Kinetics of Intraparenchymal Mononuclear Cells in A Murine Model of Pulmonary Fibrosis Induced by Bleomycin

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Bleomycin (BLM), an anti-tumor drug, has been observed to cause interstitial pneumonia followed by subsequent fibrosis. In order to elucidate the cellular mechanism in the fibrotic process, we examined inflammatory cells in the lungs of mice after intratracheal administration of BLM. Microscopic observation of May-Giemsa-stained cells demonstrated that the number of macrophages remained at the basal level as of day 3, then increased and peaked on days 7 to 14, while the number of lymphocytes increased as early as day 1, peaked on day 7, and then gradually decreased. In flow cytometric analysis, the numbers of both B and T cells, including both CD4+ and CD8+ T cells, showed a rapid increase after administration of BLM. The T cells were activated, as indicated by the induction of IL-2 receptor (IL-2R) and the augmented expression on their surface of leukocyte function associated antigen-1 (LFA-1), which has also been regarded as a T cell activation marker. In addition, marked accumulation of $\gamma \delta$ T cells was observed in the lungs of mice treated with BLM, although it has not been elucidated whether these cells were involved in the pathogenesis. These results suggest that the increase of intraparenchymal macrophages and lymphocytes and the activation of T cells are prerequisite for the development of pulmonary fibrosis.

Key Words : bleomycin, IL-2 receptor, LFA-1, $\gamma \delta$ T cells

INTRODUCTION

Bleomycin (BLM), an anti-tumor drug which has been used in the clinical treatment of neoplasms, has been observed to cause interstitial pneumonia followed by subsequent fibrosis in a dose-dependent manner[1,2]. This consequence has often restricted its clinical use, but the drug has often been used in animal models of pulmonary fibrosis, the pathogenesis of which in human remains to be elucidated[3,4].

Several studies using mice depleted of T cells demonstrated that T cells have important functions the fibrotic process. The pulmonary fibrosis induced by BLM is attenuated in mice depleted of CD4+ and CD8+ T cells by injection of monoclonal antibodies against these molecules[5], thymectomized mice[6], those treated with anti-lymphocyte immunoglobulin[7], and athymic nude mice[8]. In other studies, however, neither depletion of T cells by injection of anti-Thy1, CD4 and CD8 monoclonal antibodies[9] nor athymic *nu/nu* mutation[10] was observed to affect the fibrotic change of the lung induced by BLM. Thus, the role of T cells in the pathogenesis of pulmonary fibrosis is not fully understood.

Various cytokines have been demonstrated to be involved in the fibrotic process via activating fibroblasts to proliferate and produce fibronectin, procollagen I and III, and elastin[11,12]. Previous studies[5,13,14] have revealed that IL-1, TNF-α and TGF-β are produced in the lung tissue after treatment with BLM, and that administration of anti-TNF-α immunoglobulin prevents the development of pulmonary fibrosis.

In the present study, we analyzed intraparenchymal leukocytes in the lung, rather than bronchoalveolar lavage (BAL) cells, to examine the pathogenesis in BLM-induced pulmonary fibrosis, because we considered that the tissue cells would more directly reflect the histological changes in the lung than would do BAL cells. We found that activated T cells and $\gamma \delta$ T cells as well as macrophages accumulated in the lung after administration of BLM.

MATERIALS and METHODS

Animals and reagents. In each experiment, 7-to 9-week-old female C57BL/6 mice, designated specific pathogen-free and obtained from Japan SLC Inc. (Shizuoka, Japan,) were used. This strain has been reported to be high responders in BLM-induced pulmonary fibrosis[15,16]. All mice were housed in a pathogen-free environment and received sterilized food and water at the Laboratory Animal Center for Biomedical Science of Nagasaki University. Bleomycin sulfate was generously donated by Nippon Kayaku Co.

Ltd. (Tokyo, Japan)

Administration of BLM. BLM was dissolved in 0.9 %
NaCl at the concentration of 0.4 U/ml. The mouse was
anesthetized by intraperitoneal injection of 70 mg/ml of
pentobarbital (Abbott Lab., North Chicago, IL) and
restrained on a small board. BLM (0.4 U/ml) was admin-
istered at 50 μl by inserting a 25-gauge needle which had
been cut down to the tip into and parallel with the trachea.

Preparation of pulmonary intraparenchymal leukocytes.
Mice were sacrificed at various times, and pulmonary
intraparenchymal leukocytes were prepared as we have
described previously[17]. Briefly, the chest was opened, and
the lung vascular bed was flushed by injection of 2 or 3 ml
of chilled physiological saline into the right ventricle. The
lungs were then excised, and washed in physiological
saline. The lungs, after teasing with a stainless mesh, were
incubated in RPMI1640 with 5 % of FCS, 100 U/ml penicil-
lin G, 100 mg/ml streptomycin, 10 mM HEPES, 50 mM
2-mercaptoethanol, and 2 mM L-glutamine, containing
20 U/ml collagenase (Sigma Chemical Co.) and 1 mg/ml
DNaseI (Sigma Chemical Co.). A volume of 25 ml was used
for three to six sets of lungs. After incubation for 60 min
at 37°C with vigorous shaking, the tissue fragments and
the majority of the dead cells were removed by passing
through the 100 μm-nylon mesh. After centrifugation, the
cell pellet was resuspended in 4 ml of 40 % (v/v) Percoll
(Pharmacia, Uppsala, Sweden) and layered onto 4 ml of
80 % (v/v) Percoll. After centrifugation at 600 × g for
20 min at 15 °C, the cells at the interface were collected,
washed twice, and counted with a hemocytometer.

Analysis of leukocyte fraction. About 4 × 10⁴ cells were
centrifuged onto a glass slide at 1,100 rpm for 2 min using
Cytospin 2 (SHANDON) and stained using a May-Giemsa
technique. At least 300 cells were examined for differential
count of the cellular fraction by photomicroscopic obser-
vation.

Antibodies. The following monoclonal antibodies
(MAb) were used in this study : FITC-conjugated 30-H12,
rat anti-mouse Thy1.2 (purchased from Becton Dickinson,
Mountain View, CA) ; FITC-conjugated 53-6.7, rat anti-
mouse CD8 (purchased from Becton Dickinson) ; FITC-
conjugated 7D4, rat anti-IL-2 receptor (purchased from
PharMingen, Sandiego, CA) ; phycoerythrin (PE)-con-
jugated Ly5, rat anti-mouse B220 (purchased from Coulter
Immunology, Hiarleah, FL) ; PE-conjugated GK-1.5, rat
anti-CD4 (purchased from Becton Dickinson) ; PE-con-
jugated 30-H12, rat anti-mouse Thy1.2 (purchased from
Coulter Immunology) ; PE-conjugated 2D7, rat anti-mouse
LFA-1 α (purchased from PharMingen) ; and PE-
conjugated GL3, hamster anti-mouse γ δTCR (purchased
from PharMingen).

Immunofluorescence. Cell suspensions were stained
with various surface markers using direct dual stain
method in the presence of heat-inactivated mouse serum.
Flow cytometry was then carried out with a FACScan
analyzer (Becton Dickinson), and the data were processed
with the CONSORT 30 program. Data were collected for
10,000 cells using the parameters of forward scatter (FSC)
and side scatter (SSC) to set a gate on lymphocytes.

RESULTS

***Pulmonary intraparenchymal leukocytes in mice intra-
trachelly administered BLM.***

In mice intratracheally administered BLM, the total
number of pulmonary intraparenchymal leukocytes in-
creased with time and peaked on day 7, when it was about
four-fold that on day 0, and then remained constant or
began to decline (Fig. 1a). Microscopic observation of
May-Giemsa-stained cells showed that the lymphocyte
count increased as early as day 1, peaked on day 7, and then
gradually declined. The macrophage count remained at the
basal level until day 3, then increased, and peaked on days
7 to 14. The neutrophil count did not markedly increase
from day 1 through day 14. The neutrophil count was not
examined during the first 24 h after administration.

***Pulmonary intraparenchymal T lymphocytes in mice
intratracheally administered BLM.***

The change of T and B cell number was examined by flow
cytometric analysis of the pulmonary leukocytes stained
with anti-Thy1 and B220. After administration of BLM,
both T and B cell counts increased with time, peaked on
day 7, and gradually declined (Fig. 1b). The CD4+ and
CD8+ subsets of T cells were also examined. In normal
mice, the proportion of CD4+ T cells was larger than that
of CD8+ T cells (CD4+/CD8+ ratio, 1.55). After admini-
stration of BLM, the numbers of both CD4+ and CD8+ T
cells increased overtime. Beginning on day 7, the CD8+ T
cell count showed a distinct increase compared with the
CD4+ T cell count, and the ratio of CD4+ and CD8+ T
cells diclined to 0.48 on day 7 (Fig. 1c).

***Activation of T cells in the lung after administration of
BLM.***

The expression of IL-2R on pulmonary intraparenchymal
T cells was examined by flow cytometric analysis. The
proportion of IL-2R+ T cells (8.9 % in untreated mice)
was increased 3 days after administration of BLM, and
peaked on day 7, when 58.6 % or T cells expressed IL-2R
(Fig. 2a). This result suggests that T cells accumulated in
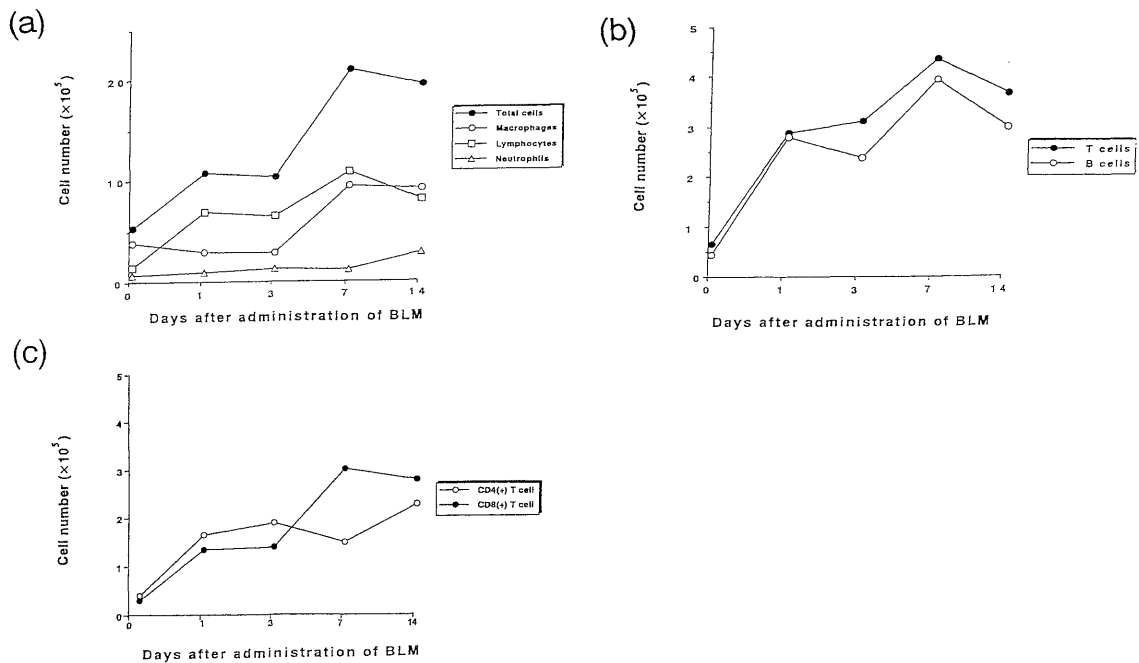the lung by local expansion in an IL-2-dependent manner.

Fig. 1 Pulmonary intraparenchymal leukocytes in mice intratracheally administered with BLM.

Pulmonary intraparenchymal leukocytes were prepared at various times after intratracheal administration of BLM. The total numbers of cells were counted, and (a) differential counts of each fraction were determined by morphological analysis of about 300 cells stained using a May Giemsa technique. (b) T cells and B cells counts; staining with FITC-conjugated anti-Thy1.2 and PE-conjugated anti-B220 (c) CD4+ , and CD8+ T cell counts ; staining with FITC-conjugated anti-Lyt 2 and PE-conjugated anti-L3T4 antibodies. The lymphocyte populations gated on the FSC and SSC parameters were analyzed using FACScan. The data in untreated mice are designated as those on day 0. The numbers of cells of each population were calculated as follows : percentage of each population × total number of lymphocytes/100. Each experiment was repeated three times, with similar results.
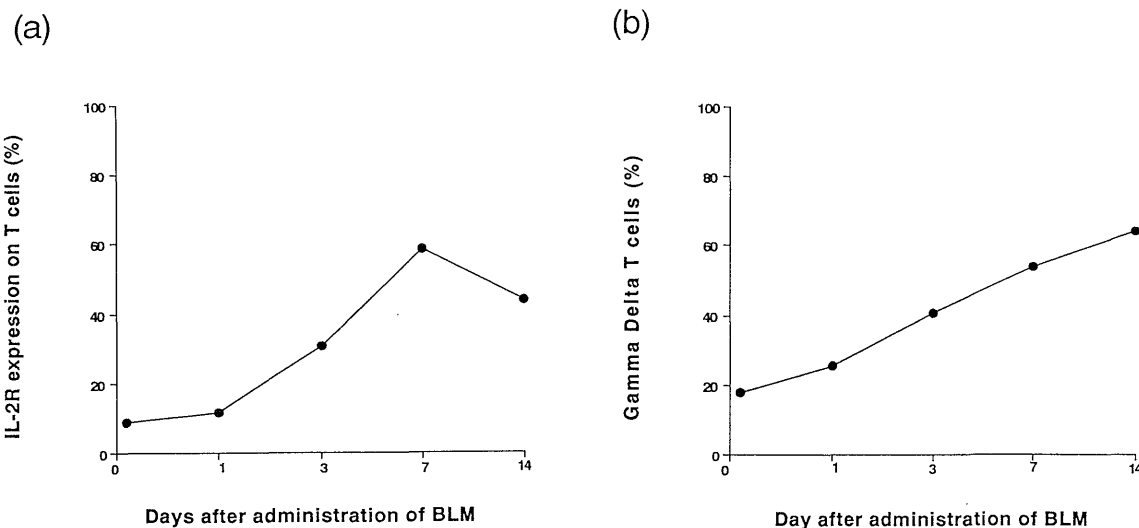

Fig. 2 Induction of IL-2R expression on T cells and $\gamma\,\delta$T cells in the lung after administration of BLM.

pulmonary intraparenchymal leukocytes were prepared at various times after administration of BLM, and (a) stained with FITC-conjugated anti-IL-2R and PE-conjugated anti-Thy1.2 antibodies. The T cell populations, gated on the expression of Thy1.2 molecule, were analyzed for the expression of IL-2R using FACScan, and (b) stained with FITC-conjugated anti-Thy1.2 and PE-conjugated anti- $\gamma\,\delta$TCR antibodies. The T cell populations, gated on the expression of Thy1.2 molecule, were analyzed for the expression of $\gamma\,\delta$TCR using FACScan, the results are expressed as a percentage of positive cells. Each experiment was repeated three times, with similar results.
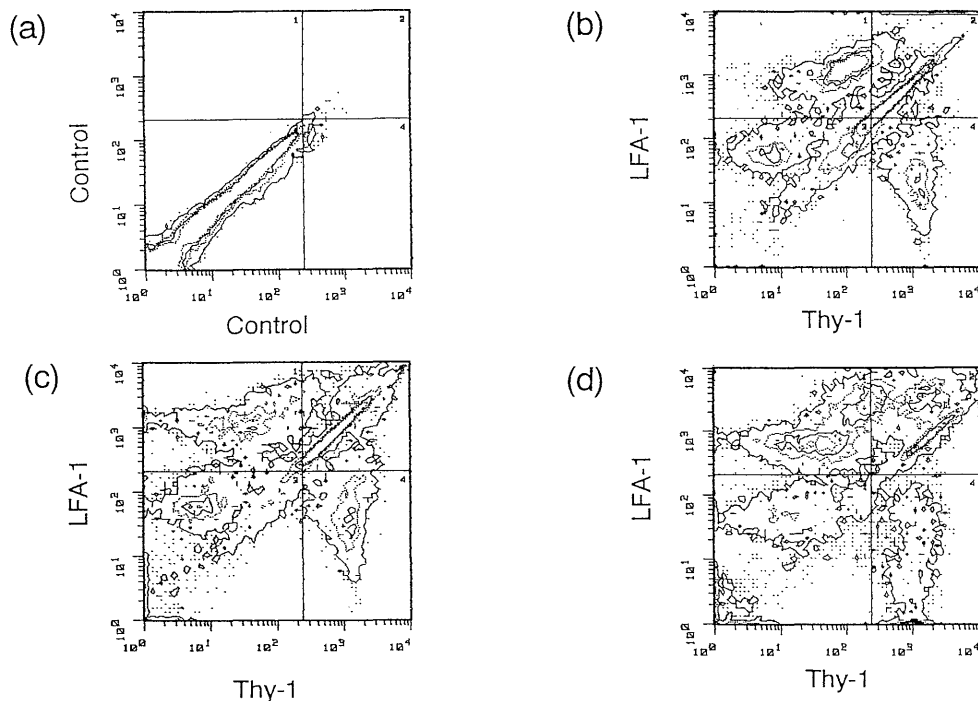
Fig. 3 Expression of LFA-1 on intraparenchymal T cells in the lung after administration of BLM.

The pulmonary intraparenchymal leukocytes from untreated mice were analyzed without staining to show their autofluorescence (a) and those from untreated mice (b) 3 days (c) and 14 days (d) after treatment with BLM. The cells were stained with FITC-conjugated anti-Thy1.2 and PE-conjugated anti-LFA-1 antibodies, and analyzed for the expression of these molecules using FACScan. The live data are presented as plotted data points without setting any gates. Each experiment was repeated three times, with similar results.

LFA-1, a familiar adhesion molecule[18], has also been regarded as an activation marker of T cells[19]. We examined, next, the expression of this molecule as an activation marker of T cells in the lung after administration of BLM. The pulmonary intraparenchymal cells derived from both untreated, (Fig. 3a) and treated mice showed strong autofluorescence and in the staining profile of the treated mice, the upregulated portion of LFA-1 on Thy1+ population overlay the autofluorescence (Fig. 3b, c), which made it difficult to analyze the specific staining. Thus, the live data are presented as plotted data points without setting any gates. In untreated mice, the majority of T cells expressed LFA-1 at a low level (Fig. 3b). After administration of BLM, this expression in both Thy1 positive and negative populations increased with time as shown in Figure 3c and 3d.

Induction of γ δ T cells in the lung after administration of BLM.

γ δ T cells have been known to be distributed in various organs including intestine, skin, lung and reproductive organs[20], and have been demonstrated to play important

roles in the host defense against various pathogens[21, 22]. To evaluate the possible involvement of these cells in BLM-induced pulmonary fibrosis, we examined the expression of γ δ TCR on T cells in the lung by flow cytometric analysis. As shown in Figure 2b, the proportion of γ δ T cells in the lungs of untreated mice was 17.9 %, and that on day 14 after administration of BLM was increased to 65 %.

DISCUSSION

The development of BLM-induced pulmonary fibrosis is thought to be divided into the three phases of acute injury, inflammatory response, and chronic response[10, 11]. In previous studies, macrophages have been found to play an important role in the second phase[23]. Macrophages accumulate in the lung rapidly after administration of BLM and modulate the fibrogenic process by releasing various cytokines which mediate inflammatory response[23-25], prostaglandins[26] and fibroblast growth factors[27, 28]. In contrast, the role of lymphocytes in the pathogenesis of BLM-induced pulmonary fibrosis remains controversial. In several studies, pulmonary fibrosis by BLM was found to

be attenuated after depletion of T cells by injection of monoclonal antibodies against their surface molecules[5], thymectomy[6], treatment with anti-lymphocyte immuno-globulin[7], and in athymic nude mice[8]. In other studies, however, depletion of T cells by either injection of anti-T cell monoclonal antibodies[9] or athymic *nu/nu* mutation[10] showed no effect on the extent of fibrotic change of the lung.

In most previous studies[9, 29], BAL cells were examined to analyze the kinetics of inflammatory cells in lung after treatment with BLM. However, in an early study using a rat model of BLM-induced pulmonary fibrosis, R. S. Thrall et al.[30] demonstrated that the specific lymphocyte populations in lung tissue changed during the development of the fibrotic process, whereas this shift in populations was not observed in the lymphocyte populations found in BAL fluid. Our unpublished results also indicate that the kinetics in BAL cells are not always correlated with those in lung tissue cells after intratracheal inoculation of *Peumocystis carinii*. Thus, we considered that the tissue cells reflect the histological change in the lung after administration of BLM more directly than do the BAL cells, and adopted the lung tissue cells in the present study to analyze the kinetics of inflammatory cells and define the role of T cells.

The flow cytometric analysis demonstrated that pulmonary intraparenchymal T cells were activated after administration of BLM, as indicated by the expression of IL-2R and the augmented expression of LFA-1. Consistent with our result, Karpel et al.[31] previously found that BAL lymphocytes from BLM-treated rabbits showed nearly four-fold greater proliferative response after incubation with IL-2 than those from untreated rabbits, suggesting that the expression of functional IL-2R was induced on T cells in the lung by treatment with BLM. On the other hand, LFA-1, a familiar adhesion molecule[18], has been shown to interact with its counterpart molecule, intercellular adhesion molecule-1 (ICAM-1)[18], and to play an important role in cell-to-cell contact with endothelial cells for the extravasation of T cells into inflammatory sites[32], and has also been regarded as an activation marker of T cells[19]. Thus, the very early accumulation of T cells on day 1 might be due to their influx from the circulation. Together with these findings, our results suggest the following two possible mechanisms for the accumulation of T cells in the lung : expansion in situ of specific T cell populations in response to an as yet undefined antigen, and recruitment of T cells from the circulation.

γ δ T cells have been found to be distributed in various organs, including the intestine, skin, lung and reproductive organs[20], and have been demonstrated to play important roles in the host defense against various pathogens[21,22]. In the present study, we found that this cell population was markedly increased in the lungs of mice treated with BLM. To our best knowledge this is the first report of this finding. Because these cells have been observed to show a proliferative response to some stress proteins[20], it is possible that γ δ T cells accumulate in response to an undefined stress protein induced on epithelial cells damaged by exposure to BLM. At present, the significance of these cells in the pathogenesis of BLM-induced pulmonary fibrosis remains to be elucidated.

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