

Evaluation of Ia positive cells in the canine lung transplantation

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ABSTRACT : The rejection process related to Ia-positive cells consisting of the lymphocytes of bronchoalveolar lavage (BAL) was mainly evaluated at rejection of lung allotransplantation and also the inhibitory effect of CsA on occurring rejection is assessed.

- 1) In BAL taken from the transplanted lung, the increase in Ia positive rate and cell population was noted. Meanwhile in B-cells, activated T lymphocytes were remarkably increased in number.
- 2) The Ia positive rate in alveolar macrophages was the highest in the rejected lung. On the other hand in monocytes of the peripheral blood it was apparently reduced with statistical significance.
- 3) In vitro study, the Ia positive rate of the lymphocytes in BAL was increased by PHA stimulation and significantly reduced by the addition of CsA. The Ia positive rate of macrophages in BAL also tended to be inhibited by adding CsA. The result enabled me to conclude that CsA acts as the elimination of Ia-positive rate in BAL lymphocytes and macrophages.
- 4) In the rejected lung a cluster of Ia-positive cells was seen not only around small vessels but in the alveolar space. Of interest is the fact that the role of Ia-positive cells is crucial to rejection of lung allografts.

INTRODUCTION

Graft rejection mechanism after organ allotransplantation has not been made clear yet. The major roles were said to be activity of antigen-presentation of the macrophage, production of activating factor by T-cell (interleukin 1)¹⁾ and cellular immune response of T-cell infiltration into the graft. It is well known that Ia antigen, which indicates a key antigenicity to immune response, has a cross reaction each other regardless of animal species²⁾. The aim of this study is to clarify the rejection mechanism by comparing with the appearance rate of Ia-positive cells of macrophages, and BAL lymphocytes as

well as monocytes in the peripheral blood and also to make clear the drug effects of cyclosporin A (CsA) at lung allotransplantation. Furthermore, attention has been focused on the question as to whether measurement of Ia-cells in BAL and the peripheral blood is of benefit to know ongoing rejection process following lung allotransplantation.

MATERIAL AND METHOD

- 1) Surgery for transplantation & use of immunosuppressive drugs

Lung auto- and allotransplantations were performed in mongrel dogs, weighing eight to 16 kg. After intravenous administration of pen-

tobarbital (25 mg/kg), intubated into the trachea and controlled respiration was maintained by Harvard respirator at a rate of 12 to 14/min with 300 to 400 ml of ventilation volume.

Left lung was removed under heparinization by left thoracotomy at the fifth intercostal space. The steps of transplantation was made in order of the anastomoses to left atrium, pulmonary artery and bronchus. 5-0 proline was used for anastomoses to the left atrium and the pulmonary artery, and also 4-0 nylon for the bronchus. The ischemic time of the donor lung was within 60 min in all dogs. The animals were divided into the following three groups, that is, group 1 : autotransplantation in nine dogs, group 2 : allotransplantation without giving any immunosuppressive drug in 10 dogs, group 3 : allotransplantation with giving 20 mg/kg of CsA daily in 19 dogs and continuously to autopsy on day three to 14.

2) Separation of monocyte from peripheral blood and BAL in normal and transplanted dogs

Peripheral blood was taken prior to transplantation and BAL also was collected from contralateral lower lobe. At autopsy BAL was taken from bilateral lower lobes with BAL 50 ml of saline, filtrated four times with sponge, centrifuged at 1700 rpm for 30 min. The monocyte was counted by staining with 0.2% trypan-blue. Confirmation of lymphocyte and macrophage was made by staining in accordance with May-Gimsa' method.

3) Measurement of Ia-positive cell in BAL and monocyte in peripheral blood

The count of monocyte in BAL was adjusted to 1×10^6 and 25 ul of 1% aggregated dog IgG was added to block the action of Fc-receptor of macrophage and cultured at 4°C for 10 min. One hundred ul of PBS also was added to prepared peripheral blood. About 5 ul of OKIa1 was added as being primary antibody although 5 ul of mouse IgG was used for the negative control and cultured at 4°C for 30 min. As secondary antibody, additional 100 ul of FITC conjugated Goat antimouse IgG was added and cultured followed by making hemolysis. Mononuclear cells derived from normal BAL were separated into two groups on the chart of cyto-gram used by Spectrum III at shown in Fig 1-A.

After culture in 5% CO₂ of FCS coatplastic dish at 37°C for 2 hours, mononuclear cells were separated into adherent and nonadherent cells (Fig 1C and D).

There were confirmed macrophage and lymphocyte. Positive rate of Ia and the cell count were calculated.

4) Measurement of B cell in BAL

After washing 1×10^6 monocytes, these were cultured at 37°C for 30 min with 2 ml of PBS. Ig-production cell by FITC conjugated anti-dog IgG (Miles Scientific Co.) was regarded as B cell.

5) Effect of immunosuppressant on BAL, lymphocyte and macrophage

A) Effect of CsA on Ia positive rate of BAL lymphocyte

The mononuclear cells collected from BAL were incubated at 37°C in 5% CO₂ for two hrs, BAL lymphocytes in 10% FCS RPMI medium adjusted to 1×10^6 cells were placed in round bottom microtiter plate (Corning Co. #25802) and 100 ul phytohemagglutinin (PHA-PDIFCO Co.) diluted at various concentration were added and cultured at 37°C in 5% CO₂ for 72 hrs.

³H-thymidine (1 μCi/ml) was added to each 24 hrs before harvesting. Thereafter the sample were transferred to scintillation vials and the radioactivity was measured in ALOKA Liquid Scintillation System (SC-903). Optimal concentration of PHA was confirmed at a concentration of 10 μg/ml. In this sample, CsA (0.1, 0.5, 1.0 and 5.0 μg/ml) were added and lymphocyte blastogenesis was measured. At the same time, BAL lymphocytes (1×10^6 /ml) were incubated with PHA-P (10 μg/ml) and CsA at various concentration for three days. Ia positive rate of BAL lymphocytes were read.

B) Effect of CsA on Ia positive rate of BAL macrophage

As the control, BAL mononuclear cells (1×10^6 cells/ml) were cultured in 100% FCS RPMI 1640 medium. An optimal dosis of PHA-P (10 μg/ml) and lipopolysaccharide (LPS : SIGMA) 20 μg/ml were added with CsA (0.1, 5.0 μg/ml) of various concentration and Ia positive rates of BAL and macrophage were compared.

6) Histologic evaluation of rejection

A) At sacrifice, histologic examination of

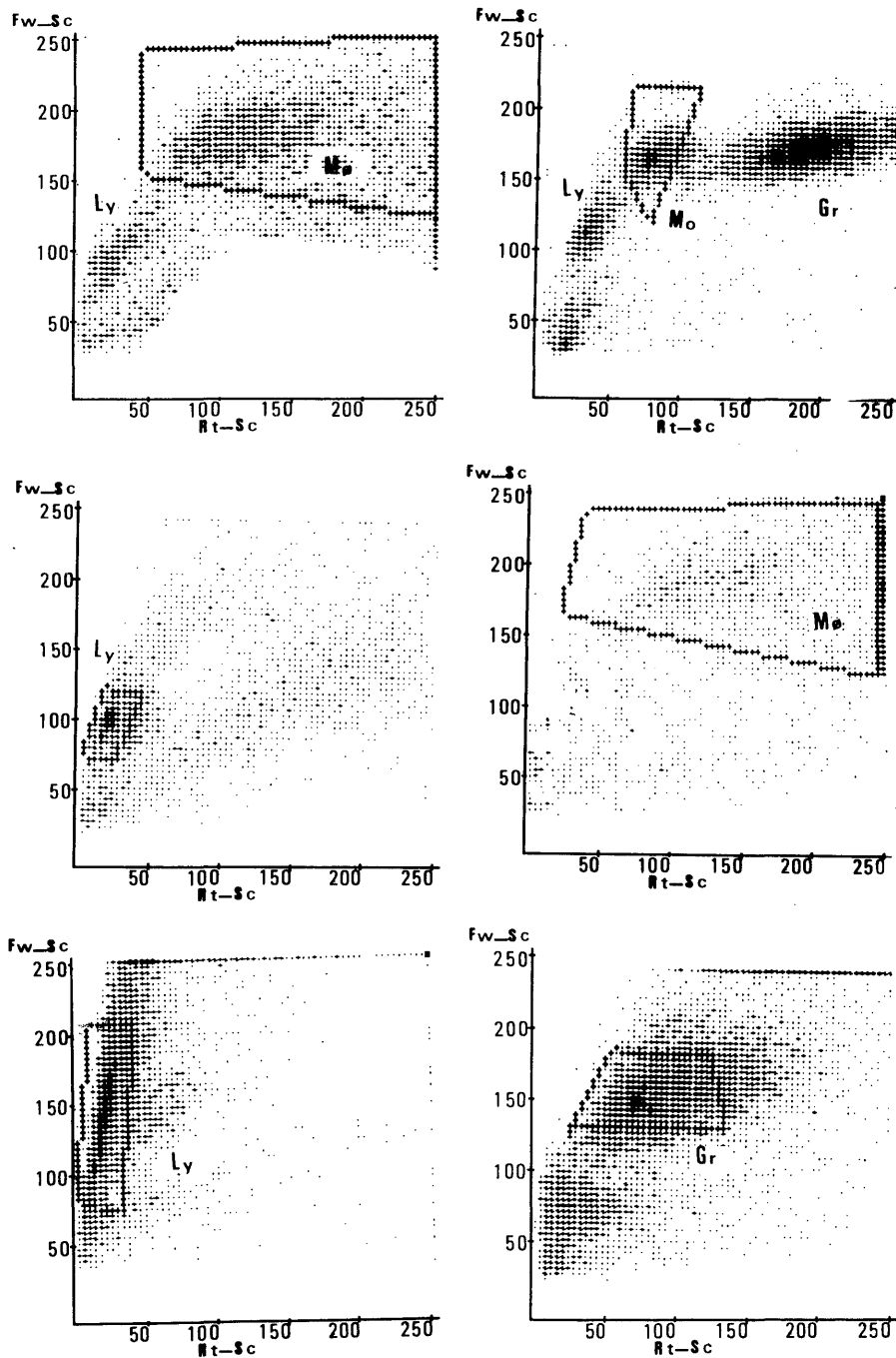


Fig. 1. Cytogram by Spectrum III

- A) normal BAL mononuclear cells
- B) peripheral blood leucocytes
- C) BAL non adherent cells in normal lung : lymphocytes
- D) BAL adherent cells in normal lung : macrophages
- E) BAL non adherent cells in rejected lung : large lymphocytes are observed
- F) BAL in infected lung : neutrophils

the lungs on both sides was made by staining with Hematoxylin-Eosin. It was graded by the degree of perivascular mononuclear cell cuffing formation, (-) : nothing of perivascular cuffing which is one of the main finding of rejection (\pm) : a few infiltration, (+) : considerably prominent infiltration, (++) : a lot of infiltration.

Finally rejection response was determined by the finding of grade (+) or (++) of perivascular cuffing with destruction of alveolar structure and progression of fibrosis according to SHIRAKUSA¹ classification³.

B) Local finding of Ia-positive cell in rejection lung

In the resected rejection lung, OCT compound was intratracheally infused and 0.5 cm blocks were embedded in OCT compound, rapidly freeze-d by liquid nitrogen and sectioned by cryostat. After drying at -30°C all day, on the next day fixed in acetone for 10 min. As a primary antibody, OKIa 1 was added and mouse IgG was used for secondary antibody for 20 min. Thereafter, Avidin Biotin affinity was added with 3-aminh-9-ethylcarbazon (AEC) used for glitter.

Poststaining of a 5-10 sec duration was completed by hematoxylin and examined microscopically. It was confirmed that in the negative control with the addition of mouse IgG there was no positive Ia-staining. Statistical significance of the date was estimated by Wilcoxon¹ test and the values were represented by mean \pm standard deviation.

RESULT

1) Change in Ia-positive rate of peripheral monocytes

Positive rate of Ia cell was preoperatively 72.0 \pm 4.90%. In group 1, slight decrease was seen on day one. However, on day four to seven there were not significant variation. In group 2, decrease on day one and four was marked with significance as compared with group

2) It reverted on day four to seven as shown in Fig. 2.

3) Changes in mononuclear cell count in BAL before and after lung transplantation

BAL taken from the rejected lung contained large lymphocyte which suggested of blastoid

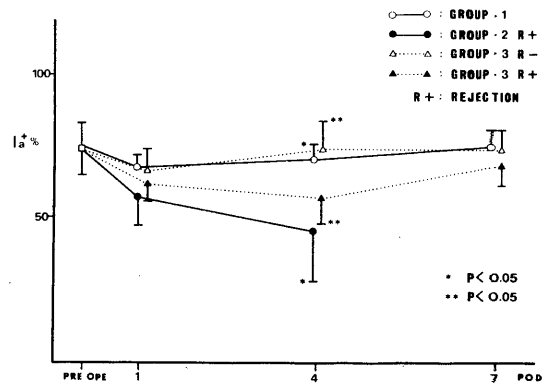


Fig. 2. Ia positive rate of peripheral blood monocytes

formation as shown in Fig. 1E on cytogram.

Mononuclear cells in BAL were presented as the lymphocyte, and the macrophage (Ly/M ϕ + Ly \times 100, M ϕ /M ϕ + Ly \times 100%). The counts of cell also were presented in 200 ml BAL. There was a significant difference in large population of lymphocyte (72.8%) in rejected lung of group 2 with a 60 times increase of cell counts although macrophage was predominant in BAL taken from the right lungs in group 1 and 2 and the not-rejected lung in group 3 as shown in Table 1.

Table 1. Cell compositions in BAL and number of BAL mononuclear cells

		Ly \times 100%	Ly counts in 200ml
		M ϕ + Ly	BALF ($\times 10^6$)
Normal Control		14.86 \pm 7.78	1.58 \pm 1.12
Group1	Left	13.06 \pm 3.29	1.17 \pm 0.47
	Right	12.63 \pm 4.33	1.05 \pm 0.41
Group2	R(+) D	72.82 \pm 8.37	90.40 \pm 34.03
	R	16.56 \pm 4.23	2.14 \pm 1.54
Group3	R(-) D	19.32 \pm 7.84	2.12 \pm 0.98
	R	12.62 \pm 3.09	1.20 \pm 0.36
Group3	R(+) D	54.04 \pm 15.75	46.81 \pm 38.03
	R	19.52 \pm 10.00	1.99 \pm 1.41

		M ϕ \times 100%	M ϕ counts in 200ml
		M ϕ + Ly	BALF ($\times 10^6$)
Normal Control		84.90 \pm 7.66	8.81 \pm 4.39
Group1	Left	86.94 \pm 3.29	8.87 \pm 1.22
	Right	87.38 \pm 4.32	7.31 \pm 1.56
Group2	R(+) D	27.19 \pm 8.35	43.87 \pm 20.54
	R	83.44 \pm 4.23	11.12 \pm 4.41
Group3	R(-) D	80.68 \pm 7.84	13.07 \pm 7.54
	R	12.62 \pm 3.09	8.54 \pm 2.54
Group3	R(+) D	47.96 \pm 15.75	44.12 \pm 23.49
	R	81.63 \pm 10.81	5.60 \pm 3.13

R(-) : Rejection(-) D : Donor
R(+) : Rejection(+) R : Recipient

On the other hand, as shown in Fig. 1F, there was difficulty to separate mononuclear cells because of an increase in neutrophile to 10 to 100 fold. As a result, Ia positive cell could not be measured in infected lungs. Furthermore, in case with thrombus formation in the pulmonary vein, it was not feasible to detect enough to measure Ia-positive cells due to stiffness of the lung tissue.

4) The rate of Ia-positive lymphocyte in BAL to B-cell

The positive rates of Ia-cell in BAL of normal lung was $18.55 \pm 9.86\%$. That of group 1 ($16.63 \pm 3.93\%$) was similar to normal lung. It increased to $61.69 \pm 12.27\%$ ($p < 0.01$) in group 2 as well as in group 3 ($63.05 \pm 6.53\%$) although group 3 showed $24.49 \pm 8.71\%$ of Ia-positive rate as shown in Fig. 3.

The count of B-cell increased to $25.90 \pm 4.83\%$ in group 2 and to $24.47 \pm 5.95\%$ in group 3, although it remained within normal range in group 1.

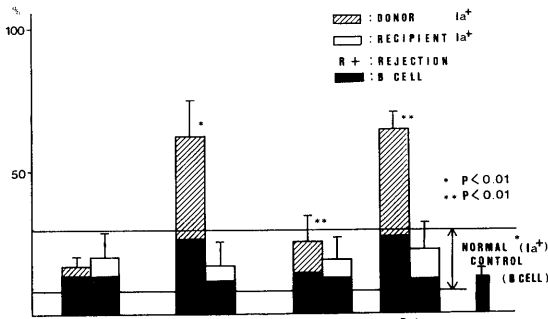


Fig. 3. Ia positive rate and B lymphocyte rate in BAL lymphocytes

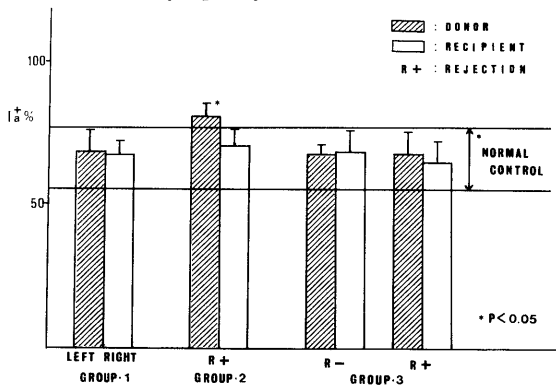


Fig. 4. Ia positive rate in BAL macrophages

5) Ia-positive rate of macrophage in BAL

The rates of Ia positive cell population increased to 69.16 ± 7.68 in group 1 and to $81.48 \pm 5.52\%$ in group 2 with statistical significance ($p < 0.05$), although it showed the normal range in group 1 as shown in Fig. 4.

However, there was no prominent change in group 3 between rejected lung ($66.65 \pm 8.30\%$) and non-rejected one ($68.92 \pm 3.23\%$).

On the other hand, the rate of Ia positive cell population on the contralateral lungs did not indicate the significant changes, that is, 68.04 ± 5.37 in group 1, 71.37 ± 6.35 in group 2, 65.64 ± 8.52 in group 3 and $69.26 \pm 8.20\%$ in contralateral lung of not rejected lung in group 3.

6) Influence of CsA on lymphocyte and macrophage in BAL

A) lymphocyte in BAL: It was confirmed beforehand that blastoid response of the lymphocyte in BAL reached a maximum by $10 \mu\text{g}$ PHA. After $10 \mu\text{g}$ PHA was mixed with $0.1, 0.5$ and $5.0 \mu\text{g}$ CsA in the lymphocyte in BAL, the Ia positive rates were $40.6 \pm 13.6\%$ at $0.1 \mu\text{g}$ CsA, $27.7 \pm 4.0\%$ at $0.5 \mu\text{g}$ CsA and $22.6 \pm 3.5\%$ at $5.0 \mu\text{g}$ with statistically significant reduction ($p < 0.05$) as shown in Fig. 5.

B) Macrophage in BAL: stimulation by a mixture of $10 \mu\text{g}$ PHA and $20 \mu\text{g}$ LPS for macrophage in BAL was carefully observed. The Ia positive rate increased to $70.1 \pm 7.0\%$ in contrast with $59.0 \pm 12.0\%$ in the control.

In the course of this response, the Ia positive rates were changed into $63.3 \pm 5.7\%$ by the addition of 0.1 ng CsA, $60.5 \pm 10.1\%$ by $0.5 \mu\text{g}$ CsA and $55.7 \pm 9.3\%$ by $1.0 \mu\text{g}$ CsA and $52.9 \pm$

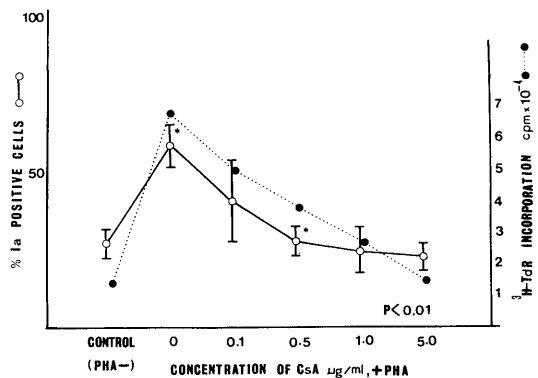


Fig. 5. Effect of CsA on Ia positive rate in BAL lymphocytes (in vitro)

11.4% by 5.0 μ g CsA, demonstrating some degree of inhibitive effect as shown in Fig. 6.

7) Histologic study at rejection

A) Evaluation of rejection response by H-E staining: According to the grades of the finding of perivascular mononuclear cell cuffing. In group 1, normal lung tissue structure was sustained. In group 2, there were two pneumonia, and by grading a finding of perivascular cuffing grade 1 was in one, grade 2 or 3 in eight. On the other hand, in group 3, one pneumonia, grade 0 in five, grade 1 in five and grade 2 or 3 in eight respectively.

B) Location of Ia positive cell in rejected lung: It was realized that the concentration of Ia-positive cells was consistent with the degree of the finding of perivascular cuffing in HE-staining and it was defined that Ia positive cells mainly concentrated to the perivascular

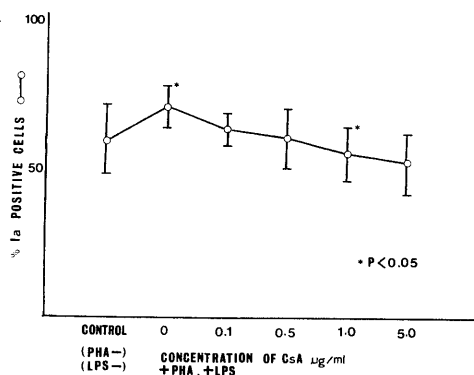


Fig. 6. Effect of CsA on Ia positive rate in BAL macrophages (in vitro)

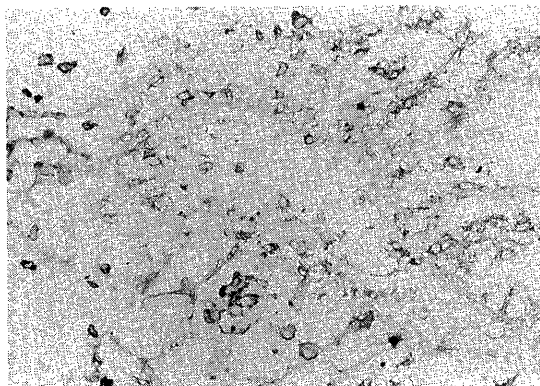


Fig. 7. Ia positive cells existed in a large part of perivascular mononuclear cells and occupied the alveolar space

mononuclear cells. However, Ia positive cells were in large part consisted of histiocyte and large lymphocyte as shown in Fig. 7 and also alveolar macrophages and large lymphocytes, which entered into the alveolar space, were selectively stained by Ia-staining.

On the contrary, when CsA was administered the Ia staining of perivascular monocyte in particular in the alveolar space was markedly inhibited and it was never potentiated even in the endothelial cells of vessels.

DISCUSSION

It is difficult to clarify the mechanism of rejection response following lung allotransplantation as compared with renal and heart transplantation.

It is characteristic of pulmonary infection which is susceptible due to immunosuppression as well as direct communication with contamination of air. It is not easy to discriminate rejection from lung infection. VEITH⁴⁾ reported that determination of rejection response at lung allotransplantation should be based on decrease of PaO₂, fever, leucocytosis, change in bacterial flora in sputum, increased shadow of the lung on chest x-p and cytology of BAL. FUJIMURA⁵⁾ stressed that spontaneous blastogenesis of lymphocyte is of most use to decide early. However, at present confirmation of rejection response depends on open lung biopsy. It is major concern about surgical insult as compared with needle biopsy which is applied to renal transplantation.

It is well known that there is major histocompatibility complex which plays a key role in causing organ rejection after allotransplantation. It is recognized that antigen complex is divided into the two antigens. These are called as class I and II antigen respectively⁶⁾. The class I antigen is widely distributed over almost all organs and cells although the class II antigen is limited in its distribution. On the other hand, it is noted that Ia-antigen, one of class II antigen, is presented by antigen presenting cells (APC) such as B-lymphocyte, activated T cell, a few macrophages, a few dendritic cells and also limited to endothelial cells of vessels and parenchymal cells of organ Ia anti-

gen not only acts as strong antigen against organ transplantation but also plays a major role in recognizing T-T, T-B, T-MO and T-APC each another.

Needless to say, it is defined that antj-Ia antigen can crossreact to Ia-antigen over different animal species²⁾. BAL technique is now used for clinical use in determining prophlogistic bacillus for lung infection, detecting malignant cells for malignant disease and diagnosing hypersensitivity and interstitial pneumonitis by using monoclonal antibody.

In the same way, application of flow cytometry is developing in the field of research in lung transplantation in terms of subset of lymphocyte, cellular RNA content of BAL & macrophage and phagocytic activity⁷⁾. It is apparent that changes in subset of lymphocytes is valid to elucidate immune response in the research area of renal and cardiac transplantation as cited by HIGASHI⁸⁾ *et al.* who insisted on valid monitoring of the ratio of helper/suppressor-cytotoxic T (TH/TS-C) which increased on day four, remained the same as prior value on day five and decreased on and after day six. However, there is few report concerning availability for application of Ia value of monocyte in the peripheral blood as well as BAL. It is generally accepted that some of Ia-cells represent strong phagocytosis with no antigen-presentation, others strong antigen-presentation with weak phagocytosis.

In the present study, the appearance rate of the Ia-cells in the monocytes of the peripheral blood decreased in the rejected lung on day four although it increased in macrophage in BAL and in the tissues. It means that there was a recruitment of Ia positive cells from the peripheral blood to the rejected lung. Iro⁹⁾ pointed out that macrophages must be much more activated for the sake of phagocytosis despite an decrease in production of intracellular RNA. It is consistent with significant elimination of the appearance rate of Ia cells in the monocyte of the peripheral blood.

Therefore, the measurement of Ia positive cells is of value to monitor the immune response with less trouble based on the methods.

In this study, it also is emphasized that cell analysis of BAL by means of spectrum III is

compatible with cell infiltration, filling with the rejected lung as seen by histologic examination. Furthermore, it is of interest to consider that the origin of increasing Ia-cells in the rejected lung is derived from activated T cells which is converted into Ia antigen bearing cells¹⁰⁾ on the basis of the result that Ia positive cells of the lymphocytes obviously increased in the rejected lung with statistical significance in spite of slight increase in B-cell count.

The main cells, fulfilled the transplanted organs, are activated cytotoxic T (Tc) as shown in lowering TH/TS-C¹¹⁾. It is obviously noticed that B-cell also plays a key role in the rejection process of transplanted organ by interaction of lymphotoxin which depends on complement. It appears that an increase in B-cell population in BAL may be a sign of rejection.

It is well known that the role of the macrophage is to present antigenicity to Th and to activate Th by interaction of interleukin 1 (IL-1)¹²⁾.

CsA is effective in inhibiting the appearance of Ia-cells and the recruitment of activated T-cells. It is assumed that CsA may blockade the antigenic signal which is transmitted to the cells and may selectively inhibit Tc action¹³⁾. According to the other consideration, CsA may be inhibit an interrelation between the lymphocytes by inhibition of IL-2 production from Th¹⁴⁾.

It is accepted that CsA acts to inhibit the release of IL-1 from the macrophage. Meanwhile, the appearance of Ia-positive cells was suppressed by CsA in this series.

It also is generally known that the action of OK-Ia1, which is known to be one of the parameters which indicates lymphocyte activity, proportionates to the response to PHA¹⁵⁾, which responds over animal species to the lymphocytes among man, rat and dog one another. It is confirmed in this study that there is close relationship in the appearance rate between activated lymphocyte and Ia-positive cells. MIYAGAWA *et al.*¹⁶⁾ reported that the appearance rate of Ia-cells in the lymphocytes activated by PHA was inhibited by giving 5 μ g/ml CsA. It is consistent with a result obtained in this study. The inhibition of the appearance rate of the macrophage activated by CsA is similarly

shown by in vitro as well as in vivo study. It also is reported that an expression of Ia-antigen by macrophage is not influenced by 90% inhibition of it¹⁷⁾. From the standpoint of the role of the lymphocyte and the macrophage concerning exhibition of Ia antigen by the macrophage is of great benefit not only in monitoring the rejection response after organ transplantation but also in treating with anti-Ia antibody as OTSUBO *et al.*¹³⁾ have treated in the field of renal transplantation in dogs.

It is defined that perivascular cuffing referred to as a histologic finding of rejection¹⁹⁾ is composed of large number of small lymphocytes with a few plasma cells and large blastoid cells which are liable to be stained by pyronin²⁰⁾. Advances in rejection process makes cell infiltration of perivascular cuffing progressed into the bronchial wall and lumen with destruction of the bronchial wall, finally invading into the alveolar space. This fact suggests that cell population and identification of BAL are of help to know the ongoing rejection response in conjunction with histologic examination. It is of interest to emphasize that we can be aware of the initiation of rejection process by a finding of increasing appearance of Ia cells, which is marked in grade 2 or 3 in spite of reversible fluctuation in grade 1³⁾, RNA index and phagocytic activity⁷⁾. The Ia cells are commonly appearing in rejected renal grafts, demonstrating that T-cells tend to be activated when exposed to antigen. It is explained by ISHIKURA²¹⁾ that the immune response is transmitted to Th by passenger and/or host strain dendritic cells, thereafter, Tc precursors is changed into Tc by humoral factor produced by Th and consequently Tc attacks the target cells. It is generally accepted that Ia antigenicity is induced by lymphokine²²⁾. In the mixed culture of the endothelium of the vessels with the lymphocytes the supernatant revealed a high positive rate of Ia, which is inhibited by the addition of CsA. Ia antigen is presented in the vessel endothelium and methangium in the rejected renal graft. The augmentation of Ia antigenicity in the endothelium of the vessels and in the epithelium of the renal tubulae, which is caused by the release of INF- γ is one of the rejection signs. It is a reflection that matching of Ia

antigenicity between donor and graft is essential to get favorable graft survival.

As a result of study on the origin of antigen-presenting cells (DC) it is defined that donor DC is required for induction, maintenance and acceleration of the rejection response²³⁾, that is donor passenger DC is necessary for initiation of acute rejection response²⁴⁾ and it migrates via vascular network of the kidney, spleen and bone marrow²⁵⁾, activating T-lymphocyte and recruiting the recipient DC. It seems worth while to consider that large number of donor DC migrate to the spleen, so is splenectomy effective to extend the graft survival time on day 3 to 5 following lung allotransplantation.

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