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

Dae Joong Kim,1 Beom Seok Han, Byeongwoo Ahn, Kwang Sik Choi,
Jong Koo Kang* and Joon Sup Lee**

Department of Pathology, National Institute of Safety Research, Seoul 122-020
*Department of Veterinary Histology, College of Veterinary Medicine,
Chungbuk National University, Cheongju 360-763
**Department of Veterinary Histology, College of Veterinary Medicine,
Seoul National University, Suwon 440-744

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ABSTRACT: Present studies were carried out in order to
establish the bronchoalveolar lavage method and to exa-
mine the response of bleomycin and peplomycin on the total
cell number and the subpopulations of bronchoalveolar la-
vage fluid. A total of 24 male F344 rats, weighing 300~350
mg, were divided into 3 groups. Animals received either bleo-
mycin (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 ste-
rise saline lacking drugs for controls (group 1). Animals were
sequentially killed 1, 3, 5, and 7 days after bleomycin, pepl-
omycin or saline treatment. Bronchoalveolar cells were col-
ceted by bronchoalveolar lavage and were determined the total
number and differential cell counts of lavage cells after cyto-
centrifugation. 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 ma-
crophages 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 inflamma-
tory phase, however, the cellular size of the alveolar macro-
phage population and the cytoplasmic vacuoles were increa-
sed. Peplomycin caused mildly to reduce the viability of peri-
toneal exudate macrophages dose- and time- dependently
like a bleomycin. Changes in the cell subpopulation composi-
tion could have dramatic effects on the inflammatory and

1Correspondence should be addressed
fibrotic responses of the lung. Thereafter bleomycin and peplo- 
mycin may act as a modulator in the lung inflammatory 
reaction.

**Key words:** Bleomycin, Peplomycin, Bronchoalveolar La- 
vage 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 demonstra-
ted that cells of the inflammatory/immune system are under the regulation of 
alveolar macrophages (AM) (Bittermann et al., 1983; Hunninghake, et al., 1980; 

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 

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 remod-
elling 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 modula-
tors of events associated with pulmonary fibrosis.

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

**MATERIALS AND METHODS**

**Animals and Chemicals**

A total of 24 male F344 rats, weighing 300~350 gm, were supplied from Na-

tional Institute of Safety Research, Seoul, Korea and were housed in polycarbonate 
cages with hard wood chips in an air-conditioned room (23±2°C, 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 sterile 0.9% sodium chloride solution for intratracheal injection.

**Treatments**

A total of 24 male F344 rats were divided into 3 groups. Under pentobarbital anesthesia, animals received intraperitoneally either BLM (0.75 mg/0.2 ml in saline/rat), PLM (0.25 mg/0.2 ml 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).

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>0</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>BLM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>PLM</td>
<td></td>
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</tbody>
</table>

Animals: F344 male rats, 10 weeks old

- Intratracheal injection of saline (0.2ml/rat)
- Bleomycin (BLM; 0.75mg/rat, i.t.)
- Pepleomycin (PLM; 0.25mg/rat, i.t.)

Sequential sacrifice after i.t. injection (1, 3, 5 and 7)

**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 mIL; Sigma Co., U.S.A.). Fetal calf serum (FCS; Gibco Lab., U.S.A.) was inactivated by heating at 56.5°C 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).

recovery of BAL with cold PBS(-) (90%)
↓
centrifuge 1500 rpm, 10 min at 4°C
↓
hemolysis of RBC with 0.83% NH₄Cl-Tris buffer
↓
wash with PBS(-)
↓
centrifuge 1500 rpm, 3 times
↓
resuspend in RPMI 1640 medium
↓
cell count, viability determination by trypan blue exclusion
↓
incubation for 2 hrs at 37°C
cytocentrifuge(5 × 10⁵ ml)
↓
remove nonadherent cells
differential count with Wright-Giemsa
↓
adherent alveolar macrophages

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 preparations (Shandon Cytospin III, Shandon Instr., U.K.) were also prepared from the cell suspensions (1 × 10⁵ 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°C/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°C, 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°C). 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 $1 \times 10^6$ PEM/ml. The PEM cells were further cultured with the RPMI 1640 complete medium with FCS. Two ml 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, $1 \times 10^3$ mg/ml), PLM (medium dose, $1 \times 10^2$ mg/ml) and PLM (high dose, $1 \times 10^1$ mg/ml). 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 1.

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell type</th>
<th>Days after BLM or PLM administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>PBS(−)</td>
<td>Total No.</td>
<td>1.3 a</td>
</tr>
<tr>
<td>BLM alone</td>
<td>Total No.</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>Alv. Mφ</td>
<td>17 b</td>
</tr>
<tr>
<td></td>
<td>Neutrophil</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Lymphocyte</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Eosinophil</td>
<td>0</td>
</tr>
<tr>
<td>PLM alone</td>
<td>Total No.</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>Alv. Mφ</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Neutrophil</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Lymphocyte</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Eosinophil</td>
<td>0</td>
</tr>
</tbody>
</table>

a Total number of cells ($1 \times 10^6$) 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 peptomycin in a time-course response. The macrophages of control group (PBS (-)) showing increased cell viability [A], while those cells exposed to peptomycin at high dose (×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-dependent manner. Wright-Giemsa. ×400.

Figure 7. Cell viability of peritoneal exudate macrophages exposed to peptomycin in a dose-course response. The macrophages exposed to peptomycin at low dose (1×10\(^{-1}\) 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 collagen 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 F344 rats after bleomycin or plomycin 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 plomycin- 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 plomycin- 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 administrated 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.

REFERENCES


