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.

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 ip 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,

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).

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Table 1. Comparison of the BAL parameters between BALB/c and C57BL/6 mice

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<th>BALB/c</th>
<th>C57BL/6</th>
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<tr>
<td>Eosinophils, $\times 10^5$ cells/ml</td>
<td>0.4 ± 0.1</td>
<td>0.6 ± 0.2</td>
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<tr>
<td>IL-4, pg/ml</td>
<td>672 ± 52</td>
<td>111 ± 9*</td>
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<tr>
<td>IL-5, pg/ml</td>
<td>158 ± 10</td>
<td>52 ± 4*</td>
</tr>
<tr>
<td>Cellular fibronectin, ng/ml</td>
<td>25 ± 3</td>
<td>&gt;10</td>
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Data are means ± SE of 6 mice in both strains. Mice were killed 1 day after challenge, and bronchoalveolar lavage (BAL) was performed. IL, interleukin. *$P < 0.05$ compared with BALB/c mice.

Fig. 1. Time course of bronchoalveolar lavage (BAL) cellular infiltration following acute and chronic challenges. Sensitized mice were challenged once (acute, A) or challenged daily for 5 consecutive days (chronic, B) with ovalbumin (OA) and then killed at specified time points. No significant variation in the number of macrophages was observed in either protocol. Data shown are from 2 different experiments, each including 5–6 mice per group, and are expressed as means ± SE. *Significance ($P < 0.05$) was determined between OA-challenged mice and nonchallenged mice (before).
hematoxylin. All slides were coded and counted blindly, under oil immersion, using a ×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...
obtained after five challenges (5.8 ± 0.4 × 10⁵ eosinophils/ml, n = 5). A diminished BAL eosinophilia was observed after 7 challenges (3.4 ± 0.3 × 10⁵ eosinophils/ml, n = 6) and disappeared after 12 challenges (0.1 ± 0.1 × 10⁵ eosinophils/ml, n = 4). Because allergen-induced lung inflammation wanes after 1 wk of allergen exposure, the chronic protocol was established as five challenges.

**BAL inflammatory cell counts.** As shown in Fig. 1, both the acute and chronic OA challenges induced neutrophil and eosinophil infiltration into the BAL. In the acute protocol, neutrophils were apparent at 6 h postchallenge, peaked at day 1, and resolved by day 3. The BAL eosinophilia was delayed, appearing on day 1, peaking at day 3, and lasting through to day 14. In the chronic protocol, a significant BAL neutrophilia was observed from the first time point studied up to the 6-h time point. BAL eosinophils were already present at the first time point studied. Thereafter, the kinetics of cell infiltration and resolution were similar to those observed in the acute protocol. However, eosinophilia was much more pronounced in the chronic model compared with the acutely challenged animals (e.g., a 4- to 5-fold increase at day 3). BAL lymphocytes were increased only following chronic challenge, and a significant increase was observed from day 1 until day 14. In 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 Penh 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 Penh 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 asthma. 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-
ever, in the acute protocol, the mice were only hyper-
sensitive 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 air-
way 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 mod-
els. 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 ob-
served 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 ex-
tracellular 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 as-
ppect 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 prolif-
eration was observed only in the chronic protocol;
however, the relevance of this observation is not
clear, since alveolar cell hyperplasia was never de-
scribed in human asthma. Although smooth muscle
hyperplasia and epithelial desquamation are charac-
teristic features of human asthma (23), such a phe-
nomenon was not evident in the present study. How-
ever, 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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<th>Untreated/PBS</th>
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<td>Acute Protocol</td>
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<td>Chronic Protocol</td>
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<td>7</td>
<td>6</td>
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<td>Eosinophils, ×10⁵ cells/ml</td>
<td>0 ± 0</td>
<td>0.86 ± 0.18*</td>
<td>0.08 ± 0.02†</td>
<td>0 ± 0</td>
<td>8.72 ± 1.12*</td>
<td>0.77 ± 0.15†</td>
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<tr>
<td>Neutrophils, ×10⁵ cells/ml</td>
<td>0.01 ± 0.01</td>
<td>0.11 ± 0.02*</td>
<td>0.04 ± 0.01†</td>
<td>0.01 ± 0.01</td>
<td>0.15 ± 0.05</td>
<td>0.12 ± 0.08</td>
</tr>
<tr>
<td>Lymphocytes, ×10⁶ 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>
<td>0.33 ± 0.05*</td>
<td>0.02 ± 0.01†</td>
</tr>
<tr>
<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>
<td>555.2 ± 58.2*</td>
<td>47.2 ± 4.2†</td>
</tr>
<tr>
<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>
<td>0.46 ± 0.04*</td>
<td>0.29 ± 0.02†</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>
<td>9.8 ± 1.2</td>
<td>26.5 ± 4.6*</td>
<td>12.5 ± 2.1†</td>
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</table>

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.
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.

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Table 3. Effect of dexamethasone on BAL cytokine levels

<table>
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<tr>
<td></td>
<td>Untreated/PBS</td>
<td>PBS/OA</td>
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<tr>
<td><strong>IL-5, pg/ml</strong></td>
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</tr>
<tr>
<td>PBS</td>
<td>&lt;5</td>
<td>190.5±48.2*</td>
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<tr>
<td>OA</td>
<td>7.5±5.2</td>
<td>554.6±37.1*</td>
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<tr>
<td><strong>IL-4, pg/ml</strong></td>
<td></td>
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<tr>
<td>PBS</td>
<td>7.5±5.2</td>
<td>554.6±37.1*</td>
</tr>
<tr>
<td>OA</td>
<td>7.5±5.2</td>
<td>554.6±37.1*</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


