Detection of Peripheral Blood and Bronchoalveolar Lavage Fluid Lymphocytes in Rat Lung Transplantation for Early Diagnosis of Rejection

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SAITO, R., KONDO, T., FUJIMURA, S., HANDA, M., ICHINOSE, T. and SHIRAISHI, Y. Detection of Peripheral Blood and Bronchoalveolar Lavage Fluid Lymphocytes in Rat Lung Transplantation for Early Diagnosis of Rejection. Tohoku J. Exp. Med., 1990, 160 (3), 231-249 — Left lung transplantation was performed in two combinations of rat strains. In group 1, lung grafts were rejected within 7 day postoperatively, and in group 2, grafts were rejected within 18 day postoperatively. The histological appearance of rejection was classified into 4 stages, and lymphocytes from peripheral blood (PB) and bronchoalveolar lavage fluid (BALF) were collected at each stage. In group 1, a significant decrease in the number of lymphocytes in PB was observed as the rejection progressed, whereas the number of lymphocytes in PB increased slightly in group 2. The number of total cells in BALF increased significantly as the rejection progressed in both groups. A marked increase in the value of spontaneous blastogenesis (SB) was observed in stage 2 in BALF lymphocytes, whereas in PB lymphocytes was found to increase after stage 3 in both groups. The ratio of T-helper/T-nonhelper did not change significantly in PB lymphocytes in both groups. On the other hand, a significant decrease in the value of T-helper/T-nonhelper (<1.0) was observed in BALF at stage 3 and 4 in both groups. These results show that the studies of BALF lymphocytes were very useful for early diagnosis of rejection in lung transplantation.

It is most important to detect rejection response in the early stage of the adequately immunosuppression in clinical lung transplantation. We have established the model of rat lung transplantation (Kondo et al. 1986; Saito 1987) considering it not to be proper to assess immunological changes after lung trans-
plantation using mongrel dogs (Fujimura and Suzuki 1967; Fujimura et al. 1970, 1983). In this article, we assessed functional and morphological changes of lymphocytes in bronchoalveolar lavage fluid (BALF) and peripheral blood (PB) after lung transplantation using our rat model.

**MATERIALS AND METHODS**

**Animals**

Male WKAH (RT1<sup>a</sup>) rats, weighing 150-240 g (Shizuoka Laboratory Animal Center Japan, Hamamatsu) and male WKY (RT1<sup>i</sup>) rats, weighing 140-260 g (Hoshino Laboratory Animal Japan, Yashio) were used as donors, and male F344 (RT1<sup>v</sup>) rats, weighing 140-270 g (Shizuoka Laboratory Animal Center Japan), were used as recipients. All animals were specific pathogen free and inbred. Left lung transplantation was performed 29 times in group 1 (WKAH as donors and F344 as recipients) and 31 times in group 2 (WKY as donors and F344 as recipients).

**Monoclonal antibodies**

T cell subset in BALF and PB was assessed using W3/25 (anti-rat helper T cell glycoprotein mouse IgG<sub>1</sub>, Serotec, London, England) and OX-8 (anti-rat cytotoxic/suppressor T cell glycoprotein mouse IgG<sub>1</sub>, Serotec) according to microcytotoxicity test.

**Transplantation**

Left lung was transplanted orthotopically according to the method reported previously (Shionozaki et al. 1982). Briefly, after an intramuscular injection of 0.01 mg of atropine sulfate, 5 mg/100 g body weight of pentobarbital sodium and 0.3 mg/100 g body weight of methylprednisolone were administered via the tail vein under slight anesthesia by inhalation of enflurane. A Teflon tube of 1.1 mm in diameter and 6 cm in length without cuff was intubated under the operation microscope. Ventilation was maintained by a Harvard Model 680 ventilator with 2 ml of the tidal volume and 100/min of the frequency. Positive endexpiratory pressure of 5 cmH<sub>2</sub>O was applied to prevent the lung collapse. The depth of anesthesia was adjusted by the intermittent inhalation of enflurane as necessary. All the operation procedure was performed under the operation microscope. The left 4th intercostal space of the recipient was opened and hilar soft tissues were separated following the dissection of the pulmonary ligament. The bronchus, pulmonary artery and pulmonary vein (left atrial cuff) were clamped and transected in this order. Donors are administered atropine sulfate, pentobarbital sodium and methylprednisolone in the same way followed by left thoracotomy through the 5th intercostal space. After an intracardiac injection of 200 units of heparin, the dissection of hilar tissue was done in the same manner. After the transection of the pulmonary artery and vein, the bronchus was transected in order to keep the blood from flowing into the air way. All hilar structures were anastomosed the 9-0 nylon (Dermalon TE143; Davis and Geck, New York, NY, USA) by continuous sutures. Immediately after the completion of the bronchial anastomosis, ventilation of the graft was restored. The thorax was closed leaving the chest drainage tube, which was removed when rat awoke from anesthesia. 5 ml of saline and 20 mg of AB-PC was administered subcutaneously and the same dose of AB-PC was administered intramuscularly for subsequent 3 days.

**Serial chest x-rays**

In 7 rats of each group chest x-ray films were taken everyday after transplantation to know the day when chest infiltrate appeared.
Peripheral blood lymphocytes (PBL) and BALF lymphocytes

Necropsy was done 2, 3, 4 and 5 days postoperatively in group 1, and 1 to 17 days postoperatively in group 2. Under slight anesthesia with inhalation of enflurane, 1 ml of PB was collected from both external jugular veins to count the number of white blood cells with an automatic blood cell counter.

Differential count of white blood cell was also performed using smeared material with Giemsa stain.

Another 2 ml of PB was taken for the separation of lymphocytes. Following this procedure, the rat was euthanized by the exsanguination and heart-lung block was put out. BALF was obtained by washing the graft with 1 ml of phosphate buffered saline (PBS) which was injected into the graft's bronchus and aspirated. This procedure was repeated until the amount of collected BALF reached 5 ml. BALF was filtrated with gauze to eliminate mucous and centrifuged at 300 × g. The pellet was resuspended in RPMI 1640 with 2% FBS. Small aliquots were put on the hemocytometer to calculated the cell number, and 1 ml was fixed by Sacomano’s solution followed by Papanicoleu’s stain in order to obtain the differential count of leucocyte in BALF.

Rest of BALF was put on Ficoll-Paque to separate lymphocytes by the gravity centrifugation. Lymphocytes in PB were also separated in the same way. Washed graft was fixed by formalin to assess the degree of the rejection response. The histological appearance of the rejection was classified into 4 stage according to the classification which we reported before (Fujimura 1967) with some modification. Briefly, stage 1 is called “minimal change” which includes normal appearance, reimplantation response and finding of small amount of mononuclear cells around vessels and bronchi (Fig. 1a, b). Stage 2 is called “mild rejection” with the histological finding of mild to moderate infiltration of mononuclear cells around vessels and bronchi without any changes in alveolar architecture (Fig. 2a, b). Stage 3 is called “moderate rejection” in which thickening of alveolar wall with the infiltration of mononuclear cells and occasional destruction of the alveolar wall and pronounced perivascular and peribronchial infiltration of mononuclear cells are included (Fig. 3a, b). Stage 4 is called “severe rejection” with widespread destruction of alveolar architectures (Fig. 4a, b).

Spontaneous blastogenesis (SB) of PBL and BALF lymphocytes

Separated lymphocytes were resuspended in PRMI1640 supplemented with 10% FBS at a concentration of 1 × 10⁶/ml. 100 μl aliquots were pipeted into 96-well, flat bottomed microplate (Corning, New York, NY, USA) with 1 μCi of ³H-thymidine in each well and put into 24-hr culture at 37°C under an atmosphere of 5% CO₂ in air. Cultured cells were collected by the automatic cell harvester and ³H activity was counted with a Beckman liquid scitillation counter. This radioactivity represents the value of SB in lymphocytes.

T cell subsets in PBL and BALF lymphocytes

Helper and non-helper T cells were counted using W3/25 and OX-8 in PBL and BALF lymphocytes by microcytotoxicity test. Briefly, 1 μl of lymphocyte suspension at a concentration of 1-2 × 10⁶/ml was pipeted into Poly-L-Lysin coated microplate. A 5 μl aliquot of 20-fold diluted MoAb was added to each well and incubated at 37°C for 30 min in CO₂ incubator. Anti-mouse IgG1, goat serum was further added to potentiate the ability of the complement activation for IgG1. Following the incubation, low toxic rabbit compliment was added in each well and incubated 20 min more. 10 μl of 0.64% trypan blue solution was subsequently added in each well and after washing with the PBS with 0.2% gelatin, the number of the alive and dead cells were counted.
RESULTS

Postoperative course

Six of 29 in group 1 died within 24 hr from such causes as bleeding, air way obstruction by the sputum, technical failure, overdose of anesthetic agent and others. In group 2, all 31 animals survived operative course. Operation time ranged from 68 to 105 min (mean 92 min) in group 1, and from 79 to 110 min (mean 96 min) in group 2. Warm ischemic time of grafts of group 1 and 2 were 38 min in the mean value, respectively.

Postoperative chest x-ray films

In group 1, diffuse infiltrative shadows appeared on the 4th postoperative day in the left lung (graft), which became completely radiopaque the 7th day (Figs. 5, 6, 7). On the contrary, the same chest x-ray appearance was noticed around the 14th day postoperatively in the left lung, which became completely radiopaque by the 18th day in group 2.

Histological appearance

All animals in both group were classified into 4 groups by their histologic appearance of the rejection (Table. 1). In group 1, the rapid progression of the rejection response was observed, in which before forming “perivascular cuffing” by lymphocytes in stage 2, stage 3 with the involvement of alveolar wall appeared.

Fig. 5. Chest x-ray film in group 1, postoperative 2 day; Airation in the left lung (graft) is good.
Fig. 6. Chest x-ray film in group 1, postoperative 4 day; Diffuse infiltrative shadows appeared in the left lung (graft).

Fig. 7. Chest x-ray film in group 1, postoperative 7 day; Infiltrative shadow in the left lung (graft) became completely radiopaque, the airation of the left chest is the mediastinal lobe of the right lung.
Lymphocytes in PB

In group 1, significant decrease in the number of lymphocytes in PB was observed as the rejection progressed, however, the number of lymphocytes in PB increased slightly in group 2 (Fig. 8).

Cell profiles in BALF

The number of total cells in BALF increased significantly as the histological change of the rejection progressed with the remarkable increase of the fraction of the lymphocyte and monocyte (Table 2).

Changes in the value of SB

The value of SB was compared with the histologic stages. The significant
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Increase in the value of SB was observed as early as in stage 2 in BALF compared with that in PBL which was found to increase only after stage 3. These results were found in both groups which had different postoperative course (Fig. 9 A, B).

T cell subset in PB and BALF

The ratio of T-helper/T-nonhelper, which was above 1 in the perioperative
period, did not change significantly in PBL in both groups. However, a significant decrease in the value of T-helper/T-nonhelper was observed in BALF stage 3 and 4 in both groups (Fig. 10A, B).

DISCUSSION

Before 1980, 38 cases of clinical lung transplantation were performed, but only 3 cases survived over 2 months (Veith et al. 1982, 1983a).

After 1981, clinical outcome of lung transplantation has greatly improved (Gunby 1982) especially with the application of cyclosporine for the immunosuppressive agent. Effective and successful single lung transplantation has been achieved at the Toronto Lung Transplant Group. Their longest survivor is the patient who survives as long as 4 years after single lung transplantation.

While improvements in technique and immunosuppression (Koerner et al. 1976, 1982; Kamholtz et al. 1983; Veith et al. 1983b) have led to major advances in experimental and clinical lung transplantation, it is also true that there remains many problems to be resolved. Among these problems, early and accurate diagnosis of rejection is important for proper immunosuppression. The most reliable way for this remains the open lung biopsy, but this method is too dangerous for the patient with single lung transplant. The specific, prompt and less invasive way for detection of rejection must be established.

We confirmed the uniformity of rejection response in rat lung transplantation model, and investigated functional and morphological changes in lymphocytes in

Fig. 10. (A) Rejection and T-helper/T-nonhelper ratio in group 1. (B) Rejection and T-helper/T-nonhelper ratio in group 2. Otherwise, the same as in Fig. 9.
BALF and PB which were compared with histologic stages of rejection.

The number of lymphocytes of PB gradually decreased with the marked accumulation of lymphocytes in grafts in group 1. This phenomenon considered to occur with the rapid recruitment of lymphocytes in graft without a sufficient supply, because such a phenomenon was not observed in group 2 in which rejection progressed gradually and slowly. The rapid onset of rejection have to be considered when the sudden decrease in the number of lymphocytes of PB observed. Results on morphological and functional changes of lymphocytes in BALF were almost same in both groups. The number of total cells, especially the fraction of lymphocytes and monocyte increased at stage 2. These cells are considered to originate from bronchus-associated lymphoid tissue (BALT) (Plesch 1982) which is widely located in the bronchial wall, because neither the destruction of the alveolar architecture nor the marked lymphocyte infiltration into the alveolus were observed in stage 1 and 2 histologically. BALT without epithelial linings on it were also encountered. Microscopic examination revealed the high cellularity in BALT as early as in stage 2 in which even BALT without epithelial linings on it was also seen. As the rejection progressed, especially in stage 3 and 4, cells in BALF are thought to originate also from destructed alveoli.

BALT is an important immune system of bronchus (Binenstock et al. 1976). In lung transplantation, lymphocytes of the recipient may encounter the donor antigens at first in this system (Prop et al. 1985a). In addition, lymphocytes in BALF from the graft are thought to be those of recipient, because lymphocytes of the donor is reported markedly decrease in the number in the graft within first 24 hr (Prop et al. 1985b). This may be the reason why functional and morphological changes of lymphocytes in BALF preceded those in PB in this experiment. The discrepancy of the change in T cell subsets between BALF and PB also supports this idea.

From these results obtained in this experiment, we concluded that the observation of functional and morphological changes in lymphocytes in BALF will be a great help in clinical lung transplantation.

References


Fig. 1a. Minimal change of group 1 — Finding of small amount of mononuclear cells around vessels and bronchi (hematoxylin-eosin; original magnification ×40).

Fig. 1b. Minimal change of group 2 — Finding of small amount of mononuclear cells around vessels and bronchi (hematoxylin-eosin; original magnification ×40).
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Fig. 2a. Mild rejection of group 1 — Finding of mild infiltration of mononuclear cells around vessels and bronchi (hematoxylin-eosin; original magnification ×40).

Fig. 2b. Mild rejection of group 2 — Finding of moderate infiltration of mononuclear cells around vessels and bronchi (hematoxylin-eosin; original magnification ×40).
Fig. 3a. Moderate rejection of group 1 — Finding of mild infiltration of mononuclear cells around vessels and bronchi, and thickening of alveolar wall with occasional destruction of the alveolar wall (hematoxylin-eosin; original magnification $\times 40$).

Fig. 3b. Moderate rejection of group 2 — Finding of moderate infiltration of mononuclear cells around vessels and bronchi, and thickening of alveolar wall with occasional destruction of the alveolar wall (hematoxylin-eosin; original magnification $\times 40$).
Fig. 4a. Severe rejection of group 1 — Finding of widespread destruction of the alveolar architectures (hematoxylin-eosin; original magnification ×40).

Fig. 4b. Severe rejection of group 2 — Finding of widespread destruction of the alveolar architectures (hematoxylin-eosin; original magnification ×40).