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CHANGES IN SUBPOPULATION OF BRONCHOALVEOLAR LAVAGE FLUID IN THE PULMONARY FIBROSIS INDUCED BY BLEOMYCIN OR PEPLOMYCIN

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ABSTRACT: *Present studies were carried out in order to estabilish the bronchoalveolar lavage method and to examine the response of bleomycin and peplomycin on the total cell number and the subpopulations of bronchoalveolar lavage fluid. A total of 24 male F_{344} rats, weighing 300~350 mg, were divided into 3 groups. Animals received either bleomycin (BLM; 0.75 mg/0.2 ml/rat), peplomycin (PLM; 0.25 mg/0.2 ml/rat) for groups 2 and 3 or an equal volume of sterile saline lacking drugs for controls (group 1). Animals were sequentially killed 1, 3, 5, and 7 days after bleomycin, peplomycin or saline treatment. Bronchoalveolar cells were collected by bronchoalveolar lavage and were determined the total number and differential cell counts of lavage cells after cytocentrifugation. Cell viability of peritoneal exudate macrophages was tested. The differential cell counts revealed that the predominant cell type in bronchoalveolar lavage of BLM- or PLM-treated rats was neutrophils (83%) and followed by macrophages in the initial stage. However, macrophages were the predominant cell type and followed by neutrophils and lymphocytes in the final stage at day 7. Acute inflammatory phase was characterized by the predominance of small- and medium-sized alveolar macrophages. In subacute inflammatory phase, however, the cellular size of the alveolar macrophage population and the cytoplasmic vacuoles were increased. Peplomycin caused mildly to reduce the viability of peritoneal exudate macrophages dose- and time- dependently like a bleomycin. Changes in the cell subpopulation composition could have dramatic effects on the inflammatory and*

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fibrotic responses of the lung. Thereafter bleomycin and peplomycin may act as a modulator in the lung inflammatory reaction.

Key words: *Bleomycin, Peplomycin, Bronchoalveolar Lavage Fluid(BALF), Alveolar Macrophage, Pulmonary Fibrosis.*

INTRODUCTION

Fibrotic processes are generally associated with inflammatory responses. Factors that contribute to the control and modulation of the pulmonary fibrosis are poorly understood, however, and the relationship between inflammatory responses and subsequent fibrogenesis is not clear. Nonetheless recent studies have demonstrated that cells of the inflammatory/immune system are under the regulation of alveolar macrophages (AM) (Bittermanm *et al.*, 1983; Hunninghake, *et al.*, 1980; Kaelin, *et al.*, 1983).

Alveolar macrophages are the major mononuclear phagocyte system of the lung, the primary defense against airborne particles, and exhibit a variety of functions, including modulation of pulmonary inflammation and fibrosis (Hocking, *et al.*, 1979a, b). The number of AM in the lung is increased after treatment of lung inflammatory agents in many inflammatory lung disorders (Daniele, *et al.*, 1980; Hunninghake, *et al.*, 1981; Weinberger, *et al.*, 1980). Moreover, using bleomycin-induced pulmonary fibrosis (Snider, , *et al.*, 1978a, b) as a model for human interstitial fibrosis, previous studies have shown alterations in the numbers and functions of AM (Kaelin, *et al.*, 1983; Kovacs, *et al.*, 1985; Thrall, *et al.*, 1982).

Recent data indicate that AM represent a heterogeneous population of cells that can be separated into a number of discrete subpopulations that differ biochemically, morphologically, and immunologically (Chandler, *et al.*, 1986a, b; Chandler and Fulmer, 1987; Everson and Chandler, 1992).

It remains unclear as to whether monocytes or monocyte-derived inflammatory macrophages are profibrogenic (Crystal, *et al.*, 1984) and involved in the remodeling of tissue during acute and chronic inflammatory states in the lung. Recent studies have focused on the role of alveolar macrophages (AM) to act as modulators of events associated with pulmonary fibrosis.

The purpose of these studies was carried out to establish the bronchoalveolar lavage method and to examine the respose of bleomycin and peplomycin on the subpopulations of bronchoalveolar lavage fluid.

MATERIALS AND METHODS

Animals and Chemicals

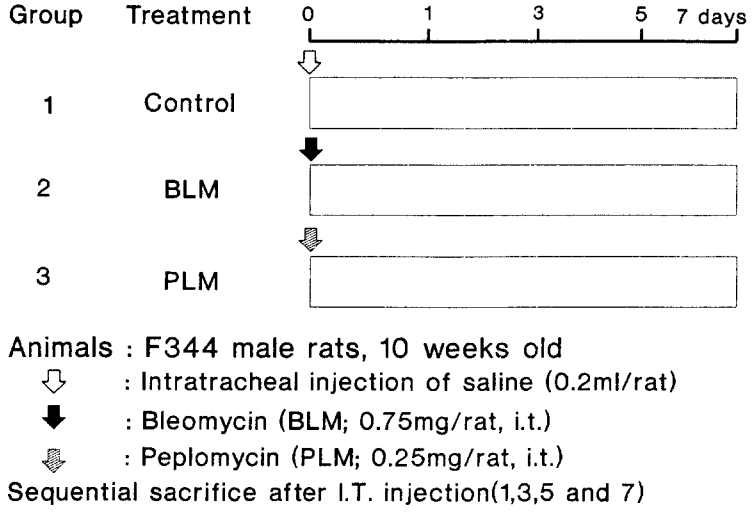
A total of 24 male F_{344} rats, weighing 300~350 gm, were supplied from National Institute of Safety Research, Seoul, Korea and were housed in polycarbonate cages with hard wood chips in an air-conditioned room (23± 2℃, 55± 10% R.H.) with a 12 h light/12 h dark cycle. Diet (Jeil Sugar Co., Seoul, Korea) and drinking

water were available *ad libitum*. All animals were fasted for 24 hours prior to sacrifice.

Bleomycin hydrochloride (BLM, CAS No. 9041-93-4) and peplomycin sulfate (PLM) were obtained from Nippon Kagaku Pharmaceutical Co. Ltd., Japan. BLM or PLM were dissolved in strile 0.9% sodium chloride solution for intratracheal injection.

Treatments

A total of 24 male F_{344} rats were divided into 3 groups. Under pentobarbital anesthesia, animals received intraperitoneally either BLM (0.75 mg/0.2 m*l* in saline/rat), PLM (0.25 mg/ 0.2 m*l* in saline/rat) for groups of 2 and 3 or an equal volume of sterile saline lacking drugs for control (group 1) by established techniques (Kim and Lee, 1988). Animals were killed 1, 3, 5, and 7 days after saline, bleomycin, or peplomycin treatment (Text-Figure 1).



Text-Figure 1. Experimental design.

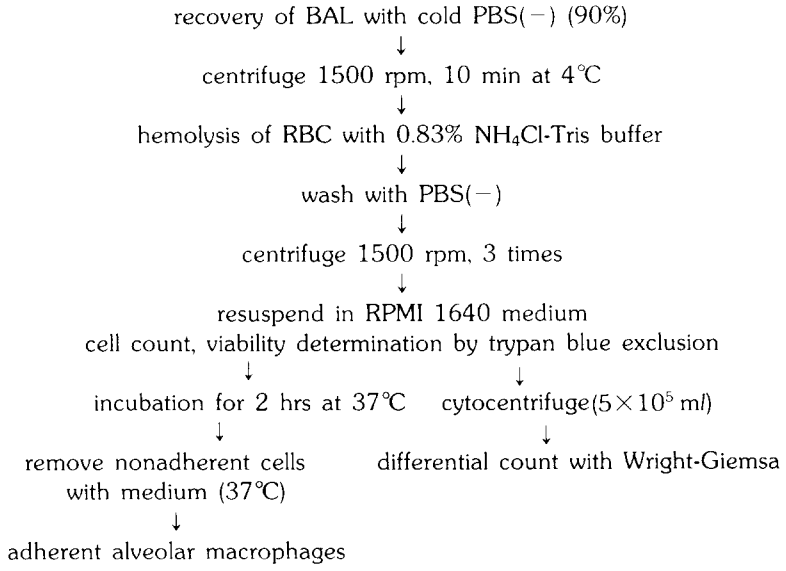
Culture Media and Reagents

RPMI 1640 complete medium with L-glutamine (R-6504, Sigma Co., U.S.A.) was prepared from powder mixture and supplemented with Penicillin/Streptomycin (P-0904, 5000 IU, 20 m*l*/L; Sigma Co., U.S.A.). Fetal calf serum (FCS; Gibco Lab., U.S.A.) was inactivated by heating at 56.5℃ for 60 minutes and added to 10% concentration. Phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS(−); 1000-3, pH 7.4, Sigma Co., U.S.A.) was used. Thioglycollate medium (T-9032, Sigma Co., U.S.A.) were prepared at 3% concentration for harvest of peritoneal exudate cells.

Isolation of Alveolar Macrophages

Bronchoalveolar cells were collected by bronchoalveolar lavage (BAL) as previously described (Chandler, *et al.*, 1986a, b; Chandler and Fulmer, 1987) Briefly,

under ether anesthesia animals were killed by exsanguination. The trachea was cannulated with polypropylene tubing and lungs were lavaged in situ with 10 ml×twice aliquots of PBS(−). The nucleated cell yield was determined on an aliquot of lavage fluid, diluted in 0.83% ammonium chloride-Tris buffer for red cell lysis, with a modified Neubauer hemocytometer. Cell viability was assessed by trypan blue dye exclusion (Text-Figure 2).



Text-Figure 2. Isolation on alveolar macrophages from bronchoalveolar lavage.

Analysis of Bronchoalveolar Cells

Values of total cell counts in lavage fluid were corrected for the corresponding fluid recovery, and all values were calculated as cell counts per lavage fluid recovered. Cytospin prepartions (Shandon Cytospin III, Shandon Instr., U.K.) were also prepared from the cell suspensions (1×10^5 cells/ml) for 10 minutes at 2,000 rpm. Differential cell counts of lavage cells were determined on approximately 500 cells from Wright-Giemsa stained Cytospin preparations.

Isolation of Peritoneal Exudate Macrophages

Peritoneal exudate cells were obtained by recovery cells using PBS(−) at 4℃/ after 4 days intraperitoneal treatment of 3% thioglycollate (5ml). Lavage fluid was pooled and centrifuged for 5 minutes at 1500 rpm, the supernatant was discarded, and cells were resuspended in RPMI 1640 containing 10% FCS and antibiotics. After 2 hours at 37℃, the nonadherent cells are removed and adherent cells in the petri dishes (35 mm in diameter) with cover glass washed twice with RPMI 1640 medium (37℃). There adherent cells were used as a peritoneal exudate macrophages (PEM) (Text-Figure 3).

3% thioglycollate (i.p.)
↓ after 4 days
peritoneal exudate cells
↓ 2 hrs incubation
peritoneal macrophages
↓
cell viability

Text-Figure 3. Isolation of macrophages from peritoneal exudate.

Cell Viability of Peritoneal Exudate Macrophages

The remaining PEM cell suspension was washed in 0.83% ammonium chloride then in PBS(−) (pH 7.4) before being resuspended in PBS(−) to a cell count of 1×10^6 PEM/m*l*. The PEM cells were further cultured with the RPMI 1640 complete medium with FCS. Two m*l* of the suspension was placed onto 35 mm plastic petri dishes (Corning Co., U.S.A.) with cover glass. The suspension were divided into 4 groups: control (PBS(−)), PLM (low dose, 1×10^3 mg/m*l*), PLM (medium dose, 1×10^{-2} mg/m*l*) and PLM (high dose, 1×10^{-1} mg/m*l*). The coverslips were washed three times with PBS(−), air dried, fixed and stained by Wright-Giemsa solution of days 1, 2, 3 and 4 days after exposure of PLM or PBS(−).

RESULTS

Effects of bleomycin or peplomycin treatment on cell subpopulation in bronchoalveolar lavage

The total number of cells and the cell subpopulation present in bronchoalveolar lavage fluid from rats dosed with bleomycin or peplomycin were shown in Table

Table 1. Cell subpopulation present in Bronchoalveolar Lavage Fluid from Rats Dosed with Bleomycin and Peplomycin.

Group	Cell type	Days after BLM or PLM administration			
		Day 1	Day 3	Day 5	Day 7
PBS(−)	Total No.	1.3[a]	1.3	1.3	1.2
BLM alone	Total No.	2.6	1.8	1.6	1.2
	Alv. Mφ	17[b]	27	75	65
	Neutrophil	83	71	23	24
	Lymphocyte	0	0	2	9
	Eosinophil	0	2	0	2
PLM alone	Total No.	1.7	1.8	1.3	1.2
	Alv. Mφ	13	56	65	59
	Neutrophil	83	44	18	23
	Lymphocyte	4	0	15	16
	Eosinophil	0	0	2	2

[a] Total number of cells(1×10^6) in BAL were measured using a hemocytometer.
[b] Differential cell counts(%) in BAL were done. BLM or PLM represent intracheally administration of bleomycin (0.75 mg/ rat) or peplomycin (0.25 mg/ rat).
The majority of subpopulation in BAL of control rats dosed with PBS( ) were alveolar macrophages (>95%) followed by neutrophils (<4%) and lymphocytes (<1%).

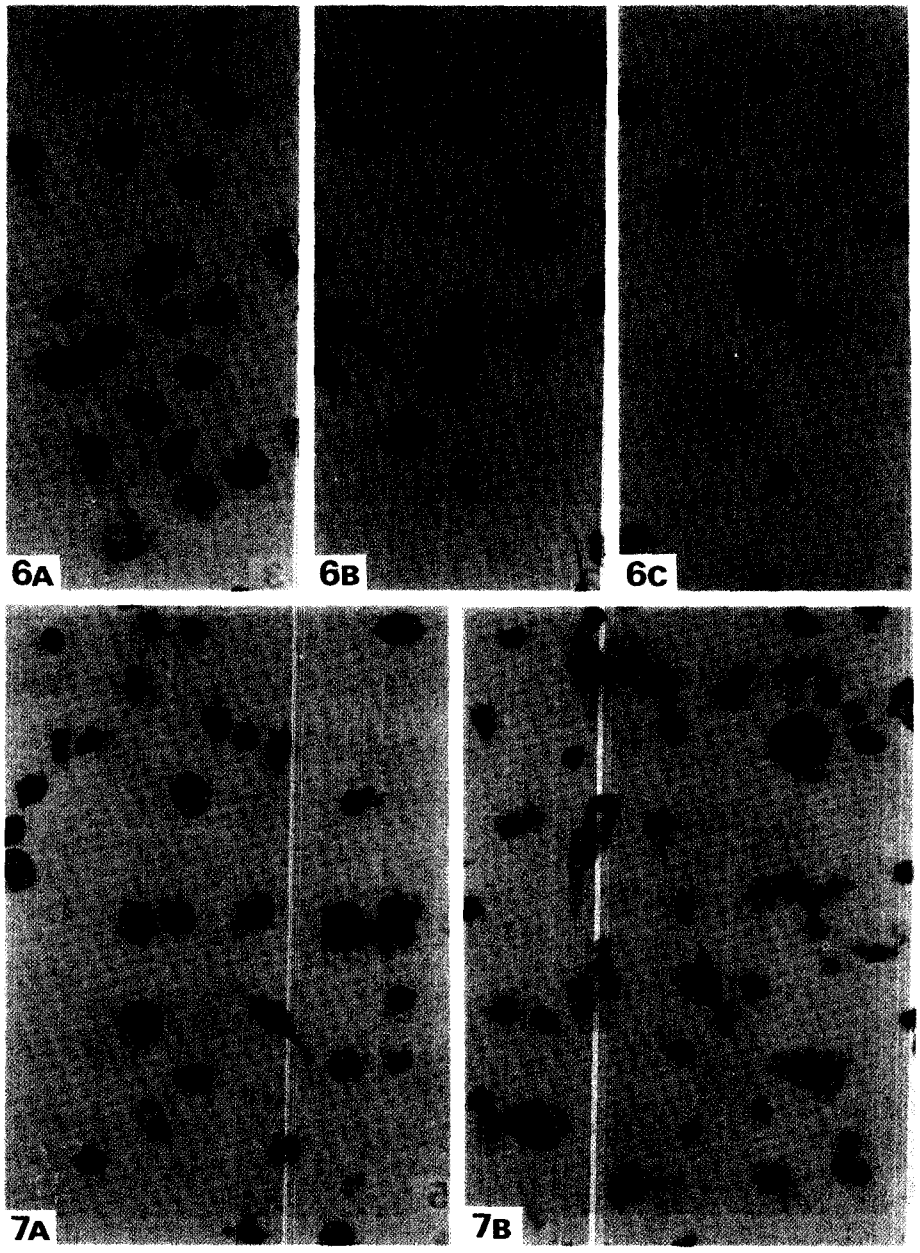


Figure 6. Cell viability of peritoneal exudate macrophages exposed to peplomycin in a time-course response. The macrophages of control group (PBS (−)) showing increased cell viability [A], while those cells exposed to peplomycin at high dose ($\times 10^{-1}$ mg/ml) for 24 hrs[B⟩, and at high dose (1×10^{-1} mg/ml) for 96 hrs[C], showing decreased cell viability in a time-depedent manner. Wright-Giemsa. ×400.

Figure 7. Cell viability of peritoneal exudate macrophages exposed to peplomycin in a dose-course response. The macrophages exposed to peplomycin at low dose (1×10^{-3} mg/ml) for 72 hrs [A], and at high dose (1×10^{-1} mg/ml) for 72 hrs [B]. Showing decreased cell viability in a dose-dependent manner. Wright-Giemsa. ×400.

dramatic effects on the inflammatory and fibrotic responses of the lungs.

Several studies have shown that alveolar macrophages produce factors that suppress fibroblast growth and collagen production by proliferating lung fibroblasts (Clark, *et al.*, 1983; Elias, *et al.*, 1985). In more recent studies, alveolar macrophages have been demonstrated to synthesize factors that stimulate collgen production by lung fibroblasts (Kovacs and Kelley, 1985; Clark and Greenberg, 1987). These diverse capacities of alveolar macrophages suggest that they are composed of discrete subpopulations of cells having different functions. Recent findings have shown that the alveolar macrophage population is composed of several subpopulations that differ biochemically, morphologically, and immunologically (Kovacs and Kelley, 1985; Thrall, *et al.*, 1982; Chandler, *et al.*, 1986a, b; Chandler and Fulmer, 1987; Everson and Chandler, 1992). Changes in the alveolar macrophage subpopulation composition and function could have dramatic effects on the inflammatory and fibrotic responses of the lung. Everson and Chandler (1992) suggested that composition and function of the alveolar macrophage subpopulation are altered during the development of bleomycin-induced fibrosis.

The present study reports the changes in the bronchoalveolar lavage cell population in F_{344} rats after bleomycin or peplomycin administration lung damage which was followed by a rapid influx of neutrophils and lymphocytes into the lung. While the presence of neutrophils in the lung is transient, alveolar macrophages are increased in the lung during the fibrotic process. Lymphocytes are gradually increased in the late stage. Our findings are similar to those reported by Thrall *et al.* (1982) and Giri *et al.* (1986) in that neutrophil was the first cell type to appear and constituted the major cell type in bronchoalveolar lavage of bleomycin-treated rats or hamsters. Our findings, however, are at variance with respect to the second most common cell type in bronchoalveolar lavage of bleomycin-treated animals. Thrall *et al.* (1982) and Kim and Lee (1992) reported lymphocytes second, whereas we found that monocytes were the second cell type in the both of treatment groups.

In the present study, bleomycin- or peplomycin- treated animals had peak influx of inflammatory cells in bronchoalveolar lavage at 1 or 3 days after treatment, when the total cell count was approximately two- or one and half-folds over control, respectively. Thereafter, the total cell numbers in bronchoalveolar lavage of bleomycin- or peplomycin- treated rats decreased. The dynamics of this marked reduction in total number of inflammatory cells are not known. An increase in the amount of non-cellular protein in the bronchoalveolar lavage of bleomycin-treated animals provides an index of increased pulmonary vascular permeability (Thrall, *et al.*, 1982; Giri, *et al.*, 1981, 1986). It is well documented that bleomycin is toxic to pulmonary endothelial cells administratered intratracheally (Catravas, *et al.*, 1983; Bae *et al.*, 1988).

Peplomycin caused mildly to reduce the viability of peritoneal exudate macrophages dose- and time- dependently like a bleomycin (Kim and Lee, 1992).

CONCLUSION

The differential cell counts revealed that the predominant cell type in bronchoalveolar lavage of BLM- or PLM-treated rats was neutrophils (83%) and followed

by macrophages in the initial stage. However, macrophages were the predominant cell type and followed by neutrophils and lymphocytes in the final stage at day 7. Acute inflammatory phase was characterized by the predominance of small- and medium-sized alveolar macrophages. In subacute inflammatory phase, however, the cellular size of the alveolar macrophage population and the cytoplasmic vacuoles was increased. Peplomycin caused mildly to reduce the viability of peritoneal exudate macrophages dose- and time- dependently like a bleomycin.

Changes in the cell subpopulation composition could have dramatic effects on the inflammatory and fibrotic responses of the lung. Thereafter bleomycin and peplomycin may act as a modulator in the lung inflammatory reaction.

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