

Mucosal inflammation in severe glucocorticoid-dependent asthma

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Mucosal inflammation in severe glucocorticoid-dependent asthma. B. Vrugt, S. Wilson, J. Underwood, A. Bron, R. de Bruyn, P. Bradding, S.T. Holgate, R. Djukanovic, R. Aalbers. ©ERS Journals Ltd 1999.

ABSTRACT: To improve our understanding of the inflammatory mechanisms underlying severe disease, a biopsy study was performed comparing 15 clinically unstable glucocorticoid-dependent asthmatics, 10 mild asthmatics, and 10 control subjects.

Compared with mild asthma, severe asthma was characterized by reduced mucosal eosinophilia. Whilst no significant differences were found in the numbers of mast cells, neutrophils, CD3+ and CD4+ T-cells between the three groups, up to a 4-fold increase in the numbers of activated T-lymphocytes bearing the interleukin (IL)-2 receptor (IL-2R) was found in the mucosa in severe asthma compared to mild asthma ($p=0.03$) and control subjects ($p=0.003$). Compared to control subjects, the mucosa of severe asthmatics contained significantly ($p=0.02$) higher numbers of IL-5+ cells, with no differences between mild and severe disease. In contrast, staining for the anti-IL-4 monoclonal antibody 3H4 revealed that biopsies from mild asthmatics contained more IL-4+ cells than biopsies from severe asthmatics and control subjects ($p=0.0008$). In the severe asthmatics, a close correlation ($r_s=0.76$, $p=0.005$) was found between the numbers of IL-2R-bearing cells and the variability in peak expiratory flow.

In conclusion, persistent T-cell activation is a prominent feature of severe asthma. These results also indicate that interleukin-5, and not interleukin-4, is upregulated in severe disease.

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The application of fiberoptic bronchoscopy, endobronchial biopsy, and bronchoalveolar lavage (BAL) to the study of mild and moderately severe asthma has established mucosal inflammation as a hallmark of atopic asthma (reviewed in [1]). Characteristic findings include increased T-cell activation [2, 3], mast cell degranulation [4], and accumulation of activated eosinophils [2–5] in the airways, which correlate with the two main physiological abnormalities, variable airflow limitation, and bronchial hyperresponsiveness. More recently, evidence has pointed to the production of a skewed profile of cytokines in atopic asthma bearing a resemblance to the T-helper (Th)2-type profile described in mice, with increased production of interleukin (IL)-3, IL-4, and IL-5 [6]. In addition, increased numbers of mast cells and eosinophils containing IL-4 and IL-5, cytokines that are relevant for the initiation and maintenance of the allergic asthmatic response, have been described in the bronchial mucosa from mild asthmatics [7]. In contrast, current knowledge of mucosal pathology in severe asthma is mainly limited to *post mortem* studies of subjects who have died during an exacerbation [8, 9]. As an alternative means of documenting increased inflammatory cell activity in severe asthma, studies of peripheral blood have shown increased numbers of CD4+ T-cells bearing surface activation markers, such as the interleukin IL-2 receptor (IL-2R) and the major histocompatibility complex (MHC) class-II antigen, human leukocyte antigen (HLA)-DR [10], raised

plasma concentrations of soluble IL-2R [10], and elevated serum levels of IL-5 [11]. A proportion of severe asthmatics are relatively resistant to the anti-inflammatory actions of glucocorticoids, with either reduced affinity or numbers of glucocorticoid receptors [12], and reduced suppression of *in vitro* proliferative T-cell responses to mitogens [13].

Summarizing these studies point to an important role for activated T-cells in maintaining the eosinophilic inflammatory response in severe asthma. To test whether these observations could be extended to the bronchial mucosa, a detailed bronchoscopic and clinical study of severe glucocorticoid-dependent asthmatics has been undertaken, and immunohistochemistry applied to resin-embedded endobronchial biopsies obtained by fiberoptic bronchoscopy to determine the extent of mucosal inflammation and cell activation. Furthermore, an investigation of whether IL-4 and IL-5 are upregulated in severe asthma was performed. In order to understand the relevance of the pathological changes to disease severity, mild asthmatics were also studied as well as healthy control subjects.

Subjects and methods

Subjects

Fifteen subjects with chronic severe asthma (two males and 13 females, median (range) age 24 (13–66) yrs), 10

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atopic mild asthmatics (five males and five females, median age 27 (21–42) yrs) and 10 nonatopic, healthy subjects (five males and five females, median age 27 (21–50) yrs) were studied. All the asthmatic subjects had a history of bronchial asthma according to the criteria of the American Thoracic Society [14]. Asthma severity was scored according to WOOLCOCK [15] and classified according to the World Health Organization (WHO) National Heart, Lung, and Blood Institute (NHLBI) Technical Report on a Global Strategy for Asthma [16]. The subjects' atopic status was confirmed by positive skin tests to two or more common aeroallergens, increased total immunoglobulin (IgE), and/or positive radioallergosorbent test (RAST). None of the studied individuals had ever smoked or experienced respiratory infections within 6 weeks prior to bronchoscopy.

All the severe asthmatics had been referred to the Dutch Asthma Centre in Davos for evaluation of poorly controlled asthma. All the subjects had been treated with high doses of oral and inhaled glucocorticoids for at least 1 yr before admission. Each subject was followed closely during a period of at least 3 months for the presence of unidentified allergies and potential aggravating factors (gastro-oesophageal reflux, obstructive sleep apnoea, hyperventilation, drug intolerance, and the presence of chronic sinus disease). In addition, self-management and

compliance were optimized. In contrast to the mild asthmatics, all the severe asthmatic subjects had frequent nocturnal symptoms characterized by awakening with dyspnoea and/or wheezing and were treated with high doses of both oral (median 40 mg·day⁻¹ prednisolone, range 2.5–100 mg·day⁻¹) and inhaled glucocorticoids (median 2,400 µg·day⁻¹ budesonide, range 2,000–4,000 µg·day⁻¹) at the time of study (table 1).

The mild atopic asthmatics and the healthy nonatopic control subjects were studied in Southampton, UK, for comparison using the same methods (table 1). None of the mild asthmatics had received inhaled or systemic glucocorticoids for at least 2 months prior to bronchoscopy. All the mild asthmatics were sensitive to house dust mite and other aeroallergens. All the healthy subjects were nonatopic and nonasthmatic as judged by negative skin prick tests to a panel of aeroallergens and history.

The study was approved by the Ethics Committee of the Dutch Asthma Centre and the Southampton Hospitals and University Ethics Committee, and all the subjects gave their written informed consent.

Clinical and physiological measurements

Asthma severity was assessed during a 2-week period preceding bronchoscopy as previously described [15]. A

Table 1. – Subject characteristics of severe and mild asthmatics

	FEV ₁ % pred	%PEF var	Salbutamol inhalation- day ⁻¹	Symptom score	Total score	IgE IU·L ⁻¹	Inhaled GC doses mg·day ⁻¹	Oral GC doses mg·day ⁻¹
Severe asthmatics								
1	68	34.6	10	7.3	12	156	2	100
2	91	26.7	8	6.9	12	126	2	60
3	82	64.1	7	4.7	12	72	4	35
4	53	21.4	7	5.7	10	1168	2	60
5	74	26.6	8	5.6	12	361	4	50
6	77	20.4	14	11.1	12	42	4	50
7	93	52.2	10	8.9	12	2735	2.4	90
8	40	21.4	8	4.9	12	1078	2	75
9	87	17.8	6	1.7	9	2000	2.4	5
10	96	26	6	3.1	12	53	2.4	5
11	95	21.9	5	4	11	869	3	5
12	86	20.7	7	7.6	12	104	2	12.5
13	71	16.4	6	5.1	10	150	3.2	2.5
14	67	22.2	6	4.6	10	6	4	7.5
15	94	20.8	5	5.5	10	34	4	7.5
Mean±sd/ median (range)	78±16.4	27.3±13.3	7 (5–14)	5.5 (1.7–11.1)	12 (9–12)	150 (6–2735)	2.4 (2–4)	40 (2.5–100)
Mild asthmatics								
1	114	5	0	0.8	2	264	0	0
2	80	23.4	0	3.8	5	1980	0	0
3	124	3.7	1	7	5	285	0	0
4	106	15	5	2.1	8	91	0	0
5	128	4.1	1	4.9	4	245	0	0
6	120	4.2	0	24	1	123	0	0
7	122	1.6	0	0.7	1	227	0	0
8	127	6.7	1	2.7	5	152	0	0
9	97	10.7	3	5.9	5	800	0	0
10	77	11.1	2	1.3	5	430	0	0
Mean±sd/ median (range)	109±19	8.6±6.6	1 (0–5)	2.5 (0.7–7)	5 (1–8)	254 (91–1980)		
p-values	0.0006	0.001	0.003	0.03	0.0003	NS		

FEV₁: forced expiratory volume in one second; PEF var: peak expiratory flow variability; IgE: immunoglobulin E; GC: glucocorticoid; NS: not significant.

total clinical severity score was given based on a combination of symptom scores, frequency of bronchodilator use, and variability in peak expiratory flow (PEF), each ranging 0–4. Subjects were asked to record daily asthma symptoms on a diary card using a 0–3 scoring system (0=no symptoms, 1=mild, 2=moderate, 3=severe). A total of six respiratory symptoms were recorded: nocturnal dyspnoea, nocturnal coughing, wheezing and coughing during the day, sputum production, and the effect of the asthma on daily activities. The mean of 14 daily asthma scores was calculated and used in the total score. All the subjects recorded PEF twice daily before and after bronchodilator medication using a mini-Wright peak flow meter (Airmed, London, UK). Variability in PEF was calculated by the difference between the maximum and minimum of the four PEF results each day and expressed as a percentage of the maximum [17]. The mean of 14 daily values was calculated and used in the total score. In addition, subjects were asked to record their daily use of salbutamol as the numbers of puffs and the frequency of nebulized salbutamol use. Prebronchodilator forced expiratory volume in one second (FEV₁) was determined using a pneumotachometer (Jaeger Masterlab, Würzburg, Germany) and a dry wedge spirometer (Vitalograph, Buckinghamshire, UK). Values were expressed as a percentage of the predicted values.

Fibreoptic bronchoscopy and tissue analyses

Fibreoptic bronchoscopy was performed according to previously published methods [18] and in adherence with the most recent NHLBI guidelines [16]. The biopsies were processed into glycol methacrylate (GMA) resin for immunohistochemistry, as previously described [19]. The following monoclonal antibodies were used: AA1 (directed against tryptase; Dako, Cambridge, UK) for mast cells, EG2 (directed against the cleaved form of eosinophilic cationic protein; Pharmacia, Milton Keynes, UK) for eosinophils, anti-neutrophil elastase (Dako, High Wycombe, UK) for neutrophils, anti-CD3 (Dako) for total T-cells, anti-CD4 (Becton Dickinson, Abingdon, UK) for helper/inducer T-cells, anti-CD8 (Dako) for suppressor/cytotoxic T-cells, and anti-CD25 (Dako) for IL-2R-bearing cells. The concentrations of the antibodies were determined previously by titration. Nonspecific binding was controlled for by using subclass-matched immunoglobulins at the same titre. Because IL-2R can be found on activated T-cells, eosinophils, monocytes, and B-cells, adjacent 2 µm thick sections were stained with cell-specific antibodies and anti-CD25 antibodies. Using the camera lucida method [7], CD25-positive staining was colocalized with cell-specific staining.

To assess the contribution of cytokines to the pathogenesis of severe asthma, IL-4 and IL-5 were chosen to be studied as these are believed to be central to atopy and eosinophilic inflammation [6, 7, 11]. For IL-4, monoclonal antibodies 4D9 and 3H4 (donated by C. Heusser, Ciba, Basle, Switzerland) previously shown to distinguish between intracellular and secreted IL-4 were used [20]. For IL-5, Mab-7 (L.A. McNamee, Glaxo, Greenford, Middlesex, UK) was used to detect cellular IL-5 [21].

All sections for each antibody were coded, stained and analysed simultaneously by one single, blinded investigator (B. Vrugt). Only nucleated, positively stained cells were counted at ×40 magnification. The area of the submucosa was measured using the Colourvision 1.6-4SR software package on a Macintosh computerized image analyser (Improvision, Birmingham, UK) excluding the blood vessels and smooth muscle. The total count of cells was expressed as the mean number of cells·mm⁻² of submucosa in two sections.

Data analyses

FEV₁ was expressed as mean±SD percentage of predicted and PEF variability as mean±SD. Comparison between groups in lung function results was performed using the unpaired Student's t-test. The coefficients of variation for three repeat measurements of a single section stained with CD3 and Mab-7 were 6 and 8.5%, respectively. The mean coefficients of variation within the same biopsy block were 12% for CD3 and 31% for Mab-7. As the immunohistochemical data were not normally distributed, differences in these variables were analysed first using the Kruskal–Wallis test (K–W) and, if significant, further tested for significance using the Mann–Whitney U-test (M–W). A Bonferonni type correction for multiple comparisons was applied by multiplying the obtained p-values by the number of tests performed. Correlations between cellular and lung function parameters were sought by Spearman's rank correlation test. Differences were considered significant at p<0.05.

Results

All bronchoscopies were performed without complications, and in no case was termination of the procedure necessary.

Clinical data

The total clinical severity score, FEV₁, PEF variability, and symptom score were significantly higher in the severe asthmatics compared to the mild asthmatics (table 1). The FEV₁ was significantly lower in the severe asthmatics than in the healthy control subjects (116±11.6%, p=0.0003). The median level of serum IgE in the severe asthmatics was 150 IU·L⁻¹ (range 6–2,735), which was not different from that in the mild asthmatics (254 IU·L⁻¹, range 91–1,980) (p>0.05). Both the severe asthmatics and mild asthmatics had significantly higher levels of IgE than the healthy control subjects (32.5 IU·L⁻¹, range 10–75) (K–W, p=0.0007).

Immunohistochemical analyses

The bronchial biopsies of the mild atopic asthmatics contained higher median (range) numbers of EG2+ cells (14.9 cells·mm⁻² (6.4–40.3)) than those from the severe asthmatics (4.4 cells·mm⁻² (0–43.6)) and the control subjects (1.2 cells·mm⁻² (1–4.6); K–W, p=0.0003). Although a

prominent mucosal eosinophilia comparable with mild asthmatics was observed in the biopsies of four severe asthmatics (subjects 8, 9, 10, and 15, table 1) (fig. 1a), eosinophil numbers in the group as a whole were not significantly higher compared to the healthy control subjects (M–W, $p=0.13$). The median (range) number of AA1+ mast cells was higher in the mild asthmatics (35.4 cells·mm⁻² (4.5–52.8)) compared to the severe asthmatics (17.5 cells·mm⁻² (2.5–56.4)) and the control subjects (17.1 cells·mm⁻² (5.5–61)); K–W, $p=0.04$) (fig. 1b), but utilizing the M–W U-test with the Bonferonni's correction no significant differences were found between the individual groups ($p>0.05$). There were also no significant differences between the three groups in the numbers of neutrophils: 23.4 cells·mm⁻² (11.4–174) in the severe asthmatics, 41.5 cells·mm⁻² (8.3–48.7) in the mild asthmatics, and 11.6 cells·mm⁻² (6.8–34.8) in the control subjects (K–W, $p=0.07$).

IL-4 immunoreactivity was observed in the bronchial mucosa both in the normal and asthmatic subjects. The two anti-IL-4 antibodies gave a different staining pattern. The 4D9 antibody was visible as a granular, cytoplasmic staining, whereas the 3H4 antibody gave a membranous

ring staining. Biopsies from the mild asthmatics (13.5 cells·mm⁻² (0–32.9); M–W, $p=0.01$) contained more 4D9+ cells than biopsies from the control subjects (3.7 cells·mm⁻² (1.2–7.8)). No significant differences were seen between the severe asthmatic group (5.0 cells·mm⁻² (1.6–21.3)) and the mild asthmatics (M–W, $p=0.10$) or healthy control subjects (M–W, $p=0.15$). The numbers of 3H4+ cells were significantly higher in the mild asthmatics (16.1 cells·mm⁻² (4.2–47.6); K–W, $p=0.0008$) than in the severe asthmatics (3.8 cells·mm⁻² (0.8–13.9)) and control subjects (3.7 cells·mm⁻² (2–5)), with no significant differences between the latter two groups (fig. 2). In six of the 10 mild asthmatics, monoclonal antibody 3H4 stained more cells than 4D9, while the converse was true in 14 of the 15 severe asthmatics.

Quantitative analysis of the tissue stained with the anti-IL-5 antibody Mab-7 revealed significantly higher numbers of Mab-7+ cells in the severe asthmatics (4.1 cells·mm⁻² (1.5–11.9)) compared to the healthy control subjects (2.3 cells·mm⁻² (0–4.3); K–W, $p=0.02$), with no significant difference between the mild asthmatics (5.4

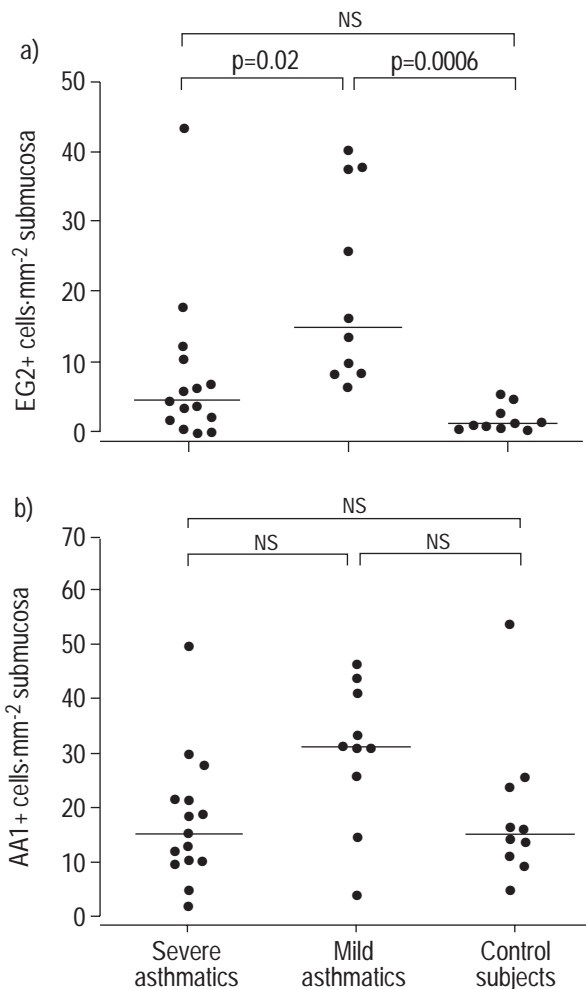


Fig. 1. – EG2+ eosinophil (a) and AA1+ mast cell (b) counts in the submucosa of severe asthmatics and healthy nonatopic control subjects. Comparison between groups was by Mann–Whitney U-test with Bonferonni's correction.

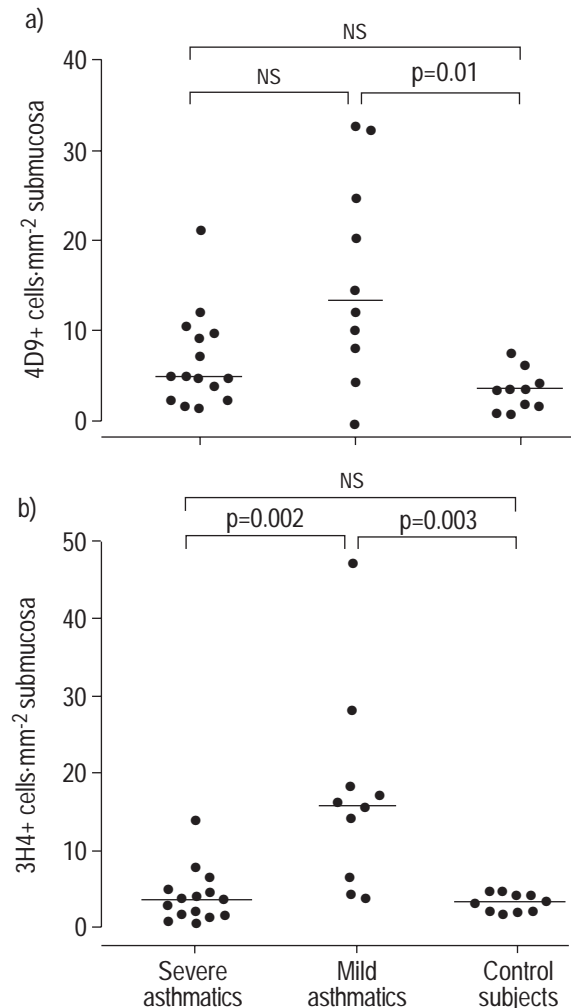


Fig. 2. – The numbers of cells staining positively for interleukin (IL)-4 in severe asthmatics, mild asthmatics and healthy control subjects. The monoclonal antibody 4D9 (a) gives granular staining and identifies intracellular IL-4, whilst the monoclonal antibody 3H4 (b) gives ring staining, identifying secreted IL-4. Comparison between groups was by Mann–Whitney U-test with Bonferonni's correction.

cells·mm⁻² (0–12.2)) and the control subjects (M–W, $p=0.15$) or the severe asthmatics and the mild asthmatics (M–W, $p=0.50$) (fig. 3).

The median (range) numbers of CD3+ T-cells in the severe asthmatics was (63.1 cells·mm⁻² (29.2–366.3)), which was not statistically different from mild asthmatics (84.2 cells·mm⁻² (25.7–232)) and control subjects (76.2 cells·mm⁻² (31.9–150.3); K–W, $p=0.60$). No significant differences were found between the median (range) numbers of CD4+ T-cells in the severe asthmatics (28.7 cells·mm⁻² (11.7–165)) when compared to the mild asthmatics (45.5 cells·mm⁻² (21.6–126.8)) or the control subjects (40.0 cells·mm⁻² (10.8–103); K–W, $p=0.67$) (fig. 4a). Similarly, no significant differences in CD8+ cells could be demonstrated between the severe asthmatics (20.6 cells·mm⁻² (6.9–167)), the mild asthmatics (37.3 cells·mm⁻² (12.3–128)), and the control subjects (35.4 cells·mm⁻² (12.6–88.2); K–W, $p=0.42$). However, there was an average 2.5-fold increase in the numbers of CD25+ cells in the severe asthmatics (6.8 cells·mm⁻² (0.8–19.7)) compared to the subjects with mild disease (2.6 cells·mm⁻² (0.6–7.3)) and a 4-fold increase compared to the normal subjects (1.6 cells·mm⁻² (1.0–4.6); K–W, $p=0.001$) (fig. 4b). The expression of CD25 was not significantly different between the mild asthmatics and the control subjects (M–W, $p=0.23$). Analyses of the serially-cut sections stained with antibodies directed against CD3, EG2, and CD25 revealed that all CD25+ cells were CD3+ cells.

A highly significant correlation was found between the numbers of CD25+ cells in the submucosa and the variability in PEF in the overall group of asthmatics ($r_s=0.59$, $p=0.004$) and particularly in the group of severe asthmatic subjects ($r_s=0.76$, $p=0.005$) (fig. 5).

Discussion

In this study of airways pathology in severe asthma, evidence has been found of striking T-cell activation which was related to disease activity and correlated with PEF

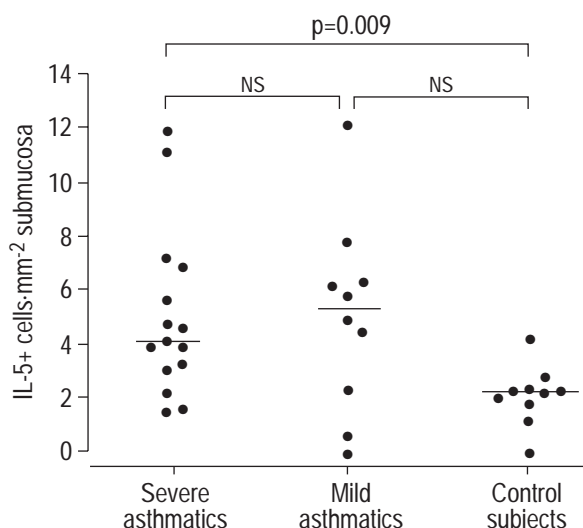


Fig. 3. – Numbers of cells staining positively for Mab-7 (anti-interleukin-5) in the submucosa of severe asthmatics, mild asthmatics, and control subjects. Comparison between groups was by Mann–Whitney U-test with Bonferonni's correction.

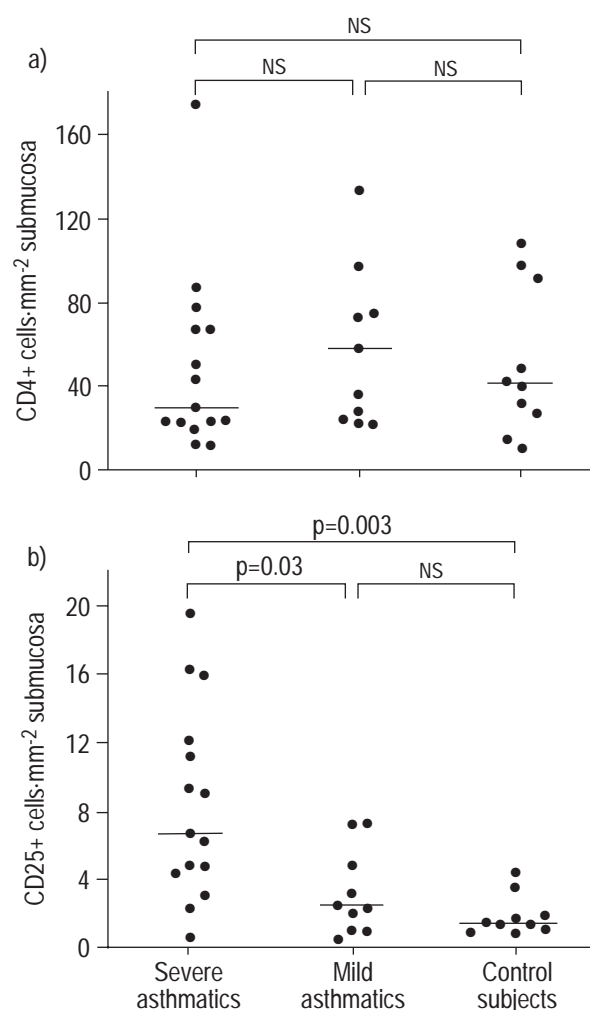


Fig. 4. – CD4+ T-cells (a) and activated (interleukin-2 receptor-bearing) T-cells (b) in bronchial biopsies of severe asthmatics, mild asthmatics and healthy control subjects. Comparison between groups was by Mann–Whitney U-test with Bonferonni's correction.

variability, an index of airways irritability [17]. In contrast, the total number of eosinophils and, to a lesser extent, mast cells were reduced in severe asthma as compared to mild disease, an effect that is most likely to be due to the suppressive action of glucocorticoids. In addition, numbers of IL-5 immunoreactive cells were increased in severe asthmatics in comparison with control subjects despite treatment with high doses of glucocorticoids.

It is well known that a spectrum of clinical responses is seen in asthmatics when treated with glucocorticoids. Whereas the majority of patients respond positively to glucocorticoids, a minority of subjects continue to display evidence of ongoing immune activation, with the glucocorticoid-resistant patient being the most extreme example [10, 12, 13]. The primary goal of this study was to investigate whether differences in inflammatory cell profiles between severe and mild asthmatics could account for differences in clinical presentation, and therefore patients were specifically selected who represent the two extremes of the disease spectrum. Because it is difficult to uncouple the attributes of more severe disease from those of glucocorticoid exposure, studying additional groups of

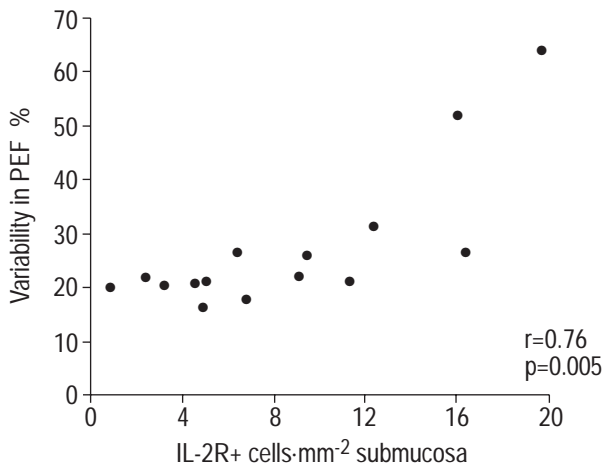


Fig. 5. – Relationship between numbers of CD25+ cells and disease severity as determined by the variability in peak expiratory flow (PEF). Correlation coefficient (r_s) and statistical significance were calculated using the Spearman's rank correlation test. IL-2R: interleukin-2 receptor.

mild asthmatics treated with inhaled glucocorticoids or severe asthmatics who are well-controlled on high doses of glucocorticoids might have given more information concerning the relevance of these findings. However, previous studies have already shown that when mild and moderately severe asthmatics are treated with anti-inflammatory drugs, a decrease in indices of cellular activation is readily achieved [22–25], consisting of marked depletion of eosinophils, reduction in mast cell numbers [22, 23], downregulation of T-lymphocyte activation [24] and messenger ribonucleic acid (mRNA) transcription for cytokines [25]. In contrast to results reported by others [3], the present study could not demonstrate a significant difference in mucosal T-cell activation between mild asthmatics and control subjects. This may be attributed to differences in processing of the tissue, thickness of the sections, the method of counting, and the nature of the disease in the current mild asthmatics.

The cellular mechanisms which underlie the persistent symptoms in severe asthma remain unclear. Recent studies addressing severe asthma pathogenesis have shown increased percentages and numbers of neutrophils in bronchoalveolar lavage (BAL) and biopsies from patients with severe glucocorticoid-dependent asthma while eosinophil counts were reduced in most cases [26, 27]. In addition, increased levels of leukotrienes in BAL fluid have been demonstrated which may be important to severe asthma pathogenesis since the production of these potent bronchoconstrictive mediators appears to be relatively insensitive to the inhibitory effects of glucocorticoids [28]. Despite large numbers of neutrophils present in the lumen of submucosal vessels, which were not included in the present counts, no increase in mucosal neutrophil numbers was seen in severe asthma when compared to mild disease or normal subjects. However, since glucocorticoid therapy is associated with peripheral neutrophilia and increased susceptibility to (airway) infections, the significance of increased neutrophil numbers is hard to assess, especially since no correlations have been demonstrated between neutrophil numbers and indices of asthma severity in the studies to date.

The observation of increased CD25 expression on T-cells in the airways of severe asthmatics extends previous observations made in peripheral blood. In these studies, increased expression of the IL-2R and HLA-DR activation antigens were found on circulating T-cells in subjects with glucocorticoid-resistant asthma [10]. It has been shown that poorly-controlled asthma is associated with reduced glucocorticoid-receptor binding affinity of peripheral blood mononuclear cells *in vitro*, which reverses to normal in the presence of medium but is sustained when incubated with IL-2 and IL-4 [12]. In addition, a recent study employing *in situ* hybridization has demonstrated a marked increase in BAL cells positive for IL-2 and IL-4 mRNA transcripts in glucocorticoid-resistant asthmatics as compared to those who are glucocorticoid-sensitive [29]. These observations support the concept that ongoing T-cell activation and cytokine secretion may be associated with persistent airway inflammation that is relatively insensitive to the immunosuppressive effects of glucocorticoids. Although five subjects in the present study have been tested and found to be less responsive to the suppressive action of dexamethasone *in vitro* (data not shown), the observation of increased expression of CD25 on mucosal T-cells is compatible with a relative glucocorticoid unresponsiveness.

The finding of reduced mucosal eosinophilia despite increased T-cell activation is consistent with previous observations made by WENZEL *et al.* [27]. However, this does not necessarily exclude a role for the eosinophil in severe asthma pathogenesis. In a previous study, persistence of eosinophil granule heterogeneity on electron microscopic examination was observed in biopsies from mild asthmatics after treatment with high doses of inhaled glucocorticoids, while numbers of EG2+ cells were reduced after treatment [22]. This suggests that glucocorticoids exert their effects primarily on eosinophil numbers without affecting eosinophil activation. In this study, the mucosal and peripheral blood eosinophilia as well as the increased levels of eosinophil cationic protein (ECP) observed in a proportion of the severe asthmatics suggests that, for whatever reason, eosinophils appear to be activated and relatively resistant to glucocorticoids in some subjects. Future studies of eosinophil markers of activation, such as major basic protein (MBP) and ECP, in BAL fluid and induced sputum should help to elucidate the contribution of the eosinophil to the pathogenesis of severe asthma.

The mechanisms by which eosinophils are recruited are complex but appear to involve the elaboration of cytokines such as IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF), as well as the CC-chemokines "regulated on activation, normal T-cells expressed and secreted" (RANTES), monocyte chemoattractant protein (MCP)-3, and MCP-4 [30, 31]. In particular, IL-5 selectively promotes eosinophil function [32] and together with IL-3 and GM-CSF, activates eosinophils to release more sulphidopeptide leukotrienes and secrete cationic proteins toxic to the airway epithelium [30]. The present study raises the possibility that in severe asthma, the relatively high numbers of activated T-cells represent a major source for cytokines such as IL-4 and IL-5. This hypothesis requires confirmation using *in situ* hybridization, a technique that cannot be applied to GMA-embedded tissue. There is accumulating evidence that

substantial amounts of IL-5 can also be generated by other cells, including mast cells and eosinophils [7]. Although the cellular source of IL-5 protein was not specifically addressed in this study, it is conceivable that in severe asthma most of the IL-5 protein is stored in eosinophils and mast cells with a relatively higher proportion of these cells expressing IL-5. Upon activation, these cells are capable of generating IL-5, thereby contributing to eosinophil survival and activation in an autocrine fashion. This may partly explain the mucosal eosinophilia observed in some of the severe asthmatics. Alternatively, a recent finding of increased amounts of IL-8 in serum, peripheral blood mononuclear cells, and biopsies from severe asthmatics when compared with mild asthmatics and normal subjects may provide an additional mechanism for the initiation of eosinophil recruitment in severe asthma despite high-dose glucocorticoid treatment [33].

In contrast to IL-5, the numbers of IL-4 immunoreactive cells were not elevated in severe asthmatics when compared with control subjects. IL-4 plays a pivotal role in IgG-IgE isotype switch [34] and preferential accumulation of eosinophils in asthmatic airways [30]. Although the implications of the reduction in numbers of IL-4+ cells for disease severity is unclear, the majority of the present patients continued to produce significant amounts of IgE. It is possible that the increased IgE synthesis in these patients is related to persistent secretion of IL-4 by activated T-cells and/or a higher sensitivity of interferon- γ relative to IL-4 to the inhibitory effects of glucocorticoids [29]. Another explanation for persistent IgE production is upregulation of the IL-13 gene [34]. Although in this study immunohistochemistry was not applied to sequential sections to identify the cellular source of IL-4, the decrease in 3H4 immunoreactivity in severe asthma compared to mild disease, suggests that IL-4 production is downregulated in severe disease. Whether this is the consequence of high-dose glucocorticoid therapy or an intrinsic feature of severe asthma awaits further investigation.

In conclusion, this study has shown evidence suggestive of selective responsiveness of inflammatory cells resident in the airways mucosa of severe atopic asthmatics, with an increased proportion of activated T-lymphocytes and immunoreactivity for interleukin-5, but not interleukin-4, being the most prominent features of severe disease. Although in severe asthma raised eosinophil counts were seen in some of the subjects, the absence of a prominent mucosal eosinophilia in the group as a whole indicates that additional factors, such as airway remodelling, may also be relevant to the clinical presentation of severe disease.

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