ORIGINAL ARTICLE

Natural allergen exposure does not diminish the sensitivity of cytokine production to glucocorticosteroids in blood cells of seasonal allergic asthma and rhinitis patients

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Abstract   Glucocorticosteroid (GCS) inhibition of cytokine production is a major anti-inflammatory mechanism. However, increased production of pro-inflammatory cytokines during allergic airway inflammation has been proposed to reduce GCS effects. This study aimed to investigate whether allergic airway inflammation due to natural allergen exposure might decrease the sensitivity of granulocyte–macrophage colony-stimulating factor (GM-CSF) production to GCS in blood cells. Blood samples were collected from patients with seasonal allergic asthma ($n = 10$) and rhinitis ($n = 8$) and healthy subjects ($n = 9$), before, during, and after the birch pollen season. Whole blood cultures were stimulated with LPS (10 ng/ml) and treated with budesonide (10^{-11}–10^{-7} M) for 20 h. GM-CSF levels were analysed using immunoassay. Birch pollen exposure did not alter LPS-stimulated GM-CSF production, although disease symptoms and blood eosinophils increased in the patients. There were no significant differences in budesonide inhibition of GM-CSF production by blood cells of asthma and rhinitis patients compared with cells of healthy subjects before, during or after the birch pollen season and no change in response to allergen exposure. A concentration of 1 nM budesonide inhibited GM-CSF production by more than 50% at all time points. In conclusion, natural allergen exposure did not reduce the sensitivity of GM-CSF production to GCS inhibition in blood cells of seasonal allergic asthma and rhinitis patients. © 2002 Elsevier Science Ltd.

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INTRODUCTION

Increased production of pro-inflammatory cytokines, such as interleukin-2 (IL-2), IL-4 and IL-13 during airway inflammation has been proposed to reduce the effects of glucocorticosteroids (GCS) and induce the GCS resis-

tance observed in certain asthma patients (1,2). Pre-treatment of mononuclear blood cells from healthy subjects with IL-2 and IL-4 *in vitro* reduces the sensitivity of granulocyte–macrophage colony-stimulating factor (GM-CSF) production to GCS inhibition (3,4), and other pro-inflammatory cytokines e.g. IL-13 or tumour necrosis factor-alpha (TNF-α) can also reduce GCS effects (2,5). The capacity for production of these pro-inflammatory cytokines may be elevated by mononuclear blood cells of patients with mild asthma and rhinitis, especially after allergen exposure (6,7). These findings indicate that endogenous inflammatory mediators may

modulate the sensitivity of cytokine production to GCS inhibition.

GCS inhibition of cytokine production is an anti-inflammatory mechanism, which might be of great importance in the treatment of asthma and allergic rhinitis (8, 9). Reduced GCS inhibition of pro-inflammatory and eosinophilopoietic cytokines, such as GM-CSF, might therefore have negative consequences for the outcome of GCS therapy. Whether the sensitivity of cytokine production to GCS may be reduced during pollen exposure due to an allergen-induced increase in pro-inflammatory inflammatory mediators is not known. In the present study, we therefore compared the sensitivity of GM-CSF production to GCS inhibition in blood cells of patients with seasonal allergic asthma and rhinitis before, during, and after natural allergen exposure, in order to examine whether increased production of endogenous inflammatory mediators may decrease the sensitivity of cytokine production to GCS. GM-CSF was selected since it is an eosinophilopoietic cytokine (10,11), the LPS-stimulated production of this cytokine is very sensitive to GCS (12,13) and its sensitivity to GCS inhibition changes in response to the pro-inflammatory cytokines IL-2 and IL-4 (3,4).

MATERIALS AND METHODS

Study design

Three parallel groups of asthmatic, rhinitic, and healthy men were studied during 1 year. Patients and healthy controls were recruited from Lund University Hospital and by advertisement in a local newspaper. Blood samples were collected before (March), at the beginning (late April) and 2 weeks into (mid-May) the birch pollen season, at the end of the grass pollen season (August), in the autumn (October) and winter (December) of 1998, in Lund, Sweden. Variations of *in vitro* GM-CSF production and GCS (budesonide) effects on GM-CSF production were studied in whole blood cultures. In addition, asthma and rhinitis symptoms, blood cell numbers and differentials were recorded at all visits. The study was approved by the University Hospital's ethics committee and all patients and controls gave their written consent.

Study subjects

Ten male patients (age 26–45 years; median 36.5) with a history of allergic asthma according to the ATS-definition, and eight male patients (age 22–44 years; median 28) with a history of allergic rhinitis were included. They all had a history of birch pollen allergy for more than two seasons and a positive skin-prick test to birch pollen. The asthma patients had FEV_1 of 83–100 % of predicted (median 92). All patients were skin-prick tested against birch, timothy, mugworth, dog, cat, *D. Pteronyss* and *D. Farinae*

(ALK, Denmark). Four rhinitis and four asthma patients were skin-prick positive to grass pollen (timothy). Patients were excluded if they had a history of allergy to house dust mite or furred animals, received hypo-sensibilisation therapy, or any medication other than short-acting β_2-agonists. None of the patients had used glucocorticosteroids, oral or inhaled, or anti-allergy medication within at least 2 months prior to the first blood sampling. Most of the asthma patients also had a history of birch pollen allergic rhinitis, and are therefore termed asthma–rhinitis. None of the rhinitis patients had a history of asthma. Nine healthy men (age 21–38 years; median 27) without any known allergy, asthma, rhinitis or eczema were included as controls. None of the enrolled subjects were smokers. Subjects planning travels outside the Northern hemisphere were not included. Women were excluded due to indications that cytokine production may vary during the menstrual cycle (14,15). During the pollen season, loratadin tablets (Clarityn®, 10 mg/day; Schering-Plough) and levocabastin eye drops (Livostin®, 0.5 mg/ml, one drop/eye, two to four times daily; Janssen-Cilag) were allowed as rescue medication for severe rhinitis symptoms. Asthma patients were also allowed to use short-acting, inhaled β_2-agonists when needed.

Upper and lower airway symptom scores

At each visit all subjects, patients and healthy controls, were asked to grade their rhinitis symptoms (sneezing; congestion; runny nose) during the last week as no symptoms (0), mild (1), moderate (2) or severe (3). A total upper airway symptom score with a maximum of 9 was calculated. All subjects were also asked to grade their asthma symptoms (shortness of breath or chest tightness; wheeze; cough; shortness of breath when walking or exercising; waking up at night due to shortness of breath) on a similar scale. A total lower airway symptom score with a maximum of 15 was calculated. Medication use, colds and other illnesses were also recorded.

Blood sampling

Venous blood was collected into sterile, heparin-containing Venoject® tubes (sodium heparin, 15 IU/ml) at six visits during 1 year (March, April, May, August, October and December, 1998). Blood was collected between 8 and 10 a.m., always at the same time for each subject, and from all subjects within 1 week in each sampling period. Patients and controls were asked not to drink alcohol later than 24 h and not to use acetylsalicylic acid or paracetamol later than 48 h before blood sampling. On each sampling day, blood samples were collected from subjects representing all three study groups to get simi-

lar day-to-day variation in pollen exposure in the different groups. The samples at visit 3 were taken 2 weeks after the samples at visit 2.

Whole blood cultures

The blood samples were diluted 1:5 in sterile RPMI 1640 with HEPES buffer (25 mM) and L-glutamine and supplemented with penicillin (100 U/ml) and streptomycin (0.1 mg/ml) (Life Technology, Rockville, U.S.A.). Aliquots of 1 ml were then incubated in 24-well culture plates with or without lipopolysaccharide (LPS; 10 ng/ml; *E. coli* 0.26:B6, Difco, Detroit, U.S.A.) and budesonide (10^{-11}–10^{-7}M; AstraZeneca R&D, Lund, Sweden), at 5% CO_2, 37°C, 98% RH. After 20 h, the cell culture plates were centrifuged for 10 min, at 1500 rpm (500 g), at 4°C. The cell-free supernatants were collected and stored at −70°C until analysis. Proceeding the present study, LPS dose–response and time kinetics were studied for several cytokines, including GM-CSF, and compared with cultures of separated mononuclear blood cells. Based on these experiments, the optimal dose of LPS (10 ng/ml) and incubation time (20 h) were selected for the present study.

GM-CSF analysis

To analyse GM-CSF levels in the whole blood supernatants an ELISA was developed. Briefly, 96-well micro plates were coated over night with a monoclonal antibody (1 μg/ml; M-500A-E; Endogen, Woburn, U.S.A.) in a carbonate buffer of pH 9.7. The coating antibody was removed and the plates were blocked with 4% BSA in PBS for at least 1 h. After three washes with TRIS +0.2% Tween-20, samples or recombinant standard (7.8–500 pg/ml; Endogen, Woburn, U.S.A.) were added together with biotinylated detection antibody (0.25 μg/ml in PBS with 4% BSA; M-501-B; Endogen, Woburn, U.S.A.). The plates were incubated for 2 h and then washed. Streptavidin-conjugated horseradish peroxidase and tetramethylbenzidine-peroxidase were used for colour development. Absorbance was measured at 450 nm with wavelength correction at 540 nm. Sample concentrations were calculated from the standard curve using computer software with a four-parameter logistic curve fit. Inter-assay variation of internal controls was 11% CV. Heparin did not interfere with the assay. The detection level of the assay was 7.8 pg/ml. All samples were analysed using antibodies from the same batches.

Calculation of GM-CSF levels

The whole blood cultures were obtained by diluting the blood five times in cell culture medium. Therefore, the cultures contain one-fifth of endogenous blood plasma

and need to be assayed for baseline plasma levels of cytokines to obtain true values of stimulated cytokine production. In the present study GM-CSF was detected in unstimulated cultures of blood of nine of the 27 subjects (two healthy, four rhinitis, and three asthma subjects). All these subjects also had detectable levels of GM-CSF in their plasma at all six visits that did not vary between visits [16]. The GM-CSF levels in the unstimulated blood cultures were equal to one-fifth of the plasma GM-CSF levels of the same subjects, and are thus likely to have originated from plasma and do not represent the unstimulated production of GM-CSF. These GM-CSF levels were considered as baseline levels derived from plasma and subtracted from LPS-stimulated GM-CSF levels in control cultures before the effect of budesonide was calculated. GM-CSF levels are expressed as mean and standard error of the mean (SEM).

Statistical analysis

Eosinophil numbers, and GM-CSF production were compared using mixed linear models with visit and group as fixed effects and patient (group) as random effect (repeated measure ANOVA; SAS computer software, version 6.12). Pair-wise comparisons were performed using *t*-type tests (SAS Proc Mixed). *P*-values < 0.05 were considered significant. The effect of budesonide on LPS-stimulated GM-CSF production was calculated as per cent of control, i.e. LPS-stimulated GM-CSF production in a culture without budesonide. Data are presented as mean and standard error of the mean (SEM). The budesonide doses at which maximum inhibition of GM-CSF production was achieved were compared between study groups and sampling periods using Kruskal–Wallis test. *P*-values < 0.05 were considered significant.

RESULTS

Pollen counts

According to local pollen reports, birch pollen counts were moderate or high (> 100 birch pollen grains/m³ air) during the week before visit 2 (April), peaked at very high levels (> 1000 birch pollen grains/m³ air) during visit 2 and declined after visit 3 (mid May) (data not shown). Grass pollen counts peaked in late June (> 100 grass pollen grains/m³ air), but were present from late May until August (visit 4).

Allergy symptoms and eosinophil numbers

Upper airway symptoms increased significantly at visits 2 and 3 in the rhinitis patients ($P < 0.001$) and at visit 3 in the asthma-rhinitis patients ($P < 0.001$) compared with those at visit 1. Lower airway symptoms increased significantly at visit 3 in both asthma-rhinitis ($P < 0.001$) and
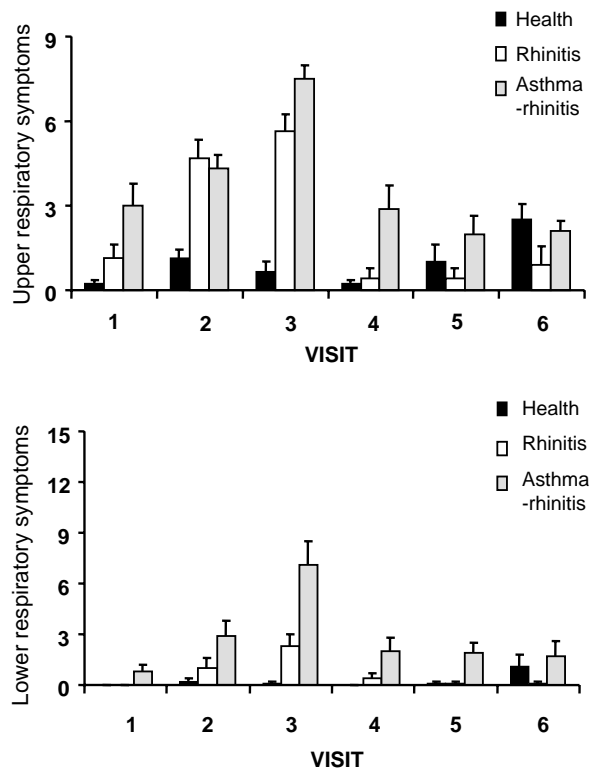
Fig 1. Upper and lower airway symptom scores in birch pollen allergic rhinitics, asthmatic–rhinitic and healthy controls before (visit 1: March), during (visit 2: April; visit 3: May) and after (visit 3–6: August, October, December) the birch pollen season in Lund, Sweden, 1998. The total maximum scores were 9 for upper respiratory symptoms and 15 for lower respiratory symptoms.

rhinitis patients ($P < 0.001$) compared with these at visit 1 (Fig. 1). FEV_1 in the asthma-rhinitis patients did not change significantly between visits ($P < 0.078$) (data not shown). Peripheral eosinophil numbers increased significantly at visits 2 and 3 in asthma−rhinitis patients ($P=0.029$; $P < 0.001$), and at visit 3 in rhinitis patients ($P=0.006$) compared with those at visit 1. The asthma−rhinitis patients had significantly higher eosinophil numbers than the rhinitis patients ($P=0.029$) (Table 1). Monocyte, lymphocyte, neutrophil and total leukocyte numbers did not differ significantly between groups or visits (data not shown).

Budesonide effects on GM-CSF production

There were no significant differences in LPS-stimulated GM-CSF production between visits within any group, i.e. LPS-stimulated GM-CSF production during natural allergen exposure did not differ significantly from GM-CSF production during allergen-free periods.

In addition, there were no significant differences in LPS-stimulated GM-CSF production between the asthma−rhinitis (19.3 (1.8) pg/ml) and the rhinitis (16.2 (1.3) pg/ml) groups compared with the control group (21.6 (2.0) pg/ml; $P=0.59$, $P=0.99$). LPS-stimulated GM-CSF production was below the detection limit of the assay in cultures of blood obtained from three asthma−rhinitis patients at all visits. In blood cell cultures from two asthma−rhinitis patients, one rhinitis patient, and four healthy subjects, GM-CSF production was below the detection limit of the assay at one or two visits. Budesonide inhibition of LPS-stimulated GM-CSF production was therefore compared with blood culture samples from five asthma−rhinitis patients, seven rhinitis patients and five healthy subjects from all visits. Budesonide caused a concentration-dependent inhibition of GM-CSF production by cells from all these patients and healthy subjects at all visits. The mean dose−response curves for the budesonide inhibitory effects in the asthma-rhinitis, the rhinitis, and the control groups overlapped at all visits (Figs 2(a−f)). The budesonide doses at which maximum inhibition of GM-CSF production was achieved did not differ between patients and healthy controls or between visits ($P > 0.05$). A concentration of 1 nM budesonide inhibited GM-CSF production by 50% or more in blood cells of patients as well as healthy subjects at all visits.

DISCUSSION

In the present study, we have demonstrated that natural allergen exposure does not diminish the sensitivity of GM-CSF production by blood cells from allergic asthma and rhinitis patients to GCS inhibition *in vitro*. These data are of interest with respect to the mechanisms of GCS action and the therapeutic use of these drugs.

Patients with birch pollen allergic asthma and rhinitis develop allergic airway inflammation during the re-

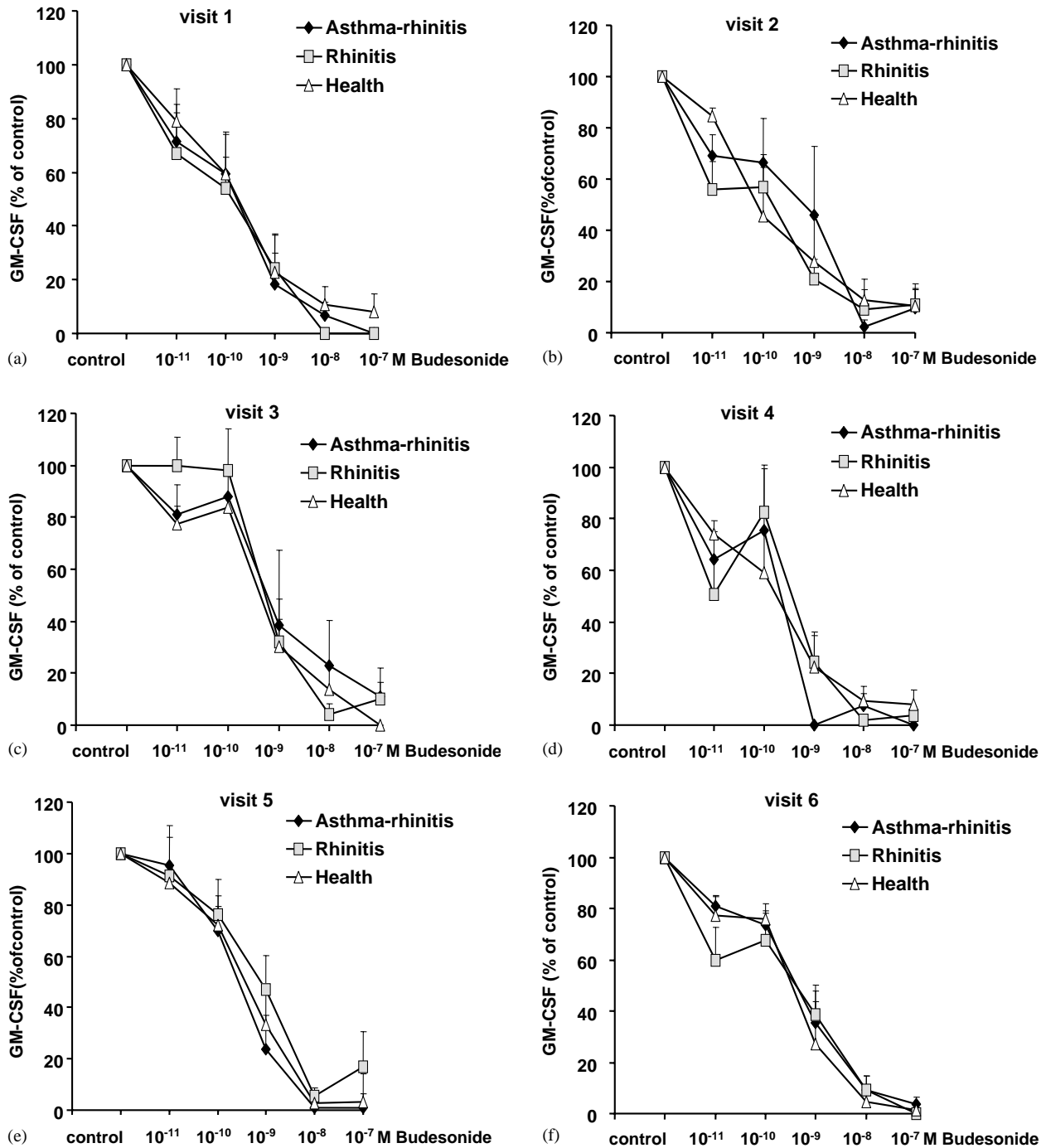|	Visit 1 (March)	Visit 2 (April)	Visit 3 (May)	Visit 4 (August)	Visit 5 (October)	Visit 6 (December)
Table 1 Circulating eosinophil numbers. Values are expressed as mean and (SEM)						
Eosinophils (10^9/L)						
Asthma−rhinitis	0.21 (0.03)	0.32 (0.03)	0.52 (0.07)	0.29 (0.04)	0.23 (0.03)	0.24 (0.03)
Rhinits	0.17 (0.03)	0.22 (0.03)	0.31 (0.05)	0.22 (0.05)	0.19 (0.03)	0.18 (0.03)
Health	0.29 (0.05)	0.24 (0.04)	0.22 (0.03)	0.24 (0.04)	0.19 (0.01)	0.21 (0.04)

FIG. 2.(a–f) GCS inhibition of GM-CSF production in cultures of whole blood from seasonal allergic asthma-rhinitis patients ($n = 5$), seasonal allergic rhinitis patients ($n = 7$) and healthy subjects ($n = 5$). Cultures of whole blood were obtained before a) (visit 1; March), during b and c) (visits 2–3; April and May) and after (d–f) (visits 4–6; August, October and December) the birch pollen season of 1998 in Lund, Sweden. The budesonide effect on LPS-stimulated GM-CSF production was calculated as per cent of control, i.e. LPS-stimulated GM-CSF production in a culture without budesonide. Data are presented as mean and standard error of the mean.

stricted time period when birch pollen is present. These patients are therefore especially useful in studies comparing allergic mechanisms during symptomatic and asymptomatic periods of allergic disease. The patients with asthma and rhinitis enrolled in the present study,

had increased airway symptoms and blood eosinophil numbers during the birch pollen season. The increase in these parameters indicated that allergic inflammation was indeed developed in response to natural allergen exposure.

GM-CSF is a cytokine that induces proliferation and differentiation of granulocytic and monocytic stem cells (10) and stimulates eosinophil activity and survival (11). GM-CSF may therefore contribute to the eosinophilic airway inflammation observed in asthma and allergic rhinitis patients. Another reason for studying GM-CSF production is that the production of this cytokine is very sensitive to GCS inhibition (12,13) and the GCS sensitivity changes in response to the pro-inflammatory cytokines IL-2 and IL-4 (3,4). IL-2 and IL-4 and other pro-inflammatory cytokines are produced at increased levels by blood cells from allergic individuals during the allergen season (6).

In the cultures of whole blood obtained from the asthma−rhinitis and rhinitis patients in the present study, LPS-stimulated GM-CSF production did not differ from GM-CSF production in cultures of whole blood from healthy subjects and was not altered due to birch pollen exposure. Although cells in culture may exhibit functions not fully concordant with those *in vivo* (17), we demonstrated that GM-CSF levels in plasma of these patients did not change either (16). Thus, neither *in vivo*, nor *in vitro* GM-CSF production was altered by natural allergen exposure in the current study.

Isolated mononuclear blood cells are commonly used in studies of cytokine production. In the present study, however, whole blood cultures were selected instead of cultures of separated mononuclear blood cells for several reasons. Whole blood cultures are much quicker to set up than cultures of isolated blood cells. This diminishes the time from blood sampling to experiment and whole blood cultures give very reproducible results when cytokine production is studied (18, 19). In addition, blood serum with endogenous proteins, such as inflammatory mediators and cytokines, is maintained in the cell cultures, and all blood cell types are present at physiological ratios. However, levels of cytokines already present in the blood samples may influence the baseline cytokine levels in the whole blood cultures and need to be carefully monitored and corrected for if *in vitro* cytokine production is studied. Hence, in the present study plasma GM-CSF levels were analysed and corrected for, as described in methods. Since cell composition may vary between various sampling periods, white blood cell numbers and differentials need to be monitored as well. In the current study, no other blood leukocytes (monocytes, lymphocytes or neutrophils) than eosinophils differed significantly between study groups and sampling periods. What also might be a disadvantage with whole blood cultures is that cell concentrations in the cell cultures are lower compared with cultures of isolated cells and thereby give lower concentrations of produced cytokines that in some cases might be difficult to analyse. Still, whole blood cultures are more similar to *in vivo* conditions than cultures of isolated cells.

No reductions in GCS inhibition on GM-CSF production by blood cells of allergic subjects were demonstrated in the present study, despite increased airway symptoms and blood eosinophilia. Both during natural allergen exposure and during allergen-free periods a concentration of 1 nM budesonide inhibited GM-CSF production by at least 50% and the maximal inhibitory effects of budesonide on GM-CSF production were reached at 10 nM. Although examined in small groups of patients, the high reproducibilty of the dose−response effects of budesonide on GM-CSF production at the different visits and in the different study groups strengthens the results. Thus, the sensitivity of GM-CSF production to GCS in blood cells from patients with seasonal allergic asthma and rhinitis seems not to diminish due to increased production of inflammatory mediators and cytokines during allergen exposure, or differ between patients and healthy controls. These results suggest that it is possible that levels of inflammatory mediators high enough to decrease GCS effects are not reached *in vivo* and that other mechanisms are more important in causing GCS resistance. This has, however, to be more carefully investigated in GCS-resistant patients.

The GCS concentrations used were selected with reference to clinical studies. After inhalation of a clinically relevant dose of budesonide (500 μg), the blood concentration is approximately 1 nM for several hours (20), whereas the concentration in lung tissue is ten times higher (21). In the present study, 1 nM budesonide inhibited GM-CSF production by at least 50% in blood cells from asthmatic−rhinitic, rhinitic, and healthy subjects at all visits, and no differences were observed in the inhibitory effects of budesonide before, during, and after an intense pollen season. This suggests that inhalation therapy with GCS might diminish the production of GM-CSF by blood cells from asthma and rhinitis patients both during asymptomatic and symptomatic periods.

In conclusion, the sensitivity of the pro-inflammatory and eosinophilopoietic cytokine GM-CSF to GCS was not diminished by naturally induced allergic airway inflammation. This is in agreement with the knowledge that GCS therapy is very efficient in the majority of patients with allergic asthma and rhinitis.

Acknowledgements

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