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Increase in airway neutrophils after oral but not inhaled corticosteroid therapy in mild asthma

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Summary *Background*: Neutrophils, in addition to eosinophils, are prominent in the airways of patients with severe asthma who are usually on long-term oral and inhaled corticosteroid treatment. We determined whether inhaled or oral corticosteroid therapy can induce airway neutrophilia.

Methods: We performed two separate placebo-controlled studies in which patients with mild asthma were treated with either prednisolone (30 mg per day for 7 days; $n = 9$) or placebo tablets ($n = 8$), or with either inhaled budesonide (800 μg twice daily for 4 weeks; $n = 6$) or inhaled placebo ($n = 6$). Fiberoptic bronchoscopy was performed before treatment and at day 7 of oral treatment, and at day 28 of inhaled therapy. Bronchial sections were immunostained with an antibody to major basic protein for eosinophils, and with an antibody to neutrophil elastase for neutrophils. Induced sputum was obtained in the prednisolone study.

Results: Neutrophils in airway submucosa increased after prednisolone from median 76 to 140/mm^2 ($P = 0.05$); this change was higher than that after placebo ($P = 0.04$). Eosinophils decreased from 24 to 9/mm^2 ($P = 0.03$), but this was not significantly different from placebo. Eosinophils and neutrophils, and levels of IL-8 and myeloperoxidase in induced sputum did not change after prednisolone. There was no change in neutrophil counts after budesonide, but the reduction in eosinophils was greater than placebo ($P = 0.05$). Budesonide improved bronchial responsiveness, but prednisolone did not.

Conclusion: Corticosteroid therapy by the oral but not inhaled route can induce neutrophil recruitment into the airways of patients with mild asthma. This could explain the increase in airway neutrophils observed in severe asthmatics treated with oral corticosteroids.

Abbreviations: MPO, Myeloperoxidase; MBP, Major basic protein; NE, Neutrophil elastase; PC_{20}, Provocative concentration of methacholine needed to cause a fall in FEV_1 of 20%

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Introduction

The association of airway eosinophilic inflammation and asthma has been known for more than a 100 years, and the number of eosinophils in bronchial tissues, in bronchoalveolar lavage and in sputum correlates with symptoms, bronchial hyperresponsiveness and airflow obstruction.[1] More recently, the presence of eosinophils in sputum has been shown to indicate asthma instability rather than asthma severity.[2] This association of eosinophils with asthma is further strengthened by the observation that inhaled corticosteroids, the mainstay of asthma treatment, suppress eosinophil infiltration in bronchial tissues, bronchoalveolar lavage fluid and sputum with improvement in symptoms, airflow obstruction and bronchial hyperresponsiveness.[3–5] The significance of neutrophil infiltration in asthma is less well documented. Increased neutrophilic inflammation has been reported in the airways of patients with sudden-onset fatal asthma, acute severe asthma and in nocturnal asthma, and also in patients with severe asthma.[6–11] It is not known how corticosteroid therapy, which is the mainstay of treatment of patients with severe asthma, can influence neutrophilic trafficking in the airways. Indeed, corticosteroids may decrease programmed cell death or apoptosis of the neutrophil, while increasing apoptosis in the eosinophil.[12] These divergent effects may lead to a greater persistence of neutrophils and to a reduction of eosinophils in the asthmatic airway exposed to corticosteroids.

In order to determine the effect of corticosteroid therapy on neutrophilic inflammation, we performed fiberoptic bronchoscopic studies in patients with mild-to-moderate asthma following treatment with inhaled and with oral corticosteroids, and measured neutrophil counts in the airways submucosa. Our studies indicate that corticosteroid administration by the oral route, but not by the inhaled route, increases airway neutrophils; however, inhaled corticosteroids were more effective in reducing airway eosinophils.

Methods

Patients and study design

Patients were enrolled in two double-blind, randomized, placebo-controlled studies, one for an oral prednisolone study and one for an inhaled budesonide study (Table 1). Both studies were approved by the Ethics Committee of the Royal Brompton Hospital and National Heart and Lung Institute and all patients gave their informed consent.

Stable, non-smoking patients with asthma, not treated with inhaled or oral steroids in the previous 3 months were recruited. Patients had a history of asthma of at least 2 years' duration and demonstrated either reversible airways disease or bronchial hyperresponsiveness with a $PC_{20} \leqslant 4$ mg/l to methacholine. Airway reversibility was defined by either an increase in absolute $FEV_1 \geqslant 12\%$ after inhaled short-acting β agonist, salbutamol (400 μg), or a variation of peak flow of $\geqslant 20\%$ over a 2-week period within the past 12 months. All patients were atopic, as defined by two or more positive skin prick tests to common allergens. For the oral prednisolone study, patients were randomized to receive either prednisolone 30 mg per day for 7 days or matched placebo for 7 days. Lung function, induced sputum and fiberoptic bronchial biopsies were performed at baseline and repeated at day 7.

For the budesonide study, patients were randomized to receive either inhaled budesonide (800 μg twice daily) for 4 weeks or inhaled placebo via a multidose dry powder inhaler (Turbohaler^R, Astra Draco, Sweden). Lung function and fiberoptic bronchial biopsies were performed at baseline and repeated on day 28.

Fiberoptic bronchoscopy and biopsies

Fiberoptic and bronchial biopsies were carried out according to conventional guidelines. To minimize bronchoconstriction, all patients were pretreated with nebulized salbutamol (2.5 mg). Oxygen (3 l/min) was administered via nasal prongs throughout the procedure and oxygen saturation was monitored with a digital oximeter. Topical anesthesia of the upper airways and larynx was achieved using lidocaine 2%. Biopsies were taken from the segmental and subsegmental carinae in the right lung and were immediately placed in optimal cutting temperature (OCT) embedding media, then snap-frozen in isopentane pre-cooled with liquid nitrogen and stored at $-70\,^{\circ}C$, before sectioning on a cryostat and immunostaining.

Lung function

Baseline spirometric parameters were recorded using a dry wedge spirometer (Vitalograph, Buckingham, UK). Standardized bronchial provocation protocol was performed using increasing doubling concentrations of nebulized isotonic metacholine solutions. FEV_1 was measured after each concentration of metacholine. The challenge was stopped

Table 1 Patient characteristics.

	Oral Prednisolone		Inhaled Budesonide	
	Treated group	Control group	Treated group	Control group
Age (years)	25.0(22.8–26.0)	22.0(21.5–24.0)	26.5(24.0–27.0)	28.5(27.0–30.0)
Gender (M/F)	4/5	7/1	5/1	4/2
FEV_1 (l)	3.31(2.42–3.63)	3.52(2.73–4.23)	4.42(3.75–4.76)	3.63(3.37–3.96)
FEV_1 (% predicted)	80.0(71.0–88.8)	80.5(69.5–88.5)	98.7(89.5–106.8)	92.8(84.5–101.2)

Data are expressed as median (25–75 percentiles).

when a 20% fall in FEV_1 was achieved. PC_{20}, the concentration of metacholine needed to cause a 20% fall in the post-saline FEV_1, was calculated by interpolation of the concentration/FEV_1 response.

Immunostaining

Consecutive 6 μm frozen bronchial biopsies sections were immunostained with mouse monoclonal anti-human antibodies for neutrophil elastase (NE, Dako, A/S, Denmark) in 1:100 dilution, and major basic protein (MBP, Monosan, Sanbio AM Uden Netherlands) in 1:50 dilution. Following 0.3% hydrogen peroxide in methanol to block endogenous peroxidase for 30 min, and 5% horse serum for 20 min to block non-specific cross-species reactions, sections were incubated with primary antibodies for 1 h at room temperature. Subsequently, a biotinylated horse anti-mouse immunoglobulin followed by a preformed avidin and biotinylated horseradish peroxidase macromolecular complex was used as secondary antibody (Vectastain ABC kit, Vector Laboratories, Burlingame, CA 94010, USA). Chromogen fast diaminobenzidine was used to visualize specific immunoreactivity and the slides were counterstained in 20% hematoxylin and mounted in dextropropoxyphene.

Cell counts

Counting of positive immunoreactive cells was performed on the whole section, at $\times 400$ magnification of a microscope (Zeiss, Germany). Cells were counted throughout the section including the epithelium. Results were expressed as total number of immunoreactive cells per mm^2 area. All counts were made by the same observer without knowledge of the treatment of individual patients. Total area was measured with KS300 Imaging System 3.0 (Zeiss, Germany).

Induced sputum and processing

Sputum induction was performed before and after treatment period in the oral prednisolone study only. The method of sputum induction and processing has been previously described.[13] Differential cell counts were performed on cytospin slides stained with May–Grunwald–Giemsa. Four hundred inflammatory cells on each of two slides were counted by an observer blinded to the clinical characteristics of the subjects. Sputum supernatants were aliquoted in 2 ml tubes and stored at $-70\,^{\circ}C$.

Sputum supernatant assays

IL-8 assays: IL-8 concentrations were measured with an amplified sandwich-type enzyme-linked immunosorbent assay (ELISA). Ninety-six-well microtiter plates (IL-8 DuoSet Kit, R&D systems, Abingdon, UK) were coated with mouse anti-human IL-8 as previously described.[14] Duplicate results were averaged.

Myeloperoxidase (MPO) assays: MPO concentrations were determined with a commercially available ELISA kit (Calbiochem, San Diego, Ca, USA) according to the manufacturer's instructions. The detection limit of the assay was 1.5 ng/ml. Results from both assays were adjusted according to a standard curve determined with 1% dithiotreitol added in an equivalent concentration to the samples.

Data analysis

Data are presented as median (25th–75th percentiles). Differences between groups were assessed with non-parametric Mann–Whitney *U*-tests, and differences before and after treatment were also assessed with non-parametric Wilcoxon test. PC_{20} data were $\log_{10}$-transformed prior to analysis. Statistical analyses were performed with Statview

5.0 software (SAS Institute Inc, SAS Campus Drive, NC, USA). A *p*-value of less or equal to 0.05 was taken as significant.

Results

There were no differences in age and baseline FEV_1 between the placebo and actively treated groups in both studies (Table 1). The oral prednisolone patients had a lower FEV_1 than the budesonide group.

Prednisolone study

Bronchial responsiveness

There were no significant changes in log PC_{20} following oral prednisolone or placebo treatment. Changes in log PC_{20} were not significantly different before and after treatment in between-group comparison (Table 2).

Neutrophil elastase immunoreactive cell count

Neutrophil elastase (NE) immunoreactive cell counts were comparable at baseline in both groups. NE immunoreactive cells per mm^2 increased significantly after oral prednisolone from 76 (67–98) to 140 (103–208) ($P = 0.05$), but not after placebo from 69 (58–107) to 64 (45–120) (Fig. 1A). The change in neutrophil counts was significantly higher in the prednisolone group compared to the placebo group (74 (7–123) versus –25 (−4–6), $P = 0.04$, Fig. 1B).

Major basic protein immunoreactive cell count

Major basic protein (MBP) immunoreactive cell counts were comparable in both groups at baseline (24 (15–55) versus 28 (10–47)). MBP immunoreactive cells decreased significantly after oral prednisolone from 24 (15–55) to 9 (5–24) ($P = 0.03$), but not after placebo from 28 (10–47) to 22 (10–35) (Fig. 1C). The change in MBP immunoreactive cells was not different between groups: Prednisolone: −19 (−39–−7) versus placebo: –3 (−28–11).

Sputum cell counts and sputum IL-8 and MPO concentrations

At baseline, sputum eosinophils (%) were significantly lower in the prednisolone group compared to placebo group ($P = 0.02$). There were no significant changes observed for total, neutrophil and eosinophil counts in sputum before and after treatment in either group. There were no significant differences in IL-8 and MPO concentrations before and after treatment in either group. There were no correlations between sputum eosinophils and tissue eosinophils, and between sputum neutrophils and tissue neutrophils (Table 3).

Budesonide study

Both groups were comparable at baseline for NE and MBP immunoreactive cell counts.

Bronchial responsiveness

Budesonide caused a significant improvement in log PC_{20} ($P < 0.05$), while placebo had no effect. Change in log PC_{20} was significantly higher in the budesonide-treated compared to the placebo-treated group ($P = 0.01$) (Table 2).

Neutrophil elastase immunoreactive cell counts

No significant changes were observed in NE immunoreactive cells/mm^2 after budesonide or placebo treatment. The changes before and after treatment were not different in between-group comparison : 78 (23–169) versus 24 (11–38) (Fig. 2A).

Major basic protein immunoreactive cell counts

MBP immunoreactive cells/mm^2 were comparable at baseline. MBP cells/mm^2 decreased significantly

Table 2 Log_{10} PC_{20}(mg/ml) during prednisolone and budesonide studies.

	Prednisolone		Budesonide	
	Prednisolone	Control	Budesonide	Control
Pre	0.05(−0.57;0.56)	−0.14(−0.45;−0.03)	−0.17(−0.84;0.03)*	0.45(0.06;0.58)
Post	−0.12(−0.70;0.46)	−0.43(−0.75;−0.15)	1.18(0.98;1.49)	0.36(0.00;0.56)
Change (post–pre)	0.0(−0.5; 0.1)	−0.1(−0.7; 0.2)	1.16(0.97;1.49)†	−0.1(−1.1;0.8)

Results expressed as median (25;75 percentiles). Abbreviation: PC_{20}=provocative concentration of methacholine causing a 20% drop in FEV_1.

*$P < 0.05$ compared to post-values.

†P=0.01 compared to control.

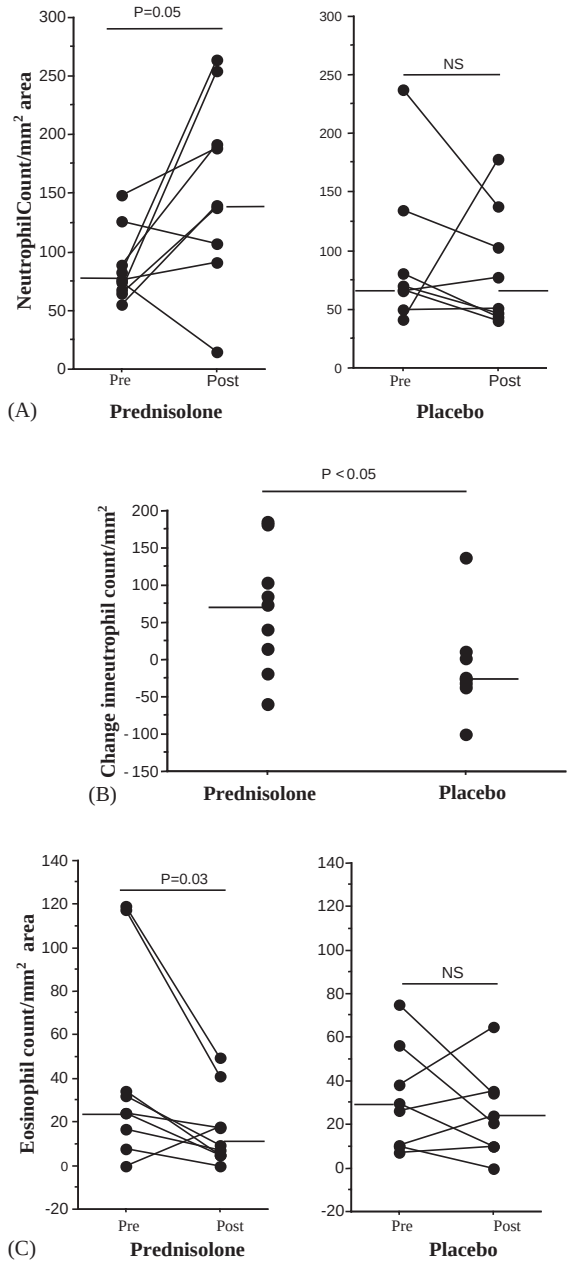

Figure 1 Effects of oral prednisolone on total neutrophil (Panel A), change in neutrophil (Panel B) and eosinophil (Panel C) counts in bronchial biopsies. Data shown as pre- and post-treatment with prednisolone or placebo. In Panel B, change in neutrophil counts represent post-treatment counts minus pre-treatment counts. Horizontal bars represent median values.

after inhaled budesonide from 31 (14–86) to 6 (4–8) ($P = 0.03$), but not after placebo from 6 (5–37) to 26 (14–35) (Fig. 2B). The change in MBP immunoreactive cell counts was significantly higher in the budesonide group compared to the placebo group (−16 (−79—9) versus 7 (−11–19), $P = 0.05$, Fig. 2C).

Discussion

We determined whether inhaled or oral corticosteroid therapy in patients with mild-to-moderate asthma was associated with an increase in mucosal neutrophils. We observed an increase in the number of neutrophils measured in bronchial biopsies, associated with a decrease in airway mucosal eosinophils following prednisolone taken for 7 days, while no changes in mucosal neutrophil counts were observed after inhaled budesonide for 28 days. The inhibition of eosinophilic inflammation by corticosteroid therapy in patients with asthma is well described,[4–5,15] but the effect of corticosteroids on neutrophilic inflammation has been less well studied. In accord with our results, one study found no increase in bronchial tissue neutrophils, associated with a decrease in tissue eosinophils, after 8 weeks of inhaled beclomethasone therapy in mild-to-moderate asthma patients.[16] There have been no previous reports on the effect of oral corticosteroid therapy on airway neutrophils.

We did not include a control non-asthmatic group in our study, and therefore we were not able to tell whether the degree of neutrophilia observed was abnormal. However, we have recently obtained neutrophil elastase-positive cells in bronchial biopsies in a non-asthmatic cohort of nine subjects, and counted 5–75 (median = 38) neutrophils/mm^2 (Niimi A and Chung KF, *unpublished observations*). The baseline neutrophil counts seen in this study in the two groups of mild asthma (prednisolone study; $n = 17$, and budesonide study; $n = 12$) were all significantly higher than that in this normal non-asthmatic group ($P = 0.004$ and 0.02, respectively), indicating that the presence of neutrophilic inflammation in mild asthma. This information indicates that neutrophilia is not only a feature of severe asthma, but also of mild asthma. Whether the submucosal neutrophilia is higher in severe asthma compared in mild asthma is not known.

We used the results of Hoshino et al.[16] to calculate the number of subjects needed to demonstrate a significant change in mucosal neutrophils after corticosteroid therapy. We calculated that, on the basis of an increase in neutrophil count of at least 25 per mm^2 mucosal tissue area with a 5% error risk and 95% power, at least 5–6 patients were needed in each group. Therefore, with the number of patients actually enrolled in each study, the statistical analysis was powerful enough to detect increases in neutrophil counts in both studies. In addition, we opted to measure neutrophil infiltration at day 7 of a 7-day course of oral prednisolone, and at day 28 of a 28-day course of

Table 3 Cells counts and supernatant IL-8 and MPO concentrations in induced sputum.

	Oral prednisolone		Placebo	
	Before	After	Before	After
Total cell count (10^5/ml)	2.6 (1.9–5.3)	2.6 (1.9–3.9)	2.2 (1.4–2.8)	1.5 (1.3–2.2)
Neu (%)	21.0 (11.2–43.7)	18.3 (10.2–41.2)	26.7 (24.4–40.0)	15.0 (11.2–25.0)
Eos (%)	5.3* (2.9–6.7)	4.0 (1.9–9.1)	10.0* (6.8–15.4)	8.2 (5.2–10.7)
IL-8 (pg/ml)	664.8 (477.1–1089.4)	681.2 (522.1–1200.8)	950.0 (213.9–1835.7)	575.2 (116.2–1403.2)
MPO (ng/ml)	589.6 (281.2–1143.0)	648.0 (292.8–755.0)	636.8 (425.2–1539.8)	278.4 (201.6–705.4)

Abbreviations: Neu=neutrophils, Eos=eosinophils, IL-8=interleukin-8, MPO=myeloperoxidase.
*P=0.02. Results are expressed as median (25–75 percentiles).

inhaled budesonide, when there was significant suppression of eosinophil mucosal counts in both studies, as may be expected.

In contrast to the changes in the mucosal biopsy counts, we did not observe any changes in eosinophil and neutrophil cell counts in induced sputum after 1 week of oral prednisolone. A similar discrepancy between the changes in eosinophils in bronchial tissue and those recovered in bronchoalveolar lavage fluid has been reported following a 4-week oral prednisolone treatment of asthma patients;[15] thus, no changes in eosinophil counts in bronchoalveolar lavage fluid were found while in tissue, there was a significant fall in eosinophil counts. This may be a reflection of a lesser potent effect of systemic steroid administration on the luminal compartment than on airway wall compartment. Sputum eosinophil counts did not reflect changes in the number of eosinophils within the airway mucosa.

We found that oral corticosteroids (prednisolone 30 mg per day) taken for 7 days did not change bronchial responsiveness when measured on the 7th day. It is likely that the effect of corticosteroids in improving bronchial hyperresponsiveness is a matter of time and duration of treatment. In a previous study, after 14 days of prednisone (15 mg per day), no significant improvement in PC_{20} was observed, while a 14-day treatment with inhaled beclomethasone was found to be more efficacious.[17] A 3-week period of treatment with 12.5 mg per day of prednisone failed to improve bronchial hyperresponsiveness,[18] but a 2-week treatment with 20 mg prednisolone per day followed by 4 weeks of 10 mg per day, significantly reduced bronchial hyperresponsiveness.[15] On the other hand, we observed a significant improvement in bronchial responsiveness following 1 month treatment with budesonide, consistent with results of previous studies.[18,19]

The mechanisms underlying the divergent effects of corticosteroid therapy on neutrophilic and eosinophilic inflammation are unclear, but one attractive hypothesis supported by strong *in-vitro* evidence is the opposing effect of such therapy on neutrophil and eosinophil apoptosis or programmed cell death. Dexamethasone promotes eosinophil apoptosis while inhibiting neutrophil apoptosis both in vitro and in vivo.[12,20–22] Treatment of asthma exacerbations with corticosteroids increased the number of apoptotic eosinophils in sputum.[23] Thus, differences in the regulation of pro- and anti-apoptotic factors may explain the divergent effects of corticosteroids on the regulation of apoptosis in these two different granulocytes.[24] Enhanced corticosteroid-induced neutrophil survival would delay their tissue removal and the contribution of the neutrophil to the chronic inflammatory process of asthma may be enhanced.

Increased numbers of neutrophils in airways mucosa or recovered from within the lumen has been described in sudden-onset fatal asthma,[6,25] in intubated patients with status asthmaticus[7] and in patients with severe asthma.[10,11,26,27] Airway neutrophils are also elevated in nocturnal asthma[28] and during asthma exacerbations.[9] Other studies have reported a negative association of sputum neutrophils with FEV_1.[29,30] Of interest, we found in this study that there were a substantial number of neutrophils in the biopsies of our mild asthmatic patients, levels that were higher than the corresponding number of eosinophils. This also indicates that the recruitment of neutrophils to the airways of patients with mild asthma is not entirely due to corticosteroid therapy. In the studies where

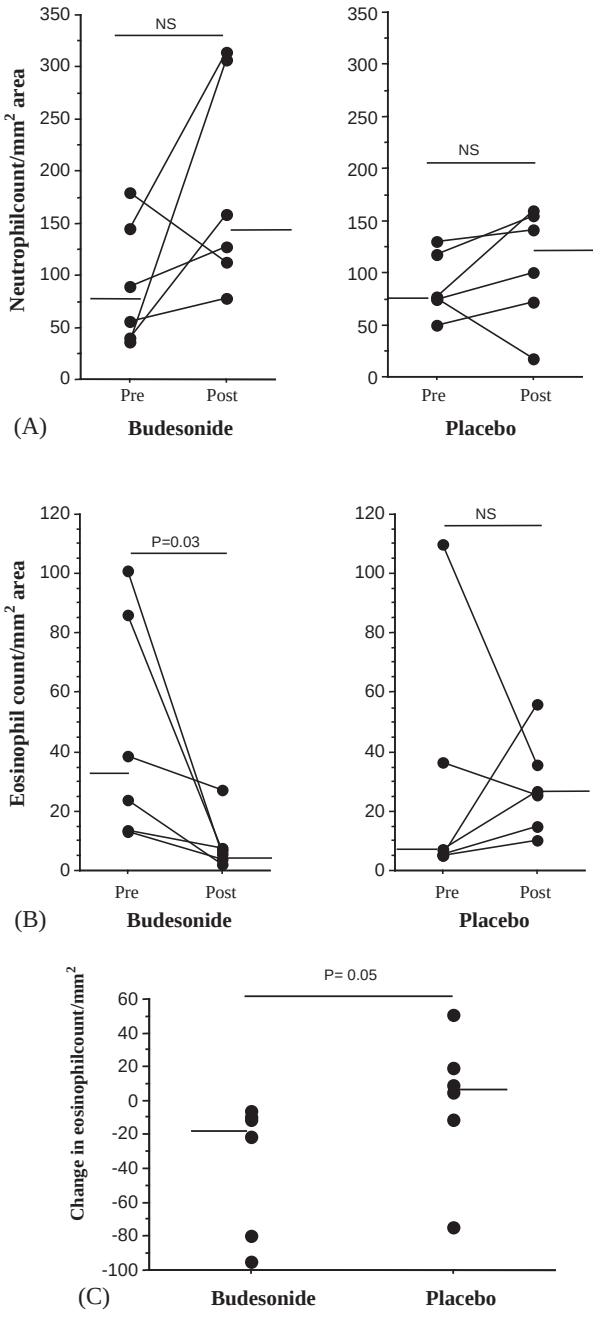

Figure 2 Effects of inhaled budesonide on neutrophil (Panel A), eosinophil (Panel B), and change in eosinophil (Panel C) counts in bronchial biopsies. Data shown as pre- and post-treatment with inhaled budesonide or placebo. Change in eosinophil counts represents post-treatment counts minus pre-treatment counts. Horizontal bars represent median values.

neutrophils have been associated with several types of severe asthma, the potential effects of oral corticosteroid therapy in increasing mucosal neutrophils cannot be definitely excluded since most of these patients are usually on maintenance corticosteroid therapy. Therefore, it is not possible to determine directly whether the increased neutrophil count is truly a marker of asthma severity in these groups.

Whether any functional significance should be attached to the increased recruitment of neutrophils induced by oral corticosteroid therapy is debatable. Neutrophils have been implicated in the pathogenesis of bronchial hyperresponsiveness induced by ozone in animal models.[31,32] Levels of neutrophil myeloperoxidase, an enzyme released from activated neutrophils, in supernatants from induced sputum did not change following oral prednisolone treatment. No changes in sputum and blood myeloperoxidase concentrations have been reported after inhaled corticosteroid therapy,[26,33] supporting the in vitro finding that the increased neutrophil survival induced by dexamethasone was not associated with cell activation as assessed by superoxide production or release of IL-8.[20] On the other hand, in severe asthma, there is evidence of activation of sputum-recovered neutrophils.[10]

In summary, our studies indicate that oral but not inhaled corticosteroids in mild steroid-naive asthmatics increased airway neutrophil infiltration. Although, the response of patients with mild asthma to corticosteroid therapy may not be the same as in severe asthma, our study suggests that oral corticosteroid therapy may be a contributory factor in the increased number of airway neutrophils observed in patients with more severe asthma who are usually on oral corticosteroid therapy. However, such studies need to be performed in severe asthmatic patients.

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