

Depletion of CD8⁺ T cells enhances pulmonary inflammation but not airway responsiveness after antigen challenge in rats

Sophie Laberge, MD, Louis Wu, Ronald Olivenstein, MD, Li-Jing Xu, MSc, Paolo M. Renzi, MD, and James G. Martin, MD Montreal, Quebec, Canada

Background: CD8⁺ (OX-8⁺) T cells may suppress airway inflammation and airway responsiveness after allergen challenge.

Objective: We studied the effects of depletion of OX-8⁺ T cells on allergen-induced lung eosinophilia and airway responsiveness in the Sprague-Dawley rat.

Methods: Sprague-Dawley rats were sensitized to ovalbumin and challenged by aerosol 14 days later. Test animals received either low-dose (2 mg, n = 9) or high-dose (3 mg, n = 7) OX-8 monoclonal antibody (mAb), whereas controls (n = 8) received BALB/c ascites fluid. A fourth group of animals (n = 10) was not sensitized to ovalbumin and also received ascites fluid. Twenty-four hours after ovalbumin challenge, responsiveness to methacholine was measured, and lung inflammation was assessed in the large airways and small airways and parenchyma.

Results: Circulating and airway CD8⁺ T cells were decreased by OX-8 mAb administration with greatest changes in animals treated with high-dose OX-8 mAb compared with controls (blood: 1.0% ± 3.6% vs 18.7% ± 3.9%, p < 0.05); (large airways: 2.5% ± 1.2% vs 13.8% ± 1.2%, p < 0.05). Ovalbumin challenge resulted in increases in macrophages and neutrophils in the small airways and parenchyma of sensitized compared with unsensitized rats (p < 0.05). High-dose OX-8 mAb further increased total leukocytes, attributable to increases in neutrophils and eosinophils, retrieved from the large airways and small airways and parenchyma compared with other groups (p < 0.05). Airway responsiveness to methacholine was not significantly different between control and ovalbumin-challenged animals and was not augmented by OX-8 pretreatment.

Conclusion: CD8⁺ T cells modulate the extent of allergen-induced airway inflammation. However, the enhancement of inflammation was not sufficient to affect airway responsiveness. (*J Allergy Clin Immunol* 1996;98:617-27.)

Key words: Sprague-Dawley rats, methacholine challenge, eosinophils, T lymphocytes

Evidence is accumulating for a pivotal role of T lymphocytes in the initiation and propagation of airway inflammation in asthma. Activated T lymphocytes

From Meakins-Christie Laboratories, Royal Victoria Hospital, McGill University; and the Respiratory Health Network of Centres of Excellence, Montreal.

Supported by Medical Research Council of Canada grant no. MT-10381. Dr. Laberge is a recipient of a Medical Research Council of Canada Fellowship, Dr. Olivenstein was a recipient of a Canadian Lung Association Fellowship, and Dr. Renzi is a Chercheur Boursier of the Fonds de Recherche en Santé du Québec.

Received for publication Apr. 27, 1995; revised Jan. 29, 1996; accepted for publication Feb. 2, 1996.

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

Copyright © 1996 by Mosby-Year Book, Inc.
0091-6749/96 \$5.00 + 0 1/1/72535

Abbreviations used

ANOVA:	Analysis of variance
BN:	Brown Norway
EC200R _L :	Concentration causing a doubling in pulmonary resistance
mAb:	Monoclonal antibody
PBS:	Phosphate-buffered saline
R _L :	Pulmonary resistance
SD:	Sprague-Dawley

phocytes are found in bronchoalveolar lavage^{1,2} and in bronchial biopsy specimens from subjects with mild asthma.³ After bronchial antigen challenge, CD4⁺ T cells are depleted in the peripheral blood and accumulate within the lung tissues.^{4,5} The release of specific cytokines related to a

T_{H2} -like subset of $CD4^+$ T cells could contribute to the recruitment and activation of immune effector cells, including eosinophils within the airways.⁶⁻⁸ IL-5, a prominent T_{H2} -type cytokine, in particular appears to be responsible for the preferential eosinophil accumulation in the airways.^{9,10}

Although considerable interest has recently been focused on the role of $CD4^+$ T cells in asthma and allergic disorders, the involvement of $CD8^+$ T cells has received less attention. Some reports have documented a decreased number of circulating $CD8^+$ T cells in both allergic¹¹ and nonallergic asthma.^{12,13} Deficient concanavalin A-induced suppressor cell activity has been reported in asthma.¹⁴⁻¹⁶ Increased numbers of $CD8^+$ T cells have been retrieved in bronchoalveolar lavage from human subjects who have isolated early airway responses as opposed to subjects who have late allergic responses, suggesting a protective role for $CD8^+$ T cells in allergen-induced airway responses.¹⁷ Interestingly, in the same study, bronchoalveolar eosinophils were increased in dual responders but not in subjects with only an early response. These observations have given rise to the hypothesis that impaired suppressor cell effects may account for an abnormally prolonged inflammatory response in asthma.

We have previously shown that in vivo depletion of $CD8^+$ T cells with a mouse anti-rat OX-8 monoclonal antibody (mAb) increased the late allergic responses after ovalbumin challenge in Sprague-Dawley (SD) rats.¹⁸ The OX-8 surface antigen is expressed on rat cytotoxic/suppressor T cells,¹⁹ as well as on a subpopulation of natural killer cells.²⁰ The SD rat was chosen because it is not a high IgE producer and it has a low prevalence of late responses after allergen challenge. These features are in contrast to the Brown Norway (BN) rat, which is a high IgE producer and has frequent late responses.²¹ These rat strains represent rodent equivalents of atopic and nonatopic subjects. Interestingly, the SD rat has a low $CD4^+$ to $CD8^+$ ratio¹⁸ compared with the BN rat,²² which led us to postulate that the difference between the two rat strains might reside in differences in the balance between lymphocyte subsets. It is a plausible hypothesis that the difference between SD and BN rats in responses to allergen challenge is determined by the characteristics of their respective $CD8^+$ cells. In this study we used the OX-8 mAb to selectively deplete the $CD8^+$ T cells and further explore the immunoregulatory roles of these cells in allergen-induced airway responses in the SD rat. We postulated that depletion of $CD8^+$

T cells would enhance allergen-induced lung inflammation, specifically the extent of eosinophilic infiltration, and would increase the cholinergic bronchial responsiveness that is usually associated with allergen exposure of sensitized animals,²³⁻²⁵ converting the responses of the SD rat to those of the BN rat.

METHODS

Animals and sensitization

Fifty-four male SD rats (8 to 10 weeks old, ranging in weight from 250 to 330 gm) and six male BN rats (6 to 8 weeks old) were obtained from a commercial source (Harlan, Indianapolis, Ind.). Rats were housed in a conventional animal facility at McGill University (Montreal, Quebec, Canada).

Chemicals and drugs

Ovalbumin, collagenase, casein, biotinamidocaproate n-hydroxysuccinimide ester, p-nitrophenyl phosphate disodium, and 2-mercaptoethanol were purchased from Sigma Chemical Company (St. Louis, Mo.). *Bordetella pertussis* vaccine was obtained from Institut Armand Frappier (Laval-des-Rapides, Quebec, Canada). W3/25 and OX-8 antibodies for staining were obtained from Dimension Labs (Mississauga, Ontario, Canada); 341 mAb was purchased from Pharmingen (San Diego, Calif.); fluorescein isothiocyanate-conjugated rabbit anti-mouse mAb was obtained from Kappel, Organon Teknika (Scarborough, Ontario, Canada); mouse anti-rat IgE mAb was obtained from Zymed Laboratories Inc. (San Francisco, Calif.); and BALB/c mouse control ascites fluid was obtained from Cedarlane Laboratories (Hornby, Ontario, Canada). Fetal bovine serum, penicillin, streptomycin, McCoy 5A medium, Hanks' balanced salt solution, and trypan blue were obtained from Gibco Laboratories (Grand Island, N.Y.). Tween-20 was purchased from Fisher Scientific (Montreal, Quebec, Canada). Ficoll-Hypaque was obtained from Pharmacia (Montreal, Quebec, Canada). May-Grünwald stain was purchased from BDH Inc. (Toronto, Ontario, Canada), and Giemsa stain was obtained from Harleco, EM Diagnostic System (Gibbstown, N.J.). Alkaline phosphatase-conjugated streptavidin was purchased from Jackson ImmunoResearch (West Grove, Pa.). We used the following drugs: xylazine (Chemagro Limited, Etobicoke, Ontario, Canada), sodium pentobarbital (MTC Pharmaceuticals, Cambridge, Ontario, Canada), aluminum hydroxide moist gel (BDH Chemicals Ltd., Poole, England).

Study protocol

Thirty-nine SD rats were sensitized with a subcutaneous injection of 1 mg of ovalbumin precipitated in 3.48 mg of aluminum hydroxide gel in 1 ml of 0.9% saline solution (wt/vol). *Bordetella pertussis* vaccine, 0.5 ml, containing 10^{10} heat-killed bacilli per milliliter, was injected intraperitoneally as an adjuvant. Fifteen control

SD rats were not sensitized. All BN rats were similarly sensitized to ovalbumin.

Animals were divided into four groups. Ten unsensitized rats were given four intravenous injections of control antibody every other day and were challenged with ovalbumin on day 6. Nine rats that were sensitized to ovalbumin received an intravenous injection of 0.5 mg of control antibody on days 8, 10, 12, and 14 after sensitization. Nine rats also sensitized to ovalbumin were given an intravenous injection of 0.5 mg of OX-8 mAb specific to rat CD8⁺ T lymphocytes on days 8, 10, 12, and 14 after sensitization. Eight additional animals were similarly sensitized but received 1 mg of OX-8 mAb on days 10, 12, and 14 after sensitization.

On day 14, blood was obtained before the ovalbumin challenge for determination of antigen-specific IgE, and subsequently, all animals were challenged with aerosols of ovalbumin. Twenty-four hours after antigen challenge, airway responsiveness to methacholine was determined, and the lung inflammatory response was assessed by means of lung digestion.²² At the same time, lymphocyte subsets were determined in the blood and lung tissues.

Monoclonal antibodies

OX-8 antibodies were obtained from an OX-8 hybridoma cell line (American Type Culture Collection, Rockville, Md.), which produces an mAb with specificity for rat T-suppressor/cytotoxic lymphocytes. Frozen OX-8 cells were thawed and cultured in complete RPMI-1640 medium (Gibco Laboratories) until dense layers were obtained. The culture was divided every 2 to 3 days, and the culture medium was pooled and centrifuged at 4000 rpm for 15 minutes at 4°C to collect the supernatant. The supernatant protein was precipitated with saturated ammonium sulfate and dialyzed against phosphate-buffered saline (PBS). IgG was purified on a protein A/G agarose affinity column, and the OX-8 fraction was again dialyzed against PBS before finally being concentrated with polyethylene glycol. The protein content was 4.63 mg/ml. The mAb was passed through a 0.2 µm Millipore filter (Millipore Corp., Bedford, Mass.) and stored at -80°C until use.

Ascites from BALB/c mice that were injected intraperitoneally with SP2/0 myeloma cells was used as control antibody.

Antigen challenge

Fourteen days after sensitization, rats were anesthetized with xylazine (7 mg/kg) and pentobarbital (50 mg/kg), administered intraperitoneally. Animals were intubated endotracheally with a 6 cm length of polyethylene tubing (PE-240; Commercial Plastics, Montreal, Quebec, Canada). Intubated animals were connected to a small Plexiglas box (265 ml in volume) and aerosolized with ovalbumin (5% wt/vol in saline solution) for 5 minutes. Aerosols were generated by using a Hudson nebulizer (Hudson Inc., Temecula, Calif.) with an out-

put of 0.25 ml/min connected to one side port of the box. After aerosolization, the animals awakened spontaneously and were extubated.

Measurement of airway responsiveness to methacholine

Animals were anesthetized and intubated as described previously and placed in the left lateral decubitus position with the end of the endotracheal tube connected to the Plexiglas box. A water-filled esophageal catheter attached to a pressure transducer (Transpac II; Sorenson, Abbott, Ill.) was used to determine changes in pleural pressure. Airflow was measured by a pneumotachograph (Fleisch 00; Bionetics, Montreal, Quebec, Canada) coupled to a differential transducer (PX 170-14 DV; Omega Engineering, Stamford, Conn.) attached to the Plexiglas box. From flow, volume, and transpulmonary pressure, pulmonary resistance (R_L) was determined by fitting the equation of motion of the lung to the data by multiple linear regression analysis²⁶ by using a commercial software package (RHT InfoDat Inc., Montreal, Quebec, Canada).

Rats inhaled aerosols of PBS and progressively doubling concentrations of methacholine from 0.0625 to 32 mg/ml in PBS for 30 seconds. Aerosols were generated by using a Hudson nebulizer with an output of 0.25 ml/min. The peak value for R_L was measured before and after inhalation of PBS and after each concentration of methacholine, and cumulative concentration-response curves were constructed. Responsiveness to methacholine was expressed as the concentration required to increase R_L to 200% of the value measured after PBS aerosolization (EC_{200R_L}), calculated by interpolation from the concentration-response curve.

Lung mincing and digestion

Lung mincing and digestion were performed as previously described.²² After anesthesia was induced, the chest wall was opened, and each animal was exsanguinated by cardiac puncture. Blood was kept for analysis of lymphocyte subsets by flow cytometry. The pulmonary vasculature was flushed with PBS ($\pm$ 10 ml) until the effluent was clear. Large airways, which comprised the lower 1 cm of the trachea and the large bronchi until the fifth generation approximately, were separated from the small airways and parenchyma, washed in balanced salt solution, and incubated for tissue digestion. Tissue digestion was performed by three consecutive 20-minute incubations in McCoy 5A medium (supplemented with 100 U/ml of penicillin, 100 µg/ml streptomycin, 10% heat-inactivated fetal calf serum, and 7.8 µg/ml 2-mercaptoethanol) containing 20 U/ml collagenase. After each incubation, cells were separated from tissue by filtration with a no. 60 stainless steel sieve (Sigma Chemical Co.). Total cell counts were performed with a hemocytometer. Differential cell counts were made from cytopspin preparations (Cytospin-3; Shandon, Cheshire, England) stained with May-Grünwald-Giemsa stain.

Immunofluorescent staining of lymphocyte subsets

Analysis of lymphocyte subsets was done in sensitized and ovalbumin-challenged rats. Blood mononuclear cells were isolated by a standard Ficoll-Hypaque method and prepared for flow cytometry. Large airway cells retrieved from tissue digestion were also prepared for flow cytometry. Cells were washed three times and then suspended in Hanks' balanced salt solution. The cells were then incubated for 30 minutes at 4° C with the mAbs W3/25 and OX-8, which recognize the CD4 and the CD8 α surface antigens, respectively, and washed three times with the same buffer. Fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG antibody was added, and the cells were incubated for another 30 minutes at 4° C, washed three times in Hanks' balanced salt solution, and fixed with 1% paraformaldehyde in saline solution. The fixed cells were stored at 4° C in the dark until analysis. The immunofluorescence analysis was performed with a FACSCAN flow cytometer (Becton Dickinson, FACS Division, Sunnyvale, Calif.) focused on the lymphocyte cluster.

To ascertain the adequacy of CD8⁺ T-cell depletion, blood mononuclear cells were isolated from additional rats pretreated either with the control antibody ($n = 2$) or the high-dose OX-8 mAb ($n = 2$) according to the study protocol described previously. The cells were incubated at 4° C with the W3/25, OX-8, and 341 mAbs, which recognize the CD4, CD8 α , and the CD8 β surface antigens, respectively. Immunofluorescent staining and flow cytometry were performed as described previously. Immunostaining with a control antibody was performed as well.

Ovalbumin-specific immunoglobulin determination

Blood was collected by orbital bleeding 14 days after sensitization and before the antigen challenge. Serum was obtained by centrifugation and frozen at -30° C until use. Specific IgE to ovalbumin was determined by ELISA. Plates (Immulon 2; Dynatech Laboratories, Chantilly, Va.) were coated overnight at 4° C with mouse mAb to rat IgE diluted 1:500 in carbonate-bicarbonate buffer. Blocking was done with PBS-0.5% casein-0.1% Tween-20. Plates were then incubated with sera diluted 1:50 in PBS-0.5% casein-0.1% Tween-20. Subsequently, biotin-labeled ovalbumin diluted 1:50 in PBS-0.5% casein-0.1% Tween-20 was added. These steps were performed for 2 hours at 37° C. Alkaline phosphatase-conjugated streptavidin in PBS-0.5% casein-0.1% Tween-20 (1:500 dilution) was added for 30 minutes. Between steps, plates were washed with PBS-0.5% casein-0.1% Tween-20. After addition of p-nitrophenyl phosphate disodium, plates were developed at room temperature and read with an ELISA plate reader (model 400 ATC, SLT; Lab Instruments, Pittsburgh, Pa.) at 405 nm. Rat IgE specific to ovalbumin was not commercially available, so the results are expressed in

units of absorbance. Plates were developed to give a reading of 1 unit of absorbance for a positive control sample from a sensitized BN rat. Sera obtained from three ovalbumin-sensitized BN rats were also included in the assay for comparison.

Intradermal skin tests

Intradermal skin testing was performed in unsensitized SD rats ($n = 5$), sensitized untreated SD rats ($n = 4$), sensitized and OX-8-treated (high-dose) SD rats ($n = 5$), and sensitized BN rats ($n = 3$). A range of ovalbumin concentrations from 1 μ g/ml to 50 mg/ml (in PBS) was tested. A 20 μ l volume of each solution was injected intradermally in the dorsal skin. Fifteen minutes later, 1 ml of 2% Evans blue dye was given intravenously, and the magnitude of the wheal was evaluated from the area of the extravasated dye. An area of blue coloration of more than 3 mm in two diameters was considered a positive reaction. Compound 48/80 (Sigma Chemical Co.) and serotonin were used as positive controls.

Data analysis

Group results are expressed as means $\pm$ SEM, except for values of EC200R_L, which are reported as geometric means. Comparisons among groups for baseline R_L, blood and lung lymphocyte subsets, lung tissue leukocyte numbers, and immunoglobulin levels were done by using one-way analysis of variance (ANOVA) and the Newman-Keuls test for multiple comparisons when the values were normally distributed. When data were not normally distributed, comparisons were made by using a nonparametric ANOVA (Kruskal-Wallis), followed by Fischer's least-significant difference test. Comparisons for EC200R_L were done on log-transformed values. Statistical significance was accepted at the 5% level of confidence.

RESULTS

Blood lymphocyte subsets

The blood CD4⁺ lymphocytes, bearing the W3/25 marker, in the lower dose OX-8-treated animals ($44.6\% \pm 5.2\%$, $n = 8$) were similar to those of the controls ($32.5\% \pm 5.2\%$, $n = 6$), but they were somewhat higher in the higher dose OX-8 group ($62.4\% \pm 6.4\%$, $n = 8$, $p < 0.05$). In contrast, blood CD8⁺ lymphocytes, bearing the OX-8 surface marker, were significantly decreased in both groups of OX-8-treated animals (low-dose: $3.0\% \pm 3.8\%$, $n = 7$ and high dose: $1.0\% \pm 3.6\%$, $n = 8$) compared with controls ($18.7\% \pm 3.9\%$, $n = 7$, $p < 0.05$).

To ascertain the adequacy of CD8⁺ T-cell depletion, immunofluorescent staining was performed by using the W3/25, OX-8, and 341 mAbs in additional control rats and additional OX-8-treated rats. The OX-8 and the 341 mAbs detect the same populations of cells in the rat but recog-

nize two different epitopes of the CD8 cell surface antigen. As shown in Fig. 1, similar immunostaining profiles were obtained by using either the OX-8 (CD8 α) or the 341 (CD8 β) mAbs both in the control animals (Fig. 1, A, $n = 2$) and the OX-8-treated animals (Fig. 1, B, $n = 2$). Surface expression of CD8 α and CD8 β in OX-8-treated rats was similar to the immunofluorescent staining (background) observed with the control mAb.

Large airway lymphocyte subsets

Large airway CD4⁺ lymphocytes did not differ between OX-8-treated animals (low-dose: 31.5% $\pm$ 5.7%, $n = 8$ and high-dose: 34.04% $\pm$ 6.6%, $n = 6$) compared with controls (34.9% $\pm$ 6.6%, $n = 6$). In contrast, airway CD8⁺ lymphocytes were reduced in both groups of OX-8-treated animals (low-dose: 2.8% $\pm$ 1.2%, $n = 8$ and high-dose: 2.5% $\pm$ 1.2%) compared with controls (13.8% $\pm$ 1.4%, $n = 6$, $p < 0.05$).

Small airway and parenchymal cellularity

The cellular populations retrieved from the small airways and parenchyma 24 hours after the antigen challenge are shown in Fig. 2. The numbers of macrophages, neutrophils, and lymphocytes were significantly elevated in the sensitized animals treated with control antibodies compared with unsensitized animals but did not differ from the numbers obtained from the lower dose OX-8-treated animals. In contrast, neutrophils ($30.01 \times 10^6 \pm 3.14$) and eosinophils ($5.60 \times 10^6 \pm 1.15$) were retrieved in larger numbers from animals treated with the higher dose of OX-8 that resulted in complete depletion of OX-8⁺ T cells compared with numbers retrieved from untreated animals ($15.63 \times 10^6 \pm 2.96$ and $1.02 \times 10^6 \pm 1.15$, respectively; $p < 0.05$).

Large airway cellularity

The cellular populations retrieved from the large airways are shown in Fig. 3. Only macrophage numbers increased in the sensitized animals treated with control antibodies ($0.58 \times 10^6 \pm 0.08$) compared with unsensitized animals ($0.14 \times 10^6 \pm 0.09$, $p < 0.05$). Cellular populations did not differ in sensitized animals pretreated with a lower dose of OX-8 mAbs compared with untreated animals. However, marked increases in neutrophils ($1.77 \times 10^6 \pm 0.18$), eosinophils ($0.13 \times 10^6 \pm 0.02$), and lymphocytes ($0.53 \times 10^6 \pm 0.08$) were observed in the higher dose OX-8-treated animals compared with untreated animals ($0.33 \times 10^6 \pm 0.18$, 0.01

$\times 10^6 \pm 0.02$, and $0.24 \times 10^6 \pm 0.08$, respectively; $p < 0.05$).

Airway responsiveness to methacholine

The values of R_L before challenge were similar among the four groups of rats. Likewise, after ovalbumin challenge, there were no significant differences in airway responsiveness among the groups (Fig. 4.).

Ovalbumin-specific IgE

Levels of ovalbumin-specific IgE were not detectably elevated above the background absorbance recorded for unsensitized animals: unsensitized, 0.059 ± 0.021 ; sensitized, 0.041 ± 0.021 ; sensitized and OX-8-pretreated (low-dose), 0.049 ± 0.018 ; sensitized and OX-8-pretreated (high-dose), 0.026 ± 0.012 . Levels of ovalbumin-specific IgE measured in BN rats ($n = 3$) reached 0.905 ± 0.252 absorbance units.

Intradermal skin tests

Unsensitized rats did not show any skin reactions to concentrations of ovalbumin below 2 mg/ml (Table I). Three of four sensitized and untreated SD rats had positive skin reactions to 1 mg/ml ovalbumin ($p < 0.05$ compared with unsensitized SD rats). Five of five OX-8-treated SD rats had positive skin reactions to 1 mg/ml ovalbumin ($p < 0.005$ compared with unsensitized SD rats). All three sensitized BN rats reacted positively to 1 mg/ml ovalbumin. Only sensitized and OX-8-treated SD rats had significant positive skin test responses to 1 μ g/ml ovalbumin compared with unsensitized rats ($p < 0.005$). Positive reactions were elicited to serotonin and compound 48/80 in all animals, and there was no evidence of a treatment effect on the reactions to these controls.

DISCUSSION

Airway hyperresponsiveness has been associated with the inflammatory process present in the airways of subjects with asthma. In an attempt to better understand the link between the development of airway hyperresponsiveness and the pattern of cellular infiltration within the lungs, we have studied the role of CD8⁺ T cells in the regulation of antigen-induced airway responses in the rat. We hypothesized that CD8⁺ T-cell depletion would enhance the antigen-induced airway eosinophilia in the low IgE responder SD rat strain and would increase the cholinergic bronchial responsiveness that is usually associated with allergen exposure of sensitized animals. The results of

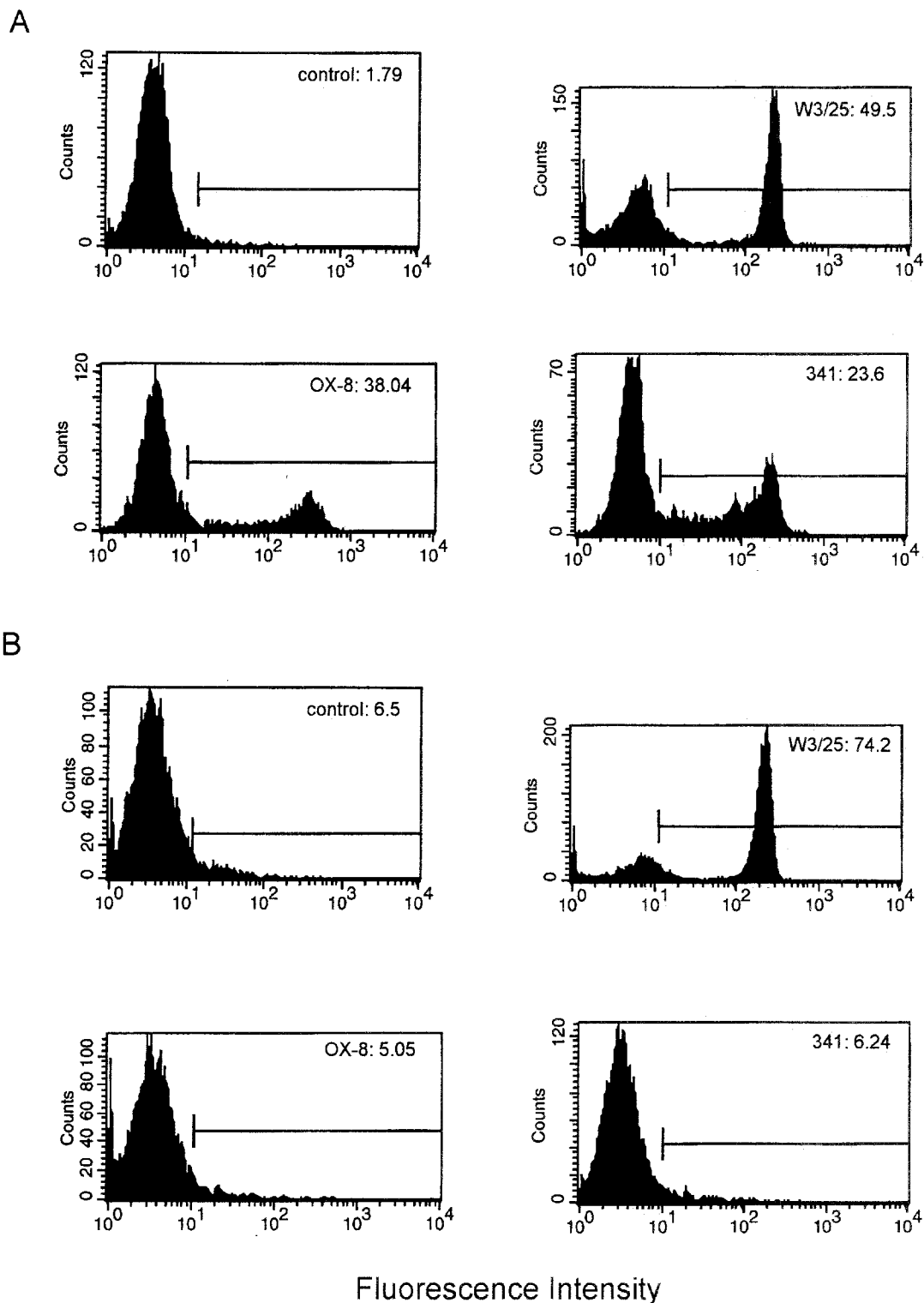


FIG. 1. FACS analysis of the immunofluorescent staining of blood lymphocytes in control rats (**A**) and high-dose OX-8-treated rats (**B**). Blood lymphocytes were isolated and incubated with control IgG, OX-8 (CD8 α), 341 (CD8 β), and W3/25(CD4) antibodies and then with a secondary fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG antibody. Histograms show the expression of CD4(W3/25), CD8 α (OX-8), and CD8 β (341) on gated lymphocytes. Numbers refer to the percentage positive cells for each cell surface marker. Immunofluorescent staining with a control mAb is included. Data from one of two experiments are shown, but results were similar for the other experiment also.

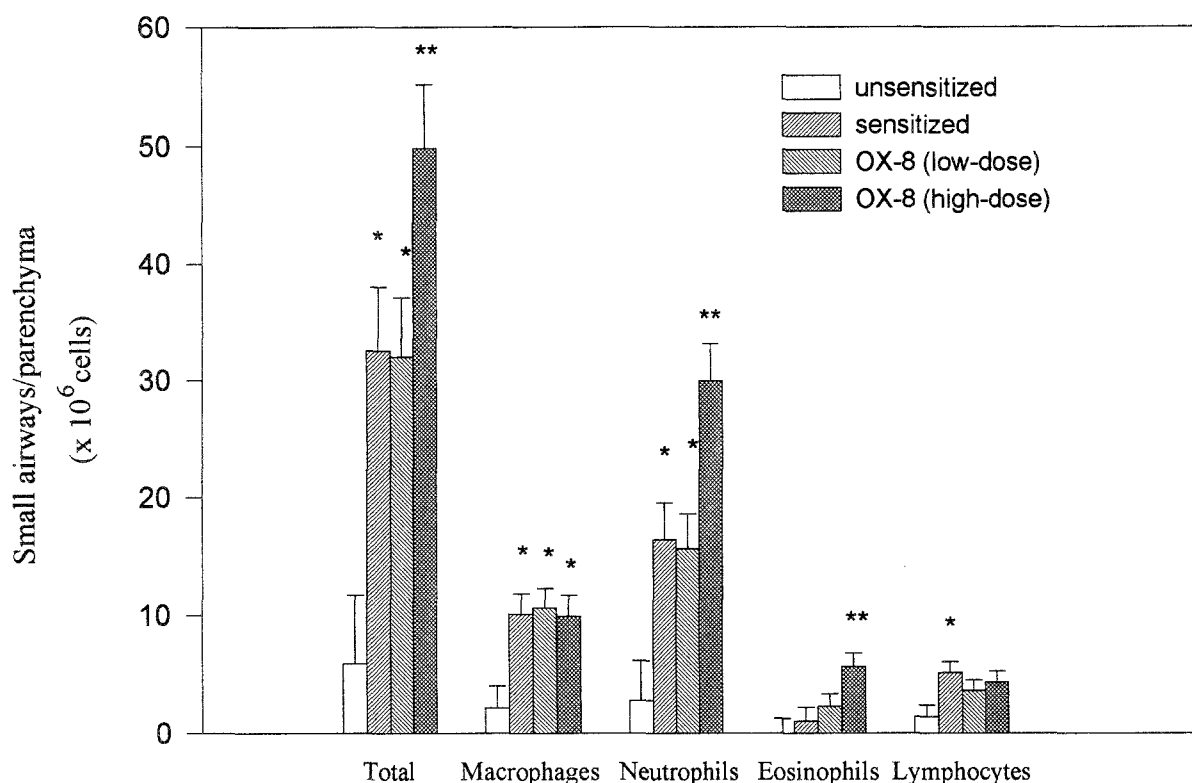


FIG. 2. Cellular populations retrieved from small airways and parenchyma 24 hours after antigen challenge. Antigen challenge induced increases in absolute numbers of total leukocytes, macrophages, neutrophils, and lymphocytes in sensitized animals ($n = 8$) compared with unsensitized animals ($n = 7$) (* $p < 0.05$ by ANOVA). High-dose OX-8 mAb ($n = 8$) resulted in further increases of total leukocytes, neutrophils, and eosinophils compared with sensitized and untreated animals, as well as low-dose OX-8 ($n = 9$) (** $p < 0.05$ by ANOVA).

this study indicate that CD8⁺ T cells are importantly involved in the regulation of allergen-induced airway inflammation in SD rats. A single allergen challenge of sensitized animals induced an inflammatory response characterized by an influx of leukocytes comprising macrophages, neutrophils, and lymphocytes to the lung tissues at 24 hours. Selective depletion of CD8⁺ T lymphocytes from the blood and the airways further amplified the inflammatory response and promoted eosinophil accumulation within the lung tissues. However, cholinergic airway responsiveness was not significantly increased by allergen challenge and was not affected by the fivefold increase in eosinophilia caused by OX-8 pretreatment.

In these studies we used the technique of *in vivo* cell depletion to investigate the role of CD8⁺ T cells in the antigen-induced airway responses. Consistent with previous reports,^{18, 27-29} adequate depletion of CD8⁺ T cells was achieved by the administration of the OX-8 mAb both in the peripheral blood and in the airways, as demonstrated in immunofluorescent staining by a marked decrease in the percent of

OX-8⁺ cells. To evaluate the possibility that OX-8 mAb administration resulted in a reduction of CD8⁺ cell numbers and not simply a masking of the OX-8 surface antigen, rat mononuclear cells were also immunostained with the 341 mAb, which recognizes a different epitope of the CD8 surface molecule. Similar immunostaining profiles were found by using both the OX-8 and 341 mAbs, which recognize the α and β chains of the OX-8 surface molecule, respectively, in both control and OX-8-treated rats, suggesting that OX-8 mAb treatment did not simply mask the OX-8 surface molecule but resulted in a virtually complete depletion of CD8⁺ cells. Moreover, the current findings of significant alteration in the inflammatory cell populations of the lung and the reported increase in late airway bronchoconstriction¹⁸ in OX-8-treated rats suggest that the function of suppressor cells was altered in rats that received the OX-8 mAb.

Elimination of CD8⁺ T cells resulted in substantial differences in the inflammatory cells in both the small airways and parenchyma and the large airways. Twofold and fivefold increases in neutro-

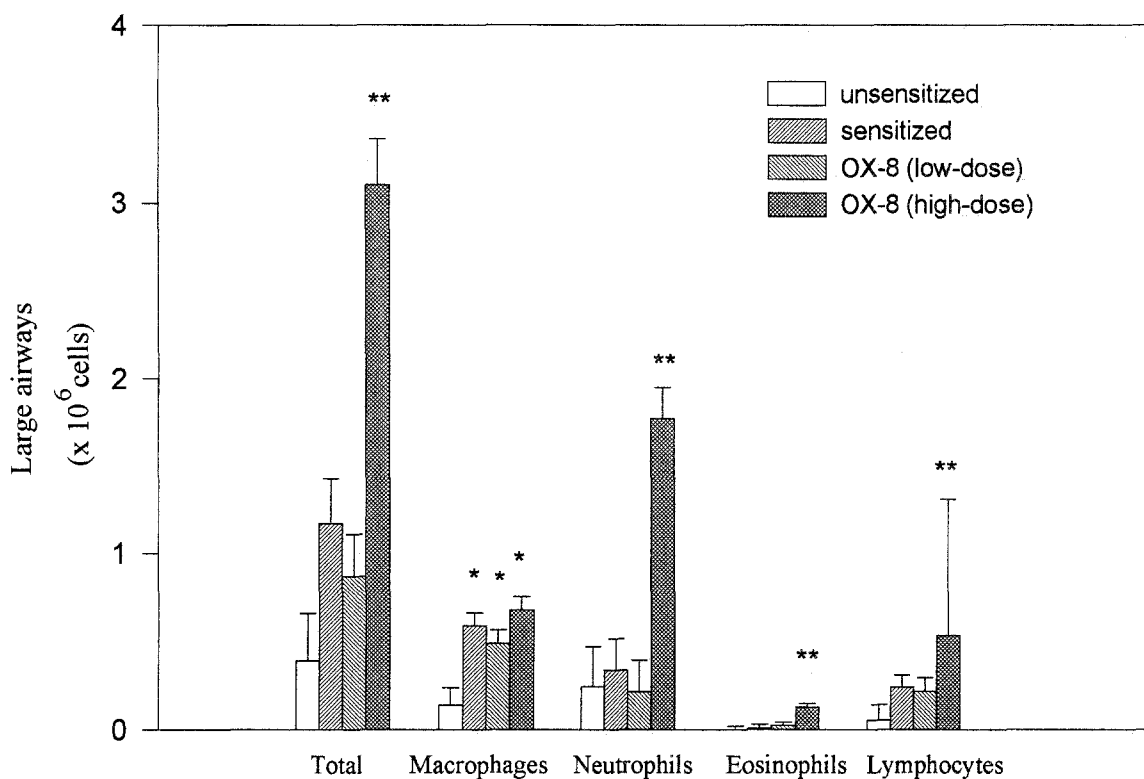


FIG. 3. Cellular populations retrieved from large airways 24 hours after antigen challenge. Increases in all leukocyte subtypes were observed in animals treated with high-dose OX-8 mAb compared with other groups. ** $p < 0.05$ (ANOVA) versus all groups. * $p < 0.05$ (ANOVA) versus unsensitized rats.

phil and eosinophil numbers, respectively, were detected in the small airways and parenchyma of animals pretreated with the higher dose of OX-8 mAb compared with untreated animals. More striking changes were observed in the large airways with a twofold increase in lymphocytes, a fivefold increase in neutrophils, and an increase of more than 10-fold in the eosinophil numbers. The findings in this study are consistent with a previous report, which demonstrated significant increases of bronchoalveolar lavage macrophages, neutrophils, and lymphocytes 8 hours after an ovalbumin challenge in SD rats pretreated with OX-8 mAb.¹⁸ The current data indicate that CD8⁺ T cells also have an inhibitory effect on eosinophil accumulation in the lung tissues, as assessed at a later time point. We have previously shown that the changes observed in the inflammatory cell populations cannot be attributed to the administration of the mAb per se because no cellular alteration occurred within the lungs of sham-sensitized animals after a similar treatment.¹⁸

Despite striking enhancement of the inflammatory response and accumulation of a large number

of eosinophils in the airways after depletion of CD8⁺ T cells, development of antigen-induced airway hyperresponsiveness to methacholine was not observed. We were not surprised that the ovalbumin-challenged SD rats not pretreated with OX-8 did not have an increased responsiveness to inhaled methacholine because these animals have very small and infrequent late airway responses.¹⁸ We anticipated an increase in responsiveness after OX-8 treatment, however, in view of the fact that late responses occur in these animals.¹⁸ In addition to late responses, airway inflammation, including eosinophilia, is also enhanced. Late allergic responses and airway hyperresponsiveness have been considered as closely related phenomena.^{30, 31} The current data do not exclude relationships among the late allergic response and cholinergic hyperresponsiveness, but they point to the complexity of the relationship between late allergic response, airway inflammation, and airway hyperresponsiveness.

The pathogenesis of airway hyperresponsiveness has been linked to airway inflammation, although the precise mechanisms underlying this phenome-

TABLE I. Intradermal skin test results

Group	Ovalbumin (1 mg/ml)	Ovalbumin (10 µg/ml)
Unsensitized SD rats	0/5	0/5
Sensitized + untreated SD rats	3/4*	2/4
Sensitized + OX-8-treated SD rats	5/5*	5/5*
Sensitized BN rats	3/3	2/3

A reaction of more than 3 mm in two diameters was considered positive.

Data are expressed as number of animals with positive skin reactions divided by total number of animals studied.

* $p < 0.05$ compared with unsensitized SD rats by Fisher's exact test.

non have not been fully elucidated.³² More specifically, eosinophils have a postulated role in the development of airway hyperresponsiveness after allergen challenge.^{33, 34} Only a few studies have examined the control of antigen-induced airway hyperresponsiveness by T-cell subsets. In a murine model of asthma, depletion of CD4⁺ T cells prevented the development of antigen-induced airway hyperresponsiveness, which was associated with the attenuation of airway inflammation and reduction of infiltrating eosinophils.³⁵ Gavett et al.³⁵ postulated a link between eosinophilia and hyperresponsiveness. Underwood et al.³⁶ have reported that elimination of CD8⁺ T cells by ricin in rats resulted in increased airway eosinophilia in ricin-treated rats compared with control rats, which was not accompanied by a further increase in airway hyperresponsiveness. In these studies, ricin-induced CD8⁺ T-cell depletion was associated with a decreased capacity for splenic T cells to produce interferon- γ but an increased ability to express the T_{H2}-like cytokines IL-4 and IL-5 messenger RNA in splenic T cells.^{37, 38} Recently, Renz et al.³⁹ reported that transfer of purified spleen CD8⁺ T cells from ovalbumin-sensitized mice to sensitized recipients prevented the increase in airway responses to electrical field stimulation that usually occurs in sensitized animals. It was argued that interferon- γ production by CD8⁺ cells mediated the inhibitory effects. Significant reduction in IgE was also reported, but possible effects on the pattern of allergic inflammation were not investigated. It is difficult to reconcile the reported absence of an augmentation of allergen-induced eosinophil infiltration in the trachea by depletion of CD8⁺ T cells in mice⁴⁰ with the observations of inhibitory actions of transferred CD8⁺ cells.³⁹ One

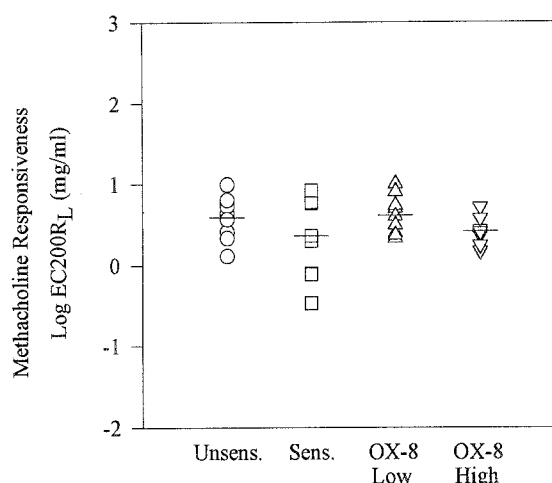


FIG. 4. Airway responsiveness to methacholine 24 hours after antigen challenge in unsensitized (*Unsens.*), sensitized and untreated (*Sens.*), and sensitized and OX-8-treated SD rats.

possible explanation is that the effect of CD8⁺ cells on hyperresponsiveness in the mouse may not be mediated by the eosinophil.

Mechanisms by which CD8⁺ T cells modulate airway responses to antigen are unclear. Lymphocytes bearing the CD8 cell surface molecule include the cytotoxic T lymphocytes and the antigen-specific suppressor T cells. CD8⁺ T cells can suppress immune responses in a variety of ways including cytotoxicity, release of soluble antigen-specific suppressive factors, and release of inhibitory cytokines.^{41, 42} In vitro studies showed that CD8⁺ T cells downregulate antigen-induced T-cell proliferative responses⁴³ and regulate IgE production in vivo.⁴² Although stimulated CD8⁺ T cells secrete lymphokines, their repertoire of lymphokine production is generally more restricted than that of CD4⁺ T cells. It has been shown that circulating CD8⁺ T cells are a major source of interferon- γ in other low-IgE-responder rat strains.⁴⁴ Interferon- γ inhibits proliferation of murine⁴⁵ T_{H2} clones in vitro and downregulates T_{H2}-type responses, such as IgE production, in vivo.^{44, 46} Interestingly, administration of recombinant murine interferon- γ prevented the allergen-induced eosinophil infiltration in the mouse trachea, whereas anti-interferon- γ promoted eosinophil and CD4⁺ T-cell infiltration in the trachea.⁴⁷ It is therefore possible that depletion of certain functional subsets of CD8⁺ T cells favored the emergence of some T_{H2}-type-like effects, such as eosinophil accumulation within the airways, in this study.

However, marked depletion of CD8⁺ lympho-

cytes did not convert the responses of the "non-atopic" SD rat to those of the "atopic" BN rat. Indeed, almost complete depletion of CD8⁺ T cells and antigen challenge did not evoke a degree of eosinophilia comparable to that observed in the BN rat^{24,25} and did not induce airway hyperresponsiveness. Likewise, the IgE was still virtually undetectable by ELISA after pretreatment with OX-8 antibody, indicating a major difference in the quantity of IgE produced by the SD rat compared with that produced by the BN rat. Successful sensitization of the SD rat was nonetheless confirmed by intradermal skin testing. These observations argue that the "atopic" behavior of the BN rat does not reside only in altered responses of its CD8⁺ cells but must involve other mechanisms. Presumably, these other mechanisms are also responsible for the airway hyperresponsiveness that follows allergen challenge in BN rats²³⁻²⁵ and may explain the strain differences in allergen-induced airway responses. In this regard, the pattern of cytokine secretion in response to specific allergens might be important. Although the SD rat has not been fully characterized from the standpoint of predominant lymphokines secreted by T cells, a preliminary report indicates that SD rats express interferon- γ in the lung after ovalbumin challenge, whereas BN rats do not.⁴⁸

In conclusion, the results of this study support an active contribution of the CD8⁺ T cells in mediating the allergen-induced pulmonary responses in the SD rat. The results indicate that the virtual absence of CD8⁺ T cells, although evoking eosinophilic airway inflammation, is not in itself sufficient to induce airway hyperresponsiveness to inhaled methacholine. Furthermore, the intervention does not convert the SD rat to one with the same characteristics as the BN rat.

REFERENCES

1. Wilson JW, Djukanovic R, Howarth PH, Holgate ST. Lymphocyte activation in bronchoalveolar lavage and peripheral blood in atopic asthma. *Am Rev Respir Dis* 1992;145:958-60.
2. Walker C, Kaegi MK, Braun P, Blaser K. Activated T cells and eosinophilia in bronchoalveolar lavages from subjects with asthma correlated with disease severity. *J Allergy Clin Immunol* 1991;88:935-42.
3. Assawi M, Bradley B, Jeffery PK, et al. Identification of activated T lymphocytes and eosinophils in bronchial biopsies in stable atopic asthma. *Am Rev Respir Dis* 1990;142:1407-13.
4. Metzger WJ, Zavala D, Richerson HB, et al. Local allergen challenge and bronchoalveolar lavage of allergic asthmatic lungs. *Am Rev Respir Dis* 1987;135:433-40.
5. Gerblich AA, Salik H, Schuyler MR. Dynamic T-cell changes in peripheral blood and bronchoalveolar lavage after antigen bronchoprovocation in asthmatics. *Am Rev Respir Dis* 1991;143:533-7.
6. Wieranga EA, Snoek M, de Groot C, et al. Evidence for compartmentalization of functional subsets of CD4⁺ T lymphocytes in atopic patients. *J Immunol* 1990;144:4651-6.
7. Robinson DS, Hamid Q, Ying S, et al. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med* 1992;326:298-304.
8. Walker C, Virchow J-C Jr, Bruijnzeel PLB, Blaser K. T cell subsets and their soluble products regulate eosinophilia in allergic and non-allergic asthma. *J Immunol* 1991;146:1829-35.
9. Corrigan CJ, Haczku A, Gemou-Engesaeth V, et al. CD4 T-lymphocyte activation in asthma is accompanied by increased serum concentrations of interleukin-5. Effect of glucocorticoid therapy. *Am Rev Respir Dis* 1993;147:540-7.
10. Van Oosterhout AJM, Ladenius ARC, Savelkoul HFJ, Van Ark I, Delsman KC, Nijkamp FP. Effect of anti-IL-5 and IL-5 on airway hyperreactivity and eosinophils in guinea pigs. *Am Rev Respir Dis* 1993;147:548-52.
11. Engel P, Huguet J, Sanosa J, Sierra P, Cols N, Garcia-Calderon PA. T cell subsets in allergic respiratory disease using monoclonal antibodies. *Ann Allergy* 1984;53:337-40.
12. Walker C, Bode E, Boer L, Hansel TT, Blaser K, Virchow J-C Jr. Allergic and nonallergic asthmatics have distinct patterns of T-cell activation and cytokine production in peripheral blood and bronchoalveolar lavage. *Am Rev Respir Dis* 1992;146:109-15.
13. Kus J, Tse KS, Vedral S, Chan-Yeung M. Lymphocyte sub-population in patients with allergic and non-allergic asthma. *Clin Allergy* 1985;15:523-9.
14. Rola-Pleszczynski M, Blanchard R. Suppressor cell function in respiratory allergy. Modulation by aminophylline and isoproterenol. *Int Arch Allergy Appl Immunol* 1981;64:361-70.
15. Harper TB, Gaumer HR, Waring W, Branon RB, Salvaggio JE. A comparison of cell-mediated immunity and suppressor T-cell function in asthmatic and normal children. *Clin Allergy* 1980;10:555-63.
16. Hwang K-C, Kikrig SM, Friedman HM, Gupta S. Deficient concanavalin-A-induced suppressor-cell activity in patients with bronchial asthma, allergic rhinitis and atopic dermatitis. *Clin Allergy* 1985;15:67-72.
17. Gonzalez MC, Diaz P, Galleguillos FR, Ancic P, Cromwell O, Kay AB. Allergen-induced recruitment of bronchoalveolar helper (OKT4) and suppressor (OKT8) T-cells in asthma. Relative increases in OKT8 cells in single early responders compared with those in late-phase responders. *Am Rev Respir Dis* 1987;136:600-4.
18. Olivenstein R, Renzi PM, Yang JP, et al. Depletion of OX-8 lymphocytes from the blood and airways using monoclonal antibodies enhances the late airway response in rats. *J Clin Invest* 1993;92:1477-82.
19. Brideau RJ, Carter PB, McMaster WR, Mason DW, Williams AF. Two subsets of rat T lymphocytes defined with monoclonal antibodies. *Eur J Immunol* 1980;10:609-15.
20. Cantrell DA, Robins RA, Brooks CG, Baldwin RW. Phenotype of rat natural killer cells defined by monoclonal antibodies marking rat lymphocyte subsets. *Immunology* 1982;45:97-103.
21. Eidelman DH, Bellofiore S, Martin JG. Late airway responses to antigen challenge in sensitized inbred rats. *Am Rev Respir Dis* 1988;137:1033-7.

22. Renzi PM, Olivenstein R, Martin JG. Inflammatory cell populations in the airways and parenchyma after antigen challenge of the rat. *Am Rev Respir Dis* 1993;147:967-74.
23. Elwood W, Barnes PJ, Fan Chung K. Airway hyperresponsiveness is associated with inflammatory cell infiltration in Brown-Norway rats. *Int Arch Allergy Immunol* 1992;99: 91-7.
24. Renzi PM, Olivenstein R, Martin JG. Effect of dexamethasone on airway inflammation and responsiveness after antigen challenge of the rat. *Am Rev Respir Dis* 1993;148: 932-9.
25. Laberge S, Rabb H, Issekutz TB, Martin JG. Role of VLA-4 and LFA-1 in allergen-induced airway hyperresponsiveness and lung inflammation in the rat. *Am J Respir Crit Care Med* 1995;151:822-9.
26. Shardonofsky FR, Sato J, Bates JH. Quasi-static pressure-volume hysteresis in the canine respiratory system in vivo. *J Appl Physiol* 1990;68:2230-6.
27. Like AA, Biron CA, Weringer EJ, Byman K, Sroczynski E, Guberski DL. Prevention of diabetes in BioBreeding/Worcester rats with monoclonal antibodies that recognize T lymphocytes or natural killer cells. *J Exp Med* 1986;164: 1145-59.
28. Stitz L, Sobbe M, Bilzer T. Preventive effects of early anti-CD4 or anti-CD8 treatment on Borna disease in rats. *J Virol* 1992;66:3316-23.
29. Sedgwick JD. Long-term depletion of CD8+ T cells in vivo in the rat: no observed role for CD8+ (cytotoxic/suppressor) cells in the immunoregulation of experimental allergic encephalomyelitis. *Eur J Immunol* 1988;18:495-502.
30. Cockcroft DW, Ruffin RE, Dolovich J, Hargreave FE. Allergen-induced increase in non-allergic bronchial reactivity. *J Clin Allergy* 1977;7:503-13.
31. Cartier A, Thompson NC, Frith PA, Roberts R, Hargreave FE. Allergen-induced increase in bronchial responsiveness to histamine: relationship to the late asthmatic response and change in airway caliber. *J Allergy Clin Immunol* 1982;70:170-7.
32. Djukanovic R, Roche WR, Wilson JW, et al. Mucosal inflammation in asthma. *Am Rev Respir Dis* 1990;142:434-57.
33. Gleich GJ. The eosinophil and bronchial asthma: current understanding. *J Allergy Clin Immunol* 1990;85:422-36.
34. Gundel RH, Letts LG, Gleich GJ. Human eosinophil major basic protein induces airway constriction and airway hyperresponsiveness in primates. *J Clin Invest* 1991;87:1470-3.
35. Gavett SH, Chen X, Finkelman F, Wills-Karp M. Depletion of murine CD4+ T lymphocytes prevents antigen-induced airway hyperreactivity and pulmonary eosinophilia. *Am J Respir Cell Mol Biol* 1994;10:587-93.
36. Underwood SL, Kemeny DM, Lee TH, Raeburn D, Karlsson J-A. IgE production, antigen-induced airway inflammation and airway hyperreactivity in the Brown-Norway rat: the effects of ricin. *Immunology* 1995;85:156-61.
37. Noble A, Staynov DZ, Lee TH, Kemeny DM. Elimination of IgE regulatory rat CD8+ T cells in vivo differentially modulates interleukin-4 and interferon-gamma but not interleukin-2 production by splenic T cells. *Immunology* 1993 78:513-9.
38. Noble A, Staynov DZ, Diaz-Sanchez D, Lee TH, Kemeny DM. Elimination of IgE regulatory rat CD8+ T cells in vivo increases the co-ordinate expression of Th2 cytokines IL-4, IL-5 and IL-10. *Immunology* 1993;80:326-9.
39. Renz H, Lack G, Saloga J, et al. Inhibition of IgE production and normalization of airways responsiveness by sensitized CD8 T cells in a mouse model of allergen-induced sensitization. *J Immunol* 1994;152:351-60.
40. Nakajima H, Iwamoto I, Tomoe S, et al. CD4+ T-lymphocytes and interleukin-5 mediate antigen-induced eosinophil infiltration into the mouse trachea. *Am Rev Respir Dis* 1992;146:374-7.
41. Murphy DB. T cell mediated immunosuppression. *Curr Opin Immunol* 1993;5:411-7.
42. Kemeny DM, Noble A, Holmes BJ, Diaz-Sanchez D. Immune regulation: a new role for the CD8+ T cell. *Immunol Today* 1994;15:107-10.
43. Gershon RK, Cohen P, Hensin R, Liebhaver S. Suppressor T cells. *J Immunol* 1972;108:586-90.
44. McMenamin C, Holt PG. The natural immune response to inhaled soluble protein antigens involves major histocompatibility complex (MHC) class I-restricted CD8+ T cell-mediated but MHC class II-restricted CD4+ T cell-dependent immune deviation resulting in selective suppression of immunoglobulin E production. *J Exp Med* 1993;178:889-99.
45. Gajewski TF, Joyce J, Fitch FW. Antiproliferative effect of IFN-gamma in immune regulation. III. Differential selection of TH1 and TH2 murine helper T lymphocyte clones using recombinant IL-2 and recombinant IFN-gamma. *J Immunol* 1989;143:15-22.
46. Finkleman FD, Katona IM, Mosmann TR, Coffman RL. IFN- γ regulates the isotypes of Ig secreted during in vivo humoral immune responses. *J Immunol* 1988;140:1022-7.
47. Iwamoto I, Nakajima H, Endo H, Yoshida S. Interferon- γ regulates antigen-induced eosinophil recruitment into the mouse airways by inhibiting the infiltration of CD4+ T cells. *J Exp Med* 1993;177:573-6.
48. Renzi PM, Yang JP, Yasruel Z, Martin JG, Hamid Q. Rats which do not develop late airway responses express mRNA for the TH1 type of cytokines in lungs after antigen challenge [abstract]. *J Allergy Clin Immunol* 1995;95:275.