Tuberculosis and Respiratory Disease 결핵 및 호흡기질환, Vol. 42, No. 4, August, 1995

□종 설□

Cytokines Stimulate Lung Epithelial Cells to Release Nitric Oxide

Richard A. Robbins* and O Jung Kwon**

Research Service, Omaha VA Medical Center, Omaha, NE 68105
**Pulmonary and Critical Care Medicine, Department of Internal Medicine, University of Nebraska Medical Center, Omaha, NE 68198-5300*
***Division of Pulmonology, Department of Medicine, Samsung Medical Center, Seoul, Korea*

INTRODUCTION

Nitric oxide(NO) is an important mediator of vasoregulation[1]. Many recent reports reflect an increasing awareness that NO also plays important roles in a number of physiologic and pathophysiologic processes[1]. Among these proposed roles for NO are modulation of edema formation, inflammation, and regulation of non-vascular smooth muscle tone. Many of these would implicate NO in basic allergic mechanisms and disorders.

NO is formed when the guanido group of the essential amino acid L-arginine is cleaved forming NO and L-citrulline[1]. The reaction is catalyzed by nitric oxide synthase(NOS) and several cofactors. NOS exists in several forms[2]. A constitutive NOS (cNOS) has been identified in endothelial and a variety of non-endothelial cells, which is a constitutively expressed, calcium-dependent enzyme accounting for the baseline production of small, picomolar amounts of NO[3~6]. An inducible form of NOS(iNOS) has also been detected in a variety of tissues and organs[6~9]. iNOS is not usually expressed in most tissues, but is induced by lipopolysaccharide(LPS) or cytokines resulting in the formation of larger, nanomolar amounts of NO[1,2].

NO is a highly reactive gas with a estimated half life of seconds in biological tissues[10]. NO reacts rapidly with 0_2^- to form peroxynitrite($OONO^-$)[11~13]. Peroxynitrite, a strong oxidizing agent, spontaneously forms hydroxyl anions(OH) and nitrite(NO_2-) through several intermediate reactions[13,14]. Nitrite is stable for several hours in water and plasma but is rapidly converted to nitrate in whole blood[2,15].

A mechanism with potential relevance in asthma has been the observation that macrophages from asthmatics spontaneously release cytokines such as TNF and IL-1[16,17]. These macrophage-derived cytokines may in turn interact with airway epithelium resulting in a number of proinflammatory events including secondary release of cytokines such as interleukin-8 and RANTES[18,19]. Airway blood vessel dilatation and edema have been proposed to play a major role in the airway obstruction in asthma. Paré and colleagues, through the use of mathematical modeling, have demonstrated that small amounts of airway edema can result in significant airway narrowing, resulting in airway obstruction[20,21]. NO might contribute to edema formation and narrowing. In support of this concept, NO inhibitors reduce microvascular permeability of guinea pig airways in response to inhalation

of histamine, platelet activating factor, substance P, A23187, or ovalalbumin in sensitized animals[22] and expression of iNOS has been demonstrated in bronchial epithelial cells in patients with asthma[23].

In the above context we made the hypothesis that cytokines released from stimulated alveolar macrophages in disorders such as asthma might interact with airway epithelial cells resulting in induction of iNOS, increased NO formation, and resultant edema and airway narrowing(Fig. 1).

ALVEOLAR MACORPHAGE STIMULATION OF NO PRODUCTION IN LUNG EPITHELIAL CELLS

In order to determine the capacity of alveolar macrophages to stimulate iNOS in lung epithelial cells, bronchoalveolar lavage was performed in 7 normal, nonsmoking adults[24]. The resultant cell population consisted of >85% alveolar macrophages(AM) with occasional ciliated cell, neutrophil, eosinophil, or other cell type identified. The AMs were suspended at 1×10^6 cells/ml in RPMI-1640 with 10% fetal calf serum and cultured for 24 hours in the presence of opsonized zymosan[25]. After centrifuging to remove the zymosan, 100μl of the AM supernatant was added to confluent cultures of the human lung epithelial cell line, A549, or the murine lung epithelial cell line, LA-4, with 900 μl of Ham's F-12 medium. After 24 hours, the supernatant fluids were harvested and evaluated for nitrite and nitrate.

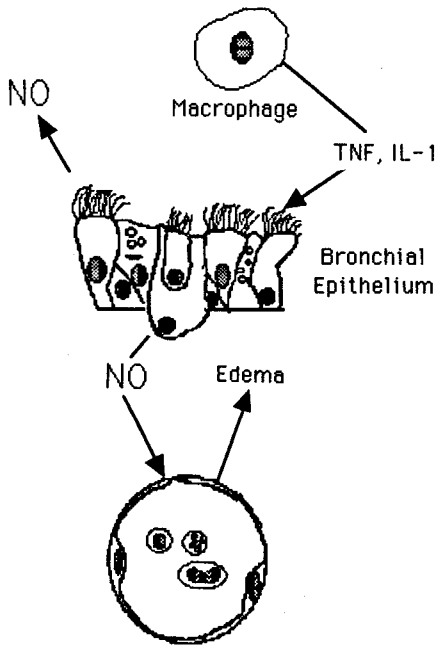

Fig. 1. Proposed role for NO in asthma. Alveolar macrophages are stimulated to release cytokines such as tumor necrosis factor α(TNF) and interleukin-1β(IL-1). These cytokines increase nitric oxide(NO) production by bronchial epithelial cells resulting in increased airway edema formation and increased NO detected in exhaled air.

Nitrite was measured by a sensitive(<1 nM) method employing conversion of nitrite to NO in glacial acetic acid with 1% KI under a nitrogen stream[26]. NO was detected with a chemiluminescence analyzer (Model 270B, Sievers, Boulder CO). Nitrite + nitrate was similarly measured except that nitrate was first converted to nitrite by incubating the sample with *E. coli* nitrate reductase(0.05 unit/ml, final concentration).

Nitrite was increased in the A549 culture supernatant compared to the AM culture supernatants. However, there was no increase compared to A549 cells cultured in media alone(Fig. 2). In contrast, nitrite + nitrate was markedly increased in the A549 supernatant fluids obtained from cells cultured with the AM supernatant compared to cells cultured in media alone.

LA-4 cells cultured for 24 hours had marked increases in nitrite and nitrite + nitrate compared to cells culture in media alone(Fig. 2).

Further experiments were done with the LA-4 cells to determine if macrophage derived cytokines such as TNF or IL-1 contributed to the AM-induced increases in nitrite. Antihuman TNFα and/or monoclonal mouse

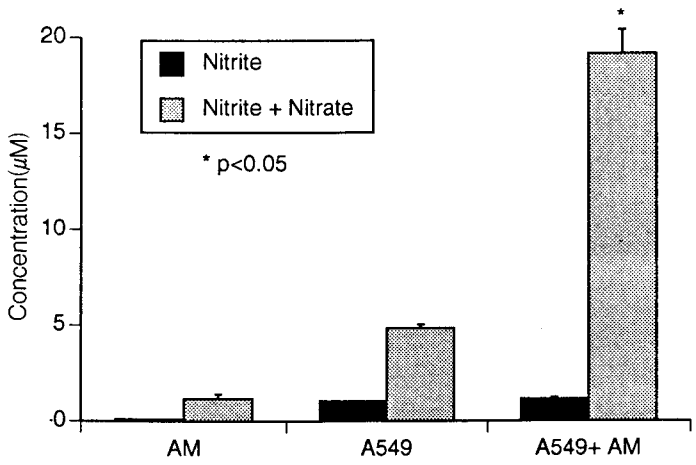

Fig. 2. Nitrite(solid bars) and nitrite + nitrate(shaded bars) in alveolar macrophage culture supernatant fluids(AM), A549 cells cultured in media alone for 24 hours(A549), or A549 cells cultured with added AM supernatant (A549 + AM). n=7 each data point. *p<0.05 compared to A549 cells cultured in media alone.

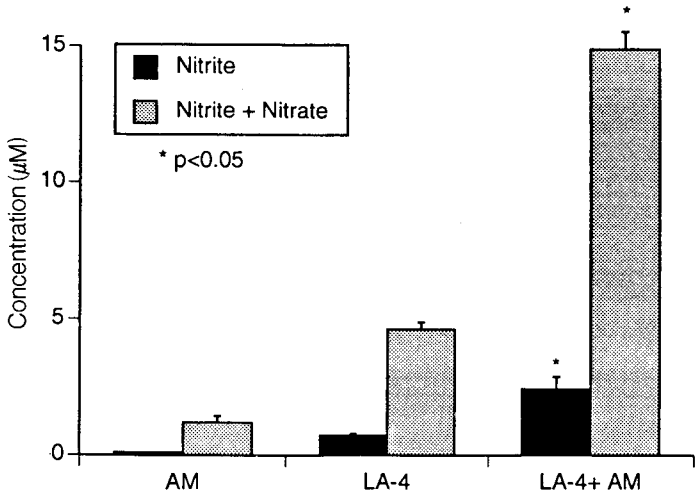

Fig. 3. Nitrite(solid bars) and nitrite + nitrate(shaded bars) in alveolar macrophage culture supernatant fluids(AM), LA-4 cells cultured in media alone for 24 hours(LA-4), or LA-4 cells cultured with added AM supernatant(LA-4 + AM). n=7 each data point. *p<0.05 compared to LA-4 cells cultured in media alone.

antihuman IL-1β antibodies were preincubated with an AM supernatant fluid and the capacity of the fluid to stimulate NO production by the LA-4 cells evaluated by measuring nitrite concentrations in the supernatant fluids after 24 hours as above. In addition, the capacity of the competitive nitric oxide synthase inhibitor N^G-monomethyl-L-arginine(L-NMMA) to inhibit the nitrite accumulation in the LA-4 culture

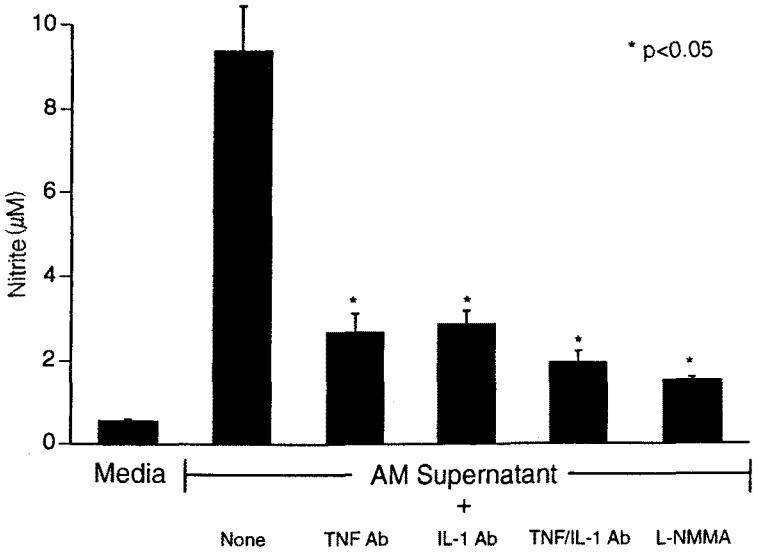

Fig. 4. Nitrite concentrations in culture supernatant fluids from LA-4 cells cultured in media alone or with an alveolar macrophage supernatant (AM Supernatant). The AM supernatant was incubated with media alone(none), a tumor necrosis factor α antibody(TNF Ab), and interleukin-1β antibody(IL-1 Ab), both antibodies(TNF/IL-1 Ab), or N^G-monomethyl-L- arginine(L-NMMA, 10^{-4}M). n=3 each data point. *$p<0.05$ compared to LA-4 cells cultured with AM supernatant alone.

supernatant fluids was assessed as a negative control.

The antibody blocking experiments demonstrate that both the anti-TNF and/or anti-IL-1 antibodies reduced nitrite accumulation in the LA-4 culture supernatant fluids(Fig. 4). The reducton in nitrite seen with the antibody blocking experiments approximated the reduction seen with the NOS inhibitor, L-NMMA.

CYTOKINE STIMULATION OF iNOS IN LUNG EPITHELIAL CELLS

Based on the studies with the AM culture supernatant fluids, further studies were done with purified cytokines to determine if the increased accumulation of nitrite in the airway epithelial cell culture supernatant fluids was secondary to increased expression of iNOS[26]. LA-4 cells were stimulated with cytomix, a combination of IL-1β, TNFα, and interferon-gamma, all at 10 ng/ml final concentration. Consistent with the experiments with the AM culture supernatant fluids, cytomix increased nitrite levels by 873% in the culture supernatant fluids. An increased number of cells were stained for iNOS on immunocytochemistry. An increase in iNOS mRNA was also observed(Fig. 5). Dexamethasone decreased the cytokine-induced increase in nitrite levels, NOS activity, iNOS immunoreactivity, and mRNA.

Similarly, cytomix increased inducible nitric oxide synthase(iNOS) expression in the human lung epithelial cell line, A549, and primary cultures of human bronchial epithelial cells[27]. Cytomix induced a time-dependent increase in nitrite levels in culture supernatant fluids($p<0.05$). Increased iNOS mRNA level was detected in the cytokine-stimulated cells com-

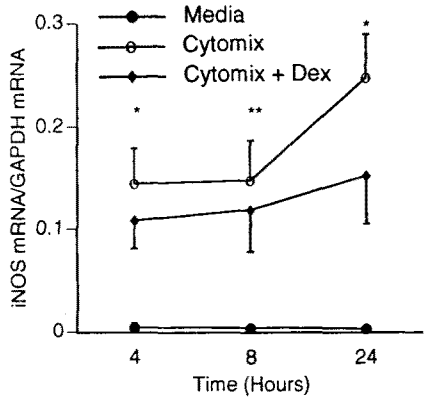

Fig. 5. Northern blot determination of iNOS mRNA levels from LA-4 cells cultured at 4, 8, or 24 hours in the presence of media, cytomix, or cytomix + dex(dexamethasone 10^{-6} M) expressed as the ratio of optical density of scanning laser densitometry of iNOS mRNA to the house keeping gene glyceraldehyde 3-phosphate dehydrogenase(GAPDH) mRNA. n=3 each data point. *p<0.05 cytomix compared to media and cytomix compared to cytomix + dex, **p<0.05 cytomix compared to cytomix + dex.

pared to control(Fig. 6). Dexamethasone diminished the cytokine-induced increase in nitrite, iNOS by immunocytochemistry, and iNOS mRNA.

The above data demontrate that cytokines which may be released by cells within the lung can induce lung epithelial iNOS expression and NO release which is attenuated by dexamethasone.

EXHALED NITRIC OXIDE

The data above suggest that AMs releasing cytokines such as TNF and IL-1 may induce lung epithelial cells to increase expression of iNOS resulting in increased nitric oxide production. Asthma, but not COPD, is a disorder which has been associated with an increase in AM release of TNF and IL-1[16,17]. Therefore, we hypothesized that increased airway epithelial cell NO production would be associated with increased exhaled NO in asthmatics not recei-

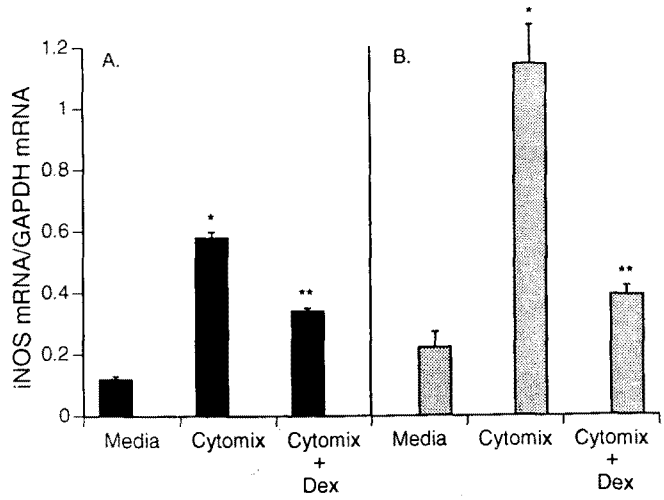

Fig. 6. Northern blot determination of iNOS mRNA levels from A549 cells(Panel A) or primary cultures of human bronchial epithelial cells (Panel B) cultured for 24 hours in the presence of media, cytomix, or cytomix + dex(dexamethasone 10^{-6} M). iNOS mRNA is expressed as the ratio of optical density on scanning laser densitometry of iNOS mRNA to the house keeping gene glyceraldehyde 3-phosphate dehydrogenase(GAPDH) mRNA. n=3 each data point. *p<0.05 cytomix compared to media and **cytomix compared to cytomix + dex compare to cytomix.

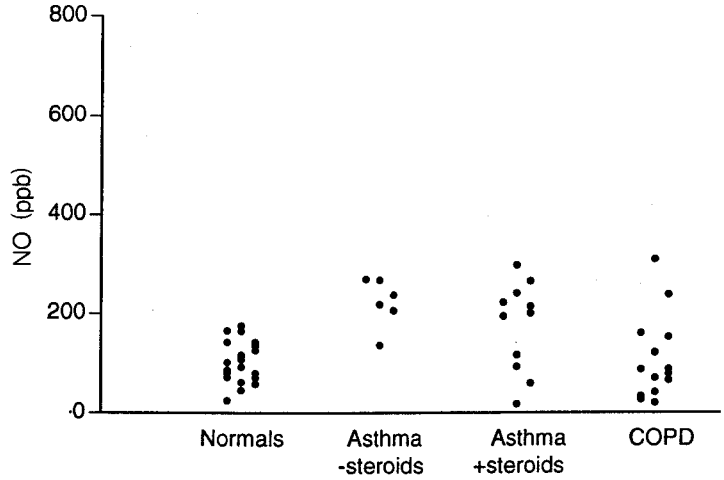

Fig. 7. Peak exhaled NO levels in normal, nonsmoking controls(normals, n=23), asthmatics not receiving corticosteroids(Asthma -steroids, n=7), asthmatics receiving corticosteroids(Asthma + steroids, n=11), and parients with chronic obstructive pulmonary disease(COPD, n=14). The asthmatics receiving corticosteroids had significantly greather exhaled NO levels than the other 3 groups($p<0.05$).

ving corticosteroids compared to nonasthmatic controls, asthmatics receiving corticosteroids, or COPD subjects.

To test this hypothesis, exhaled NO was measured using a chemiluminescence analyzer(Model 270B, Sievers). Exhaled NO measurements were performed by having the subjects perform a slow vital capacity maneuver directly into the analyzer[28]. Exhaled NO was expressed as the peak NO concentration detected during the slow vital capacity maneuver in 23 normal subjects, 18 asthmatics(11 receiveing corticosteroids), and 14 patients with COPD.

Exhaled NO was increased in the asthmatics not receiving corticosteroids(Fig 7. $p<0.05$ compared to the other groups). Peak exhaled NO levels were increased in asthmatics not taking corticosteroids (n=7) compared to normal controls(280 ± 77 vs. 106 ± 8 ppb, $p<0.05$) but not in asthmatics taking corticosteroids(n=11, 174 ± 27 ppb, $p>0.05$) or COPD, a disorder not associated with alveolar macrophage cytokine release(n=14, 106 ± 22 ppb, $p>0.05$). The asthmatics receiving corticosteroids and the COPD patients did not differ from the normal nonsmoking controls.

SUMMARY

Cytokine release from alveolar macrophages and subsequent interaction of these cytokines with the bronchial epithelium can induce epithelial cells to release inflammatory mediators. Nitric oxide(NO), a highly reactive gas formed from arginine by nitric oxide synthase(NOS), is known to be involved in inflammation and edema formation, and the inducible form of NOS(iNOS) can be increased by cytokines. In this context, we hypothesized that lung epithelial cells could be stimulated by cytokines released by alveolar macrophages to express iNOS. To test this hypothesis, the murine lung epithelial cell line, LA-4, or the human lung epithelial cell line, A549, were stimulated with culture supernatant fluids from alveolar macrophages. NO production was assessed by

evaluating the culture supernatant fluids for nitrite and nitrate, the stable end products of NO. Both murine and human cell culture supernatant fluids demonstrated an increase in nitrite and nitrate which were time- and dose-dependent and attenuated by TNFα and IL-1β antibodies($p<0.05$, all comparisons). Consistent with these observations, cytomix a combination of TNFα, IL-1β, and γ-interferon, stimulated the lung epithelial cell lines as well as primary cultures of human bronchial epithelial cells to increase their NO production as evidenced by an increase in nitrite and nitrate in their culture supernatant fluids, an increase in the iNOS staining by immunocytochemistry, and an increase in iNOS mRNA by Northern blottin($p<0.05$, all comparisons). The cytokine effects on iNOS were all attenuated by dexamethasone. To determine if these *in vitro* observations are reflected *in vivo*, exhaled NO was measured and found to be increased in asthmatics not receiving corticosteroids. These data demonstrate that alveolar macrophage derived cytokines increase iNOS expression in lung epithelial cells and that these *in vitro* observations are mirrored by increased exhaled NO levels in asthmatics. Increased NO in the lung may contribute to edema formation and airway narrowing.

REFERENCES

1) Moncada S, Higgs A: The L-arginine-nitric oxide pathway. N Engl J Med **329**:2002-2012, 1993
2) Förstermann U, Schmidt HHHW, Pollock JS, Sheng H, Mitchell JA, Warner TD, Masaki N, Murad F: Isoforms of nitric oxide synthase: characterization and purification from different cell types. Bioch Pharmacol **10**:1849-1857, 1991
3) Lamas S, Marsden PA, Li GK, Tempst P, Michel T: Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct constitutive enzyme isoform. Proc Natl Acad Sci USA **89**:6348-6352, 1992
4) Hiki K, Yui Y, Hattori R, Eizawa H, Kosuga K, Kawai C: Three regulation mechanisms of nitric oxide synthase. Eur J Pharmacol 206:163-164, 1991
5) Robbins RA, Hamel FG, Floreani AA, Gossman GL, Nelson KJ, Belenky S, Rubinstein I: Bovine bronchial epithelial cells metabolize L-arginine to L-citrulline: possible role of nitric oxide synthase. Life Sciences **52**:709-716, 1993
6) Kobzik L, Bredt DS, Lowenstein CJ, Drazen J, Gaston B, Sugarbaker D, Stamler JS: Nitric oxide synthase in human and rat lung: immunochemical and histochemical localization. Am J Respir Cell Mol Biol **9**:371-377, 1993
7) Hibbs JB, Taintor RR, Vaurin Z: Macrophage cytotoxicity: role of L-arginine deimunase activity and imino nitrogen oxidation to nitrite. Science **235**:473-476, 1987
8) Nakayama DR, Geller DA, Lowenstein CJ, Davies P, Pitt BR, Simmons RL, Billiar TR: Cytokines and lipopolysaccharide induce nitric oxide synthase in cultured rat pulmonary artery smooth muscle. Am J Respir Cell Mol Biol **7**:471-476, 1992
9) Geller DA, Lowenstein CJ, Shapiro RA, Nussler AK, Disilvio M, Wang SC, Nakyama D, et al: Molecular cloning and expression of inducible nitric oxide synthase from human hepatocytes. Proc Natl Acad Sci USA, **90**:3491-3495
10) Stamler JS, Singel DJ, Loscalzo J: Biochemistry of nitric oxide and its redox-activated forms. Science **258**:1898-1902, 1992
11) Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitirite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA **87**:1620-1624, 1990
12) Blough NV, Zafiriou OC. Reaction of superoxide with nitric oxide to form peroxynitrite in alkaline

aqueous solution. Inorg Chem **24**:3502-3504, 1985

13) Saran M, Michel C, Bors W: Reaction of NO with O_2^-: implications for the action of endothelium-derived relaxing factor(EDRF). Free Fadical Res Commun **10**:221-226, 1990

14) Radi R, Beckman JS, Bush KM, Freeman BA: Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. Arch Biochem and Biophys **288**: 481-487, 1991

15) Kelm M, Feelisch M, Grude R, Motz W, Strauer BE: Metabolism of endothelium-derived nitric oxide in human blood. In: Moncada S, Marletta MA, Hibbs JB Jr, Higgs EA, eds. The biology of nitric oxide. Vol. 1. London: Portland Press, 319-322, 1992

16) Ohna I, Ohkawara Y, Yamauchi K, Tanno Y, Takishima T: Production of tumor necrosis factor with IgE receptor triggering from sensitized lung tissue. Am J Respir Cell Mol Biol **3**:285-289, 1990;

17) Borish L, Mascali JJ, Dishuck J, Beam WR, Martin RJ, Rosenwasser LJ: Detection of alveolar macrophage-derived IL-1β in asthma: inhibition with corticosteroids. J Immunol **149**:3078-3082, 1992

18) Kwon OJ, Jose PJ, Robbins RA, Schall TJ, Williams TJ, Barnes PJ: Dexamethasone inhibition of RANTES expression in human lung epithelial cells. Am J Resp Cell Mol Biol **12**:488-496, 1995

19) Kwon OJ, Au BT, Collins PD, Mak JC, Robbins RA, Chung KF, Barnes PJ: Tumor necrosis factor-induced interleukin-8 expression in pulmonary cultured human airway epithelial cells. Am J Physiol **267**:L398-L405, 1994

20) Paré PD, Wiggs BR, James A, Hogg JC, Bosken C: The comparative mechanics and morphology of airways in asthma and in chronic obstructive pulmonary disease. Am Rev Respir Dis **143**: 1189-1193, 1992

21) Wiggs BR, Bosken C, Paré PD, James A, Hogg JC: A model of airway narrowing in asthma and in chronic obstructive pulmonary disease. Am Rev Respir Dis **145**:1251-1258, 1992

22) Sherwood J, Kreutner W: Microvascular permeability of guinea pig airways is modulated by nitric oxide formation. FASEB J **8**:A383, 1994

23) Hamid Q, Springall DR, Riveros-Moreno V, et al: Induction of nitric oxide synthase in asthma. Lancet **342**:1510-1513, 1993

24) Rennard SI, Ghafouri M, Thompson AB, Linder J, Vaughan W, Jones K, Ertl RF, Christensen K, Prince A, Stahl MG, Robbins RA: Fractional processing of sequential bronchoalveolar lavage to separate bronchial and alveolar samples. Am Rev Resp Dis **141**:208-17, 1990

25) Robbins RA, Russ WD, Thomas KR, Rasmussen JK, Kay HD: Complement component C5 is required for release of macrophage-derived neutrophil chemotactic activity. Am Rev Respir Dis **135**:659-64, 1987

26) Robbins RA, Springall DR, Warren JB, Kwon OJ, Buttery LDK, Wilson AJ, IM Adcock IM, Rivers-Moreno V, Moncada S, Polak J, Barnes PJ: Inducible nitric oxide synthase is increased in murine lung epithelial cells by cytokine stimulation. Biochem Biophys Res Commun **198**:835-843, 1994

27) Robbins RA, Barnes PJ, Springall DR, Warren JB, Kwon OJ, Buttery LDK, Wilson AJ, Geller DA, Polak JM. Expression of inducible nitric oxide in human lung epithelial cells. Biochem Biophys Res Commun **203**:209-218, 1994

28) Kharitonov SA, Yates D, Robbins RA, Logan-sinclair R, Shinebourne EA, Barnes PJ: Increased nitric oxide in exhaled air of asthmatic patients. Lancet **343**:133-135, 1994