Dysregulation of Interleukin 4, Interleukin 5, and Interferon γ Gene Expression in Steroid-resistant Asthma

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Summary

In steroid-resistant (SR) asthma, there is a lack of clinical responsiveness to oral prednisone. Previous studies indicate that this may be explained by the effect of the combination of interleukin 2 (IL-2) and IL-4 on glucocorticoid receptor binding affinity. By contrast, steroid-sensitive (SS) asthmatics respond well to glucocorticoids, and this is accompanied by a decrease in the numbers of bronchoalveolar lavage (BAL) messenger RNA$^+$ (mRNA$^+$) cells expressing IL-4 and IL-5, and an increase in interferon γ (IFN-γ) transcripts. In the present study, we hypothesized that SR asthma is associated with alterations in T helper types 1/2 (Th2/Th1)-type cytokine gene expression. BAL was performed in six SR asthmatics and six SS asthmatics, before and after a 1-wk course of 40 mg daily prednisone. mRNA$^+$ cells for IL-2, IL-4, IL-5, and IFN-γ was measured by in situ hybridization using ^{35}S-labeled RNA probes. Before prednisone therapy, there were significantly greater numbers of BAL cells (per 1,000) expressing IL-2 mRNA (p <0.01) and IL-4 mRNA (p <0.05) in SR asthmatics as compared with SS asthmatics, but no differences between the two groups in the numbers of BAL cells expressing IFN-γ or IL-5 mRNA expression were observed. After a 1-wk course of prednisone, IL-2 expression was not altered in either group. However, SS asthmatics had a significant decrease in the numbers of BAL cells expressing mRNA for IL-4 (p <0.01) and IL-5 (p <0.001), and a rise in the numbers of IFN-γ mRNA$^+$ cells (p <0.01). In contrast, after prednisone treatment, SR asthmatics had no significant change in either the number of BAL cells expressing mRNA for IL-4 or IL-5. Of note, there was an unexpected decrease in the numbers of IFN-γ mRNA$^+$ cells (p = 0.05). Our current findings indicate that SR asthma is associated with a dysregulation of the expression of the genes encoding for Th2/Th1 cytokines in airway cells and is compatible with the concept that a combination of IL-2 and IL-4 induce glucocorticoid (GR) binding affinity and T cell responsiveness to glucocorticoids.

R ecent National Institutes of Health (NIH) guidelines for the treatment of asthma have focused on early intervention with antiinflammatory therapy, particularly inhaled glucocorticoids (1). However, a subset of asthmatics fails to demonstrate a satisfactory response even to systemic glucocorticoid therapy (2, 3) and has been termed "steroid resistant" (SR)[1]. It is important to recognize these patients early because failure to respond to steroids often leads to courses of very high dose glucocorticoid therapy and consequent ad-

verse effects despite persistent airway compromise (4). Indeed the documented rise in asthma mortality during the past decade, despite the use of glucocorticoids for asthma therapy in many countries, raises the question of whether a proportion of these patients were indeed SR. Since these patients likely evolve over time, understanding mechanisms involved in glucocorticoid resistance could lead to earlier identification of this challenging group of asthmatics and provide insight for new directions in the management of severe chronic asthma.

The actual mechanism by which glucocorticoids reduce airway inflammation in asthma remains poorly understood. However, at least one aspect of its action likely relates to the modulation of cytokine production (5). In this regard, recent bronchoalveolar lavage (BAL) studies in atopic asthmatics

have demonstrated increased numbers of T cells expressing mRNA for IL-4 and IL-5 (6). These two cytokines play a critical role in the induction of IgE synthesis as well as the recruitment, activation, and survival of eosinophils, a cell thought to play an important role in mediating inflammation and epithelial damage in asthmatic airways (7, 8). Recent studies indicate that steroid-sensitive (SS) asthmatics respond to prednisolone therapy with a reduction in eosinophils as well as a decrease in activated T cells expressing IL-4 and IL-5 mRNA transcripts but an increase in IFN-γ (9). To gain further insight into potential mechanisms by which glucocorticoids act in vivo, it is also of interest to study patients who fail to respond to steroid therapy.

Previous studies on PBMCs from patients with SR asthma have demonstrated that glucocorticoids fail to inhibit their mitogen-induced T cell proliferation and cytokine secretion in vitro (10, 11). In addition, T cells from peripheral blood of SR asthmatics, but not SS asthmatics, are persistently activated despite continued treatment with aggressive steroid therapy (12). Recently, we have found that the majority of patients with SR asthma have a reversible defect in glucocorticoid receptor (GR)-binding affinity which can be sustained in vitro by the addition of the combination of IL-2 and IL-4 but not the individual cytokines (13). Furthermore, in vitro incubation of normal T cells with the combination of IL-2 and IL-4, in the absence of IFN-γ, reduces their GR-binding affinity to the level seen in SR asthma (14). These in vitro data suggested that the pattern of cytokine gene expression may play a role in determining steroid resistance. In the current study, we therefore used the technique of mRNA in situ hybridization to examine whether airway cells from SR asthmatics vs. SS asthmatics expressed different patterns of cytokines, particularly after treatment with oral steroids.

Materials and Methods

Patients. 12 consecutive patients with a diagnosis of asthma, based on The American Thoracic Society (ATS) criteria (15), who fulfilled the entry criteria of this study and were willing to undergo the procedures related to this protocol were studied. At baseline, all subjects had a morning prebronchodilator forced expiratory volume in 1 s (FEV$_1$) <70% of predicted values and a ≥15% increase in FEV$_1$ after two inhalations of albuterol (90 μg/actuation). None of these patients had evidence for other types of lung disease, pregnancy, suspected noncompliance with medical care, or concurrent therapy with medications that alter glucocorticoid metabolism, such as anticonvulsants or erythromycin.

Study Design. Informed consent, approved by the National Jewish Center Institutional Review Board, was obtained from all patients before their entry into this study. All patients entering into this study agreed to undergo fiberoptic bronchoscopy with BAL and flow spirometry, immediately before and after a 1-wk course of oral prednisone taken 20 mg twice per day. Flow spirometry was carried out in accordance with the ATS standardized guidelines (16). At each timepoint, patients had three FEV$_1$ measurements, and the best of two FEV$_1$ measurements, which were within 5% of each other, was used. BAL fluid was analyzed for cytology, numbers of T cells expressing the HLA-DR activation antigen by flow cytometry, and cytokine gene expression by in situ hybridization. BAL fluid was analyzed for cytology, numbers of

T cells expressing the HLA-DR activation antigen by flow cytometry, and cytokine gene expression by in situ hybridization.

Patients were classified as SS or SR based on their prebronchodilator morning FEV$_1$ and response to a course of oral prednisone in the current study. Compliance with prednisone therapy was documented by monitoring plasma morning cortisol levels and prednisolone levels (the active metabolite of prednisone) as indicated in Table 1. Asthmatic patients were defined as SR if they failed to improve their morning prebronchodilator FEV$_1$ by ≥15% after a 1-wk course of oral prednisone (13, 16). Patients were classified as SS if they had an increase in FEV$_1$ ≥30% above baseline.

Fiberoptic Bronchoscopy. Each subject had a baseline and post-treatment bronchoscopy performed at the same time of day. The baseline bronchoscopy occurred on the day that prednisone treatment was initiated. The final bronchoscopy was performed within 12 h after the last dose of prednisone. The fiberoptic bronchoscopy was well tolerated by all subjects without any significant side effects during or after the procedure.

Bronchoscopy with BAL was performed by our previously described method (17). Prebronchoscopy medications for each subject included intramuscular injections of 60 mg codeine and 0.6 mg atropine, 4% xylocaine nebulized to the nasopharynx, 5–8 mg of intravenously administered midazolam, a total of 6 ml of 1% xylocaine directly applied to the laryngeal area via the bronchoscope, and 2 ml of 1% xylocaine directly applied to each of the trachea, right mainstem bronchus, and opening of the right middle lobe. Nasal oxygen at 4 liter/min was used throughout the procedure. A different segment of the right middle lobe was lavaged at each bronchoscopy, using five 60-ml aliquots of 36°C sterile normal saline after the bronchoscope was wedged into a subsegment. The BAL fluid was harvested by immediate, gentle-hand suction applied to each instilling syringe.

Cellular Analysis of BAL Cells. BAL fluid analysis was performed immediately after the bronchoscopy and was independently verified by two technicians blinded to the subjects' condition. The total cell count was determined by using the original lavage fluid suspension, and the differential cell analysis was prepared by the cytocentrifuge method using the Diff-Quik® stain (American Scientific Products, McGraw Park, IL) (18). For analysis of the number of activated T cells expressing the HLA-DR surface antigen, aliquots of 5 × 10^5 BAL cells were incubated for 30 min at 4°C with FITC-conjugated anti-HLA-DR and PE-conjugated anti-CD3 or respective IgG$_1$-FITC and IgG$_2$-PE isotype controls from Becton Dickinson & Co. (Mountain View, CA). After two washes in medium, fluorescence intensity was analyzed with a FACScan® flow cytometer (Becton Dickinson & Co.) as previously described (19, 20).

In Situ Hybridization. BAL cells suspended at a concentration of 10^5 cells/ml in RPMI 1640 (Flow Laboratories, McLean, VA) were cytospun at 800 rpm for 5 min onto poly L-Lysine coated slides. Cytospins were then air-dried for 5 min, fixed in 4% paraformaldehyde in PBS (pH 7.2) for 30 min, followed by two changes of 15% PBS-sucrose for 1 h each. Slides were then incubated at 37°C, and stored at −80°C before hybridization.

In situ hybridization was performed as previously described (6, 21, 22). Briefly, ^{35}S-UTP-labeled RNA probes were prepared from cDNA for IL-2, IL-4, IFN-γ, and IL-5. To avoid nonspecific binding of ^{35}S-labeled RNA probes, incubation with N-ethyl maleimide, iodoacetamide, and triethanolamine was included in prehybridization steps, and dithiothreitol (150 mM) was included in the hybridization mixture (Sigma Chemical Co., St. Louis, MO). As a negative control, preparations were hybridized with sense probes or pretreated with RNase before hybridization (Promega, Southampton, UK). Positive controls were prepared from a concanavalin A–stimulated

Table 1. *Patient Characteristics**

Parameter	SS asthma	SR asthma
No. of Subjects	6	6
Age	31 ± 3	30 ± 2
Sex (M/F)	4/2	5/1
Mean weight (kg)	73 ± 7	72 ± 5
Inhaled steroids (yes/no)	3/3	3/3
Systemic steriods (yes/no)	0/6	0/6
Duration of asthma (yr)	23 ± 2	26 ± 2
Allergen prick skin test	6/6 positive	6/6 positive
Baseline FEV_1 (percent pred)[‡]	47 ± 3	56 ± 2
FEV_1 after prednisone (percent pred)[§]	78 ± 2	61 ± 3
Baseline AM cortisol (mcg/dl)	10.6 ± 4.7	15.2 ± 2.6
AM cortisol after prednisone	0[‖]	0.2 ± 0.2[‖]
Baseline prednisolone levels (ng/ml)	0	0
Prednisolone levels after prednisone	131.5 ± 37.6[‖]	96.1 ± 35.8[‖]
Percent BAL eosinophils (baseline)	16 ± 4	10 ± 2
Percent BAL eosinophils after prednisone	8 ± 2[¶]	11 ± 5
Percent BAL HLA-DR$^+$ T cells (baseline)	30 ± 11	42 ± 10
Percent BAL HLA-DR$^+$ T cells after prednisone	9 ± 2[*]	50 ± 12

* All values represent mean ± SEM.
‡ Mean AM prebronchodilator FEV_1 value of patients before burst.
§ Mean AM prebronchodilator FEV_1 value following a 1-wk course of prednisone 20 mg orally twice daily. The SS, but not the SR, asthma group demonstrated a significant ($p < 0.01$) rise in FEV_1 after prednisone therapy.
‖ After prednisone therapy, AM cortisol levels decreased significantly ($p < 0.01$) and prednisolone levels increased significantly ($p < 0.05$) in the SS and SR asthma groups. Plasma cortisol and prednisolone levels were determined by a high performance liquid chromatography method described in reference 31.
¶ After prednisone therapy, the percentages of eosinophil in BAL decreased significantly in SS asthma ($p < 0.05$) as compared with SR asthma.
** After prednisone therapy, the percentages of activated HLA-DR$^+$ T cells in BAL decreased significantly ($p < 0.05$) in SS asthma, but not SR asthma.

human T cell clone known to produce IL-2, IL-4, and IL-5, or from PHA-stimulated mononuclear cells for IL-2 and IFN-γ.

Counting was done in a blinded fashion without knowledge of treatment groups or diagnosis. Hybridization between cytokine mRNA and cRNA probes was identified as dense collections of silver grains overlying cells. Hybridization signals were assessed by counting mRNA positive cells/1,000 total BAL cells on at least two slides for each cytokine probe and counts were performed in triplicate. The intraobserver coefficient of variation was <5%.

Statistical Analysis. Statistical comparison of BAL differential counts, T cells expressing HLA-DR and in situ hybridization results was performed with the Wilcoxon Signed Rank Test within groups and the Wilcoxon Rank Sum Test between groups. *p* values ≤0.05 were considered statistically significant.

Results

Clinical Features and Physiologic Response To Prednisone. Patient characteristics are summarized in Table 1. SR and SS asthmatics were similar for all parameters with one exception: although the SR asthmatics had a higher baseline FEV_1 (*p*

<0.05) before the prednisone course, as compared with the SS asthma patients, their FEV_1 after treatment with prednisone was significantly lower than SS asthmatics ($p < 0.01$). Of note, none of the patients entered in this study had received a course of prednisone for at least 2 mo before enrollment.

Compliance with prednisone therapy was documented by obtaining AM plasma cortisol levels and prednisolone levels before and after the course of prednisone. AM plasma cortisol levels were similar at baseline for each group. As shown in Table 1, after prednisone therapy, both study groups had a significant decrease in morning cortisol ($p < 0.01$) as well as significant increase in plasma prednisolone levels ($p < 0.05$).

Analysis of BAL Cells. At baseline, there was no significant difference between total white cell counts, percentage of eosinophils, or activated T cells in BAL fluid from the SR asthma group versus the SS asthma group (Table 1). However, after prednisone therapy, there was a significant decrease in the percentage of BAL eosinophils ($p < 0.05$) as well as percentage of BAL-activated T cells ($p < 0.05$) in the SS asthma group.
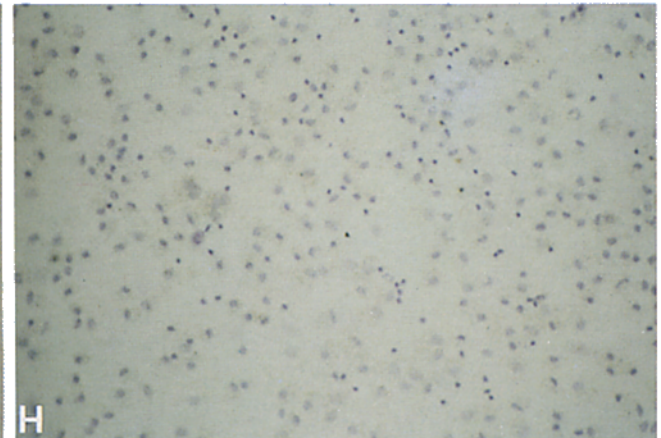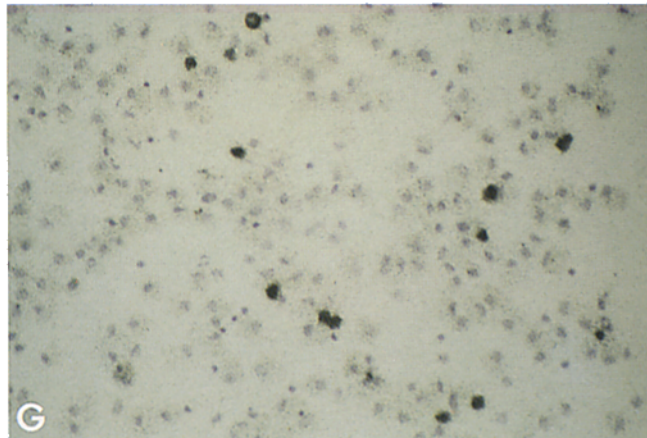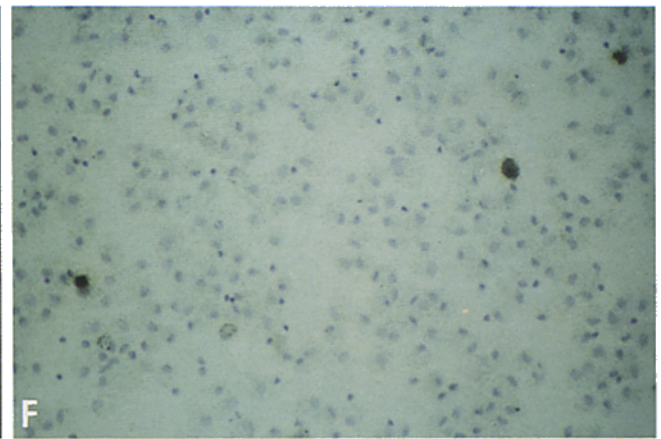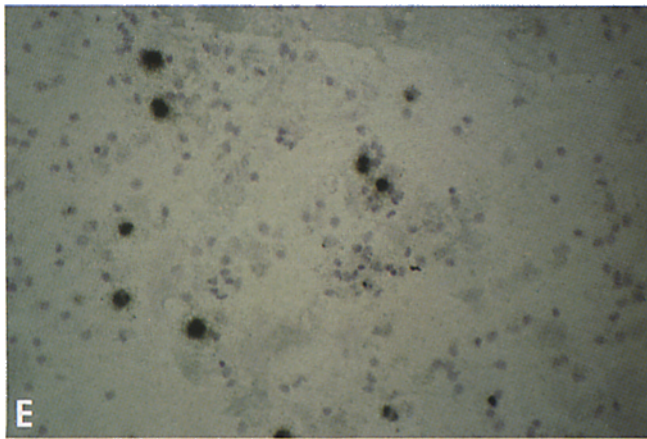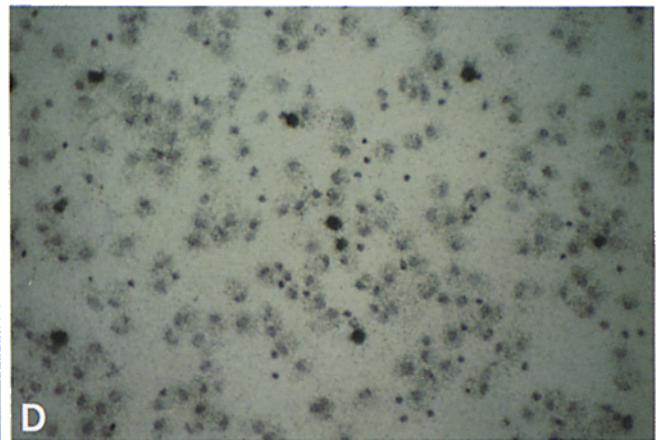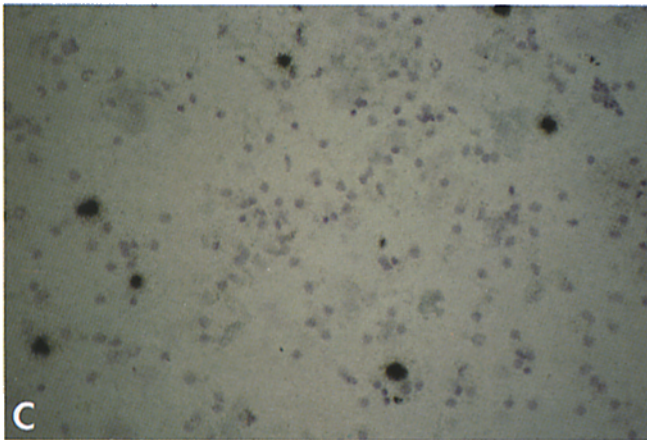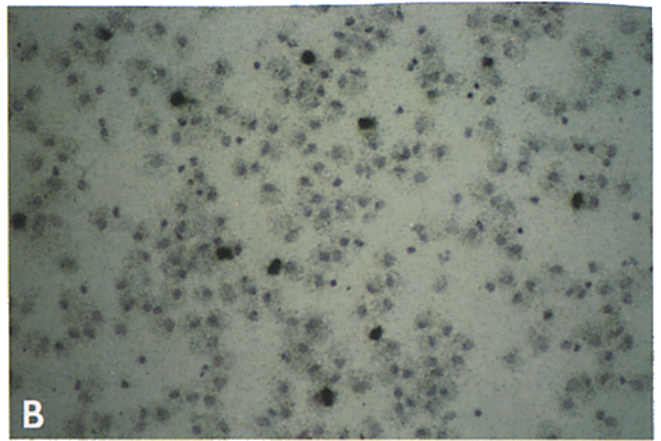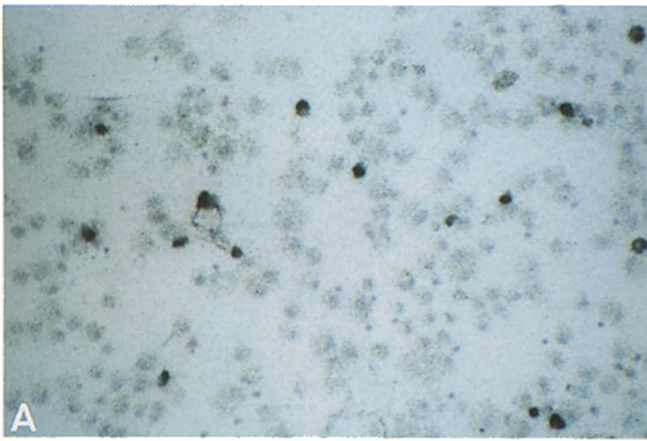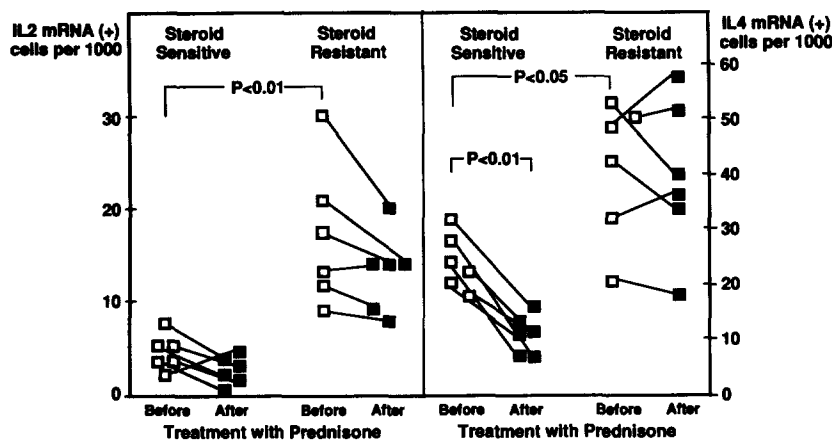
Figure 2. Number of cells with positive hybridization signals for IL-2 and IL-4 mRNA in BAL fluid from six patients with SS asthma and six patients with SR asthma obtained before and after treatment with prednisone. Before prednisone therapy, there were significantly more cells expressing IL-2 (p <0.01) and IL-4 (p < 0.05) mRNA in the BAL fluid of patients with SR asthma as compared with SS asthma. Values were compared by the Mann-Whitney U test.

In contrast, in the SR asthma group, prednisone therapy was not accompanied by any significant changes in percentage of BAL eosinophil counts or BAL-activated T cells.

Cytokine Gene Expression in the Airways of SS vs. SR Asthmatics. Positive in situ hybridization signals for cytokine mRNA were observed as dense collections of silver grains overlying BAL cells on autoradiographs only when antisense probes were used. No signals were observed on cytospins hybridized with sense probes, nor from preparations pretreated with RNase. Representative examples of autoradiographs showing signals from BAL cells of SS asthmatics or SR asthmatics hybridized with antisense riboprobes to IL-4 and IL-5 are shown in Fig. 1. A variable number of positive hybridization signals were observed for each cytokine in the SS vs. SR asthma groups depending on whether the sample was obtained before or after prednisone therapy.

As shown in Fig. 2, before prednisone therapy, BAL cells from patients with SR asthma, as compared with cells from SS asthma, had a significantly higher number of cells expressing positive hybridization signals for IL-2 mRNA (p <0.01) and IL-4 mRNA (p <0.05). However, no significant differences between these two patient populations were observed in the
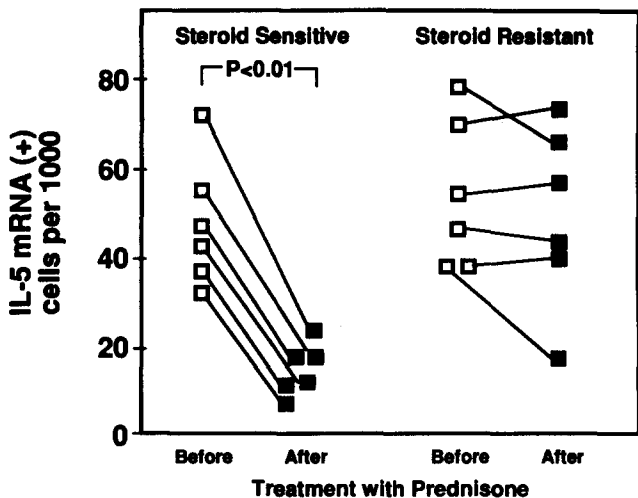

Figure 3. Number of cells with positive hybridization signals for IL-5 mRNA in BAL fluid from six patients with SS asthma and six patients with SR asthma obtained before and after treatment with prednisone. Before prednisone therapy, there were no significant difference in numbers of IL-5 mRNA-expressing cells (p = 0.7) in the BAL fluid of patients with SR asthma as compared with SS asthma. After prednisone therapy SS asthma, but not SR asthma, was associated with a significant decrease in numbers of IL-5 mRNA(+) cells (p <0.001).
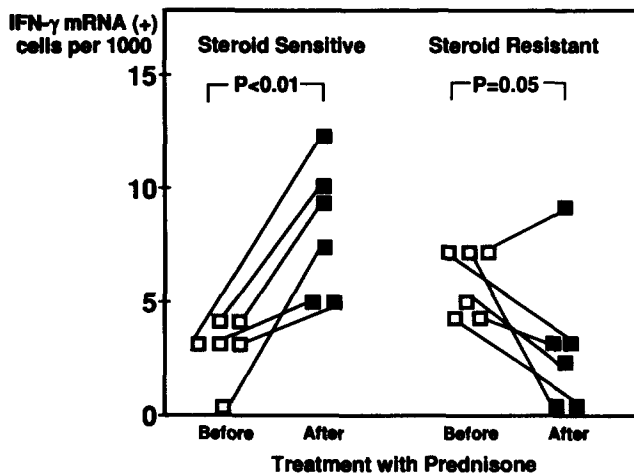

Figure 4. Number of cells with positive hybridization signals for IFN-γ mRNA in BAL fluid from six patients with SS asthma and six patients with SR asthma obtained before and after treatment with prednisone. After prednisone therapy, there was a significant rise (p <0.01) in IFN-γ mRNA+ cells in SS asthma, but a decrease in numbers of IFN-γ mRNA+ cells (p = 0.05) in SR asthma.

Figure 1. Autoradiographs of cytospin preparations of BAL cells from a SR asthmatic hybridized with an antisense riboprobe to IL-4 before (*A*) and after (*B*) prednisone therapy; a SR asthmatic hybridized with an antisense riboprobe to IL-5 before (*C*) and after (*D*) prednisone therapy; a SS asthmatic hybridized with an antisense riboprobe to IL-5 before (*E*) and after (*F*) prednisone therapy; a SS asthmatic hybridized with IFN-γ antisense probe after prednisone therapy (*G*); a representative control example of a BAL preparation from a SR asthmatic tested with a sense IL-4 probe (*H*). The positive hybridization signals are the dense collections of silver grains overlying BAL cells.

expression of baseline IL-5 mRNA (Fig. 3) and IFN-γ mRNA (Fig. 4). After prednisone therapy, BAL cells from SS asthmatics demonstrated a significant decrease in the number of cells expressing IL-4 mRNA (p <0.01, Fig. 2) and IL-5 mRNA (p <0.001, Fig. 3). In contrast, after prednisone therapy, BAL fluid from patients with SR asthma did not demonstrate any significant alteration in the number of BAL cells expressing either IL-4 mRNA or IL-5 mRNA. Furthermore, although small reductions in the numbers of BAL cells/1,000 positive for IL-2 mRNA accompanied prednisone therapy in each patient group, these differences were not significant (Fig. 2).

As shown in Fig. 4, treatment of SS asthmatics with prednisone resulted in a significant rise in the numbers of BAL cells expressing mRNA for IFN-γ (p <0.01). In contrast, when SR asthmatics were treated with prednisone, their BAL cells demonstrated a decrease in the numbers of IFN-γ mRNA$^+$ cells (p = 0.05). These divergent responses resulted in a highly significant difference in IFN-γ gene expression after prednisone treatment between the SS vs. SR asthmatic groups (p <0.01).

Discussion

This is the first study demonstrating that the BAL cells from the airways of patients with SR asthma have a distinct pattern of cytokine gene expression, and response to prednisone which differs from that found in SS asthma. Of note we found that, both before and after prednisone therapy, BAL cells from SR asthmatics had a significantly higher level of IL-2 and IL-4 gene expression than BAL cells from SS asthmatics. This is of particular interest because previous in vitro studies have demonstrated that the combination of IL-2 and IL-4 is required to reduce the GR-binding affinity in normal peripheral blood T cells (13). Furthermore, T cells from the majority of patients with SR asthma have a GR binding defect which reverses in culture. Although, this defect is sustained in vitro by the presence of the combination of IL-2 and IL-4, incubation with IL-2 or IL-4 alone is not sufficient to mediate an effect on GR binding affinity (12).

The method of in situ hybridization is semiquantitative and it is possible that mRNA expression may not always lead to cytokine production. Analyses of cytokine protein in concentrated BAL fluid from atopic asthmatics have, however, revealed a pattern similar to that detected at the level of mRNA (23). Therefore, it is most likely that our current data on cytokine mRNA expression reflects cytokine production in the airways of SS vs. SR asthmatics.

We have previously reported that IL-5 has no effect on GR binding affinity (12). However, T cells are a major source of IL-5 in asthmatic airways. Therefore, the failure of prednisone to inhibit airway T cell activation in SR asthma (Table 1) as well as peripheral blood T cell activation (see references 9 and 10), may have important pathologic consequences in that IL-5 gene activation will be sustained and therefore continue to promote eosinophil differentiation and survival in the airways of SR asthmatics. Indeed, our current findings

provide an immunologic basis for the original description by Schwartz et al. (2), in 1968, of SR asthma where he noted that prednisone did not suppress peripheral eosinophil counts in these patients.

An interesting finding in this study was the observed decrease in the number of BAL cells expressing IFN-γ mRNA in SR asthmatics after treatment with prednisone. A previous study in SS asthmatics demonstrated that improvement in pulmonary function after prednisolone treatment was accompanied by a significant increase in the number of cells expressing mRNA for IFN-γ (9). Our current study confirms this observation in that we also found an increased number of BAL cells expressing IFN-γ mRNA in SS asthmatics after prednisone therapy as compared with baseline values (Fig. 4). These data suggest that IFN-γ producing type 1 T helper (Th1)-like cells, as compared with IL-4 or IL-5 secreting type 2 T helper (Th2)-like cells, may be more sensitive to the inhibitory effects of glucocorticoids. Although a previous study (6) of patients with chronic allergic asthma demonstrated that IL-4 and IL-5 expression were primarily localized to T cells, recent reports indicate that other cells can also express these cytokines to varying degrees (24, 25). Future studies are therefore needed to determine the cell source of these various cytokines in SR asthma, and their relative sensitivity to the effects of glucocorticoids.

The precise mechanisms by which different patterns of cytokines or immune activation might result in decreased steroid responsiveness in asthma remain to be elucidated. We have previously reported that PBMCs from the majority of patients with SR asthma have reduced GR binding affinity and have postulated that these may contribute to decreased responses to corticosteroids (13). Furthermore, we found a marked difference between the nuclear and cytosolic GR-binding parameters of peripheral blood T cells from SR asthma. In this regard, it is well known that the GR changes its structure and/or conformation when translocated between the cytosol and nucleus (reviewed in reference 26). The activated GR nuclear complex regulates transcription by binding to specific DNA sequences, called glucocorticoid-responsive elements. The induction or repression of GR target genes ultimately results in the altered expression of glucocorticoid-regulated proteins (27). This latter action appears to be mediated via direct binding of the modulatory domain of the GR with transcriptional factors, such as AP-1. However, overexpression of AP-1 or other transcription factors has been found to interfere with GR function (28). Since our current studies demonstrate that SR asthma is associated with much higher levels of cytokine activation than SS asthma (Fig. 2), the activation of particular transcription factors such as AP-1 (29) may provide a plausible explanation for the nuclear localization of the GR-binding defect in SR asthma and differences in patterns of steroid responsiveness in asthma.

Our study also raises the intriguing possibility that SR asthma may be the end result of ongoing immune activation. It has been previously demonstrated that PHA-induced T cell proliferation and cytokine production by PBMC from patients with SR asthma is poorly inhibited by the addition

of dexamethasone or methylprednisolone in vitro (9, 10). Recently, we reported several SR patients whose asthma came under control with the combination of troleandomycin and methylprednisolone therapy and this resulted in normalization of T cell sensitivity in vitro to the inhibitory effects of methylprednisolone on T lymphocyte proliferation (11). Furthermore, cyclosporin A, a drug whose major action is inhibition of T cell proliferation and cytokine secretion, was reported to improve the clinical symptoms of several patients with SR asthma (30). These observations are consistent with the notion that ongoing asthma inflammation and cytokine secretion may contribute to the acquired GR defect found in SR asthma.

In conclusion, our current findings may have several important implications toward understanding the pathophysiology of chronic asthma. First, with the increasing use of corticosteroids in the treatment of asthma, it is important to recognize that such patients may vary in their response to steroids. Second, the observation of a distinctive pattern of cytokine gene expression in SR asthma is consistent with the possibility that different patterns of cytokine expression alter response to therapy. While many of these patients may respond to an extremely high dose of glucocorticoids such treatment carries the risk of serious side effects. Continued research is needed to define the mechanisms of action for ongoing airway immune activation, correlation of response to particular treatment strategies and thereby identify the most appropriate treatment regimen for a particular pathway of cellular activation. An understanding of the mechanisms by which glucocorticoids fail to resolve inflammation in asthma may provide important insight into its pathogenesis, especially as related to progressive pulmonary deterioration. This new information may then trigger new approaches for the management of this challenging respiratory condition.

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