Time course of inflammatory and remodeling events in a murine model of asthma: effect of steroid treatment

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Trifilieff, Alexandre, Ahmed El-Hashim, and Claude Bertrand. Time course of inflammatory and remodeling events in a murine model of asthma: effect of steroid treatment. Am J Physiol Lung Cell Mol Physiol 279: L1120–L1128, 2000.—The kinetics of airway inflammation and remodeling processes following ovalbumin aerosol challenge in sensitized BALB/c mice was studied. Mice were exposed to either single or five ovalbumin challenges over 5 days. In both protocols, time-dependent increases in bronchoalveolar lavage (BAL) cellular fibronectin, neutrophils and eosinophils were observed. The kinetics of these events were similar in both protocols; however, the magnitude of the response was much greater following repeated challenges. BAL protein levels and lymphocyte numbers were increased only following repeated challenges, whereas interleukin (IL)-5 and IL-4 were increased in both protocols. Histological analysis revealed a time-dependent increase in epithelial cell proliferation and in mucus-producing epithelial cells. Proliferation of alveolar cells was observed only following repeated challenges. Airway hyperreactivity was observed in both protocols but was much greater following repeated challenges. Pretreatment with dexamethasone fully inhibited the inflammatory response and airway hyperreactivity but only partially inhibited the remodeling process. These data suggest that glucocorticoids, although potent anti-inflammatory agents, may not be potent in reducing the lung remodeling process associated with asthma.

Airway hyperreactivity; dexamethasone; eosinophils

IT IS THOUGHT THAT CHRONIC INFLAMMATION of the asthmatic airways is responsible for the reversible airway obstruction and the nonspecific bronchial hyperresponsiveness observed in these patients (5). In addition to the inflammatory process, another regular feature of asthma is a significant airway remodeling that leads to structural lung changes. These changes include basement membrane thickening due to collagen and fibronectin deposition (21), fibroblast proliferation (2), airway smooth muscle thickening as a result of both smooth muscle cell hyperplasia and hypertrophy (11), and excessive production of mucus glycoproteins (24). All these modifications lead to the thickening of asthmatic airway walls, which in turn could explain the hyperresponsiveness observed in this disease (11, 27).

Although lung remodeling is a constant observation in chronic asthma (23), very few studies have attempted to develop an animal model to study this process (18, 19, 22). In this study, we developed a murine model of lung inflammation using sensitized mice and ovalbumin (OA) aerosol challenge, and we used this model to study the airway hyperresponsiveness and the kinetics of lung inflammation and remodeling, including inflammatory cell influx, interleukin (IL)-4, and IL-5 levels, plasma leakage, cellular proliferation, cellular fibronectin production, and mucus secretion. Moreover, we also studied the effect of a glucocorticosteroid, dexamethasone, given 1 h before each aerosol exposure on all these parameters.

METHODS

Experimental design. Male BALB/c mice or C57BL/6 (25–30 g) were immunized intraperitoneally with 10 μg of OA (grade V; Sigma, St. Louis, MO) in 0.2 ml of alum (Serva, Heidelberg, Germany) on days 0 and 14. On day 20, in some of the mice, ALZET minipumps (model 2002; Charles River, St. Aulbin-les-Elbeuf, France) filled with 5-bromo-2'-deoxyuridine (BrdU; 10 mg/ml; Sigma) were implanted subcutaneously in the scapular region. The BrdU minipumps lasted 2 wk and were replaced on day 34. Mice were challenged with a nebulized solution of either OA (50 mg/ml of PBS) or PBS alone for 20 min as described previously (4). One group was challenged once on day 21 (acute protocol), and a second group was challenged daily between days 21 and 25 (chronic protocol). At specified time points after the last challenge, mice were killed by an injection of 0.2 ml of pentobarbital sodium (60 mg/kg). Once deeply anaesthetized, mice were used either for bronchoalveolar lavage (BAL; 5–6 mice) or for tissue collection (3–4 mice).

In another set of experiments, mice were treated with an injection of 3 mg/kg ip of water-soluble dexamethasone (Sigma) in PBS 1 h before each challenge. Control mice received 0.1 ml of PBS. For BAL cellular fibronectin and protein levels, BAL cellular content, and total serum IgE, mice were killed 3 days after the last challenge. For all the others parameters, mice were killed 7 days after the last challenge.

Assessment of BAL inflammatory cell infiltration. After anesthesia, the trachea was cannulated, and BAL was performed by injecting 0.3 ml of PBS, kept at room temperature,
into the lung via the trachea. The fluid was withdrawn and stored on ice. This procedure was repeated four times. Total cell count was measured, and cytospin preparation (Shandon Scientific, Cheshire, UK) was performed. Cells were stained with Diff-Quik (Baxter Dade, Dudingen, Switzerland), and a differential count of 200 cells was performed using standard morphological criteria. The remaining BAL fluid was centrifuged (300 g for 10 min), and the supernatant was collected and stored at −80°C for soluble mediator measurements.

**BAL soluble mediator measurement.** Protein concentration was measured using the bicinchoninic acid protein assay according to the manufacturer’s instructions (Pierce, Rockford, IL).

**BAL cytokine levels** [IL-5, IL-4, and interferon-γ (IFN-γ)] were measured using commercially available kits (Endogen, Woburn, MA). The sensitivity of these assays was 15 pg/ml for IFN-γ and 5 pg/ml for IL-5 and IL-4.

To measure the BAL cellular fibronectin content, an ELISA procedure modified from Rennard and colleagues (20) was used. Briefly, 96-well plates were coated overnight at 4°C with a solution of human cellular fibronectin (150 ng/ml; Sigma). BAL samples, at appropriate dilution, were incubated overnight at 4°C with a mouse anti-cellular fibronectin antibody (1:10,000; Sigma) and then transferred to the fibronectin-coated wells. After the wells were washed, antibodies that did not react with fibronectin in the BAL samples were revealed by sequentially adding a biotinylated secondary anti-mouse IgM (1:1,000; Sigma) and a streptavidin-horseradish peroxidase complex (1:1,000; Amersham, Little Chalfont, UK). The substrate 2,2-azino-bis(3-ethylbenzthiazoline 6-sulfonic acid) diammonium (Sigma) was then added for 5 min, the reaction was stopped with 10% SDS, and the optical density was measured at 405 nm. Using these procedures, the detection limit was 10 ng/ml. No signal was observed when the plates were coated with collagen types I and IV or with laminin.

**Determination of total serum IgE levels.** Following anesthesia, blood was taken from the aorta, the serum was prepared, and the antibody titer was determined by ELISA as described previously (15).

**Determination of airway reactivity.** Airway reactivity was measured using barometric plethysmography and whole body plethysmography (8). Twenty-four hours after the final challenge, unrestrained conscious mice were placed in a plethysmographic chamber (Buxco Electronics, Sharon, CT), and respiratory parameters of each animal were measured in response to increasing doses (0–0.3 M) of aerosolized methacholine dissolved in sterile PBS. The resistance was expressed as enhanced pause (Penh) according to the manufacturer’s instructions.

**Tissue preparation.** After anesthesia, the lungs were inflated through the trachea with 4% buffered Formalin solution in PBS (pH 7.4) under a constant pressure of 150 mmHg. After 2 h, the lungs were removed from the thoracic cavity, cleared of nonlung tissue, and immersed in 4% Formalin for 1 h. As a positive control for BrdU incorporation and alcian blue-periodic acid-Schiff staining, a section of gut was removed, perfused with 1 ml of 4% Formalin, and immersed in fixative solution for 3 h. Lungs and gut were routinely embedded in paraffin, and 4-μm sections were cut and mounted on glass slides precoated with poly-L-lysine (Sigma).

**BrdU and fibronectin immunostaining on lung sections.** Sections were deparaffinized for 20 min in xylene, dehydrated for 10 min in 100% ethanol, and then washed with PBS for 10 min. For BrdU staining, slides were treated for 20 min with 0.2% trypsin (Zymed, San Francisco, CA) at 37°C and washed under running tap water. After a 2 M HCl treatment for 30 min, sections were neutralized for 5 min in sodium borate (0.1 M, pH 8.5) and washed in PBS. Endogenous peroxidase activity was inhibited with 2% H2O2 in PBS for 30 min. After the blocking solution was applied (1% sheep serum in PBS) for 15 min, sections were incubated with a rat anti-BrdU antibody (1:50; abV Immune Response, Derry, UK) for 1 h, incubated with biotinylated sheep anti-rat antibody (1:100; Amersham) for another 1 h, incubated with streptavidin-biotinylated horseradish peroxidase complex (1:300; Amersham) for 30 min, incubated with diamobenzidine substrate for 10 min, and counterstained with Harris.
hematoxylin. All slides were coded and counted blindly, under oil immersion, using a $\times 400$ magnification length. The bronchial epithelium proliferation rate, previously shown to be a good index for the measurement of lung cell proliferation (19), was measured, as well as BrdU incorporation in alveolar cells as an index of the changes occurring in the deep parenchyma. Care was taken to exclude all the infiltrating inflammatory cells. In preliminary experiments, airways were characterized according to the basement membrane length and defined as large (>2 mm), medium (1–2 mm), or small (<1 mm). BrdU-positive epithelial cells were expressed as a percentage or as cells per millimeter of basement membrane. In either case, no difference between the different airway sizes was observed. Epithelial nuclear labeling index was expressed as a percentage of BrdU-positive nuclei vs. total nuclei in at least 20 randomly chosen airways. For alveolar cell proliferation, 1,000 cells were counted in randomly chosen fields. Systemic distribution was confirmed by intense BrdU staining in the gut of all animals.

Alcian blue-periodic acid-Schiff staining. Sections were deparaffinized and immersed for 10 min in a solution of alcian blue (1% in 3% acetic acid, pH 2.5). After a prolonged washing in running tap water, sections were treated with 0.5% periodic acid for 5 min, washed with several changes of distilled water, placed in Schiff solution (Sigma) for 10 min, rinsed with running tap water, and mounted without any counterstain. Section analysis was performed in a blind fashion using a qualitative scoring system (0–4), where 0 = no epithelial staining, 1 = slight epithelial staining, 2 = moderate epithelial staining, 3 = heavy epithelial staining, and 4 = massive epithelial staining.

Data analysis. Data, expressed as means ± SE, were analyzed by ANOVA. A value of $P < 0.05$ was taken as significant.

RESULTS

In preliminary experiments the response of both BALB/c and C57BL/6 mice to a single challenge of OA was compared. As shown in Table 1, C57BL/6 mice had significantly decreased responses to OA compared with BALB/c mice for all the inflammatory parameters examined, with the exception of the BAL eosinophilia. More importantly, increased BAL fibronectin levels were observed only in BALB/c mice. On the basis of these data, BALB/c mice were chosen for study rather than C57BL/6 mice. The influence of the number of challenges on the BAL eosinophilic influx by exposing sensitized BALB/c mice to five challenges per week over 3 wk was examined. Maximum response was
both protocols, no change was observed in the number of macrophages (data not shown). In the acute protocol, no increase in the BAL protein content was observed, whereas in the chronic protocol, protein content increased from day 1, peaked at day 3, and resolved by day 7 (Fig. 2). An increase in BAL T helper cell type 2 (Th2) cytokines (IL-4 and IL-5) was observed in both protocols as early as 0 and 6 h for the chronic and acute protocols, respectively. By day 3, no more Th2 cytokines were detectable (Fig. 3). In both protocols, no IFN-γ was detected in the BAL (data not shown). At day 1 postchallenge in both protocols, no significant increase in BAL inflammatory cell infiltration, protein, or cytokine levels were observed in sensitized mice challenged with PBS (data not shown).

**Total serum IgE.** The immunization procedure induced a time-dependent increase in the level of total serum IgE, which peaked at day 9 following the initial sensitization. Following the boost, on day 14, a more rapid and dramatic increase in total serum IgE was observed (Fig. 4). No further increase was induced by a single OA challenge (Fig. 5). However, repeated OA challenges induced a further significant increase of total serum IgE that started 1 day following the last challenge and peaked at 3 days. Total serum IgE levels had begun to return to basal values after 14 and 21 days for the acute and chronic protocols, respectively (Fig. 5).

**Lung remodeling.** BAL cellular fibronectin content was measured as a marker of extracellular matrix component production. Using either protocol, a similar time-dependent BAL cellular fibronectin increase was observed. However, the level of BAL cellular fibronectin was much more elevated in the chronic protocol than in the acute protocol (Fig. 6).

Both acute and chronic OA challenge induced a significant increase in the number of BrdU-positive epithelial cells compared with PBS challenge (Fig. 7A). This effect was rapid in onset as evident from the significant proliferation observed at the first time point studied (3 days after the last challenge). During the acute protocol, OA-specific cellular proliferation increased until day 14 and then appeared to plateau at day 21. In contrast, during the chronic protocol, the antigen-induced cellular proliferation was much more pronounced on the 3rd day following the last challenge but did not show any further increase (Fig. 7A).
When compared with PBS challenge, the OA challenge did not increase alveolar cell proliferation in the acute protocol (Fig. 7B). In contrast, significant OA-specific alveolar cell proliferation was observed in the chronic protocol. As observed for the epithelial cells, this effect was rapid in onset (3 days after the last challenge) and plateaued from day 7 (Fig. 7B).

In PBS-challenged mice, either no or very few alcian blue-periodic acid-Schiff positive epithelial cells were observed. However, in contrast, OA provocation induced a dramatic change in the secretory phenotype of the epithelium in both protocols (Fig. 8). This secretory phenotype was mainly observed at the level of the large bronchi. A semiquantitative analysis of the epithelial mucus secretory phenotype showed that it was maximal at the first time point studied (3 days after the last challenge) and thereafter decreased through to day 21. The mucin secretory phenotype was more intense in the chronic protocol but was more prolonged in the acute protocol (Fig. 9).

Measurement of airway reactivity. Animals acutely challenged with OA showed a significant increased P_{enh} in response to increasing doses of methacholine compared with PBS-challenged animals. However, no significant difference was observed at the highest dose of methacholine (0.3 M). The increased P_{enh} to the dose response of methacholine observed following chronic challenge was much more pronounced. Moreover, this hyperreactivity was also present at the highest dose of methacholine. Dexamethasone (3 mg/kg ip) fully inhibited the hyperreactivity seen in the acute and chronic protocols (Fig. 10).

**Effect of dexamethasone on inflammatory events and lung remodeling.** At day 3 postchallenge, in both protocols, dexamethasone (3 mg/kg ip) fully inhibited the inflammatory parameters found to be increased (BAL eosinophil and neutrophil numbers in the acute protocol; BAL eosinophil and lymphocyte numbers, BAL protein levels, and total serum IgE in the chronic protocol). Other cell types were not affected by this treatment (Table 2). In the same way, at day 1 postchallenge, BAL IL-4 and IL-5 levels were also fully inhibited in both protocols (Table 3). In contrast to the inflammatory parameters, cellular fibronectin content was only partially inhibited (Table 2).

At day 7 postchallenge, dexamethasone significantly reduced the allergen-induced epithelial cell proliferation in both protocols. However, it did not fully reverse...
this effect (Fig. 11). Similar results were obtained for proliferation of alveolar cells in the chronic protocol (Fig. 11). No allergen-specific alveolar cell proliferation was observed during the acute protocol, and dexamethasone had no effect on the basal proliferation of these cells (Fig. 11). The epithelial mucus secretory phenotypes induced by OA challenge were also attenuated, but not completely abrogated, in the acute and chronic protocols (data not shown).

**DISCUSSION**

There is widespread evidence to support an important role for airway wall remodeling in chronic asthma patients (23). However, probably because of the lack of experimental tools, the mechanisms leading to this phenomenon are still not fully elucidated. In the present study, we have characterized an allergen-driven murine model of lung inflammation and have shown that the airway inflammation is associated with some of the remodeling features typically seen in asthmatics. Nonmurine antigen-driven models have been used to model this feature of human asthma (18, 19, 22). However, the increasing number of reagents capable of probing the murine immune system and its genetic variants may be helpful to unravel the events leading to airway remodeling.

We report an allergen-driven murine model of lung inflammation that simulates many of the characteristic features of human asthma. On sensitization and aerosol challenge, the mice developed an inflammatory cell infiltration that became more pronounced with repeated aerosol exposure to the allergen. In the acute protocol, the inflammatory cells present in the BAL were mainly neutrophils and eosinophils, whereas an influx of lymphocytes was observed only following chronic challenge. Consistent with this lung eosinophilic inflammation, a similar increase in BAL Th2 cytokines was observed in both protocols. Although repeated challenges clearly increased the intensity of the lung inflammatory cell infiltration, they also induced new inflammatory processes compared with the acute allergen exposure. In addition to the lymphocytic BAL infiltration, an allergen-induced plasma leakage as measured by BAL protein levels was observed only after repeated exposure. Similarly, an increase in total serum IgE over the sensitization level following aerosol exposure of the allergen was evident only in the chronic protocol. Overall, the pattern of the inflammation obtained in the chronic protocol was closer to what is observed in human asthma (5).

In both protocols, the allergen challenge induced an increased airway responsiveness to methacholine when compared with PBS-challenged animals. How-
however, in the acute protocol, the mice were only hypersensitive to methacholine (no difference was observed at the highest dose of methacholine). In contrast, in the chronic protocol, mice were both hypersensitive and hyperreactive to methacholine, and the magnitude of the response was much higher when compared with the acute protocol. Although the mechanisms of airway hyperreactivity in human asthma are not fully understood, many studies have tried to address this problem using murine models of asthma. Both IgE (7) and T cells (9) have been implicated as major contributors to airway hyperreactivity in these models. In our model, the BAL lymphocytic influx and increase in total serum IgE, observed only following repeated challenge, could at least partially explain the increase in severity of the hyperreactivity observed in the chronic protocol. In addition to the immunological component, lung structural changes have also been suggested to play a role in airway hyperreactivity (6, 18). Accordingly, in our model, the parenchymal cell proliferation and the plasma leakage, observed only in the chronic protocol, may also play a role in the increased hyperreactivity.

One of the characteristics of the lung remodeling in human asthma is basement membrane thickening due to extracellular matrix protein deposition (21). In our model, we do not have evidence for deposition of extracellular matrix in the lung. However, the increased cellular fibronectin levels observed in the BAL could be the first step of a cascade, eventually leading to extracellular matrix protein deposition in the tissue. Indeed, using a similar model, a previous study has suggested that subepithelial fibrosis is only apparent after 4–6 wk of allergen exposure (25). Another aspect of the lung remodeling is the proliferation of various cellular types that have been reported in both asthmatic individuals (2, 10) and animal models (18, 19, 22). Our data clearly show that allergen challenge induced epithelial cell proliferation in both protocols. It has to be noted that alveolar cell proliferation was observed only in the chronic protocol; however, the relevance of this observation is not clear, since alveolar cell hyperplasia was never described in human asthma. Although smooth muscle hyperplasia and epithelial desquamation are characteristic features of human asthma (23), such a phenomenon was not evident in the present study. However, all these features may be related to the severity and the chronicity of the disease (12), and, despite the chronic allergen challenges, we still may have induced an acute and mild inflammatory response in our model.

### Table 2. Effect of dexamethasone on BAL inflammatory cell infiltration and BAL protein and fibronectin levels

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<td></td>
<td>n</td>
<td>5</td>
<td>7</td>
<td>6</td>
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<td>Eosinophils, $\times 10^5$ cells/ml</td>
<td>0 ± 0</td>
<td>0.86 ± 0.18*</td>
<td>0.08 ± 0.02†</td>
<td>0 ± 0</td>
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<td>Neutrophils, $\times 10^6$ cells/ml</td>
<td>0.01 ± 0.01</td>
<td>0.11 ± 0.02*</td>
<td>0.04 ± 0.01†</td>
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<tr>
<td>Lymphocytes, $\times 10^6$ cells/ml</td>
<td>0.01 ± 0.01</td>
<td>0.05 ± 0.02</td>
<td>0.02 ± 0.01</td>
<td>0.01 ± 0.01</td>
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<td>Cellular fibronectin, ng/ml</td>
<td>5.9 ± 1.3</td>
<td>48.2 ± 4.2*</td>
<td>18.3 ± 1.6†</td>
<td>6.3 ± 1.9</td>
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<td>Proteins, mg/ml</td>
<td>0.19 ± 0.03</td>
<td>0.25 ± 0.02</td>
<td>0.28 ± 0.03</td>
<td>0.23 ± 0.05</td>
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<td>Serum IgE, µg/ml</td>
<td>10.2 ± 1.8</td>
<td>11.5 ± 1.4</td>
<td>8.9 ± 1.8</td>
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Data are means ± SE; n, no. of mice. Dexamethasone (Dex; 3 mg/kg ip) was applied 1 h before each challenge. Mice were killed 3 days after the last challenge, and BAL was performed. *P < 0.05 compared with untreated/PBS-challenged animals. †P < 0.05, dexamethasone-treated/ovalbumin (OA)-challenged animals vs. PBS-treated/OA-challenged animals.
It has been suggested that airway smooth muscle thickening may be the most important determinant of airway responsiveness alterations (14). However, an increase in airway submucosal area (11) or an increase in adventitial thickness (16) could also exaggerate airway narrowing. Our data have demonstrated, using the chronic protocol, a significant increase in BrdU incorporation in the alveolar cells that may account for an increased thickness of the alveolar wall. This, in turn, may decrease the elastic load of the parenchyma on smooth muscle, eventually resulting in airway obstruction (16). This concept is further supported by the fact that the airway hyperreactivity observed following repeated challenges was much more pronounced compared with the acute protocol.

Mucus hypersecretion (24) is also thought to contribute to the structural changes occurring in asthmatic lungs. Excessive production of mucus glycoproteins may lead to a decrease in airway caliber, airway obstruction, and progressive respiratory insufficiency. However, very few studies have attempted to understand the mechanism(s) responsible for this increase in mucus production. Whether the increase in mucus-producing cells observed in the present study is related to proliferation of secretory cells or to differentiation of other epithelial cells to a secretory type remains to be determined. However, the high proliferative rate observed in the epithelium following allergen challenge, plus the fact that secretory cells are known to be able to divide (1), may favor of the first hypothesis.

Steroids are the most effective class of drugs to inhibit the inflammatory reaction in asthma (3), but the question of whether they are also able to inhibit the airway remodeling in human asthma is still controversial (13, 17, 26). In this study, intraperitoneal administration of dexamethasone (3 mg/kg) before each challenge fully inhibited the inflammatory reaction and the airway hyperreactivity but only partially affected the remodeling process in both protocols. These data suggest that steroids may be at least partly effective in reducing the airway remodeling seen in asthmatic patients.

Although we were unable to demonstrate a complete picture of the asthmatic airway remodeling, most probably due to the fact that this process is related to the chronicity of the disease, we believe that the allergic murine models described in the present study may be useful to study the initial events leading to this process. Further studies using these models combined with genetically modified mice and/or specific receptor antagonists may prove useful in determining the link between the allergic airway response and tissue remodeling in diseases such as asthma.

Part of this work was performed at Novartis (Basel, Switzerland) with the technical assistance of Antje Holle, Marinette Erard, Isabelle Bruckhardt, and Junko Tsuyuki.

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Table 3. Effect of dexamethasone on BAL cytokine levels

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<td>IL-5, pg/ml</td>
<td>&lt;5</td>
<td>190.5 ± 48.2*</td>
<td>9.3 ± 3.2†</td>
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<tr>
<td>IL-4, pg/ml</td>
<td>7.5 ± 5.2</td>
<td>554.6 ± 37.1*</td>
<td>15.4 ± 8.5†</td>
<td>9.2 ± 4.5</td>
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Data are means ± SE; n, no. of mice. Dexamethasone (3 mg/kg ip) was applied 1 h before each challenge. Mice were killed 1 day after the last challenge, and BAL was performed. *P < 0.05 compared with untreated/PBS-challenged animals. †P < 0.05, dexamethasone-treated/OA-challenged animals vs. PBS-treated/OA-challenged animals.

Fig. 11. Effect of dexamethasone on proliferation of epithelial (A) and alveolar (B) cells. One hour before each challenge, mice were treated intraperitoneally with 0.1 ml of PBS (open bars) or 3 mg/kg of dexamethasone (hatched bars). PBS-challenged mice (cross-hatched bars) were not treated. Seven days after the last challenge, mice were killed, and BrdU-positive cells were counted. Data shown are from 4 mice per group and expressed as means ± SE. *P < 0.05.
REFERENCES


