Antibody to Very Late Activation Antigen 4 Prevents Antigen-induced Bronchial Hyperreactivity and Cellular Infiltration in the Guinea Pig Airways

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Summary

This report examines the effect of an anti-VLA-4 monoclonal antibody (mAb) HP1/2 on antigen-induced bronchial hyperreactivity to methacholine, and on eosinophil and T lymphocyte infiltration in the airways of guinea pigs sensitized and challenged by aerosolized ovalbumin and used 24 h thereafter. The intravenous administration of 2.5 mg/kg of HP1/2, but not of its isotype-matched mAb 1E6, 1 h before and 4 h after antigen inhalation, markedly inhibited the increased bronchopulmonary responses to intravenous methacholine, as well as airway eosinophilia in bronchoalveolar lavage (BAL) fluid and in bronchial tissue. HP1/2 also suppressed the antigen-induced infiltration of the bronchial wall by $CD4^+$ and $CD8^+$ T lymphocytes, identified by immunohistochemical technique using specific mAbs that recognize antigenic epitopes of guinea pig T cells. Treatment with HP1/2 also resulted in a significant increase in the number of blood eosinophils, suggesting that inhibition by anti-VLA-4 mAb of eosinophil recruitment to the alveolar compartment may partially account for their accumulation in the circulation. These findings indicate that eosinophil and lymphocyte adhesion and subsequent infiltration into the guinea pig airways that follow antigen challenge are mediated by VLA-4. Furthermore, concomitant inhibition of antigen-induced bronchial hyperreactivity and of cellular infiltration by anti-VLA-4 mAb suggests a relationship between airway inflammation and modifications in the bronchopulmonary function.

Airway infiltration by inflammatory cells, particularly eosinophils and T lymphocytes, and an exaggerated sensitivity of the airways to different stimuli, namely bronchial hyperreactivity, are characteristic features of asthma (1, 2). The participation of eosinophils in the development of bronchial hyperreactivity is supported by their presence in high numbers in bronchial biopsies and bronchoalveolar lavage (BAL)[1] fluid from asthmatics (1). Furthermore, the observation that most of the infiltrating eosinophils appear degranulated, as a result of in situ activation (3), suggests an important role for the eosinophil-derived cationic proteins, including major basic protein (MBP) and eosinophil peroxidase (EPO), in bronchial asthma. Indeed, these proteins appear to be responsible for the shedding and lysis of the respiratory epithelium (4–6), which contributes to increase the airway reactivity to bronchoconstrictor stimuli.

Activated T lymphocytes are also commonly found in the bronchial submucosa of asthmatic patients (7–9). It was suggested that switching of $CD4^+$ T lymphocytes for the preferential production of Th2-derived cytokines (10–12), such as IL-5, a cytokine able to promote the growth, differentiation, and survival of eosinophils (13–15), may induce the recruitment and activation of these cells at the site of allergic reactions. Nevertheless, mobilization of leukocytes into the inflamed tissues is initiated by their adhesion to endothelial cells. This phenomenon is mediated by the binding of LFA-1 (CD11a–CD18 complex) to intercellular adhesion molecule 1 (ICAM-1), which is present on various cell populations, including lymphocytes (16), mononuclear cells (17), neutrophils (18), and eosinophils (19). However, the further demonstration that lymphocytes and eosinophils, but not neutrophils, express very late activation antigen 4 (VLA-4) and can thus bind to vascular cell adhesion molecule 1 (VCAM-1) (20), suggests a selective mechanism for their localization in inflammatory sites.

Recently, Weg et al. (21) demonstrated that the in vitro preincubation of ^{111}In-labeled guinea pig eosinophils with

[1] *Abbreviations used in this paper:* APAAP, alkaline phosphatase antialkaline phosphate; BAL, bronchoalveolar lavage; EPO, eosinophil peroxidase; ICAM-1, intercellular adhesion molecule 1; MBP, major basic protein; VCAM-1, vascular cell adhesion molecule 1; VLA-4, very late activation antigen 4.

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the mouse anti-human mAb HP1/2, which is directed against the $\alpha 4$ subunit of VLA-4 (22), inhibited their accumulation induced by the intradermal administration of various inflammatory agents. In the same study, HP1/2 was shown to bind to guinea pig eosinophils, but not to neutrophils (21), allowing its use in this species as a tool to investigate the role of VLA-4 in the inflammatory and immune reactions involving eosinophil mobilization.

In the present study, we show that ovalbumin inhalation by aerosol-sensitized guinea pigs is followed, 24 h later, by increased sensitivity of the airways to bronchoconstrictor responses induced by methacholine. Bronchial hyperreactivity is accompanied by increased numbers of eosinophils in the airways and in BAL fluid as well as by a rise in the EPO levels in the supernatant of BAL fluid, as a marker of in situ activation. Antigen-challenged guinea pigs also exhibit increased numbers of T lymphocytes in their bronchial wall, mostly consisting of the $CD4^+$ subtype. Treatment of sensitized guinea pigs with HP1/2 mAb abrogates antigen-induced bronchial hyperreactivity to methacholine and the cellular infiltration in the airways, suggesting the involvement of VLA-4 in these processes. Our results clearly indicate that VLA-4 inhibitors may have a promising future to counteract allergic airway inflammation and the accompanying alterations in the bronchopulmonary function.

Materials and Methods

Sensitization Procedure and Antigen Challenge. Male Hartley guinea pigs (400–600 g; C. Lebeau, Gambais, France) were immunized by aerosolized ovalbumin (Miles, Naperville, IL), at 1% in sterile 0.9% NaCl (saline) for 30 min. The procedure was repeated 48 h later and the animals were exposed to antigen challenge 14–17 d after the first inhalation. The day of antigen provocation, guinea pigs were exposed for 15 min to five successive solutions of ovalbumin of 0.001, 0.01, 0.1, 0.5, and 1%, respectively (modified from reference 23). In rare cases, when the concentration of ovalbumin reached 0.1%, animals exhibited labored breathing and coughing.

Control animals were sensitized as above and exposed to aerosolized sterile saline for 30 min. The aerosol was delivered into a 36 L plexiglass chamber using an ultrasonic nebulizer (Ultra-Neb 99; De Vilbiss Medical, Arcueil, France), which produced particles of a mass diameter averaging between 0.5 and 3 μm. The liquid output of the nebulizer was 0.6 ml/min. The animals were used for the in vivo experiments 24 h after challenge with saline or ovalbumin.

mAbs. HP1/2 is a mouse IgG1 mAb directed against the $\alpha 4$ chain (CD49d) of VLA-4 (22). The cell line was grown as ascites and mAbs were purified by protein A and gel filtration chromatography under endotoxin-free conditions, as described (21). Isotype-matched mAb 1E6 was purified as above.

Treatments. HP1/2 was injected intravenously to sensitized guinea pigs at the dose of 2.5 mg/kg, 1 h before and 4 h after antigen challenge. This dose and route of administration were selected on the basis of results showing that HP1/2 inhibited eosinophil accumulation in the guinea pig skin induced by the intradermal injection of different inflammatory agonists (21). Control animals were treated intravenously twice with 2.5 mg/kg of the isotype-matched mAb 1E6. Aliquots of HP1/2 and 1E6 were stored at −20°C and diluted in sterile saline immediately before use.

In Vivo Experiments. The day of the experiment, guinea pigs were anesthetized by an intraperitoneal injection of 1.2 mg/kg ethyl urethane (Sigma Chemical Co., St. Louis, MO), tracheae were cannulated, and the animals were prepared for the recording of bronchial resistance to inflation. The jugular vein was cannulated to allow drug injections. Spontaneous breathing was suppressed by the intravenous injection of 4 mg/kg of pancuronium bromide (Organon Teknika, Fresnes, France). At least 1 h after surgery, bronchial reactivity was tested by the intravenous administration of 1–64 μg methacholine (Sigma Chemical Co.) at 15-min intervals. Changes in bronchial resistance to inflation were measured by a pressure transducer (Gould Electronics BV, Bilthoven, The Netherlands) placed between the trachea and the respiratory pump (Palmer BioScience, Sheerness, UK) and continuously recorded on a dynograph (model R511; Beckman Instruments, Schiller Park, IL).

Results are expressed as the effective dose of methacholine, calculated from the dose-response curve, required to increase the bronchial resistance to inflation by 30% of the maximal response obtained by clamping the tracheal cannula (ED30).

Blood Leukocyte and Bronchoalveolar Cell Counts and Differentiation. In separate experiments, sensitized guinea pigs challenged either with saline or ovalbumin and treated with HP1/2 or 1E6, were anesthetized as above and tracheae and carotid arteries were cannulated. Aliquots of arterial blood (200 μl) were collected for total leukocyte counts, using a Coulter Counter (model ZM; Coultronics, Margency, France), and for differential counts after staining with Diff-Quik stain (Merz & Dade AG, Baxter Dade AG, Duedingen, Switzerland). Results are expressed as number of each cell population/μl blood. Bronchoalveolar cells were collected in five successive lavages using 10-ml aliquots of sterile saline at room temperature injected and recovered through a polyethylene tracheal cannula. The lavage fluid was stored on ice and total cell counts were performed as above. An aliquot of the suspension was then diluted to reach a final concentration of 1.5×10^5 cells/ml and cytospin preparations were performed (Hettich Universal, Tuttingen, Germany). Differential cell counts were obtained by counting 300 cells after staining with Diff-Quik stain. The results are expressed as the concentration of each cell population/ml BAL. Aliquots of 1 ml of the remaining lavage fluid were centrifuged at 200 g for 15 min at 4°C and the supernatant was collected and stored at −20°C until the determination of EPO, as described below. The corresponding pellet was resuspended in 1 ml of 0.5% solution of NP-40 (Sigma Chemical Co.) in distilled H_2O and then stored at −20°C until the measurement of EPO activity.

EPO Determination. The levels of EPO in the pellet and in the supernatant of BAL fluid from saline- or ovalbumin-challenged guinea pigs treated with HP1/2 or 1E6, as described above, were determined by a previously described method (24). Briefly, 100 μl of the substrate solution consisting of 0.1 mM *o*-phenylenediaminedihydrochloride (OPD) in 0.05 M Tris-HCl containing 0.1% Triton X-100, and 1 mM hydrogen peroxide (all from Sigma Chemical Co.) were added to 100 μl of the samples in microtiter plate wells. The plates were left at 37°C for 30 min before stopping the reaction by the addition of 50 μl of 4 M sulfuric acid (Sigma Chemical Co.). The optical densities were measured at 490 nm (OD_{490}) with an automatic microplate-reader (Dinatech MR 5000; Dinatech Laboratories, Saint-Cloud, France).

Immunohistochemistry. After the BAL collection was performed as described above, guinea pigs were exsanguinated via the abdominal aorta and the contents of the thoracic cavity resected "en bloc." The lungs were inflated via the trachea with 3 ml Histocon (Polysciences, Inc., Warrington, PA), the lobes dissected and mounted over cork disks, covered by optimum cutting temperature com-

pound (BDH Chemicals Ltd., Poole, UK), and snap frozen in isopentane (Prolabo, Paris, France) cooled by liquid nitrogen. The frozen blocks were kept at −80°C before use. Sections alongside the main intrapulmonary bronchus were cut in a cryostat kept at −21°C and collected on glass slides previously coated with poly-L-lysine (Sigma Chemical Co.), fixed in chloroform-acetone (vol/vol; Merck, Darmstadt, Germany) for 10 min, wrapped in a plastic film, and kept at −20°C before use. Representative sections of each block were also stained with hematoxylin-eosin (Rhône-Poulenc, Viliers-Saint Paul, France) for conventional histology.

Consecutive sections of each block were stained with the following mAbs, as described (25): H159, rat anti-guinea pig T lymphocytes, staining 90% circulating T cells (26); CT5, mouse anti-guinea pig T lymphocytes, staining also macrophages and some B cells (27); H155, rat anti-guinea pig CD4, recognizing helper T lymphocytes (28), and CT6, mouse anti-guinea pig CD8, recognizing suppressor/cytotoxic T cells (27). For the rat antibodies, immunoperoxidase staining was performed, employing the Vectastain ABC *Elite* Kit (Vector Laboratories, Inc., Burlingame, CA); biotinylated rabbit anti-rat Ig (BA-4001; Vector Laboratories, Inc.) and swine anti-rabbit Ig conjugated to horseradish peroxidase (P217; Dakopatts a/s, Copenhagen, Denmark) were used together with Vectastain. All stainings were followed by diaminobenzidine (DAB) (Sigma Chemical Co.) development and light hematoxylin counterstaining. For the murine antibodies (CT5 and CT6), alkaline phosphatase antialkaline phosphatase (APAAP) staining procedure was performed using rabbit Ig to mouse Ig (Z259; Dakopatts) and mouse APAAP (D651; Dakopatts), followed by incubation with the substrates Fast Red TR (Sigma Chemical Co.) and naphtol AS MX phosphate (Sigma Chemical Co.), and light hematoxylin counterstaining. Cyanide-resistant EPO activity, employing potassium cyanide (Merck), DAB, and hydrogen peroxide (Merck), was used to stain the eosinophils (25, 29, 30).

At least two sections stained with each antibody or EPO technique, were coded and read in a "blind" fashion. Positive cells were enumerated in the bronchial submucosa (between the basal lamina and the smooth muscle), and in the bronchial adventitia (between the smooth muscle and the cartilage), by means of an eyepiece graticule composed of 100 squares of known area. The area of the compartments and the number of positive cells were determined on each microscope field, and at least 10 fields were analyzed. The results of each stained slide were expressed as the number of positive cells per unit area (6.25×10^4 μm^2, the total area of the graticule). Results were calculated per mAb for each experimental group.

Statistical Analysis. Results are expressed as mean ± SEM of the indicated number of experiments. One-way analysis of variance (ANOVA) was used to determine significance among the groups. If a significant variance was found, an unpaired Student's *t* test was used to assess comparability between means. A value of $p \leq 0.05$ was considered significant.

Results

Effect of mAb HP1/2 on Antigen-induced Bronchial Hyperreactivity to Methacholine. Ovalbumin inhalation by sensitized untreated- or 1E6 (2.5 mg/kg, i.v.)-treated guinea pigs was followed by an augmented bronchoconstrictor response to intravenous methacholine, as shown by a significant decrease in ED30 values, as compared with saline-challenged guinea pigs (Fig. 1). The dose of 2.5 mg/kg HP1/2, administered

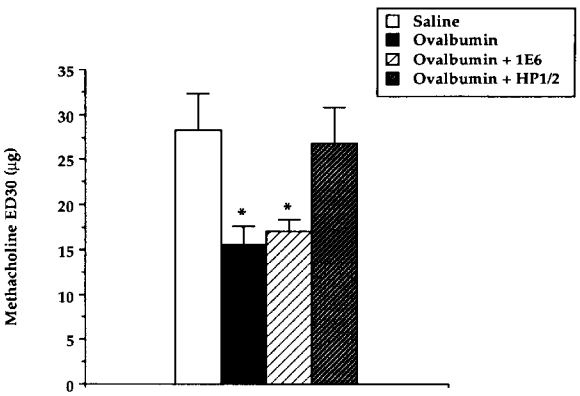


Figure 1. Effect of HP1/2 on antigen-induced bronchial hyperreactivity to methacholine in sensitized guinea-pigs. Bronchial responsiveness to methacholine was evaluated in saline- and ovalbumin-challenged guinea pigs, either untreated or treated intravenously with 2.5 mg/kg 1E6 or HP1/2, 1 h before and 4 h after antigen challenge. The baseline bronchial resistance to inflation was 6.90 ± 0.30, 6.90 ± 0.37, 7.65 ± 0.66, and 7.40 ± 0.47 cmH_2O for saline- and ovalbumin-challenged, untreated or 1E6- or HP1/2-treated-guinea-pigs, respectively (differences not statistically significant). Results are expressed as the effective dose of methacholine (in micrograms), calculated from the dose-response curve, required to increase the bronchial resistance to inflation by 30% of the maximal response obtained by clamping the tracheal cannula (ED30). Values are means ± SEM of 6–9 experiments.
* $p < 0.05$, as compared with saline-challenged guinea pigs.

1 h before and 4 h after antigen challenge, inhibited by 92% ($n = 6–9$, $p = 0.046$) bronchial hyperreactivity to methacholine in immunized guinea pigs (Fig. 1).

Effect of mAb HP1/2 on Antigen-induced Changes in Blood Cell Numbers. Antigen challenge in sensitized guinea pigs induced a slight increase in the total number of leukocytes, which was more pronounced in 1E6-injected animals (Table 1). This resulted from a rise in the number of neutrophils and mononuclear cells, particularly in antigen-challenged untreated animals (Table 1). Treatment with HP1/2 was followed by a significant increase in the number of circulating eosinophils, without changes in that of other cell types (Table 1).

Effect of mAb HP1/2 on Antigen-induced Changes in Cell Composition and EPO Levels in BAL Fluid. A significant increase in the total cell and eosinophil numbers was observed in the BAL fluid from antigen-challenged untreated or 1E6-treated guinea pigs, as compared with controls (Table 2). Neutrophil numbers slightly increased after ovalbumin inhalation, and a statistically significant difference was achieved when saline- and ovalbumin-challenged 1E6-treated animals were compared (Table 2). No significant changes in the number of lymphocytes were observed, even though a trend toward an augmentation was noted in antigen- as opposed to saline-challenged animals (Table 2). Treatment of sensitized guinea pigs with HP1/2 significantly inhibited total cell and eosinophil numbers in the BAL fluid, without modifying the other cell populations (Table 2).

Ovalbumin inhalation was also followed by a rise in EPO

Table 1. *Cellular Distribution in Blood Collected from Sensitized Guinea Pigs Exposed, 24 h Earlier, to Aerosolized Saline or Ovalbumin and Treated Intravenously with 1E6 or HP1/2, 1 h before and 4 h after Antigen Challenge*

Treatment		Cell Type/μL blood			
Aerosol	Intravenous	Total cells	Eosinophils	Neutrophils	Mononuclear cells
Saline	None	5,511.5 ± 545.6	111.3 ± 38.9	2,544.6 ± 425.6	3,007.3 ± 89.0
Ovalbumin	None	8,752.0 ± 1,843.1	197.0 ± 28.6	3,661.3 ± 1,279.3	4,829.3 ± 669.3
Ovalbumin	1E6	7,926.4 ± 808.3*	72.6 ± 14.2	1,823.0 ± 315.8	5,954.6 ± 751.4*
Ovalbumin	HP1/2	8,791.8 ± 871.7*	421.0 ± 89.3*‡§	2,015.4 ± 378.8	6,091.4 ± 706.4*

Values are means ± SEM of five experiments.
* $p < 0.05$, as compared with saline-challenged guinea pigs.
‡ $p < 0.05$, as compared with ovalbumin-challenged guinea pigs.
§ $p < 0.05$, as compared with ovalbumin-challenged 1E6-injected guinea pigs.

Table 2. *Cellular Distribution in BAL Fluid Collected from Sensitized Guinea Pigs Exposed, 24 h before, to Aerosolized Saline or Ovalbumin and Treated Intravenously with 1E6 or HP1/2, 1 h before and 4 h after Antigen Challenge*

Treatment		Cell type				
Aerosol	Intravenous	Total cells	Macrophages	Eosinophils	Neutrophils	Lymphocytes
				× 10^5/ml BAL		
Saline	None	5.63 ± 1.01	4.10 ± 0.77	0.98 ± 0.12	0.15 ± 0.08	0.14 ± 0.07
Ovalbumin	None	11.01 ± 1.03*	6.88 ± 0.85	2.83 ± 0.64*	1.10 ± 0.50	0.28 ± 0.11
Ovalbumin	1E6	10.96 ± 0.65*	7.00 ± 0.41*	2.78 ± 0.64*	0.63 ± 0.16*	0.22 ± 0.11
Ovalbumin	HP1/2	7.88 ± 1.16§	5.96 ± 0.60	1.06 ± 0.22‡§	0.70 ± 0.14*	0.16 ± 0.09

Values are means ± SEM of five experiments.
* $p < 0.05$, as compared with saline-challenged guinea pigs.
‡ $p < 0.05$, as compared with ovalbumin-challenged guinea pigs.
§ $p < 0.05$, as compared with ovalbumin-challenged 1E6-injected guinea pigs.

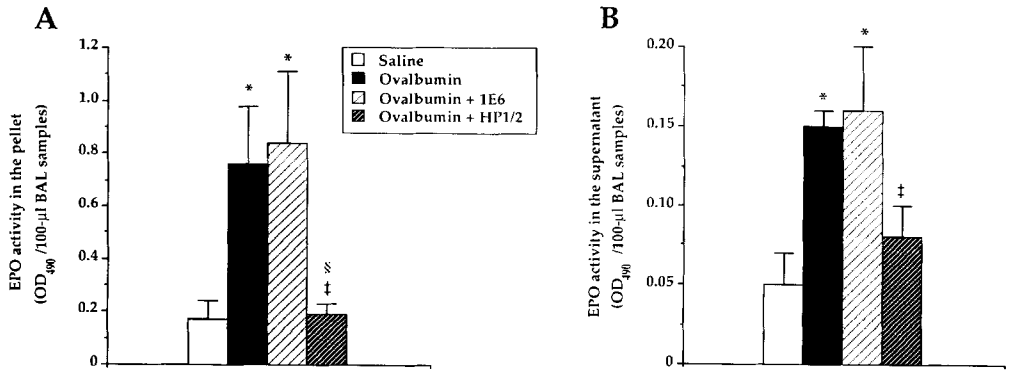


Figure 2. Effect of HP1/2 on antigen-induced EPO accumulation in the BAL fluid from sensitized guinea pigs. EPO activity was measured in the lysed pellet (*A*) and in the supernatant (*B*) of BAL fluid from sensitized saline- or ovalbumin-challenged guinea pigs, either untreated or treated intravenously with 2.5 mg/kg 1E6 or HP1/2, 1 h before and 4 h after antigen challenge. Results are expressed as the mean ± SEM of five experiments for each group.
* $p < 0.05$, as compared with saline-challenged guinea pigs.
‡ $p < 0.05$, as compared with ovalbumin-challenged guinea pigs.
§ $p < 0.05$, as compared with ovalbumin-challenged 1E6-injected guinea pigs.

levels in the pellets and in the supernatants of BAL fluids. These effects were markedly inhibited by the administration of HP1/2 to sensitized animals (Fig. 2).

Effect of mAb HP1/2 on Antigen-induced Changes in the Number of Eosinophils and T Lymphocytes in the Bronchial Wall. A marked infiltration of eosinophils was seen in the bronchial mucosa of ovalbumin-challenged untreated or 1E6-treated animals, particularly next to the epithelium (Fig. 3). Most of the positive cells were found in the bronchial submucosa than in the adventitia (Fig. 4). The intravenous treatment with 2.5 mg/kg HP1/2 blocked the increase in the number of eosinophils in both compartments (Figs. 3 and 4).

Ovalbumin inhalation by sensitized untreated or 1E6-treated guinea pigs was also followed by T cell infiltration in the bronchial submucosa and the adventitia, as detected by the mAb H159 and CT5 (Figs. 5 and 6). This increment was

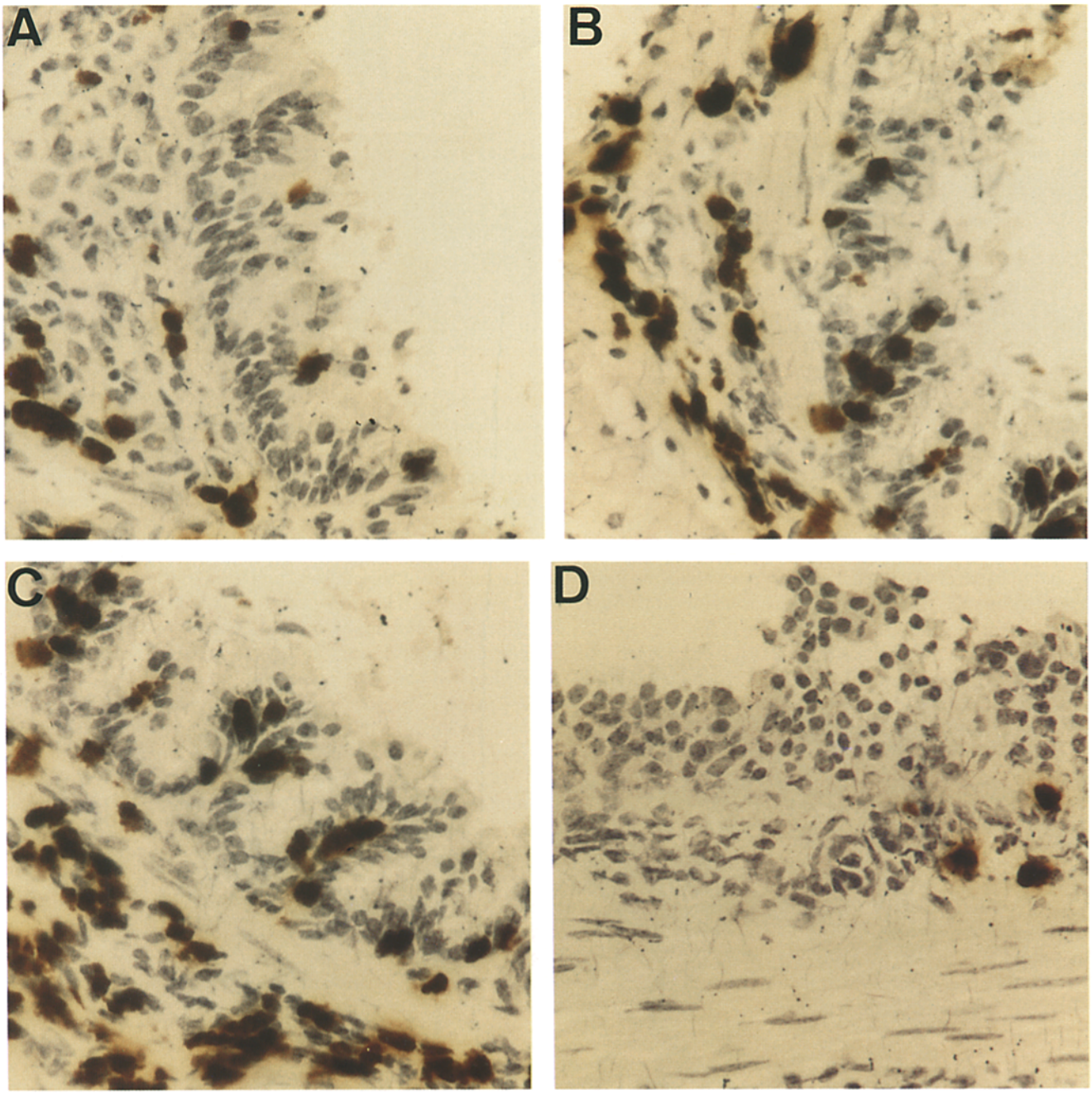


Figure 3. Cyanide-resistant eosinophil peroxidase activity in the bronchial wall of sensitized saline- or antigen-challenged guinea pigs. (*A*) saline-challenged animal, showing scant positive cells in the bronchial submucosa; (*B*) ovalbumin-challenged guinea pig, with intense EPO^+ cells infiltrating the bronchial submucosa; (*C*) ovalbumin-challenged 1E6-treated animal, also showing many EPO^+ cells in the bronchial wall; (*D*) ovalbumin-challenged HP1/2-treated guinea pig with few positive cells in the bronchial submucosa (original magnifications ×400).

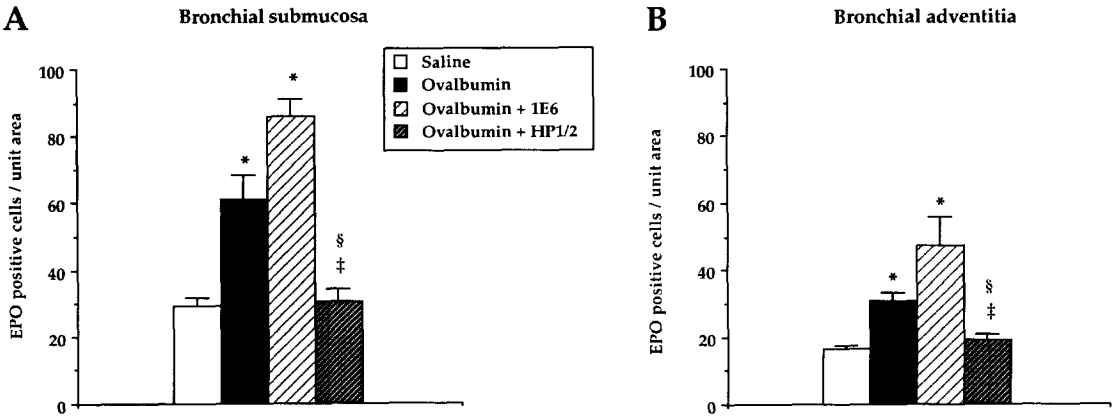


Figure 4. Effect of HP1/2 on eosinophil infiltration into the bronchial lumen of antigen-challenged guinea pigs. EPO$^+$ cells in the bronchial submucosa (*A*) and in the bronchial adventitia (*B*) of sensitized guinea pigs, challenged with saline or ovalbumin and treated intravenously with 2.5 mg/kg 1E6 or HP1/2, 1 h before and 4 h after antigen challenge. Results represent the number of positive cells ± SEM/unit area of bronchial wall compartment (6.25×10^4 μm^2). n = 5 experiments for each group.
* p <0.05, as compared with saline-challenged guinea pigs.
‡ p <0.05, as compared with ovalbumin-challenged guinea pigs.
§ p <0.05, as compared with ovalbumin-challenged 1E6-injected guinea pigs.

mostly due to CD4$^+$ T cells, even though a significant increase in the number of CD8$^+$ T cells was observed in the bronchial submucosa of ovalbumin-, as opposed to saline-challenged animals (Fig. 6). HP1/2 suppressed total T cell recruitment, as well as that of CD4$^+$ and CD8$^+$ T lymphocytes in both bronchial compartments (Figs. 5 and 6).

Discussion

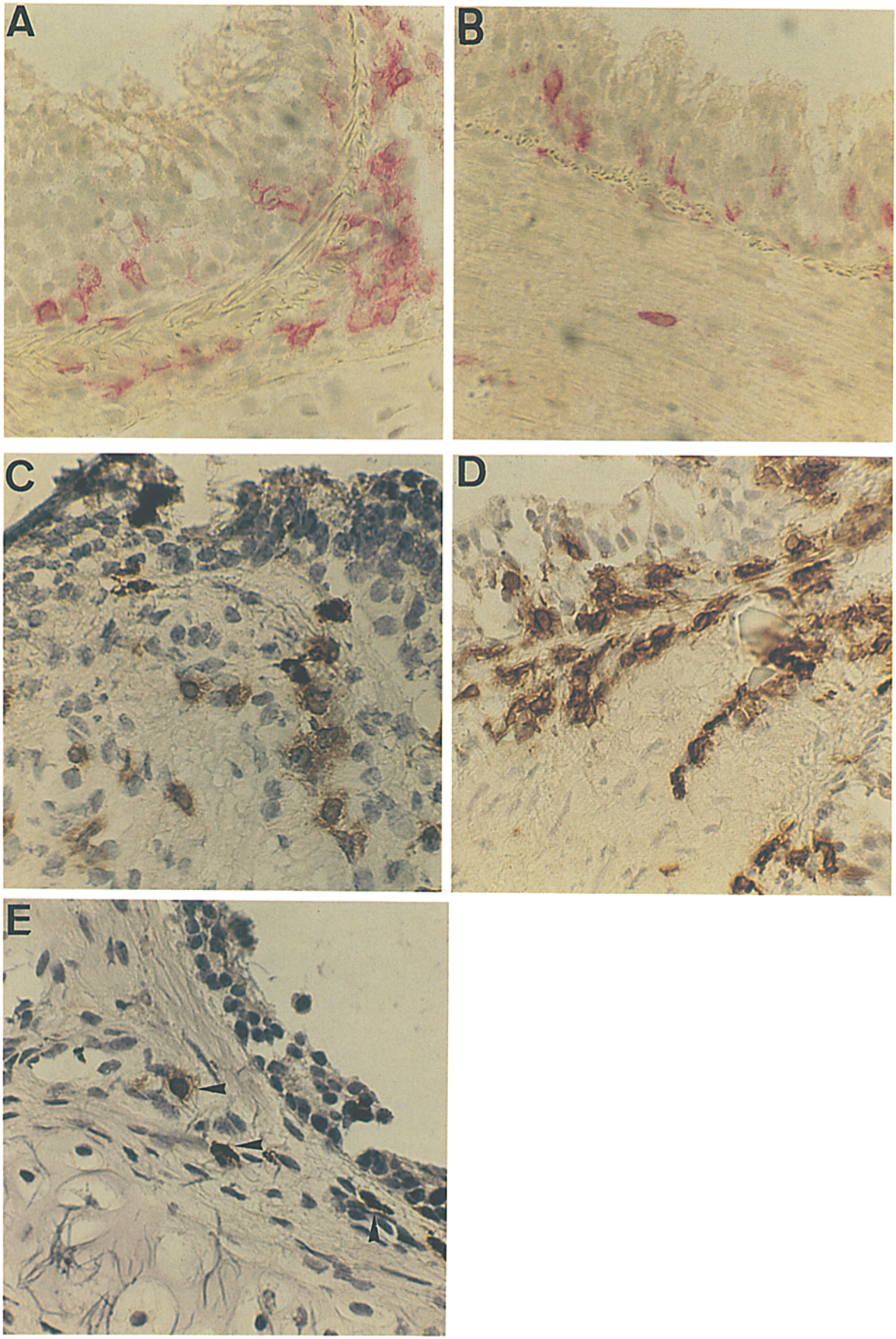
Increasing evidence suggests that the frequently observed association between activated T lymphocytes and eosinophils plays a major role in the development of airway inflammation and in the accompanying bronchial hyperreactivity (7–9, 31). The concomitant presence of infiltrating eosinophils and T lymphocytes in the bronchi of asthmatics may result from their common pathway of adherence to endothelial cells. Indeed, eosinophils bind to activated endothelial cells by adhering to VCAM-1 (20), a ligand also involved in lymphocyte adherence to endothelium (32). Furthermore, adhesion of eosinophils and lymphocytes to VCAM-1 is mediated by the binding of VLA-4 present on their surface (20, 33), which underlines the selectivity of the mechanisms involved in their migration into inflamed tissues.

Upon reaching the airways, eosinophils can release a variety of cationic proteins, such as EPO and MBP, that are responsible for injury and shedding of airway epithelium (4–6). Disruption of the epithelium leads to the exposure of the underlying mucosal structures and sensory nerve endings to allergen and irritants, contributing to the developing of nonspecific bronchial hyperreactivity.

In the present study, we demonstrate that antigen inhalation by aerosol-sensitized guinea pigs is followed by a two-fold increase in the bronchial reactivity to methacholine. This is accompanied by intense eosinophil infiltration in the bronchopulmonary tissue and accumulation in the BAL fluid. Under these conditions, treatment of the sensitized animals with the anti-VLA-4 mAb HP1/2 results in a marked inhibition of antigen-induced bronchial hyperreactivity and eosinophil infiltration and accumulation in the bronchial wall. This suggests that anti-VLA-4 antibody exerts its effect by preventing binding of VLA-4 located on eosinophils to endothelium-associated VCAM-1, leading to the inhibition of the recruitment of eosinophils into the bronchopulmonary tissue. In support, suppression of antigen-induced airway eosinophilia in HP1/2-treated animals was accompanied by a rise in the number of eosinophils in the blood.

Weg et al. (21) showed that VLA-4 is involved in eosinophil recruitment at the site of antigen stimulation in the guinea pig skin. Here we highlight other facets of VLA-4–mediated allergic inflammation, such as antigen-induced bronchial hyperreactivity and in situ eosinophil activation. In our model, eosinophil degranulation was detected by increased concentrations of EPO in the BAL fluid from antigen-challenged guinea pigs. We have previously validated the use of EPO as a marker for eosinophil activation and showed that the levels of EPO and MBP, a cationic protein specifically stored

Figure 5. Identification of T lymphocytes and subsets in the bronchial compartments of sensitized saline- or antigen-challenged guinea-pigs treated with 1E6 or HP1/2. (*A*) marked T lymphocyte infiltration in the bronchial submucosa of ovalbumin-challenged 1E6-treated guinea pig (CT5, APAAP); (*B*) as in *A*, ovalbumin-challenged HP1/2-treated guinea pig, showing few positive cells. (*C*) CD4$^+$ T lymphocytes in the bronchial mucosa of lungs from sensitized saline-challenged guinea pigs (H155, immunoperoxidase), showing scant positive cells in the bronchial submucosa; (*D*) as in *C*, ovalbumin-challenged 1E6-treated guinea pig with intense positive CD4$^+$ T lymphocytes infiltrating the bronchial submucosa; (*E*) as in *C* and *D*, ovalbumin-challenged HP1/2-treated guinea pig with few positive cells in the bronchial submucosa (*arrows*). Original magnifications ×400.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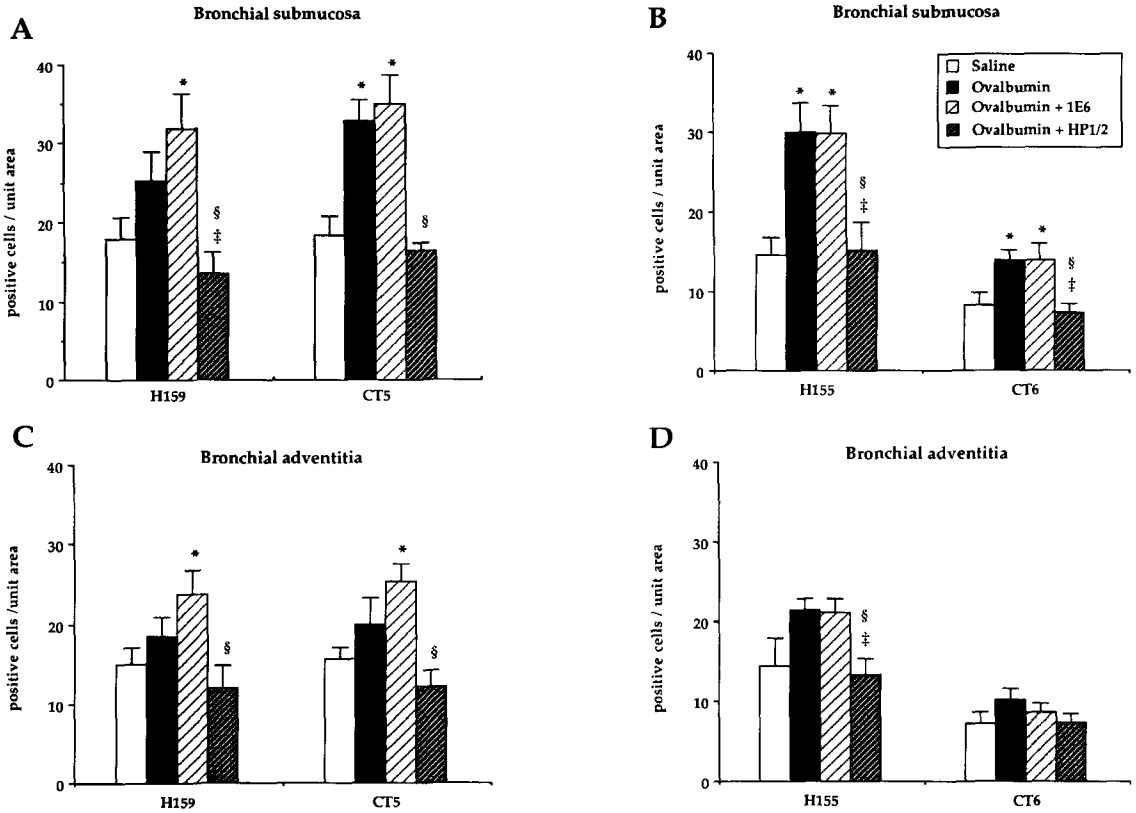

Figure 6. Effect of HP1/2 on antigen-induced T lymphocytes recruitment into the bronchial compartments of sensitized guinea pigs. T lymphocytes (H159, immunoperoxidase and CT5, APAAP) and their subsets (H155, immunoperoxidase and CT6, APAAP) were identified in the bronchial submucosa (*A* and *B*) and in the bronchial adventitia (*C* and *D*) of sensitized guinea pigs, challenged with saline or ovalbumin and treated intravenously with 2.5 mg/kg 1E6 or HP1/2, 1 h before and 4 h after antigen challenge. Results represent the number of positive cells ± SEM/unit area of bronchial wall compartment (6.25×10^4 μm^2). $n = 5$ experiments for each group.
* $p < 0.05$, as compared with saline-challenged guinea pigs.
‡ $p < 0.05$, as compared with ovalbumin-challenged guinea pigs.
§ $p < 0.05$, as compared with ovalbumin-challenged 1E6-injected guinea pigs.

in the eosinophils, correlated significantly (34). A marked decrease in the amounts of EPO in the BAL fluid of antigen-challenged guinea pigs was observed in HP1/2-treated animals, indicating that the eosinophils present in the airways after antigen challenge fail to undergo activation and thus do not release cationic proteins.

Inhibition of eosinophil secretion by HP1/2 may reflect the block of the interaction between VLA-4 and fibronectin (35). Indeed, it was recently reported that adherence of eosinophils to extracellular matrix proteins, such as fibronectin, induced their activation, as expressed by enhanced leukotriene (LT)C_4 production (31). The hypothesis that activation of airway eosinophils is required for the development of bronchial hyperreactivity is supported by our recent study showing that antigen inhalation by systemically sensitized guinea pigs is followed by a large infiltration of nonactivated eosinophils into the BAL fluid, without any change in bronchial reactivity to methacholine (34). However, bronchial hyperreactivity did occur when these eosinophils were stimulated in situ by the intratracheally administered LTB_4. This maneuver was translated into the release of large amounts of MBP and EPO in the BAL fluid, suggesting that activation of and secretion by eosinophils recruited to the lungs upon antigen challenge were necessary to induce bronchial hyperreactivity in the guinea pig.

Very recently, Abraham et al. (36) showed that HP1/2 inhibits antigen-induced late phase and bronchial hyperreactivity in the sheep, without reducing significantly the recruitment of eosinophils to the BAL fluid. However, the effect of HP1/2 on the activation status of eosinophils in vivo was not examined. Our results showing that HP1/2 suppresses in vivo antigen-induced eosinophil degranulation as well as the accompanying bronchial hyperreactivity confirm our previous proposal that these events are associated (34) and underline the requirement role of VLA-4 for their induction.

In our hands, only a slight, but significant increase in the number of neutrophils was observed in the BAL fluid 24 h after antigen challenge, which confirm previous studies showing a short-lived rise in this cell population after antigen exposure (23, 37). Interestingly, HP1/2 did not affect the number of neutrophils in BAL fluid, since these figures were still significantly different than those of saline-challenged

animals (Table 2). This result confirms the specificity of the anti-VLA-4 mAb that does not affect neutrophil, as opposed to eosinophil, mobilization.

The participation of T lymphocytes in the pathogenesis of bronchial asthma and the accompanying bronchial hyperreactivity, has been widely demonstrated (31). Indeed, activated $CD4^+$ T lymphocytes are found in the blood and bronchial lumen from asthmatics (7–9, 38) and their numbers decreased in parallel with clinical improvement upon therapy with corticosteroids (39).

Recently, interest has been focused on the characterization of $CD4^+$ T lymphocytes based on their repertoire of secreted cytokines and its possible role in the pathogenesis of allergic disorders. Thus, $CD4^+$ T cells from asthmatics preferentially elaborate Th2-derived cytokines, such as IL-4 and IL-5, which have been shown to enhance IgE synthesis (40), and to act specifically on eosinophil survival, activation, and secretion of proinflammatory mediators (14). Furthermore, IL-4 is highly effective in inducing VCAM expression on endothelial cells (41), which, in turn, facilitates the infiltration of lymphocytes and eosinophils into the inflamed tissues. In this respect, mice transgenic for IL-4 exhibit marked eosinophilic tissue infiltration (42). It is interesting to speculate that inhibition of $CD4^+$ T lymphocyte accumulation by anti-VLA-4 antibody would result in decreased IL-4 secretion, which in turn may reduce VCAM-1 expression.

Large numbers of T lymphocytes, mainly of the $CD4^+$ subset, have been identified in the bronchial mucosa of antigen-challenged guinea pigs (25, 43), even though no temporal relationship with bronchial hyperreactivity and eosinophil infiltration and activation in the airways were demonstrated until now. Here, using immunohistochemical techniques and mAbs specific for guinea pig T cell epitopes, we extended our previous observation of the presence in the bronchial mucosa of systemically sensitized and boosted guinea pigs of increased numbers of $CD4^+$ T lymphocytes and eosinophils (25). In the present study, however, the increased numbers of T cells in the bronchial wall, particularly in the bronchial submucosa, was concomitant with marked bronchial hyperreactivity to methacholine and with eosinophil degranulation. The increase in T cells was mostly accounted for by the $CD4^+$ subset, even though a rise in the number of $CD8^+$ T lymphocytes was observed in the bronchial submucosa. The invasion of the airways by $CD8^+$ may be related to the observation that asthmatics developing early phase responses after allergen inhalation exhibit increased numbers of suppressor T cells in the BAL fluid accompanied by their decrease in the circulation (44). In the present study, treatment of sensitized guinea pigs with HP1/2 suppressed antigen-induced T lymphocyte infiltration in both bronchial compartments examined. Inhibition of T cell influx resulted from the decrease in the number of the $CD4^+$ and $CD8^+$ subclasses, as indicated by the staining with mAb H155 and CT6, respectively. Furthermore, recent data have shown that activated T lymphocytes express fibronectin at their surface (45), another site of interaction for VLA-4. This suggests that sites of intervention for anti-VLA-4 mAb, other than VLA-4/VCAM-1 interaction, may be involved in the migration and activation of leukocytes, particularly of T lymphocytes, to inflammatory sites. Taken together, our results showing the concomitant inhibition of eosinophil and T cell recruitment into the airways of antigen-challenged animals, confirm the involvement of VLA-4 in the mobilization of these two cell types at the site of allergic inflammation.

The role of adhesion molecules in experimental asthma was first demonstrated by Wegner et al. (46), who showed attenuation of antigen-induced airway eosinophilia in *Ascaris*-sensitive monkeys upon treatment with an anti-ICAM-1 antibody. In this study, bronchial hyperreactivity was also decreased, suggesting again that airway inflammation and alterations in lung function are parallel events. Increased expression of adhesive proteins on endothelial cells during allergic reactions has also been reported (47–50). Finally, Hansel et al. (51) demonstrated that sputum eosinophils from asthmatics, but not from normal individuals, express ICAM-1 at their surface. These results indicate that upregulation of adhesive proteins on leukocytes, on one side, and on endothelial cells, on the other, is a common feature of allergic inflammation. Thus, antagonism of adhesion molecules and/or of their respective ligands may have important implications in cellular networks involved in the development and perpetuation of inflammatory and allergic disorders.

In conclusion, our findings demonstrate that, as in human asthma, activated eosinophils and increased numbers of T lymphocytes in the bronchial mucosa, particularly of the $CD4^+$ phenotype, accompany bronchial hyperreactivity in antigen-challenged guinea pigs. These modifications are prevented by treating the animals with a specific anti-VLA-4 mAb, suggesting that interventions designed to selectively prevent eosinophil and T lymphocyte influx and activation in target tissues, may inhibit allergic inflammation and its consequences on the bronchopulmonary function.

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