

## Ia Expression and RNA Content in BALF of Allotransplanted lungs in Dogs

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**ABSTRACT:** Ia expression and RNA content in BALF and peripheral blood were evaluated on the condition of combination therapy of Cyclosporine (CsA), Azathioprine (Azp), and Predonine (Pr) following lung allotransplantation in dogs.

1) Ia expression of lymphocytes in BALF was facilitated according to the appearance of rejection and depressed according to the disappearance of rejection. Therefore, it is indicated that Ia expression is one of the valuable monitorings to determine the appearance of rejection.

2) The low RNA content of macrophages in BALF and monocytes in the peripheral blood showed regardless of the appearance of rejection or not. It was of no use to determine rejection on the condition of prescribing CsA.

### INTRODUCTION

Much progress has been made towards improvement of the result of lung transplantation according to prevalence of the use of CsA as an immunosuppressive drug. Therefore, the incidence of graft failure has become diminished. The major items of concern involve how to early and accurate detection for rejection of which histologic finding of bronchiolitis obliterance is taken into consideration. It is accepted that histologic finding from the specimen obtained by open lung biopsy, is valid to precisely make a diagnosis for rejection. However, it is necessary for detecting less invasive and less simple method than open lung biopsy<sup>1)</sup>. Nakano<sup>2)</sup> reported that positive rates of Ia-expression of lymphocytes in BALF are increased at rejection, and Itoh<sup>3)</sup> reviewed that RNA content of BALF macrophages increased with rejection.

The purpose of this study is to certify significance of the measurement of Ia-expression and RNA content in macrophages and lympho-

cytes in BALF under the combination therapy of CsA, Azp and Pr to detect rejection at early stage of lung transplantation in dogs.

### MATERIAL AND METHOD

#### 1. Transplantation

Left lung allotransplants were performed in 25 mongrel dogs (each weighing 8 to 16kg). After the animals were anesthetized with intravenous administration of pentobarbital (25mg/kg). The dogs were mechanically ventilated by Haverd ventilator through the tracheal tubes intubated orotracheally at a rate of 12 to 14/min, and a respiratory volume of 300 to 400ml. Left thoracotomy at the fifth intercostal space was performed, and left lung in recipient was resected. After the left lung was dissected under heparinization, the graft was transplanted by anastomosing left atrium, pulmonary vein and bronchus of graft to recipient. 5-0 proline was used for anastomosis to the left atrium, 6-0 proline for pulmonary vein, and also 4-0 proline for bronchus. The ischemic time of the donor lung was

within 60min in all dogs. Through upper abdominal laparotomy after lung transplantation, the omental pedicle was prepared for wrapping of bronchial anastomosis.

All recipient had plain chest roentgenograms performed every other days after trasplantation, and every days after 15 th p. o. d.

After fiberoptic bronchoscope was performed befor and 7, 14, 21, days after operation, and pre and post pulse therapy. BALF was colcted through the channel of bronchoscope and peripheral blood was taken. Ia pasitive rate and RNA content of BALF and peripheral blood measured. At the same time, open lung biopsy was performed.

Antibiotic; AB-PC 1gr was administered for 7days after transplantation, and for 3days after open lung biopsy.

## 2. Use of immunosuppressive drugs

Immunosuppressive regimen was administered orally every days from transplantation to death including sacrifice. transplantation~7P. O. D.

CsA	20mg/kg/day
AZP	2.5mg/kg/day

8P. O. D.~14P. O. D.

CsA	20mg/kg/day
AZP	2.5mg/kg/day
Pr	0.5mg/kg/day

15P. O. D.~sacrificial day

CsA	10mg/kg/day
AZP	2.5mg/kg/day
Pr	0.5mg/kg/day

After day 15 CsA was reduced. Recipient was treated with intraveous methylprednisolone, 250mg/day daily for 3days when episodi of allograft rejection was suspected by chest roentgenograms.

## 3. Collection of BALF

BALF was taken under general anesthesia through fiberoptic bronchoscope. After bronchoscope was inserted until lobal bronchus of allograft, 30ml saline at once was injected through channel of bronchoscope, and sucked until 100ml of recovery were obtained.

## 4. Separation of mononuclear cell from peripheral blood and BALF

The samples of BALF filtrated three times with sponge, and peripheral blood were putted up Ficoll-Conray fluid, adjusted to 1.077 specific

gravity and p.H 7.2, and centrifuged at 1700 rpm for 30min. Mononuclear cell alone was separated, and floated in PBS.

## 5. Measurement of Ia positive rate in lymphocytes and monocytes of peripheral blood

10 $\mu$ l of OKIa (O1th. Co.), as being primary antibody through 10 $\mu$ l of mouse IgG was added to 200 $\mu$ l of peripheral blood and cultured at 4°C for 30min. Then as scndary antibody, additional 100 $\mu$ l of FITC, conjugated Goat antimouse IgG, was added, and cultured at 4°C for 30min. Only secondary antibody was added and cultured at 4°C for 30min for the negative control. After making hemolysis, 2ml of a medium 10%FCS-RPMI 1640 was added, and Ia positive rate was measured by Spectrum III (Flowcytometry, O1th. Co.).

## 6. Measurement of Ia positive rate in lymphocytes and macrophages of BALF

10 $\mu$ l of OKIa antibody was added to 200 $\mu$ l of BALF, that was adjusted to 5 $\times$ 10<sup>6</sup>/ml, and cultured at 4°C for 30min, as same as peripheral blood. Then 100 $\mu$ l of FITC conjugated Goat antimouse IgG, as secondary antibody, was added and cultured at 4°C for 30min. Only secondary antibody was added to 200 $\mu$ l of BALF and cultured at 4°C for 30min for the negative control. After this reaction, 2ml of 10%FCS-RPMI medium was added, and Ia positive rate measured by Spectrum III.

## 7. Measurement of RNA content in monocytes of prephral blood and macrophanges of BALF

2 $\times$ 10<sup>6</sup> mononuclear colls were fixed in ethanol-aceton (ethanol: aceton=1:1), and stained with acridine orange<sup>4)</sup>. RNA content was measured by FACS IV (Flowcytometry Becton Dickinson. CO.). **Fig. 1** represented DNA histogram and RNA histogram of BALF macrophages by FACS IV. RNA Peakchannel regaded as RNA content.

## 8. Histologic evaluation of rejection

Any specimen of allograft, which was taken by open lung biopsy, were fixed in 10%formalin. Histologic examination of the lungs tissue was made by staining with Hematoxillin-Eosin for episod and degree of rejection. The degree of rejection was evaluated by Venth's classification<sup>5)</sup>.

## 9. Analysis of data

All data were represented by mean $\pm$ standard deviation, and estimated by Wilcoxon test.

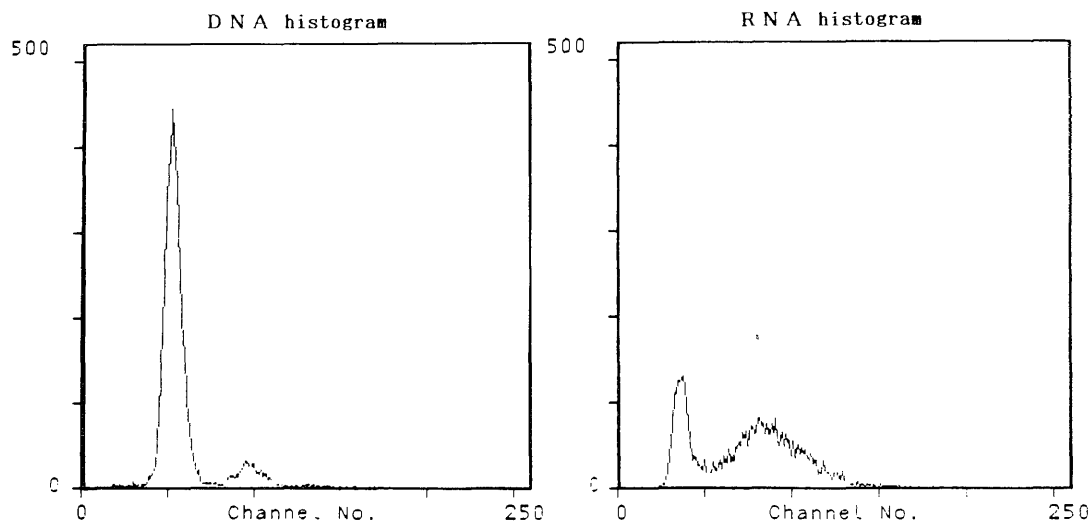


Fig. 1. DNA histogram and RNA histogram of BALF cell

## RESULT

1) Ia-expression of lymphocytes in peripheral blood (Fig. 2)

The positive rate of Ia-expression of lymphocytes showed  $61.0 \pm 13.7\%$  ( $n=7$ ) preoperatively. On day 7 after lung transplantation, under double therapy, CsA and AZP, it was  $72.6 \pm 14.2\%$  in case without rejection and  $71.4 \pm 13.3\%$  in case with rejection, indicating a slight increase in positive rate of Ia-expression without statistically significant difference.

On the other hand, on day 14, it was  $55.5 \pm 30.0\%$  in case without rejection and  $73.0 \pm 17.8\%$  in case with rejection when used combination with pr.

Meanwhile, only the dosis of CsA was reduced on day 21, it was  $70.0 \pm 4.7\%$  in case without rejection and  $76.8 \pm 10.4\%$  in case with rejection. There was not significant differences between cases with and without rejection.

2) Ia-expression of monocytes in peripheral blood (Fig. 3)

The positive rate of Ia-expression of monocytes in the peripheral blood was  $35.4 \pm 10.0\%$  prior to lung transplantation. On day 7, it was  $47.3 \pm 12.8\%$  in case without rejection and  $43.2 \pm 10.0\%$  in case with rejection, showing the slight increase in the Ia-expression rate. In contrast,

on day 14, it was  $40.3 \pm 24.4\%$  in case without rejection and  $46.7 \pm 4.6\%$  in case with rejection, thereafter, on day 21 it was  $64.7 \pm 7.7\%$  in case without rejection, and  $46.5 \pm 7.7\%$  in case with rejection indicating definite increase without significant variation.

3) Ia-expression of lymphocytes in BALF (Fig. 4)

The positive rate of Ia-expression of lymphocytes in BALF was  $6.5 \pm 3.5\%$  prior to lung

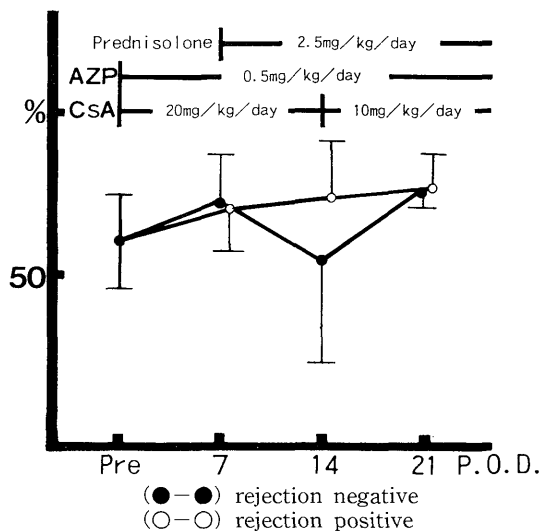


Fig. 2. Ia positive rate of peripheral blood lymphocyte

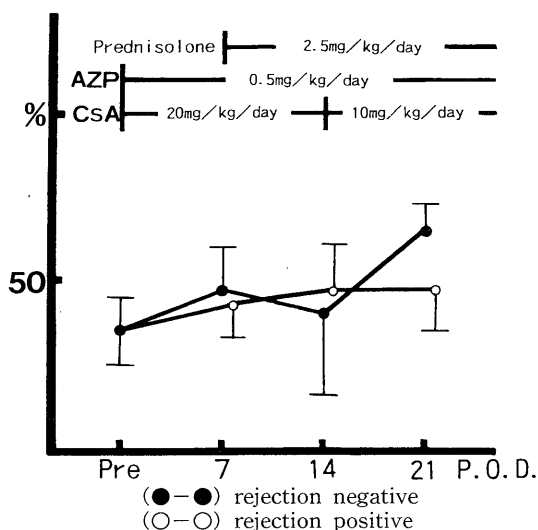


Fig. 3. Ia positive rate of peripheral blood monocyte

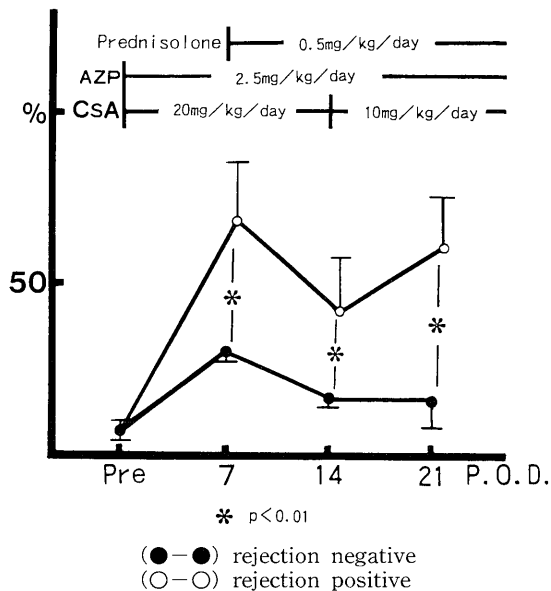


Fig. 4. Ia positive rate in BALF lymphocyte

transplantation. On day 7 it increased to  $30.5 \pm 3.0$  in case without rejection and  $68.1 \pm 17.2\%$  in case with rejection, showing statistically significant difference ( $p < 0.05$  and  $p < 0.005$ ) respectively. On day 14, it was  $16.3 \pm 1.5\%$  in case without rejection and  $41.7 \pm 15.4\%$  in case with rejection with elapse of 7 days when Prd was prescribed ( $p < 0.02$ , and  $p < 0.05$ ). On day 21, it was  $16.4 \pm 8.0$  in case without rejection and  $62.2 \pm 15.2\%$  in case with rejection, showing significant increase in those with rejection.

There showed statistically significant difference ( $p < 0.01$ ) between cases with and without rejection in every measurement.

Fig. 5 showed a relationship between Ia-expression and rejection response. On day 7, the positive rate of Ia-expression much more increased in the group with severe rejection than with slight one. On the contrary, on day 14, and 21 much more decreased in severe one than in slight one without statistically significant difference.

4) Ia-expression of macrophages in BALF (Fig. 6)

The positive rate of Ia-expression was  $62.3 \pm 25.6\%$ . On day 7, it was  $15.0 \pm 4.8\%$  in case without rejection and  $26.8 \pm 14.4\%$  in case with rejection with statistical significance ( $p < 0.025$ ). On the other hand, on day 14, it was  $82.6 \pm 14.7\%$  in case

without rejection and  $47.1 \pm 23.5\%$  in case with rejection, indicating recovery to the preoperative values. On day 21, it was  $63.5 \pm 2.5\%$  in case without rejection and  $52.3 \pm 15.0\%$  in case with rejection, showing an increase as compared with those with positive cases, in contrast, a decreased as compared with those with negative cases. However, there was no definitive difference between with and without rejection.

5) RNA content of monocytes in the peripheral blood (Fig. 7)

The RNA content of monocytes in the peripheral blood was  $45 \pm 8.5$ . On day 7, it was  $47 \pm 12.1$  in case without rejection,  $53 \pm 11.3$  with rejection, on day 14,  $48 \pm 5.9$  and  $50.5 \pm 16.3$ , and on day 21  $43 \pm 13.3$  and  $45 \pm 12.3$ , indicating not significant difference and not any difference between with and without rejection.

6) RNA content of macrophages in BALF (Fig. 8)

RNA content of macrophages was  $87 \pm 24.5$  prior to transplantation, on day 7, it was  $77 \pm 21.9$  in case without rejection and  $53.8 \pm 10.9$  in case with rejection, on day 14,  $61 \pm 15.5$  and  $73.8 \pm 31.3$  and also on day 21,  $61 \pm 15.5$  and  $73.8 \pm 31.3$  and also on day 21,  $61 \pm 2.8$  and  $38.3 \pm 5.8$ , demonstrating a decrease with time following lung transplantation without significant difference as well as not significant difference bet-

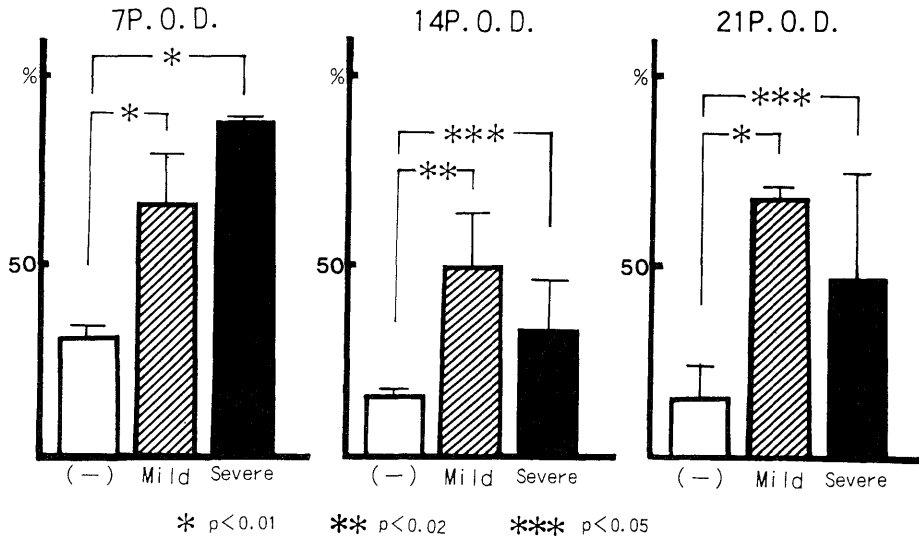


Fig. 5. Ia positive rate in BALF lymphocyte

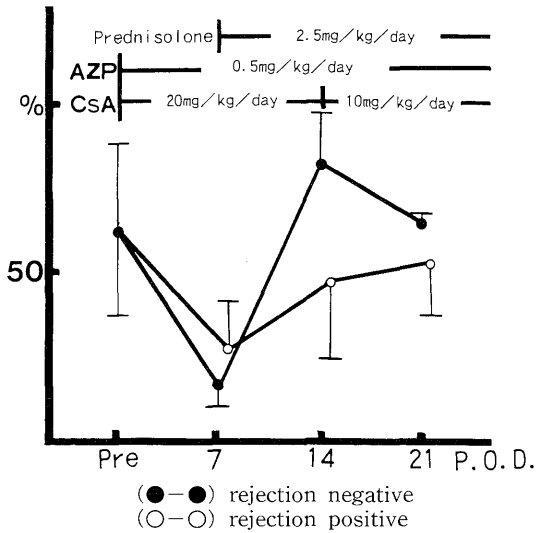


Fig. 6. Ia positive rate in BALF macrophage

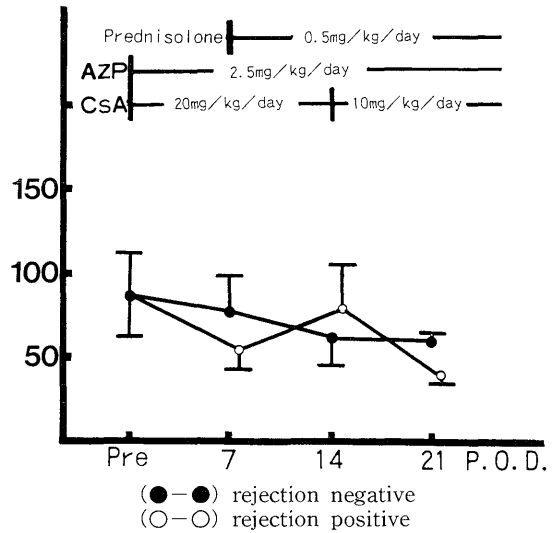


Fig. 7. RNA content of peripheral blood monocyte

ween with and without rejection.

7) Changes in Ia-expression by steroid pulse therapy (Fig. 9)

Histologic finding prior to prescription of steroid pulse therapy showed the slight degree of perivascular cuffing in two dogs and slight pneumonia in the remaining one. Following prescribing steroid pulse therapy, chest xp film showed elimination of infiltrative shadow and also disappearance of histologic findings, which

confirmed the appearance of rejection.

The positive rate of Ia-expression of macrophages in BALF and peripheral blood changed with a varying variety. In contrast, that of lymphocytes in BALF was reduced after completion of steroid pulse therapy.

DISCUSSION

It is well known that the outcome of organ

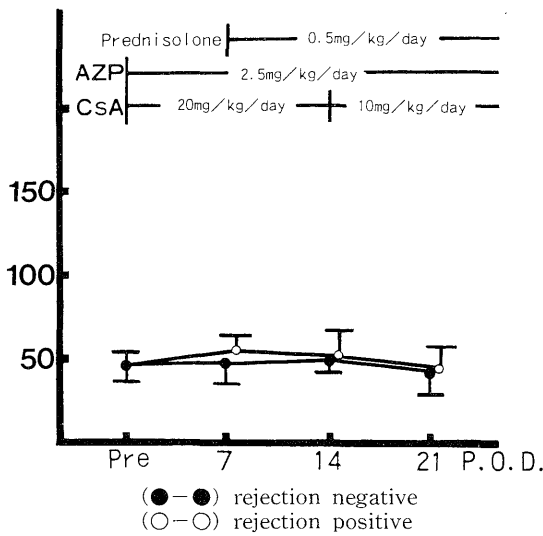


Fig. 8. RNA content in BALF macrophage

transplantation is closely related to antigenicity of major histocompatibility between a donor and a recipient.

Antigens of histocompatibility are classified into the two groups of class I and II according to functional characteristics, molecular structure and genetic structure.

It is generally accepted that class I antigen widely distributes to all organs and cells. On the contrary, Ia-antigen which is included in class II antigen is present in antigen — presenting

cells of B cells, a part of macrophages and dendritic cells, immunocompetent cells including a part of activated T cells, endothelial cells and parenchymal cells.

It is worthy of notice that Ia-expression is not only strong transplantation antigen but also plays a immunologically key role in recognition of mutual immunocompetent cells and interaction of mixed lymphocyte reaction and cell mediated lymphocytolysis<sup>6)</sup>.

It is well known that rejection phenomenon is produced by recognition of antigenicity by phagocytizing macrophages.

Antigens, which are phagocytized by macrophages, are digested into lysosomes. These again appear on the surface of cell membrane of macrophages as the Ia-antigen complex, presenting antigen to helper T(T<sub>H</sub>) and synthesize and generate interleukin 1(IL-1)<sup>7)</sup>. And so antigen-presented T<sub>H</sub> cells recognize Ia-antigen and are activated by stimulation of IL-1.

As a result, activated T<sub>H</sub> cells generate IL-2, followed by generation of antibodies by B-cells, proliferating and differentiating precursor cytotoxic T lymphocytes (P-CTL) into CTL. P-CTL are activated by homologous antigens on the surface of target cells and cause damage to target cells with help of CTL which is activated by IL-2<sup>8)</sup>.

Meanwhile, RNA synthesis which plays a key role in protein synthesis is enhanced at G<sub>1</sub> phase

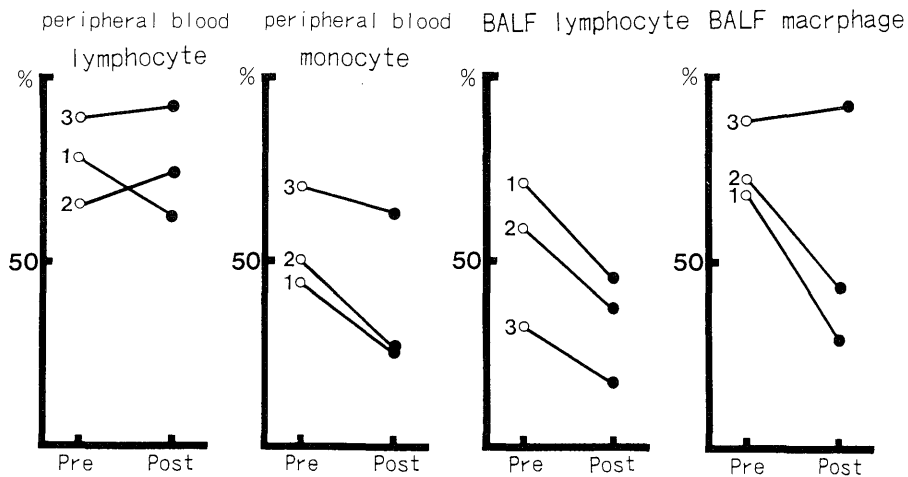


Fig. 9. Ia positive rate of peripheral lymphocyte, monocytes, BALF lymphocytes and BALF macrophage under the pulse therapy

of cell cycle<sup>9)</sup>. Most of lymphocytes and macrophages are present at G<sub>0</sub> phase. When these are activated by IL-1 and protein synthesis is initiated, transferring to G<sub>1</sub> phase and activating RNA synthesis including m-RNA. In matter of fact, RNA content has become increased, reflecting the grade of cell activation. Acute rejection phenomenon following lung transplantation is initiated by dotted findings of perivascular cuffing, followed by lung edema, fibrin deposition, alveolar bleeding and tissue necrosis<sup>10)</sup>.

Nakano<sup>2)</sup> reported that Ia-positive cells are present in the perivascular area at rejection and a presence of Ia-positive cells is inhibited by prescribing CsA. At present various immunosuppressive drugs are clinically used. However, CsA is of great value to improve the outcome of organ transplantation<sup>10, 11)</sup>. It is well known that CsA plays an important role in suppressing the immune response by inhibition of IL-1 generation and in releasing it from macrophages<sup>12)</sup>, IL-2 generation and release from activated T<sub>H</sub><sup>13)</sup> and do damage to activation of CTL (generation of IL-2 and mRNA<sup>14)</sup>) and also CsA is of great effect to inhibit RNA synthesis by macrophage, Ia expression by macrophages in BALF, lymphocytes and T-cell in the peripheral blood<sup>2, 3, 15)</sup>.

It is also accepted that Azp prevents DNA synthesis and directly caused damage to DNA synthesis in macrophages and T-cells, indicating immunosuppressive effects<sup>16)</sup>. On the other hand, although the action of steroid upon depression of the immune response is not so clear, much research work has been detailed that IL-1 generation by macrophage<sup>17)</sup>, IL-2 generation by T<sub>H</sub><sup>18)</sup>, glucose and protein metabolism in lymphocytes<sup>19)</sup> are uniformly inhibited. Meanwhile, many reports<sup>8, 15, 17, 20, 21)</sup> certifies that Ia expression of macrophages and lymphocytes also is depressed. In this study, Ia expression was depressed with elapsed time of 8 days following prescribing predonine.

In contrast, various kinds of immunosuppressive drugs have the side effects of nephrohepatotoxicity by CsA, bone marrow depression and hepatic dysfunction by Azp and delay in wound healing and disruption following infection at bronchial anastomosis by steroid<sup>22)</sup>.

Combination therapy of CsA, Azp and Pr is now prevalent.

In Toronto Lung Transplant Group<sup>23)</sup>, combination use of CsA and Azp is preoperatively applied and added anti-lymphocyte globuline in postoperative period. It is generally thought that one hesitate to use predonine until healing of bronchial anastomosis completes. In contrast, in case that rejection repeats on day 5 following allotransplantation, methylpredonine is used.

It is required that precise diagnosis of rejection should be done in early stage, identified it from infection. At present it is made by transbronchial, transcutaneous needle and/or open lung biopsies<sup>22)</sup>. Needless to say, open lung biopsy is the best way to precisely diagnose rejection<sup>24)</sup> although surgical insult is grave and frequent attempts are difficult to practically use. It is well known that the lung is directed outwards and has many chances to be affected by infection. BALT plays a significant role in defense mechanism against infection<sup>25)</sup> and lymphocytes in BALT are replaced with lymphocytes from a recipient and provide a chance in close contact with antigens<sup>26)</sup>. It is suggested that intrabronchial BALF originated from protruded BALT is of great benefit to determine the appearance of rejection. Saito<sup>27)</sup> pointed out the fact that the ratio of lymphocytes to total cells in BALF increases according to occurrence of rejection and also the ratio of T<sub>H</sub> to non-helper T(T<sub>n</sub>) decreases. Matsumura<sup>28)</sup> reported that the ratio of CD<sub>4</sub> to CD<sub>8</sub> in lymphocytes of BALF decreases in the progression of rejection. Other reports clarified that donor specific reactions to CML in lymphocytes of BALF and primed lymphocyte test are facilitated in accordance with progression of rejection<sup>29)</sup> and active oxygen species production in macrophages of BALF was increased with pneumonia and rejection<sup>30)</sup>. The fact that RNA content in macrophages in BALF and monocytes in the peripheral blood falls down in the combination therapy is indicating no valuable contribution to determination of the appearance of rejection phenomenon. It also indicates that there is no difference in Ia-expression between drugs used and the intensity of rejection and also no significant contribution to determination of rejection by the

examination of immunocompetent cells in the peripheral blood.

Ia-expression of BALF macrophages was significantly diminished at 7th day under therapy of CsA and AZP, and was recovered after used predonine together.

A few days following lung allotransplantation accord with the time when macropahges recognize the histocompatibility antigen they activate phagocytic activity and dissolve into peptide at the time of Ia-negative period<sup>7)</sup>. Therefore, one takes it into consideration that Ia-positive macrophages in BALF are very few.

On days 5 to 7 following lung allotransplantation, macropahges exhibit antigen on the surface of cells as a Ia-antigen complex. On day 7 and later, it is easily thought Ia-positive macrophages have become increased. In this series, Ia expression of lymphocytes in BALF was inhibited by instillation of steroid and increased by occurrence of rejection. It did not vary greatly regardless of the slight or severe degress of rejection. It is concluded that the measurement of Ia expression is of great value to know the appearance of rejection.

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