

Pulmonary Dendritic Cell Distribution and Prevalence in Guinea Pig Airways: Effect of Ovalbumin Sensitization and Challenge¹

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ABSTRACT

We characterized the localization and prevalence of dendritic cells (DC) in guinea pig airways before and after s.c. sensitization and aerosol challenge with ovalbumin (OVA). DC, eosinophils, macrophages, T cells and B cells in lung and trachea were identified and quantified in frozen sections using monoclonal antibodies and computer-assisted image analysis. Airway reactivity of conscious animals to inhaled methacholine was examined. In unsensitized animals, DC were localized primarily within the lamina propria of the trachea and bronchi, in the submucosa of the trachea and in the adventitia of the bronchi. In contrast to reported studies on rats, few DC were noted in the epithelium. After OVA challenge, sensitized animals demonstrated an early obstructive response and a late-phase response that was well developed by 18 hr. Challenge with OVA

increased DC prevalence in the lamina propria and submucosa of the trachea and in the lamina propria and adventitia of the bronchi. There was widespread eosinophilia throughout the airways, but no changes in B cells or T cells were evident. Macrophages were increased in the epithelium of both OVA-treated and saline-treated animals. At 18 hr after challenge, sensitized guinea pigs but not saline-treated controls were hyperreactive to inhaled methacholine. Except for macrophages, none of these effects were observed after saline treatment. Our findings indicate that inflammation in the airways of OVA-sensitized guinea pigs involves infiltration of DC, which is seen at the time animals are hyperreactive to inhaled methacholine.

Asthma is a chronic lung disease characterized by reversible airway obstruction and nonspecific airway hyperreactivity (Sears, 1993). Pathophysiologic features include desquamation of the respiratory epithelium, goblet cell metaplasia, increase in mucous gland number, hypertrophy and hyperplasia of airway smooth muscle, inflammation of the airways and airway hyperreactivity (Corrigan and Kay, 1991). In asthmatic patients, two phases of response to antigen challenge can be observed (O'Byrne *et al.*, 1987; Busse and Sedgwick, 1992). The immediate or early-phase response is characterized by bronchoconstriction, which can be inhibited through the administration of β_2 -adrenoceptor bronchodilators. The second, or late-phase response, occurs 4 to 8 hr after antigen exposure. This response is less affected by bronchodilators (Busse and Sedgwick, 1992) and is associated with inflammation in the airways and airway hyperresponsiveness.

Pulmonary eosinophilia is prominent in asthmatic airways and is considered important in the pathogenic changes asso-

ciated with the disease. Development of bronchial hyperreactivity (Pretolani *et al.*, 1994) and damage to the respiratory epithelium (Frigas *et al.*, 1991) have been correlated with the release of major basic protein and other granule constituents from activated eosinophils. Recruitment of eosinophils into airways during the late-phase inflammatory response to antigen involves the sensitization of T lymphocytes to the antigen with subsequent release of cytokines (Kay *et al.*, 1991), particularly interleukin-3, interleukin-5 and granulocyte-monocyte colony-stimulating factor.

Inflammation of the airways in response to antigen requires the presentation of the antigen to T lymphocytes and their subsequent activation. Accessory cells present within the lung that are capable of antigen presentation to T cells include alveolar macrophages (Holt and Batty, 1980), B lymphocytes (Kammer and Unanue, 1980) and DC (Steinman and Nussenzweig, 1980). DC are found in various lymphoid and nonlymphoid tissues (Schuler and Steinman, 1985). They are characterized by their long, slender processes and high MHC Class II expression (Steinman, 1991). DC are present in the airways of numerous species, including rat, guinea pig and human (Holt and Schon-Hegrad, 1987; Lapa

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ABBREVIATIONS: DC, dendritic cells; OVA, ovalbumin; PBS, phosphate-buffered saline; APAAP, alkaline phosphatase anti-alkaline phosphatase; MCh, methacholine; SRaw, specific airway resistance.

e Silva *et al.*, 1993, Nicod *et al.*, 1987). *In vivo* studies in the rat airways have revealed a network of MHC Class II-positive DC in the epithelium (Holt *et al.*, 1990), predominantly within the upper airways. The prevalence of DC has been shown to increase by 50% after exposure to wood shavings derived from *Pinus radiata* (Schon-Hegrad *et al.*, 1991). The location of DC near the air-tissue interface in the lung suggests a role for the cells as a first line of defense against pathogenic and nonpathogenic antigens present within inspired air.

Pulmonary DC have been studied extensively in other species, such as the rat (Holt *et al.*, 1990; McWilliam *et al.*, 1994; Nelson *et al.*, 1994). However, there is a paucity of information about the localization and role of DC in guinea pig airways, despite the widespread use of the guinea pig for studies on mechanisms of inflammation and airway hyper-reactivity in asthma. We hypothesized that a DC response in the airways would accompany inflammation and airway hyper-reactivity. Therefore, in the present study, we characterized and quantified the distribution of DC in guinea pig trachea and bronchi and examined the airways for changes in DC location and number in guinea pigs that were sensitized and challenged with OVA. Alterations in DC were examined in relation to changes in other inflammatory cells and to changes in basal pulmonary function and reactivity to inhaled MCh aerosol in conscious animals.

Materials and Methods

OVA sensitization and challenge. The method for sensitizing and challenging guinea pigs with OVA in this study was chosen after systematically comparing and evaluating four different protocols (Warner *et al.*, 1995). Dunkin Hartley guinea pigs (Harlan; Indianapolis, IN) (300–350 g) were sensitized with OVA (Sigma Chemical Co., St. Louis, MO) on day 0 and day 14 by s.c. injection, in the nuchal region, of 10 µg OVA and 1 mg Al(OH)₃ dispersed in 0.5 ml of 0.9% NaCl. On day 21, animals were challenged with aerosols of OVA in saline until desired pulmonary obstructive endpoints were reached (see below). Animals were placed either in a whole-body plethysmograph or in a two-chamber plethysmograph (see below). Control animals received saline aerosol on day 21. Another group of animals, the naive animals, were not exposed to any aerosol.

Unrestrained whole-body plethysmography. Two-chamber plethysmography is not suitable for long-term pulmonary function measurements, because the animal must be restrained, and food and water are not available. Therefore, to evaluate obstructive responses over 24 hr, bias-flow-ventilated whole-body plethysmography (Chand *et al.*, 1992, 1993) was utilized on conscious, unrestrained guinea pigs to examine the time course of pulmonary function changes after inhalation challenge with inhaled saline or OVA aerosols. Whole-body plethysmographs (Buxco Electronics Inc., Troy, NY; XA software) provided flow-derived parameters from which enhanced pause (Penh), an indirect parameter of pulmonary obstruction, was calculated by the software according to the relation

$$\text{Penh} = [(\text{Te}/\text{RT}) - 1][(\text{PEF}/\text{PIF})]$$

where Te = expiratory time, RT = relaxation time (time from beginning of expiration until the end of the end expiratory pause), PEF = peak expiratory flow and PIF = peak inspiratory flow. These parameters were averaged over and logged at 30-sec intervals; only Penh will be reported here. After acclimation of the animal to the chamber, baseline data were logged for 10 to 15 min before the experiment.

Two-chamber whole-body plethysmography. In order to examine further the obstructive responses of conscious animals in response to inhaled OVA aerosol or to develop dose-response rela-

tionships for obstructive responses to inhaled MCh aerosol, we measured SRaw changes using a two-chamber whole-body plethysmograph (Thorne and Karol, 1988) connected to a noninvasive airway mechanics analyzer (Model LS-20; Buxco). This method is utilized for short time periods with little stress on the animal. All animals had been acclimated to the apparatus before the experiment. SRaw data were logged at 6-sec intervals; the mean of 10 consecutive interval averages was calculated as the measurement at each time-point. Calculation of SRaw was based on the previous mathematical description by Pennock *et al.* (1979). SRaw provides an indirect index of obstruction in the lower airways (Finney and Forsberg, 1994).

For the two-chamber plethysmograph, aerosols of OVA, MCh and saline solutions generated by a nebulizer (Ultra Neb 99, Devilbiss Co., Somerset, PA) were driven to a 3l Plexiglas chamber in which the mass concentration of the aerosol was monitored (in mg/m³) by a Miniram Aerosol Monitor (Model PDM-3, MIE, Bedford, MA). Ten-second averages of the aerosol mass concentration were used by a computer to adjust the flow of dilutant air automatically before the aerosol entered the head chamber of the two-chamber whole-body plethysmograph in which the conscious guinea pigs were placed. It had been verified in extensive preliminary experiments that the mass concentrations of the aerosols were not affected by the concentrations of solute employed in this study.

Procedures and endpoints during OVA aerosol challenge. When animals were placed in the two-chamber plethysmograph for challenge, the OVA aerosol was delivered until a sign of obstruction or altered breathing was observed. The signs occurred at different times in different animals, and they consisted of cough, increases in breathing frequency, increases in SRaw and/or changes in the breath waveform displayed on the computer screen. When one of these events occurred, the aerosol was stopped and the head chamber was flushed with air. Saline-exposed control animals were administered saline aerosol for 3 min.

When the whole-body plethysmography was employed to examine the time course of obstructive responses to OVA, sensitized animals were challenged with nebulized 0.1% OVA solution, generated by a nebulizer (Ultra Neb 99) and driven to the chamber, until the breath waveform was altered on the computer screen or for 4 min, whichever occurred first. The chamber was flushed with air for 1 min. If the animal did not respond to the challenge with 0.1% OVA, the procedure was repeated after 5 min with nebulized 0.5% OVA solution. By this concentration, all animals except one responded. (We observed that precipitous responses and high mortality occurred if exposures were begun with 0.5% or 1% OVA solutions.) After 30 min to 1 hr, food was added to the chamber, having been withheld earlier; water was always available. Data were recorded for 23 to 24 hr. Saline-exposed controls were exposed to nebulized saline aerosol for 3 min, followed by flushing of the chamber with air for 1 min.

***In vivo* reactivity to inhaled MCh.** The effect of OVA sensitization and challenge on reactivity to MCh was assessed before the animals were sensitized and again 18 hr after challenge with OVA. That is, each animal served as its own control for the effect of OVA or saline treatment; this made possible a within-animal paired experimental design. (The 18-hr time point was established from the time courses of changes in Penh after OVA challenge over a 24-hr period (see fig. 1), which showed that the animals were experiencing the late-phase response at this time.) Each guinea pig was placed in a two-chamber plethysmograph and, after a 10-min acclimation period, was exposed to aerosolized saline and, subsequently, to increasing concentrations of aerosolized MCh (Sigma Chemical Co., St. Louis, MO; 0.1–80 mg/ml) for 3 min *via* a side port in the head chamber of the plethysmograph. SRaw readings were taken at 6-sec intervals for 1 min after exposure to saline and the individual MCh aerosols. MCh aerosols were administered in increasing concentrations until SRaw was raised to approximately 3 times the baseline reading, which was taken as that which occurred after exposure to saline aerosol.

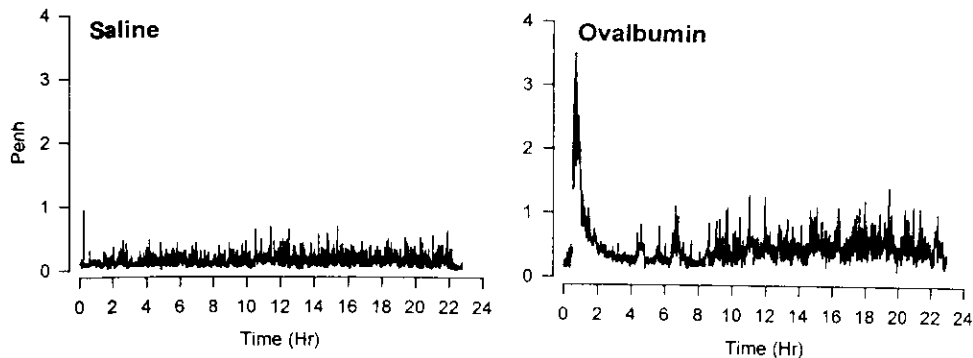


Fig. 1. Time course of postchallenge pulmonary function changes in conscious, freely moving guinea pigs using whole-body plethysmography. Left panel, saline-treated animal administered saline aerosol; right panel, OVA-sensitized animal administered OVA aerosol. Penh, enhanced pause. After recording of control values for 10 to 15 min to establish base line (beginning at $t = 0$), animals were exposed to saline aerosol for 3 min or to OVA aerosol. The particular animal shown in the right panel was exposed to OVA aerosol of 0.1% for 4 min without a visible or measurable response. After washout with air, the animal was exposed to 0.5% OVA aerosol for 52 sec, at which time the pattern of breaths and the amplitude of chamber pressure deflections were noted on the computer screen to be markedly changed. In the left panel, the "spike" near $t = 0$ reflects the artifacts created by delivery of saline aerosol. In the right panel, the break in the tracing near $t = 0$ reflects rejection of breaths by the software, during which time data are not logged. Note also that there are many data points after OVA challenge that are at or very close to the prechallenge base line; some of these thousands of data points are resolvable in this figure, but many are not distinguishable. This experiment was repeated using 10 saline-treated and 14 OVA-treated animals.

Lung removal and fixation. Microscopic examination of the airways and lungs was done entirely with animals that had been challenged in the two-chamber plethysmograph. Eighteen hours after challenge, guinea pigs were anesthetized i.p. with sodium pentobarbital (The Butler Co., Columbus, OH; 100 mg/kg) and exsanguinated. For histochemical and immunohistochemical studies, a 3-cm length of trachea was dissected out. The lungs were inflated with Histocon (Polysciences Inc., Warrington, PA) at a pressure of 30 cm H_2O and removed *en bloc*. The trachea and right diaphragmatic and left cardiac lobes of the lung were snap-frozen in isopentane cooled by liquid nitrogen. Sections of the trachea were prepared by cutting tangentially through the tracheal wall parallel to the longitudinal axis; lung lobes were cut such that the largest airways were included in the sections. Tissue sections 6 μm in thickness (Hacker-Bright Micro Cryostat 2122, Hacker Instruments Inc., Fairfield, NJ) were cut at $-20^\circ C$, collected on Vectabond-coated slides (Vector Laboratories Inc., Burlingame, CA) and air-dried.

Detection of dendritic cells, B cells, T cells and macrophages in histological sections. Because there are no available antibodies specific for guinea pig DC, the cells were characterized according to the following criteria: 1) positive staining with the anti-guinea pig monoclonal antibody Cl.13.1 (Harlan Bioproducts for Science, Indianapolis, IN), a marker for MHC Class II; 2) negative staining with MR-1 (Kraal *et al.*, 1988) (Harlan), a marker for phagocytic tissue macrophages; 3) dendritic morphology. These criteria were assessed routinely by preparing consecutive sections of trachea and lung from the same animal and comparing the localization of Cl.13.1 in relation to MR-1-positive cells.

Sections were analyzed for B cells by using the anti-guinea pig monoclonal antibody MsGp9 (Harlan), a marker primarily for but not absolutely specific for B lymphocytes, which may react with some macrophages and DC. Again, consecutive sections were employed to assess the degree of cross-reactivity.

T lymphocyte density and distribution were determined by incubation with the anti-guinea pig monoclonal antibody CT7 (Harlan), a marker that recognizes $CD4^+$ T cells (T helper cells) (Tan *et al.*, 1985; Antoniou *et al.*, 1986).

Monoclonal antibodies were diluted in PBS, pH 7.55. After fixing in cold acetone for 10 min, consecutive sections of the lung and trachea were incubated for 30 min with 200 μl of monoclonal antibody solution. The slides were rinsed in PBS and postfixated in a 1:1 acetone/formalin mixture, pH 6.6, for 1 min. Sections were then placed in Tris-buffered saline, pH 7.6. The primary antibodies were localized by utilization of the APAAP staining technique (DAKO Kit

K670, Dako Corp., Carpinteria, CA) (Cordell *et al.*, 1984). Positive cells were visualized by incubation with Fast Red TR substrate for 20 min, which produced a red reaction product. Sections were counterstained with Gill's III hematoxylin (Polysciences Inc.) and mounted with Crystal Mount (Biomedica Corp, Foster City, CA).

Negative controls were processed routinely, and they consisted of sections that were incubated without primary antibody. Spleen sections were processed routinely with antibody as positive controls for cell selectivity because of the natural localization of cells expressing the cell surface markers of interest within the spleen. It was observed that T cells were localized adjacent to central arteries in the white pulp. B cells were also localized in the white pulp, immediately outside the T lymphocyte-enriched region. Together, the B lymphocytes and the T lymphocytes formed what is commonly known as the periarteriolar lymphatic sheath. Macrophages and DC were localized primarily within the red pulp and in the marginal zone between the red and white pulp.

Several techniques used in other species to optimize DC detection were found in preliminary experiments to be ineffective in guinea pig airways. For example, cold ethanol fixation before freezing, suggested by Schon-Hegrad *et al.* (1991) to provide superior antigen preservation, provided poor structural preservation and thus was not used. Additionally, the immunoperoxidase technique used by others (Holt *et al.*, 1988; Maarten *et al.*, 1994) for visualization of inflammatory cells could not be utilized in the guinea pig airway tissues because of the presence of large amounts of endogenous peroxidase activity associated with eosinophils in guinea pigs.

Detection of eosinophils in histological sections. Cyanide-resistant eosinophil peroxidase activity was employed to stain sections for eosinophils (Yam *et al.*, 1971). Frozen sections were incubated in 0.1 M Tris \cdot HCl buffer, pH 7.2, containing 0.5 mg/ml diaminobenzidine (DAB), 0.015% H_2O_2 and 0.1 M KCN for 5 min at room temperature and counterstained with Harris hematoxylin (Polysciences Inc.).

Quantitative (immuno)histochemistry. No epithelial shedding or obvious damage was observed in paraffin sections of lung and trachea from OVA-treated and control animals at 18 hr. Computer-assisted image analysis (Optimas Corp., Edmonds, WA) was employed either to count individual cells (DC, macrophages, T cells and B cells) or to quantify the cellular response by area of staining (eosinophils; see below). After rigorous preliminary experiments indicated that it was justified to do so, several compartments in the trachea and lung were designated as regions of interest for localizing and quantitating inflammatory cells, according to the criteria de-

scribed earlier. Slides were coded and read in a "blind" fashion. For cell counting, regions of the airway wall were chosen at random in the sections. With the assistance of a cursor, a field of interest was outlined, the cells that yielded a red stain for the cell marker of interest within the field were marked and the cells in a given field were automatically counted *via* the image analysis program. This yielded in each region of interest the number of cells per square millimeter. At least three replicate measurements were made for each region of interest from each slide.

OVA treatment brought about such a robust influx of eosinophils into the tracheal epithelium that the boundaries of cells could not be discriminated. Being unable to count individual cells, we quantitated the eosinophil response with computer-assisted image analysis by setting color parameters and thresholds for the brown DAB product indicative of eosinophils and calculating the percentage of the area of epithelium that stained positively for DAB. The region of interest bracketed the entire epithelial cell layer from apical to basolateral surfaces. This was an advantageous method for quantifying the eosinophil response, because the prevalence of the cells was thus normalized for the entirety of the section through the epithelium for any section, irrespective of any variation in the angle of cutting to prepare the tangential section. The percentage of the epithelial region of interest, defined with a cursor on the computer screen, that stained positively for DAB is referred to as the "eosinophil influx index." The eosinophil response in bronchial epithelium was likewise robust; in this case, eosinophil influx response was not enumerated either by counting of individual cells or by calculation of DAB-positive area, because there was extensive folding of the bronchial epithelium.

For quantification of DC localized in the trachea, positive cells were localized and enumerated in 1) the epithelium, between the lumen and the basement membrane, 2) the lamina propria, between the basement membrane and the smooth muscle, and 3) the submucosa, between the smooth muscle and the cartilage. Positive DC in the bronchi (Bai *et al.*, 1994) were localized and enumerated in 1) the lamina propria, between the basement membrane and the smooth muscle, and 2) the adventitia, outside the smooth muscle. Counting of DC in the bronchial epithelium was prevented by extensive folding of the epithelial layer, which obscured the individual cells; in addition, the DC were not stained intensely enough to allow calculation of DC area, the approach that had been used successfully with eosinophils. Because the APAAP staining techniques did not lead to clearly defined cell borders, quantitation of DC cell number was determined by counting the nuclei that were contained within cells that were APAAP-positive. The area of the analyzed regions and the number of positive cells in those areas were determined by computer-assisted image analysis. The results were expressed as number of positive cells per square millimeter of region area.

The remaining inflammatory cells were easily visualized with APAAP staining. They were outlined within random fields of interest in the airways and counted.

Analysis of results. The results are presented as mean \pm S.E.M. Differences in inflammatory cell prevalence among OVA-treated, saline-treated and nontreated animals were determined by the Mann-Whitney ranked-sum test for nonparametric data. Changes in eosinophil prevalence were evaluated for significance by one-way ANOVA with *post-hoc* comparisons by the Student-Newman-Keuls test. Treatment effects on basal SRaw were analyzed using one-way ANOVA for repeated measures. For *in vivo* studies on reactivity to MCh, SRaw values for each aerosolized MCh concentration were averaged over 1 min, and the average value was plotted *vs.* the MCh concentration. The MCh concentration that doubled SRaw (the PC₂₀₀ concentration) was determined by linear interpolation. Changes in reactivity to MCh were analyzed by a paired *t* test. *n* is the number of separate experiments. *P* < .05 was considered significant.

Results

Time course of pulmonary response to inhaled OVA; whole-body plethysmography. Monitoring Penh over a 23- to 24-hr period in 14 animals revealed two patterns of response to OVA challenge. An initial, rapid increase in P_{enh} lasting approximately 1 to 2 hr occurred in every animal. In 7 of the 14 animals, a late-phase response also was observed, whereas in the remaining animals, the late-phase response did not develop. Figure 1 shows a typical experiment from one of the animals in which both phases appeared. As shown in the figure, the late response typically was less severe, the obstruction ensued at approximately 8 to 12 hr, it was well developed by 18 hr and it lasted until the end of the recording period. No responses were elicited by saline aerosol in non-sensitized animals. On the basis of these results, all subsequent experiments utilized the 18-hr post-exposure period for examination of histological and functional changes.

Effect of OVA challenge on SRaw; double-chamber plethysmography. Before challenge with saline, saline-treated animals had a prechallenge basal SRaw of 1.8 ± 0.2 cm H₂O · s. Before OVA challenge, the SRaw values of OVA-sensitized animals were not different from these values. Aerosolized saline had no effect on basal SRaw immediately after or 18 hr after challenge (data not shown; *n* = 6), this parameter increased significantly immediately after OVA challenge (fig. 2) and returned to prechallenge levels by 18 hr.

Effect of OVA challenge on reactivity to inhaled MCh. Airway reactivity to MCh was measured in saline-treated and OVA-treated animals; representative concentration-response curves are shown in figure 3. Three general observations were made during the development of these curves. 1) The first effective dose of a series of MCh concentrations often evoked a very large SRaw response. 2) The curves were very "steep." Administering more than two or three effective doses led to asphyxia and death, even though the concentration increment between doses was small and the maximal response was not achieved. Because EC₅₀ values could not, therefore, be calculated, linear interpolation was used to calculate the MCh concentration that produced a 2-fold increase in SRaw above basal level, which was designated [MCh] PC₂₀₀. 3) There was appreciable variability in the control level of reactivity to MCh between animals, even from a given shipment. However, reactivity in each animal

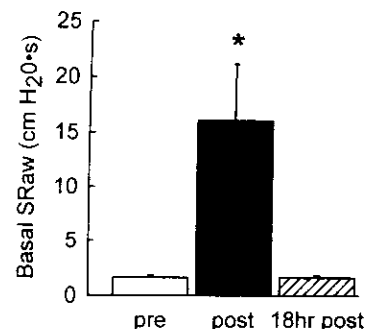


Fig. 2. Effect of OVA aerosol challenge on basal SRaw of OVA-sensitized guinea pigs. "pre," 1 min before challenge; "post," 1 min after challenge; "18 hr post," 18 hr after challenge. *n* = 6. **P* < .05 compared with "pre" values.

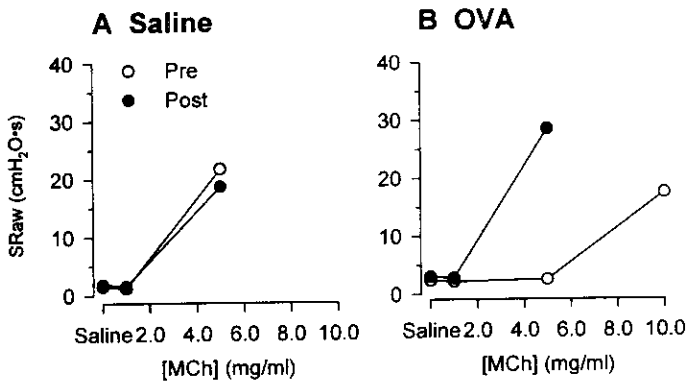


Fig. 3. Representative concentration-response curves for inhaled MCh in saline-treated (panel A) and OVA-treated (panel B) guinea pigs before treatment (○) and 18 hr after challenge with saline or OVA (●). Both curves in each panel were obtained from the same animal, the first before treatment and the second 18 hr after challenge, *i.e.*, each animal served as its own control. There was no change in MCh reactivity in the saline-treated animal. However, there was a leftward shift of the MCh dose-response curve in the OVA-treated animal 18 hr after OVA challenge.

was observed to be reproducible during the treatment period, so each animal could serve as its own control.

The [MCh] PC₂₀₀ of sensitized animals was decreased significantly 18 hr after OVA challenge; there was no change in reactivity in the saline-treated animals (fig. 4).

Dendritic cells. Cells that stained positively for MHC Class II antibody, stained negatively for tissue macrophage antibody and had a dendritiform appearance were scored as DC. In naive, untreated animals, the cell bodies of these cells were seen primarily within the lamina propria of trachea and bronchi, as well as in the submucosa of the trachea and in the adventitia of the bronchi, particularly clustering around blood vessels (fig. 5). Comparatively few DC were seen in the guinea pig epithelium, which in rats appears to be their primary location (Holt *et al.*, 1990). In the trachea, the order of abundance normalized to area was lamina propria = sub-

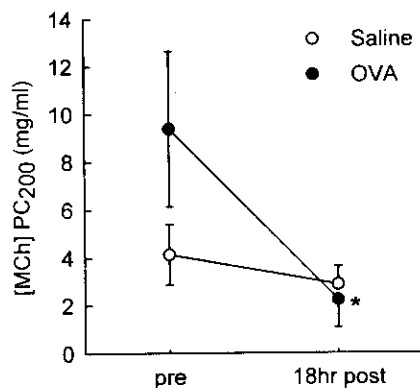


Fig. 4. Effect of OVA sensitization and challenge on reactivity to inhaled MCh. [MCh] PC₂₀₀ (mg/ml) is the provocative concentration of MCh that doubled the baseline value of SRaw. MCh dose-response curves were generated before treatment and 18 hr after challenge in saline-treated (○) and OVA-treated (●) guinea pigs. There was a significant decrease in [MCh] PC₂₀₀ 18 hr after treatment in OVA-treated but not the saline-treated animals. There was no significant difference in the basal values of SRaw for the saline- and OVA-treated groups before treatment and before OVA-challenge. A decrease in [MCh] PC₂₀₀ indicates an increase in airway reactivity. $n = 8$ for saline- and OVA-treated groups. * $P < .05$ compared with values before treatment.

mucosa > epithelium. Endothelial cells of blood vessels and Type I and Type II alveolar cells expressed weak MHC Class II positivity.

The effects of OVA treatment on the density of DC in the epithelium, lamina propria and submucosa of the trachea were examined in detail. There was no effect of saline inhalation on the abundance of MHC Class II-positive cells in any region when compared with the trachea of naive (untreated) animals (fig. 6). OVA treatment resulted in significant increases in DC in the epithelium, lamina propria and submucosa when compared with the tracheas of naive animals (fig. 5C; 6), but the difference in DC prevalence after OVA treatment in epithelium did not reach significance when compared with that of saline-treated animals (fig. 6). In the lamina propria and submucosa, OVA treatment increased the number of positive cells, particularly around blood vessels.

In the bronchi, the density of DC in the lamina propria and adventitia was not affected by saline treatment (fig. 7). However, OVA treatment resulted in significant increases in DC number in both regions when compared with both naive and saline-treated animals (fig. 1D; fig. 7).

OVA treatment had no effect on MHC Class II staining of endothelial cells and Type I and II alveolar cells.

Macrophages, T cells, B cells. Cells staining positively for the tissue macrophage antibody were localized primarily in the epithelium near the basement membrane (fig. 5E). Tissue macrophage density was found to increase in the tracheal epithelium after OVA treatment. However, saline challenge also increased macrophage cell number in the tracheal epithelium (fig. 8). T helper cells were found in all regions of interest in the trachea and bronchus. However, OVA treatment had no effect on T cell density in any region of the trachea or bronchus (fig. 9). No B cells were detected in tracheal or bronchial sections from naive animals. A few cells were noted in the saline-treated and OVA-treated animals, but no differences in density were noted (data not shown).

Eosinophils. OVA challenge resulted in a significant increase in the degree of eosinophilia within the tracheal epithelium (fig. 10; 11B). Eosinophils also were observed in the adventitia between smooth muscle fascicles (data not shown). In the bronchial epithelium, eosinophilia was observed after OVA challenge (fig. 11), but the obvious influx was not amenable to quantitation (see "Materials and Methods").

Discussion

In recent years, the significant role of the DC as an antigen-presenting cell (Levin *et al.*, 1993) and its potential role in asthma have become increasingly appreciated. Therefore, our study utilized quantitative immunohistochemical analysis of trachea and bronchi to gain insight into the DC response in the context of other inflammatory cell changes after OVA treatment of guinea pigs. We examined these changes in animals that had been determined at 18 hr after OVA challenge to be hyperreactive to inhaled MCh. At this time, a late-phase obstructive response was also observed in many of the animals. That it did not occur in all of the animals is similar to a pattern of human response to occupational sensitizers (Perrin *et al.*, 1991).

There have been numerous studies of the localization and function of DC in the lungs of the rat, but this cell has

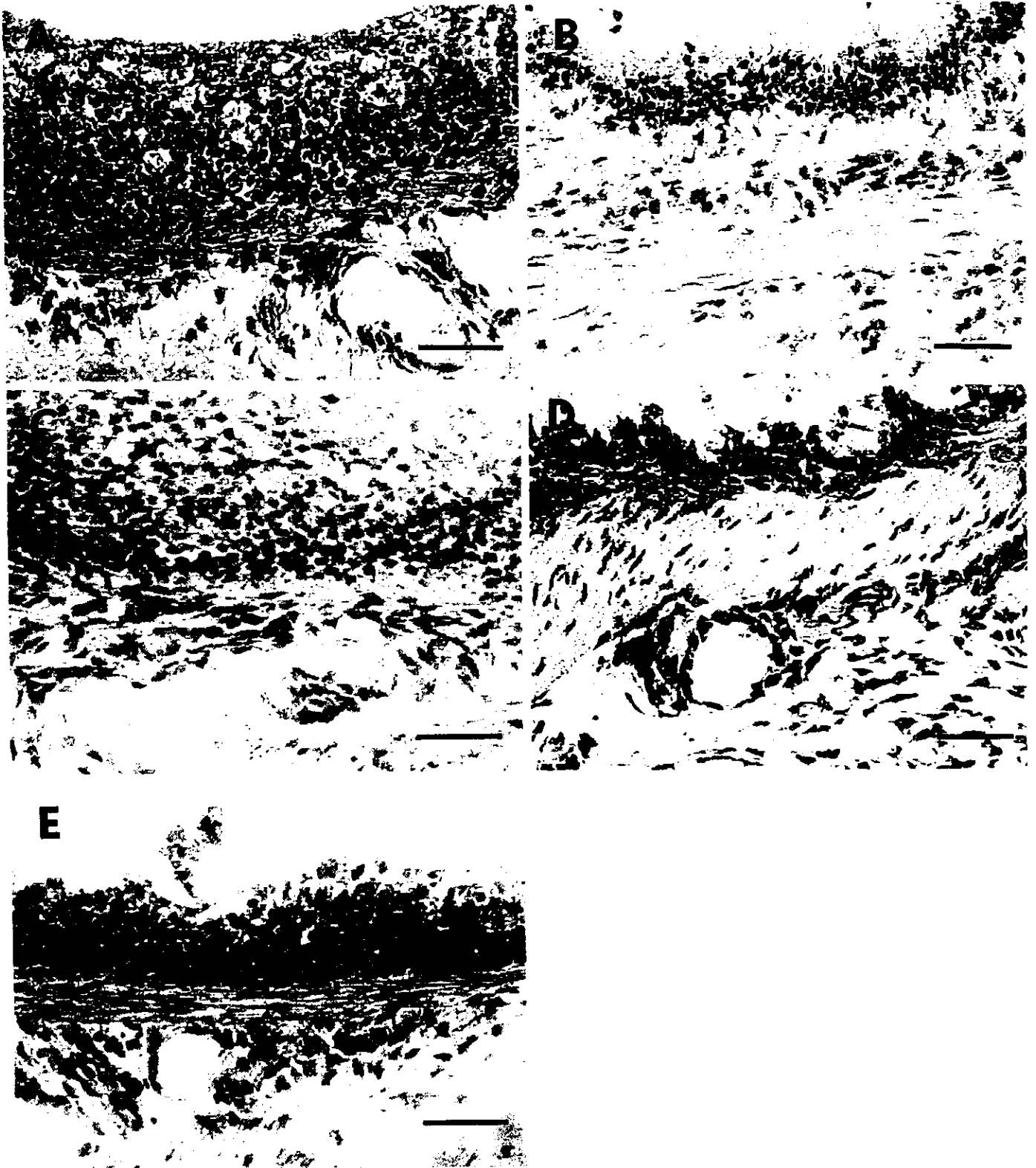


Fig. 5. Distribution of MHC Class II-positive DC in the trachea of saline-treated (panel A) and OVA-treated (panel C) guinea pigs and in the bronchi of naive (panel B) and OVA-treated (panel D) guinea pigs. These MHC Class II-positive DC were also negative for tissue macrophage antibody and are visualized in red. Panel E shows tissue macrophage distribution in tracheal epithelium of OVA-treated guinea pigs. Bar = 50 μ m.

received little attention in guinea pigs. We observed many differences between rat and guinea pig airways with respect to DC. For example, in the rat, pulmonary DC are localized primarily within the tracheal epithelium at a density of

$\sim 600/\text{mm}^2$ (Schon-Hegrad *et al.*, 1991; Holt *et al.*, 1994). The large number of DC present in the extensive network in rat airway epithelium is readily visualized in tangential sections (Holt *et al.*, 1990). In contrast, in the guinea pig trachea, DC

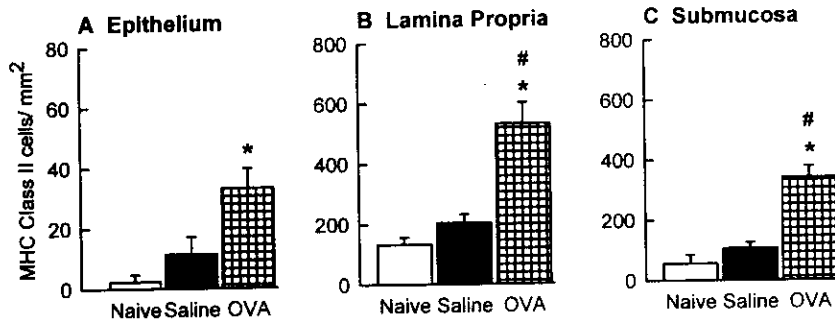


Fig. 6. Effect of OVA treatment on DC abundance in the epithelium (panel A), lamina propria (panel B) and submucosa (panel C) of the trachea. "Naive," untreated animals ($n = 4$); "Saline," saline-treated animals ($n = 6$); "OVA," OVA-treated animals ($n = 6$). The results are represented as the number of MHC Class II-positive cells per square millimeter of tracheal region. * $P < .05$ compared with naive animals; # $P < .05$ compared with saline-treated animals.

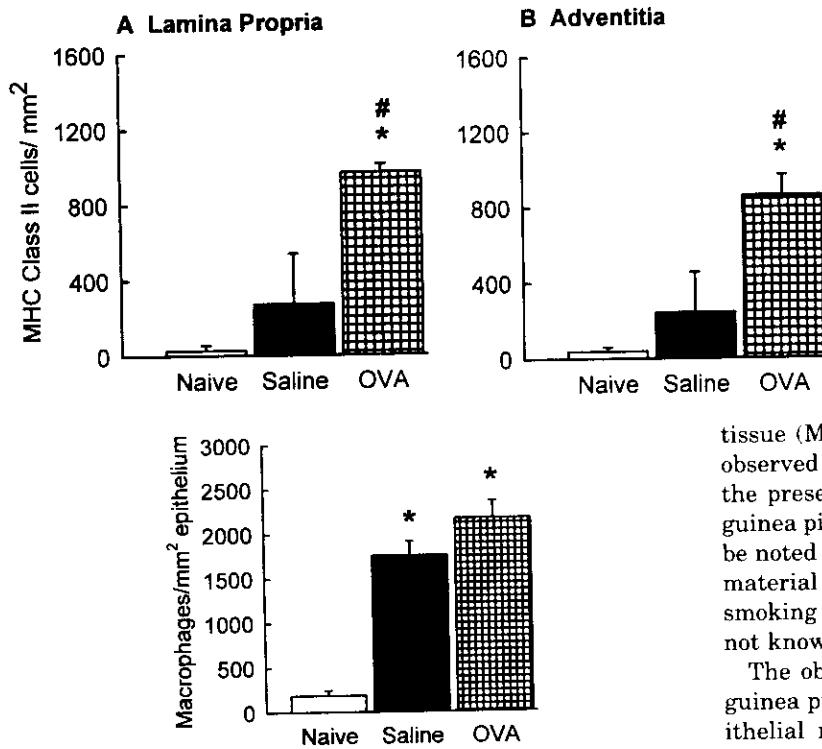


Fig. 7. Effect of OVA treatment on DC abundance in the lamina propria (panel A) and adventitia (panel B) of the bronchi. "Naive," untreated animals ($n = 4$); "Saline," saline-treated animals ($n = 6$); "OVA," OVA-treated animals ($n = 6$). The results are represented as the number of positive cells per square millimeter of bronchial region. * $P < .05$ compared with naive animals; # $P < .05$ compared with saline-treated animals.

Fig. 8. Effect of OVA treatment on tissue macrophage abundance in the epithelium of the trachea. "Naive," untreated animals ($n = 4$); "Saline," saline-treated animals ($n = 6$); "OVA," OVA-treated animals ($n = 6$). The results are represented as the number of positive cells per square millimeter of epithelium. * $P < .05$ compared with naive animals.

were localized largely in the lamina propria ($203/\text{mm}^2$). In tangential sections of the guinea pig trachea, very few MHC Class II-positive cells were detected within the epithelium. These differences are not due to methodological or technical differences, however, because we have used the same procedures on tracheas from the Brown Norway rat and confirmed the localization of numerous DC in the epithelium (unpublished observations). Therefore, the paucity of DC in guinea pig epithelium reflects a species difference. Our findings are in agreement with the previous qualitative study of DC in guinea pigs (Lapa e Silva *et al.*, 1993). The distribution and density of DC in guinea pigs are very similar to those obtained from human bronchial biopsy material (Maarten *et al.*, 1994). In human airways, the density of MHC Class II-positive cells in the epithelium ($245/\text{mm}^2$) was considerably less than that in rat, and the dense network of DC seen in the rat (Schon-Hegrad *et al.*, 1991) was not observed in the human airways. The greatest density of DC in the human biopsy material ($320/\text{mm}^2$) was found in the subepithelial

tissue (Maarten *et al.*, 1994), which is similar to what was observed in the lamina propria and submucosa of trachea in the present study. Overall, the density of DC was lower in guinea pig airways than in rat and human airways. It should be noted that the studies on airways involved human biopsy material from smokers (Maarten *et al.*, 1994); the effect that smoking could have had on DC density in these patients is not known.

The observation that the cell bodies of pulmonary DC in guinea pigs and humans reside predominantly in the subepithelial regions, whereas dendritic extensions are not observed in the epithelium, is of interest in the context of these cells playing an important role as a first line of defense against incoming antigens. The presence of dendritic extensions throughout epithelium in rats implies that antigen uptake and processing must occur very close to the air interface. Perhaps this different spatial relationship connotes different mechanisms and sites of antigen delivery and processing in human and guinea pig airways, with antigen having to cross the epithelium to be taken up and processed by DC. Alternatively, the dendritic extensions are present in the epithelium but are not visualized by the APAAP procedure in frozen sections. The advent of more suitable antibodies for guinea pig DC may increase resolution of dendritic processes and assist in clarifying these alternatives.

Although OVA treatment results in an increase in DC density, our experiments do not enable us to distinguish whether the number of DC in the airways was increased or whether MHC Class II expression on resident DC had increased. Although these alternatives have never been directly assessed, studies on airway epithelial DC after irradiation in rats (Holt *et al.*, 1994) have shown that resident DC have a turnover rate of approximately 3 days and that there is a renewal from incoming DC precursors derived from the bone marrow. The migration of DC into the airways could

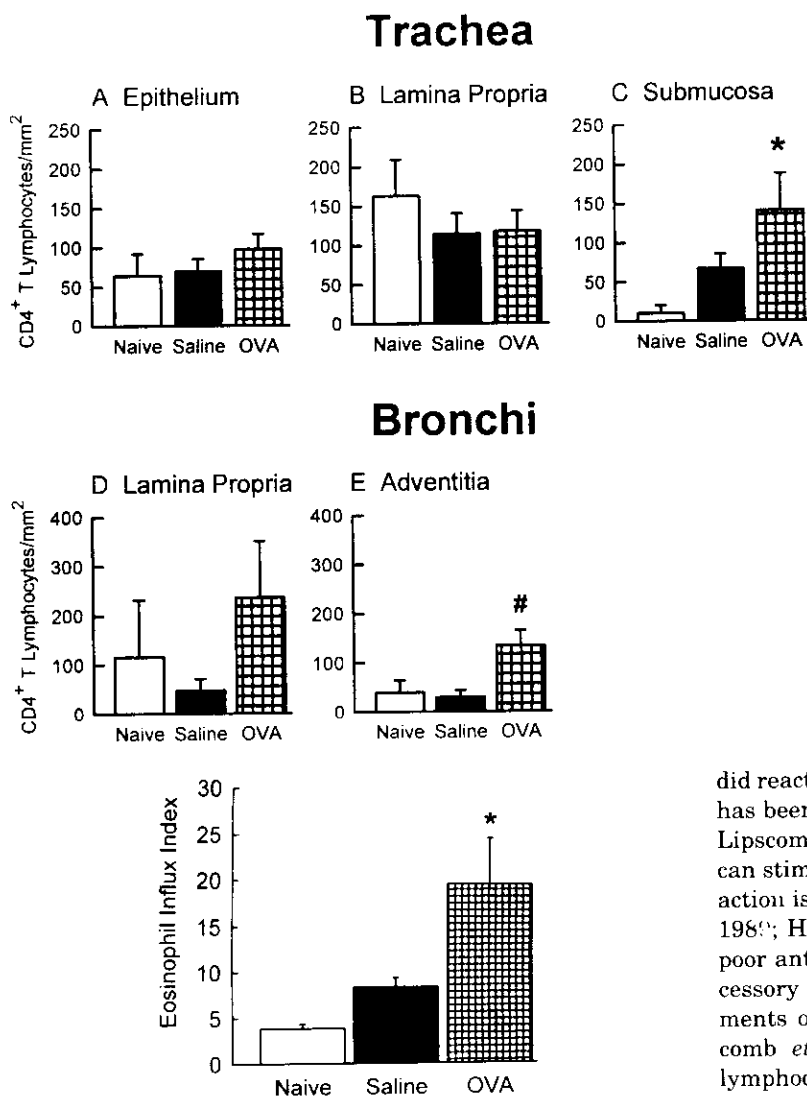


Fig. 10. Effect of OVA treatment on eosinophil abundance in trachea. "Naive," untreated animals ($n = 4$); "Saline," saline-treated animals ($n = 6$); "OVA," OVA-treated animals ($n = 6$). The results are represented as the percentage of epithelial area that was DAB-positive. * $P < .05$ compared with naive animals; # $P < .05$ compared with saline-treated animals.

thus increase their numbers. A study of DC recruitment after bacterial exposure (McWilliam *et al.*, 1994) showed that an increase in the number of MHC Class II-positive cells with rounded morphology was seen 2 hr after exposure. Between 8 and 24 hr after exposure, the morphology of the cells changed to a more dendritic nature without any further increase in number. This suggests that immature DC are recruited into the airways and undergo maturation after reaching the pulmonary tissue. At 18 hr after OVA treatment, we observed a clustering of DC around pulmonary blood vessels, which could suggest migration of DC into the airways. It is therefore likely that the increase in MHC Class II-positive cells in the airways of the guinea pig reflects, at least in part, homing of DC to the airways.

Interstitial macrophages were present within the epithelium, but they did not show MHC Class II-positivity. This suggests these cells did not play a role in OVA presentation to T lymphocytes. Alveolar macrophages, on the other hand,

Fig. 9. Effect of OVA treatment on CD4⁺ T lymphocyte abundance in the epithelium (panel A), lamina propria (panel B) and submucosa (panel C) of the trachea and in the lamina propria (panel D) and adventitia (panel E) of the bronchi. "Naive," untreated animals ($n = 4$); "Saline," saline-treated animals ($n = 6$); "OVA," OVA-treated animals ($n = 6$). The results are represented as the number of positive cells per square millimeter of epithelium. * $P < .05$ compared with naive animals; # $P < .05$ compared with saline-treated animals.

did react with MHC Class II antibodies in our experiments. It has been previously reported (Gorenberg and Daniele, 1978; Lipscomb *et al.*, 1981) that guinea pig alveolar macrophages can stimulate T lymphocyte proliferation *in vitro*. This interaction is different from that in rat and mouse (Thepen *et al.*, 1980; Holt *et al.*, 1993), in which alveolar macrophages are poor antigen-presenting cells and can down-regulate the accessory cell activity of DC. However, the previous experiments on guinea pigs (Gorenberg and Daniele, 1978; Lipscomb *et al.*, 1981) were performed with lymph node T lymphocytes. In a study (Hill and Burrell, 1979) investigating the stimulation of T lymphocytes isolated from lung tissue by guinea pig alveolar macrophages, a suppression of antigen-stimulated proliferation of T lymphocytes was observed. In addition, the previous guinea pig studies utilized an isolation technique for alveolar macrophages that involves harvesting cells that are adherent to plastic Petri dishes. However, DC also are loosely adherent to plastic (Nicod *et al.*, 1987; Wilkes and Weissler, 1994), and some DC may have been present in Gorenberg and Daniele's (1978) and Lipscomb *et al.*'s (1981) alveolar macrophage preparations.

T helper cells present in the airways were not significantly increased 18 hr after OVA challenge. This is in agreement with the findings of Lapa e Silva *et al.* (1993), who noted no change in numbers of CD4⁺ T lymphocytes 24 hr after antigen challenge. This result differs from a significant increase in CD3⁺ T-cells 17 hr after antigen challenge observed in other studies in the guinea pig (Frew *et al.*, 1990). It can be hypothesized that the peak T lymphocyte response, *i.e.*, activation and migration to regional lymph nodes (Holt *et al.*, 1991), may have already occurred by the 18-hr time-point used in our experiments.

We observed a large influx of eosinophils into the epithelium of the trachea and bronchi 18 hr after OVA challenge that is characteristic of the late-phase inflammatory re-

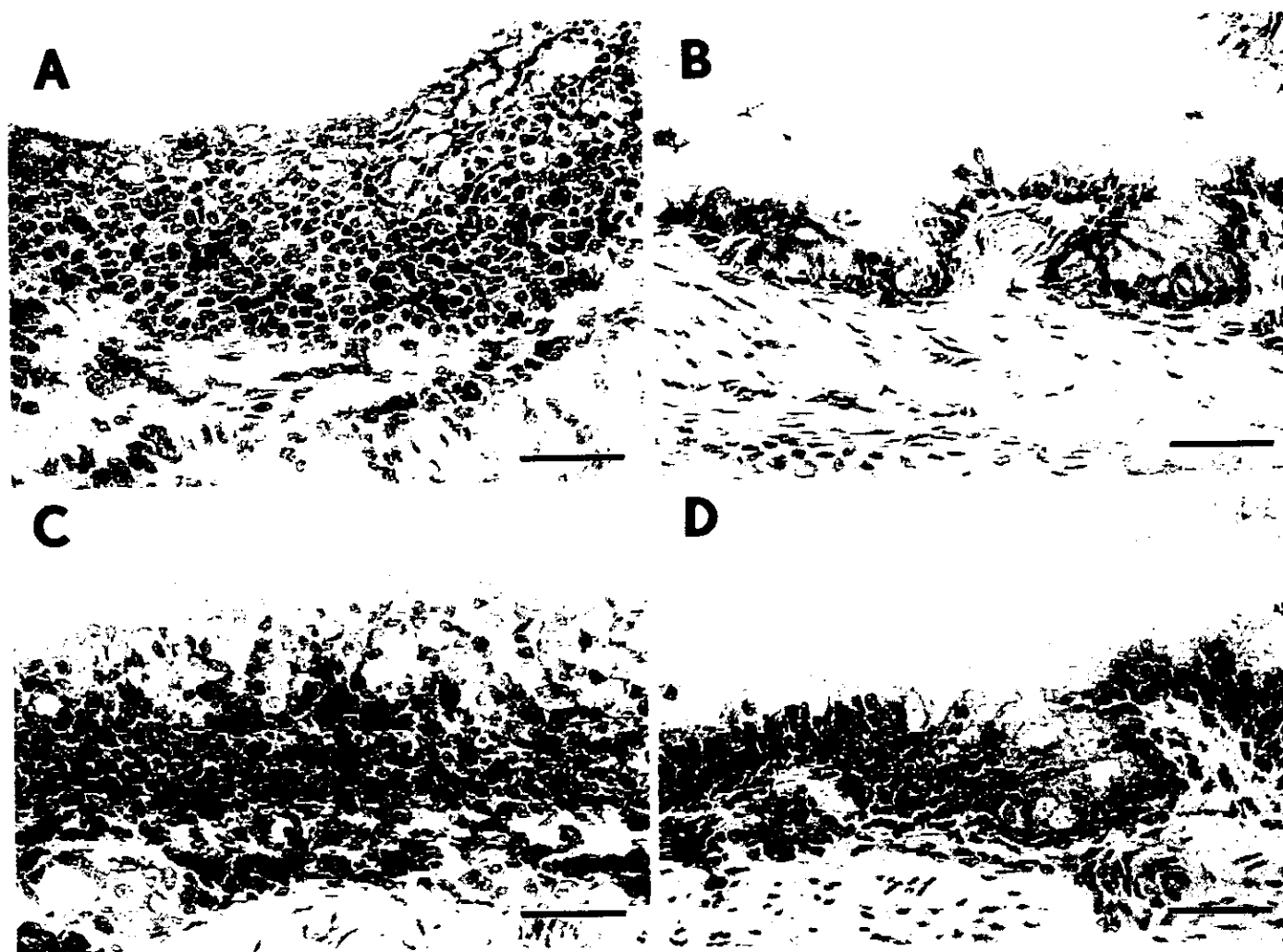


Fig. 11. Eosinophils in tracheal epithelium of naive (panel A) and OVA-treated (panel C) guinea pigs and in bronchial epithelium of naive (panel B) and OVA-treated (panel D) guinea pigs. Panels C and D depict the characteristic infiltration of DAB-positive cells into the epithelium. Although OVA-treatment gave rise to an obvious and large eosinophilic response, the eosinophilic infiltration into the bronchial epithelium could not be quantitated by image analysis ("Materials and Methods"). Bar = 50 μ m.

sponse in guinea pigs (Hutson *et al.*, 1988; Underwood *et al.*, 1992). Thus the elevations in DC number seen in the present study occur at a time of profound epithelial eosinophilia. The guinea pig has been widely used to investigate allergen-provoked changes in pulmonary function and inflammatory cell influx because of the similarities that exist with the human asthmatic response, the mediators involved and the reactivity of the airways to bronchoactive agents. The localization of DC in the guinea pig airways, which agrees with pulmonary DC distribution in humans, and the increases in DC density within the airways of OVA-treated animals suggest that the guinea pig may provide much insight into the role of DC in the development of inflammation in human airways. Further quantitative studies on the time courses of DC changes *vis à vis* changes in other inflammatory cells will provide more insight into key sequential intercellular interactions between DC, T cell and B cells, which will help clarify the relationship between DC and pulmonary dysfunction.

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