Airway Hyperresponsiveness and Remodeling in Antigen-challenged Guinea Pigs

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Abstract

Airway hyperresponsiveness (AHR) is the main feature of allergic subjects/animals, and its underlying mechanism is not clear. We explored whether antigen-induced AHR is associated with cytokine generation, inflammatory cell infiltration, and/or remodeling of airway smooth muscle. Guinea pigs were divided into three groups: control-1, control-2, and ovalbumin (OA). Animals in the control-1 group were not sensitized, while those in the control-2 and the OA group were sensitized with OA. Forty to forty-two days after the initial sensitization or equivalent time, animals in the control-2 group inhaled saline aerosol and those in the OA group inhaled OA aerosol for 30 min. Twenty-four h after OA challenge or equivalent time, animals in each group were further divided into two subgroups: methacholine and hyperventilation. Functional tests were carried out before and after the methacholine or hyperventilation treatment. Immediately after the functional study, bronchoalveolar lavage fluid was collected for determination of inflammatory cells and tumor necrosis factor-α (TNF-α). The trachea was then removed to determine smooth muscle mass. In both the methacholine and hyperventilation subgroups, significantly more severe airway constriction was found in the OA group, indicating OA-induced AHR. Eosinophil accumulation increased in the control-2 group and this increase was further augmented in the OA group. In addition, TNF-α level and smooth muscle mass significantly increased in the OA group. These results suggest that OA challenge-induced AHR is associated with increases in TNF-α level, cellular infiltration, and airway smooth muscle mass.

Key Words: airway reactivity, antigen challenge, cytokines, inflammatory cells, airway smooth muscle mass

Introduction

Airway hyperresponsiveness (AHR), an increase in airway responsiveness to a fixed stimulus, is the main feature of allergic subjects/animals and its underlying mechanism is not clear. Several studies (6, 9, 10, 15, 17, 19, 21) suggest that sensitization and challenge with antigen induced AHR in animals and thus mimicked the asthmatic attack in human subjects. There are various types of AHR. Here, we examined methacholine- and hyperventilation-induced AHR. The former is a cholinergic response and methacholine acts on the muscurinic receptor, while the latter is a noncholinergic reaction and its transmitters are primarily tachykinins (mainly substance P and neurokinin-A). This study was carried out to test whether allergic animals react differently to methacholine and hyperventilation. In addition, we investigated if antigen challenge-induced AHR is related to elevated cytokine (tumor necrosis factor-α, TNF-α), bronchoalveolar lavage (BAL) cell counts, and/or airway smooth muscle mass.

Materials and Methods

Animal Preparations

Fifty young Hartley strain guinea pigs weighing 433 ± 8 g were divided into three groups: control-1 (n = 16); control-2 (n = 16); and ovalbumin (OA, n = 18). Each animal in the control-1 group was not sensitized, while that in the control-2 and OA groups was

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sensitized with an intraperitoneal injection of 1 ml mixture of OA (10 μg/animal), pertussis vaccine (10^{10} U/animal), and $Al(OH)_3$ (1 mg/animal), according to the method of Watson et al. (21). 40- to 42-days after the initial sensitization or equivalent time, animals in the control-1 and control-2 groups inhaled saline aerosol and those in the OA group inhaled OA aerosol for 30 min. Saline or OA aerosol was generated, respectively, from 8 ml saline or OA (1 mg/ml) solution using a nebulizer (Ultra-Neb99, DeVilbiss Co., Somerset, PA). 24-h after OA challenge or equivalent time, animals in each group were further divided into two subgroups: methocholine and hyperventilation. For the methacholine subgroup, each animal was injected intravenously with methacholine (10 μg/kg) to induce airway constriction. Each animal in the hyperventilation subgroup was subjected to a previously reported hyperventilation maneuver (5) to initiate airway constriction. Bronchial tests were carried out before and 1-20 min after the methacholine or hyperventilation treatment.

For the bronchial testing, each animal was anesthetized with sodium pentobarbital (30-40 mg/kg). Subsequently, each animal's trachea, carotid artery and jugular vein were cannulated. After being paralyzed with gallamine triethiodide (4 mg/kg), the animal was artificially ventilated. To ensure that the animal was anesthetized during paralysis, gallamine was administered according to the following plans. Gallamine was only given when its active period (40 min) was within the effective duration of pentobarbital (1-2 h). If it was necessary to administer gallamine beyond this effective period of anesthetic, supplemental doses of pentobarbital were given before any more gallamine treatment.

Evaluation of Bronchial Function

During the baseline period, we first performed the full maximal expiratory flow-volume (MEFV) maneuver 2-3 times to obtain baseline total lung capacity [TLC, lung volume at airway opening pressure (Pao) = 30 cm H_2O] (12). Subsequently, the partial MEFV maneuver was carried out 1-20 min after methacholine or hyperventilation to examine the degree of airway constriction. At peak volume when Pao = 10 cm H_2O (instead of the usual Pao = 30 cm H_2O for the full MEFV maneuver), the inflation valve was shut off and immediately another solenoid valve (connected to a negative pressure of 40 cm H_2O) for deflation was automatically turned on and thus initiated a MEFV curve. Dynamic respiratory compliance (Crs), forced expiratory volume in 0.1 sec ($FEV_{0.1}$), and maximal expiratory flow at 30% baseline total lung capacity ($\dot{V}max_{30}$) were obtained according to our previous method (5) and used as indicators of airway constriction.

Bronchoalveolar Lavage (BAL) Cell Counts

On the day of the study, 10 ml warm (37°C) saline with bovine serum albumin (0.025%) was instilled into the lung of the anesthetized animal via trachea at an appropriate time after saline (control), methacholine, or hyperventilation. The fluid in the bronchoalveolar space was withdrawn 40 s after the instillation. One ml of BAL fluid was used to analyze cell counts. The BAL fluid was centrifuged at 4°C for 10 min, and the cell pellet was resuspended in Hank's balanced salt solution; 100 μl of Turk's solution was added to the same volume of cell suspension. After mixing, 10 μl of the mixture was placed in a hemacytometer for total cell counts. Differential cell counts were determined from cytospin preparations of 500 cells stained with Liu stain (Handsel Technologies, Inc., Taipei, Taiwan).

Tumor Necrosis Factor-α

The rest of BAL fluid was employed to analyze TNF-α using a Human TNF-α enzyme immunoassay kit (R & D System, Minneapolis, MN, USA). The bottom of the plate was coated with the mouse anti-human TNF-α monoclonal antibody. After TNF-α in the sample reacted with the antibody at room temperature for 2 h, the unbound TNF-α was washed away with wash buffer. Then, the second horseradish peroxidase-linked polyclonal anti-TNF-α antibody was added for 2 h at room temperature. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution of horseradish peroxidase, tetramethyl- benzidine, was added for 20 min. Finally, 2 N sulfuric acid was added for 30 min to stop reaction. The OD of the plate was read under the wave length at 450 nm. The concentration of TNF-α was obtained using a standard curve for human TNF-α.

Tracheal Smooth Muscle Mass

At the conclusion of the experiment, the tracheal segment was isolated. After its surrounding connective tissues were removed, the segment was then immersed in the buffered neutral formalin solution. After decalcification, dehydration, clearing, paraffin embedding, and sectioning, the sample was stained with hematoxyline-eosin. Subsequently, histology of the tracheal section was analyzed with a photomicrography digitalized integrate system (MGDS). The image under a light microscope (Zeiss Axiophot) was stored with a digital camera and a Magneto Optical disk. The smooth muscle area in the section was then computed using the Image-Pro Plus (Media

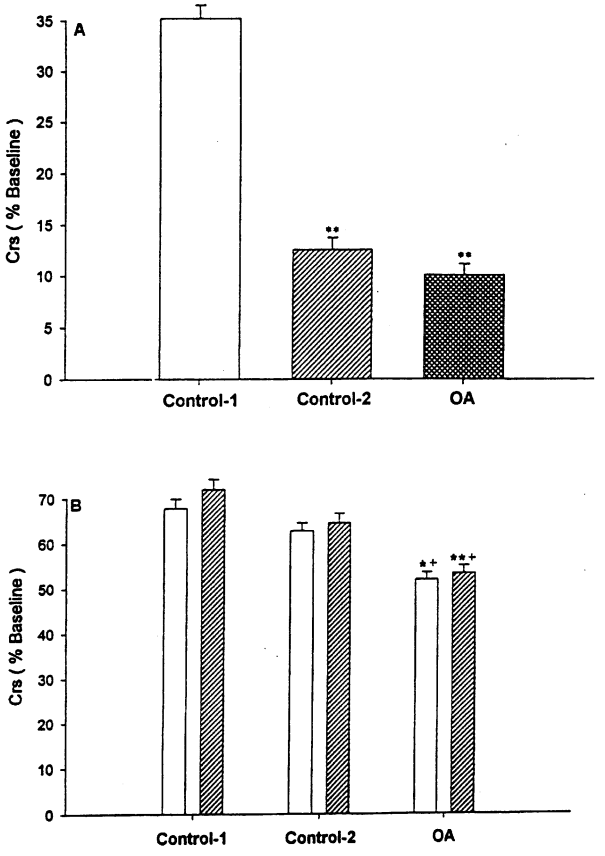

Fig. 1. Methacholine- (*A*) and hyperventilation-induced (*B*) decreases in dynamic respiratory compliance (Crs), expressed as percent of baseline values, in three groups of guinea pigs. OA = ovalbumin; Values in *B* were obtained at 3-min into the recovery period (following the hyperventilation period) (*open bars*) and at 8-min into the recovery period (following the hyperventilation period) (*stripped bars*). Significant differences compared to the control-1 group: $*P < 0.05$; $**P < 0.01$. $^{+}$Significant difference ($P < 0.05$) compared to the control-2 group.

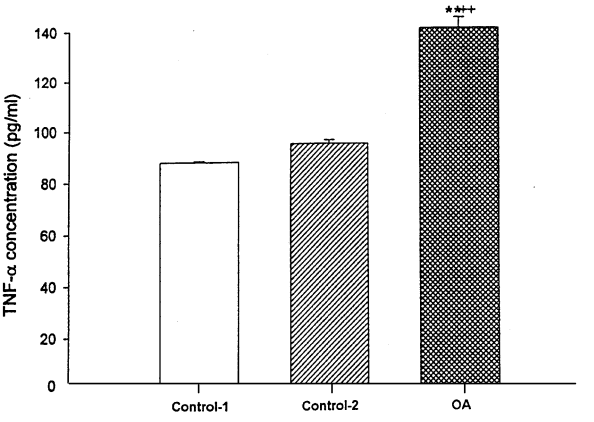

Fig. 2. Tumor necrosis factor-α (TNF-α) concentration of bronchoalveolar lavage fluid in the methacholine subgroup. OA = ovalbumin. **Significant difference ($P < 0.01$) compared to the control-1 group. $^{++}$Significant difference ($P < 0.01$) compared to the control-2 group.

Cybernetics, Silver Spring, MD, USA) software.

Data Analysis

All data are reported as means ± SE. One-way analysis of variance was used to establish differences among groups or subgroups. If significant difference existed among groups or subgroups, the Student-Newman-Keul's test was used to differentiate differences between any two of them. Difference was considered significant when $P < 0.05$.

Results

Baseline dynamic respiratory compliance (Crs) values for the control-1, control-2, and OA groups were 0.48 ± 0.02, 0.41 ± 0.22, and 0.41 ± 0.02 ml/cmH_2O, respectively. No significant difference in Crs was detected among these groups. Figure 1 shows that methacholine- (A) and hyperventilation challenge- (B) induced decrease in Crs in three groups of guinea pigs. Methacholine caused a marked decrease in Crs, indicating methacholine-induced airway constriction. This constriction was significantly augmented in the control-2 and OA groups. Compared to methacholine, hyperventilation challenge induced a smaller degree of airway constriction (Fig. 1B), which was significantly enhanced in the OA group. Similar results to those of Crs were obtained in terms of $FEV_{0.1}$ and $\dot{V}max_{30}$.

The OA challenge significantly increased BAL TNF-α concentration in the methacholine subgroup (Fig. 2). Similarly, the OA challenge caused an increase in BAL TNF-α concentration in the hyperventilation subgroup (not shown). Significant increases in total cells, eosinophils, and lymphocytes were found in the control-2 group of the methacholine subgroup (Fig. 3). Furthermore, for the same subgroup, all cell types increased in the OA group (Fig. 3). For most cases, similar increases in inflammatory cells were found in the hyperventilation subgroup (not shown).

For the methacholine subgroup, smooth muscle area increased in both the control-2 and OA groups (Fig. 4). These increases were similarly occurred in the hyperventilation subgroup (not shown).

Discussion

We demonstrated in this study that antigen (OA) challenge caused increases in BAL TNF-α concentration and cell counts, enlarged airway smooth muscle mass, and augmented methacholine- and hyperventilation-induced airway constriction. We will now discuss the relationship between allergic airway responses, TNF-α, and airway smooth muscle mass.

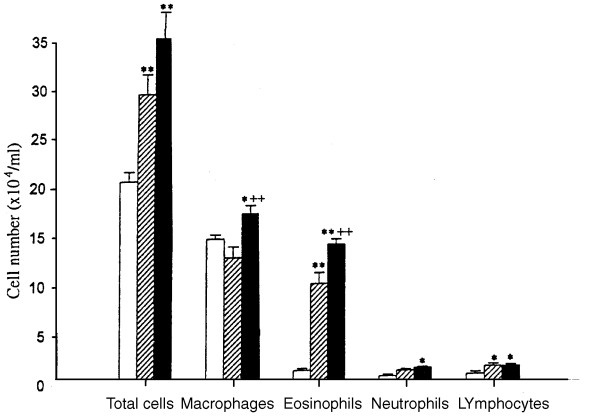

Fig. 3. Bronchoalveolar lavage cell numbers in the methacholine subgroup (the control-1 group, *open bars*, the control-2 group, *stripped bars*; and the OA group, solid bars). OA = ovalbumin. Significant differences compared to the control-1 group: $*P < 0.05$; $**P < 0.01$. $^{++}$Significant difference ($P < 0.01$) compared to the control-2 group.

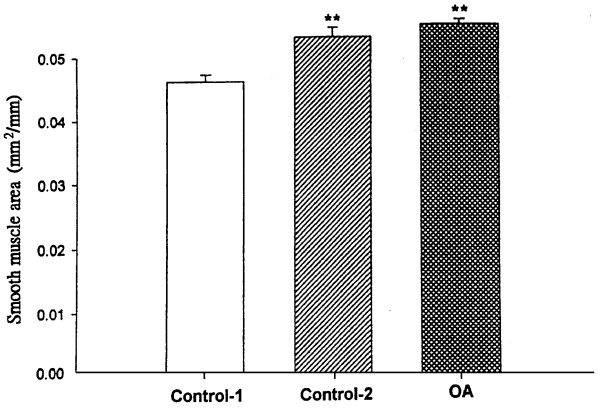

Fig. 4. Airway smooth muscle area in the methacholine subgroup. OA = ovalbumin. **Significant difference ($P < 0.01$) compared to the control-1 group.

Antigen Challenge and TNF-α

We showed that OA challenge, but not OA sensitization alone, increased BAL TNF-α level (Fig. 2). TNF-α is an important cytokine and is produced from macrophages, mast cells, T lymphocytes, eosinophils, neutrophils, epithelium, and smooth muscle cells (11). Elevated TNF-α causes neutrophil and eosinophil infiltration and an increase in the production of adhesion molecules (14). TNF-α at concentrations ranging from 0.3 to 30 pM can stimulate airway smooth muscle proliferation via the TNF-α p55 receptor (1). In addition, TNF-α has been shown to induce hyperresponsiveness of airway smooth muscle (2).

Antigen Challenge and Inflammatory Cells

We demonstrated that OA challenge increased BAL total cells, macrophages, neutrophils, eosinophils, and lymphocytes; while OA sensitization alone elevated BAL total cells, eosinophils, and lymphocytes (Fig. 3). Similarly, it was shown previously that there is an elevated inflammatory cell in BAL samples (6, 17), in epithelium (9), or in the sputum (8) of asthmatic patients. Antigen-induced cytokines can recruit inflammatory cells during allergic condition. Subsequently, increased inflammatory cells can produce cytokines, mediators, enzymes, and reactive oxygen species to augment the inflammation process. For example, eosinophils have various contents including basic proteins, mediators, enzymes, and reactive oxygen species. All of these eosinophil contents are closely related to antigen challenge-associated airway constriction and AHR (18).

Antigen Challenge and Airway Smooth Muscle Mass

We demonstrated that there was increased airway smooth muscle mass in OA-sensitized and OA-challenged guinea pigs (Fig. 4). This increase in smooth muscle mass could be due to hyperplasia and/or hypertrophy of airway smooth muscle. In addition to hypertrophy, Panettieri et al. (15) demonstrated that hyperplasia of airway smooth muscle occurs following antigen challenge. Similarly, Dunnill et al. (3), Heard and Hossain (7), and Ebina et al. (4) demonstrated that there is an increase in bronchial smooth muscle mass or volume in patients died with asthma. Following sensitization-challenge with antigen, there is an increase in mitogenic or proliferative agents, which in turn cause proliferation of airway smooth muscle cells. These mitogenic factors are produced by mast cells, eosinophils and macrophages. Several mitogenic factors such as thrombin, platelet-derived growth factor, leukotriene D_4, and thromboxane augment the proliferation of airway smooth muscle (20). Furthermore, other mediators such as histamine, endothelin-1, TNF-α, interleukin-1 (IL-1) and IL-6 enhance also the proliferation of airway smooth muscle.

Antigen Challenge and AHR

We performed airway responsiveness to two types of challenges (methacholine and hyperventilation) prior to and after antigen sensitization-challenge. The responsiveness to methacholine is mainly a test of cholinergic reaction while that to hyperventilation is a noncholinergic reaction (5, 16). Both OA-sensitization and OA-challenge enhanced methacholine-induced airway constriction (Fig. 1). However, only OA-challenge, but not OA-sensitization, augmented hyperventilation-induced airway constriction (Fig.

4). The increased cholinergic airway response following antigen sensitization-challenge could be caused by two mechanisms (10): increased bronchial response to a fixed level of cholinergic transmitter, and/or an increase in muscarinic receptors in airways. Antigen challenge-induced increases in TNF-α, inflammatory cells and airway smooth muscle mass, singly or in combination, should result in AHR. As indicated above, TNF-α can augment airway constriction, airway smooth muscle proliferation, and/or AHR. Increased inflammatory cells can release several types of cytokines, mediators, enzymes, and reactive oxygen species, which in turn cause airway constriction, airway smooth muscle proliferation, and/or AHR. Increased proliferation of airway smooth muscle results in thickened bronchial wall, which in turn augments airway constriction, resulting in AHR (13).

Compared to antigen challenge, antigen sensitization alone could cause less TNF-α production, which in turn may augment less hyperventilation-induced ARH. Thus, repeated antigen treatment was more effective in inducing inflammation-related alterations than that of a single antigen administration (19). Another comparison is the airway constriction induced by methacholine and hyperventilation. Compared to methacholine-induced airway constriction, hyperventilation caused much less severe constriction (Fig. 1). This discrepancy might be due to the fact that hyperventilation induces the release of less potent and/or less amount of endogenous constrictor(s) in comparison to the exogenously applied methacholine.

In summary, we showed that OA challenge caused increases in TNF-α, inflammatory cells, airway smooth muscle mass, and airway responsiveness to methacholine and hyperventilation. Therefore, AHR is likely to be induced by multiple factors associated with allergic inflammation.

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References

1. Amrani, Y., Panettieri, R.A., Jr., Frossard, N. and Bronner, C. Activation of the TNFα-p55 receptor induces myocyte proliferation and modulates agonist-evoked calcium transients in cultured human tracheal smooth muscle cells. *Am. J. Respir. Cell Mol. Biol.* 15: 55-63, 1996.
2. Anticevich, S.Z., Hughes, J.M., Black, J.L. and Armour, C.L. Induction of human airway hyperresponsiveness by tumor necrosis factor-α. *Eur. J. Pharmacol.* 284: 221-225, 1995.
3. Dunnill, M.S., Massarella, G.R. and Anderson, J.A. A comparison of the quantitative anatomy of the bronchi in normal subjects, in status asthmaticus, in chronic bronchitis, and in emphysema. *Thorax* 24: 176-179, 1969.
4. Ebina, M., Takahashi, T., Chiba, T. and Motomiya, M. Cellular hypertrophy and hyperplasia of airway smooth muscles underlying bronchial asthma: a 3-D morphometric study. *Am. Rev. Respir. Dis.* 148: 720-726, 1993.
5. Fang, Z.X. and Lai, Y.-L. Oxygen radicals in bronchoconstriction of guinea pigs elicited by isocapnic hyperpnea. *J. Appl. Physiol.* 74: 627-633, 1993.
6. Gonzalo, J.A., Lloyd, C.M., Kremer, L., Finger, E., Martinez, C.A., Siegelman, M.H., Cybulsky, M. and Gutierrez-Ramos, J.C. Eosinophil recruitment to the lung in a murine model of allergic inflammation: The role of T cells, chemokines, and adhesion receptors. *J. Clin. Invest*. 98: 2332-2345, 1996.
7. Heard, B.E. and Hossain, S. Hyperplasia of bronchial muscle in asthma. *J. Pathol.* 110: 319-331, 1973.
8. Huber, H.L. and Koessler, K.K. The pathology of bronchial asthma. *Arch. Intern. Med*. 30: 689-760, 1922.
9. Ishida, K., Kelly, L.J., Thomson, R.J., Beattie, L.L. and Schellengerg, R.R. Repeated antigen challenge induces airway hyperresponsiveness with tissue eosinophilia in guinea pigs. *J. Appl. Physiol*. 67: 1133-1139, 1989.
10. Kageyama, N., Igarashi, A., Ichinose, M., Miura, M., Yamauchi, H., Tomaki, M., Ishikawa, J., Sasaki, Y. and Shirato, K. Chronic allergen exposure enhances cholinergic neurotransmission in sensitized guinea-pigs. *Eur. Respir. J.* 8: 752-754, 1995.
11. Kips, J.C. and Pauwels, R.A. Proinflammatory cytokine. In: Asthma. Edited by Barnes, P.J., Grunstein, M.M., Leff, A.R., and Woolcock, A.J. Philadelphia: Lippinocott-Raven Publishers. 1997, chapter 49, pp. 653-662.
12. Lai, Y.-L. Maximal expiratory flow in the guinea pig. *Lung* 166: 303-313, 1988.
13. Lambert, R.K., Wiggs, B.R., Kuwano, K., Hogg, J.C. and Pare, P. D. Functional significance of increased airway smooth muscle in asthma and COPD. *J. Appl. Physiol*. 74: 2771-2781, 1993.
14. Lukacs, N.W., Strieter, R.M., Chensue, S.W., Widmer, M. and Kunkel, S.L. TNF-α mediates recruitment of neutrophils and eosinophils during airway inflammation. *J. Immunol.* 154: 5411-5417, 1995.
15. Panettieri, R.A., Jr., Murray, R.K., Eszterhas, A.J., Bilgen, G. and Martin J.G. Repeated allergen inhalations induce DNA synthesis in airway smooth muscle and epithelial cells in vivo. *Am. J. Physiol.* 274: L417-L424, 1998.
16. Ray, D.W., Hernandez, C., Leff, A.R., Drazen, J.M. and Solway, J. Tachykinins mediate bronchoconstriction elicited by isocapnic hyperpnea in guinea pigs. *J. Appl. Physiol*. 66: 1108-1112, 1989.
17. Sanjar, S., Aoki, S., Kristersson, A., Smith, D. and Morley, J. Antigen challenge induces pulmonary airway eosinophil accumulation and airway hyperreactivity in sensitized guinea-pigs: the effect of anti-asthma drugs. *Br. J. Pharmacol.* 99: 679-686, 1990.
18. Strek, M.E. and Leff, A.R. Eosinophils. In: Asthma. Edited by Barnes, P.J., Grunstein, M.M., Leff, A.R., and Woolcock, A.J. Philadelphia: Lippincott-Raven Publishers. 1997, chapter 30, pp. 399-418.
19. Trifilieff, A., El-Hashim, A. and Bertrand C. Time course of inflammatory and remodeling events in a murine model of asthma: effect of steroid treatment. *Am. J. Physiol.* 279: L1120-L1128, 2000.
20. Wang, C.G., Du, T., Xu, L.J. and Martin, J.G. Role of leukotriene D_4 in allergen-induced increase in airway smooth muscle in the rat. *Am. Rev. Respir. Dis.* 148: 413-417, 1993.
21. Watson, M.L., Smith, D., Bourne, A.D., Thompson, R.C. and Westwick, J. Cytokines contribute to airway dysfunction in antigen-challenged guinea pigs: inhibition of airway hyperreactivity, pulmonary eosinophil accumulation, and tumor necrosis factor generation by pretreatment with an interleukin-1 receptor antagonist. *Am. J. Respir. Cell Mol. Biol.* 8: 365-369, 1993.