

Effect of inhaled beclomethasone dipropionate on expression of proinflammatory cytokines and activated eosinophils in the bronchial epithelium of patients with mild asthma

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Increasing evidence suggests that cytokines play a role in airway inflammation by attracting and activating inflammatory cells. This may lead to epithelial cell damage and airway hyperresponsiveness. Bronchial provocative concentration of histamine causing a 20% fall in forced expiratory volume in 1 second was measured in patients with mild asthma, and bronchial biopsy specimens were stained for granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-8, and activated eosinophils (EG2) in the bronchial epithelium. The effect of inhaled beclomethasone dipropionate was also assessed in a placebo-controlled double-blind manner. There was a correlation between GM-CSF expression and EG2-staining cells ($r = 0.484$, $p < 0.05$) in the epithelium. Provocative concentration of histamine causing a 20% fall in forced expiratory volume in 1 second was correlated with GM-CSF expression ($r = -0.462$, $p < 0.05$). Treatment with inhaled beclomethasone dipropionate 500 μ g twice a day led to a significant decrease in both the expression of GM-CSF ($p < 0.01$) and IL-8 ($p < 0.02$) and the number of EG2-staining cells ($p < 0.01$) in the epithelium. The changes in GM-CSF ($r = 0.798$, $p < 0.01$) and IL-8 ($r = 0.653$, $p < 0.02$) expression were correlated with the changes in EG2-staining cells after treatment. These results suggest that GM-CSF may influence eosinophil activation in the epithelium in vivo and participate in the etiology of bronchial hyperresponsiveness in mild asthma. Also, beclomethasone dipropionate may inhibit eosinophil activation partly by downregulating the expression of GM-CSF and IL-8 in the bronchial epithelium. (J ALLERGY CLIN IMMUNOL 1994;94:1025-34.)

Key words: Bronchial epithelium, GM-CSF, IL-8, eosinophil, beclomethasone dipropionate, mild asthma

In fatal asthma there is severe damage to the epithelium, but this has not been consistently demonstrated in mild asthma.¹⁻⁷ Activated eosinophil infiltration of the epithelium and submucosa is well recognized in the bronchi of patients with asthma.^{1-5, 8-10} Eosinophils may be responsible for

Abbreviations used

BDP:	Beclomethasone dipropionate
CI:	Confidence interval
FEV ₁ :	Forced expiratory volume in 1 second
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
IL:	Interleukin
PC ₂₀ :	Provocative concentration causing a 20% fall in FEV ₁

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epithelial damage in asthma, and their presence is associated with opening of tight junctions and intercellular spaces.⁴ Several eosinophil granule-associated proteins, such as major basic protein, eosinophil cationic protein, and eosinophil peroxidase, which are released on activation of eosino-

phils, are capable of damaging airway epithelium¹¹⁻¹⁶ and are thought to contribute to airway hyperresponsiveness.^{4, 5, 11, 16} The specific mechanisms underlying the recruitment and activation of eosinophils in the epithelium, however, are not well understood. Studies with asthmatic bronchial epithelial cells in culture have demonstrated that these cells are capable of synthesizing different proinflammatory cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-8, and IL-6.¹⁷ More recently, Sousa et al.¹⁸ have shown upregulation of GM-CSF in the bronchial epithelium of patients with asthma *in vivo*.

In view of these findings, we have hypothesized that synthesis of proinflammatory cytokines by airway epithelial cells may contribute to eosinophilic bronchial inflammation in asthma and possibly lead to increased bronchial responsiveness. In this study we have investigated expression of GM-CSF and IL-8 and activation of eosinophils in the bronchial epithelium of patients with mild asthma and assessed their associations with bronchial responsiveness. We have also assessed the effects of 4 months of treatment with inhaled beclomethasone dipropionate (BDP) 500 μ g twice a day on GM-CSF, IL-8, and activated eosinophils in the bronchial epithelium in a placebo-controlled double-blind manner.

METHODS

Patients

Twenty-five nonsmoking volunteers with mild asthma (age range, 18 to 45 years) were recruited into this double-blind placebo-controlled study. All patients had forced expiratory volume in 1 second (FEV₁) greater than 60% of predicted normal value and provocative concentration causing a 20% fall in FEV₁ (PC₂₀) less than 2 mg/ml histamine (equivalent to 11 μ mol cumulative dose) when the tidal breathing method was used.¹⁹ None received corticosteroids, sodium cromoglycate, or nedocromil sodium in the preceding 6 months. Only inhaled salbutamol was used as required as a bronchodilator in the preceding 6 weeks. Patients were randomized to receive either BDP 500 μ g twice a day or placebo for 4 months. The study was approved by the ethics committee of St. Bartholomew's Hospital, and signed informed consent was obtained from each patient.

Study design

Patients were randomized to two parallel treatment groups. Each patient received a 16-week course of either BDP 500 μ g twice a day through a metered-dose inhaler or matched placebo. Patients were provided

with a salbutamol metered-dose inhaler for use as required. All patients underwent lung function tests and histamine bronchial provocation tests at the time of recruitment. Bronchoscopy and bronchial biopsy were performed 10 days later, immediately before commencing treatment. All tests were repeated at the end of the treatment period; the laboratory tests preceded bronchoscopy by 10 days, as before.

Measurement of provocation concentration

Subjective rating of asthma symptoms was recorded at each visit. Bronchial responsiveness to histamine was measured by using the tidal breathing method described by Bennett and Davies.¹⁹ The PC₂₀ was calculated by linear interpolation of the last 2 points on the logarithmic dose-response curve.

Biopsy, fixation, and staining of bronchial mucosa

Biopsy specimens were obtained from segmental carinae on the right side by fiberoptic bronchoscopy, while patients were under local anaesthesia with topical lidocaine; specimens were immediately placed in either Carnoy's fixative (absolute alcohol, 60 ml; chloroform, 30 ml; glacial acetic acid, 10 ml) or 10% buffered formaldehyde for 3 hours. All specimens were then processed in a Shandon Citadel 1000 automatic tissue processor (Shandon Southern Products Ltd., Runcorn, England) and finally embedded in paraffin. The tissue was cut to 3 μ m thickness, and sections were stained for the presence of GM-CSF, IL-8, or activated eosinophils.

Staining for GM-CSF, IL-8, and activated eosinophils

In the first instance, biopsy sections were stained on 1 day for EG2-staining cells in batches, which included both pretreated and posttreated samples from each patient. All samples were subsequently stained for either GM-CSF or IL-8 on the same day.

Activated eosinophils were determined by staining in formalin-fixed tissue. Sections were treated with 0.5% hydrogen peroxide in methanol for 15 minutes to block endogenous peroxidase activity and then for a further 15 minutes with 1% normal horse serum to block nonspecific binding of EG2 monoclonal antibody. EG2 monoclonal antibody (Pharmacia, Milton Keynes, England), diluted 1:40 was then added, and all sections were incubated for 2.0 hours at room temperature, and then with biotinylated rabbit anti-mouse antibody E354 (DAKO Ltd., High Wycombe, England) for 30 minutes. The sections were then incubated further with streptavidin-peroxidase for 30 minutes and washed with phosphate-buffered saline. The sections were then incubated in the presence of freshly prepared diaminobenzidine-hydrogen peroxide substrate (Sigma Chemical Co., Poole, England) for up to 5 minutes to develop color and counterstained with Mayer's hematoxylin.

GM-CSF and IL-8 were determined in Carnoy's

TABLE 1. Subject characteristics

Characteristics	Treatment group	
	Placebo	BDP
No. of patients	12	13
Mean age (yr)	26.7	30.6
95% CI	22.3-31.0	24.9-36.3
Age range (yr)	18-24	18-45
Sex ratio (M:F)	4 : 8	3 : 10
Mean FEV ₁ (%)	84.8	85.2
95% CI	75.1-95.4	76.9-93.5
Range	63-106	62-106
Geometric mean PC ₂₀	0.11	0.12
95% CI	0.04-0.28	0.06-0.23
Range	0.015-1.5	0.015-0.52

fixed tissue. Sections treated with hydrogen peroxide and normal horse serum, as described above, were incubated with monoclonal mouse anti-human GM-CSF antibody (monoclonal antibody (ZM-213; NBS Biologicals, Hatfield, U.K.) diluted 1:75, for 2.5 hours at room temperature. After washing with phosphate-buffered saline, the sections were incubated for 45 minutes with peroxidase-conjugated rabbit anti-mouse antibody P260 (DAKO Ltd.) and visualized for color by incubation with diaminobenzidine–hydrogen peroxide solution and Mayer’s hematoxylin, as described above.

IL-8 was stained by a similar protocol, except that the tissues were incubated primarily for 2.5 hours in the presence of a 1:10 goat anti-human IL-8, polyclonal antibody BDA 26 (British Biotechnology Products Ltd., Abingdon, England). After incubation for 45 minutes with a secondary peroxidase-conjugated rabbit anti-goat antibody P160 (DAKO Ltd.), the sections were developed with freshly prepared diaminobenzidine–hydrogen peroxide and counterstained with Mayer’s hematoxylin, as described above.

Negative controls for GM-CSF/EG2 and IL-8 staining were prepared with sections incubated and stained in the presence of mouse IgG₁ MOPC21, and goat IgG I5256 (Sigma Chemical Co.), respectively, instead of the specific primary antibodies. Positive control sections were prepared by use of intestinal tissue affected by Crohn’s disease for EG2 staining and tonsil tissue for GM-CSF and IL-8 staining.

Quantitation of GM-CSF, IL-8, and activated eosinophils in the epithelium

All sections were observed and analyzed by an individual blinded to the treatment regimens. Expression of GM-CSF and IL-8 in the bronchial epithelium was quantified by means of a “FREELANCE” computerized color image analysis and processing system (Sight System, Hove, England), according to a modification of the technique described by Poston and Gall.²⁰ Briefly, each section was viewed through an Olympus BH2 micro-

scope (Olympus Optical Co. Ltd., Tokyo, Japan), at ×40 magnification, and the viewed image was transposed onto a high-intensity color monitor, via a JVC TK-1070E color video camera (JVC Professional Products Co., Elmwood Park, N.J.). The area of the epithelium on the color monitor was outlined by means of an “electronic mouse cursor,” and the color produced by peroxidase reaction within the epithelium was detected and measured as pixels of hue, saturation, and intensity. The epithelium staining in each section was assessed by viewing several high-magnification fields covering the entire section, and the results are expressed as the percentage of the total epithelial area staining for either GM-CSF or IL-8.

Sections stained for activated eosinophils were also observed in a blinded manner, and the total epithelial area in each section was measured as outlined above. The total numbers of EG2-staining cells in the epithelium were counted, and the results are expressed as the number of cells per square millimeter.

Statistical analysis

Results were tested for normality with a normal probability plot and the Shapiro-Wilk test. GM-CSF results (normally distributed) and EG2 results (normally distributed after log-transformation) were subjected to paired Student’s *t* test for within-group comparisons and to two-sample *t* test for between-group comparisons. IL-8 results (nonnormally distributed) were subjected to Wilcoxon test for within-group comparison and to Mann-Whitney U test for between-group comparison. Linear regression was used to assess correlations of GM-CSF, EG2-staining cells, PC₂₀, and correlation of changes of GM-CSF, IL-8, and EG2-staining cells after treatment.²¹ All statistical tests were performed with Minitab statistical computer software (Minitab Inc., State College, Pa.).

RESULTS

Twelve patients were treated with placebo and 13 with BDP during the study. All of the volun-

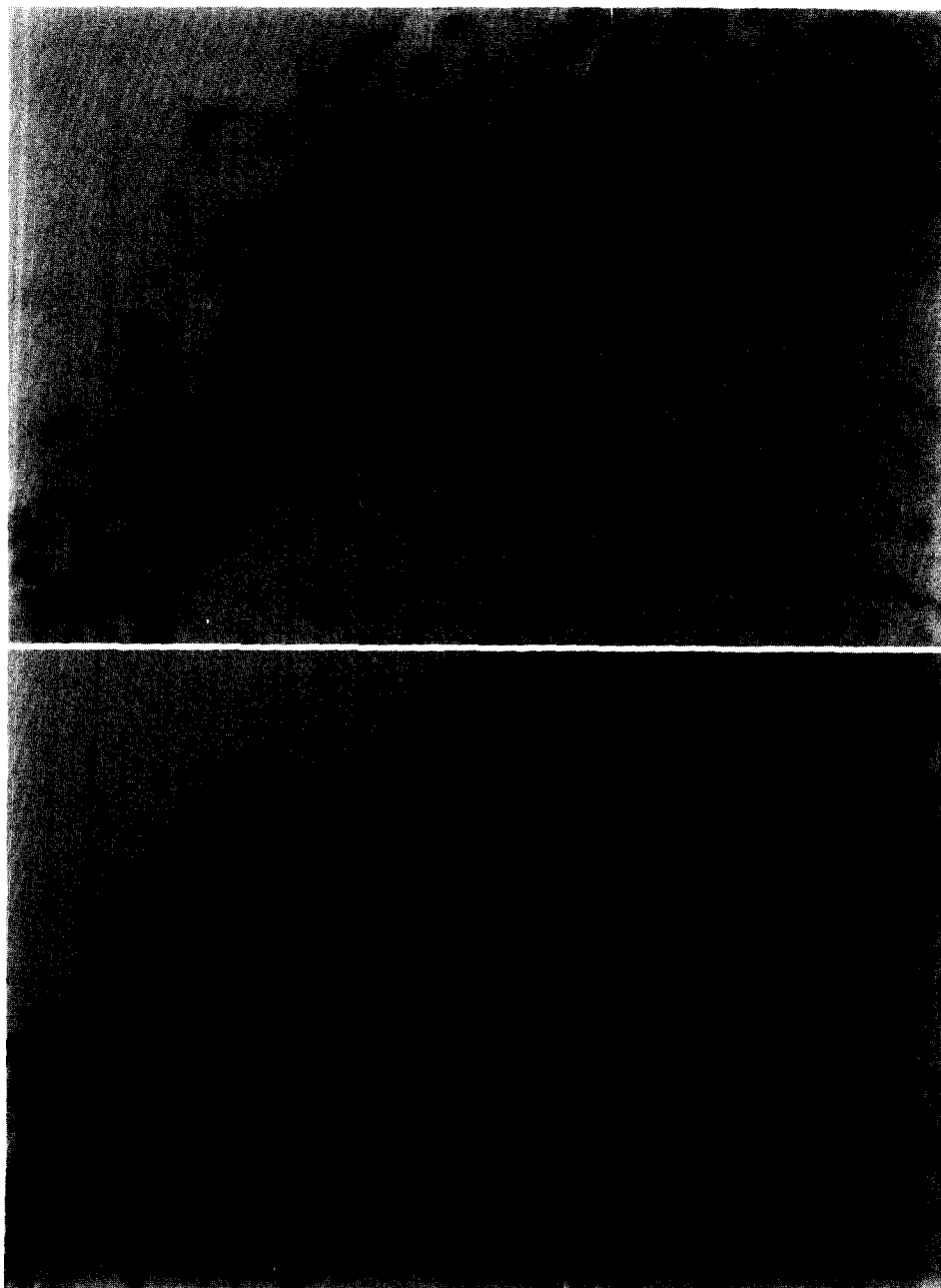


FIG. 1. Expression of GM-CSF in the bronchial epithelium from patients with mild asthma (**A**) and negative control (**B**).

teers showed bronchial hyperresponsiveness to inhaled histamine with a PC_{20} less than 2 mg/ml. There was no significant difference between the two groups in terms of age, gender, resting FEV_1 , or PC_{20} (Table I).

Eleven placebo-treated and 12 BDP-treated patients completed the study. Symptom scores did not change significantly in the placebo-treated

patients. However, seven of the BDP group, but none of the placebo group, became free of symptoms. There was an improvement in FEV_1 (liter) in both the placebo-treated (mean, pre: 2.989, post: 3.286) and BDP-treated patients (mean, pre: 2.728, post: 3.021). The difference between the improvement after the two treatments was not significant. There was a trend toward improve-

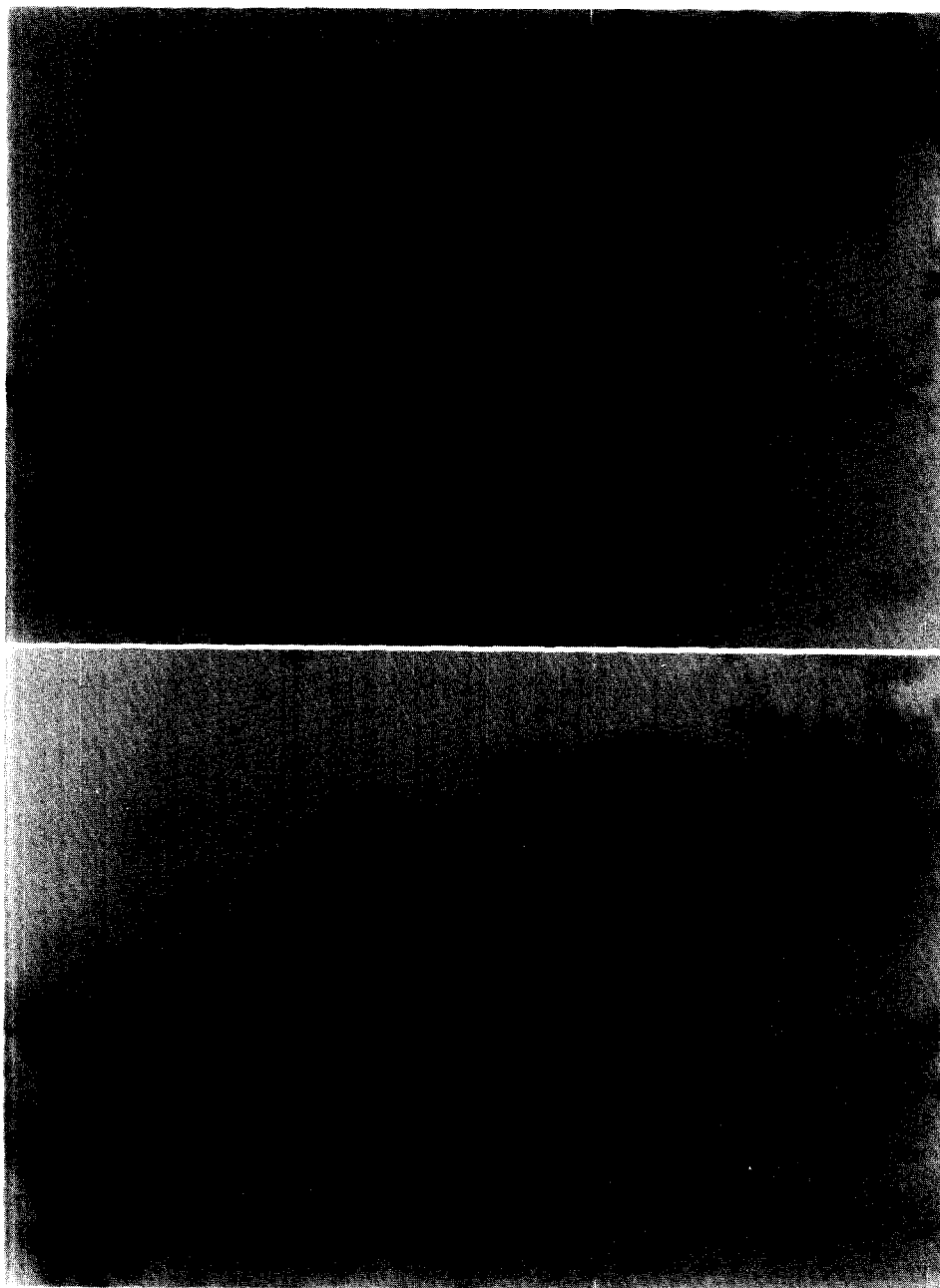


FIG. 2. Expression of IL-8 in the bronchial epithelium from patients with mild asthma (**A**) and negative control (**B**).

ment in PC_{20} in both placebo (geometric mean, pre: 0.100, post: 0.222) and BDP (geometric mean, pre: 0.131, post: 0.412) treatment groups, which did not reach significance within or between groups.

Staining for GM-CSF and IL-8 in bronchial biopsy specimens was extensively localized in the epithelium (Figs. 1 and 2). However, some inflam-

matory cells and endothelial cells in the biopsy specimens were also stained. EG2-staining cells were also abundant in the bronchial epithelium (Fig. 3). Epithelial expression of GM-CSF was correlated weakly but significantly with both PC_{20} in response to histamine ($r = -0.462$, $p < 0.05$) and EG2-staining cells in the epithelium ($r = 0.484$, $p < 0.05$). However, no correlation was



FIG. 3. EG2-staining cells in the bronchial epithelium from patients with mild asthma.

found between PC_{20} and EG2-staining cells in the bronchial epithelium. There was no significant association of IL-8 expression with GM-CSF expression, EG2-staining cells, or bronchial responsiveness.

For the purpose of statistical analysis of the effect of treatment on the epithelial expression of GM-CSF, IL-8, and EG2-staining cells in the epithelium, both pretreatment and posttreatment biopsy specimens with an intact epithelium were needed. Consequently, measurements of GM-CSF and IL-8 were carried out in six such sets of biopsy specimens with an intact epithelium from the placebo-treated group and eight sets from the BDP-treated group. Similarly, estimation of EG2-staining cells was carried out in eight sets of biopsy specimens with an intact epithelium from the placebo-treated group and 11 sets from the BDP-treated group. There were significant reductions in the expression of GM-CSF ($p = 0.008$) and IL-8 ($p = 0.014$) in the epithelium after 4 months of inhaled BDP treatment (Figs. 4 and 5). Mean percentage expression of GM-CSF decreased from $24.7\% \pm 7.4\%$ to $12.8\% \pm 2.2\%$ (mean $\pm$ 95% confidence interval [CI]). Expression was significantly reduced in the BDP group compared with the placebo group (two sample t test, $p < 0.02$). Median percentage IL-8 expression decreased from 27.10% to 14.65%. Expression

was significantly reduced in the BDP group compared with the placebo group (Mann-Whitney U test, $p < 0.02$).

The numbers of EG2-staining cells were also substantially reduced after 4 months of treatment with inhaled BDP from geometric mean $790.1/\text{mm}^2$ (geometric 95% CI, 529.5-1179.0) to geometric mean $203.3/\text{mm}^2$ (geometric 95% CI, 124.6-331.7) ($p = 0.003$) (Fig. 6). EG2-staining cells were significantly reduced in the BDP group compared with the placebo group (two-sample t test, $p < 0.02$). The changes in expression of GM-CSF and IL-8 in the epithelium were independently correlated with changes of EG2-staining cells after treatment ($r = 0.798, p < 0.01$ and $r = 0.653, p < 0.02$, respectively). However, there was no correlation between the changes in GM-CSF and the changes in IL-8.

DISCUSSION

In this study we were interested principally in comparing the effect of BDP and placebo treatment on indices of bronchial epithelial inflammation in patients with mild asthma. We used immunohistochemical methods to investigate the expression of epithelial cell GM-CSF and IL-8 and activation of eosinophils in the bronchial epithelium of these individuals.

By using computerized color image analysis,

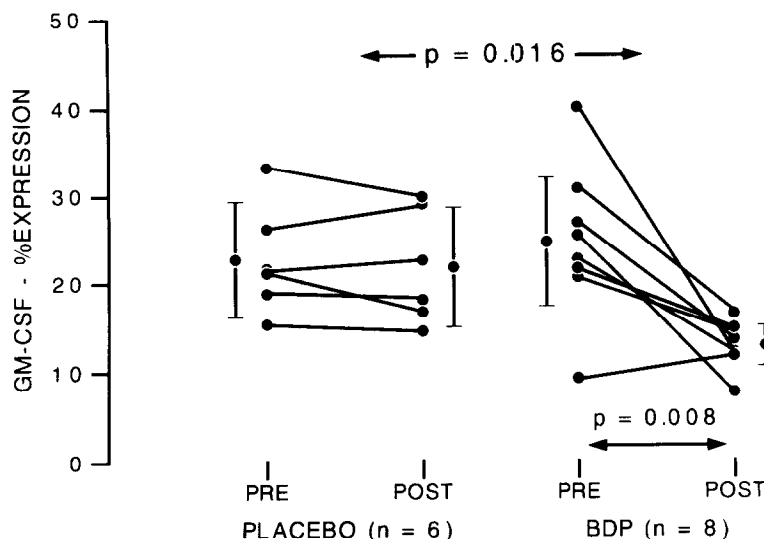


FIG. 4. Percentage GM-CSF expression in the bronchial epithelium from patients with mild asthma. After treatment with BDP, expression of GM-CSF was significantly lower than after treatment with placebo. Bars indicate the mean and 95% CIs. (Two-sample *t* test was applied to determine significance in difference between response to the two treatments; paired *t* test was applied to determine significance in the change within each treatment group).

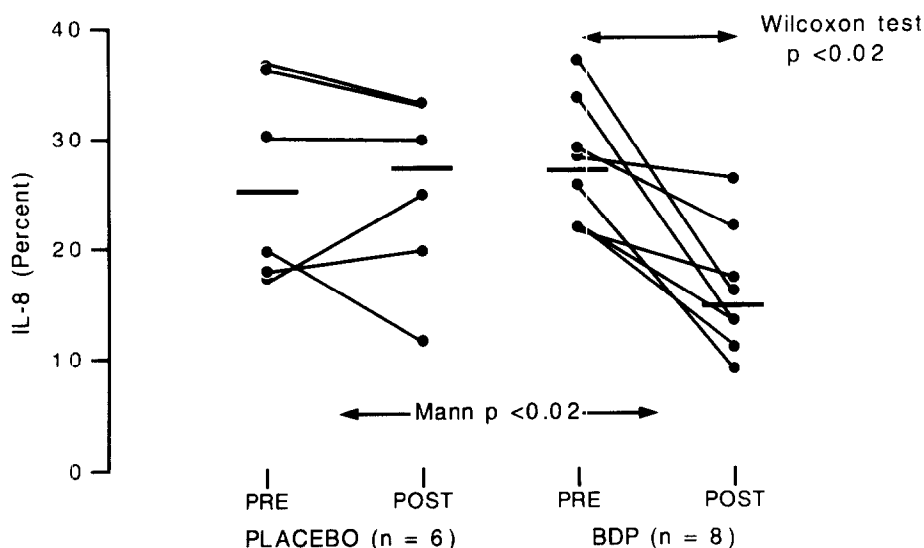


FIG. 5. Percentage IL-8 expression in the bronchial epithelium from patients with mild asthma. After treatment with BDP, expression of IL-8 was significantly less than after treatment with placebo. (Mann-Whitney U test was applied to determine significance in the difference between response to the two treatments; Wilcoxon signed-rank test was applied to determine significance in the change within each treatment group).

which allows a more objective quantitative assessment of the expression of cytokines and inflammatory cells in the epithelium, we have established correlations between: (1) expression of epithelial cell GM-CSF and number of EG2-staining cells (activated eosinophils) and (2) expression of

epithelial cell GM-CSF and PC_{20} in these patients. Expression of epithelial cell IL-8, however, was not correlated with the expression of epithelial cell GM-CSF, activated eosinophils, or bronchial responsiveness. The finding that expression of IL-8 in the epithelium of these patients was not

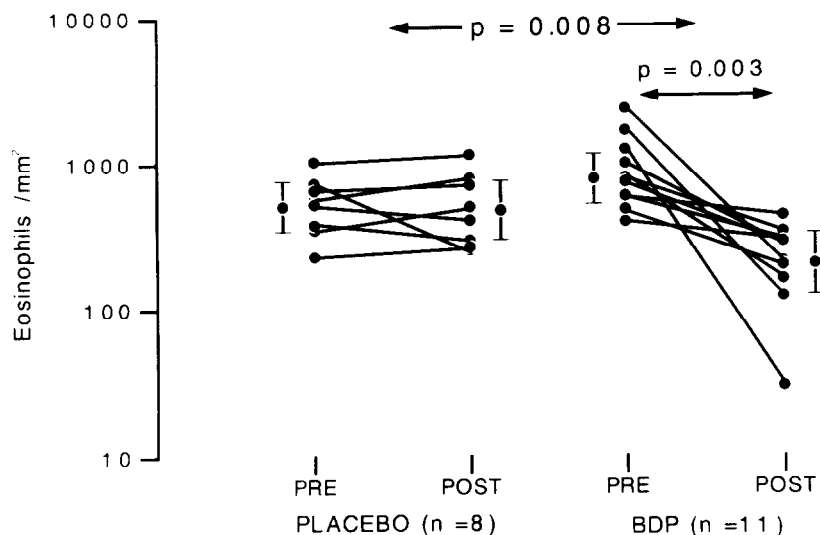


FIG. 6. Numbers of EG2-staining cells per square millimeter of bronchial epithelium. BDP treatment led to a significant decrease in EG2-staining cells. Bars indicate the geometric mean and 95% CIs. (Statistics as in legend to Fig. 3).

correlated specifically with activation of eosinophils is not altogether surprising because this cytokine has been shown to be more selective as an attractant and an activator of neutrophils.²²

This study suggests that the expression of GM-CSF in the bronchial epithelium of patients with asthma may influence, at least in part, both the number of activated eosinophils and bronchial hyperresponsiveness *in vivo*. This is in agreement with collective evidence from several animal and human studies, which have independently demonstrated that local production of specific cytokines regulates inflammatory cell infiltration and activation *in vivo*,²³ and that this may contribute to bronchial hyperresponsiveness.^{24, 25} Several recent studies have shown that cytokines, such as GM-CSF, IL-3, and IL-6, prolong survival of eosinophils and increase their growth and activation *in vitro*.^{16, 26-32} Although the production of these cytokines has generally been attributed to fibroblasts, lymphocytes, macrophages, eosinophils,^{33, 34} mast cells,³⁵ and endothelial cells,³⁶ recent evidence suggests that airway epithelial cells are also capable of synthesizing cytokines. Studies with human bronchial epithelial cells in culture have demonstrated that these cells are capable of synthesizing several proinflammatory cytokines including GM-CSF, IL-1 β , IL-6, IL-8, and tumor necrosis factor- α .^{17, 28, 29, 37} and that GM-CSF, in particular, is important for the survival of eosinophils.²⁹

Our studies of the effect of BDP treatment have

demonstrated that there were significant decreases in both the expression of IL-8 and GM-CSF and EG2-staining cells after treatment with BDP but not after treatment with placebo and that the changes in the expression of IL-8/GM-CSF and EG2-staining cells were strongly correlated after the treatment. However, multiple regression analysis suggests that the correlation of changes in EG2 cells is mainly accounted for by changes in GM-CSF expression. These findings suggest that BDP may attenuate the activation and infiltration of the eosinophils partly by downregulating the expression of GM-CSF and possibly IL-8 in the bronchial epithelium. Our finding of a downregulation of GM-CSF expression in the epithelium of patients with mild asthma after treatment with BDP is in agreement with the finding of Sousa et al.¹⁸ These authors have investigated the expression of GM-CSF in the bronchial epithelium of patients with mild asthma and have demonstrated that this could be downregulated after treatment for 8 weeks with BDP 500 μ g twice a day. These authors did not, however, study the effect of corticosteroid treatment on either the expression of any other cytokines or on the activity of inflammatory cell types. Although Jourens et al.³⁸ and Marini et al.¹⁷ have shown that corticosteroids can decrease IL-8 levels in the circulation of patients undergoing cardiopulmonary bypass and from cultured human bronchial epithelial cells *in vitro*, respectively, to our knowledge the effect of corticosteroid treatment on the expression of IL-8 in airway tissue *in vivo* has not

been previously demonstrated. Studies by Djukanovic et al.³⁹ in patients with asthma and by Lozewicz et al.⁴⁰ in patients with allergic rhinitis have demonstrated that treatment for 2 to 6 weeks with glucocorticoid decreased, respectively, the numbers of activated eosinophils in the bronchial and nasal mucosa of these patients. Similarly, studies investigating the mechanisms of glucocorticoid action have suggested that these may inhibit activated eosinophil infiltration either directly by preventing them from responding to GM-CSF^{29, 41-43} or indirectly by decreasing gene expression and local production of GM-CSF.³⁶

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