Inhibition of allergic airways inflammation and airway hyperresponsiveness in mice by dexamethasone: Role of eosinophils, IL-5, eotaxin, and IL-13

Seok-Yong Eum, MD, Karim Maghni, PhD, Qutayba Hamid, MD, PhD, David H. Eidelman, MD, Holly Campbell, BSc, Susumu Isogai, MD, and James G. Martin, MD *Montreal, Quebec, Canada*

Background: Glucocorticoids inhibit allergen-induced airway eosinophilia and airway hyperresponsiveness (AHR). Whether glucocorticoids mediate their effects on AHR by inhibiting eotaxin and IL-5, 2 of the principal mediators of eosinophilia, or through IL-13, an important mediator of AHR, has not been established.

Objective: We sought to investigate the effects of glucocorticoids on airway eosinophilia and the expression of IL-5, eotaxin, and IL-13 in relation to the induction of AHR in a murine model of allergic asthma.

Methods: Dexamethasone (4 mg/kg) and mAbs against eotaxin (80 □g/kg) and IL-5 (100 □g/kg) singly and in combination were administered to immunized mice before antigen challenge. Airway responsiveness to methacholine was measured in anesthetized and mechanically ventilated animals. Eotaxin, IL-5, and IL-13 in bronchoalveolar lavage fluid (BALF), lung homogenates, or both were measured by means of ELISA.

Results: A single antigen challenge induced AHR that lasted at least 10 days. Eotaxin protein and mRNA levels increased in lung tissue but not in BALF after challenge. IL-5 protein and mRNA levels increased both in BALF and in lung tissue. Dexamethasone reduced airway eosinophilia, AHR, and protein and mRNA for eotaxin and IL-5. Anti-murine eotaxin and anti-IL-5 antibodies alone and in combination reduced the ovalbumin-induced airway eosinophilia significantly but failed to inhibit AHR. Both dexamethasone and anti-IL-5/anti-eotaxin inhibited the increases in lung IL-13 levels after ovalbumin challenge to a similar extent.

Conclusion: These findings suggest that the inhibition of AHR by the glucocorticoid dexamethasone does not appear to be explained by effects on eosinophilia, eotaxin, IL-5, or IL-13. (J Allergy Clin Immunol 2003;111:1049-61.)

From Meakins-Christie Laboratories, Department of Medicine, McGill University, Montreal.

Dr Eum is supported by a grant from Merck Inc and CIHR grant no. MOP-10381. Dr Maghni is the recipient of an MRC fellowship award. Dr Eidelman is the recipient of a scholarship award of the Fonds de Recherche en Santé du Québec.

Received for publication March 14, 2002; revised January 19, 2003; accepted for publication January 21, 2003.

Reprint requests: James G. Martin, MD, Meakins-Christie Laboratories, McGill University, 3626, rue St-Urbain, Montreal, QC, Canada H2X 2P2.

0091-6749/2003 $30.00 + 0
doi:10.1067/mai.2003.1416

Key words: *Asthma, glucocorticoid, eotaxin, IL-5, murine model*

Glucocorticoids are among the first-line agents used in the treatment of asthma. The mechanisms of action of these molecules are potentially several,[1-4] but the predominant site of action that accounts for their therapeutic efficacy is still a matter of controversy. Recruitment of eosinophils to the airways is a characteristic of asthma, and the degree of eosinophilia is correlated with the severity of this disease.[5] These cells are often considered to play a major role in inducing airway hyperresponsiveness (AHR), potentially by means of the secretion of their toxic cationic proteins, which might damage the airway mucosa.[6] The mechanisms that regulate the selective accumulation of eosinophils involve eosinophil hematopoiesis, adhesion to endothelium, chemotaxis, and survival. Glucocorticoids potently inhibit airway eosinophilia by actions at all stages of the abovementioned processes.[1,7-9]

Eosinophilic inflammation is regulated to a major extent by activated T lymphocytes in the airways that secrete the T_H2 cytokine IL-5. This cytokine is a central mediator in the regulation of eosinophilic inflammation through effects on the proliferation, differentiation, and activation of eosinophils,[10,11] as well as providing a signal for the rapid mobilization of eosinophils from the bone marrow.[11] Eosinophil adhesion and locomotion are predominantly controlled by chemoattractants. There is increasing recognition of the importance of chemokines released by the epithelium and by other airway cells in response to allergic challenge[12] in the recruitment of leukocytes to the sites of inflammation. The C-C chemokines are most closely related to allergic inflammation[13] and include eotaxin, an eosinophil-specific chemoattractant.[14,15] Eotaxin also has a potent and selective effect in mobilizing bone marrow eosinophils into the blood.[16] The question of whether the reduction of eosinophilia by glucocorticoids is sufficient to explain their efficacy in inhibiting allergen-induced AHR formed the basis for the current study. We hypothesized that dexamethasone prevents AHR by downregulating eotaxin and IL-5 expression and, in so doing, by reducing

Abbreviations used
BAL: Bronchoalveolar lavage
BALF: Bronchoalveolar lavage fluid
MBP: Major basic protein
OVA: Ovalbumin
R_{rs}: Respiratory system resistance

eosinophilia. We also selectively reduced airway eosinophilia using specific mAbs against eotaxin and IL-5 to an extent that was comparable in magnitude with the reduction caused by dexamethasone. We confirmed the efficacy of our interventions in reducing airway tissue eosinophilia by means of morphometry. Having established conditions for the comparable inhibition of eosinophilia by means of both approaches, we then compared the effects of dexamethasone treatment on allergen-induced AHR with those of anti-eotaxin and anti-IL-5 antibody treatments. We also compared the effects of dexamethasone and antibody treatments on the levels of IL-13 in lung homogenates because IL-13 is a candidate cytokine for the mediation of allergen-induced AHR,[17] the inhibition of which might provide an alternate explanation for the effects of dexamethasone.

METHODS

Immunization and antigen challenge of mice

Male A/J mice 6 to 8 weeks of age were purchased from Harlan Sprague Dawley, Inc. The protocols were approved by an institutional animal care committee. Mice were immunized twice 7 days apart with 100 μg of ovalbumin (OVA) administered subcutaneously in 0.4 mL of a 4 mg/mL suspension of $Al(OH)_3$. One week after the second injection, mice were challenged with 10 μg of OVA in 50-μL sterile saline administered intranasally after achievement of light anesthesia.

Evaluation of airway responsiveness

Airway responsiveness was measured 24, 48, 96, and 240 hours after a single OVA challenge in mice that were sedated (xylazine, 8 mg/kg administered intraperitoneally), anesthetized (pentobarbital, 70 mg/kg administered intraperitoneally), tracheostomized, paralyzed (doxacurium, 0.5 mg/kg administered intraperitoneally), and attached to a small-animal ventilator (Flexivent, SCIREQ). Animals were ventilated quasisinusoidally (150 breaths/min, 6 mL/kg, positive end-expiratory pressure of 1.5 hPa). After a standard volume history, small-amplitude volume oscillations at frequencies of 0.9, 4.8, and 10.4 Hz were applied at a constant lung volume to the tracheal opening for 16 seconds, and respiratory system resistance (R_{rs}) was measured. Responses at 0.9 Hz exceeded those at other frequencies so that only results for this frequency of oscillation are reported. Methacholine was injected through the jugular vein every 5 minutes at doses of 3.3, 10, 33, 100, 330, and 1000 μg/kg, and measurements of R_{rs} were made immediately afterward.

Bronchoalveolar lavage

After measurement of airway responses, bronchoalveolar lavage (BAL) was performed by using 9 aliquots (0.5 mL) of PBS. The first 0.5-mL BAL fluid sample was retained for measurements of IL-5 and eotaxin by means of ELISA, and the subsequent 8 volumes (4.0 mL) were used for other measurements. Total cell numbers were counted with a hemacytometer. The cytospin slides of BAL cells (Cytospin model II, Shandon) were stained with May-Grünwald-Giemsa stain. Differential cell counts were based on a count of at least 200 cells. Absolute cell numbers were also calculated as the product of the total and differential cell counts.

Evaluation of airway tissue eosinophilia

The eosinophil numbers in the airway walls were evaluated on lung tissues that were fixed with 10% buffered formalin at a pressure of 25 cm of H_2O. The tissues were immunostained with a rabbit anti-mouse polyclonal antibody for major basic protein (MBP; kindly provided by Dr G. Gleich, Mayo Clinic, Rochester, Minn) and developed by using swine anti-rabbit biotin-conjugated secondary antibody (DAKO Canada Inc) and the alkaline phosphatase–antialkaline phosphatase method. Slides were counterstained with hematoxylin. The airways were examined by means of light microscopy (magnification 400×), and the number of eosinophils present in 3 randomly selected airways was determined. The total number of nuclei was also counted, and the eosinophil count was expressed as a percentage of the total cells in the airway.

Treatments with anti-IL-5 and anti-eotaxin antibodies and dexamethasone

Monoclonal anti-IL-5 (TRFK-5) and anti-eotaxin antibodies (R&D Systems, Inc) were injected alone or in combination into immunized mice at 100 μg/kg (TRFK-5)[18] or 800 μg/kg (anti-eotaxin)[19] intravenously 30 minutes before antigen challenge. For control mice, the same quantity of rat IgG isotype was injected. Dexamethasone phosphate was injected at a dose of 4 mg/kg in 100 μL of sterile saline intraperitoneally 1 hour before antigen challenge. For control mice, the same volume of saline was injected. The evaluation of BAL fluid (BALF) and AHR was performed 48 hours after antigen challenge.

Eotaxin, IL-5, and IL-13 levels in BALF, lung tissues, or both

The levels of IL-5 and eotaxin in BALF and in lung homogenates and IL-13 in lung homogenates were determined by means of ELISA. Lungs were homogenized in PBS (1 mL/50 mg of lung tissue), and the supernatant was stored at –80°C until use. Ninety-six-well plates were coated with an anti-IL-5 mAb (TRFK-5, R&D Systems, Inc), an anti-eotaxin polyclonal antibody (R&D Systems, Inc), or an anti-mouse IL-13 mAb (R&D systems, Inc) at 4°C overnight. The plates were blocked with PBS containing 10% complement-depleted FBS and incubated with the supernatants of BALF or lung homogenates at room temperature. The plates were then incubated with biotinylated anti-IL-5 mAb (TRFK-4, Pharmingen), anti-eotaxin polyclonal antibody (R&D Systems, Inc), or anti-mouse IL-13 polyclonal antibody (R&D systems, Inc) at room temperature. Then the plates were incubated with streptavidin–horseradish peroxidase (Pharmingen), and ABTS substrate solution containing H_2O_2 was added to the wells. The plates were read at an optical density of 405 nm. A standard curve was generated with varying concentrations of each recombinant cytokine. The limit of detection was at least 10 pg/mL for IL-5, eotaxin, and IL-13.

RT-PCR analysis

Total RNA was extracted from BAL cells and lung tissues with TRIZOL (Gibco BRL), as previously described.[19] RNA pellets were dissolved in RNAse- and DNAse-free water (Ambion Inc). Strand cDNA was made in a 20-μL reaction by use of 2 μg of total RNA as template and oligo$(dT)_{12\text{-}18}$ primer and Superscript II enzyme in the presence of acetylated BSA (Gibco BRL) and RNAguard ribonuclease (Pharmacia Biotech) as enzyme inhibitors. The

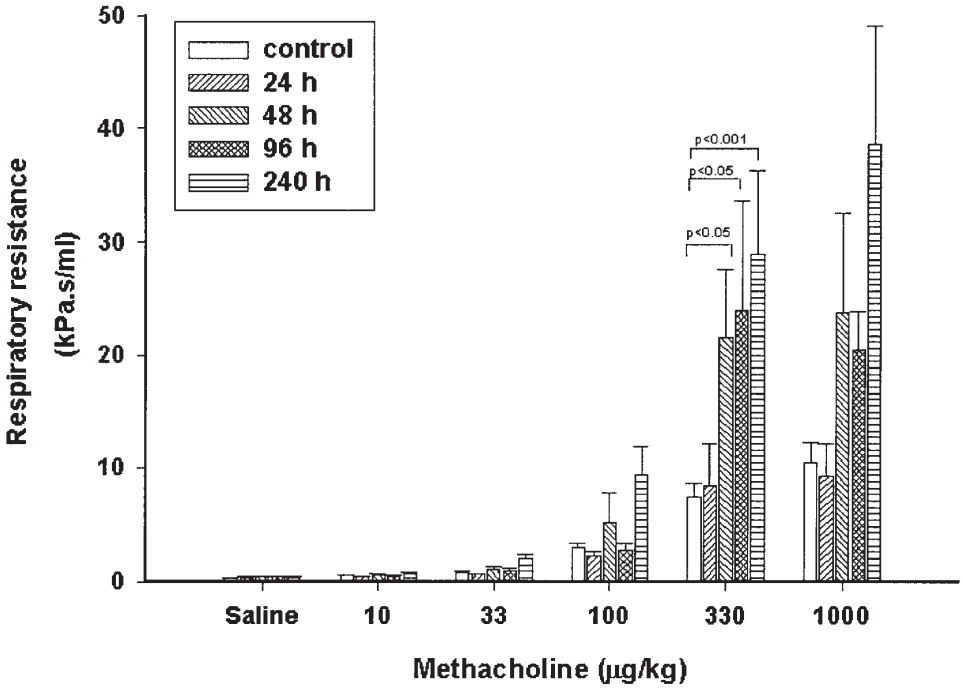

FIG 1. Effect of antigen challenge on the increase of airway responsiveness to methacholine with time in immunized A/J mice. Control animals *(saline)* were saline challenged. Each value represents the mean ± SEM of 6 to 7 mice. Significant differences at 330 μg/kg methacholine compared with the saline-challenged control group are indicated.

PCR mixture consisted of 1.5 mmol/L $MgCl_2$, 1× PCR buffer, 0.2 mmol/L deoxyribonucleoside triphosphate mixture, 2.5 units of Platinum Taq polymerase (Gibco BRL), 20 pmol of the upstream and downstream primers, as well as the synthesized cDNA strand. The primers used were 5′-ACTCTCAGCTGTGTCTGGG-3′ (sense) and 5′-GCCCACTCTGTACTCATCAC-3′(antisense) for IL-5, 5′-TTCTATTCCTGCTGCTCACG-3′(sense) and 5′-TTATG-GTTTTGGAGTTTGGAG-3′(antisense) for eotaxin, and 5′-GGT-CAACCCCACCGTGTTCTTCG-3′(sense) and 5′-GTGCTCTC-CTGAGCTACAGAAGG-3′(antisense) for the housekeeping gene cyclophilin. The samples were amplified in a Programmable Thermal Controller (PTC-100, MJ Research Inc) for 35 cycles (1 minute of denaturation at 92°C, 2 minutes of annealing at 56°C, and 3 minutes of extension at 72°C) for IL-5, 30 cycles for eotaxin, and 28 cycles for cyclophilin (1 minute of denaturation at 92°C, 2 minutes of annealing at 60°C, and 3 minutes of extension at 72°C). The PCR products were visualized by means of ethidium bromide staining after agarose gel (2%) electrophoresis, and the size of the bands was determined by means of comparison with DNA molecular weight markers (Roche Molecular Biochemicals). The results are expressed as the densitometric ratios of IL-5 or eotaxin mRNA bands compared with those of the corresponding cyclophilin bands, which were determined by using a FluorChem imaging system (Alpha Innotech Corp). PCR primers were synthesized and purified by means of FLPC at the Sheldon Biotechnology Centre.

Statistical analysis

The data are presented as means ± SEM. Statistical comparisons were performed by using 1-way ANOVA, followed by the Fisher least significant difference test. For measurements of airway responsiveness, the analysis was performed at 330 or 1000 μg/kg methacholine because the largest differences between the groups were seen at these doses. For comparisons of data that were not normally distributed, a Kruskal-Wallis test followed by a Mann-Whitney *U* test was performed. A *P* value of less than .05 was considered significant.

RESULTS

Characterization of temporal changes in allergic airways inflammation and AHR

OVA challenge increased total cell and eosinophil numbers in BALF in a time-dependent manner. Cell numbers peaked at 48 hours and decreased subsequently to reach similar levels to those in control animals by 240 hours (Table I). Lymphocytes were increased significantly in the time period from 48 to 240 hours after OVA challenge, whereas there were no significant increases in either macrophage or neutrophil numbers. In saline-challenged mice greater than 90% of total BALF cells were macrophages, whereas eosinophils numbered less than 1%.

In all groups of mice, there were dose-dependent increases in R_{rs} to intravenously injected methacholine over the range of frequencies examined. There were no differences between baseline R_{rs} and the responses at 3.3 μg of methacholine between saline- and OVA-challenged animals (data not shown). However, at higher doses, R_{rs} increased after methacholine injections, and in OVA-challenged mice the increase in R_{rs} to methacholine was significantly higher than that in saline-challenged mice, confirming the development of AHR. AHR was observed 48 hours after antigen challenge and was sustained at least to 240 hours (Fig 1).

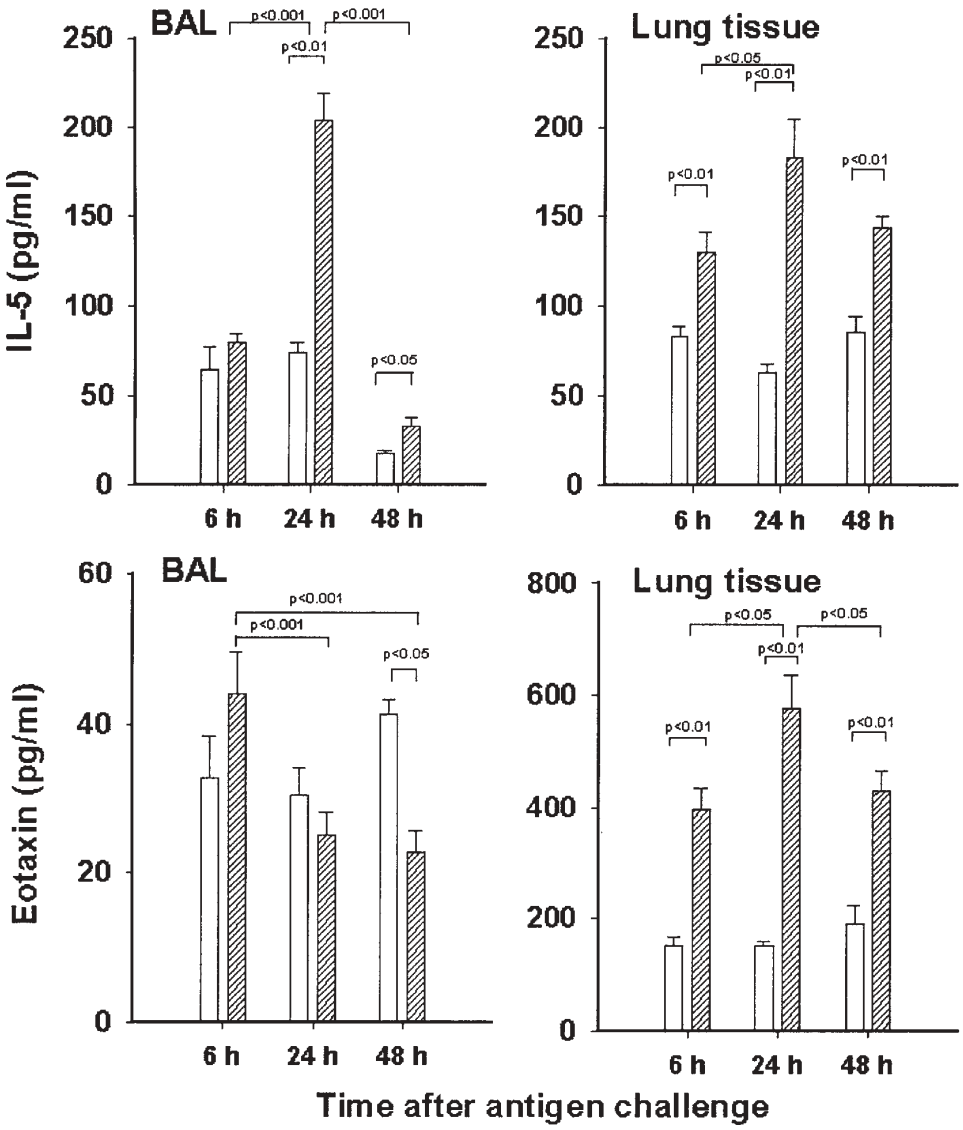

FIG 2. Effects of saline and OVA challenge on IL-5 and eotaxin levels in BALF and lung tissue. Samples were obtained at 6, 24, and 48 hours after challenge. The number of mice per group ranged from 5 to 12. *Open bars* represent saline-challenged animals, and *hatched bars* represent OVA-challenged animals.

The kinetics of antigen-induced lung eotaxin and IL-5 expression

Eotaxin and IL-5 protein levels were measured in BALF and in lung homogenates after antigen challenge (Fig 2). In nonimmunized mice approximately 16.5 ± 0.8 pg/mL eotaxin was detected in BALF. There were significant increases after immunization and challenge, but no difference was observed between the sham-challenged and OVA-challenged groups. Indeed, at 48 hours, levels of eotaxin were higher in sham-challenged mice than in the OVA-challenged group. In lung tissue, however, the levels of eotaxin in OVA-challenged mice exceeded those in the saline-challenged group from 6 to 48 hours. The IL-5 level in the BALF of nonimmunized mice was 38.5 ± 3.5 pg/mL. After immunization and antigen challenge, this amount increased time dependently, reaching a peak at 24 hours. However, even at 48 hours, IL-5 was still significantly higher than that of saline-challenged mice. In lung tissue the IL-5 levels in OVA-challenged mice were significantly higher than those in saline-challenged animals from 6 to 48 hours after challenge.

Modulation of airway eosinophilia and AHR by eotaxin, IL-5, and dexamethasone

After the confirmation of increased levels of immunoreactive eotaxin and IL-5 in BALF and lung tissue after a single antigen challenge, we next evaluated the effects of neutralizing mAbs against eotaxin and IL-5 (TRFK-5) on airway eosinophilia and AHR. All the measurements were performed at 48 hours after antigen challenge because the maximal eosinophil numbers were

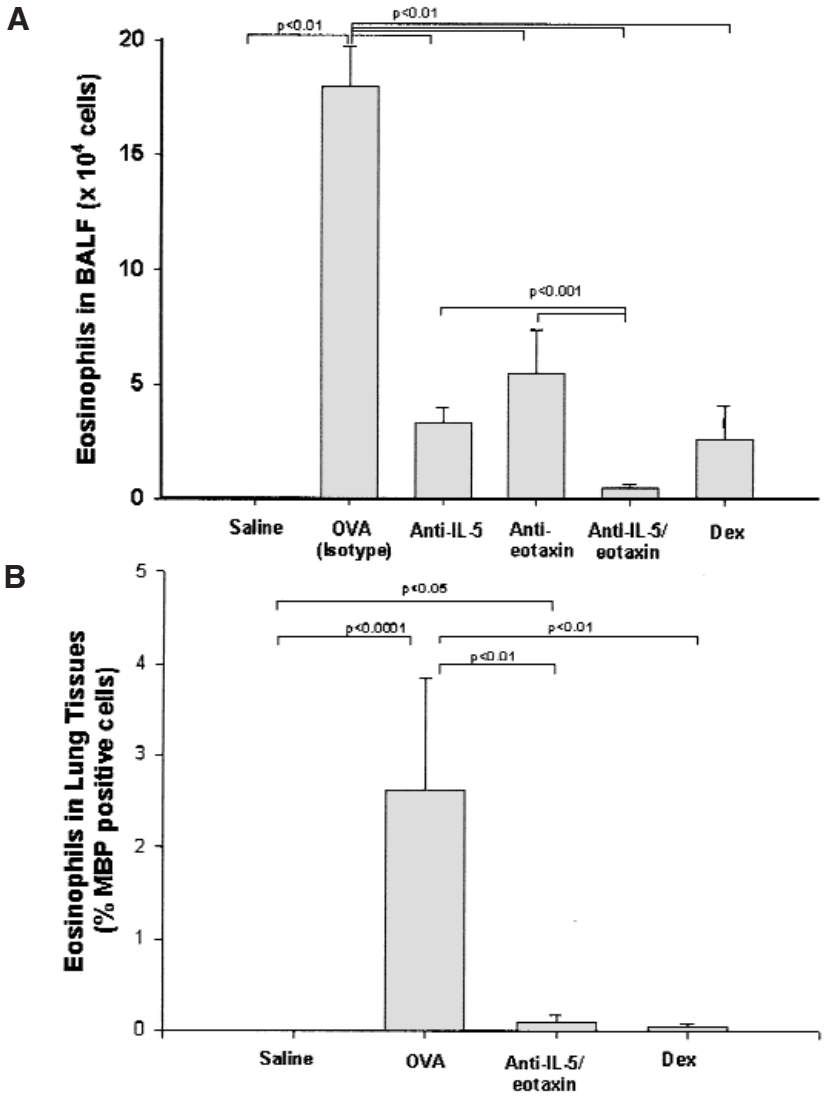

FIG 3. Effects of anti-IL-5, anti-eotaxin, or dexamethasone treatment on eosinophil recruitment into BALF **(A)** and lung tissues **(B)**. Antibodies were injected intravenously separately or in combination to immunized mice 30 minutes before OVA challenge. Dexamethasone was injected intraperitoneally 1 hour before challenge. An isotype control antibody (rat IgG) was injected into control mice. BALF and lung tissues were obtained 48 hours after challenge. Each value represents the mean ± SEM of 5 to 6 mice.

observed in BALF at this time point. Similar to the findings from animals studied to establish the kinetics of changes in AHR, 48 hours after OVA challenge, the eosinophil numbers were increased in BALF (17.9 ± 1.7 □ 10^4 cells) compared with those in saline-challenged mice (0.03 ± 0.02 □ 10^4 cells, $P < .001$). Lymphocyte numbers were also significantly increased (from 0.01 ± 0.01 □ 10^4 cells to 0.28 ± 0.06 □ 10^4 cells, $P < .05$). Pretreatment with anti-eotaxin antibody significantly reduced eosinophils in BALF (5.5 ± 1.9 □ 10^4 cells, $P < .001$; Fig 3, *A*), whereas macrophages, neutrophils, and lymphocytes were unaffected (data not shown). Treatment with TRFK-5 also reduced eosinophil numbers significantly (3.4 ± 0.7 □ 10^4 cells, $P < .001$; Fig 3, *A*), whereas there were no significant changes in other leukocytes (data not shown). Administration of anti-eotaxin and TRFK-5 in the same doses but in combination almost completely blocked the recruitment of eosinophils (0.5 ± 0.2 □ 10^4 cells, $P < .001$; Fig 3, *A*). However, despite the marked reduction in eosinophils in BALF by anti-eotaxin and TRFK-5 administered singly or in combination, no inhibition of AHR was observed (Fig 4).

Dexamethasone (4 mg/kg administered intraperitoneally) administered 1 hour before antigen challenge markedly reduced the eosinophil numbers in BALF 48 hours after antigen challenge (from 17.9 ± 1.7 □ 10^4 cells to 2.6 ± 1.5 □ 10^4 cells, $P < .001$; Fig 3, *A*). Lymphocyte numbers were also reduced significantly after antigen challenge in dexamethasone-treated mice compared with in saline-treated animals (from 0.3 ± 0.1 □ 10^4 cells to

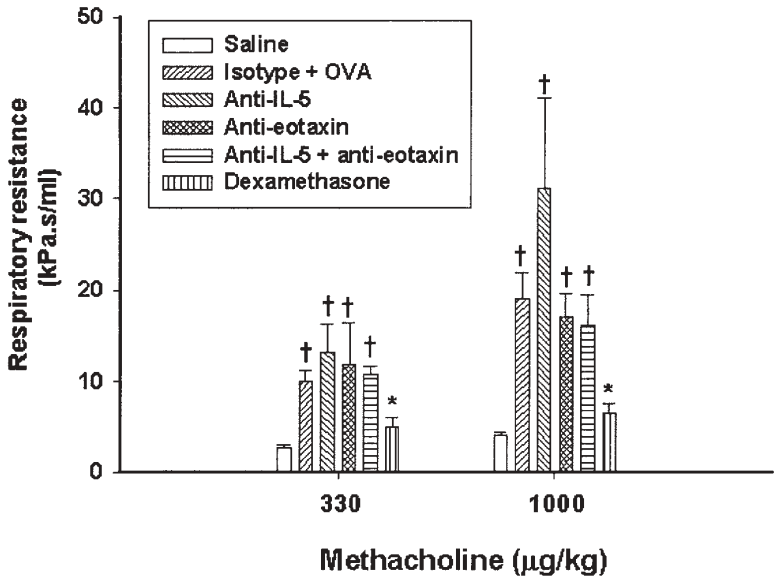

FIG 4. Effect of anti-IL-5, anti-eotaxin, or dexamethasone on AHR. The results at 330 and 1000 □g/kg methacholine are shown because the biggest differences were observed at these doses. AHR was evaluated at 48 hours after airway challenge. Each value represents the mean of 6 animals for each of the treatment groups and 21 animals for the control group. Comparisons with saline-challenged animals are indicated as follows: †$P < .001$. Differences between isotype-treated and OVA-challenged animals and dexamethasone-pretreated animals are indicated as follows: *$P < .05$.

TABLE I. BAL cells (□ 10^4/mL) after antigen challenge in immunized A/J mice*

Time after challenge	Total cells	Macrophages	Eosinophils	Neutrophils	Lymphocytes
Saline	6.5 (0.5)	6.33 (0.54)	0.01 (0.003)	0.13 (0.03)	0.01 (0.005)
24 h	11.6 (2.4)†	6.19 (1.07)	4.64 (1.41)‡	0.69 (0.24)†	0.07 (0.04)
48 h	22.7 (5.6)‡	7.79 (1.70)	14.41 (3.93)‡	0.28 (0.08)	0.4 (0.11)‡
96 h	9.2 (1.6)†	6.24 (0.40)	4.07 (1.05)‡	0.14 (0.05)	0.22 (0.14)†
240 h	8.0 (0.4)	7.49 (0.31)	0.28 (0.12)†	0.11 (0.03)	0.13 (0.09)†

*The data are based on measurements in groups of 6 to 7 mice. SEMs are shown in parentheses. Significant differences between OVA-challenged animals and the control groups are shown for each time point: †$P < .05$ and ‡$P < .001$.

0.02 ± 0.01 □ 10^4 cells, $P < .05$). In contrast to the treatment with eotaxin and IL-5 antibodies, dexamethasone significantly reduced AHR (Fig 4).

To ensure that BALF eosinophil counts were representative of the tissue cell numbers, we performed a morphometric analysis of tissue eosinophils. We found that the analysis of MBP immunoreactive cells in lung tissues yielded similar results to the analysis of BALF. Three airways were analyzed for each animal from each of 4 groups (Fig 3, *B*): saline-challenged animals (n = 6), OVA-challenged animals (n = 6), anti-IL-5/anti-eotaxin–treated and OVA-challenged animals (n = 4), and dexamethasone-treated and OVA-challenged animals (n = 4). There were no eosinophils (expressed as a percentage of the total airway cells) in the saline-challenged group. The number of eosinophils was markedly increased in the OVA-challenged group, and the numbers were markedly reduced by antibody and dexamethasone treatments. Only the OVA-challenged group had significantly different counts from the other groups.

Effects of dexamethasone on antigen-induced eotaxin and IL-5 expression (mRNA and protein) in BALF cells and lung tissue

The effect of dexamethasone on the eotaxin and IL-5 mRNA and protein levels was examined in BALF cells and lung tissue harvested at 24 hours after antigen challenge to address the question of whether dexamethasone was likely to mediate its effects on eosinophilia through the inhibition of eotaxin and IL-5. This time point was chosen because the maximal levels of eotaxin and IL-5 were detected at this time point. Eotaxin mRNA was undetectable in BALF cells (not shown) but was found in the lung tissues and was significantly higher in OVA-challenged mice compared with saline-challenged animals (Fig 5). Dexamethasone almost completely inhibited the eotaxin mRNA levels in lung tissue. IL-5 mRNA increased after antigen challenge in both BALF cells (Fig 6) and lung tissue at 24 hours (Fig 7) and was also significantly reduced by dexamethasone.

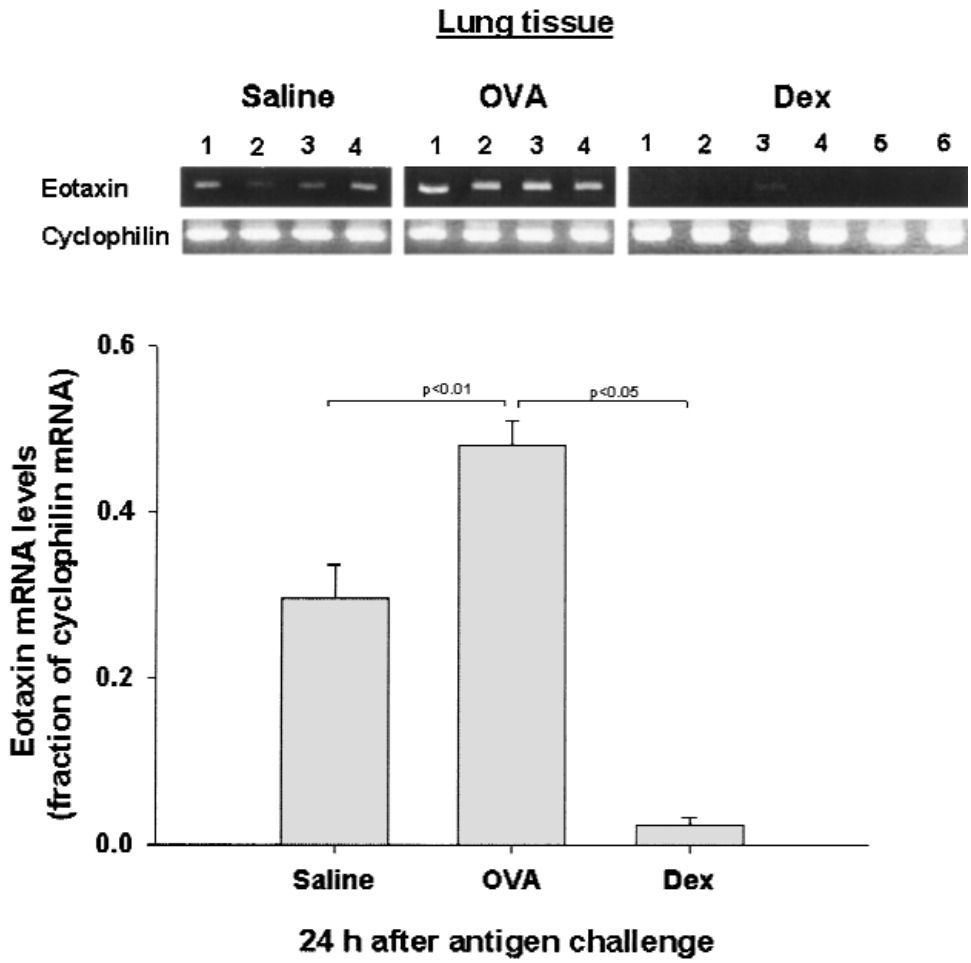

FIG 5. RT-PCR analysis of eotaxin mRNA expression in lung tissues after saline (n = 4), OVA challenge (n = 4), or OVA challenge plus dexamethasone pretreatment (n = 6). The *top panel* shows the agarose gel stained with ethidium bromide and visualized by means of UV light, and the *bottom panel* shows densitometry for eotaxin mRNA relative to cyclophilin mRNA.

We measured protein levels for IL-5 and eotaxin in BALF and lung tissue by means of ELISA (Fig 8). Dexamethasone was again administered 1 hour before challenge, and BALF and lung homogenates were obtained 24 hours after antigen challenge. At 24 hours after antigen challenge, dexamethasone reduced BAL eosinophils from 7.8 ± 2.8 to 0.05 ± 0.01 □ 10^4 cells/mL ($P < .001$). Dexamethasone reduced eotaxin levels in lung tissue to less than the basal values (Fig 8). In contrast, there was a slight increase in BALF eotaxin levels after treatment with dexamethasone. IL-5 levels were reduced by dexamethasone in both BALF and lung tissue. Combined anti-IL-5/anti-eotaxin antibody treatment also reduced IL-5 levels in BALF to levels (62.9 ± 8.9 pg/mL) similar to baseline (76.7 ± 6.2 pg/mL), but eotaxin was unaffected. The effects of antibody treatment on lung tissue IL-5 and eotaxin levels were not tested.

Effects of dexamethasone and anti-IL-5/anti-eotaxin on antigen-induced IL-13 protein in lung tissue

To explore the possibility that a reduction in IL-13 expression by dexamethasone might have accounted for the inhibitory effects of dexamethasone on AHR, we measured IL-13 protein in the lung tissues of animals after saline challenge, OVA challenge alone, and OVA challenge with dexamethasone or anti-IL-5/anti-eotaxin antibody treatments. IL-13 was detectable in all groups and was significantly increased after OVA challenge. The levels were lower in both dexamethasone- and antibody-treated groups of animals than in the OVA-challenged but OVA-untreated animals (Fig 9). There was no difference in the effect of dexamethasone and antibody treatments on the IL-13 levels, however.

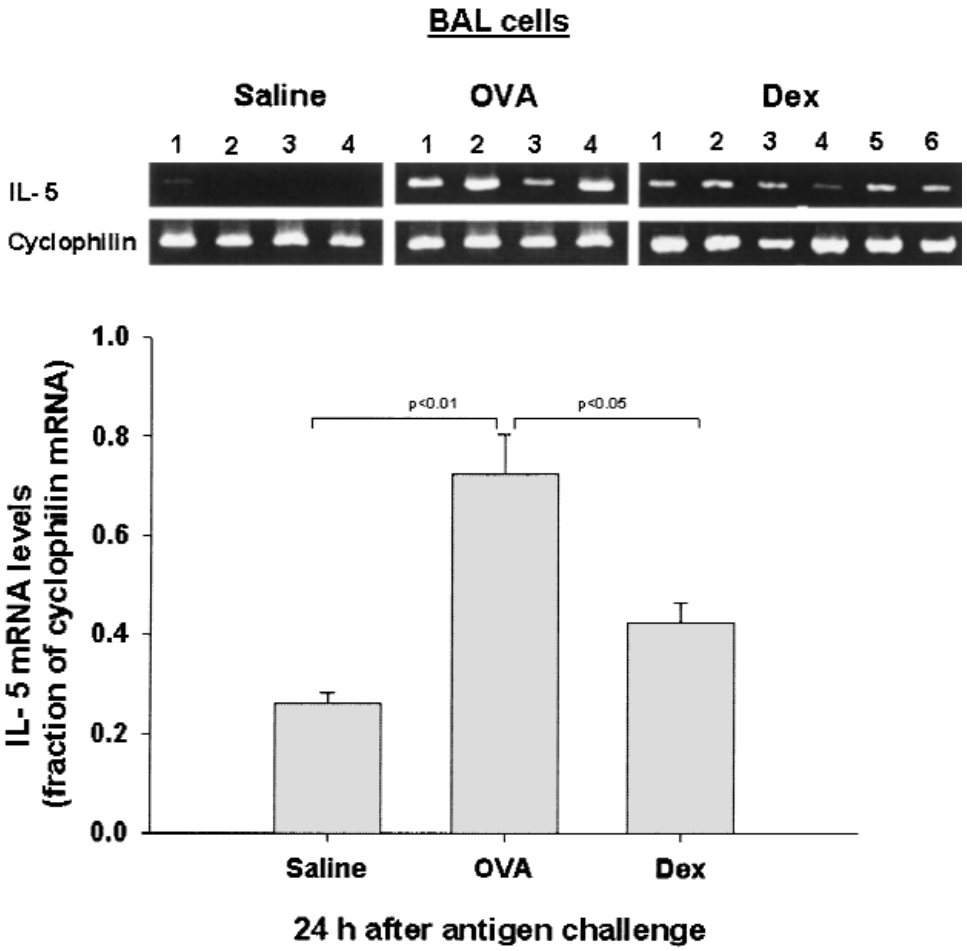

FIG 6. RT-PCR analysis of IL-5 mRNA expression in BAL cells. The BAL cells were harvested at 24 hours after challenge with saline, OVA, or OVA after dexamethasone pretreatment. The *top panel* shows the agarose gel stained with ethidium bromide and visualized with UV light. The *bottom panel* shows densitometry for IL-5 mRNA relative to cyclophilin mRNA.

DISCUSSION

In this study we examined the airway responses of the sensitized mouse to allergen challenge. We were specifically interested in the roles of eosinophilia, eotaxin, and IL-5 in the inhibitory effects of dexamethasone on allergen-induced AHR. To address the issue, we used a murine model that involved only a single airway exposure to OVA that resulted in AHR by 48 hours and that persisted for at least 10 days. The duration of AHR contrasted with the kinetics of eosinophilia, which reached a peak by 48 hours but virtually resolved by 10 days after challenge. Dexamethasone inhibited both IL-5 and eotaxin expression, as well as eosinophilia. Inhibition of eosinophilia by antibodies against eotaxin and IL-5 effected an inhibition of eosinophilia of equal or greater magnitude in both BALF and lung tissues but failed to influence AHR, suggesting that dexamethasone is unlikely to exert its effects on AHR by modulating IL-5, eotaxin, or eosinophilia. Given the lack of support for the importance of eosinophils and eosinophil-related cytokines in AHR, we examined the possibility that a reduction in IL-13 might result from dexamethasone treatment but not from anti-IL-5/anti-eotaxin treatment. However, we found that anti-IL-5/anti-eotaxin treatment and dexamethasone were equally effective in reducing lung tissue IL-13 levels. This strongly argues against reductions in IL-13 alone as accounting for the effects of dexamethasone.

Eotaxin is a potent and specific eosinophil chemoattractant[14,15] that also activates eosinophils, increasing both leukotriene C_4 synthesis and eosinophil peroxidase activity.[20] Aerosol exposure of naive guinea pigs to eotaxin induces BAL eosinophilia.[21] Eotaxin seems to be most important in the early phases of eosinophil recruitment after allergen challenge.[22] In the present study OVA challenge increased eotaxin levels in lung tissue from 6 to 48 hours after challenge. Interestingly, BALF levels of eotaxin did not show antigen-specific induction. It appears that eotaxin might be released from airway epithelial cells[23] into the airway lumen by the nonspecific stimulus, saline, but the levels of secreted protein in

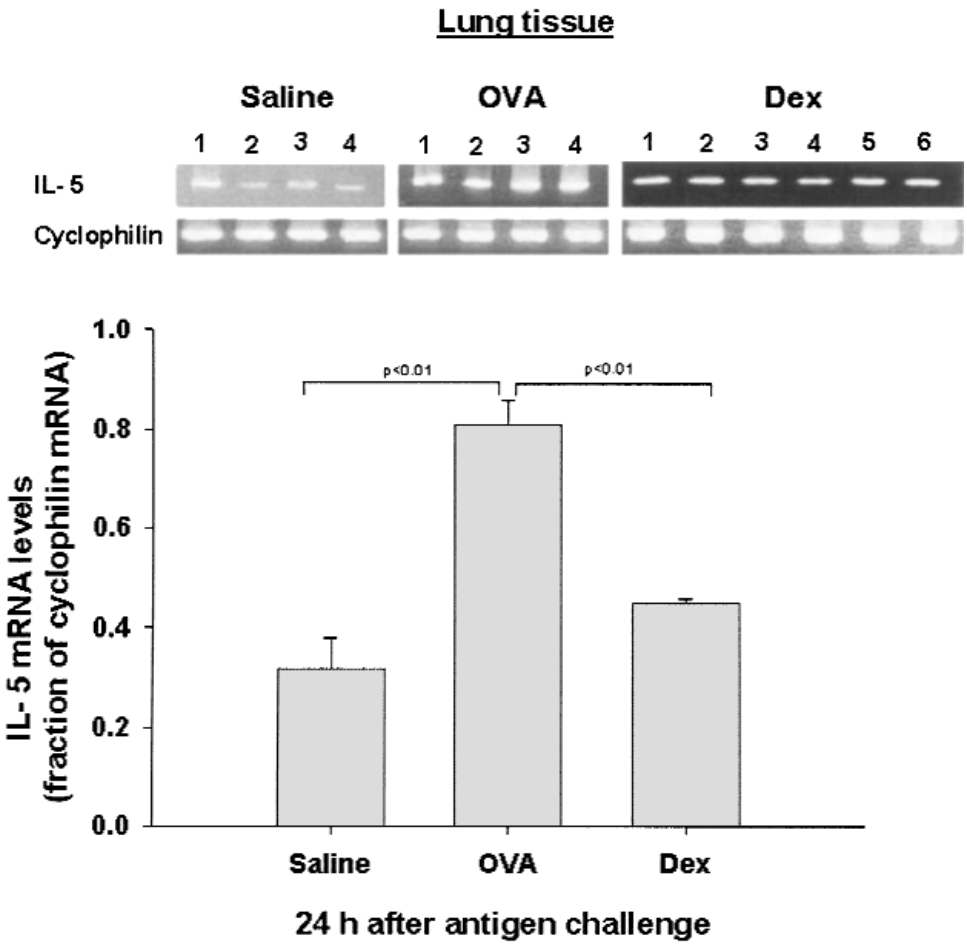

FIG 7. RT-PCR analysis of IL-5 mRNA expression in lung tissues after saline or OVA or dexamethasone challenge in immunized A/J mice. The *top panel* shows the agarose gel stained with ethidium bromide and visualized with UV light, and the *bottom panel* shows the densitometry for IL-5 mRNA relative to cyclophilin mRNA.

the BALF were not sufficient to cause eosinophilia, presumably because other factors, such as IL-5, are required. The neutralization of eotaxin with an mAb inhibited the recruitment of eosinophils into the BALF after OVA challenge, in agreement with other reports,[8] but not AHR. Eotaxin has been previously implicated in allergen-induced AHR.[9] However, the relationship between eotaxin and AHR is complex, and a recent study using recombinant vaccinia virus to overexpress eotaxin in the airways of the mouse has helped to clarify the issue.[24] In that study eotaxin caused substantial airway eosinophilia and in conjunction with IL-5 caused an even more marked increase in eosinophils. However, the eosinophilia alone did not cause AHR but did enhance AHR when accompanied by allergen exposure.[24] The lack of relationship between eotaxin and AHR in the current study might reflect quantitative differences in eotaxin expression and the choice of mouse strain (ie, A/J compared with C57BL/6) in the study of Mould et al[24] or methodological differences, such as the protocol of sensitization and challenge, as well as the technique used to evaluate airway function.

IL-5 mRNA expression by BAL cells was found at 24 hours after OVA challenge, suggesting that BAL cells might contribute to the IL-5 measured in BALF. IL-5 mRNA in the lung tissues also increased after OVA challenge. IL-5 protein followed a similar pattern of changes and was an important signal for eosinophilia in these experiments because anti-IL-5 mAb inhibited the development of eosinophilia. When anti-IL-5 was combined with anti-eotaxin treatment, inhibition was even more pronounced. Such an interaction between IL-5 and eotaxin has been previously reported.[25,26] Interestingly, anti-IL-5 and anti-eotaxin antibodies also reduced IL-5, but not eotaxin, levels in BALF. This finding indicates the presence of a feed-forward mechanism in the regulation of IL-5 levels. Such a mechanism is consistent with the idea that eosinophils themselves might be contributing significantly to IL-5 levels in the BALF. Eotaxin mRNA was virtually undetectable in the BAL cells of both saline- and OVA-challenged mice at 24 hours after challenge but was readily detectable in lung tissues, suggesting that airway epithelial cells and not immune effector cells within the airway lumen are likely to be major

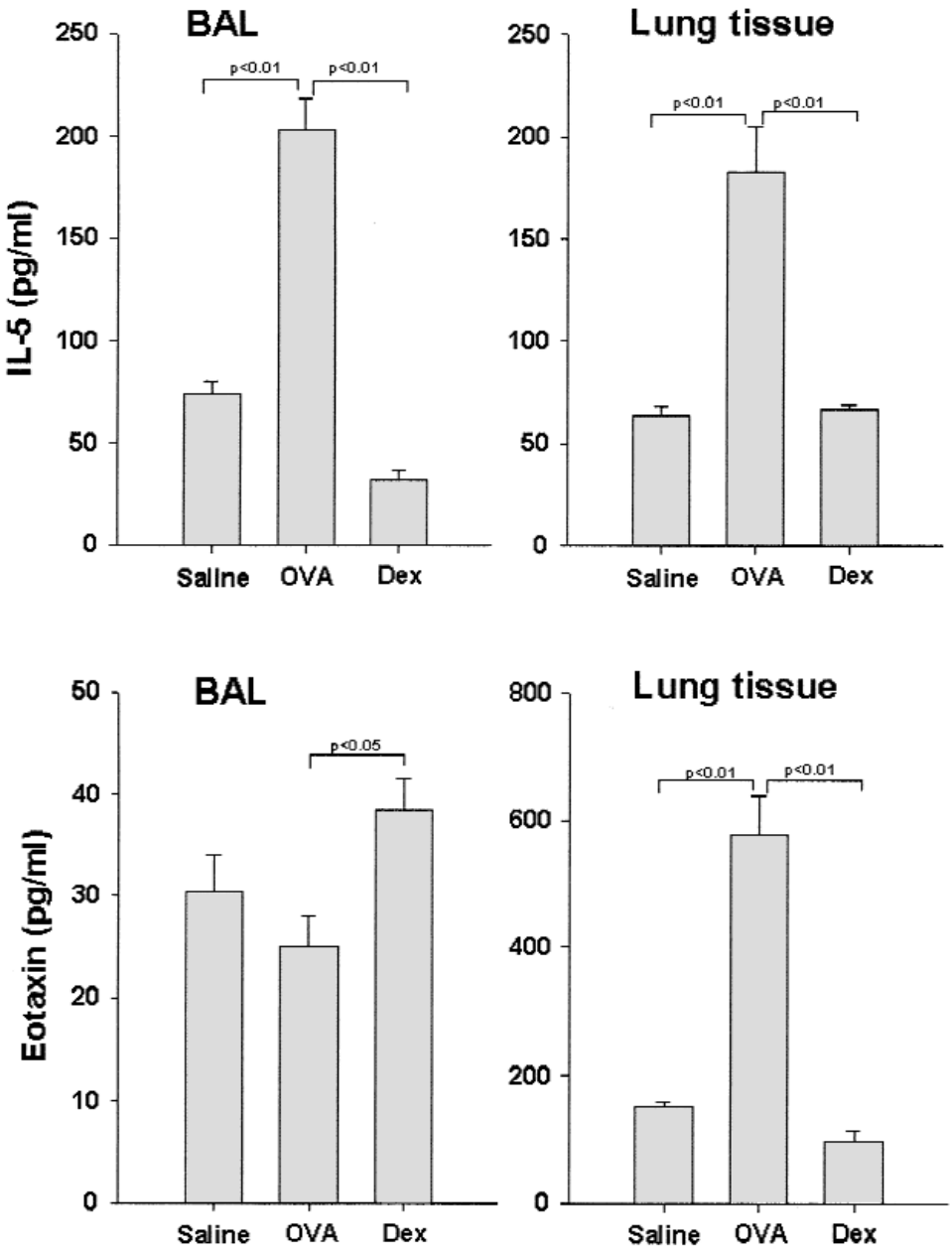

FIG 8. IL-5 and eotaxin levels in BALF and lung tissues after saline challenge, OVA challenge, or OVA challenge after dexamethasone pretreatment. Each *bar* represents the mean of data from 5 to 6 animals.

sources of eotaxin.[23] The significance of the IL-5 expression for AHR is less clear. Previous studies have linked IL-5 and eosinophilia to AHR,[27-29] whereas other studies have reported a dissociation between the two,[30,31] suggesting alternative pathways for the mediation of AHR. IL-5 has also been linked to AHR through mechanisms unrelated to eosinophilia. The IL-5 receptor is expressed on airway smooth muscle and is responsible for IL-1–induced AHR.[32] Despite such evidence, under the conditions of our experiment, IL-5 was not necessary for the development of AHR in the murine model. Indeed, in human subjects IL-5 might also not be necessary for allergen-induced changes in airway function. A recent clinical trial of an anti-IL-5 mAb administered to human asthmatic subjects before allergen challenge showed that reductions in sputum and peripheral blood eosinophilia were effected, but the late asthmatic response to allergen challenge was unaltered.[33]

Glucocorticoids are potent inhibitors of allergen-induced airway responses, and their inhibitory actions on the recruitment of eosinophils into the airways are often presumed to be an important component of their therapeutic effect. Dexamethasone reduced IL-5 levels in BALF and lung tissues from allergen-challenged mice.

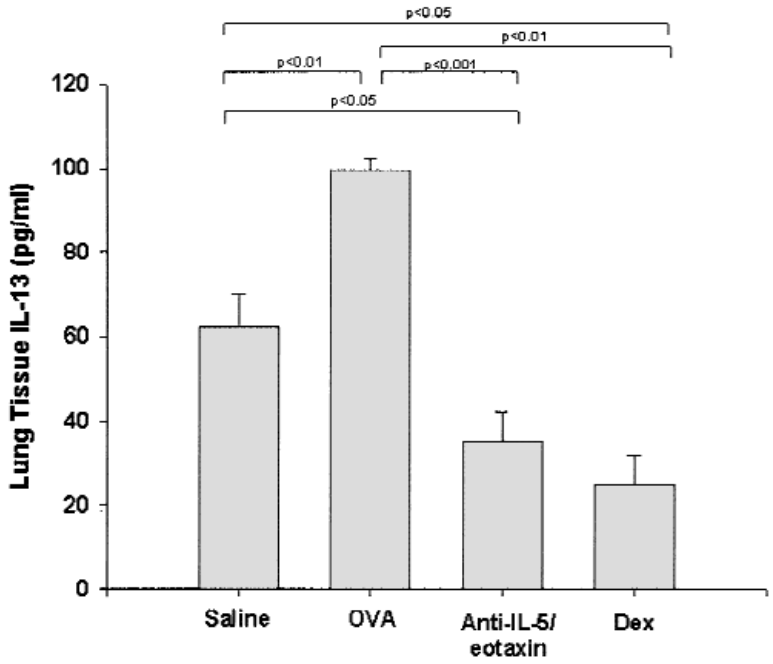

FIG 9. IL-13 levels in lung tissue after saline challenge, OVA challenge, or OVA challenge with anti-IL-5/anti-eotaxin antibody treatment or dexamethasone treatment. Each *bar* represents the mean of data from 7 to 9 animals.

Our results indicate that eotaxin protein and mRNA in the lung tissue compartment were also substantially reduced by dexamethasone in vivo. Curiously, there was a slight increase of eotaxin protein levels in the BALF after dexamethasone treatment, and we are not certain of the significance of this finding. In vitro, dexamethasone has been shown to inhibit cytokine-induced increases in eotaxin mRNA in human lung epithelial cells, as well as eotaxin protein production and secretion.[23] Therefore as expected dexamethasone inhibited the recruitment of eosinophils after OVA challenge, although less completely than the combined antibodies. Despite the similarities between the effects of dexamethasone and anti-IL-5/anti-eotaxin treatments on eosinophilia and its regulation by IL-5 and eotaxin, dexamethasone differed in reducing AHR to control levels in allergen-challenged mice. These results suggest that the effects of dexamethasone on AHR are unlikely to be related to alterations in eosinophilia, IL-5 levels, or eotaxin levels. Established airway eosinophilia and AHR can be reversed also by dexamethasone, whereas anti-IL-5 antibody reduces only eosinophil numbers and not AHR.[31,34] To ensure the adequacy of inhibition of tissue eosinophilia by both modalities of treatment, we performed an analysis of MBP immunoreactive cells in lung tissues, and we found that anti-IL-5/anti-eotaxin antibody treatment was as effective as dexamethasone in reducing eosinophil numbers.

The effects of dexamethasone might be mediated by actions on the expression of other cytokines. For example, IFN-γ a T_H1 cell–derived cytokine induces AHR without affecting eosinophilia in mice,[35] and dexamethasone reduces IFN-γ levels in BALF, whereas an anti-IL-5 antibody does not.[34] Glucocorticoids downregulate the activation of both T_H1 and T_H2 cells.[36] Indeed, in this study the lymphocyte numbers were increased in BALF after OVA challenge, and of the interventions tested, only dexamethasone significantly reduced their numbers. Recently, a novel T cell–regulated mechanism modulating allergen-induced AHR in mice was proposed by Hogan et al.[37] In their model inhibition of the action of IL-5, IL-4, or both did not abolish AHR despite the diminution of airways inflammation. AHR was reduced only by anti-CD4 mAb treatment, leading the authors to conclude that a pathway intimately regulated by CD4 T cells but independent of IL-4 and IL-5 underlies AHR. One of the T_H2 cell–derived cytokines, IL-13, might be a candidate for such an effect because the administration or overexpression of this cytokine was sufficient to induce AHR.[17,38] Exogenous IL-13 administered to mice induces AHR that can be evoked in IL-5– and eotaxin-deficient animals.[39] Interestingly IL-13 also increased in lung tissues after OVA challenge of the A/J mice in the current study. However, dexamethasone and anti-IL-5/anti-eotaxin treatments were of comparable efficacy in reducing IL-13 to less than baseline levels. A dependence of IL-13 production in mice on eotaxin and IL-5 has been recently described.[40] This dependence is eosinophil mediated, suggesting an interaction between these cells and the T cells that produce IL-13. However, because antibody treatment reduced IL-13 levels as much as dexamethasone but was without an effect on AHR, it seems unlikely that dexamethasone is mediating its effects through inhibition of IL-13.

In conclusion, eotaxin, an eosinophil-specific chemokine, is clearly involved in a cooperative fashion with IL-5 in the accumulation of eosinophils in the airways after allergen challenge but does not mediate the induction of AHR. The glucocorticoid dexamethasone inhibits airway eosinophilia and AHR and reduces the mRNA and protein levels of eotaxin and IL-5 in the lung. However, antibodies to IL-5 and eotaxin that are highly effective in reducing airway eosinophilia do not abolish allergen-induced AHR. These results suggest that the effects of dexamethasone on AHR are unlikely to be related to alterations in eosinophilia, eotaxin levels, or IL-5 levels. The differences in the actions of dexamethasone and antibody treatments are also not accounted for by IL-13, which is equally inhibited by the treatments. Insofar as murine models are applicable to human asthma, our results suggest that therapeutic strategies on the basis of the inhibition of release or actions of IL-5 and eotaxin are unlikely to be successful in altering AHR in asthmatic subjects.

We acknowledge the excellent technical assistance of Dr Rame Taha and Ms Jamilah Saeed.

REFERENCES

1. Leung DYM, Bloom J. Update on glucocorticoid action and resistance. J Allergy Clin Immunol 2003; 111:•••.
2. Stellato C, Beck LA, Gorgone GA, Proud D, Schall TJ, Ono SJ, et al. Expression of the chemokine RANTES by a human bronchial epithelial cell line. Modulation by cytokines and glucocorticoids. J Immunol 1995;155:410-8.
3. Berkman N, Jose PJ, Williams TJ, Schall TJ, Barnes PJ, Chung KF. Corticosteroid inhibition of macrophage inflammatory protein-1 alpha in human monocytes and alveolar macrophages. Am J Physiol 1995;269:L443-52.
4. Pitzalis C, Pipitone N, Bajocchi G, Hall M, Goulding N, Lee A, et al. Corticosteroids inhibit lymphocyte binding to endothelium and intercellular adhesion: an additional mechanism for their anti-inflammatory and immunosuppressive effect. J Immunol 1997;158:5007-16.
5. Bousquet J, Chanez P, Lacoste JY, Barneon G, Ghavanian N, Enander I, et al. Eosinophilic inflammation in asthma. N Engl J Med 1990;323:1033-9.
6. Gleich GJ, Frigas E, Loegering DA, Wassom DL, Steinmuller D. Cytotoxic properties of the eosinophil major basic protein. J Immunol 1979;123:2925-7.
7. Schleimer RP, Bochner BS. The effects of glucocorticoids on human eosinophils. J Allergy Clin Immunol 1994;94(suppl):1202-13.
8. Li D, Wang D, Griffiths-Johnson DA, Wells TN, Williams TJ, Jose PJ, et al. Eotaxin protein and gene expression in guinea-pig lungs: constitutive expression and upregulation after allergen challenge. Eur Respir J 1997;10:1946-54.
9. Gonzalo JA, Lloyd CM, Kremer L, Finger E, Martinez A, Siegelman MH, et al. Eosinophil recruitment to the lung in a murine model of allergic inflammation. The role of T cells, chemokines, and adhesion receptors. J Clin Invest 1996;98:2332-45.
10. Yamaguchi Y, Suda T, Suda J, Eguchi M, Miura Y, Harada N, et al. Purified interleukin 5 supports the terminal differentiation and proliferation of murine eosinophilic precursors. J Exp Med 1988;167:43-56.
11. Lopez AF, Sanderson CJ, Gamble JR, Campbell HD, Young IG, Vadas MA. Recombinant human interleukin 5 is a selective activator of human eosinophil function. J Exp Med 1988;167:219-24.
12. Baggiolini M, Dewald B, Moser B. Human chemokines: an update. Annu Rev Immunol 1997;15:675-705.
13. Baggiolini M, Dahinden CA. CC chemokines in allergic inflammation. Immunol Today 1994;15:127-33.
14. Jose PJ, Griffiths-Johnson DA, Collins PD, Walsh DT, Moqbel R, Totty NF, et al. Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea pig model of allergic airways inflammation. J Exp Med 1994;179:881-7.
15. Rothenberg ME, Luster AD, Leder P. Murine eotaxin: an eosinophil chemoattractant inducible in endothelial cells and in interleukin 4-induced tumor suppression. Proc Natl Acad Sci U S A 1995;92:8960-4.
16. Palframan RT, Collins PD, Williams TJ, Rankin SM. Eotaxin induces a rapid release of eosinophils and their progenitors from the bone marrow. Blood 1998;91:2240-8.
17. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, et al. Interleukin-13: central mediator of allergic asthma. Science 1998;282:2258-61.
18. Kung TT, Stelts DM, Zurcher JA, Adams GK III, Egan RW, Kreutner W, et al. Involvement of IL-5 in a murine model of allergic pulmonary inflammation: prophylactic and therapeutic effect of an anti-IL-5 antibody. Am J Respir Cell Mol Biol 1995;13:360-5.
19. Gonzalo JA, Lloyd CM, Wen D, Albar JP, Wells TN, Proudfoot A, et al. The coordinated action of CC chemokines in the lung orchestrates allergic inflammation and airway hyperresponsiveness. J Exp Med 1998;188:157-67.
20. Sporn PHS, Fries FP, Anderson JA. Eotaxin enhances synthesis of leukotriene C4 synthesis by human blood eosinophils [abstract]. Am J Respir Crit Care Med 2000;161:A709.
21. Griffiths-Johnson DA, Collins PD, Rossi AG, Jose PJ, Williams TJ. The chemokine, eotaxin, activates guinea-pig eosinophils in vitro and causes their accumulation into the lung in vivo. Biochem Biophys Res Commun 1993;197:1167-72.
22. Rothenberg ME, MacLean JA, Pearlman E, Luster AD, Leder P. Targeted disruption of the chemokine eotaxin partially reduces antigen-induced tissue eosinophilia. J Exp Med 1997;185:785-90.
23. Lilly CM, Nakamura H, Kesselman H, Nagler-Anderson C, Asano K, Garcia-Zepeda EA, et al. Expression of eotaxin by human lung epithelial cells: induction by cytokines and inhibition by glucocorticoids. J Clin Invest 1997;99:1767-73.
24. Mould AW, Ramsay AJ, Matthaei KI, Young IG, Rothenberg ME, Foster PS. The effect of IL-5 and eotaxin expression in the lung on eosinophil trafficking and degranulation and the induction of bronchial hyperreactivity. J Immunol 2000;164:2142-50.
25. Collins PD, Marleau S, Griffiths-Johnson DA, Jose PJ, Williams TJ. Cooperation between interleukin-5 and the chemokine eotaxin to induce eosinophil accumulation in vivo. J Exp Med 1995;182:1169-74.
26. Mould AW, Matthaei KI, Young IG, Foster PS. Relationship between interleukin-5 and eotaxin in regulating blood and tissue eosinophilia in mice. J Clin Invest 1997;99:1064-71.
27. Lee JJ, McGarry MP, Farmer SC, Denzler KL, Larson KA, Carrigan PE, et al. Interleukin-5 expression in the lung epithelium of transgenic mice leads to pulmonary changes pathognomonic of asthma. J Exp Med 1997;185:2143-56.
28. Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. J Exp Med 1996;183:195-201.
29. Eum SY, Haile S, Lefort J, Huerre M, Vargaftig BB. Eosinophil recruitment into the respiratory epithelium following antigenic challenge in hyper-IgE mice is accompanied by interleukin 5- dependent bronchial hyperresponsiveness. Proc Natl Acad Sci U S A 1995;92:12290-4.
30. Hamelmann E, Oshiba A, Loader J, Larsen GL, Gleich G, Lee J, et al. Antiinterleukin-5 antibody prevents airway hyperresponsiveness in a murine model of airway sensitization. Am J Respir Crit Care Med 1997;155:819-25.
31. Corry DB, Folkesson HG, Warnock ML, Erle DJ, Matthay MA, Wiener-Kronish JP, et al. Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity. J Exp Med 1996;183:109-17.
32. Hakonarson H, Maskeri N, Carter C, Chuang S, Grunstein MM. Autocrine interaction between IL-5 and IL-1beta mediates altered responsiveness of atopic asthmatic sensitized airway smooth muscle. J Clin Invest 1999;104:657-67.
33. Leckie MJ, ten Brinke A, Khan J, Diamant Z, O'Connor BJ, Walls CM, et al. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. Lancet 2000;356:2144-8.
34. Mathur M, Herrmann K, Li X, Qin Y, Weinstock J, Elliott D, et al. TRFK-5 reverses established airway eosinophilia but not established hyperre-

sponsiveness in a murine model of chronic asthma. Am J Respir Crit Care Med 1999;159:580-7.
35. Hessel EM, Van Oosterhout AJ, Van A, Van Esch B, Hofman G, Van Loveren H, et al. Development of airway hyperresponsiveness is dependent on interferon- gamma and independent of eosinophil infiltration. Am J Respir Cell Mol Biol 1997;16:325-34.
36. Braun CM, Huang SK, Bashian GG, Kagey-Sobotka A, Lichtenstein LM, Essayan DM. Corticosteroid modulation of human, antigen-specific Th1 and Th2 responses. J Allergy Clin Immunol 1997;100:400-7.
37. Hogan SP, Matthaei KI, Young JM, Koskinen A, Young IG, Foster PS. A novel T cell-regulated mechanism modulating allergen-induced airways hyperreactivity in BALB/c mice independently of IL-4 and IL-5. J Immunol 1998;161:1501-9.
38. Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, et al. Requirement for IL-13 independently of IL-4 in experimental asthma. Science 1998;282:2261-3.
39. Yang M, Hogan SP, Henry PJ, Matthaei KI, McKenzie AN, Young IG, et al. Interleukin-13 mediates airways hyperreactivity through the IL-4 receptor-alpha chain and STAT-6 independently of IL-5 and eotaxin. Am J Respir Cell Mol Biol 2001;25:522-30.
40. Mattes J, Yang M, Mahalingam S, Kuehr J, Webb DC, Simson L, et al. Intrinsic defect in T cell production of interleukin (IL)-13 in the absence of both IL-5 and eotaxin precludes the development of eosinophilia and airways hyperreactivity in experimental asthma. J Exp Med 2002; 195:1433-44.