Pulmonary Fibrosis in BALB/c Mice Caused by Long-Term Instillation of Bleomycin

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ABSTRACT. It is well-known that bleomycin-induced pulmonary fibrosis is difficult to produce in BALB/c mice. To see whether bleomycin could induce pulmonary fibrosis in BALB/c mice, we repeatedly administered this drug through the airway for 10 consecutive days. Our experiment clearly showed that fibrosis can be induced, although it was rather mild, and also suggested that the bronchiolar and alveolar epithelia are the primary sites of injury. We believe this experimental model may be useful in studying the early stage of intraluminal polypoid fibrosis.

Key words: bleomycin — lung — pulmonary fibrosis — repeated instillations — BALB/c mice

Bleomycin is an antibiotic antitumor derived from the Streptomyces verticullus strain B-80-Z2[1] and is often used to treat a variety of tumors[2] because of its DNA-damaging effects.[3-5] However, like other chemotherapeutic agents, bleomycin also adversely affects normal cells. A major side effect of bleomycin is the development of interstitial and intra-alveolar fibrosis in the lung, which often progress into diffuse pulmonary fibrosis.[6] Many experimental models of bleomycin-induced pulmonary fibrosis have been reported, using a variety of animal species and methods of administration, including transnasal, intratracheal, intravenous, intraperitoneal, intramuscular, and subcutaneous routes.[7-13]

Evidence suggests that the degree of bleomycin damage varies among animal species and that pulmonary fibrosis is difficult to induce in BALB/c mice.[14-17] To our knowledge, however, there have been no reports concerning histological changes after its repeated instillation. In BALB/c mice, bleomycin has not been given in multiple daily doses, and, therefore, it remains whether it can induce fibrosis in the BALB/c mouse lung. For this reason, we undertook this experiment to produce pulmonary fibrosis in BALB/c mice instilling bleomycin transnasally once a day for 10 consecutive days.

MATERIALS AND METHODS

Seven male BALB/c mice (Clea Japan, Inc.), six to seven weeks old, were utilized in each study group. They received food and water *ad libitum* throughout the period of the experiment. To avoid possible complications of respiratory infection, mice from a specific pathogen-free colony were used. They were kept in a sterile condition with filter tops under isolated temperature

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control (22 to 26°C), fed sterile foods, and handled carefully with sterile gowns, masks and caps. Therefore, no spontaneous mortality was observed in any of the mice handled.

Bleomycin was instilled transnasally once a day for 10 consecutive days. Under light anesthesia with diethyl ether (Nacalai Tesque Inc.) bleomycin dissolved in sterile saline at a concentration of 0.625 mg/ml (Bleomycin ; Bleo, Nippon Kayaku Company Ltd., Tokyo, Japan) was given in a final volume of 40 μl. The dates mentioned hereafter indicate the days after the initial instillation. The mice were sacrificed on days 24 and 38. Two mice to which the same volume of saline (40 μl) had been instilled in the same manner were used as a control and were sacrificed on the same days.

For the light microscopic examination, mice were exsanguinated under dimethyl ether anesthesia. A midline ventral incision was made from the lower abdomen through the suprasternal region. The trachea was cannulated with a fine teflon tube through which 10% buffered formalin was instilled under a constant 10 cm water pressure.

Paraffin-embedded blocks were made in a standard manner. Four μm thick sections were stained with hematoxylin-eosin, Van Gieson's elastic stain, and Masson's trichrome stain.

RESULTS

On day 24, a few lymphocytic infiltrates were observed in alveolar, bronchial, and bronchiolar walls as well as adventitia of small vessels. In the alveolar walls, lymphocytes tended to accumulate in the walls near bronchi or bronchioles with a little fibroblastic proliferation. The walls of medium-and large-sized vessels were slightly edematous in places. The alveolar spaces contained polypoid fibrous tissues which were mostly separated from the alveolar walls by small air spaces but which were connected to parts of the alveolar duct walls (Fig. 1 a,b). A few foamy macrophages and lymphocytes were seen within the alveolar spaces, especially, near bronchioles and bronchi (Fig. 2). These macrophages were larger than usual and their cytoplasm was slightly vacuolated. In some areas with a little fibrosis, the alveolar spaces were shrunken with non-foamy macrophages, and lymphocytes were also scattered. The bronchiolar epithelia, particularly in their proximal portion, were cuboidal in shape and their nuclei were enlarged with increased chromatin and prominent nucleoli. These cuboidal epithelia had spread peripherally along and covered the alveolar walls. The bronchiolar and alveolar walls were slightly thickened with a few lymphocytes and fibroblasts (Fig. 3). These changes tended to be seen near proximal bronchi.

On day 38, morphological changes were essentially similar to those on day 24. However, the lesions were wider, and the cuboidal bronchiolar epithelia had become flatter than on day 24 (Fig. 4). Mild fibrosis was still observed in the alveolar spaces. The number of macrophages within the alveolar spaces and of lymphocytes in the alveolar walls were unchanged in comparison with on day 24. Lymph follicles were noticed in a few portions of pleura (Fig. 5). There were no pathological changes in the control mice, and not even slight edema was seen in the walls of small vessels.

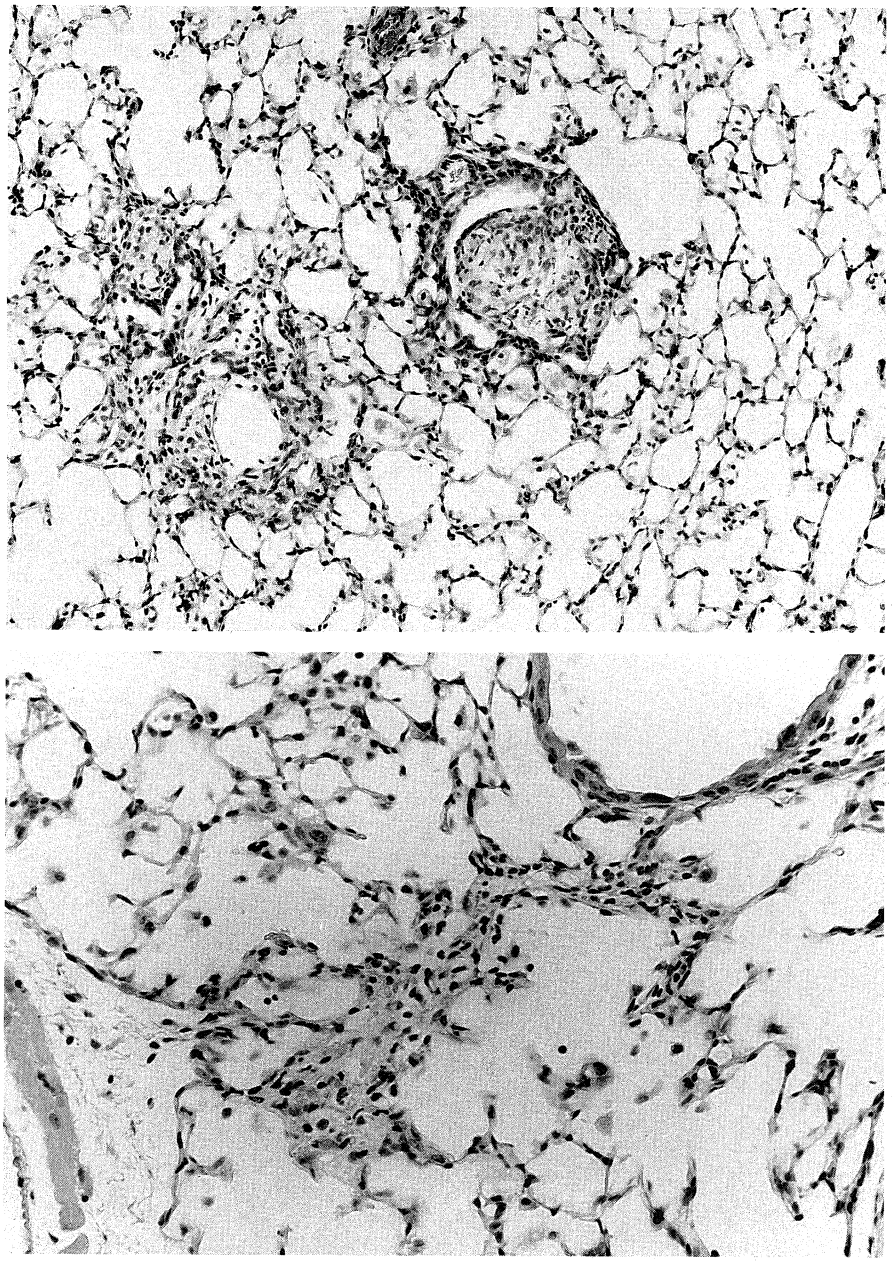

Fig. 1. On day 24, polypoid fibrous tissues are present in alveoli (a) and alveolar duct (b).

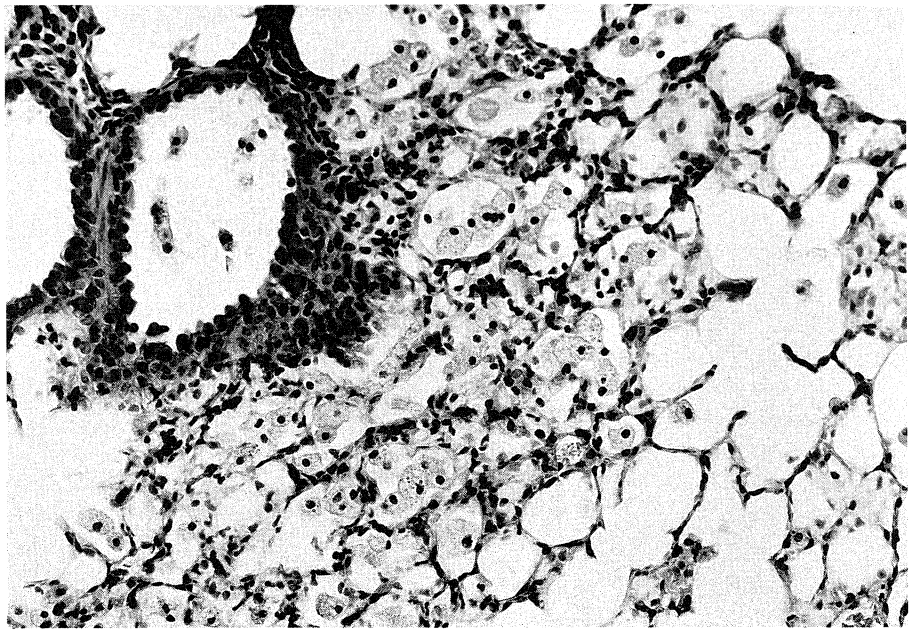

Fig. 2. Day 24. A few foamy macrophages and lymphocytes aggregate in the alveolar spaces near bronchioles.

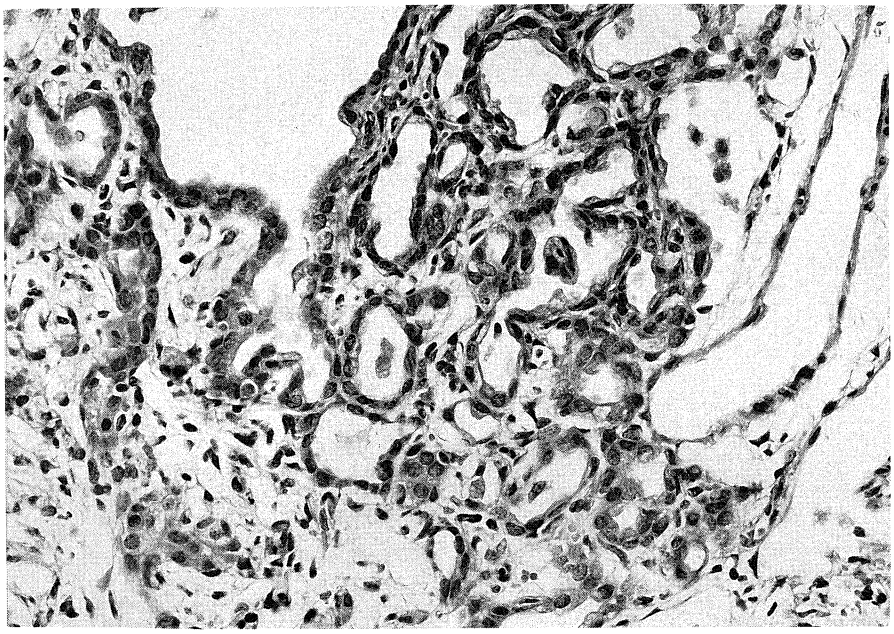

Fig. 3. On day 24, cuboidal bronchiolar epithelia with enlarged and hyperchromatic nucleoli grow, spread along and cover alveolar walls with fibrous thickening and lymphocytic infiltration.

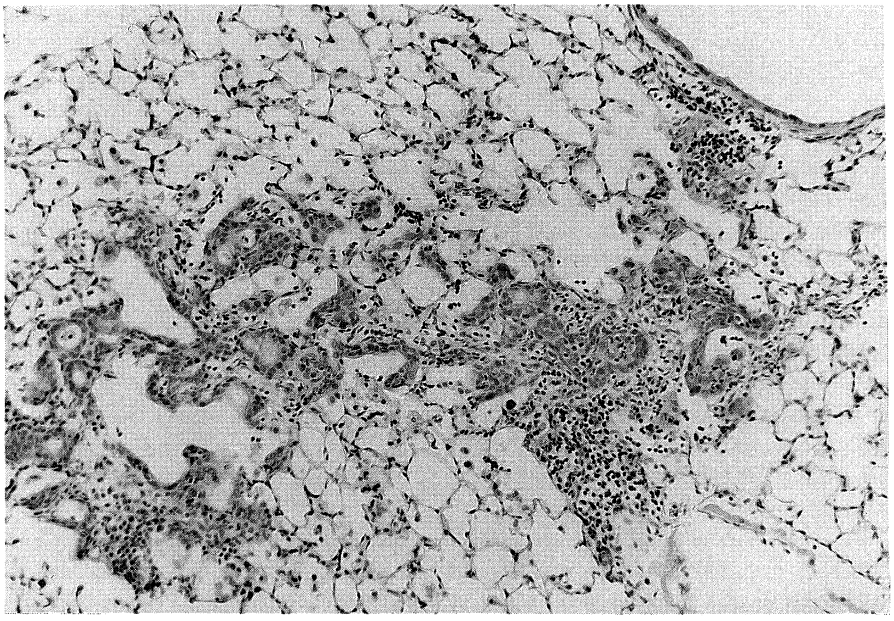

Fig. 4. On day 38, lesions are wider, and the cuboidal bronchiolar epithelia are flatter than on day 24.

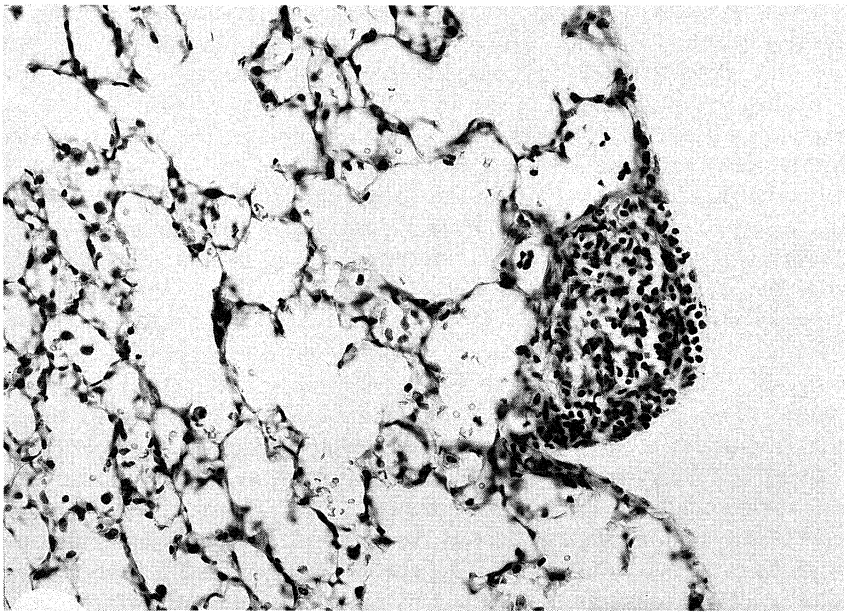

Fig. 5 Pleural change on day 38. Note the presence of a lymph follicle.

DISCUSSION

It is well known that the severity of bleomycin-induced pulmonary fibrosis varies among animal species and strains. ICR, C3H and C57BL/6 mice, for instance, sustain severe pulmonary fibrosis, but Swiss, DAB/2 and BALB/c mice do not.[14-17] In fact, in our preliminary experiment with BALB/c mice, single transnasal administration of bleomycin did not induce fibrosis (unpublished data).

Generally, fibrosis is an end result of tissue injury and chronic inflammation. In certain situations, a single insult may cause fibrosis, while repeated tissue damage is necessary to cause fibrosis in other circumstances. The reason why we repeatedly administered bleomycin to BALB/c mice in this experiment is because these mice are known to be resistant to bleomycin, and seldom develop bleomycin-induced pulmonary fibrosis. To date most experiments have involved single administration of bleomycin through the respiratory tract (either transnasally or intratracheally). Therefore, to see whether bleomycin could induce pulmonary fibrosis in BALB/c mice, we decided to instill bleomycin repeatedly for 10 days. If indeed it could induce fibrosis we were also interested in seeing what types of fibrosis would appear in the mice after administration of the drug.

In our experiment, we successfully showed that bleomycin can induce fibrosis in the BALB/c mouse lung, although the fibrosis was rather mild. In addition, one of the most prominent morphological changes seen in the present experiment was alveolar bronchiolization. Cuboidal bronchiolar epithelia which had apparently descended from proximal bronchioles, covered the alveolar walls, particularly in the centriacinar zone. The features of alveolar bronchiolization have been already observed in previous reports of bleomycin-induced pulmonary fibrosis. Generally, alveolar bronchiolization is recognized as a tissue reaction in which the injured alveolar epithelium is replaced by descending regenerative bronchiolar epithelium. In other words, the presence of alveolar bronchiolization indicates that the bronchiolar and alveolar epithelia have been damaged. The intraluminal polypoid fibrosis seen on day 24 also suggests the past presence of epithelial damage, particularly damage to the bronchiolar and alveolar duct epithelia.

Manabe *et al.*[18] postulated on the pathogenetic mechanism of intraluminal fibrosis. They considered alveolar duct damage to be a prerequisite for the development of this type of fibrosis. Exudates from alveolar duct wall may be incorporated as polypoid fibrous tissues by organization. Why, then, doesn't this fibrosis progress or at least persist in BALB/c mice? At present, we do not have an answer to this question except to observe that BALB/c mice are resistant to bleomycin. Several hypotheses have been proposed for this resistance. The epithelium of BALB/c mice may be resistant to bleomycin because it contains a large amount of bleomycin-hydrolase as Umezawa *et al.* and others[17,19,20] suggested. Fibroblastic proliferation in this mouse strain is limited in comparison with other strains. An abundance of antioxidant enzyme[21] and immunological factors such as T lymphocytes, macrophages, neutrophils, cytokines, and other unknown factors,[22-36] and a reduced capacity to repair the DNA damage[37] produced by bleomycin may all participate in this process. Finally, instead of epithelial cells, endothelial cells may play a more

important role in the development of fibrosis. Then, alveolar duct damage may be a necessary but not sufficient condition for the development of intraluminal fibrosis. A great deal of investigation must be done to elucidate the fibrosing mechanism in bleomycin fibrosis.

Manabe *et al.*[18] divided the morphology of intraluminal fibrosis (intraalveolar fibrosis) in humans into four patterns; intraluminal diffuse fibrosis of complete type, intraluminal diffuse fibrosis of incomplete type, intraluminal polypoid fibrosis and membranous fibrosis. In the present study, the main type of fibrosis was intraluminal polypoid fibrosis. The fibrosing mechanism of intraluminal polypoid fibrosis proposed by Manabe *et al.* is still an assumption based on the reconstruction of histological findings obtained from human materials. It must be substantiated, of course, by animal experiments. In this regard, the slow and mild course of the fibrosing process in our mice may provide a good animal model for study of intraluminal polypoid bibrosis.

In any case, in the present experiment, we have shown, that pulmonary fibrosis can be induced in BALB/c mice by repeated instillation of bleomycin, although the fibrosis was mild. Bleomycin seems to damage bronchiolar and alveolar epithelia primarily. Regeneration of epithelia is brisk and is pursued mainly by bronchiolar epithelium. This experimental model may be useful in studying the early stage of intraluminal polypoid fibrosis.

REFERENCES

1) Umezawa, H., and Maeda, K., Takeuchi, T. and Okami, Y.: New antibiotics, bleomycin A and B. J. Antibiot. **19**: 200-209, 1966
2) Blum, R.H., Carter, S.K. and Agre, K.: A clinical review of bleomycin. A new antineoplastic agent. Cancer **31**: 903-914, 1973
3) Sausville, E.A., Stein, R.W., Peisach, J. and Horwitz, S.B.: Properties and products of the degradation of DNA by bleomycin and iron (II). Biochemistry **17**: 2746-2754, 1978
4) Oberley, L.W. and Buettner, G.R.: The production of hydroxyl radical by bleomycin and iron (II). FEBS Lett. **97**: 47-49, 1979
5) Hecht, S.M.: DNA strand scission by activated bleomycin group antibiotics. Fed. Proc. **45**: 2784-2791, 1986
6) Ishizuka, M., Takayama, H., Takeuchi, T. and Umezawa, H.: Activity and toxity of bleomycin. J. Antibiot. **20**: 15-24, 1967
7) Fleischman, R.W., Baker, J.R. and Thompson, G.R., Schaeppi, U.H., Illievski, V.R., Cooney, D.A. and Davis, R.D.: Bleomycin-induced interstitial pneumonia in dogs. Thorax **26**: 675-682, 1971
8) Adamson, I.Y.R. and Bowden, D.H.: The pathogenesis of bleomycin-induced pulmonary fibrosis in mice. Am. J. Pathol. **77**: 185-198, 1974
9) Aso, Y., Yoneda, K. and Kikkawa, Y.: Morphologic and biochemical study of pulmonary changes induced by bleomycin in mice. Lab. Invest. **35**: 558-568, 1976
10) Snider, G.L., Gelli, B.R., Goldstein, R.H., O'Brien, J.J. and Lucey, E.C.: Chronic interstitial pulmonary fibrosis produced in hamsters by endotracheal bleomycin. Am. Rev. Respir. Dis. **117**: 289-297, 1978
11) Phan, S.H., Thrall, R.S. and Ward, P.A.: Bleomycin-induced pulmonary fibrosis in rats: Biochemical demonstration of increased rate of collagen synthesis. Am. Rev. Respir. Dis. **121**: 501-506, 1980.
12) Clark, J.G., Kostal, K.M. and Marino, B.A.: Bleomycin-induced pulmonary fibrosis in hamsters. J. Clin. Invest. **72**: 2082-2091, 1983
13) McCullough, B., Collins, J.F., Johanson, W.G. and Grover, F.L.: Bleomycin-induced diffuse interstitial pulmonary fibrosis in baboons. J. Clin. Invest. **61**: 79-88, 1978
14) Schrier, D.J., Kunkel, R.G. and Phan, S.H.: The role of strain variation in murine bleomycin-induced pulmonary fibrosis. Am. Rev. Respir. Dis. **127**: 63-66, 1983

15) Ekimoto, H., Takanashi, K., Matsuda, A. and Umezawa, H.: Animal model on BLM-induced pulmonary fibrosis cmparison of the systemic (intraperitoneal) administration with the local (intratracheal) instillation. Jpn. J. Cancer Chemother. **10**: 2550-2557, 1983 (in Japanese)
16) Harrison, J.H. and Lazo, J.S.: High dose continuous infusion of bleomycin in mice: A new model for drug-induced pulmonary fibrosis. J. Pharmacol. Exp. Ther. **243**: 1185-1194. 1987
17) Filderman, A.E. and Lazo, J.S.: Murine strain differences in pulmonary bleomycin metabolism. Biochem. Pharmacol. **42**: 195-198, 1991
18) Manabe, T., Moriya, T. and Sugihara, K.: Intraluminal (intraalveolar) diffuse fibrosis of the lung. Kawasaki. Med. J. **14**: 159-169, 1988
19) Umezawa, H., Takeuchi, T., Hori, S., Sawa, T., Ishizuka, M., Ichikawa, T. and Komai, T.: Studies on the mechanism of antitumor effect of bleomycin on squamous cell carcinoma. J. Antibiot. **25**: 409-420, 1972
20) Lazo, J.S. and Humphreys, C.J.: Lack of metabolism as the biochemical basis of bleomycin-induced pulmonary toxiciy. Proc. Natl. Acad. Sci. **80**: 3064-3068, 1983
21) Bryan, C.L. and Jenkinson, S.G.: Species variation in lung antioxidant enzyme activities. J. Appl. Physiol. **63**: 597-602, 1987
22) Schrier, D.J., Phan, S.H. and McGarry, B.M.: The effects of the nude (nu/nu) mutation on bleomycin-induced pulmonary fibrosis. A biochemical evaluation. Am. Rev. Respir. Dis. **127**: 614-617, 1983
23) Kaelin, R.M., Center, D.M., Bernardo, J., Grant, M. and Snider, G.L.: The role of macrophage-derived chemoattractant activities in the early inflammatory events of bleomycin-induced pulmonary injury. Am. Rev. Respir. Dis. **128**: 132-137, 1983
24) Thrall, R.S., Phan, S.H., McCormick, J.R. and Ward, P.A.: The development of bleomycin-induced pulmonary fibrosis in neutrophil-depleted and complement-depleted rats. Am. J. Pathol. **105**: 76-81, 1981
25) Thrall, R.S., McCormick, J.R., Jack, R.M., Phan, S.H., and Ward, P.A.: The effect of antilymphocyte globulin on the development of bleomycin-induced pulmonary fibrosis in the rat. Am. Rev. Respir. Dis. **119**: 83, 1979
26) Phan, S.H., Thrall, R.S. and Williams, C.: Bleomycin-induced pulmonary fibrosis. Effects of steroid lung collagen metabolism. Am. Rev. Respir. Dis. **124**: 428-434, 1981
27) Thrall, R.S., McCormic, J.R., Jack, R.M., McReynolds R.A. and Ward, P.A.: Bleomycin-induced pulmonary fibrosis in the rat. Am. J. Pathol. **95**; 117-130, 1979
28) Schrier, D.J. and Phan, S.H.: Modulation of bleomycin-induced pulmonary fibrosis in the BALB/c mouse by cyclophosphamide-sensitive T cells. Am. J. Pathol. **116**: 270-278, 1984
29) Rossi, G.A., Szapiel., S, Ferrans, V.J. and Crystal, R.G.: Susceptibility to experimental interstitial lung disease is modified by immune- and non-immune-related genes. Am. Rev. Respir. Dis. **135**: 448-455 1987
30) Piguet, P.F., Collart, M.A., Grau, G.E., Kapanci, Y. and Vassalli, P.: Tumor necrosis factor cachectin plays a key role in bleomycin-induced pneumopathy and fibrosis. J. Exp. Med. **170**: 655-663, 1989
31) Postlethwaite, A.E., Snyderman, R. and Kang, A.H.: The chemotactic attraction of human fibroblasts to a lymphocyte derived factor. J. Exp. Med. **141**: 1188-1203, 1976
32) Thrall, R.S., Barton, R.W., D'Amato, D.A. and Sulavik, S.B.: Differential cellular analysis of bronchoalveolar lavage fluid obtained at various stage during the development. Am. Rev. Respir. Dis. **126**: 488-492, 1982
33) Takahashi, K., Sasaki, H., Ito, E., Ikeda, H. and Sato, S.: Inhibitory effects of elastase on fibrous tissue formation in hamster lung treated with bleomycin. Jpn. J. Med. **21**: 318, 1982
34) Kovacs, E.J., and Kelley, J.: Secretion of macrophage-derived growth factor during acute lung injury induced by bleomycin, J. Leukoc. Biol. **37**: 1-14, 1985
35) Khalil, N., Breznay, O., Sporn, M. and Greenberg, A.H.: Macrophage production of transforming growth factor B and fibroblast collagen synthesis in chronic pulomnary inflammation. J. Exp. Med. **170**: 727-737, 1989
36) Hoyt, D.G. and Lazo, J.S.: Alterations in pulmonary mRNA encoding procollagens, fibronectin and transforming growth factor-B precede bleomycin-induced pulmonary fibrosis in mice. J. Pharmacol. Exp. Ther. **246**: 765-771,1988
37) Harrison, J.H., Hoyt, D.J. and Lazo, J.S.: Acute pulmonary toxicity of bleomycin: DNA scission and matrix protein mRNA levels in bleomycin-sensitive and -resistant strain in mice. Mol. Pharmacol. **36**: 231-238, 1989