	↑8–12% FEV ₁ ↓34% symptom scores ↓36% rescue medication steroidsparing effect: ↓50% BDP	[169] [118]
pranlukast	moderate-severe (n = 79; BDP ≥ 1500 µg/day)	6 wk (2 × 450 mg)	steroidsparing effect: ↓50% BDP	[118]
zafirlukast	moderate-severe (n = 276)	6 wk (2 × 5/10/20 mg)	↑11% FEV ₁ ↓26% daytime symptom scores ↓46% nighttime symptom scores ↓30% β ₂ -agonist use	[123]
zafirlukast	moderate (n = 9; BDP 1500 µg/day)	12 wk (1 × 20 mg)	steroidsparing effect: ↓BDP	[119]
montelukast	moderate-severe (n = 29)	10.3 days (3 × 200 mg)	↑9–14% FEV ₁ ↓day/nighttime symptom scores ↓β ₂ -agonist use	[170]
montelukast	moderate-severe (n = 681)	12 wk (1 × 10 mg)	↓31% asthma exacerbations ↑37% asthma-free days	[171]
montelukast	moderate (n = 226; ICS 300–3000 µg/day)	12 wk (1 × 10 mg)	40% (M) vs 29% (P) tapering of ICS	[120]
montelukast	mild-moderate (n = 40)	4 wk (1 × 10 mg)	↓48% sputum eosinophils	[127]

Table 2 (continued)

Compound	Asthma (n = no. of patients)	Dose, route	Effect	Ref.
zileuton	moderate (n = 10)	13 wk (4 × 400/600 mg)	↑58% cold, dry air extra for ↓15% FEV ₁ upto 10 days after cessation of therapy	[136]
zileuton	moderate-severe (n = 401)	13 wk (4 × 400/600 mg)	6% (Z) vs 16% (P) need for corticosteroids during treatment period ↑16% FEV ₁ ↓26% β ₂ -agonist use ↓28% daytime symptom scores ↓33% nighttime symptom scores	[172]
zileuton	mild-moderate (n = 373)	26 wk (4 × 400/600 mg)	↑16% FEV ₁ ↑7–10% PEF ↓37% daytime symptom scores ↓31% nighttime symptom scores ↓31% β ₂ -agonist use ↓62% need for steroids ↓ blood eosinophils	[133]

BDP, beclomethasone; ICS, inhalational corticosteroids; M, montelukast; P, placebo

Considerable dose-dependent improvements in clinical symptoms and in lung function, together with a steroid-sparing effect, have been reported in patients with mild to moderate persistent asthma after 6 months of treatment with oral zileuton [133]. Using a similar treatment protocol, a steroid-sparing effect of zileuton was also demonstrated after 13 weeks treatment in patients with moderate to severe persistent asthma [134]. Further evidence for anti-inflammatory activity of this LTSI has been provided by other long-term studies [135–139]. In aspirin-intolerant asthmatics using corticosteroids, 6 weeks of treatment with oral zileuton produced a significant, additional improvement in clinical symptoms, lung function, and non-specific BHR [135]. In patients with mild to moderate persistent asthma, 13 weeks of treatment with oral zileuton provided adequate protection against bronchoconstriction induced by cold, dry air [136]. Unexpectedly, this protection persisted for up to 10 days after discontinuation of treatment, long after the end of the washout period of this short-lived drug (half life: 2.3 h), suggesting a modulatory effect of leukotriene inhibition on airway inflammation. The ability of zileuton to reduce BHR was comparable to that achieved with high-dose inhaled corticosteroid (budesonide), and better than that achievable with theophylline, cromoglycate, or a β_2 -agonist [136]. In another study in asthmatic patients treated with inhaled corticosteroids for at least 6 months, a single oral dose of zileuton (400 mg) significantly reduced BHR to inhaled histamine (2.1 doubling doses) and to distilled water (1.3 doubling doses), without affecting baseline FEV₁ [137]. Furthermore, in atopic asthmatic patients, 8 days of pretreatment with oral zileuton not only reduced BAL fluid and urinary LTE₄ levels (by about 85%) 24 h after segmental allergen challenge, but also significantly reduced BAL eosinophil counts by over 60%, with trends to lower basophil and macrophage counts and to reduced BAL fluid levels of eosinophil cationic protein (ECP) [138]. In patients with mild to moderate persistent asthma, 7 days of treatment with zileuton significantly reduced baseline LTB₄ and cys-LT levels in BAL fluid and reduced urinary LTE₄ excretion, and showed a trend for improvement in nocturnal FEV₁ [139]. Moreover, these effects were accompanied by a significant decrease in the percentage of eosinophils both in the BAL fluid and in peripheral blood [139]. Among the group of drugs capable of reducing eosinophilia in asthma, which includes corticosteroids and cromones, only anti-LT agents have a clearly-defined and specific mechanism of action, proving that blood and airway eosinophilia in asthma are due, to a significant degree, to endogenous leukotriene production.

Adverse effects of anti-leukotriene drugs

Anti-leukotriene drugs are generally well-tolerated. Most cysLTRAs such as zafirlukast show only a low rate of side-effects including headache, gastrointestinal tract disturbance, incidental infections, and rash, and most of these adverse effects are

seen in a similar proportion of patients in the placebo group, suggesting that they are not drug-related [140]. However, about 4.5% of patients receiving the LTSI drug zileuton had elevations in hepatic aminotransferase levels compared to only 1.1% of the placebo group [134]. The elevations in hepatic enzymes were reversible either spontaneously following continued treatment for 2–3 months, or by discontinuation of treatment. In the USA, the FDA recommend regular liver function monitoring in patients receiving zileuton. Caution may also need to be exercised in reducing steroid dosage drastically if asthma symptoms improve with concomitant anti-LT drug therapy, as rare cases of re-emergence of underlying hypereosinophilia (Churg-Strauss syndrome) have been reported.

The place of anti-leukotriene therapy in current asthma management guidelines

Recently, a consensus has emerged in long-term asthma management, which emphasises the need for a stepwise approach covering the spectrum from intermittent to severe persistent asthma, with early intervention with strenuous anti-inflammatory therapy when required [1, 141]. The guidelines also stress the need for educating health professionals and patients to expect high standards of asthma control, and for self-monitoring of lung function and to allow step-down in medication levels when good control is achieved. In intermittent asthma, occasional β_2 -agonist use may be all that is required. In persistent asthma, anti-inflammatory therapy is recommended in combination with bronchodilators p.r.n., depending on the frequency and severity of the clinical symptoms and pathophysiological parameters (Tab. 3).

The anti-LT agents are an entirely new approach which combines both bronchodilator (“reliever”) and anti-inflammatory (“controller”) properties in one family of drugs. Their efficacy has been demonstrated across the spectrum of disease severity. However, their efficacy may vary between patients at each level of severity, probably linked to recent observations of a non-responder minority [142, 143], who, although asthmatic, are unable to generate leukotrienes due to polymorphism in the 5-LO gene promoter [144]. The improvements achievable within the majority “responder” group may therefore be commensurately better than anticipated from the average values reported in studies where the two sub-groups are not distinguished. Experience in clinical trials and in clinical practice suggests that anti-LT drug therapy can be discontinued if no response is apparent within the first 2 months of treatment. At the opposite extreme, spectacular improvements in objective and subjective measures of quality of life can sometimes be observed in small numbers of responder patients.

Although anti-LT drugs are very unlikely to replace the corticosteroids, particularly in the treatment of severe persistent asthma, and are likely to be used in conjunction with β_2 -agonist rescue medication, their place in the management of asthma

Table 3 - Stepwise approach to management of asthma according to modern guidelines [1]

Severity of asthma	Controller	Anti-asthma therapy	Reliever
intermittent	<ul style="list-style-type: none"> • mostly none needed*) 		short-acting inhalational β_2 -agonists, as needed (max. once a week)
mild-persistent	<ul style="list-style-type: none"> • inhaled corticosteroids (200–500 μg), cromoglycate, nedocromil, or • sustained-release theophylline 	<ul style="list-style-type: none"> • inhaled corticosteroids (800–2000 μg), and • long-acting bronchodilator: either long-acting inhaled or oral β_2-agonist, or sustained-release theophylline 	short-acting inhalational β_2 -agonists, as needed (max. 3–4 \times in one day)
moderate-persistent	<ul style="list-style-type: none"> • inhaled corticosteroids (\geq 800–2000 μg), and • long-acting bronchodilator: either long-acting inhaled or oral β_2-agonist, or sustained-release theophylline, and • long-term oral corticosteroids 	<ul style="list-style-type: none"> • long-acting bronchodilator: either long-acting inhaled or oral β_2-agonist, or sustained-release theophylline, and 	short-acting inhalational β_2 -agonists, as needed (max. 3–4 \times in one day)
severe-persistent		<ul style="list-style-type: none"> • long-acting bronchodilator: either long-acting inhaled or oral β_2-agonist, or sustained-release theophylline, and • long-term oral corticosteroids 	short-acting inhalational β_2 -agonists, as needed

* during exacerbations short-term oral corticosteroids may be needed

ma seems justified [145]. Revised guidelines for their positioning in asthma management will suggest that they are indicated as first-line treatment in specific asthma phenotypes, including most patients with aspirin-induced asthma [146, 147], and also in patients with intermittent or mild persistent asthma who regularly experience exercise-induced bronchoconstriction [136, 147]. In studies comparing the effects of pobilukast or zafirlukast with cromoglycate, these cysLTRAs provided at least equivalent protection against exercise-induced bronchoconstriction, with a better recovery than cromoglycate [148, 149]. Furthermore, in patients with more severe asthma (moderate to severe persistent), who need daily medication with high doses of inhaled or oral corticosteroids, anti-LT drugs may be useful because of their steroid-sparing effect [119, 120]. Moreover, their beneficial effects both on clinical and pathophysiological parameters, in combination with a relatively good side-effect profile, make anti-LT therapy a promising approach in children and adolescents [149, 150]. An additional advantage is the favourable pharmacokinetic profile of some of these compounds: with some cysLTRAs, a single oral dose may provide protection for 24 h [120, 127, 150]. This is likely to enhance compliance with therapy.

In summary, following years of intensive research into the inflammatory basis of asthma, a novel generation of rationally-targeted drugs has been added to the current armoury of asthma medication. Anti-LT drugs represent the first entirely novel approach to asthma therapy since the introduction of inhaled corticosteroids over 25 years ago. Their ultimate position in asthma management will depend upon their effects on the pathophysiology and histopathology of the asthmatic lung. To realise the full potential of this new development, further long-term studies are required to compare the ability of anti-LT therapy to modulate airway inflammation in asthma with that of adequate doses of corticosteroids and other current asthma therapies.

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Immunomodulators

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Introduction

Airway inflammation is fundamental to the disease process in chronic asthma. Different components of the immune system play important roles in perpetuating and orchestrating this inflammatory response. Many types of cells are involved, the airway epithelium is shed; eosinophils, T lymphocytes, polymorphonuclear cells, mast cells, and macrophages are present in an activated state and release proinflammatory mediators, cytokines and growth factors. From inflammation, a process of healing and repair may follow and this has been postulated to lead to remodeling of the airways.

This immunological basis underlying asthma is increasingly recognised and may explain many of the pathophysiological features characteristic of asthma. Recently, considerable interest and emphasis have been put on a subtype of CD4⁺T lymphocytes, termed type 2 T helper cells (Th2). These Th2 cells preferentially elaborate and release cytokines such as interleukin-4 and 5 that promote eosinophilic inflammation. Although not all asthmatics are atopic, they all share similar pathologic features, which supports the importance of these CD4⁺ T lymphocytes in the pathophysiology of chronic asthma [1].

Many of the established therapies in asthma, notably corticosteroids, have their clinical activity because they affect these immunologically important cells. Theophylline, traditionally considered a bronchodilator, has now been shown to have immuno-modulatory effects with evidence for regulation of the expression of CD4⁺ and CD8⁺ cells in the airway (see Chapter by Tenor and Schudt, this volume). The concept of modulating or suppressing the immune system using other therapeutic agents forms the theoretical basis for the use of immunosuppressants to treat patients with severe and difficult-to-control asthma.

Patients with severe chronic asthma currently represent a management problem. They may need long-term oral corticosteroids in addition to high doses of inhaled

corticosteroids and additional bronchodilators to control their asthma. Many of these individuals become chronically dependent on oral corticosteroids and suffer the side-effects arising from their chronic use. Despite this, they are often symptomatic and their daily activities are severely restricted.

Modulating the immune system in chronic asthma

Currently, drugs that suppress the immune system may be divided into three categories: first, anti-inflammatory agents such as those of the corticosteroid family and gold; secondly, cytotoxic drugs such as methotrexate and azathioprine; and thirdly, fungal and bacterial derivatives, such as cyclosporin A and tacrolimus (FK506), which inhibit signaling events by T lymphocytes.

Corticosteroids are by far the most effective agent to suppress unwanted immune responses in the airways of chronic asthmatics (see Chapter by Barnes, this volume). The introduction of inhaled steroids has revolutionized the management of asthma worldwide. Nevertheless, these agents are only partially successful in treating the more severe asthmatics, many of whom are dependent on oral corticosteroids. The use of other potent anti-inflammatory/immunosuppressive agents may have particular value in such patients.

Cytotoxic drugs inhibit all proliferating cells indiscriminately, including those of the immune system. Their toxicity and uncertain safety in long-term use would limit their use in treatment of many immune-mediated diseases. Immunosuppressants such as cyclosporin A, however, have a more selective action on immune cells, in particular T lymphocytes, and thus leave other cells relatively unaffected, potentially limiting the extent of unwanted cytotoxic side-effects.

The clinical use of intravenous serum immune globulin (IVIG) in severe asthmatics, has also received some attention in view of its increasingly recognised beneficial effect in treating other immune-mediated diseases, an effect which is distinct from simple replacement of immunoglobulins. Many of these efficacies observed are believed to be related to a variety of “immune-modulatory” activities that IVIG possesses.

Other experimental “immunomodulators” are presently being investigated. They include anti CD4⁺ lymphocyte monoclonal antibody, anti-IgE agents (see Chapter by Patalano, this volume) and cytokine receptor antagonists (see Chapter by Foster and Hogan, this volume). These new agents represent exciting challenges to our present understanding of the immunopathogenesis of severe asthma and may provide potentially effective alternative treatment for asthma patients. This chapter will focus on the use of immunosuppressants and IVIG as immunomodulators. These agents have primarily been assessed for their potential to reduce the requirements for corticosteroid (“steroid-sparing”) on which many patients become dependent.

Gold

The exact mechanism by which gold salts exert their anti-inflammatory activity is unknown. They have been shown to inhibit IgE-mediated release of histamine and leukotriene C₄ from human basophils and mast cells [2]. Gold also interferes with T cell function, which may partly explain its efficacy in rheumatoid arthritis. Otherwise its ability to arrest progression of disease and induce apparent remission (“disease modifying”) in rheumatoid arthritis is largely unexplained.

The clinical use of oral gold (auranofin) in corticosteroid-dependent asthmatics has been studied in several trials. The first [3] was an open trial involving 20 recruits who showed an improvement in symptoms and a reduced frequency of asthma attacks. They also had reduced bronchial reactivity to methacholine and a 34% reduction in corticosteroid requirement. Following this, a controlled trial [4] in 32 corticosteroid dependent asthma patients given oral gold (3 mg twice daily) or placebo for 26 weeks also showed promising results. The gold treated group showed a statistically significant reduction in oral corticosteroid use and the number of asthma exacerbations requiring rescue courses of corticosteroid, together with a small improvement in lung function compared to the placebo group. More recently, a double-blind study of the effect of oral gold (3 mg twice daily) or placebo over 12 weeks in 19 mild non-corticosteroid dependent asthma patients showed a greater reduction in bronchial responsiveness to methacholine in those treated with gold [5].

Recently, a large trial of oral gold therapy in corticosteroid-dependent asthmatics has been reported [6]. 275 patients were recruited in this placebo-controlled, double-blind, multicentre U.S. study and patients were followed-up over a 9-month period. On completion, 60% of the gold-treated patients achieved a 50% reduction in oral corticosteroid requirement, while only 32% of the placebo-treated patients reached this end point. This study showed a clear “steroid-sparing” effect with gold therapy, although improvement were not seen until after several weeks of treatment.

Gold salts are associated with side-effects. Problems include dermatitis, stomatitis, proteinuria, blood dyscrasias, deranged liver function and gastrointestinal disturbances. However they usually resolve after reducing or stopping treatment.

Hydroxychloroquine

Hydroxychloroquine can inhibit phospholipase A₂, an enzyme involved in the release of arachidonic acid from membrane phospholipids. This could potentially lead to a reduction in the production of leukotrienes and prostaglandins in the airways, many of which are bronchoconstrictor or proinflammatory. Hydroxychloro-

quine is mainly used as an antimalarial agent but has been used in rheumatoid arthritis and in systemic and discoid lupus erythematosus.

The steroid-sparing effect of hydroxychloroquine was first suggested in a case report in 1983 [7]. However, a placebo-controlled, crossover trial [8] in nine steroid-dependent asthmatics over 9 weeks did not show any steroid-sparing effect nor were there any significant difference in symptom scores, lung function or requirement for β agonists. Another open trial [9] performed in 11 asthmatic patients for 28 weeks with hydrochloroquine (300–400 mg daily) reported improvement in lung function and symptom scores. The dose of corticosteroid was reduced in seven corticosteroid-dependent asthmatics by 50%. The absence of a control group in this study makes any useful interpretation of results difficult. A serious side-effect of irreversible retinal damage caused by hydroxychloroquine is rare except in overdose.

Methotrexate

Methotrexate is an antimetabolite that acts by causing folate coenzyme deficiency in all cells. When administered in low doses (10 to 15 mg weekly), methotrexate possesses anti-inflammatory properties which can be used to treat chronic inflammatory disorders such as rheumatoid arthritis and severe psoriasis. It can inhibit neutrophil chemotaxis and interleukin-1 (IL-1) production from activated peritoneal macrophages in animal models as well as inhibit histamine release from human basophils.

The results from a number of “steroid-sparing” studies with low dose methotrexate have produced both positive and negative results. The general trend however is of a positive effect. Mullarkey and colleagues [10] performed the first randomised, double-blind, crossover study of 22 steroid-dependent asthmatics, requiring at least 10 mg prednisolone a day. They showed that treatment with oral methotrexate (15 mg/week) for 12 weeks could result in a 36% reduction in oral steroid requirement compared to placebo. There were also subjective improvements in asthma symptoms without deterioration in pulmonary function.

The largest placebo-controlled study was performed by Shiner et al. [11] involving 69 corticosteroid-dependent asthmatics randomised to receive oral methotrexate (15 mg/week) or placebo for 24 weeks. At the end of the study there was a greater reduction in prednisolone dose in the methotrexate treated group than in the placebo treated group (50% versus 14%). However this benefit was not seen in the first 10 weeks after commencing treatment compared to placebo and was not sustainable after cessation of treatment. There were fewer asthma exacerbations during methotrexate treatment while symptom scores and lung function remained stable throughout the study. Recently, in a meta-analysis of 11 controlled trials [12], the author showed that low-dose oral methotrexate used in severe asthmatics has a

statistically significant steroid-sparing effect. Overall steroid usage was decreased by 23.7% (4.37 mg prednisolone/ day) from baseline.

The adverse effects reported with the use of low dose methotrexate in these trials include nausea, anorexia, transient liver enzyme elevations, dermatitis and myelosuppression. These effects are dose-related and usually disappear when methotrexate is discontinued or the dose reduced. The more serious side-effects that have been reported with the use of methotrexate are opportunistic infections with *Pneumocystis carinii* [13, 14], varicella-zoster pneumonia [15], pulmonary cryptococcosis [16], nocardiosis [17], and pulmonary as well as hepatic fibrosis.

Azathioprine

Azathioprine is an antimetabolite which by being incorporated into cellular DNA, prevents cell division. It has been shown to suppress T cell mediated immunity and is therefore useful in prevention of allograft rejection. It is used for patients with severe rheumatoid arthritis and has also been used in chronic inflammatory bowel diseases.

It has been investigated in two short and small scale clinical trials. Asmundsson et al. [18] showed in an open study that on improvement in lung function and a reduction in asthma exacerbations were possible in selected patients with asthma, but not with chronic bronchitis, following 12-week treatment with azathioprine. However in a later study [19] where 20 patients with corticosteroid dependent asthma were randomised to receive placebo or azathioprine, 2 mg/kg or 5 mg/kg/ day for 3 and 4 weeks respectively, there were no improvements in symptoms or lung function in the actively treated group compared to placebo. Corticosteroid dose was held constant throughout the study. The adverse effects experienced included mild dyspepsia and dose-related myelosuppression. Based on its mode of action, azathioprine should be effective but in the absence of controlled trials, it is difficult to advocate its use.

Cyclosporin A

Cyclosporin A (CsA) is a cyclic undecapeptide produced by the fungus *Tolypocladium inflatum*. It inhibits the activation of T lymphocytes [20], the production of cytokines such as IL-2, IL-4, IL-5 and tumour necrosis factor- α (TNF α) and the release of histamine and leukotriene C₄ from mast cells and basophils [21].

The introduction of CsA has provided an entirely new approach to immunosuppression in organ transplantation. By virtue of its selective ability to inhibit

activation of T cells, it can be used at therapeutic concentrations which do not cause myelosuppression, unlike those of other cytotoxic immunosuppressants. This relatively selective activity has been useful in the treatment of severe psoriasis, atopic dermatitis, anterior uveitis and Crohn's disease with varying degrees of success.

The first study was a placebo-controlled, crossover study in 33 steroid-dependent asthmatics giving low dose oral CsA (5 mg/kg/day) [22]. At the end of 12 weeks, the CsA treated group showed a significant improvement in pulmonary function and a reduction in the number of asthma exacerbations requiring extra prednisolone compared to placebo treated subjects. Corticosteroid doses were kept constant throughout this period apart from when required to treat exacerbations.

Two subsequent controlled studies were performed to address the issue of "steroid-sparing" effect of CsA. Nizankowska et al. [23] in a double-blind, placebo-controlled study of 34 corticosteroid-dependent asthma patients showed that CsA therapy could only confer slight benefit over placebo treatment in terms of oral corticosteroid reduction and subjective parameters of asthma severity. Lung function showed no significant difference between the groups at the end of the study. This result is in contrast to another study [24] in which 39 corticosteroid-dependent asthmatics were randomised to receive either CsA or placebo for 36 weeks. CsA therapy resulted in reduction in median prednisolone dose of 62% (10 to 3.5 mg) compared with a decrease of 25% (10 to 7.5 mg) in placebo-treated patients. This reduction with CsA therapy was most pronounced during the last 12 weeks of treatment. There were improvements in peak expiratory flow rate despite reduction in corticosteroid use following CsA therapy. However upon stopping treatment, steroid requirements returned to baseline values.

The main adverse effect of low-dose CsA is decreased renal function. In studies to date which have been less than 12 months in duration, renal impairment has not been a significant problem especially when whole blood CsA is maintained between 100 and 200 ng/ml. Any decrease in renal function is reversible upon stopping treatment. Other side-effects however have occasionally led to discontinued treatment. These include hypertrichosis, hypertension and neuropathy.

CsA has a more selective action (i.e. to CD4⁺ helper T lymphocytes) compared to other immunosuppressants which affect all dividing cells. However it also has activity on mast cells and basophils which have recognised roles in the pathogenesis of asthma. In a recent double-blind, placebo-controlled study assessing the effect of CsA on allergen induced bronchoconstriction, it was demonstrated that the late asthmatic response (thought to be mainly T cell mediated) could be abrogated by CsA while the early asthmatic response (mainly mast cell mediated) was unaffected [25]. This lends support to the hypothesis that the effects of CsA in asthma are related to inhibition of T lymphocytes rather than mast cells. With the accumulating evidence to implicate T lymphocytes in "driving" the ongoing airway inflammation in

chronic severe asthma, CsA may be of particular value as effective alternative treatment in these difficult patients.

Intravenous Serum Immune Globulin (IVIG)

Pooled human immunoglobulin has been used to confer broad-based humoral immunity on immunodeficient patients since congenital agammaglobulinaemia was first described by Bruton in the early 1950s. These products are prepared from the blood of thousands of healthy donors and invariably contain a wide variety of antibody specificities.

Intravenous serum immune globulin (IVIG) appears to have therapeutic effects in patients with diseases mediated by immune mechanisms, most notably, autoimmune diseases such as immune thrombocytopenia [26]. These patients are not necessarily lacking in any classes of immunoglobulins. This observation has now been extended to include other immune-mediated conditions such as myasthenia gravis, bullous pemphigoid and systematic lupus erythematosus. Thus IVIG may possess immune-modulatory properties which may be beneficial in the treatment of refractory asthma.

Two early open clinical trials of IVIG in severe asthma were performed in children and involved small numbers of patients. The first [27] involved eight asthmatic children who required continuous oral corticosteroids. They were treated with IVIG at a dose of 1 g/kg for two consecutive days each month for a total of six months- a dose regime similar to those used for treating autoimmune diseases. At the end of six months treatment, prednisolone was reduced from a mean dose of 32 mg daily to 11 mg alternate-day therapy. Symptom scores and home peak expiratory flow had significantly improved. In the second open study [28], nine asthmatic adolescents who were dependent on moderately high dosage of inhaled corticosteroid alone were treated with IVIG for 5 months at a mean dosage of 0.8 g/kg on a single day each month. Of these nine patients, six were able to reduce dosages of inhaled corticosteroid from a mean of 720 mcg/day to 400 mcg/day. Their asthma symptoms as well as bronchial reactivity to histamine were also reduced. This benefit was not maintained 10 months after completion of scheduled therapy. This study suggested that the effect of IVIG was apparent even in moderately severe asthmatic patients not requiring oral corticosteroids, albeit small and short-lived.

Recently, preliminary results of a multicentre, double-blind, placebo-controlled trial of IVIG in oral corticosteroid-dependent asthmatics has been reported [29]. Forty patients were randomised to receive either placebo (albumin 2 g/kg) or IVIG at dose of 1 g/kg or 2 g/kg, administered once a month for 7 months. At the end of the scheduled therapy, both groups receiving active treatment showed a 41% reduction in their oral corticosteroid requirement, compared to a 30% reduction in placebo-treated group. This difference did not reach statistical significance. Adverse

experiences reported were similar in both actively treated groups and were more frequent than in those treated with placebo.

The results from this controlled trial, studying the “steroid-sparing” effect of IVIG in oral corticosteroid-dependent asthmatics do not seem to support its use. The authors however recognised the highly heterogenous nature of the studied population which may be reflected in the marked placebo effect. Nevertheless the effectiveness of IVIG in severe asthmatics should now be treated more cautiously in view of the findings in this controlled trial.

The most common side-effects relating to IVIG were post-infusion headache, fever and rigors. They appear to be temporary and the severity could be overcome by reducing the infusion dose of immunoglobulin. This phenomenon is largely unexplained but may be due to formation of antigen-antibody complexes. IVIG has also been associated with aseptic meningitis [30].

How may IVIG mediate improvement in asthmatics patients? Over 90% of IVIG is monomeric IgG with only a small proportion of other immunoglobulins such as IgA and IgM. Many of the effects of IVIG observed in autoimmune diseases especially in immune cytopenias appear to be mediated by the Fc portion of IgG through the Fcγ receptor on phagocytic and other cells. Binding of these Fc portions to the receptor sites can either inhibit or modulate phagocytic functions resulting in disease control. Such modulation of Fcγ receptor bearing monocytic/phagocytic cells can also affect both T and B lymphocytes, influencing both cellular and humoral immune responses.

We now know that IVIG preparations also contain many other substances such as solubilized membrane components, and possibly important solubilized receptors, as well as specific antibodies to HLA determinants and lymphocyte surface molecules. In fact solubilized CD4, solubilized CD8, HLA class I and class II determinants have been isolated from commercial IVIG preparations [31]. These cell surface molecules could interfere with antigen presentation by blocking the interaction between HLA class II and membrane-bound CD4 on T cells or between HLA class I and CD8 in T cell-mediated cytotoxicity. Specific antibodies to HLA determinants and lymphocyte surface molecules could similarly affect the immune regulation of T cells. This perhaps explains why large amounts of IVIG preparation are required for clinical benefit to be realized.

Key issues in the use of immunosuppressive therapy

All immunosuppressive therapies have potentially serious adverse effects. Therefore their clinical use should be confined to asthmatic patients who are dependent on unacceptably high maintenance doses of corticosteroids. The side-effects of corticosteroids are usually not troublesome until doses in excess of 7.5 mg prednisolone per day (above the physiologic level) are used over a long period of time.

The first question is how effective immunosuppressive agents are in patients with severe chronic corticosteroid-dependent asthma, whether they are administered as adjunctive or alternative (“steroid-sparing”) therapy. Secondly, how does the toxicity of such agents compare to the reduction in unwanted side-effects of corticosteroids? Can short-term administration of these agents provide sustainable benefits, and therefore improve the long-term outlook for these patients?

Many of the activities of these agents are felt to be “additive” to the anti-inflammatory / immunosuppressive actions aimed at by the use of corticosteroids. In doing so, some of the effects of corticosteroids can be “replaced” and their requirement reduced. Most studies primarily aimed at assessing their “steroid-sparing” efficacy. Whether there is any unique benefit conveyed from administration of one agent compared to another, is unclear at the moment until there are studies specifically designed to address this issue.

“Steroid-sparing” capability of immunosuppressive agents

Of the agents studied, methotrexate, cyclosporin A and gold provide more consistent evidence of a “steroid-sparing” benefit. At present, hydroxychloroquine have not been adequately assessed in larger, controlled trials for potential “steroid-sparing” effect, nor has azathioprine shown proof of clinical benefit when added to the steroid regime of studied populations of asthmatics. The “steroid-sparing” effect of IVIG appears to be minimal. The administration of IVIG can be costly despite the attractiveness of its relatively “benign” side-effects when compared to those observed with immunosuppressants.

The mean reduction in prednisolone use is possibly in the range of 0–9.5 mg/day with methotrexate and 0–5 mg/day with CsA and gold, as suggested from these trials. This indicates that the mean overall “steroid-sparing” effect of these immunosuppressants is modest. However, it is important to recognise that individual responses to immunosuppressive agents, whether in terms of benefit or side-effects, are unpredictable. This creates the difficulty in trying to equate benefit and risk for any single individual with certainty.

For some asthmatic responders, a small reduction of 5 mg prednisolone/day may perhaps be all that is required to significantly reduce the side-effects caused by corticosteroids whilst experiencing no adverse effects from the use of immunosuppressants. However the converse can also be true in that patients may suffer considerable side-effects from taking these immunosuppressants while experiencing little success in reducing corticosteroid dosage. This consideration may be further compounded by an observation in a recent meta-analysis of low-dose methotrexate [12], that greater steroid sparing benefit occurred in those who took lower doses of oral steroids (≤ 20 mg/day). This may be because these patients are less intractable than those requiring higher doses of corticosteroids.

Safety and clinical benefits from short-term use of immunosuppressive agents

Long-term depression of immune surveillance by immunosuppressive agents increases the incidence of malignancy seen in patients after allograft transplant [32]. However the incidence of malignancy with use of these agents in autoimmune diseases is much lower, probably due to the lower doses used and the shorter treatment periods employed. If possible, only a short duration of treatment with immunosuppressants should be used in treating severe asthma.

Unwanted side-effects associated with different immunosuppressants have been discussed earlier. They may be equally or even more unpleasant to those resulting from corticosteroids. Sometimes, they are distressing enough to warrant discontinuing treatment. The increased risk of opportunistic viral, bacterial and fungal infections should always be borne in mind when using immunosuppressants. These can develop rapidly and may on occasion be fatal. It is therefore important for both physicians and patients to be vigilant for any signs of infection and to respond appropriately.

The “steroid-sparing” effect from these immunosuppressive agents is observed to be short-lived. Corticosteroid requirement seems to return to baseline values after immunosuppressive agents are discontinued, although some studies have reported success in stopping all asthma medications or lasting improvements in lung function after gold and CsA treatment [4, 22, 33]. Some observed rebound exacerbations of asthma within 6 weeks following 18 months’ treatment with CsA [34]. However it is conceivable that such potent agents could have “reset” the level of airway inflammation and perhaps made airway disease more amenable to subsequent treatment. In fact this has been claimed to occur in patients who responded to relatively long-term therapy (18 to 28 months) of low-dose methotrexate [35]. They responded more rapidly to doses of prednisolone that were ineffective prior to the use of methotrexate and tolerated more rapid reductions in corticosteroids than before methotrexate treatment.

Practical considerations in the use of immunosuppressive therapy

The clinical use of immunosuppressive therapy should be restricted to physicians familiar with the management of chronic severe asthmatics and the pharmacology of these agents. The oral steroid dose should be reduced to the lowest possible level to maintain symptom control prior to the use of immunosuppressants. In doing so, the benefit/risk ratio of adding immunosuppressants to existing asthma medication can be accurately assessed and treatment adjusted accordingly. Prior to commencing immunosuppressants, it is essential to exclude other factors which may be responsible for poor control such as non-compliance with medication, hyperventi-

lation, occupational factors, continued exposure to specific allergens and significant gastroesophageal reflux disease. Issues concerning the pharmacokinetics of corticosteroids may require consideration especially in an individual who receives high doses of corticosteroids and fails to demonstrate any “anticipated” side-effects. This involves questions of whether there is insufficient corticosteroid absorption from the gastrointestinal tract or accelerated catabolism/elimination from the body. Here, measurement of plasma prednisolone levels may be useful. Close monitoring of routine blood and urine tests is also essential, and investigations will vary, depending on the type of immunosuppressive agents selected.

Potential benefit from immunosuppressive agents should not be dismissed until a reasonable trial period has passed. Obviously if there are unacceptable adverse effects, these agents will have to be discontinued. The steroid-sparing benefit of methotrexate appears to take effect only after prolonged treatment of at least 6 months [12]. Cyclosporin A and gold seem to act more rapidly and steroid reduction may be possible within 3 months.

In patients who respond to immunosuppressive therapy, how long should we continue the treatment? There are reports [35] to suggest that stopping methotrexate within the first month of discontinuing oral corticosteroids could lead to disease exacerbation. Hence it is reasonable to continue treatment for up to 6 months after stopping oral corticosteroids. Using this regime, some patients have managed to stay off continuous oral corticosteroids for over 2 years. Our experience with Cyclosporin A has been that patients generally relapse on stopping treatment. From the existing literature, it is unclear how long gold therapy should continue after stopping oral corticosteroids.

It is also important to remember that improvement with immunosuppressants does not obviate the need for periodic rescue courses of oral steroids during disease exacerbations. Its chief objective is to reduce dependency on oral corticosteroids and may, as earlier stated, “reset” the level of airway inflammation and influence the natural course of disease.

Future considerations in immunosuppressive/immune-modulatory therapy

Inhaled route of administration

There is obviously a need for safer immunosuppressive agents. One possibility is to deliver these agents by the inhaled route, thus maximising delivery to the target site of inflammation while reducing systemic uptake. Aerosolized cyclosporin A has been used in lung allograft recipients with some success [36]. Similarly, the development of inhaled formulations for other immunosuppressive agents could represent a way forward for better tolerated therapy.

Newer immunosuppressive agents

Rapamycin and tacrolimus (FK506) are new immunosuppressive agents presently studied in the field of organ transplantation. They share a molecular structure related to CsA and appear to be more potent, yet less toxic than CsA [37]. Tacrolimus, like CsA, exerts its effects principally through alteration of T cell gene expression, while rapamycin blocks signal transduction mediated by interleukin-2 and other cytokines, therefore inhibiting T cell activation at a later stage. Another immunosuppressant presently being evaluated is mycophenolate mofetil acid, which acts by inhibiting *de novo* synthesis of pyrimidines and purines. These agents have potential in overcoming the problems of earlier immunosuppressants and may be useful to treat severe asthmatics.

Experimental immune-modulatory agents

Specifically targeting immunologically important cells/cytokines/immune-mediated inflammatory cascade in asthma may be another way forward. These possibilities are presently being explored in animal models and as yet, their potential therapeutic role in humans is undefined.

As discussed earlier, CD4⁺ T lymphocytes are implicated in orchestrating ongoing airway inflammation, particularly in chronic asthma. By modulating their function, the airway inflammatory process may be attenuated and disease control improved. Recently we have completed a small, double-blind, placebo-controlled study assessing the effect of a humanised antiCD4 monoclonal antibody in steroid-dependent asthmatics [38]. Our results showed that following a single infusion of 3 mg/kg of the monoclonal antibody keliximab, an improvement in lung function could be observed as early as 24 h which persisted for 14 days when compared to placebo. The effect of repeated dosage and any “steroid-sparing” action were not studied. Larger, longer term trials are now being set up to investigate this further.

Potential role of immunosuppressants in corticosteroid-resistant asthma

Patients with corticosteroid-resistant asthma are fortunately few. The potential of benefit with the use of immunosuppressive therapy in this group of patients has not been thoroughly studied. The mechanism of steroid resistance is complex and is still poorly understood. Yet it is conceivable that immunosuppressive agents may benefit this group of patients because their mechanisms of action are primarily independent of the glucocorticoid receptor. In fact there is *in vitro* evidence that while stimulated T cell proliferation from corticosteroid-resistant asthmatics was relatively unaffected by corticosteroid, it was inhibited by CsA, tacrolimus and mycopheno-

late mofetil [39]. A clinical study to specifically address this question will be most interesting.

Early introduction of immunosuppressants in difficult-to-control asthma

Asthma management has changed significantly over the last few years, in particular with earlier and more aggressive use of anti-inflammatory agents. This is based on the evidence that airway inflammation is present early in the asthma disease process and early introduction of anti-inflammatory agents would be a logical step in improving the prognosis in asthma.

It is still unclear why certain asthmatics appear to have a more aggressive onset of disease. Their airway inflammation continues to be active despite being treated with high doses of corticosteroids. In patients such as these, should we consider bringing our most potent drugs, i.e. immunosuppressive drugs, into action early rather than late? Obviously the risk of potential toxicity should be weighed against the benefit of early and adequate control of airway inflammation and consequently reduction in longer term morbidity and indeed mortality from progressively destructive disease.

Interestingly, some open studies [40] have suggested that early introduction of immunosuppressants in selected groups of patients with severe asthma could reduce the incidence of corticosteroid dependency. Already other chronic autoimmune/inflammatory diseases have been managed with such an “aggressive” approach with the intention of making long-term prognosis favourable.

Conclusion

The immunological basis of the disease of asthma is now more clearly defined than before. Clinical studies with existing therapeutic drugs have in fact shown immunosuppressive or immune-modulatory activity relevant to the pathophysiology of chronic asthma. Corticosteroids remain our best “immunosuppressant”. Many potent agents have been tried as adjuncts or alternatives to corticosteroids, mostly in situations when side-effects from corticosteroids become unacceptable. Of these, methotrexate, gold salts and cyclosporin A have the best documented “steroid-sparing” effect in severe asthmatics. However, with increased potency comes the “price” of toxicity and the uncertainty of long-term beneficial effect. Their clinical use must therefore be weighed carefully and individually assessed. The clinical efficacy of intravenous serum immune globulin in severe asthma appears to be minimal despite the increasing recognition of its varied immune-modulatory effects and clear benefits in treating some autoimmune/inflammatory diseases. “Safer” and more “effective” immunosuppressive/immune-modulatory agents are always needed because of

the clear management problem in patients with severe asthma due to the lack of effective alternative treatment.

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Anti-IgE agents

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Introduction

There is little debate over the essential role of IgE in allergic reactions. During an allergic response, CD4⁺ T lymphocytes of the Th2 phenotype stimulate allergen-specific B cells to produce IgE molecules via the release of IL-4/IL-13. The receptors for IgE are found on a multitude of different cells and two different types have been identified: the high-affinity receptor (FcεRI) on mast cells, basophils, and antigen-presenting cells, and the low-affinity receptor (FcεRII or CD23) on B lymphocytes, monocytes/macrophages, eosinophils, dendritic cells, and epithelial cells. After interaction with allergens, IgE-armed cells release a number of inflammatory mediators and enhance and redirect antigen presentation. This cascade of events seems to have a crucial role in the generation and persistence of symptoms in allergic diseases. The development of drugs that interfere with IgE production and function may therefore represent a more specific approach to treat these pathological conditions [1, 2].

A number of different strategies have been devised to inhibit IgE synthesis on the basis of studies *in vitro* or in animal models of allergic inflammation. These studies have indicated that it may be possible to inhibit IgE production by using STAT-6 inhibitors, which interfere with the signal transduction of IL-4 and IL-13 in B cells [3], or molecules that interfere with the binding of IL-4 to its receptor on B cells (IL-4 antagonists and neutralizing antibodies to IL-4) [4]. IgE production has also been shown to be partially inhibited by anti-CD23 antibodies [5]. However, no clinical evidence of benefit in allergic conditions has been demonstrated with these strategies.

In 1976, a complete suppression of the IgE-mediated response to *Nippostrongylus Brasiliensis* was reported in mice following treatment with specific polyclonal antibodies [6–8]. This set the stage for studying methods of antibody-mediated IgE inhibition suitable for human use and notable progress has been made since then. At present, the use of monoclonal antibodies that inhibit IgE function by preventing the binding of IgE to effector cells represents the most promising strategy for treat-

ment of allergic diseases. In fact, it has been proven to be effective in asthma and allergic rhinitis, as described in the next sections.

Development of a non-anaphylactogenic monoclonal antibody (mAb) to human IgE

A therapeutically viable anti-human IgE mAb should have the following characteristics: (1) It should bind specifically to IgE to minimize the unwanted effects on the immune system; (2) it should not bind to IgE attached to the receptors on cell surfaces to minimize the risk of triggering cell degranulation and mediator release; (3) it should not trigger immune complex diseases.

Anti-human IgE antibodies were first described in 1981 by Hook et al. [9]. Later, anti-mouse IgE antibodies were shown to inhibit IgE binding to the FcεRI, but in so doing they induced mast cell degranulation by receptor cross-linking [10]. This was avoided by producing mAbs that recognized IgE at the same site as FcεRI and FcεRII [11–13]. These mAbs were shown to bind to free IgE. They were unable to bind to IgE attached via IgE receptors to the cell surfaces [2, 13–15].

Antibody 1-5 [12], TES-C21 [14] and MAE 11 [15] were rodent antibodies that recognized IgE at a target structure located within the Fcε3 domain containing the binding site for FcεRI. Six amino acids of the binding site (Arg-408, Ser-411, Lys-414, Glu-452, Arg-465 and Met-468) are critically involved in the binding of IgE to FcεRI and represented the ideal target for these non-anaphylactogenic anti-human IgE mAbs [15]. Since the binding site for FcεRI is contained within the same domain as the site for FcεRII, they also inhibited the binding of IgE to the low-affinity receptor [15–17].

A major factor that limited the use of these murine antibodies as therapeutic agents was the fact that an antigenic (anti-antibody) response can occur following repeated administration of xenogenic mAbs. This antigenic response can decrease the efficacy of the antibodies by reducing their half-life through the formation of antibody-anti-antibody complexes, and it carries the danger of anaphylaxis [18–22]. Since the antigenic response against the constant region of a xenogenic mAb comprises as much as 90% of the total response, the potential for an anti-antibody response was reduced by generating recombinant hybrid mAbs. These mAbs retain the mouse variable regions, and therefore retain specificity for the antigen. The constant regions of the immunoglobulins have been replaced by human constant regions. Chimeric and humanized mAbs have been generated by ligation of entire mouse V region genes with human constant region genes or by grafting the murine antigen-binding loops onto a human IgG1 subclass framework, respectively. Humanized recombinant mAbs possess little or no expected antigenicity [2, 22–29].

CGP 51901 and E25 are the chimeric and humanized constructs of the murine antibodies TES-C21 and MAE11, respectively. Most of the clinical data available have been obtained with these mAbs.

Effect of anti-human IgE antibodies in atopic subjects

The humanized antibody E25 and the chimeric antibody CGP 51901 were tested in a series of single and multiple dose clinical trials in subjects with elevated serum IgE. Their administration resulted in a dose-dependent decrease in serum free IgE levels.

In 23 atopic subjects, single doses of 3, 10, 30, and 100 mg i.v. of CGP 51901 induced an immediate fall in the concentration of circulating free IgE. Free serum IgE levels decreased by more than 95% after the highest dose, with a mean recovery to 50% of baseline levels by 39 days. Total IgE (sum of IgE-anti-IgE complexes and free IgE) increased, indicating that clearance of the immune complex is slow [30].

E25 was also shown to induce and maintain extremely low IgE levels for a long period of time. In an open trial, 45 atopic subjects received 0.015 or 0.03 mg of E25/kg/total serum IgE i.v. every 2 weeks. After 182 days of treatment, serum IgE levels were still less than 2% of the baseline value. After reducing the dose, serum IgE levels promptly began to return to pre-treatment values [31].

Antibodies against the anti-IgE mAbs were measured in all the clinical trials performed. A weak antigenic reaction was measured in only one of the subjects treated with the chimeric antibody CGP 51901 [30] and in none of those treated with the humanized mAb E25. Both antibodies were well tolerated.

Effect of anti-human IgE antibodies in allergic diseases

Allergic rhinitis

Two trials [32, 33] have been performed to test the efficacy of the anti-IgE mAbs in seasonal allergic rhinitis.

The first trial [32] was designed to evaluate the ability of CGP 51901 to prevent symptoms caused by mountain cedar pollen in subjects with a history of rhinitis upon exposure to this pollen. The administration of CGP 51901 every 2 weeks, at doses of 15, 30, or 60 mg, prevented the development of symptoms during the pollen season in a dose-dependent manner. This effect was achieved in patients whose IgE levels were reduced by at least 85 % from the baseline value. In this trial, approximately 25% of the patients treated with the highest dose did not need additional medication during the season, whereas 35 out of 36 patients in the placebo group needed symptomatic treatment.

One could speculate that repeated administration of these antibodies may allow recovery from chronic mucosal inflammation due to recurrent allergen exposure, thereby preventing or reversing some common complications of the chronic inflammatory process, particularly sinusitis and polyps.

Models of allergic asthma

Antigen challenge in the laboratory represents a model for mimicking the changes occurring in the airways of subjects with allergic asthma after natural exposure to an allergen. This model has been repeatedly used in the development of antiasthmatic drugs to predict their efficacy before embarking on large and expensive trials. Most of the drugs capable of affecting bronchial responses to allergen inhalation, particularly those affecting the late asthmatic response, have been effective in the clinical setting. This is the case with inhaled corticosteroids which have been shown to inhibit the late reaction by interfering with several steps of the associated inflammatory response [34, 35].

The role of IgE in the development of the allergen-induced early asthmatic reaction is undisputed, because the early response is the result of the release of mediators triggered by IgE cross-linking on mast cell surfaces [36]. The direct involvement of IgE in the late reaction and in the associated inflammatory and functional changes, such as tissue eosinophil accumulation and increased bronchial hyperresponsiveness, is still unclear [37]. The study of the effect of anti-IgE mAbs on allergen-induced early and late asthmatic reactions can therefore provide important information on the pathogenesis of the inflammatory response that follows allergen exposure, in addition to giving an indication of their therapeutic potential in asthma.

One randomized, double-blind study by Boulet and colleagues [38] tested the ability of E25 to affect the allergen-induced early asthmatic response. After an initial loading dose of 2 mg/kg, E25 was administered i.v. to 10 allergic asthmatic individuals, on study days 7, 14, 28, 42, 56 and 70, at a dose of 1 mg/kg. Nine individuals received placebo. The allergen concentration able of inducing a 15% fall in FEV1 (PC15) was measured on days 1, 27, 55, and 77. E25 administration resulted in a significant increase in the PC15, that was 2.2 to 2.7 doubling doses higher than the baseline value (Fig. 1). The magnitude of this increase exceeds that reported with inhaled corticosteroids [39].

In another randomized double-blind study, Fahy and colleagues [40] tested the effect of E25 on the allergen-induced early and late asthmatic responses and on some inflammatory parameters measurable in induced sputum. Either E25 (0.5 mg/kg) or placebo were administered i.v. every week for 9 weeks. Allergen challenge was carried out before and at the end of the treatment phase. E25 significantly attenuated the early asthmatic response and reduced the magnitude of the late asth-

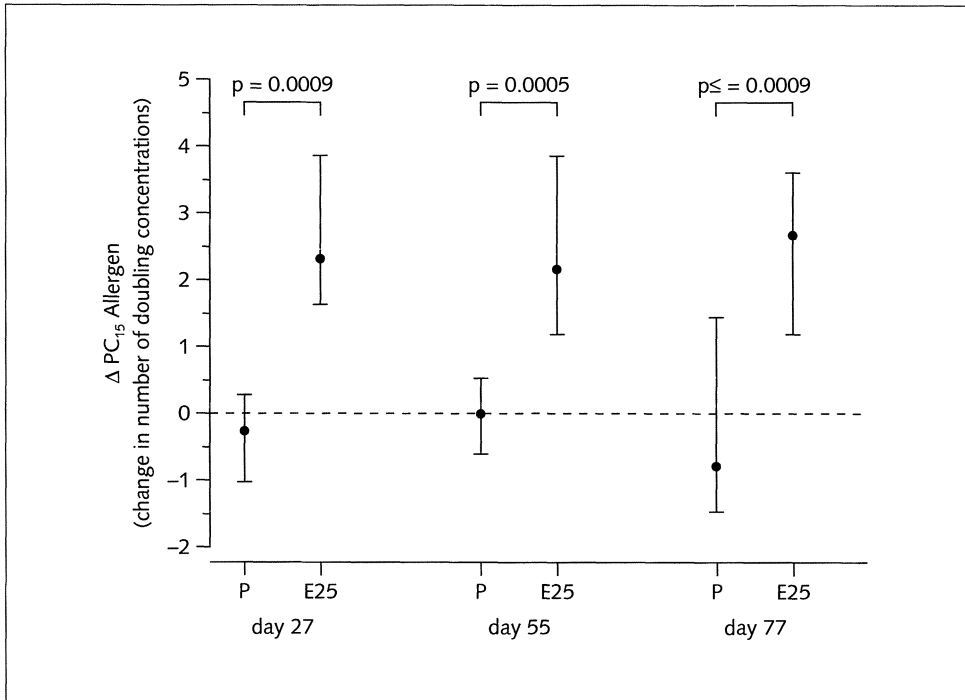


Figure 1

Effect of E25 and placebo (P) on allergen-induced bronchoconstriction measured as PC15. The change in allergen PC15 during treatment is reported as median change in doubling concentrations of allergen and 25th–75th quartiles [38]. Reproduced by permission.

matic response by more than 60% (Fig. 2). These effects were statistically significant versus placebo ($p = 0.01$ for the early response and $p = 0.047$ for the late response). In addition, the airway hyperresponsiveness to inhaled methacholine measured 24 h after allergen inhalation was significantly lower at the end of treatment with E25 than before. E25 administration had a small overall effect on inflammatory parameters as compared to placebo, probably because higher concentrations of the allergen were inhaled after E25 administration than after placebo. However, the allergen-induced increase in the number of eosinophils and the release of eosinophil cationic protein in sputum were lower after treatment with the anti-IgE mAb than before (all $p < 0.05$).

Taken together, these studies provide the first direct evidence of the involvement of IgE in both the early and late asthmatic reactions that follow allergen inhalation, and predict therapeutic utility of E25 and similar anti-IgE mAbs in asthma.

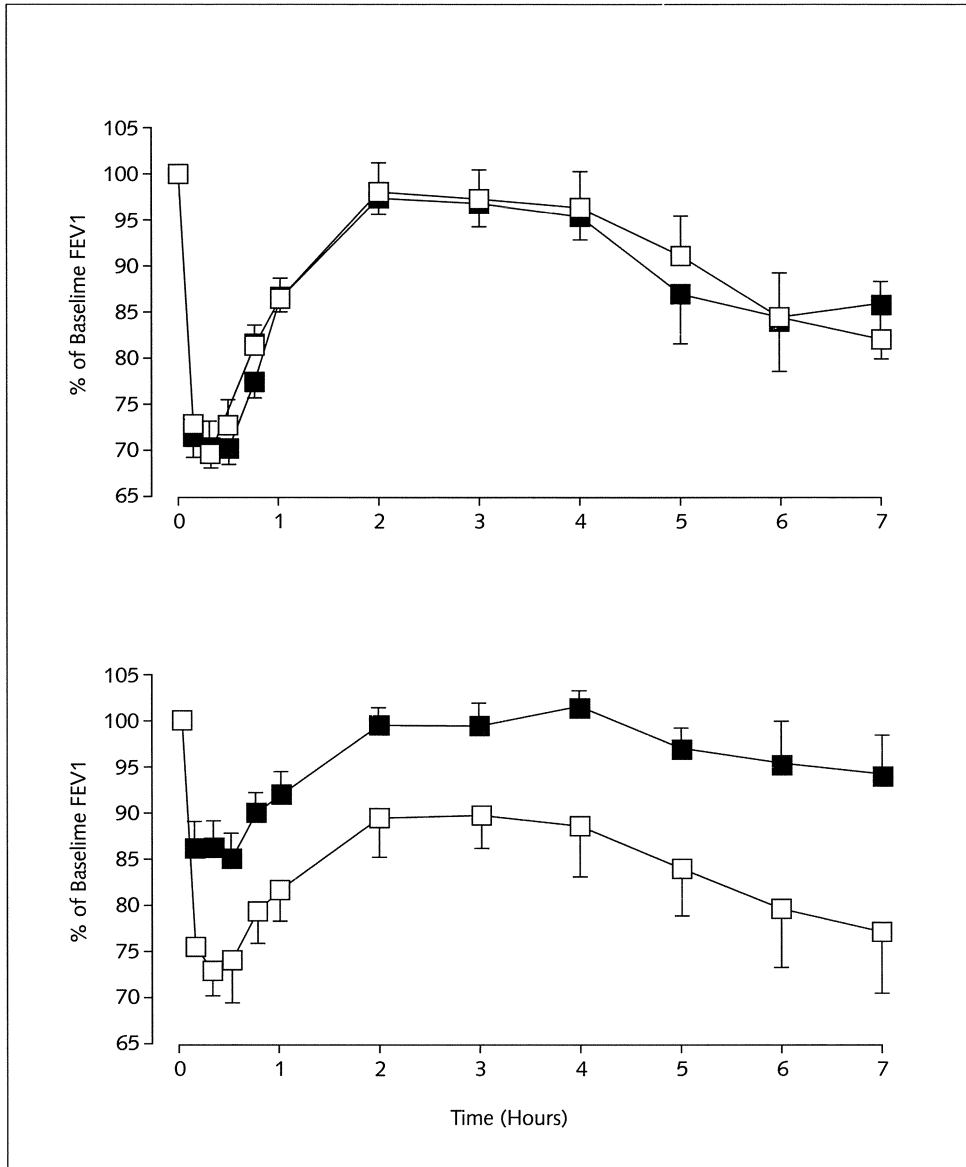


Figure 2
 Effect of E25 on the allergen-induced early and late asthmatic responses. Changes in FEV₁ in the first hour after allergen challenge (early response) and from 2 to 7 h after allergen challenge (late response) in the placebo (top panel) and E25 (lower panel) treated groups are reported as mean percent of baseline values \pm SD before treatment (open squares) and at the end of treatment (closed squares) [40]. Reproduced by permission.

Naturally occurring asthma

There is an ongoing debate on the percentage of patients with bronchial asthma who are atopic. In addition, it is difficult to estimate the pathogenetic importance of the atopic status per se. However, several investigators have found significant correlations between total serum IgE concentration, clinical parameters of disease severity [41], bronchial hyperresponsiveness [42, 43] and risk of emergency room admission [44].

E25 has been tested in asthmatic patients with positive skin prick test to at least two allergens known to be associated with bronchial asthma. Those patients were moderate to severe asthmatics treated with a standard dose of inhaled steroids and bronchodilators. A subgroup of them was receiving oral steroids. The study design involved two phases. During the first 12-week phase either E25 or placebo was added at random and double-blind to a standard treatment for moderate to severe bronchial asthma that included inhaled β_2 -receptor agonists and inhaled or oral steroids and was chosen on the basis of the current guidelines [45]. During the second 8-week phase, a forced reduction in the dose of the inhaled and oral steroids was attempted [46].

When E25 was added to the standard treatment, it significantly reduced symptoms and improved PEFr while decreasing simultaneously the use of rescue medications with β_2 -receptor agonists. The most remarkable effect of E25 during this study phase was its ability to reduce the number of asthma exacerbations by approximately 50% [45]. During the second study phase, patients receiving E25 were able to reduce their dose of inhaled and oral steroids by 50%, whereas patients in the placebo group were able to reduce the dose of inhaled steroids by 25% [46]. No reduction in the dose of oral steroids was possible in the placebo group. In spite of the reduction in the dose of steroids, the improvement observed in the patients receiving E25 during the first phase continued during the second phase, and the number of asthma exacerbations was further reduced [46].

These results demonstrate the effectiveness of E25 in chronic asthma and its ability to reduce the use of steroids in patients that otherwise need continuous administration of these agents.

In another randomized, double blind study [38], repeated administration of 1 mg/kg of E25 on study days 7, 14, 28, 42, 56 and 77, after an initial loading dose of 2 mg/kg, induced a significant reduction in the airway hyperresponsiveness to inhaled methacholine in 10 atopic asthmatics ($p = 0.048$ on day 76). As mentioned in the previous section, this study was designed to assess the effect of E25 on airway response to allergen inhalation test. The subjects recruited were therefore mild asthmatic patients with little or no symptoms and no recent or concurrent natural exposure to the relevant allergen. Since E25 is IgE-specific, it should be able to modify only allergen-induced airway inflammation and airway hyperresponsiveness. Thus, even the minimal effect on airway hyperresponsiveness observed in this study was totally unexpected.

Effect of anti-human IgE on cutaneous reactivity

As mentioned in the previous sections, the anti-IgE mAbs tested in the studies reported above do not bind to the IgE already attached to their receptors on the cell surfaces. This is the most plausible reason to explain why the administration of these agents for a short period of time does not change skin reactivity. Longer periods of treatment seem to affect allergen-induced cutaneous reactions, and a reduction in the wheal response to skin prick testing after the administration of E25 for 3 and 6 months has been reported recently [31, 47]. The apparent dissociation between the onset of the effect in the lung and in the nose and the onset of the effect in the skin is unclear. It is possible that effective IgE depletion in the skin requires prolonged administration of the mAb, or that cells bearing the receptors for IgE play different roles in the immediate reactions induced by allergens in the airway and in the skin, or that inhibition of allergen-induced immediate reactions like the cutaneous wheal-and-flare response does not represent the mechanism by which anti-IgE mAbs improve allergic rhinitis and asthma. The latter hypothesis would imply that the clinical efficacy of anti-IgE mAbs is not merely due to the inhibition of mast cell degranulation or mediator release from other cells that bind IgE, and that it depends on more complex mechanisms, such as interference with dendritic cell function and antigen presentation or eosinophil trafficking and activation.

Other effects of long-term treatment with anti-human IgE mAbs

At the time of this writing, the longest period of administration of anti-human IgE mAbs in atopic individuals has been 1 year [31]. No serious unwanted effects have been reported. The virtual absence of an antigenic (anti-antibody) reaction and the absence of any sign of deleterious effect due to the formation of immune complexes predict an excellent tolerability of these agents during longer periods of treatment.

There are many hypotheses on the consequence of prolonged administration of anti-human IgE in allergic individuals. It has been speculated that long-term treatment with these mAbs may reduce IgE synthesis through interference with a feedback mechanism mediated by IL4 [1]. Furthermore, treatment with E25 has been shown to reduce the expression of IgE receptors on the surfaces of circulating basophils [47]. On the basis of these putative mechanisms, it has been suggested that it may be possible to reduce the dose of the mAbs and achieve a more permanent effect on clinical parameters of disease severity after long-term administration. Data available up to now, however, do not support those theories. When the dose of E25 was reduced in 54 subjects after 6 months of high-dose therapy, IgE levels rose immediately. Treatment withdrawal after 1 year induced a further rise in IgE levels, and the expression of the IgE receptor returned to 80 % of the baseline value [48].

While these observations are reassuring for the long-term safety of the drug, because there is no permanent modification of the immunological characteristics, they also anticipate the necessity of prolonged administration of anti-IgE mAbs for achieving permanent remission of allergic diseases. Longer trials and observational studies will clarify this point.

Another issue concerning the long-term treatment with anti-IgE mAbs is the protection against parasitic diseases. Elevated IgE levels are an important characteristic of the immune response during and after parasitic infections, and are considered to be a host defense against secondary infections [49]. Recent epidemiological studies have also indicated a correlation between schistosome-specific IgE levels and resistance to reinfection [50, 51]. However, protection against parasites is not solely an IgE-dependent phenomenon, as it involves many cellular and humoral factors [52, 53]. In addition, several studies have demonstrated a neutral or beneficial effect of low IgE on the outcome of parasitic infection and resistance to reinfection in mice [52–54].

Some studies have suggested that the atopic status may protect against the development of cancer [55, 56]. This effect has not been related specifically to IgE levels, and other studies have failed to confirm the reduced risk of neoplasia in allergic individuals [57, 58].

Whatever role IgE molecules may play in immunological responses other than allergic reactions, it must be considered that during treatment with anti-human IgE mAbs atopic individuals have circulating levels of IgE similar to those commonly detectable in non-atopic subjects. It is therefore extremely unlikely that a population treated with anti-IgE differs from a normal population in terms of relative risk for diseases not associated with atopy.

Conclusions

IgE molecules are key mediators in the development and maintenance of allergic reactions. The inhibition of IgE function by preventing the binding of IgE to specific receptors on effector cells represents a valid therapeutic strategy in allergic diseases such as allergic asthma and seasonal allergic rhinitis. The development of chimeric and humanized anti-IgE monoclonal antibodies has provided the first agents capable of obtaining this effect efficiently and safely.

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Mast cell proteases as new targets for therapeutic intervention in asthma

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Introduction

Mast cells have long attracted attention for their potential to contribute to the disease process in asthma [1]. These cells are widely distributed throughout the body, but are particularly prevalent in tissues which form an interface with the external environment. In the lower airways mast cells are numerous in the bronchial mucosa [2], submucosa and alveolar walls [3], and are even found free in the lumen [4]. The activation of mast cells by allergen or by other stimuli is associated with the rapid release of a range of potent mediators of inflammation and bronchoconstriction.

Mast cell products include proteases, histamine, heparin, prostaglandins and a variety of cytokines [5]. Several of these have been the subject of extensive study and have become targets for drug development, but until recently proteases have been relatively little investigated. Over the past few years, however, evidence has been accumulating that these major products of the mast cell may have potent biological actions. Mast cell proteases, and in particular tryptase and chymase, may be important mediators of inflammation and are showing promise as targets for therapeutic intervention in asthma and other allergic conditions.

The proteases of human mast cells

Neutral proteases are the most abundant components of the mast cell secretory granule on a weight basis. Five distinct proteases have been localised to the human mast cell, of which three have now been purified, tryptase, chymase and carboxypeptidase (Tab. 1). Tryptase is the major protease with approximately 10 pg per lung mast cell and 35 pg per skin mast cell [6]. There are estimated to be 5–16 pg of carboxypeptidase [7] and 4.5 pg of chymase per skin mast cell [6]. A protease with some enzymatic and antigenic properties in common with neutrophil cathepsin G has also been detected [8, 9] and there are conflicting reports on the presence in mast cells of an elastase-like serine protease [8, 9]. The high concentra-

Table 1 - Human mast cell proteases

Enzyme	Molecular weight (kDa)	Subpopulation
Tryptase	132	MC _T and MC _{TC}
Chymase	30	MC _{TC}
Carboxypeptidase	34.5	MC _{TC}
Cathepsin G	ND	MC _{TC}
Elastase	ND	?

ND, not determined for the protease identified in mast cells.

tions of proteases in mast cell secretory granules would be consistent with these proteases playing a major mediator role in conditions involving mast cell activation.

Tryptase and chymase have assumed importance as biochemical markers of mast cells [10] and of mast cell heterogeneity in humans [11]. Two distinct subpopulations of mast cells have been identified, those containing both tryptase and chymase (MC_{TC}), and those possessing tryptase but not chymase (MC_T). Carboxypeptidase and the cathepsin G-like enzyme have been localised to the MC_{TC} subset [9, 12]. Mast cells of the MC_{TC} phenotype are the predominant phenotype in the skin and other connective tissues, while MC_T cells appear to be most numerous in the normal mucosal tissues of the lung and gut [11]. The defined mast cell subpopulations may reflect relative rather than absolute differences in protease composition. Thus, the results of histochemical studies with a chymotryptic substrate [13] or immunocytochemistry with a sensitive protocol for chymase detection [14] have indicated the presence of chymase, or perhaps of a new form of chymase, in the majority of cells generally classed as being of the MC_T phenotype in the gastrointestinal mucosa. Nevertheless, utilising immunocytochemistry under carefully standardised conditions, the detection of MC_T and MC_{TC} subsets can offer important information on mast cell heterogeneity and function. There is no simple relationship between mast cell functional heterogeneity and protease composition in terms of susceptibility to activation by basic peptides or control by pharmacological agents [15]. However, in the upper airways it is the MC_{TC} population which appears to be the principle store of interleukin 4 (IL-4), while IL-5 and IL-6 have been localised to the MC_T subset [16].

The relative numbers of each mast cell subset may be altered in disease. In the gastrointestinal mucosa of patients with AIDS and other immunodeficiency syndromes, a selective deficiency in cells of the MC_T phenotype has been noted [17], prompting the suggestion that the growth and survival of this subset are dependent on intact T lymphocyte function. In keeping with this idea, increased numbers of MC_T cells have been associated with inflammatory changes in seasonal allergic

rhinitis [18], atopic dermatitis [19], scleroderma [20], conjunctivitis [21] and rheumatoid arthritis [22]. However, MC_T numbers may also be elevated in osteoarthritis [23], a condition in which inflammation is not a prominent feature. In asthmatic airways there have been no detailed investigations of the distribution of MC_T and MC_{TC} cells, but the evidence available from bronchial biopsies suggests that the relative numbers of each of these subpopulations are relatively little altered [16].

Tryptase

Biochemical and enzymatic properties

Early studies with tryptase isolated from human mast cells established that it is a tetrameric serine protease with a molecular weight of approximately 130 kDa and with each of the subunits possessing a single active site [24]. Tryptase expresses optimal catalytic activity when in its tetrameric form. Initially detected in mast cells using histochemical substrates [25], tryptase was later purified to homogeneity from pulmonary mast cells [24], human skin [26] and human lung tissue [27]. Subsequently tryptase has been isolated from cells and tissues of the dog [28], rat [29], cow [30], guinea pig [31] and monkey [32]. Studies in different species have revealed major differences in the types of protease present and in their properties. Thus for example, while tryptase in the dog, rat and monkey appears to be tetrameric and with a molecular weight similar to that in humans [24], that purified from bovine tissues has a molecular weight of 360 kDa [30], and that from guinea pigs, 860 kDa, indicative of a very large structure with some 20–22 subunits [31]. On account of such species-related differences, the focus here will be on the human proteases.

Recently the crystal structure of tetrameric human tryptase has been reported [33] as a square flat ring structure composed of four subunits with the active site of each monomer facing a central pore. This arrangement will restrict access to the catalytic site and helps to explain why tryptase appears to cleave few protein substrates, and exhibits little susceptibility to inhibition by endogenous protease inhibitors. Two complementary deoxyribonucleic acid (cDNA) molecules termed α and β have been cloned from a human lung mast cell library and sequenced [34,35], while three cDNA molecules named type I, II & III have been derived from a human skin library [36]. Beta-tryptase shows 98–100% amino acid sequence homology with the three tryptases from human skin and 90% with alpha tryptase. However, although β -tryptase (or type II tryptase) and types I and III tryptase are similar in sequence, some of the differences may affect the catalytic site. While one putative glycosylation site has been identified for β -tryptase/type II tryptase the other forms possess two putative sites.

Much of the size and charge heterogeneity of purified tryptase observed following electrophoresis on SDS polyacrylamide gels can be attributed to the differences in glycosylation. The extent to which heterogeneity in sequence or glycosylation pattern of tryptase may lead to differences in function remain to be determined. However, a major difference between α -tryptase and β -tryptase-like forms has been suggested by the application of immunoassays using antibodies with different affinities for α and β -tryptase. α -Tryptase appears to be released constitutively from mast cells in an inactive form, while β -tryptase appears to represent the form which is stored in the secretory granules and released on anaphylactic degranulation [37, 38]. In biological fluids, the levels of α -tryptase are likely to reflect numbers of mast cells present in nearby tissues, whereas the concentration of β -tryptase should provide an indication of the degree of mast cell activation. It is α -tryptase which predominates in the serum of normal subjects, and it is present at much higher levels in patients with mastocytosis [37]. α -Tryptase also appears to comprise the majority of tryptase which can be detected in the synovial fluid of most patients with rheumatoid arthritis or osteoarthritis [39], conditions associated with increased numbers of mast cells in synovial tissue [23]. In contrast, tryptase in the serum of anaphylactic patients is predominantly of the β form [37].

In the asthmatic bronchi, there is an increased degree of mast cell activation, with little or no evidence of mast cell hyperplasia. This suggests that much of the tryptase released into the airways in this condition will be of the β form, though this has yet to be tested directly. As enzymatically inactive α -tryptase is not stored, there are particular difficulties in isolating and studying this form other than in recombinant expression systems. The function of α -tryptase in health and disease remains a subject for conjecture. Almost all studies to date have been with the stored forms of tryptase which must represent β or β -like tryptase, and therefore all subsequent references will be to these forms.

The detection of tryptase within mast cells using histochemical substrates [25, 40] indicates that tryptase is stored in the secretory granules in a fully-active state. However, inside the granule, tryptase will be tightly bound to heparin proteoglycan, an association which could serve to maintain the tetrameric structure and hence the stability of the enzyme following secretion into the extracellular space [41]. The high negative charge of heparin is primarily responsible for its binding to tryptase, as other proteoglycans of lower negative charge bind less efficiently [41]. Tryptase has been found to cleave chromogenic substrates with a pH optimum of pH 7 to 8 [26]. In contrast, in the acidic conditions of the mast cell granule (estimated to be in the region of pH 5.5 [42]), tryptase is likely to have relatively little activity [43]. In addition, tryptase activity in the granule will be inhibited by the presence of histamine which at high concentrations can act as a reversible inhibitor of this enzyme [44]. The main actions of tryptase are thus likely to be in the neutral pH conditions outside the cell following mast cell activation. Recently, however, fibrinogen has been identified as a substrate which tryptase may cleave more

efficiently at pH 5 [45], indicating a possible role for this protease in acidic pH conditions.

The enzymatic activity of tryptase may be inhibited by diisopropylfluorophosphate (DFP) and benzamidine and by certain natural inhibitors of proteases such as, antipain, leupeptin [27] and an inhibitor isolated from the medicinal leech [46]. However, circulating serine protease inhibitors such as α_1 -antitrypsin and α_2 -macroglobulin fail to inhibit the enzymatic activity of tryptase [47]. Secretory leukocyte protease inhibitor (SLPI) has been reported to have some inhibitory actions on tryptase [48], but little evidence has been presented to date that an endogenous human protease inhibitor may be effective as an inhibitor of tryptase. Probably of greater importance is the role of heparin in stabilising the tetrameric structure of this unstable molecule. Tryptase in the absence of heparin dissociates rapidly into inactive monomers in physiological buffers [41]. In contrast, when tryptase is in physiological buffer in the presence of heparin, it retains its activity for several hours. Divalent cations [44] or other heparin binding proteins such as antithrombin III [27] or lactoferrin [49] can serve as inhibitors of tryptase by virtue of their ability to destabilise the tryptase/heparin complex. Tryptase is secreted with proteoglycan in a complex of some 200 kDa [50]. The large size of this complex is likely to hinder diffusion away from the site of its release and it is likely that the actions of tryptase will be in the immediate vicinity of degranulating mast cells.

Extracellular substrates of tryptase

Studies with synthetic substrates have indicated that tryptase cleaves peptide and ester bonds on the carboxyl side of amino acids [26], but relatively few natural substrates have been identified. Peptide substrates of potential relevance in asthma which are efficiently cleaved by tryptase include the neuropeptides vasoactive intestinal peptide (VIP), peptide histidine methionine (PHM) [51] and calcitonin gene-related peptide (CGRP) [52]. VIP and PHM are both potent relaxants of bronchial muscle [53] and the cleavage of these peptides by tryptase has been suggested to contribute to the bronchoconstriction observed in asthmatics. CGRP is a potent vasodilator [54] and cleavage of this by tryptase results in attenuation of vasodilator activity [52].

The ability of tryptase to generate kinins remains controversial. Some workers have reported that tryptase fails to generate kinins from low molecular weight kininogen (LMWK) [55] or high molecular weight kininogen (HMWK) [56], whilst others have demonstrated kininogenase activity for tryptase with these substrates [57, 58]. Fibrinogen may be degraded by tryptase so that it no longer acts as a substrate for thrombin in the clotting cascade [59]. The anticoagulant properties of tryptase may be of particular importance in acidic conditions as the reaction is optimal at acidic pH [45].

A number of extracellular matrix (ECM) components are cleaved to some extent by tryptase. Tryptase can degrade the ECM synthesised *in vitro* by rat heart smooth muscle cells [60] and by a human lung fibroblast cell line [50]. Cleaved substrates include gelatinase/type IV collagenase and fibronectin [61]. In addition, tryptase can activate stromelysin (matrix metalloprotease 3; MMP 3) [62] resulting in the activation of latent collagenase [63], and also urokinase [64]. These actions of tryptase in degrading the ECM could facilitate the entry of cells and plasma proteins into areas of mast cell degranulation, and tryptase could be an important participant in processes of matrix remodelling and repair. Structural alterations and increased epithelial fragility are features in the bronchial tissue of asthmatics and tryptase may contribute to these.

Cellular targets of tryptase

Cells in the vicinity of a degranulating mast cell are likely to be exposed to high concentrations of tryptase and other mast cell products (Fig. 1). Recent studies have indicated the potential of tryptase to induce profound alterations in cell behaviour (Tab. 2). The early studies involved incubation of purified dog tryptase with cells of non-human origin. Canine tryptase was found to be a potent mitogen for Chinese hamster lung fibroblasts (CHL cells) and Rat-1 fibroblasts, and in both cases the effect was inhibited by protease inhibitors, suggesting dependence on an intact catalytic site [65]. Moreover, tryptase was reported to act synergistically with basic fibroblast growth factor (FGF), epidermal growth factor (EGF), insulin and thrombin on CHL cells [66]. Human tryptase has now been reported to act as potent mitogen for human lung fibroblasts [66, 67], epithelial cells [68], airway smooth muscle cells [69], including those from the dog [70], and human microvascular endothelial cells [71], but not macrovascular endothelial cells [72]. These various actions could be important in the context of tissue remodelling in asthma and other inflammatory conditions. Of particular importance could be the ability of tryptase to stimulate the synthesis and release from fibroblasts of type I collagen as well as of collagenase activity [67, 73]. In addition tryptase can stimulate fibroblast chemotaxis *in vitro* [73]. The release of tryptase into the upper airways of asthmatics could be a major factor in the induction of myofibroblast and smooth muscle cell hyperplasia and in the deposition of collagen beneath the epithelial basement membrane.

The proinflammatory potential of tryptase is becoming quite well recognised. Studies with animal models have demonstrated that the injection of human tryptase into the skin can induce microvascular leakage [74] and accumulation of eosinophils and neutrophils, and also to some extent of macrophages and lymphocytes [75]. The microvascular leakage induced by tryptase may be inhibited by pre-treatment with antihistamines, suggesting that the effect is dependent on mast cell activation by tryptase. This has been confirmed by the demonstration that human

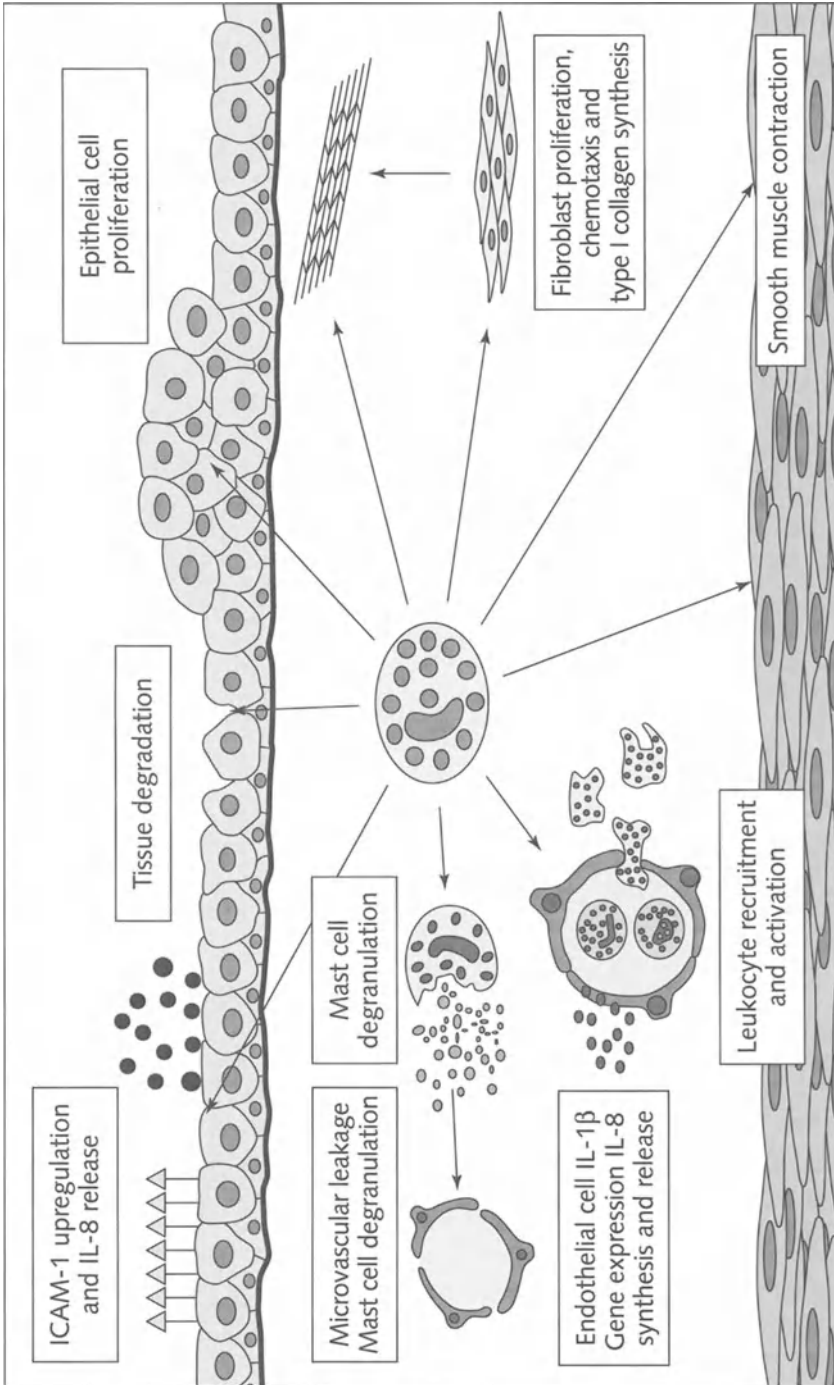


Figure 1
Tryptase actions on cell function.

Table 2 - Actions of tryptase on the behaviour of human cells

Cell type	Effect
Epithelial cell	Proliferation, IL-8 release and upregulation of ICAM-1
Fibroblast	Proliferation, chemotaxis synthesis and secretion of type I collagen synthesis
Endothelial cell	IL-1 β gene expression and IL-8 release
Airway smooth muscle cell	Proliferation
Mast cell	Histamine release
Eosinophil	Chemotaxis and ECP release
Neutrophil	Chemotaxis

tryptase can induce histamine release from guinea pig mast cells [74] and from human tonsil mast cells, though this effect has not been found with human skin mast cells [76].

The ability of tryptase to stimulate the accumulation of inflammatory cells *in vivo* is likely to involve a complex interplay between several cell types which are affected by this protease. Tryptase can stimulate the release of the potent granulocyte chemoattractant IL-8 and upregulate expression of intercellular adhesion molecule 1 (ICAM-1) in epithelial cells [68]. In addition, tryptase can induce the secretion of IL-8 and upregulate expression of mRNA for both IL-8 and IL-1 β in primary cultures of human umbilical vein endothelial cells (HUVEC) [72] and provoke the release of neutrophil chemotactic activity [77]. Potentiation of IL-6 and IL-8 release from TGF β or IL-4 stimulated fibroblasts has also been reported in a preliminary study [78]. As well as inducing the release of granulocyte chemoattractants tryptase appears to have direct effects on granulocytes, acting as a chemotactic agent for human eosinophils and neutrophils *in vitro* and provoking the release of eosinophil cationic protein [79]. This latter action is consistent with observations in a guinea pig model in which the presence of partially degranulated eosinophils has been noted at the sites where human tryptase has been injected into skin [75]. There is thus an accumulation of evidence to implicate this major mast cell product as playing a key role in mast cell dependent inflammation.

The precise mechanisms by which tryptase can alter cell function remain to be resolved and this is an issue of considerable interest. The cloning of a receptor for thrombin [80], now termed protease activated receptor-1 (PAR-1), opened the way for the identification of a family of related receptors, and has indicated the potential importance of proteases in regulating cell function. PAR-1 may be activated to some extent by tryptase when expressed in COS-1 cells, but tryptase appears not to activate this receptor on HUVEC [81]. Recently tryptase has been found to cleave PAR-2 [81], a receptor found on human endothelial cells [82], vascular smooth mus-

cle cells [83], keratinocytes [84], rat colonic myocytes [85] and neutrophils [86], but not fibroblasts [84]. The activation of PAR-2 appears to be less efficient with tryptase than with trypsin, a stimulus which is not of physiological importance outside the intestinal tract [87], and there is some uncertainty over the extent to which the actions of tryptase *in vivo* may be dependent on the activation of PAR-2. Thus a peptide agonist for PAR-2 can stimulate a strong proliferative response in HUVEC [82], yet this has not been reproduced using tryptase [72]. The activation of PAR-2 by tryptase may therefore not account for all the actions of this protease observed on cells. Recently the characterisation of a PAR-3 on HUVEC has been described, another receptor which can be cleaved by thrombin [88]. The growing number of PARs suggests that it is possible that other receptors for tryptase are yet to be discovered.

Chymase

Biochemical and enzymatic properties

Chymase is a chymotryptic-like protease which has been purified from human skin [89], heart [90] and tonsil [91] with a molecular weight of approximately 30 kDa. Although multiple amino acid sequences have been derived for chymases from rodents [92], to date the sequence of only a single human chymase has been reported from skin, heart and tonsil tissues [91, 93, 94]. This encodes a protein of 25 kDa with a net positive charge of 13. There is evidence from several sources, however, for the presence of more than one type of human chymase. Employing heparin agarose affinity chromatography, at least two distinct forms of chymase-like protease have been identified in high salt extracts of a variety of human tissues [95]. Chymase B eluted with sodium chloride concentrations of 1.0 to 1.2 M, and chymase C at 1.8 to 2.0 M. Both forms exhibited similar enzymatic properties with synthetic substrates and reacted with a range of chymase-specific antibodies, but marked differences were observed in their tissue distribution. Chymase B constitutes the predominant form in extracts of human skin, heart and synovial tissue, while chymase C is the major form in lung tissues. The extent to which differences observed between chymases B and C represent differences in primary sequence or in post-translational modifications remains to be determined. However, the isolation from lung tissue has been reported of a protease with properties similar to chymase, but with an N-terminal sequence which differed at a number of residues [96]. It is possible that as in rodents, there may be quite different chymases expressed in human mucosal and connective tissue compartments.

Understanding of the enzymatic properties of human chymase is based largely on studies with purified preparations in which chymase B is likely to represent the main constituent. This protease exhibits relatively little catalytic activity towards synthet-

ic substrates at pH 5.5 [97], the reported pH of the mast cell granule [42]. Thus, although stored in a catalytically active form in the secretory granules, the principle actions of chymase are likely to be extracellular. Moreover, heparin exerts a further inhibitory effect on chymase activity at pH 5.5, but can enhance activity at pH 7.5, the pH of the extracellular space [97]. The observation that the activation of recombinant prochymase by dipeptidyl peptidase I occurs with a pH optimum in the neutral range and is inhibited by both heparin and histamine [98] would appear to indicate that this process is restricted to the early stages of vesicle formation.

Chymase is released in a complex of 400–560 kDa with proteoglycans and carboxypeptidase, but not with trypsin [99]. Chymase is inhibited by the circulating serine protease inhibitors α_1 -antichymotrypsin, α_1 -proteinase inhibitor and α_2 -macroglobulin [100] and also by SLPI [101].

Substrates and cellular targets

Compared with trypsin, chymase appears to be much less restricted in substrate specificity. Peptide bonds are cleaved preferentially where there is a phenylalanine residue at the P₁ site and hydrophobic residues at P₂ and P₃ [102]. Angiotensin I is cleaved by chymase in this way to generate angiotensin II, a reaction which is catalysed more efficiently than with angiotensin converting enzyme itself [103]. The ability of chymase to cleave a range of substrates may suggest an important role in tissue degradation and remodelling at sites of mast cell activation. Addition of chymase to cultures of fresh human skin has been reported to result in extensive degradation of the epidermal-dermal junction [104]. There may be direct actions on components of the extracellular matrix. For example, Type I procollagen is processed by human chymase to initiate the formation of collagen fibrils [105]. A less direct contribution to matrix remodelling may involve the activation of interstitial procollagenase and other proenzymes [106].

The ability of chymase to degrade IL-4 [107], to activate the precursor form of IL-1 β [108] and to cleave and release the membrane bound form of stem cell factor (SCF) [109] suggests roles for this protease in the regulation of cytokine bioavailability. Evidence that chymase may exercise a proinflammatory role has come from studies in which the purified enzyme has been injected into laboratory animals. Injection of chymase into the skin of guinea pigs elicits a prolonged increase in microvascular permeability that is still apparent 120 min following injection, and that appears to be dependent on an intact catalytic site [110]. Unlike the skin reactions which may be provoked by trypsin in this model, those induced by chymase are unaffected by antihistamine pretreatment of the animals. This suggests that the mechanism of chymase action does not involve histamine release; a subsequent study with dispersed human lung, tonsil and skin mast cells has failed to demonstrate chymase-induced mast cell activation [111].

Several hours following the injection of chymase into guinea pig skin or into the peritoneum of mice a massive accumulation of eosinophils and neutrophils has been observed with significant increases in the numbers of lymphocytes being noted in the mouse model [112]. Quantities of chymase as low as 5ng [1.7×10^{-13} moles] were able to induce an inflammatory infiltrate, and in all cases co-injection with a protease inhibitor, or heat inactivation of the enzyme significantly inhibited the response. Chymase, like tryptase, could thus represent a potent stimulus for the recruitment of inflammatory cells following mast cell activation in bronchial asthma.

In the context of inflammatory conditions of the respiratory tract, the finding that dog chymase can stimulate mucus secretion from glandular cells *in vitro* [113] is of particular interest, and there is a clear need for the potential role of human chymase to be investigated as a stimulus of mucus secretion. The extent to which chymase may alter cell function remains largely unexplored, and it is not known if there are cell surface receptors for chymotryptic proteases.

Carboxypeptidase

Carboxypeptidase purified from human lung and skin has a molecular weight of 34 to 40 kDa [114, 115]. Sequences for carboxypeptidase cDNA derived from human lung and skin libraries encode a protein with a molecular weight of 36.1 kDa with a net positive charge of 16 [116, 117]. There is little evidence for heterogeneity, with only one sequence being derived.

Mast cell carboxypeptidase possesses a similar substrate profile to pancreatic carboxypeptidase A, although it has greater sequence homology to carboxypeptidase B. Natural substrates include neurotensin, Leu⁵-enkephalin and kinetensin [118]. On mast cell degranulation carboxypeptidase is co-released with tryptase and chymase, and it is bound to the same proteoglycan macromolecular complex as chymase [99]. It seems likely that carboxypeptidase will act in concert with other mast cell proteases.

Therapeutic potential of inhibitors of mast cell proteases

Mast cell proteases have long been neglected as potential targets for therapeutic intervention. While it has been known for several years that mast cell proteases are released in substantial quantities into the airways of asthmatic subjects, it is only now being recognised that they may possess a range of potent biological activities. The proinflammatory actions of tryptase and chymase implicate these proteases in the genesis of both early and late phase allergic reactions, and they could both play particularly important roles in mediating structural alterations in

chronic disease. A consistent finding with *in vitro* and *in vivo* experimental models has been that the biological actions of trypsin and chymase can be inhibited by protease inhibitors. However, information is sparse on the ability of protease inhibitors to modulate processes of allergic disease induced by allergen, and are restricted at present to some relatively small scale studies of the effects of trypsin inhibitors.

The synthetic protease inhibitors APC-366 and BABIM, both of which are relatively selective for trypsin, have been shown to be effective in a sheep model of asthma [119]. Their administration reduced both the early and late phase response to allergen, as well as blocking the acquired airway hyperresponsiveness. Consistent with the findings of purified trypsin in animal models [74, 75], APC-366 was also found to inhibit microvascular leakage and eosinophil accumulation. A subsequent study with the same sheep model has involved administration of a preparation of neutrophil lactoferrin, which has inhibitory activity on human trypsin [49]. While this treatment had little effect on the early reaction to allergen, both the late phase reaction and allergen-induced hyperresponsiveness were abolished.

Recently, the results have been reported of a randomised cross-over study in which APC-366 was administered by aerosol to 16 subjects with mild-to-moderate atopic asthma [120]. Following allergen challenge, administration of APC-366 resulted in a significant reduction in the late phase response compared with placebo, whether assessed as the area under the curve or as the maximum fall in FEV₁. While there was a trend for APC-366 administration to be associated with a reduced early phase reaction, this did not reach statistical significance and bronchial hyperresponsiveness appeared unaffected.

The studies to date with trypsin inhibitors have been encouraging, and it will be important to determine more precisely their anti-inflammatory properties. Of particular interest must be the ability of trypsin inhibitors to affect processes of tissue remodelling in airways disease. Observations that trypsin is a potent growth factor for fibroblasts [65, 67] and a stimulus for collagen secretion [67, 73] call attention to the potential role of this major mast cell product in inducing the structural alterations which are prominent features of asthmatic bronchi [121]. While experimental evidence with inhibitors of chymase is still lacking, the biological actions described for this protease also suggest that they could prove valuable in modifying processes of both acute inflammation and matrix remodelling in the airways and at other sites. They may also be of benefit in controlling mucus hypersecretion.

Trypsin and chymase represent promising targets for therapeutic intervention in conditions associated with an increased degree of mast cell activation. The design of selective small molecular weight inhibitors of mast cell proteases, or the preparation of certain broad spectrum endogenous protease inhibitors, or even the development of antagonists of protease activated receptors could lead to the provision of some new classes of anti-inflammatory and anti-fibrotic drug.

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Cytokine and adhesion molecule antagonists

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Introduction

The proposed central role of specific leukocyte subsets in the pathophysiology of asthma has focused attention on the development of agents that will selectively inhibit the migration of these inflammatory cells into the lung. In asthma, airway CD4⁺ Th2 type lymphocytes, mast cells and eosinophils appear to be primarily effector cells that underlie the clinical manifestations of disease [1]. The cellular and molecular mechanisms involved in the regulation of the recruitment of these inflammatory cells from the blood to sites of inflammation are complex, however, cellular migration appears to be modulated by two fundamental processes; cell-adhesion systems located in the vascular endothelium and signals elicited through cytokine and chemokine (chemoattractant cytokines) receptors. Cell-adhesion and cytokine signalling systems form networks that are elegantly coordinated to promote cellular extravasation and localisation to the site of inflammation [2, 3]. At the initiation of inflammation, cytokines and chemokines play key roles in propagating the inflammatory response by eliciting signals that activate adhesion-systems, induce the secretion of other cytokines/chemokines from the vascular bed and promote chemotaxis. The type of cytokines produced in response to a particular inflammatory stimulus are intimately involved in directing the immune response by promoting the selective mobilization, attachment and recruitment of specific leukocyte sub-sets to the site of provocation [2–4].

In asthma the inflammatory response appears to be predominantly driven by the Th2 type cytokines, in particularly interleukin-(IL)-4 and IL-5 secreted from allergen-specific CD4⁺ T cells [1, 5]. Th2 cytokines may also be derived from activated mast cells and eosinophils. These cytokines, in association with specific adhesion molecules and other inflammatory molecules (chemokines, leukotrienes, prostaglandins, and platelet activating factor) provide the basis of the molecular network that regulates the trafficking of leukocytes to the asthmatic lung.

In this chapter we provide an overview of the molecular and cellular mechanisms that regulate the recruitment of leukocytes to sites of allergic inflammation. We also

identify the cytokines and adhesion molecules that are currently thought to be the key molecular targets for the inhibition of leukocyte trafficking to the lung and describe their potential for future anti-inflammatory therapy of asthma. Mechanisms for the antagonism of cytokine and adhesion molecule function will also be discussed.

Potential roles of cytokines and adhesion molecules in asthma

Key cytokines involved in regulating allergic inflammation

The accumulation and/or activation of inflammatory cells during an asthmatic response may be initiated and partially sustained by cytokines released at sites of antigen provocation (such as the airway epithelium and from antigen presenting cells) and from T cells and mast cells after activation by specific antigens [1, 4]. B lymphocytes, basophils, monocytes and neutrophils may act in concert with T cells, mast cells and eosinophils in the lung to modulate the inflammatory responses. Cytokines in conjunction with chemokines are thought to play an integral role in orchestrating the molecular events that initially stimulate the inflammatory response and regulate leukocyte endothelium interactions, extravasation, chemotaxis and localization of inflammatory cells at the site of allergen provocation. Each of the events involved in the regulation of leukocyte migration provides an opportunity for therapeutic intervention and subsequent attenuation of the inflammatory response.

Characterisation of the immunopathogenesis of asthma suggests that Th2 type cytokines (IL-3, -4, -5, -10, -13) and granulocyte macrophage colony stimulating factor (GM-CSF) play central roles in the asthmatic response by regulating the production of IgE, the effector function of mast cells and eosinophils, and by promoting transendothelial cell migration [1, 4, 5]. In particular, IL-4 is thought to be a critical factor for the regulation of T cell commitment to the CD4⁺ Th2 phenotype and is known to regulate IgE production by B cells and mast cell function [6]. In addition, IL-4 also has potential roles in regulating the inflammatory response to recall antigens and in the production of the eosinophil-specific chemoattractant, eotaxin [6, 7]. In conjunction with other Th2 type cytokines as well as IL-1 β and tumor necrosis factor, IL-4 may also regulate leukocyte trafficking in asthma by activating adhesion systems in the vascular endothelium [8, 9].

IL-5 has also been identified as a key mediator in the aetiology of asthma [1, 5, 10]. IL-5 not only regulates the growth, differentiation, and activation of eosinophils, but also provides an essential signal for the induction of eosinophilia during allergic inflammation [11–13]. Furthermore, in conjunction with chemokines (RANTES, monocyte chemoattractant protein-3 (CP-3), macrophage inflammatory protein-1 α (MIP-1 α) and eotaxin) and lipid mediators (platelet-activating factor and leukotriene B₄), IL-5 may promote eosinophil chemotaxis [14–16]. Notably, of

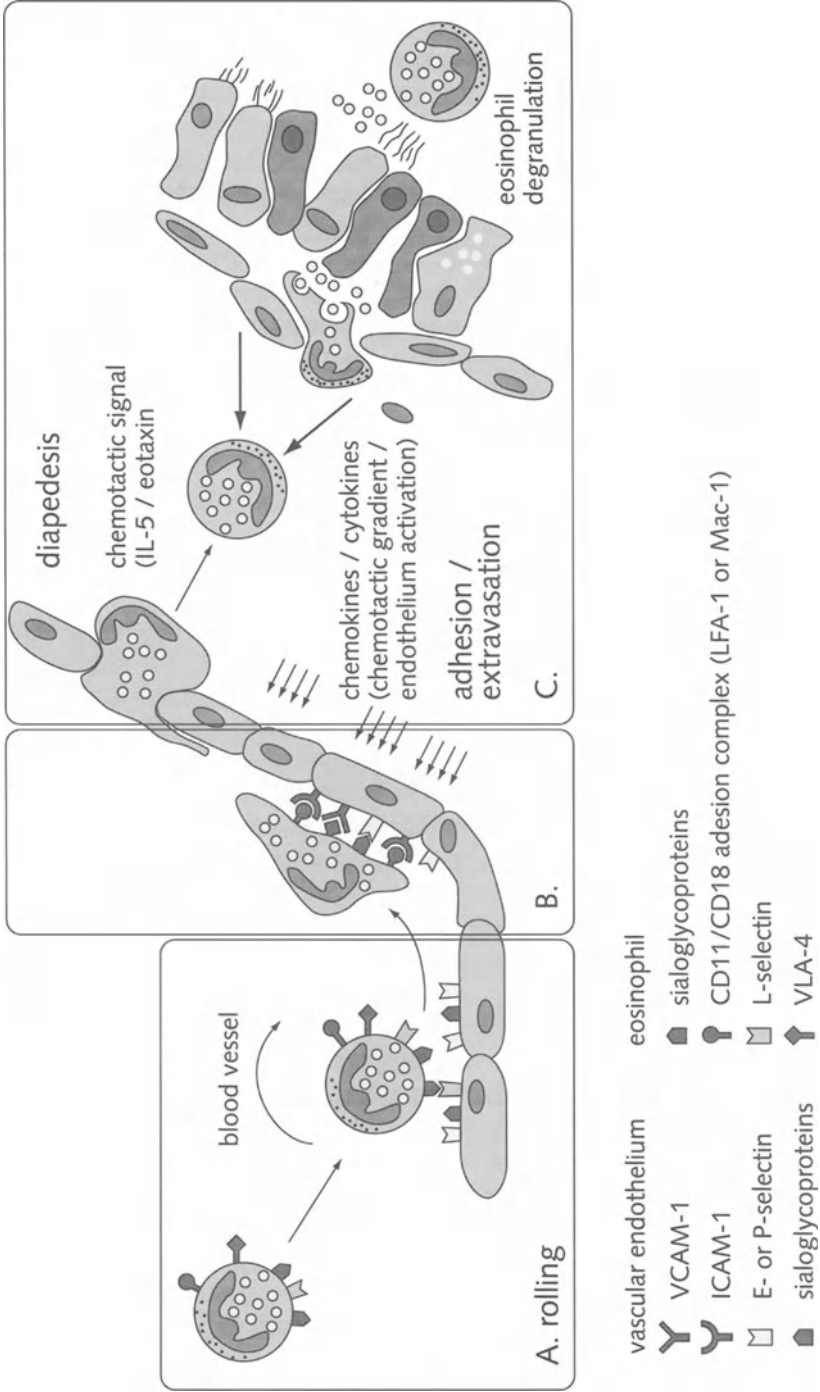
the cytokines/chemokines implicated in modulating eosinophilic inflammation, only IL-5 and eotaxin have been identified to selectively regulate eosinophil trafficking. Eotaxin is a newly identified member of the C-C branch of chemokines and is highly potent and extremely rapid in inducing pulmonary and intradermal eosinophil recruitment [7, 16] (recently, eotaxin-2 has been identified). Investigations in guinea pigs and mice suggest that eotaxin and IL-5 act cooperatively to promote the recruitment of eosinophils into tissues [7, 17, 18]. Evidence is also accumulating that eotaxin may play a role in the aetiology of eosinophil-associated allergic disease in humans.

Adhesion molecules

Adhesion molecules are glycoproteins that play intimate roles in cell assembly, cell signalling and cell-cell communication [2, 3]. The adhesion molecules that are primarily involved in regulating the migration and interaction of leukocytes during inflammation are the selectins, integrins and the cell adhesion molecules (CAMs) of the immunoglobulin superfamily. It is specific members of these families of adhesion molecules that appear to constitute the adhesion systems utilized during inflammatory responses in asthma. In particular, leukocyte endothelial-cell interactions during allergic responses are regulated through the L-, P-, and E- type selectins, vascular- and intercellular-CAMs (VCAMs and ICAMs, respectively) and the integrins, lymphocytes function-associated antigen-1 ((LFA-1), the β_2 -integrin, CD11a/CD18 adhesion complex), Mac-1 (the β_2 integrin, CD11b/CD18 adhesion complex) and very-late activation antigen 4 ((VLA-4), the β_1 -integrin, $\alpha_4 \beta_1$) (Tab. 1).

Molecular basis of leukocyte adhesion

The arrest and extravasation of circulating leukocytes at the vascular bed involves activation of adhesion molecules on the surfaces of both the endothelium and inflammatory cells. Leukocyte extravasation proceeds primarily through three steps that have been well characterised [2, 3, 19] (Fig. 1). The initial phase involves weak interactions between the endothelium and the migrating cell that are regulated through selectins (E-, P- or L-selectin) and their receptors (e.g. sialoglycoproteins), which promotes leukocyte tethering and rolling of the arrested cell on the endothelium. The second phase is coordinated by signals elicited by cytokines/chemokines produced in response to inflammation that up-regulate the expression of selectins, various CAMs and β -integrin adhesion complexes (e.g. VCAM-1/VLA-4, ICAM-1/LFA-1 or ICAM-1/Mac-1 interactions). The coordinated interaction between cytokines and adhesion systems promotes stable adherence of inflammatory cells to the vascular endothelium and allows for the final phase of migration, diapedesis.



Notably, the events leading to extravasation are sequential and are mediated by distinct molecular interactions between specific adhesion-complexes that are characteristic of the immune response.

Thus, inhibition of these molecular interactions during any phase of leukocyte-endothelial cell interactions should inhibit the process of extravasation and potentially attenuate the inflammatory response within the tissue.

Adhesion molecules and asthma

The increased expression of a number of adhesion complexes has been correlated with the clinical manifestations of asthma, which suggests a functional role for these molecules in the aetiology of disease (reviewed in [20]). Increased levels of ICAM-1 and E-selectin have been found in the blood of acute asthmatics and in the bronchial epithelium and pulmonary vascular bed of allergic and non allergic asthmatics [20–25]. The recruitment of granulocytes to the airways has also been correlated with increased levels of soluble -ICAM-1 and -E-selectin in the bronchial alveolar lavage fluid (BALF) of asthmatics after segmental antigen-challenge [26, 27]. In allergic asthmatics correlations have also been observed between elevated levels of IL-4 in BALF, eosinophil numbers in the airway wall, and increased VCAM-1 expression [28]. L-selectin has also been implicated in regulating eosinophil migration into the asthmatic lung after allergen-challenge [29].

Thus, the postulated role of leukocytes in the pathophysiology of asthma in association with evidence that suggests that cytokines and adhesion molecules are upregulated in this disease, suggests that therapeutics which target these inflammatory molecules may be effective in the relief of airways obstruction.

Figure 1

Molecular regulation of eosinophil transmigration. (A) Eosinophils express L-selectin, which promotes transient interactions with the vascular endothelium through carbohydrate ligands (sialoglycoproteins). Eosinophils also express the CD11/CD18 adhesion complex and the integrin, very-late-activation-antigen 4 (VLA-4). (B) Initial activation of the endothelium occurs through interactions with chemokines and cytokines, which promote eosinophil-endothelium interactions by chemotaxis and activating adhesion pathways. Firm adhesion to the activated endothelium may be mediated by eosinophils binding to P-selectin and E-selectin, and interactions between intercellular adhesion molecule-1 (ICAM-1) and the CD11/CD18 adhesion complex. Vascular cell adhesion molecule 1 (VCAM-1) and the VLA-4 adherence pathway may promote the initial binding of activated eosinophils to the endothelium and preferentially promote eosinophil accumulation at sites of inflammation. (C) Transmigration of eosinophils across the vascular bed occurs after priming with cytokines and in response to chemotatic signals (adapted from [19]).

Table 1 - Key adhesion molecules involved in leukocyte trafficking in asthma

Adhesion molecule subtypes	Cell localization	Ligand
<i>Immunoglobulin superfamily</i>		
VCAM-1	endothelial cells	VLA-4
ICAM-1	endothelial cells	LFA-1
	leukocytes	hyaluronic acid
	epithelium	Mac-1
	lymphocytes	LFA-1, Mac-1
<i>Selectins</i>		
P-type	endothelial cells	PSGL-1; SL-C
E-type	endothelial cells	SL-C
L-type	PMN leukocytes/lymphocytes	CD-34
		SL-C
		GlyCAM-1
		E-selection
		P-selection
<i>Integrins</i>		
β_1	VLA-4	lymphocytes, eosinophils monocytes, mast cells
β_2	LFA-1	lymphocytes, leukocytes
	Mac-1	lymphocytes/various leukocytes
		ICAM-1, fibrinogen, others

P-selectin glycoprotein ligand, (PSGL-1): sialyl lewis and other carbohydrate moieties, SL-C; integrin subsets, $\alpha_4\beta_1$ (VLA-4), CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1); polymorphonuclear leukocyte (PMN); other abbreviations see text. For detailed references see [2, 3, 20, 94].

Key cytokine targets for the inhibition of allergic airways inflammation

Glucocorticosteroids remain the current drugs of choice for the regulation of severe manifestations of asthma. These drugs may be effective, in part, because they suppress cytokine production (IL-4, IL-5 and others) by inflammatory cells and modify the participation of adhesion systems in the inflammatory response [30]. However, long-term glucocorticosteroid therapy is associated with harmful systemic effects and does not always relieve bronchial hyperreactivity. Clearly, new antagonists which target key molecules in the inflammatory cascade are required.

Clinical and experimental investigations have identified Th2-cells and -cytokines as key regulatory components of the inflammatory response associated with asthma and allergic disease. It is hoped that the delivery of specific cytokine antagonist to the airways early in an asthmatic's life will attenuate inflammatory infiltrates, the observed progressive morphological changes to the airway wall, and the development of enhanced bronchial reactivity. Specific Th2 cell suppressors may also provide a significant advance in the treatment of established disease. The immunomodulatory functions of IL-4 and IL-5 have identified these cytokines as key therapeutic targets for the relief of airways inflammation and obstruction in asthma. Furthermore, in animal models of asthma, both cytokines have been implicated in the development of airways hyperreactivity to spasmogens after antigen inhalation [13, 31–33]. Although animal models are only representative of the immunopathological process underlying asthma, they do provide important insights into the potential contribution of individual inflammatory cells and molecules to the pathogenesis of this disease which in turn allows identification of key targets for potential therapeutic intervention.

Roles of IL-4, IL-5 and eotaxin

Currently, there are no specific antagonists of IL-4, IL-5 or eosinophil-specific-chemokines available for clinical practice. However, analysis of the literature suggests that the development of such agents will provide a significant advancement in the treatment of asthma. IL-5 plays a central role in eosinophil development and activation and has been strongly implicated in the aetiology of allergic and non-allergic asthma. In transgenic mice, the overexpression of IL-5 in the respiratory epithelium results in changes to the airways pathognomic of asthma and in enhanced bronchial reactivity [32]. Investigations in IL-5 deficient mice indicate that this cytokine is critical for regulating eosinophilia during allergic inflammation [13]. Moreover in a mouse model of asthma, IL-5 was found to be essential for the development of airways epithelial cell damage and bronchial hyperreactivity in response to inhaled allergen [13]. Recently, we have also shown that IL-5 secreted from allergen specific CD4⁺ Th2 cells plays a pivotal role in the pathophysiology of allergic airways disease by regulating eosinophilia and bronchial hyperreactivity in response to allergen inhalation [34]. Notably, IL-5 deficiency does not affect the production of other cytokines or antibodies, nor significantly impair T and B cell function, suggesting that antagonism of this molecule would not significantly impair other immunological responses [35].

Interestingly, evidence is accumulating that IL-5 production can be regulated by CD4⁺ T cells in the absence of IL-4. This particular sub-population of CD4⁺ T cells develops into the Th2 phenotype (producing IL-4 and IL-5) independently of IL-4 and can provide enough IL-5 during allergic inflammation of the lung to induce

eosinophilia, bronchial hyperreactivity and morphological changes to the airways [33, 36]. Thus, two CD4⁺ T cell pathways exist for the immune system to regulate IL-5 production and eosinophilia in response to inhaled allergens; one dependent on IL-4 and the other independent of this factor. The central role of IL-5 in both T cell components of allergic disease further highlights the requirements for highly specific therapeutic agents that inhibit production and/or action of this cytokine.

In conjunction with IL-5, inhibition of the function of eotaxin may markedly suppress the movement of eosinophils into tissues. Eotaxin and IL-5 may act in synergism to regulate the homing of eosinophils to sites of allergic inflammation. IL-5 mobilizes eosinophils from the bone marrow and also promotes homing to the infected organ, while eotaxin elicits signals for cell polarization and chemotaxis within the inflamed tissue [18]. It appears that “cross talk” occurs between IL-5 and eotaxin signalling systems to uniquely and selectively promote eosinophil chemotaxis/locomotion and potentially degranulation. Investigations using inhibitory mAbs in guinea pigs indicated that this chemokine may play a major role in the movement of eosinophils from the blood to the allergic lung [37]. In contrast, eosinophilia was not significantly altered in eotaxin deficient mice during the late-phase of allergic airways inflammation [38]. Recently, the eotaxin receptor has also been identified on memory Th2 cells, suggesting that eotaxin may also play a role in the recruitment of this T cell to sites of allergic inflammation [39]. The biological functions of eotaxin suggest that this molecule, in conjunction with IL-5, will provide an important new therapeutic target for the attenuation of the progression of allergic disease.

Blocking the actions of IL-4 may also provide an effective mechanism to attenuate inflammation of the airways in asthmatics. Inhibition or deletion of IL-4 during allergic airways inflammation attenuates eosinophilia, IgE production and bronchial hyperreactivity [31, 40], while overexpression of this cytokine in the lung of transgenic mice results in morphological changes to the respiratory epithelium [41]. Recently, IL-4 was also shown to play a crucial role for the homing of Th2 cells to the allergic lung [42].

Although blocking the actions of IL-4 may provide an effective mechanism for the relief of airways obstruction in asthma, evidence from animal models of Th2 dependent allergic airways inflammation indicate that targeting this cytokine alone may not be an effective therapeutic strategy. Notably, enhanced reactivity to cholinergic stimuli and eosinophilia are still features of inflammatory responses (albeit significantly reduced) in mice treated with anti-IL-4 mAbs or deficient in this factor [31, 33]. Furthermore, unlike IL-5 transgenic mice [32], over expression of IL-4 in the airway wall does not result in the induction of airways hyperreactivity, although morphological changes in the respiratory epithelium are observed [41]. Anti-IL-4 mAbs also are only effective in attenuating aeroallergen-induced airways hyperreactivity if administered during the primary sensitisation phase, but not dur-

ing the period of direct provocation of the airways with allergen [31], suggesting that antagonists of the actions of IL-4 during an established asthmatic response may not attenuate bronchial hyperreactivity. Eosinophilia and bronchial hyperreactivity are also induced after allergen-inhalation in sensitised mast cell-, IgE- and CD40- (no production of IgE, IgG or IgA) deficient mice, components of the inflammatory response whose functions are intimately regulated by IL-4 [33, 40, 43, 44].

A number of other interleukins may also play important roles in regulating airways occlusion in asthma [4]. IL-13 has similar biological activities to IL-4 in the downregulation of proinflammatory cytokines and chemokine production, in the induction and expression of integrins and in the regulation of IgE synthesis [45, 46]. Thus, targeting both of these cytokines concurrently may be required to attenuate the asthmatic response. IL-1 β , IL-11 and TNF α have also been implicated in various mechanisms associated with leukocyte activation and trafficking in allergic inflammation [4]. However, the role of these cytokines in a broad range of immune responses suggests that these molecules would not be suitable targets for therapeutic modulation of asthma. In contrast, the Th2 cytokine IL-10 down regulates processes associated with allergic disease, such as IL-5 and IgE synthesis. Notably, IL-10 has been shown to suppress the recruitment of leukocytes into the lung during the early-phase of allergic airways inflammation in mice [47].

Although clinical and experimental investigations indicate that IL-4 and IL-5 are primary targets for the attenuation of asthma, studies in mice also suggest that directly targeting T cell activation may be required to alleviate bronchial hyperreactivity associated with allergic inflammation of the airways [18, 48–50]. Recently, we have described a novel CD4⁺ T cell pathway in BALB/c mice that modulates allergen-induced airways hyperreactivity independently of the collective actions of IL-4 and IL-5 [48]. Data indicates that CD4⁺ T cells operate at least two pathways that can act independently to induce airways hyperreactivity. The co-existence of parallel pathways may account for the dissociation of airways eosinophilia from the development of airways hyperreactivity in some cases of asthma and in animal models of this disease [48]. Notably, the transfer of enriched naïve T cell populations from a strain of mice that displayed inherent airways hyperreactivity to methacholine to a hyporeactive strain conferred enhanced airways reactivity to this spasmogen in the absence of antigen challenge [50]. Airways hyperreactivity was directly associated with CD4⁺ T cells and occurred in the absence of eosinophilia and pronounced inflammation and morphological changes to the airways. Although the mechanism of T cell activation in the absence of antigen was obscure these investigations support our conclusion that factors secreted from CD4⁺ T cells play fundamental roles in determining the level of airways reactivity to cholinergic stimuli. Thus, while a number of molecules contribute to the mechanisms underlying the regulation of bronchial hyperreactivity in mice, only CD4⁺ T cells have been shown to exclusively regulate disease pathogenesis [48, 49].

Key adhesion molecule targets for the inhibition of leukocyte-endothelium transmigration during allergic airways inflammation

The roles of various adhesion-molecules in the trafficking of leukocytes into the lung have been investigated in various animal models of asthma. Collectively, these investigations suggest that adhesion molecules play a pivotal role in controlling T cell and eosinophil trafficking into the allergic lung and in the subsequent induction of bronchial hyperreactivity in response to allergen inhalation, thus indicating the potential therapeutic value of targeting adherence pathways at the vascular endothelium for the treatment of asthma.

The potential importance of VCAM-1, ICAM-1, VLA-4, and LFA-1 on antigen-induced leukocyte recruitment to the trachea in a mouse model of allergic airways disease has been demonstrated by using inhibitory mAbs [51]. Inhibition of the VCAM-1/VLA-1 pathway, but not of ICAM-1 or of LFA-1, suppressed eosinophilia. In this model, antigen challenge induced the expression of VCAM-1, but not of ICAM-1 on the vascular endothelium. Notably, VCAM-1 expression was suppressed (27%) by anti-IL-4 mAb treatment, without significantly affecting eosinophil accumulation into the trachea. Thus, factors other than IL-4 are important in promoting VCAM-1-regulated airways eosinophilia [51]. In contrast to eosinophil transmigration, all of the adhesion molecule mAbs reduced CD4⁺ and CD8⁺ T cell infiltration into the trachea, indicating the importance of both LFA-1/ICAM-1 and VLA-4/VCAM-1 adhesion pathways for lymphocyte extravasation during allergic airways inflammation [51].

In similar studies, CD18 and VLA-4 were shown to play key roles in eosinophil extravasation in the lung and blockade of these integrins also suppressed the influx of mononuclear cells and neutrophils [52, 53].

A number of studies have attempted to correlate the effects of inhibiting adhesion molecules during allergic inflammation not only with the selective reduction in the trafficking of leukocyte subsets, but also with the level of bronchial reactivity to spasmogens. In primate, rat and guinea pig models of asthma, administration of anti-ICAM-1 or anti-VLA-4 mAbs attenuated the development of inflammation and bronchial hyperreactivity [53–55]. Inhibition of integrin signalling through Mac-1 or LFA-1 also attenuated enhanced airways reactivity in models of allergic asthma [56–59].

The mechanisms whereby adhesion molecules regulate airways hyperreactivity are not clear. Observations in a sheep model of asthma suggest a key role for α_4 integrins in the development of allergen-induced late-phase airways dysfunction independently of leukocyte recruitment [57, 60]. Systemic and airway administration of anti- α_4 mAb (MP1/2) did not alter the composition of leukocyte subsets recovered in BALF, while significantly reducing an increase in late-phase lung resistance and bronchial hyperreactivity [60]. Inhibition of ICAM-1 signalling or of integrin function (with mAbs against VLA-4, both LFA-1 and Mac-1 or with mAbs

which recognise different epitopes on the CD18 molecule) have also shown that adhesion molecules can regulate bronchial hyperreactivity in the presence of inflammatory cell influx into the airways [55–59]. Thus, adhesion systems may not only play an important role in inflammatory cell extravasation but also in their state of activation and ability to communicate directly with other inflammatory cells and pathways. This concept is supported by recent investigations which show that integrins control cell adhesion to extracellular ligands and also transduce biochemical signals both into and out of cells [61].

Mice carrying a targeted disruption of a gene encoding a particular adhesion molecule have been employed to investigate the role of the factors in leukocyte trafficking to sites of inflammation. Lymphocytes from L-selectin deficient mice do not bind to high endothelial venules of peripheral lymph nodes and total numbers of lymphocytes are decreased at these sites [62]. A central role for L-selectin in leukocyte homing to non-lymphoid tissues during contact and delayed-type hypersensitivity responses was also observed [63]. Leukocyte rolling was also significantly impaired in P- and L- selectin deficient mice [62, 64]. In P-selectin-deficient mice the accumulation of CD4⁺ T lymphocytes, monocytes and neutrophils, and the degranulation of mast cells were significantly reduced during contact hypersensitivity reactions [65]. Recently, endothelial selectins were also shown to be critical for α_4 -integrin dependent leukocyte rolling in chronically inflamed venules in mice deficient in P- and E- selectin [68]. P-selectin deficiency has also been associated with the attenuation of leukocyte recruitment to the lung and of airways hyperreactivity in a mouse model of asthma [66]. In ICAM-1-deficient mice, IL-5 and eosinophil peroxidase levels were significantly lower in BALF in comparison to wild type after sensitisation and exposure to aeroallergen [67]. Furthermore, aeroallergen-induced airways hyperreactivity was significantly attenuated in the absence of ICAM-1. Thus, ICAM-1 was considered to be an important molecule for regulating eosinophilia and enhanced bronchial reactivity during allergic responses of the airways [67]. Recent investigations suggest that adhesion mechanisms may also be able to distinguish between Th1 and Th2 subsets and mediate differential trafficking of these T cells [69]. Collectively, these investigations indicate that targeting specific adhesion-molecule complexes may be an efficient means of controlling leukocyte infiltration during late-phase asthmatic responses.

Cellular and molecular approaches to antagonise adhesion pathways and cytokine signalling

Th2 type cytokines and adhesion systems activated by these factors have become a major focus of research directed at developing novel anti-inflammatory agents for the treatment of asthma. Several cellular and molecular approaches are currently being pioneered with the view to providing new insights and principles for the devel-

opment of highly specific therapeutics. Experimental strategies currently are aimed at inhibiting cytokine, chemokine or adhesion molecule signalling processes by targeting molecular interactions between ligands and their receptors or by modifying specific components of signal transduction pathways or of mechanisms of transcription.

The molecular components (transcription factors and promoter elements) regulating cytokine-induced expression of specific adhesion molecules in endothelial cells and of cytokine expression by Th2 cells are being increasingly characterized. In particular, the transcription factor NF- κ B plays a critical role in immune and inflammatory responses. NF- κ B is regulated by the cytokine-responsive I- κ B complex (IKK α and β subunits) and a catalytically inactive form of IKK- β has been shown to block cytokine-induced NF- κ B activation [70]. Nitric oxide, by inhibiting the action of NF- κ B has also been shown to suppress cytokine induced expression of adhesion molecules and cytokine production within endothelial cells. The signal transducers and activators of transcription (STATs) provide another target for the inhibition of cytokine gene expression [71]. In particular, IL-4 signals through STAT-6 to regulate T helper development, IgE production, expression of CD23, and presentation of MHC class II antigen on B cells [71, 72]. Importantly, IL-13 also activates the STAT-6 pathway [73]. Thus, the inhibition of STAT-6 signal transduction and/or other transcription factors involved in cytokine and adhesion molecule signalling may provide a mechanism for attenuating allergic inflammation in asthma.

Cytokine or adhesion molecule function may also be antagonised by targeting specific components of their signal transduction pathways. The intracellular domains of integrins communicate with several structural and signal transduction proteins to regulate a wide variety of cellular functions associated with leukocyte activation and recruitment [61]. Recently, the Rho subfamily of small guanosine triphosphate (GTP)-binding proteins was identified as a potential pharmacological target for the uncoupling of G-protein-linked chemoattractant receptors from integrin-mediated adhesion of leukocytes [74]. Inactivation of Rho by C3 transferase coenzyme blocked chemokine induced lymphocyte $\alpha_4\beta_1$ adhesion to VCAM-1 [74]. Calreticulin has also been shown to be an essential modulator of both integrin adhesion functions and intercellular signalling [75]. Thus, disruption of calreticulin-integrin interactions may not only inhibit cell adhesion but also activation of other mechanisms associated with the inflammatory stimulus, perhaps providing a mechanism to attenuate bronchial hyperreactivity. However, the redundancy in the actions of cytokines and chemokines and the pleotropic actions of many of the transcription factors and intracellular transducing elements involved in both cytokine and adhesion molecule signalling suggests that modulation of specific components of the inflammatory response by targeting intracellular compartments will be very difficult and such approaches currently are far removed from clinical applications.

Animal studies with monoclonal antibodies (see above) suggest that targeting specific inflammatory molecules with humanised antibodies may provide an effec-

tive therapeutic approach to the treatment of asthma. The recent success at blocking chemokine receptors with low molecular weight compounds also indicates that the use of small molecules, peptide antagonists and perhaps non-signalling receptor binding analogues of specific cytokines/chemokines and adhesion molecules may also provide a more direct modality to attenuate inflammatory responses. Heterocyclic bicyclams, an 18 residue peptide and a highly cationic oligopeptide containing nine arginines were found to inhibit HIV entry of cells by interacting with the CXCR-4 (receptor 4 for CXC chemokines) (see [76] for review). The repeated delivery of truncated analogues of chemokines (MCP-1) has also been shown *in vivo* to inhibit chronic inflammatory arthritis that develops spontaneously in MRL-lpr mice [77]. Collectively, these investigations identify the potential for chemokine/cytokine receptor antagonists as anti-inflammatories (76).

Structural analysis of inflammatory molecules and their receptors is also allowing for the identification of specific motifs that can be targeted with small molecules/peptides to inhibit downstream activation of signal transduction pathways. For example, antagonists of the QIDSPV motif within domain one of VCAM indicate that this site is critical for VLA-4 binding [78, 79]. The EILDVPST sequence within the connecting segment-1 (CS-1) region of fibronectin is also recognised by VLA-4 [80]. Recently, the cyclic hexapeptide CWLDVS (TBC 772) has been shown to be a potent antagonist of α_4 integrin function; inhibiting lymphocyte interactions with fibronectin, VCAM-1 and mucosal vascular adhesion-CAM-1 (MAdCAM-1) [81, 82]. This peptide is not toxic to T cells *in vitro* and is integrin selective in its suppressive activity as co-stimulation through other molecules in the presence of TBC 772 was not impaired [81]. Notably, the antagonist peptide inhibited integrin function by a mechanism independent of competitive binding [81]. Recently, a CS-1 ligand mimic was also shown to inhibit VLA-4 mediated mast cell binding to fibronectin and attenuate degranulation [83]. These *in vitro* investigations with peptide antagonists have been extended in a sheep asthma model [84]. Aerosol administration of the CS-1 ligand mimic (phenylacetyl-L-leucyl-L-aspartyl-L-phenylalanyl-D-prolineamide) before aeroallergen-challenge significantly attenuated (40%) the early-phase and almost ablated the late-phase airways response (88%). Moreover, the VLA-4 antagonist inhibited the development of airways hyperreactivity to cholinergic stimuli. Inhibition of antigen-induced changes in airways function were directly correlated with a reduction of VLA-4 positive leukocytes (eosinophils, lymphocytes and metachromatic-staining cells) in airway biopsies taken 24 h after the challenge [84]. These investigations also provide evidence that small molecules which target specific inflammatory molecule interactions can be delivered directly to the airways to potentially inhibit asthmatic like responses. These results also support investigations with anti- α_4 -integrin mAbs and studies with other small molecule VLA-4 antagonists and suggest that inhibition of CS-1 as well as VCAM-1 binding may be important for suppression of allergic airways inflammation [85]. The potential of intravenous or perhaps subcutaneous adminis-

tration of specific carbohydrate antagonists as therapeutics for blocking leukocyte trafficking in asthma has also been demonstrated. Intravenous administration of fucoidin, a selectin-binding carbohydrate (blocking of selectin-carbohydrate interactions), markedly inhibited (>98%) leukocyte rolling flux [68].

Recently, a mutant of human IL-5, E12K was shown *in vitro* to act as a potent and specific antagonist of IL-5 dependent cell proliferation and eosinophil adhesion, and of the events inducing downstream tyrosine phosphorylation [86]. Although this mutant did not inhibit all of the actions mediated by IL-5, it demonstrated the potential for inhibiting IL-5 function with engineered structural analogues. The recent demonstration that insertion mutants of IL-5 can be expressed as monomers with biological activity similar to that of native IL-5 provides a platform for the development of non-signalling receptor binding antagonists of this cytokine [87].

Th2 cell and cytokine suppression by opposing cytokines

The molecular complexity, high receptor affinity, pleiotropic actions, and redundancy of cytokines has to date made it difficult to design pharmacological antagonists that will specifically modify the actions of an individual cytokine and/or attenuate inflammatory responses. Furthermore, once the inflammatory cascade is initiated mechanisms may operate in parallel and independently to regulate leukocyte trafficking and the induction of bronchial hyperreactivity in asthma, suggesting that strategies targeting one factor may not be sufficient to relieve airways obstruction.

Alternatively, over all suppression of a specific inflammatory response may be achieved by molecules that are normally involved in the downregulation of the particular immune response *in vivo*. The ability of cytokines to cross-regulate immune responses driven by T cells suggests a possible role for cytokine-directed therapy of the inflammatory response underlying asthma. In particular, the cytokines IFN γ , IFN α and IL-12 have been shown to potentially suppress immunological responses driven by Th2 cells [88–93]. These cytokines may act by inhibiting the proliferation of Th2-cells, by suppressing IgE and IL-4 production and by downregulating adhesion pathways. IFN α and IL-12 may exert their inhibitory effects by enhancing the production of IFN γ and also independently of this cytokine. IFN α inhibits antigen-induced eosinophil and CD4⁺ T cell recruitment into airway tissue [93]. Furthermore, both IL-12 and IFN γ are potent inhibitors of antigen-induced airways hyperreactivity and Th2 driven inflammation [90–92]. Recently, we have demonstrated the potential of cytokine directed gene therapy for the suppression of airway inflammatory responses in asthma. The delivery of viral constructs encoding IL-12 to the lung before the onset and during an established allergic response, selectively inhibited airways eosinophilia and hyperreactivity to cholinergic stimuli [93]. This effect was accompanied by a shift to Th1 type cytokine expression in lung T cells. The protective effects of IL-12 were almost completely abolished when experiments were

performed in mice with disruption of the gene encoding the IFN γ receptor. Interestingly, this strategy may promote the clearance of respiratory tract viral infections (by stimulating the proliferation of IFN γ producing CD8 $^+$ T cells) which are thought to play key roles in inducing and exacerbating asthmatic responses.

Summary

The proposed central role of airways inflammation in the induction and maintenance of the asthmatic responses has identified molecules that specifically regulate the migration and activation leukocytes as key therapeutic targets for the relief of airways obstruction. In asthma, leukocyte trafficking to the lung appears to be predominantly regulated by Th2 cytokines (in particularly IL-4 and IL-5) secreted from allergen-specific CD4 $^+$ T cells in association with specific adhesion molecules (VCAM-1, ICAM-1, VLA-4, LFA-1, Mac-1 and P-, E- and L-selectin). Clinical and experimental investigations suggest that the delivery of specific antagonists of these molecules to the airways may provide a significant advance in the treatment of asthma. Currently, a range of cellular and molecular approaches are being employed with the view to identifying novel anti inflammatory targets and agents. The complexity of the inflammatory response in asthma suggests that Th2 cell suppressers may be more efficient at attenuating the inflammatory response than targeting individual inflammatory molecules.

The immunoregulatory effects of IFN γ , IFN α and IL-12 suggest that these molecules have the potential to inhibit the activation of CD4 $^+$ Th2 cells and mast cells and suppress airways eosinophilia in asthma. The delivery of inhibitory cytokines or antagonists of cytokine and adhesion molecule signalling networks directly to the airways may provide a rapid, potent and convenient method for the treatment of developing and ongoing asthmatic responses.

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