Inhaled Budesonide Decreases Airway Inflammatory Response to Allergen

ELIZABETH A. BECKY KELLY, WILLIAM W. BUSSE, and NIZAR N. JARJOUR

Pulmonary and Critical Care Section and Allergy and Immunology Section of the Department of Medicine, University of Wisconsin, Madison, Wisconsin

To define the mechanisms by which inhaled glucocorticosteroid regulates allergen-induced airway inflammation, a double-blind, placebo-controlled, cross-over study with inhaled budesonide was conducted in 14 subjects with allergic asthma. After baseline bronchoscopy and bronchoalveolar lavage (BAL), subjects were randomized to budesonide (400 μg, twice daily) or placebo treatment for 4 wk. At the end of each treatment phase, whole-lung allergen inhalation challenge was performed, followed by BAL 48 h later. Budesonide treatment improved the FEV_1, attenuated both the immediate- and late-phase response to allergen, and prevented the increase in bronchial hyperresponsiveness after allergen challenge. Budesonide treatment also decreased allergen-induced airway eosinophilia. To determine the effects of budesonide on airway cell function, BAL cells were stimulated *ex vivo* with the T cell mitogen PHA, and cytokine generation was measured by ELISA. Budesonide decreased *ex vivo* generation of IL-5 and IFN-γ by BAL cells. *Ex vivo* IL-5 production was significantly correlated with the number of airway eosinophils ($r_s = 0.61$), and levels of eosinophil-derived neurotoxin (EDN) ($r_s = 0.57$) and IL-5 ($r_s = 0.52$) in BAL fluid. Moreover, PHA-induced IL-5 generation correlated with FEV_1 fall during the late-phase response to allergen ($r_s = 0.60$). Budesonide decreased circulating eosinophils and serum levels of IL-5, but did not reduce IL-5 generation by peripheral blood mononuclear cells. The reduction in circulating eosinophils correlated with the decrease in levels of EDN ($r_s = 0.61$) in BAL fluid and late response to inhaled allergen ($r_s = 0.51$). These findings suggest that long-term treatment with inhaled budesonide reduces airway cell generation of cytokines, specifically IL-5, which then decreases circulating eosinophils and their availability for recruitment to the airway after allergen exposure.

Inhaled corticosteroid treatment is an effective therapy in persistent asthma (reviewed in References 1 and 2). Corticosteroid treatment improves lung function (3), attenuates the early and late pulmonary response to inhaled allergen (4–7), and prevents allergen-induced airway hyperresponsiveness (5, 8). Furthermore, inhaled corticosteroid reduces airway inflammation in bronchial mucosal biopsy (9–11) and decreases allergen-induced airway eosinophilia (5, 12–15). It is proposed that corticosteroid modulates allergic inflammation by inhibiting transcription of proinflammatory cytokine/chemokine genes and, thus, the differentiation, maturation, recruitment, and/or activation of a wide variety of cells associated with asthma, including airway epithelial cells, macrophages, eosinophils, and T cells (2). These effects lead to a diminished level of airway inflammation.

We hypothesized that corticosteroid-mediated improvement in lung function is related to a decrease in the generation of interleukin 5 (IL-5) and consequently eosinophil recruitment to the airway. To test this hypothesis, whole-lung inhalation of allergen was used to model inflammatory events associated with allergic inflammation. Inhaled allergen causes most individuals with allergic asthma to develop acute airflow obstruction. The development of a late-phase response is associated with eosinophil recruitment, airway cell generation of IL-5, and a subsequent enhancement of airway responsiveness (16). It is proposed that events associated with the late-phase response to allergen exposure resemble the acute inflammatory response of asthma and serve as a model to assess the effect of intervention with inhaled budesonide on this response. To evaluate the effects of inhaled budesonide on markers of allergic inflammation in asthma, including airway eosinophilia and cytokine generation, bronchoscopy with lavage was performed after whole-lung allergen inhalation challenge.

METHODS

Subjects

Fourteen subjects with atopic asthma, including 5 males and 9 females between the ages of 21 and 42 yr, completed the study (Table 1). Each subject underwent a medical history, physical examination, and pulmonary function testing by spirometry. Selection criteria included a positive skin prick test to one or more aeroallergens, a history of asthma, $MethPC_{20}$ (provocative concentration of methacholine causing a 20% fall in FEV_1) < 8 mg/ml, and demonstration of a late-phase response (LPR) to allergen (≥ 15% fall in FEV_1 4–8 h after allergen challenge) at the screening visit. In addition, all subjects had an FEV_1 greater than 75% of predicted, were nonsmokers, were without respiratory infections within 30 d of study, and had not received antihistamines or β-agonists within 7 d, nedocromil within 14 d, or corticosteroid or sodium cromolyn within 30 d of study enrollment. The study was approved by the University of Wisconsin-Madison Center for Health Sciences Human Subjects Committee. Informed consent was obtained from each subject before participation.

Study Design

The study design was a randomized, double-blind, placebo-controlled, two-period cross-over study (Figure 1). Subjects were first screened and had a baseline evaluation, including an allergen skin prick test and inhalation challenge using a graded nebulization of an aeroallergen to establish the presence of a late-phase response to allergen. To evaluate baseline markers of airway inflammation, bronchoscopy with bronchoalveolar lavage (BAL) was performed 14–21 d after the screening visit and before any treatment (baseline value). After the screening-phase bronchoscopy, subjects were randomized to budesonide or placebo treatment. Each treatment period lasted 22–34 d, depending on subject availability for bronchoscopy at the end of the treatment period. Placebo or inhaled budesonide (400 μg twice daily) was delivered by a breath-actuated, metered-dose, powder inhaler (Turbuhaler; Astra USA, Westborough, MA). The two treatment phases were separated by a 14- to 21-d washout period. At the end of each treatment phase, airway responsiveness to a methacholine challenge was measured (*see below*), followed 24 h later by a single-dose allergen inhalation challenge with monitoring for a late-phase response (Figure 1B). A second methacholine challenge was performed 24 h after allergen challenge, and bronchoscopy with BAL was performed 24 h later. Budesonide or placebo treatment was continued through the morning of the bronchoscopy and BAL.

(*Received in original form October 19, 1999 and in revised form March 6, 2000*)

Supported, in part, by Astra USA, Inc. and National Institutes of Health Grant HL 56 396.

Correspondence and requests for reprints should be addressed to E. A. B. Kelly, Ph.D., Section of Pulmonary and Critical Care Medicine, 600 Highland Avenue, CSC H6/380, University of Wisconsin School of Medicine, Madison, WI 53972. E-mail: eak@medicine.wisc.edu

Am J Respir Crit Care Med Vol 162. pp 883–890, 2000
Internet address: www.atsjournals.org

TABLE 1
SUBJECT CHARACTERISTICS

Subject No.	Sex	Age (*yr*)	FEV_1 (*% pred*)	$MethPC_{20}$ (*mg/ml*)	Late-Phase Response (*% decrease*)	Allergen for Inhalation Challenge
1	F	32	98	7.82	16	House dust mite
2	F	42	114	2.01	25	House dust mite
3	M	32	90	1.65	16	Cat dander
4	F	24	87	0.38	28	House dust mite
5	F	26	105	1.25	20	Cat dander
6	M	21	78	4.67	17	Cat dander
7	F	23	96	1.65	24	Cat dander
8	F	25	85	0.56	16	Cat dander
9	M	23	105	1.84	25	Ragweed
10	M	30	91	8.99	16	Cat dander
11	F	21	106	0.24	15	House dust mite
12	F	31	93	0.19	21	Cat dander
13	F	30	95	1.06	24	House dust mite
14	M	25	82	2.79	21	House dust mite
	9F, 5M	25 (23, 30)	93 (87, 105)	1.65 (1.0, 2.0)	21 (16, 24)	6 house dust mite, 7 cat dander, 1 ragweed

Airway Responsiveness to Methacholine

To quantitate airway responsiveness, graded doses of methacholine were delivered through a nebulizer attached to a French–Rosenthal dosimeter (17). Baseline spirometry was measured and then redetermined 10 min after five breaths of the diluent solution. If there was no significant response to diluent challenge (FEV_1 remained within 10% of baseline), five breaths of methacholine were inhaled; spirometry was repeated 5 min later. Consecutively higher concentrations of methacholine were given until the FEV_1 fell by $\geq 20\%$ from baseline. $MethPC_{20}$ was calculated by linear interpolation of the last two doses on the dose–response curve, using a computer program (PD_{20}; Madison Scientific Software, Wexford, PA) and expressed in milligrams per milliliter.

Allergen Challenge

Allergen used in the inhalation challenge for each subject was selected from the skin test response and correlative history. To determine the dose of allergen to be given during the study phases, patients were screened with a graded nebulization of allergen as described for methacholine challenge. However, the interval between each consecutive dose of allergen was 10 min. The allergen provocative dose causing a 20% fall in FEV_1 (PD_{20}) was calculated by linear interpolation of the dose–response curve and expressed in cumulative breath units. This predetermined allergen PD_{20} was administered by a single-dose whole-lung allergen inhalation challenge during both study phases. Using a predetermined dose of allergen allowed for the evaluation of treatment both on the immediate and late-phase response to a consistent amount of allergen. Allergens used in whole-lung allergen challenge included ragweed (GS ragweed mix; Greer Laboratories, Lenoir, NC), cat dander (Bayer Allergy Products, Spokane, WA), and house dust mite (*Dermatophagoides farinae*; Miles Allergy Products, Spokane, WA).

Bronchoalveolar Lavage

A baseline bronchoscopy with lavage was performed before any treatment. At the end of each treatment phase, bronchoscopy with lavage was performed 48 h after whole-lung allergen challenge. Before bronchoscopy, subjects were treated with atropine (0.6 mg) and midazolam (1.0 mg) given by intramuscular injection; albuterol (180 μg) was administered by metered dose inhaler. BAL was performed in the right middle lobe, using six 40-ml aliquots of sterile 0.9% NaCl that were injected through the flexible fiberoptic bronchoscope and recovered by gentle hand suction.

Cellular Analysis of Blood and BAL Fluid

Peripheral blood was drawn immediately before the BAL. Total cell counts and differentials were performed on EDTA-treated whole blood. Total cell numbers were determined by hemacytometer, using Turk's counting solution containing acetic acid and methylene blue. Eosinophils were also enumerated by hemacytometer, using a Unopette pipette test containing 1% Phloxine B (Becton Dickinson; purchased through Fisher Scientific, Itasca, IL). Peripheral blood mononuclear cells were obtained from 20 ml of heparinized blood by centrifugation over Ficoll-Paque (Ficoll 400-diatrizoate; Pharmacia Biotech, Piscataway, NJ). Cells collected from the interface were then centrifuged over fetal calf serum (200 × *g* for 20 min) to remove

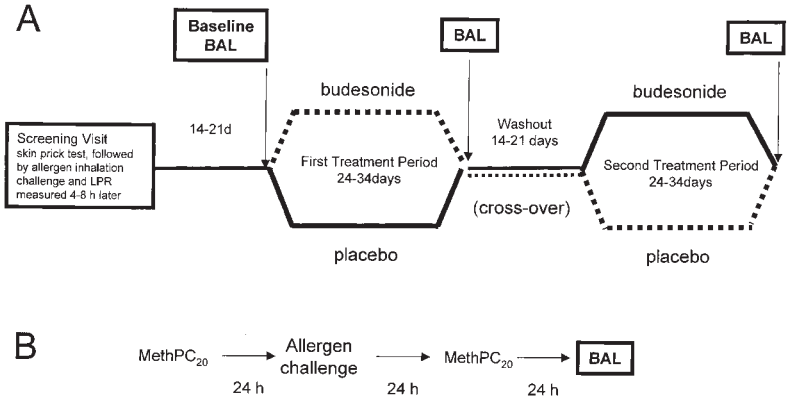

Figure 1. (*A*) Cross-over design. The study design was a randomized, double-blind, placebo-controlled, two-period cross-over study. The screening visit included a skin prick test, followed by inhalation allergen challenge and late-phase response (LPR) 4–8 h later. Baseline bronchoscopy with BAL was performed 14–21 d after the screening visit. After the baseline BAL, patients were randomized to inhaled budesonide (*dashed lines*) or placebo (*solid lines*) treatment. Each of the two treatment periods lasted 22–34 d and were separated by a 14- to 21-d washout period. BAL was performed at the end of each treatment period. (*B*) Detail of allergen challenge and BAL. At the end of each treatment phase, subjects underwent a methacholine challenge followed 24 h later by allergen inhalation challenge. A second methacholine challenge was performed 24 h after allergen challenge and bronchoscopy with BAL was performed 24 h later.

platelets. BAL cells were recovered from the lavage fluid by centrifugation at 400 × *g* for 10 min at 4° C. BAL cells were washed twice with Hanks' balanced salt solution containing 2% newborn calf serum. For differential cell counts, blood smears and cytospin preparations of BAL cells were stained with the Giemsa-based Diff-Quik stain (Baxter Scientific Products, McGaw Park, IL). BAL fluids were stored at −70° C until analyzed.

Flow Cytometric Analysis to Determine the Expression of Cell Surface Activation Markers on Eosinophils and T Lymphocytes

BAL cells (1×10^5 cells) and 100-μl aliquots of whole blood were stained by simultaneous addition of fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated antibodies specific for cell surface markers (Becton Dickinson Immunocytometry Systems, San Jose, CA) as previously described (18). For analysis, 10,000 events were collected by a Becton Dickinson FACScan II, and data analyses were performed with the CellQuest software package (Becton Dickinson). To normalize for noncellular debris that was present in some BAL preparations, the relative percentage of $CD19^+$, $CD3^+$, $CD4^+$, $CD8^+$, and natural killer ($CD3^-CD16^+CD56^+$) cells was calculated on the basis of the total number of $CD45^+$ leukocytes present within the lymphocyte gate (19). For eosinophils, an electronic eosinophil region within the forward and side scatter plot was established by back-gating on FITC–CD45 bright, PE–autofluorescent cells. At least 5,000 gated events were collected. The majority of cells within this forward and side scatter gate are eosinophils. Nonetheless, all test samples contained an FITC–CD16/FITC–CD14 cocktail, which allowed for electronic exclusion of contaminating neutrophils and monocytes, respectively.

Cell Cultures to Determine the Capacity of Airway Cells to Generate Cytokines

Peripheral blood mononuclear cells or unseparated BAL cell populations were cultured at 2×10^6 viable cells per milliliter as previously described (18). Either phytohemagglutinin (PHA; Sigma, St. Louis, MO) or lipopolysaccharide (LPS, *Escherichia coli* serotype 055:B5; Sigma) was used at a final concentration of 10 or 20 μg/ml, respectively. This approach was used to determine the capacity of airway cells, presumably lymphocytes and macrophages, respectively, to generate cytokines. All cultures were done in triplicate. Cells were cultured for 48 h at 37° C, 5% CO_2, in a humidified incubator. Culture supernatant fluids were removed and stored at −20° C until analyzed.

Analysis of Proteins in BAL Fluid

Total protein was measured in 1× BAL fluid by a Lowry assay modified for a 96-well microtiter plate and with a sensitivity of 20 μg/ml. A double-antibody competition radioimmunoassay, with radioiodinated eosinophil-derived neurotoxin (EDN), rabbit anti-EDN antibody, and burro anti-rabbit IgG (20), was used to measure EDN concentrations in 20× BAL fluids. The threshold value is 10 ng/ml. Tryptase was measured in 1× BAL fluids by a sandwich radioimmunoassay employing the murine monoclonal antibody G5 for capture and the biotinylated antibody G4 for detection. (Antibodies were produced in the laboratory of L. Schwartz, Virginia Commonwealth University, Richmond, VA). The immunoassay has a lower limit of 0.2 ng/ml. Histamine was measured in 1× BAL fluids by a radioenzymetric assay in which a histamine-specific *N*-methyltransferase is used to transfer a tritiated methyl group from *S*-adenosyl-L-methionine to histamine, forming an *N-tele*-methylhistamine (21). The radiolabeled product is isolated through a series of solvent extractions and the assay has a linear detection range of 30–50,000 pg/ml.

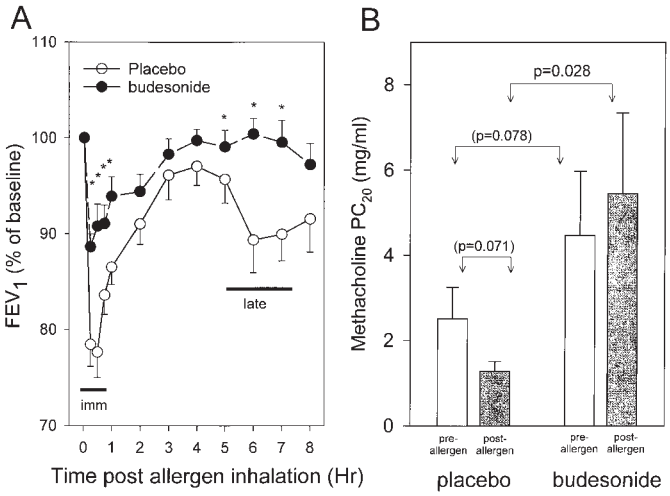

Figure 2. Effect of budesonide on early and late response to allergen and nonspecific airway responsiveness. (*A*) Airway response to allergen was determined after a 4-wk treatment with placebo (*open circles*) or budesonide (*closed circles*) and is represented as FEV_1 percent of baseline (mean ± SEM) at various times after allergen challenge. *$p < 0.05$ compared with the value for placebo phase at the same time point. (*B*) Methacholine PC_{20} (mean ± SEM) was determined before (*white bars*) and 24 h after (*dark bars*) whole-lung allergen challenge.

Cytokine ELISAs

A sensitive two-step sandwich enzyme-linked immunosorbent assay (ELISA) was used to measure cytokines in serum, diluted cell culture supernatant fluids, and 40× concentrated BAL fluids as previously described (18). Monoclonal cytokine-specific antibodies (unlabeled coating antibodies and biotinylated detection antibodies) were purchased from PharMingen (San Diego, CA). To increase ELISA sensitivity, a streptavidin–horseradish peroxide polymer, POLY-HRP-40 (Research Diagnostics, Flanders, NJ) was used for enzyme and a one-component 3,3′,5,5′-tetramethylbenzidine (TMB; Kirkegaard & Perry, Gaithersburg, MD) was used for the substrate. To determine the lowest limit of detection, six samples consisting of diluent only were analyzed in each assay, and a cutoff value was established as 2 standard deviations above the mean of the diluent controls. The sensitivity for each cytokine assay was as follows: interleukin 4 (IL-4), 0.8 pg/ml; IL-5, 3 pg/ml; interferon γ (IFN-γ), 0.8 pg/ml; regulated on activation, normal T cell expressed and secreted (RANTES), 6.0 pg/ml; granulocyte-macrophage colony-stimulating factor (GM-CSF), 1.5 pg/ml. Data are expressed as picograms per milliliter of 40× BAL fluid, pico-

TABLE 2

BLOOD AND BAL CELL NUMBERS AND DIFFERENTIAL*

	BAL Cells			Blood Cells		
	Baseline, No Allergen	48 h Post-allergen Placebo	48 h Post-allergen Budesonide	Baseline Blood, No Allergen	48 h Post-allergen Placebo	48 h Post-allergen Budesonide
Total cells, $\times 10^6$	28.8 (21.0, 33.1)	25.9 (21.8, 30.1)	29.7 (21.6, 38.3)	5.6 (4.6, 6.7)	6.9 (5.5, 5.6)	6.6 (5.6, 7.9)
Macrophages, %	86.2 (81.0, 90.9)	81.7 (74.7, 86.0)†	90.1 (88.4, 92.4)†‡	9.0 (6.0, 11.8)	8.0 (5.3, 9.0)	7.0 (5.0, 10.5)
Lymphocytes, %	10.8 (5.6, 14.2)	12.5 (8.9, 15.5)	7.8 (6.2, 9.3)‡	32.5 (28.0, 40.5)	30.5 (26.5, 35.5)	35.0 (25.3, 39.0)
Neutrophils, %	0.7 (0.3, 1.0)	1.0 (0.4, 1.9)	0.9 (0.3, 1.2)	51.0 (46.5, 56.8)	54.0 (46.0, 58.0)	55.0 (48.0, 57.8)
Eosinophils, %	0.7 (0.3, 1.9)	2.0 (1.4, 2.0)†	0.7 (0.3, 1.3)‡	5.5 (4.0, 8.8)	7.5 (6.0, 10.5)	3.5 (3.0, 4.8)

* Data are depicted as medians with 25 and 75% quartiles.
† $p < 0.05$, versus baseline.
‡ $p < 0.05$, versuss 48 h post-allergen, placebo treatment.

TABLE 3

BAL FLUID PROTEINS*

	Baseline, No Allergen	48 h Post-allergen Placebo	48 h Post-allergen Budesonide
Total protein, μg/ml	67.3 (55.6, 78.3)	80.5 (62.8, 100.9)	61.5 (52.9, 71.3)
Histamine, pg/ml	232.5 (144.0, 449.3)	260.0 (212.3, 434.8)	89.0 (74.0, 124.3)†‡
Tryptase, μg/ml	0.0 (0.0, 0.3)	0.3 (0.0, 0.4)	0.2 (0.0, 0.3)
IL-5, pg/ml of 40× BALF	8.7 (1.4, 12.5)	14.1 (5.4, 24.5)	5.7 (0.0, 22.8)
IFN-γ, pg/ml of 40× BALF	0.0 (0.0, 9.3)	6.4 (0.0, 11.1)	0.0 (0.0, 11.7)
RANTES, pg/ml of 40× BALF	67.5 (44.6, 114.8)	97.8 (80.8, 170.3)†	85.2 (56.0, 135.6)‡
GM-CSF, pg/ml of 40× BALF	10.6 (6.1, 12.4)	14.1 (8.6, 17.3)	10.8 (8.5, 13.5)

* Data are depicted as medians with 25 and 75% quartiles.
† $p < 0.05$, versus baseline.
‡ $p < 0.05$, versus 48 h postallergen, placebo treatment.

grams per milliliter of 1× culture supernatant fluid, and picograms per milliliter of undiluted serum.

Statistical Analysis

Data were expressed as medians with 25 and 75% interquartiles. A Wilcoxon signed rank test (or paired *t* test, for normally distributed data) was used to compare data. Correlations were performed with a Spearman rank order correlation test. A p value of < 0.05 was considered significant. Statistical analysis was performed with the SigmaStat software package (Jandel Scientific Software, San Rafael, CA).

RESULTS

Effect of Budesonide Treatment on Lung Function and Response to Inhaled Allergen

After 4 wk of treatment with inhaled budesonide, FEV_1 (mean ± SEM, 99.2 ± 2.9% of predicted) was significantly ($p = 0.03$) improved over baseline (94.6 ± 2.7% of predicted). Furthermore, budesonide treatment reduced both the immediate and late-phase responses to inhaled allergen (Figure 2A), and attenuated allergen-induced airway hyperresponsiveness (Figure 2B).

Effect of Budesonide on the Cellular Profile and Protein Content of BAL Fluid after Allergen Challenge

Although inhaled allergen did not increase the total number of cells in the BAL, there was a significantly greater proportion of eosinophils and a decrease in alveolar macrophages (Table 2). Budesonide treatment did not change the total number of airway cells compared with the placebo phase; however, budesonide treatment prevented the allergen-induced increase in eosinophils. The percentage of lymphocytes was also significantly reduced after budesonide treatment (Table 2).

Total protein in the BAL fluid did not increase significantly after whole-lung allergen challenge and was not affected by budesonide treatment (Table 3). With budesonide treatment, the histamine concentrations 48 h after allergen challenge were significantly reduced and were even lower than levels observed at baseline. Tryptase levels in BAL fluid were at or below the level of detection in most subjects (data not shown). Although cytokine levels in BAL fluid were quite low, use of 40× concentrated BAL fluid and an amplified ELISA made detection possible for IL-5, IFN-γ, RANTES, and GM-CSF; while IL-4 levels remained below the level of detection. After inhaled allergen challenge, IL-5 levels increased in 9 of 14 subjects, but the change in the group as a whole did not reach significance ($p = 0.18$). Only RANTES increased significantly after allergen challenge, and there was a modest, but significant, decrease in RANTES after budesonide treatment (Table 3).

Effect of Budesonide Treatment on BAL Eosinophils

Compared with placebo, budesonide treatment inhibited allergen-induced airway eosinophilia (Figure 3A) and BAL fluid concentrations of the eosinophil granule protein EDN (Figure 3B). The percentage of BAL eosinophils expressing the early activation marker CD69 was not affected by budesonide treatment (Figure 3C).

Effect of Budesonide Treatment on the Phenotype and Function of Airway Lymphocytes

Although the percentage of lymphocytes in the BAL decreased after budesonide treatment (Table 2), no change was observed in the proportion of T cell subsets or expression of activation markers on $CD4^+$ T cells. The relative percentage of

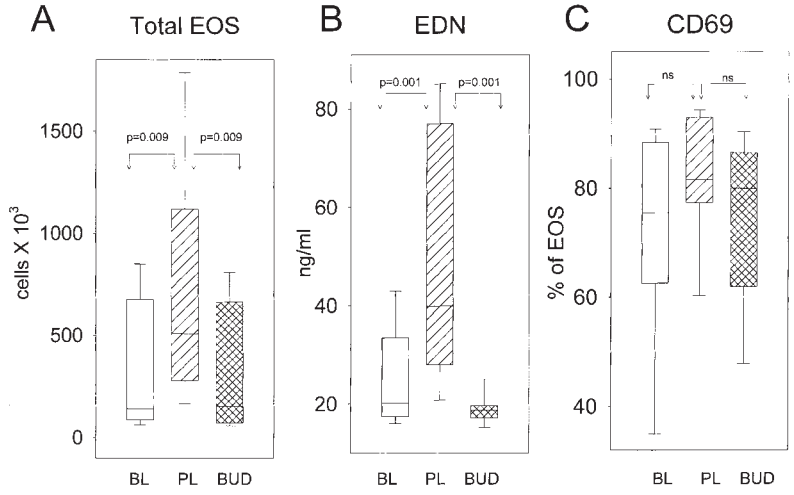

Figure 3. Effect of budesonide treatment on numbers and activation of BAL eosinophils. Numbers and activation of BAL eosinophils were determined during the baseline (BL) phase (*open bars*) and 48 h after a whole-lung allergen inhalation challenge, which was given at the end of a 4-wk treatment with placebo (PL, *diagonal-hatched bars*) or budesonide (BUD, *cross-hatched bars*). Data depict (*A*) total numbers of BAL eosinophils, (*B*) levels of eosinophil-derived neurotoxin (EDN) in BAL fluid, and (*C*) the percentage of BAL eosinophils that express the activation marker CD69. *Bars* represent medians within 25 and 75% quartiles, error bars represent the 10th and 90th percentiles.

TABLE 4

BLOOD AND BAL CELL NUMBERS AND DIFFERENTIAL*

	BAL Cells			Blood Cells		
	Baseline, No Allergen	48 h Post-allergen Placebo	48 h Post-allergen Budesonide	Baseline Blood, No Allergen	48 h Post-allergen Placebo	48 h Post-allergen Budesonide
NK	3 (2, 4)	4 (2, 5)	4 (2, 4)	7 (5, 11)	9 (5, 13)	8 (5, 10)
CD3	89 (86, 93)	94 (90, 95)	92 (89, 94)	75 (71, 79)	75 (72, 78)	76 (71, 80)
CD4	55 (44, 62)	52 (48, 58)	56 (48, 68)	46 (40, 49)	43 (41, 44)	42 (39, 47)
CD8	40 (30, 52)	37 (31, 42)	37 (29, 44)	31 (25, 35)	33 (28, 35)	30 (29, 36)
CD25, % of CD4	26 (21, 33)	26 (19, 33)	22 (19, 28)	38 (28, 41)	37 (30, 40)	36 (30, 41)
HLA-DR, % of CD4	59 (43, 70)	67 (62, 73)	68 (63, 73)	7 (6, 8)	7 (5, 8)	6 (5, 9)
CD69, % of CD4	66 (55, 72)	71 (60, 76)	71 (62, 76)	11 (9, 12)	14 (10, 17)	14 (6,17)

* Data are depicted as medians with 25 and 75% quartiles; there are no significant differences among the groups.

$CD3^+$, $CD4^+$, $CD8^+$, and natural killer cells was not changed by inhaled allergen challenge or modified by budesonide treatment (Table 4). Likewise, expression of activation markers, including the IL-2 receptor (CD25), MHC class II allergen (HLA-DR), and an early activation marker (CD69), remained unchanged by allergen challenge or budesonide treatment (Table 4).

In contrast to cell surface features for lymphocytes, budesonide caused a marked decrease in generation of IL-5 and IFN-γ by airway cells in response to *ex vivo* stimulation with T cell mitogen (PHA) (Figure 4). Budesonide treatment had no effect on PHA- or LPS-induced secretion of IL-10 or GM-CSF by BAL cells (data not shown).

Relationship of PHA-induced IL-5 Generation by BAL Cells to Airway Eosinophilia and Obstruction

When the budesonide and placebo treatment groups were analyzed as a whole, PHA-induced generation of IL-5 by airway cells obtained 48 h after allergen challenge correlated significantly with numbers of BAL eosinophils, BAL fluid levels of IL-5 and EDN, and the late-phase response to an inhaled allergen challenge (Figure 5).

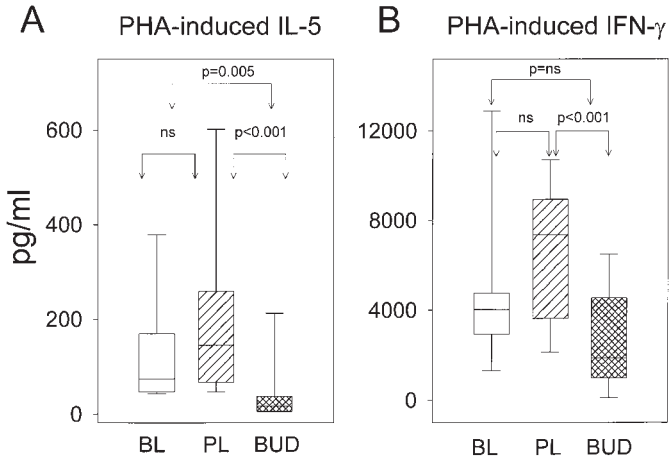

Figure 4. Effect of budesonide treatment on airway lymphocyte function. The functional capacity of airway lymphocytes to generate IL-5 (*A*) and IFN-γ (*B*) *ex vivo* was determined for BAL cells obtained during the baseline (BL) phase (*open bars*) and 48 h after a whole-lung allergen inhalation challenge, which was given at the end of a 4-wk treatment with placebo (PL, *diagonal-hatched bars*) or budesonide (BUD, *cross-hatched bars*). Data are expressed as picograms of cytokine per milliliter of culture supernatant fluid, and indicate the amount of cytokine secreted into the supernatant fluid by 2 million cells after a 48-h culture period. *Bars* represent medians within 25 and 75% quartiles, error bars represent the 10th and 90th percentiles.

Effect of Budesonide Treatment on Numbers of Peripheral Blood Eosinophils, Lymphocyte Function, and Serum Cytokine Levels

There was a trend ($p = 0.08$) toward increased numbers of circulating eosinophils and enhanced expression of CD69 ($p = 0.09$) 48 h after inhaled allergen challenge. Inhaled budesonide treatment reduced the number of peripheral blood eosinophils to below that observed before allergen challenge (Figure 6), but had no effect on the expression of CD69.

Serum levels of IL-5 were low but detectible with our enhanced ELISA. Budesonide treatment significantly reduced serum IL-5 when levels were compared with baseline values (Figure 6C). In contrast, budesonide treatment did not change concentrations of eotaxin (median within quartiles was 75 pg/ml [51, 112] for placebo and 81 pg/ml [59, 103] for budesonide, $p = 0.84$) or RANTES (median within quartiles was 73 ng/ml [47, 109] for placebo and 53 ng/ml [35, 82] for budesonide; $p = 0.37$). In contrast to effects on airway cells, treatment with budesonide did not attenuate PHA-induced generation of IL-5 (Figure 6D) or IFN-γ (median within quartiles was 1,649 pg/ml [1,221, 3,072] for placebo and 1,272 pg/ml [826, 1,885] for budesonide; $p = 0.51$) by peripheral blood mononuclear cells. Interestingly, there was a significant correlation between numbers of circulating eosinophils and BAL fluid levels of EDN and the late-phase response to allergen challenge (Figure 7).

DISCUSSION

The use of bronchoscopy and BAL after allergen challenge allowed us to evaluate the mechanisms associated with inhaled corticosteroid regulation of airway inflammation in asthma. We demonstrated that inhaled budesonide decreased *ex vivo* generation of IL-5 and IFN-γ by airway cells. The capacity of BAL cells to generate IL-5 correlated with numbers of BAL eosinophils, BAL fluid levels of EDN and IL-5, and the late-phase response to inhaled allergen challenge. In addition, we found that inhaled budesonide decreased circulating eosinophils and serum levels of IL-5. The number of circulating eosinophils was significantly correlated with the concentration of EDN in BAL fluid and the late-phase response to allergen inhalation. These findings suggest that inhaled budesonide reduces airway cell generation of IL-5, which is reflected in decreased circulating levels of IL-5 and eosinophils and, thus, a reduction in eosinophil recruitment to the airway.

The mechanisms by which inhaled budesonide reduces circulating eosinophils are unclear. Inhaled corticosteroids have

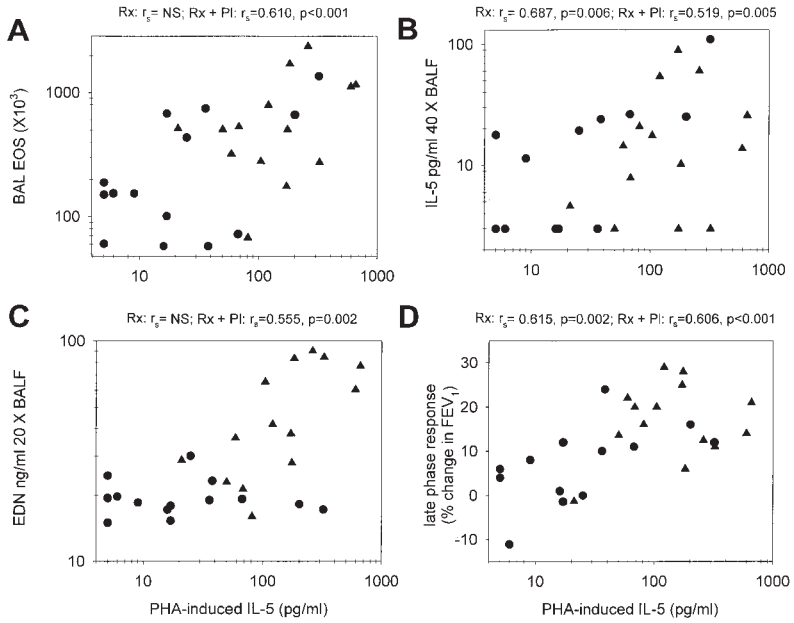

Figure 5. Correlations of PHA-induced IL-5 biological and physiological parameters. There was a significant positive correlation between the capacity of BAL cells to produce IL-5 in response to PHA and (*A*) numbers of BAL EOS. For budesonide treatment (Rx) phase, r_s = NS, for Rx + placebo (Pl) phases, $r_s = 0.610$, $p < 0.001$. (*B*) Levels of IL-5 in BAL fluid. For Rx, $r_s = 0.687$, $p = 0.006$; for Rx + Pl phases, $r_s = 0.519$, $p = 0.005$. (*C*) Levels of EDN in BAL fluid. For Rx, r_s = NS; for Rx + Pl phases, $r_s = 0.555$, $p = 0.002$. (*D*) the late-phase response to allergen. For Rx, $r_s = 0.615$, $p = 0.002$; for Rx + Pl phases, $r_s = 0.616$, $p < 0.001$. Data represent placebo (*triangles*) and budesonide (*circles*) treatment phases.

a low systemic effect (22). Budesonide treatment did not affect the expression of activation markers on circulating eosinophils, nor did it affect the number or function of circulating T cells. However, inhaled budesonide did decrease serum levels of IL-5, a cytokine that plays a key role in mobilization of eosinophils from the bone marrow (23). A study by Wood and coworkers (6) showed that treatment with a low dose (400 μg/d) of inhaled budesonide for 8 d reduced the number of IL-5-responsive progenitors cells in the bone marrow at baseline; however, it did not inhibit the allergen-induced increase in IL-5-responsive progenitors or $CD34^+$ stem cells that express the α chain of the IL-5 receptor (IL-5Rα) (6). These data raise the possibility that at low doses budesonide may not attenuate eosinophilopoiesis, rather, it may inhibit the release of mature eosinophils into the circulation and/or the recruitment of eosinophils to the airway. In our study, a 4-wk course of budesonide at 800 μg/d reduced circulating eosinophils and serum levels of IL-5, as well as the recruitment of eosinophils to the airway after allergen challenge. On the basis of these observations, we propose that at higher doses, inhaled budesonide causes changes in the microenvironment of the airway to prevent allergen-induced signals, including IL-5, to the bone marrow for production and/or maturation and release of eosinophils.

In addition to IL-5, inhaled budesonide also decreased the capacity of BAL cells to produce IFN-γ. This is in contrast to studies in which *nonchallenged* subjects with moderately severe asthma were treated for 2 wk with oral prednisolone; this resulted in decreased IL-5 and increased IFN-γ mRNA-positive cells in BAL (24) and bronchial mucosa (25). In an *ex vivo* study, Krouwels and coworkers (26) demonstrated that addition of dexamethasone (10^{-7} M) to airway- and blood-derived T cell clones reduced production of IL-5 to a greater extent

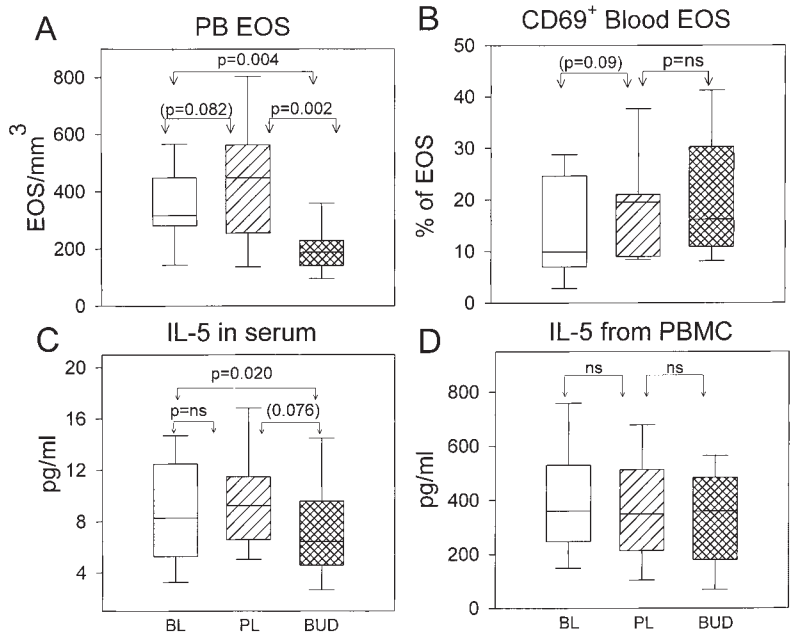

Figure 6. Effect of budesonide treatment on peripheral blood: numbers of peripheral blood eosinophils (*A*), the percentage of circulating eosinophils expressing CD69 (*B*), serum levels of IL-5 (*C*), and the capacity of peripheral blood mononuclear cells to produce IL-5 in response to PHA (10 μg/ml) (*D*). These were determined from blood samples obtained during the baseline (BL) phase (*open bars*) and 48 h after a whole-lung allergen inhalation challenge, which was given at the end of a 4-wk treatment with placebo (PL, *diagonal-hatched bars*) or budesonide (BUD, *cross-hatched bars*). *Bars* represent medians within 25 and 75% quartiles, error bars represent the 10th and 90th percentiles.

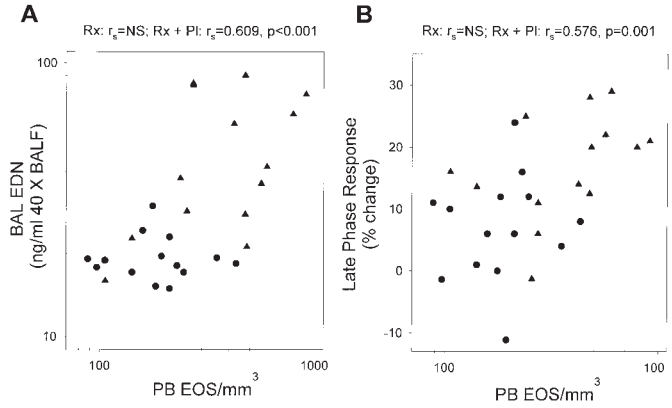

Figure 7. Correlations of numbers of circulating eosinophils and airway responses to allergen challenge. There was a significant positive correlation between numbers of circulating eosinophils and (*A*) levels of EDN in BAL fluid ($r_s = 0.609$, $p < 0.001$), and (*B*) the late-phase response to allergen ($r_s = 0.576$, $p = 0.001$). Data represent placebo (*triangles*) and budesonide (*circles*) phases.

than IFN-γ. While both these studies concluded that the helper T cell type 2 (Th2) class of cytokines are more susceptible than the Th1 class of cytokines to inhibition by corticosteroid treatment, our study provides evidence that a 4-wk treatment with inhaled budesonide attenuates both IL-5 and IFN-γ production by BAL cells.

Although the capacity of BAL cells to generate cytokines was significantly reduced by inhaled budesonide, the percentage of $CD4^+$ BAL cells that expressed the activation markers CD25 (IL-2 receptor), HLA-DR (MHC class II determinant), and CD69 (early activation marker), remained unchanged (Table 2). This is in contrast to a previous report, by Wilson and coworkers (12), that demonstrated a reduction in the number of CD25- and HLA-DR-positive BAL T cells after a 6-wk treatment of symptomatic subjects with asthma with large doses of beclomethasone (2,000 μg/d for 2 wk and 1,000 μg/d for an additional 4 wk). Interestingly, in studies using systemic corticosteroid, a reduction in numbers of T cells expressing cell surface activation markers was demonstrated in bronchial mucosa (27) and peripheral blood (28, 29), but not in the BAL (24). These data indicate that while oral corticosteroid may alter T cell phenotype, local exposure to low doses of budesonide may affect T cell function without necessarily altering the cell surface phenotype.

There are inherent limitations to our study. First, PHA is not a physiological stimulus. Nonetheless, physiological relevance is suggested by the significant correlation between generation of IL-5 by BAL cells and (*1*) the number of BAL eosinophils, (*2*) BAL fluid levels of the eosinophil product EDN, (*3*) BAL fluid concentrations of IL-5, and (*4*) the late-phase response to inhaled allergen challenge. Second, we evaluated cytokine generation in mixed populations of cells rather than purified cells or T cell clones. While there are potentially multiple cell sources of these cytokines, we have previously demonstrated that $CD4^+$ T cells are a major source of IL-5 (18). In our hands, neither purified blood nor BAL eosinophils secrete IL-5 after *ex vivo* stimulation with PHA (data not shown). Third, although subjects were selected on the basis of the presence of a late-phase response > 15% during the screening visit, a subsequent drop of 15% was not achieved in all subjects during the study phases. Nonetheless, equivalent doses of allergen were administered during the drug and placebo phase and a significant decrease in the degree of FEV_1 fall after allergen challenge was demonstrated after budesonide treatment compared with placebo. Fourth, although measures of allergen-induced airway inflammation (e.g., eosinophils, cytokine generation) were significantly lower after budesonide, the magnitude of the change was small. The low numbers of BAL eosinophils and cytokine concentrations were due to the use of whole-lung allergen inhalation challenge as opposed to segmental bronchoprovocation, which in our experience induces a more vigorous airway eosinophil response. Finally, it is also important to point out that this study was not designed to demonstrate the effect of inhaled budesonide on baseline lung function. All subjects studied had mild asthma; thus, the increase in the FEV_1 after budesonide treatment, although significant, was small.

In summary, budesonide treatment of subjects with mild asthma inhibited allergen-induced fall in pulmonary function, as well as eosinophil recruitment to the lung, *ex vivo* generation of IL-5 and IFN-γ by BAL cells, number of circulating eosinophils, and serum levels of IL-5. Taken together, these data suggest that a beneficial action of inhaled budesonide in the treatment of asthma is an inhibition of local generation of cytokines, specifically IL-5, which contributes to eosinophil recruitment to the airway. As a result, budesonide reduces allergen-induced airway inflammation and associated abnormalities in lung function.

***Acknowledgment*:** The authors thank Dr. Ross Rocklin (Astra USA) for assistance in the design of the study; Raymond Rodriguez, Cheri Swenson, and Andy Cardoni for technical expertise; Dr. Larry Schwartz for performing BAL tryptase measurements; Dr. Gerry Gleich for analysis of EDN; and Ann Dodge and Mary Jo Jackson for assistance with patient recruitment, screening, and bronchoscopies.

References

1. Boushey, H. A. 1998. Effects of inhaled corticosteroids on the consequences of asthma. *J. Allergy Clin. Immunol.* 102:S5–S16.
2. Barnes, P. J. 1998. Anti-inflammatory actions of glucocorticoids: molecular mechanisms. *Clin. Sci.* 94:557–572.
3. Busse, W. W., P. Chervinsky, J. Condemi, W. R. Lumry, T. L. Petty, S. Rennard, and R. G. Townley. 1998. Budesonide delivered by Turbuhaler is effective in a dose-dependent fashion when used in the treatment of adult patients with chronic asthma. *J. Allergy Clin. Immunol.* 101:457–463.
4. Dahl, R., and S. A. Johansson. 1982. Importance of duration of treatment with inhaled budesonide on the immediate and late bronchial reaction. *Eur. J. Respir. Dis.* 122:167–175.
5. Gauvreau, G. M., J. Doctor, R. M. Watson, M. Jordana, and P. M. O'Byrne. 1996. Effects of inhaled budesonide on allergen-induced airway responses and airway inflammation. *Am. J. Respir. Crit. Care Med.* 154: 1267–1271.
6. Wood, L. J., R. Sehmi, G. M. Gauvreau, R. M. Watson, R. Foley, J. A. Denburg, and P. M. O'Byrne. 1999. An inhaled corticosteroid, budesonide, reduces baseline but not allergen-induced increases in bone marrow inflammatory cell progenitors in asthmatic subjects. *Am. J. Respir. Crit. Care Med.* 159:1457–1463.
7. Paggiaro, P. L., F. L. Dente, M. C. Morelli, L. Bancalari, A. Di Franco, D. Giannini, B. Vagaggini, E. Bacci, L. M. Fabbri, and C. Giuntini. 1994. Postallergen inhaled budesonide reduces late asthmatic response and inhibits the associated increase of airway responsiveness to methacholine in asthmatics. *Am. J. Respir. Crit. Care Med.* 149:1447–1451.
8. Osterman, K., M. Carlholm, J. Ekelund, J. Kiviloog, K. Nikander, L. Nilholm, P. Salomonsson, V. Strand, P. Venge, and O. Zetterstrom. 1997. Effect of 1 year daily treatment with 400 microgram budesonide (Pulmicort Turbuhaler) in newly diagnosed asthmatics. *Eur. Respir. J.* 10: 2210–2215.
9. Djukanovic, R., J. W. Wilson, K. M. Britten, S. J. Wilson, A. F. Walls, W. R. Roche, P. H. Howarth, and S. T. Holgate. 1992. Effect of an inhaled corticosteroid on airway inflammation and symptoms in asthma. *Am. Rev. Respir. Dis.* 145:669–674.
10. Trigg, C. J., N. D. Manolitsas, J. Wang, M. A. Calderon, A. McAulay, S. E. Jordan, M. J. Herdman, N. Jhalli, J. M. Duddle, and S. A. Hamilton. 1994. Placebo-controlled immunopathologic study of four months of inhaled corticosteroids in asthma. *Am. J. Respir. Crit. Care Med.* 150: 17–22.

11. Lim, S., A. Jatakanon, M. John, T. Gilbey, B. J. O'Connor, K. F. Chung, and P. J. Barnes. 1999. Effect of inhaled budesonide on lung function and airway inflammation: assessment by various inflammatory markers in mild asthma. *Am. J. Respir. Crit. Care Med.* 159:22–30.
12. Wilson, J. W., R. Djukanovic, P. H. Howarth, and S. T. Holgate. 1994. Inhaled beclomethasone dipropionate downregulates airway lymphocyte activation in atopic asthma. *Am. J. Respir. Crit. Care Med.* 149:86–90.
13. Duddridge, M., C. Ward, D. J. Hendrick, and E. H. Walters. 1993. Changes in bronchoalveolar lavage inflammatory cells in asthmatic patients treated with high dose inhaled beclomethasone dipropionate. *Eur. Respir. J.* 6:489–497.
14. Jatakanon, A., S. Lim, K. F. Chung, and P. J. Barnes. 1998. An inhaled steroid improves markers of airway inflammation in patients with mild asthma. *Eur. Respir. J.* 12:1084–1088.
15. van Rensen, E. L., K. C. Straathof, M. A. Veselic-Charvat, A. H. Zwinderman, E. H. Bel, and P. J. Sterk. 1999. Effect of inhaled steroids on airway hyperresponsiveness, sputum eosinophils, and exhaled nitric oxide levels in patients with asthma. *Thorax* 54:403–408.
16. Lemanske, R. F., and M. A. Kaliner. 1993. Late phase reactions. *In* Allergy: Principles and Practice. Mosby, St. Louis. 320–361.
17. Chai, H., R. Farr, and L. A. Froelich. 1975. Standardization of bronchial inhalation challenge procedures. *J. Allergy Clin. Immunol.* 56:323–327.
18. Kelly, E. A., R. R. Rodriguez, W. W. Busse, and N. N. Jarjour. 1997. The effect of segmental bronchoprovocation with allergen on airway lymphocyte function. *Am. J. Respir. Crit. Care Med.* 156:1421–1428.
19. Givan, A. L. 1992. Cells from without: lymphocytes and the strategy of gating. *In* Anonymous Flow Cytometry: First Principles. Wiley-Liss, New York. 75–102.
20. Abu-Ghazaleh, R. I., T. Fujisawa, J. Mestecky, R. A. Kyle, and G. J. Gleich. 1989. IgA-induced eosinophil degranulation. *J. Immunol.* 142: 2393–2400.
21. Bowsher, R. R., K. M. Verburg, and D. P. Henry. 1983. Rat histamine *N*-methyltransferase: quantification, tissue distribution, purification, and immunological properties. *J. Biol. Chem.* 258:12215–12220.
22. Hogan, S. P., A. W. Mould, J. M. Young, M. E. Rothenberg, A. J. Ramsay, K. Matthaei, I. G. Young, and P. S. Foster. 1998. Cellular and molecular regulation of eosinophil trafficking to the lung. *Immunol. Cell Biol.* 76:454–460.
23. Palframan, R. T., P. D. Collins, N. J. Severs, S. Rothery, T. J. Williams, and S. M. Rankin. 1998. Mechanisms of acute eosinophil mobilization from the bone marrow stimulated by interleukin 5: the role of specific adhesion molecules and phosphatidylinositol 3-kinase. *J. Exp. Med.* 188:1621–1632.
24. Robinson, D., Q. Hamid, S. Ying, A. Bentley, B. Assoufi, S. Durham, and A. B. Kay. 1993. Prednisolone treatment in asthma is associated with modulation of bronchoalveolar lavage cell IL-4, IL-5 and interferon gamma cytokine gene expression. *Am. Rev. Respir. Dis.* 148:401–406.
25. Bentley, A. M., Q. Hamid, D. S. Robinson, E. Schotman, Q. Meng, B. Assoufi, A. B. Kay, and S. R. Durham. 1996. Prednisolone treatment in asthma. Reduction in the numbers of eosinophils, T cells, tryptase-only positive mast cells, and modulation of IL-4, IL-5, and interferon-gamma cytokine gene expression within the bronchial mucosa. *Am. J. Respir. Crit. Care Med.* 153:551–556.
26. Krouwels, F. H., J. F. van der Heijden, R. Lutter, R. J. van Neerven, H. M. Jansen, and T. A. Out. 1996. Glucocorticosteroids affect functions of airway- and blood-derived human T-cell clones, favoring the Th1 profile through two mechanisms. *Am. J. Respir. Cell Mol. Biol.* 14:388–397.
27. Djukanovic, R., S. Homeyard, C. Gratziou, J. Madden, A. Walls, S. Montefort, D. Peroni, R. Polosa, S. Holgate, and P. Howarth. 1997. The effect of treatment with oral corticosteroids on asthma symptoms and airway inflammation. *Am. J. Respir. Crit. Care Med.* 155:826–832.
28. Gemou-Engesaeth, V., A. Bush, A. B. Kay, Q. Hamid, and C. J. Corrigan. 1997. Inhaled glucocorticoid therapy of childhood asthma is associated with reduced peripheral blood T cell activation and "Th2-type" cytokine mRNA expression. *Pediatrics* 99:695–703.
29. Corrigan, C. J., A. Haczku, V. Gemou-Engesaeth, S. Doi, Y. Kikuchi, K. Takatsu, S. R. Durham, and A. B. Kay. 1993. CD4 T-lymphocyte activation in asthma is accompanied by increased serum concentrations of interleukin-5. Effect of glucocorticoid therapy. *Am. Rev. Respir. Dis.* 147:540–547.