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Interleukin-2 and -4 induce resistance of granulocyte–macrophage colony-stimulating factor to corticosteroids

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Abstract

In vitro pretreatment of human mononuclear blood cells with a combination of interleukin-2 and interleukin-4 decreases corticosteroid receptor affinity and reduces the anti-proliferative effects of corticosteroids. Similar abnormalities have been observed in mononuclear blood cells of steroid-resistant asthmatics. In vitro steroid resistance was induced by 48 h pretreatment of mononuclear blood cells from healthy individuals ($n = 10$) with interleukin-2 and interleukin-4 (500 Units (U)/ml). The effects of three structurally different corticosteroids (10^{-7}–10^{-11} M) on lipopolysaccharide-stimulated (10 ng/ml; 20 h) production of granulocyte–macrophage colony-stimulating factor (GM-CSF) were examined. GM-CSF production was efficiently inhibited by all three corticosteroids in the control cultures. Cortivazol was significantly more potent ($IC_{50} = 3 \times 10^{-11}$ M) than budesonide and tipredane ($IC_{50} = 2.5 \times 10^{-10}$ M and $IC_{50} = 2 \times 10^{-10}$ M, respectively). However, interleukin-2 and interleukin-4 pretreatment counteracted the inhibitory effects of all three corticosteroids to a similar degree. The results highlight the importance of interleukin-2 and interleukin-4 in the induction of steroid resistance, since pretreatment of mononuclear blood cells with these cytokines impaired corticosteroid inhibition of GM-CSF production.

Keywords: Steroid resistance; Corticosteroid; Cytokine; Asthma; Mononuclear blood cell

1. Introduction

Corticosteroids have long been used in the treatment of asthma due to their anti-inflammatory properties. There is, however, a subset of patients who do not show improvement of pulmonary function following corticosteroid therapy (Carmichael et al., 1981; Kamada et al., 1992; Woolcock, 1993). These patients with chronic asthma, unresponsive to high doses of corticosteroids, have been termed 'steroid-resistant'. Steroid resistance is recognized in several other inflammatory and immune diseases in addition to asthma and the mechanisms have been studied but not yet elucidated (Lamberts et al., 1992; Chikanza and Panayi, 1993; Frieri and Madden, 1993).

The airway cells of steroid-resistant asthmatics, compared with those of steroid-sensitive asthmatics, have a different pattern of cytokine gene expression and a distinct response to corticosteroid therapy (Leung et al., 1995). Steroid-resistant asthmatics have airway cells with increased expression of mRNA for interleukin-2 and interleukin-4. They also have increased T cell activation and do not show reduced numbers of eosinophils and activated T cells in bronchoalveolar lavage in response to corticosteroids (Leung et al., 1995). Several investigators have also reported data on defective responses of mononuclear blood cells to corticosteroids in vitro, which correlate with clinical resistance to corticosteroid treatment in steroid- resistant asthmatics (Kay et al., 1981; Poznansky et al., 1984; Wilkinson et al., 1989; Corrigan et al., 1991a; Alvarez et al., 1992; Corrigan et al., 1996; Spahn et al., 1996a). In addition, mononuclear blood cells of the majority of steroid-resistant asthmatics have decreased corticosteroid receptor binding affinity and increased receptor numbers in their nuclei (Kam et al., 1993; Sher et al., 1994). These abnormalities are reversible and revert to normal when mononuclear blood cells are incubated in vitro for 48 h, but persist when the combination of interleukin-2 and interleukin-4 is added (Kam et al., 1993; Sher et al., 1994).

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These effects of interleukin-2 and interleukin-4 treatment in vitro indicate that an increased local production of cytokines, including interleukin-2 and interleukin-4, might be involved in steroid resistance (Leung et al., 1995).

Furthermore, interleukin-2 and interleukin-4 treatment of normal mononuclear blood cells (i.e. a mixture of monocytes and lymphocytes) can induce corticosteroid receptor abnormalities, similar to those seen in steroid-resistant patients (Kam et al., 1993; Sher et al., 1994). These induced corticosteroid receptor abnormalities correlate with reduced inhibition of T cell proliferation in interleukin-2 and interleukin-4 treated normal mononuclear blood cells in response to corticosteroids (Kam et al., 1993). A similar reduction of the anti-proliferative effects of corticosteroids have been found in mononuclear blood cells of steroid-resistant asthmatics (Alvarez et al., 1992; Corrigan et al., 1996; Spahn et al., 1996a). Interestingly, when the T cell or the non-T cell fraction of normal mononuclear blood cells is treated separately with the combination of interleukin-2 and interleukin-4, significant receptor abnormalities develop in the T cell fraction only (Kam et al., 1993; Sher et al., 1994). However, studies of steroid resistance in rheumatoid arthritis show that the difference in corticosteroid inhibition of T cell proliferation seen in mononuclear blood cells of steroid-sensitive and steroid-resistant patients disappears when highly purified T cells are used (Chikanza and Panayi, 1993). Thus, interactions between T cells and monocytes seem to be crucial for the development of steroid resistance.

The present study aimed to investigate whether interleukin-2 and interleukin-4 pretreatment of normal mononuclear blood cells can lead to impaired inhibition of cytokine production by corticosteroids, as observed in mononuclear blood cells of steroid-resistant asthmatics (Wilkinson et al., 1989; Corrigan et al., 1991b; Adcock et al., 1995a). Granulocyte-macrophage colony-stimulating factor (GM-CSF) production was selected as a functional parameter since the production of this cytokine is sensitive to corticosteroid treatment (Linden and Brattsand, 1994) and since it is a cytokine important in the pathophysiology of asthma (Lopez et al., 1986; Djukanovic et al., 1990; Nakamura et al., 1993; Woolley et al., 1994). Three structurally different corticosteroids were selected for the study. Budesonide was selected since it is a potent and clinically well established corticosteroid for asthma therapy (Brattsand and Selroos, 1994). Cortivazol was selected since it binds to at least two different sites at the corticosteroid receptor, in contrast to other corticosteroids that bind to one site only (Thompson et al., 1989). In addition, cortivazol has been found to kill a highly dexamethasone-resistant clone of a human leukemic cell line (Ashraf et al., 1991). The third corticosteroid, tipredane, was selected because of a report on a similar inhibitory effect on proliferation of T cells of both steroid-sensitive and steroid-resistant asthmatics while there was a difference when dexamethasone was tested (Corrigan et al., 1994).

2. Materials and methods

2.1. Cell separation and culture

Venous blood (100 ml) was collected (between 7.45 and 8.30 am) from ten healthy volunteers, into sterile EDTA-containing tubes. Erythrocyte sedimentation was increased by mixing the blood 5:1 with saline containing 5% Dextran T500 (Pharmacia Biotech, Uppsala) and glucose (30 mg/ml; BDH Laboratory Supplies, Poole). After sedimentation for 30 min at room temperature the plasma fraction was layered on Ficoll Paque (Pharmacia Biotech, Uppsala) and mononuclear cells were isolated.

The mononuclear cells were washed and resuspended at a concentration of 1×10^6 cells/ml in sterile RPMI 1640 with HEPES buffer (25 mM) and L-glutamine (Gibco, Paisley). Fetal calf serum (1%, Gibco, Paisley), benzylpenicillin (0.1 mg/ml; Astra AB, Södertälje) and streptomycin sulphate (0.1 mg/ml; Sigma, St Louis MO) were added. Aliquots of 1×10^6 cells/well were cultured in 24-well culture plates (Nunc, Roskilde) at 37°C, 5% CO_2, 98% rH in the presence and absence of interleukin-2 and interleukin-4 (50 or 500 units (U)/ml; R&D Systems Europe, Abingdon). The cytokines were reconstituted in sterile phosphate-buffered saline with the addition of bovine serum albumin (0.1%; Sigma, St Louis, MO). After 48 h, lipopolysaccharide (10 ng/ml; Difco Laboratories, Detroit) and various concentrations (10^{-11}–10^{-7} M) of budesonide (Astra Draco AB, Lund), cortivazol (Roussel Uclaf, Paris) or tipredane (Astra Draco AB, Lund) were added as aliquots of 10 μl. The corticosteroids were dissolved in ethanol and diluted in RPMI 1640. The effects of the substances on cytokine production were compared with the production in control cultures. After another 20 h of incubation the whole culture plates were centrifuged at $200 \times g$ for 10 min at 4°C. The culture supernatants were transferred to plastic tubes and centrifuged again. The cell-free supernatants were decanted to new plastic tubes and stored at −70°C until analysis.

2.2. Analysis

GM-CSF levels were assayed using ELISA-kits (lower detection limit 1.5 pg/ml; R&D Systems Europe, Abingdon). All values are expressed as means ± standard error of the mean (S.E.M.). GM-CSF (% of control) was calculated as % GM-CSF in a culture with corticosteroid addition compared with a similarly pretreated culture without corticosteroid. Thus, a 'value of 0% of control' means that GM-CSF was totally inhibited and a 'value of 100% of control' that GM-CSF was not affected at all by corticosteroids.

2.3. Statistics

The first part of the study was evaluated using an analysis of variance with experiment and treatment as

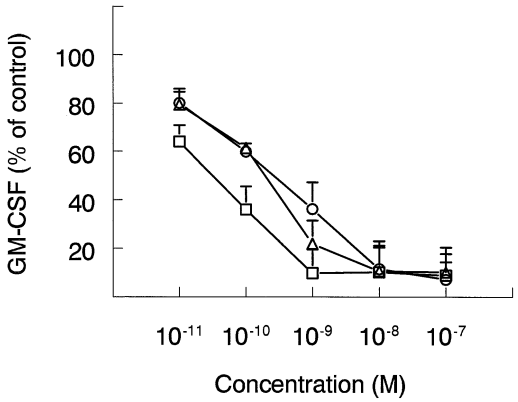

Fig. 1. Effects of budesonide (○), cortivazol (□) and tipredane (△) on GM-CSF production in lipopolysaccharide-stimulated (10 ng/ml) cultures of mononuclear blood cells. The cells were incubated in cell media alone for 48 h prior to addition of lipopolysaccharide and corticosteroids and then incubated for another 20 h. Each point represents the mean ± S.E.M. of five experiments. The IC_{50} for cortivazol was significantly lower than the IC_{50} of budesonide and tipredane.

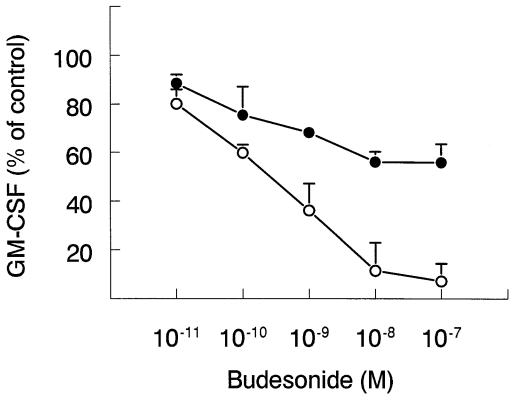

Fig. 2. Effects of budesonide on GM-CSF production in lipopolysaccharide-stimulated (10 ng/ml) cultures of mononuclear blood cells pretreated with (●) and without (○) interleukin-2 and interleukin-4 (500 U/ml). Lipopolysaccharide and corticosteroids were added after 48 h and the cultures were incubated for another 20 h. Each point represents the mean ± S.E.M. of five experiments. Pretreatment with interleukin-2 and interleukin-4 significantly decreased the inhibitory effect of budesonide.

factors, followed by pairwise comparisons. P values < 0.05 were considered significant. The second part of the study was analysed using a sigmoidal E_{max} model to describe the concentration-effect relationship. Each experiment was assumed to have its own set of parameters, although drawn from the same multinormal distribution. A numerical algorithm was used to estimate the mean parameters in the distribution (Davidian and Giltian, 1993). From the parameter estimates, the differences in maximal effect as well as relative potencies were estimated.

3. Results

3.1. Induction of steroid resistance in mononuclear blood cells

Initially, the effect of budesonide (10^{-8} M) on GM-CSF production was studied in lipopolysaccharide-stimulated (10 ng/ml) mononuclear blood cells, pretreated for 48 h with various concentrations of interleukin-2 and interleukin-4 (Table 1). Budesonide totally inhibited GM-CSF production of cells incubated with medium alone. Similarly, in the cultures pretreated with 50 U of interleukin-2 and interleukin-4, budesonide inhibited GM-CSF production totally in three of five cultures. In the remaining two cultures, GM-CSF production was reduced to 31.0% and 45.6% of control, respectively. Pretreatment with 500 U of interleukin-2 and interleukin-4 significantly decreased the inhibitory effect of budesonide compared with medium pretreatment ($P = 0.002$) and GM-CSF production was 79.3% of the control levels. Pretreatment with 500 U of interleukin-2 and interleukin-4 did not change the production of GM-CSF compared with medium alone ($P > 0.05$).

3.2. Corticosteroid effects on GM-CSF production in steroid-resistant and non-resistant mononuclear blood cells

The effects of various concentrations (10^{-11}–10^{-7} M) of budesonide, cortivazol or tipredane on GM-CSF produc-

Table 1
Effects of budesonide (10^{-8} M) on production of GM-CSF in cultures of mononuclear blood cells pretreated with and without various concentrations of interleukin-2 and interleukin-4

Pretreatment [a]	Budesonide [b] (M)	GM-CSF (pg/1 × 10^6 cells)	GM-CSF [c] (% of control)
Medium	0	26.2 ± 5.2	
Medium	10^{-8}	0	0 [e]
50 U interleukin-2 and -4	0	17.9 ± 6.4	
50 U interleukin-2 and -4	10^{-8}	5.0 ± 3.2	15.3 ± 9.7
500 U interleukin-2 and -4	0	32.6 ± 7.0 [d]	
500 U interleukin-2 and -4	10^{-8}	23.8 ± 4.4	79.3 ± 10.7

[a] Mononuclear blood cell cultures ($n = 5$) were pretreated for 48 h in RPMI-1640–1% FCS with and without addition of interleukin-2 and -4.
[b] Lipopolysaccharide (10 ng/ml) and budesonide were added after 48 h and the cells were incubated for another 20 h.
[c] GM-CSF (% of control) was calculated as % GM-CSF in a culture with budesonide compared with a similarily pretreated culture without budesonide.
[d] No significant difference in production of GM-CSF compared with pretreatment with medium only ($P > 0.05$).
[e] The inhibitory effect of budesonide differed significantly between pretreatment with medium and pretreatment with 500 U of interleukin-2 and -4 ($P = 0.002$) but not between pretreatment with medium and pretreatment with 50 U of interleukin-2 and -4 ($P = 0.34$).

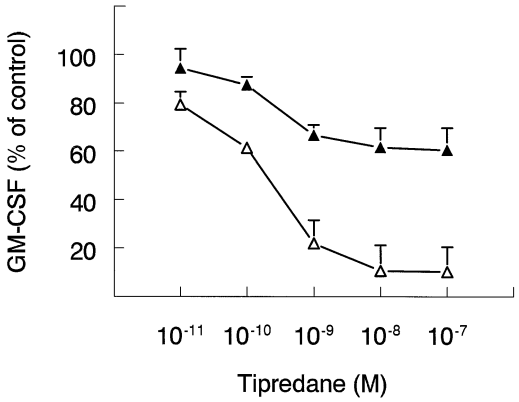

Fig. 3. Effects of tipredane on GM-CSF production in lipopolysaccharide-stimulated (10 ng/ml) cultures of mononuclear blood cells pretreated with (▲) and without (△) interleukin-2 and interleukin-4 (500 U/ml). Lipopolysaccharide and corticosteroids were added after 48 h and the cultures were incubated for another 20 h. Each point represents the mean ± S.E.M. of five experiments. Pretreatment with interleukin-2 and interleukin-4 significantly decreased the inhibitory effect of tipredane.

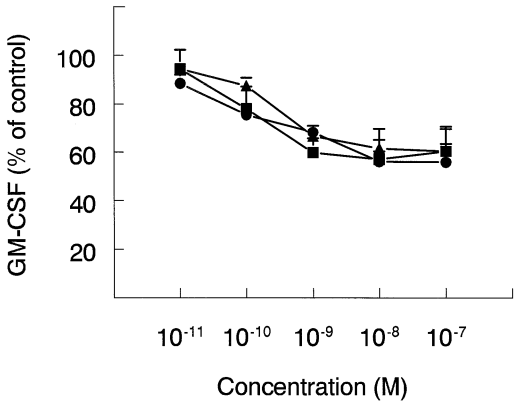

Fig. 5. Effects of budesonide (●), cortivazol (■) and tipredane (▲) on GM-CSF production in lipopolysaccharide-stimulated (10 ng/ml) cultures of mononuclear blood cells pretreated with interleukin-2 and interleukin-4 (500 U/ml) for 48 h. Lipopolysaccharide and corticosteroids were added after 48 h and the cultures were incubated for another 20 h. Each point represents the mean ± S.E.M. of five experiments. Pretreatment with interleukin-2 and interleukin-4 significantly decreased the inhibitory effects of all three corticosteroids. There were no significant differences in the inhibitory effects of the three corticosteroids.

tion were compared in lipopolysaccharide-stimulated (10 ng/ml) cultures of mononuclear blood cells pretreated for 48 h in the presence or absence of 500 U of interleukin-2 and interleukin-4. Cortivazol was significantly more potent than budesonide and tipredane in cells not pretreated with interleukin-2 and interleukin-4. The IC_{50} for cortivazol (3×10^{-11} M) was approximately eight times lower than the IC_{50} of budesonide (2.5×10^{-10} M) and tipredane (2×10^{-10} M). The IC_{50} of budesonide and tipredane did not differ significantly from each other (Fig. 1). Pretreatment with interleukin-2 and interleukin-4 resulted in a significantly decreased maximal effect of budesonide (Fig. 2), tipredane (Fig. 3) and cortivazol (Fig. 4) compared with the effect of medium pretreatment. The maximal effect of the corticosteroids on GM-CSF production was significantly decreased by pretreatment with interleukin-2 and interleukin-4 and there were no differences in inhibitory effects between the different corticosteroids studied (Fig. 5).

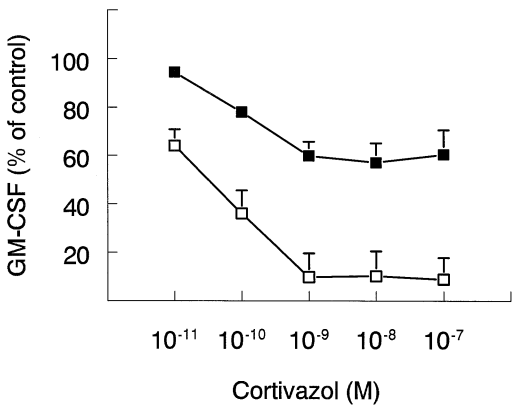

Fig. 4. Effects of cortivazol on GM-CSF production in lipopolysaccharide-stimulated (10 ng/ml) cultures of mononuclear blood cells pretreated with (■) and without (□) interleukin-2 and interleukin-4 (500 U/ml). Lipopolysaccharide and corticosteroids were added after 48 h and the cultures were incubated for another 20 h. Each point represents the mean ± S.E.M. of five experiments. Pretreatment with interleukin-2 and interleukin-4 significantly decreased the inhibitory effect of cortivazol.

4. Discussion

In the present study, we found that in vitro pretreatment of normal mononuclear blood cells with interleukin-2 and interleukin-4 counteracted the inhibitory effects of corticosteroids on GM-CSF production. We found that this induced steroid resistance was dependent on the doses of interleukin-2 and interleukin-4, in agreement with a recent study of corticosteroid receptor affinity (Raub et al., 1995). The failure of corticosteroids to efficiently suppress the in vitro production of GM-CSF in induced steroid resistance, demonstrated in the present study, was in contrast to the ex vivo findings of Lane et al. (1993). In that study however, GM-CSF production was studied in cultures of separated blood monocytes obtained from steroid-sensitive and steroid-resistant asthmatics. Concomitant corticosteroid effects on mononuclear blood cells (i.e. lymphocytes and monocytes) of these patients were not studied. It is possible that excluding lymphocytes, which are major producers of interleukin-2 and interleukin-4, influenced the outcome of these experiments. Moreover, since GM-CSF production was studied in 24-h cultures there is a risk that in vivo induced abnormalities in corticosteroid responsiveness might have normalised during the in vitro incubation. Indeed, Kam et al. (1993), as well as Sher et al. (1994), have demonstrated that corticosteroid receptor abnormalities start normalising within 24 h when mononuclear blood

cells of steroid-resistant asthmatics are incubated in vitro without interleukin-2 and interleukin-4 addition.

We selected three structurally different corticosteroids for the study. Budesonide and tipredane were equally potent to inhibit GM-CSF production in non-resistant mononuclear blood cells, while cortivazol was more potent. In the cells pretreated with interleukin-2 and interleukin-4, i.e. steroid-resistant, the inhibitory effects of all three corticosteroids were significantly less compared with those in cells not pretreated with interleukin-2 and interleukin-4. In contrast with a previous report by Ashraf et al. (1991) demonstrating that cortivazol had an effect even on a highly dexamethasone-resistant clone of a human cell line, we found that cortivazol was significantly less efficient to inhibit cytokine production in steroid-resistant mononuclear blood cells compared with non-resistant mononuclear blood cells. This suggests that different mechanisms underlie the steroid resistance of the leukemic cell line and the changed steroid responsiveness that we induced in mononuclear blood cells by cytokine pretreatment. Furthermore, Corrigan et al. (1994) reported that there were no differences in the inhibitory effects of tipredane on proliferation of T cells of steroid-resistant and steroid-sensitive asthmatics, despite significant differences when dexamethasone was tested. We found, however, that the inhibitory effect of tipredane on GM-CSF production was decreased in steroid-resistant mononuclear blood cells compared with non-resistant mononuclear blood cells. All three structurally different corticosteroids had very little effect on GM-CSF production of steroid-resistant mononuclear blood cells, i.e. cells pretreated with interleukin-2 and interleukin-4. Thus, it seems that interleukin-2 and interleukin-4 pretreatment can induce changes in functional cell responses to corticosteroids, and that the induced steroid resistance is likely to apply to all corticosteroids. The same conclusion regarding a general class effect in steroid resistance was drawn from two recent studies comparing the anti-proliferative effects of different corticosteroids in mononuclear blood cells obtained from steroid-resistant asthmatics (Corrigan et al., 1996; Spahn et al., 1996a).

The decision to use mononuclear blood cells, i.e. monocytes and lymphocytes together, for functional assay of steroid resistance in the present study was based on a study by Chikanza and Panayi (1993). If highly purified T- cells of rheumatoid arthritis patients were used, corticosteroids did not inhibit the proliferation of T cells from steroid-sensitive or steroid-resistant patients. Thus, T cells of steroid-sensitive patients became resistant to corticosteroids when accessory cells were removed. Therefore, it was suggested that corticosteroids could inhibit T cell proliferation indirectly, via an effect on accessory cell function. It was further proposed that steroid sensitivity and resistance are related to accessory cells rather than to T cells themselves, and are consequences of cytokine production. In fact, reduced corticosteroid inhibition of interleukin-2 and interleukin-4 production of mononuclear blood cells in vitro correlated with steroid resistance in the patients. Leung et al. (1995) have also suggested that increased production of cytokines, e.g. a combination of interleukin-2 and interleukin-4, is involved in steroid resistance. This is strongly supported by the results of Kam et al. (1993) and Sher et al. (1994) showing that normal mononuclear blood cells incubated with interleukin-2 and interleukin-4 develop corticosteroid receptor abnormalities similar to those observed in steroid-resistant asthmatics and that the anti-proliferative effects of corticosteroids are reduced. Furthermore, a recent study by Klemm et al. (1996) showed that mononuclear blood cells of steroid resistant-asthmatics had reduced corticosteroid receptor–DNA binding. Similar results were found after interleukin-2 and interleukin-4 treatment of normal cells.

Although the mechanisms of interleukin-2 and interleukin-4 induction of steroid resistance are unknown, the combination of the two cytokines seems to be important. Interleukin-2 and interleukin-4 alone did not change corticosteroid receptor affinity or reduce corticosteroid anti-proliferative effects in normal mononuclear blood cells (Kam et al., 1993; Sher et al., 1994). Also, Almawi et al. (1989) showed that exogenous interleukin-2 or interleukin-4 alone failed to reduce corticosteroid anti-proliferative effects. Among other pro-inflammatory cytokines, a combination of interleukin-6 + interleukin-1β + interferon-γ was studied. This cytokine cocktail had an abrogating effect on the anti-proliferative corticosteroid effects on mononuclear blood cells (Almawi et al., 1989). However, corticosteroid receptor affinity was not studied, and interleukin-6 and interleukin-1β can stimulate T cell proliferation (Holsti and Raulet, 1989). Therefore, it cannot be excluded that the effect of interleukin-6 + interleukin-1β + interferon-γ might be a stimulation of proliferation instead of a direct abrogation of corticosteroid effects. In addition, other studies on corticosteroid receptor affinity found no reduction by interleukin-6, interleukin-1β, tumour necrosis factor-α or combinations of these cytokines (Rakasz et al., 1993; Sher et al., 1994; Spahn et al., 1996b). Interleukin-13, in contrast, induced corticosteroid receptor abnormalities and reduced corticosteroid inhibition of interleukin-6 production in monocytes (Spahn et al., 1996b). This suggests that several cytokines might be involved in steroid resistance and that both monocytes and lymphocytes are affected. Another potential mechanism of steroid resistance suggested by Adcock et al. (1995b) was that steroid-resistant asthmatics have increased levels of transcription factor AP-1. Therefore, interactions between transcription factors and corticosteroid receptors rather than defective corticosteroid receptor affinity were proposed to cause steroid resistance. Effects of the combination of interleukin-2 and interleukin-4 on transcription factors remain to be studied.

The present finding of reduced corticosteroid sensitivity of GM-CSF production in in vitro induced steroid resistance in mononuclear blood cells, is of interest since

GM-CSF is a cytokine important in the pathophysiology of asthma (Lopez et al., 1986; Djukanovic et al., 1990; Woolley et al., 1994). Asthmatics have increased GM-CSF levels, which correlate with increased eosinophil numbers, both in the bronchial mucosa and in peripheral blood (Djukanovic et al., 1990; Nakamura et al., 1993; Woolley et al., 1994), and also have increased GM-CSF production by the mononuclear blood cells (Nakamura et al., 1993). In addition, the GM-CSF production of monocytes and alveolar macrophages is highly sensitive to corticosteroids (Linden and Brattsand, 1994). Therefore, based on the present results, we hypothesise that the inability of corticosteroid treatment to e.g. reduce eosinophil numbers in the airways of steroid-resistant asthmatics (Leung et al., 1995), might result from reduced inhibition of GM-CSF production.

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