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Abstract

Reported clinical and experimental observations indicate that heart grafts in combined heart-lung transplantation are less frequently rejected than heart grafts transplanted alone. In order to elucidate the mechanism of this difference, twenty-eight inbred male Lewis rats receiving heterotopic allografts from inbred male Fisher rats were evaluated for surface markers of graft infiltrating lymphocytes (GIL) and peripheral blood lymphocytes (PBL) using flowcytometry. Monoclonal antibodies investigated in this study were W3/25 (anti-helper T lymphocyte), OX8 (anti-suppressor/cytotoxic T lymphocyte), OX39 (anti-interleukin 2 receptor), and OX6 (anti-MHC class II antigen). In the acute study, a heart transplanted group (n = 7) and a heart-lung transplanted group (n = 7) without immunosuppression were studied. In the chronic study, cyclosporine (10 mg/kg/day i.m.) were administered in the heart transplanted group (n = 7) and the heart-lung transplanted group (n = 7). Both in the acute and chronic studies, the proportion of W3/25 positive cells in GIL of heart grafts of the heart transplanted group was significantly higher than that of heart grafts and lung grafts of the heart-lung transplanted group. OX8 positive cell proportion in GIL of heart grafts and lung grafts of the heart-lung transplanted group were significantly higher than that of heart grafts of the heart transplanted group. These results lead us to speculate that suppressor T lymphocytes are an important distinguishing factor in the rejection processes of heart allografts and heart-lung allografts as observed in clinical experience.

KEYWORDS: rejection, heart transplantation, heart-lung transplantation, lymphocyte subsets, flowcytometry

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Difference of Rejection between Heart and Heart-Lung Transplantation in Rats: Flowcytometric Analysis of Graft Infiltrating Lymphocyte Subsets

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Reported clinical and experimental observations indicate that heart grafts in combined heart-lung transplantation are less frequently rejected than heart grafts transplanted alone. In order to elucidate the mechanism of this difference, twenty-eight inbred male Lewis rats receiving heterotopic allografts from inbred male Fisher rats were evaluated for surface markers of graft infiltrating lymphocytes (GIL) and peripheral blood lymphocytes (PBL) using flowcytometry. Monoclonal antibodies investigated in this study were W3/25 (anti-helper T lymphocyte), OX8 (anti-suppressor/cytotoxic T lymphocyte), OX39 (anti-interleukin 2 receptor), and OX6 (anti-MHC class II antigen). In the acute study, a heart transplanted group (n = 7) and a heart-lung transplanted group (n = 7) without immunosuppression were studied. In the chronic study, cyclosporine (10 mg/kg/day i.m.) were administered in the heart transplanted group (n = 7) and the heart-lung transplanted group (n = 7). Both in the acute and chronic studies, the proportion of W3/25 positive cells in GIL of heart grafts of the heart transplanted group was significantly higher than that of heart grafts and lung grafts of the heart-lung transplanted group. OX8 positive cell proportion in GIL of heart grafts and lung grafts of the heart-lung transplanted group were significantly higher than that of heart grafts of the heart transplanted group. These results lead us to speculate that suppressor T lymphocytes are an important distinguishing factor in the rejection processes of heart allografts and heart-lung allografts as observed in clinical experience.

Key words : rejection, heart transplantation, heart-lung transplantation, lymphocyte subsets, flowcytometry

Despite improvements in immunosuppressive therapy since the introduction of cyclosporine, rejection of transplanted hearts and lungs continues to be a serious problem. Clinical and experimental observations indicate that heart grafts in combined heart-lung transplantation are

less frequently rejected than heart grafts transplanted alone (1-3). In order to elucidate the mechanism of this difference, we investigated intragraft event by flowcytometric analysis of graft infiltrating lymphocytes from rat heart and combined heart-lung allografts.

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Materials and Methods

Animals. For this study, two different strains of male inbred rats were selected: Lewis (LEW)(PT1¹) weighing 250–400 g as recipients and Fisher (F344)(PT1^{1v1}) weighing 125–250 g as donors. All animals were purchased from Charles River Japan Inc., Japan. All animals received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Science and published by National Institutes of Health (NIH Publication No. 86–23 revised 1985).

Surgical procedure. The intra-abdominal heart transplantation performed was a modification of the method described by Ono and Lindsey (4). The Fisher rat was first anesthetized by inhalation of ether. After a median laparotomy, the donor rat was heparinized at a dose of 1 mg/kg from inferior vena cava. Anterior thoracotomy was followed by a bolus injection of St. Thomas cardioplegic solution from inferior vena cava. Afterward the cardioplegia was injected from the descending aorta and cardiac arrest was accomplished. The ascending aorta and pulmonary artery were transected and the three venae cavae and left atrium were ligated and divided, and then procurement of the graft heart completed. The graft heart was then immersed in cold saline. The recipient Lewis rat was anesthetized by ether inhalation. The median laparotomy was performed and abdominal aorta and inferior vena cava were dissected freely beneath the renal branches. The abdominal aorta and inferior vena cava were cross-clamped independently. Aorto-aortic end-to-side anastomosis and pulmonocaval end-to-side anastomosis were performed with 8-0 Prolene continuous sutures.

The intra-abdominal heart-lung transplantation was performed according to the method described by Lee *et al.* (5). The donor Fisher rat was first anesthetized by inhalation of ether. After a median laparotomy, the donor rat was heparinized at a dose of 1 mg/kg from inferior vena cava. Anterior thoracotomy was followed by a bolus injection of cold saline solution from the inferior vena cava. Afterward St. Thomas cardioplegic solution was injected from descending aorta and cardiac arrest was accomplished. The thymic lobe was dissected from right superior vena cava and the vein was ligated and divided. The azygos vein with the left superior vena cava and the inferior vena cava were ligated and divided. The aorta was transected at the origin of the innominate artery. The

heart and lungs were removed after the trachea was divided at its bifurcation. The graft heart-lung mass was then immersed in cold saline. The recipient Lewis rat was anesthetized by ether inhalation. The median laparotomy was performed and the abdominal aorta was dissected freely beneath the renal branches. The abdominal aorta was cross-clamped and aorto-aortic end-to-side anastomosis was performed with 8-0 Prolene continuous suture. The trachea remained open in the abdominal cavity.

Transplanted rats were divided into 4 groups as follows: For acute study, heart allograft ($n=7$) and heart-lung allograft recipients ($n=7$) which did not receive immunosuppression. For chronic study, heart allograft recipients treated with cyclosporine 10 mg/kg/day i.m. ($n=7$) and heart-lung allograft recipients treated with cyclosporine 10 mg/kg/day i.m. ($n=7$).

Graft survival was assessed by daily palpation of heart pulsation and the cessation of pulsation was diagnosed as graft rejection. All grafts without immunosuppression were rejected and grafts were removed on the day of rejection. All grafts of immunosuppression groups achieved long-term survival of more than 70 day, and were then sacrificed by terminal ether anesthesia on 71st day and grafts were removed.

Histological examination. The basal half of the graft heart and lung were fixed by 10 % neutral buffered formalin and stained with hematoxylin and eosin for microscopic examination. Graft rejection was graded according to the International Society for Heart Transplantation (ISHT) standardized grading system(6, 7).

Lymphocytic surface marker analysis. Flow-cytometric analysis of lymphocytes, using EPICS model 753; Coulter Electronic Co., was performed using both peripheral blood lymphocytes (PBL) and graft infiltrating lymphocytes (GIL).

PBL preparation. PBL was prepared from heparinized blood using Lympholyte-R (Cedarlane Co.). Blood was overlaid on Lympholyte-R and centrifuged at 2,200 r. p. m. for 30 min in room temperature. The layer of lymphocytes was carefully aspirated. Lymphocytes were suspended in RPMI 1640 culture medium containing 1 % of fetal calf serum and 0.1 % NaN_2 .

GIL preparation. The apical half of graft heart and the small segment of graft lung were minced independently by tissue homogenizer and cells were released according to the method of Totterman *et al.* (8). The tissue homogenate was incubated with 3 ml of digestion medium (20 mM Hepes Buffer from Sigma, 136 mM NaCl, 4.7 mM KCl, 0.65 mM MgSO_4 , 1.2 mM CaCl_2 , pH 7.45)

containing collagenase 2 mg/ml, DNase 0.05 mg/ml and 1.5 % bovine serum albumin. After agitation and incubation at 37°C for 1 h, cells were filtered through nylon mesh (100 μm^2) to remove aggregates and overlaid on Lympholyte-R. After centrifugation at 2,200 r.p.m. for 20 min cells in the lymphocytic layer were carefully aspirated and suspended in the same medium as PBL (9).

Antibody incubation. The lymphocyte suspension of PBL and GIL were incubated with the optimal concentration of monoclonal antibodies at 4°C for 30 min in a dark incubation chamber. The following lymphocyte surface markers were studied: W 3/25 anti-helper T lymphocyte antibody labelled with fluorescein (FITC), OX 8 anti-suppressor/cytotoxic T lymphocyte antibody labelled with phycoerythrin (PE), OX 39 anti-interleukin 2 receptor labelled with PE, OX 6 anti-MHC class II antigen labelled with FITC. All the statistic analysis was performed by ANOVA test (10).

Results

In the acute study, all grafts were rejected. Graft survival was 16.1 ± 1.1 days in heart allograft group and 22.1 ± 1.4 days in heart-lung allograft group. The heart-lung allograft group showed a statistically significant prolongation in cardiac survival ($p < 0.05$). In the chronic study, all grafts achieved long-term survival of more than 70 days in both heart allograft group and heart-lung allograft group (Table 1).

The histological studies demonstrated that the

rejection of the heart grafts of the heart allograft groups was more advanced than the heart-lung allograft groups in both acute and chronic studies (Fig. 1).

In the acute study, the specimens of heart grafts from heart allograft (Fig. 2A) and heart grafts from heart-lung allograft (Fig. 2B) showed severe rejection. Lung graft tissues showed severe rejection (Fig. 2C). In the chronic study, the specimens of heart grafts from allograft (Fig. 3A) and heart grafts from heart-lung allograft (Fig. 3B) showed mild rejection. Lung graft tissues showed bronchiolitis obliterans and vasculitis (Fig. 3C).

Flowcytometric analysis of lymphocytic surface markers was studied in PBL, heart GIL and lung GIL. These markers did not differ significantly in flowcytometric analysis of PBL (Fig. 4). GIL surface markers showed difference between heart grafts of heart allograft and heart and lung grafts of heart-lung allograft in both acute and chronic studies. In the acute study, W 3/25 positive cell proportion in GIL, helper T lymphocyte subpopulations, were significantly

Table 1 Profile of heart and heart-lung transplantation

Allografts ^a	Graft survival (Days)
Acute study	
(No immunosuppression)	
H T ^b	15, 15, 16, 16, 16, 17, 18
H-L T ^c	20, 21, 22, 22, 23, 23, 24
Chronic study	
(Cyclosporine : 10 mg/kg/day, i. m.)	
H T ^b	All grafts > 70
H-L T ^c	All grafts > 70

a: Fisher rats are donors and Lewis rats are recipients.

b: Heart transplantation

c: Heart-lung transplantation

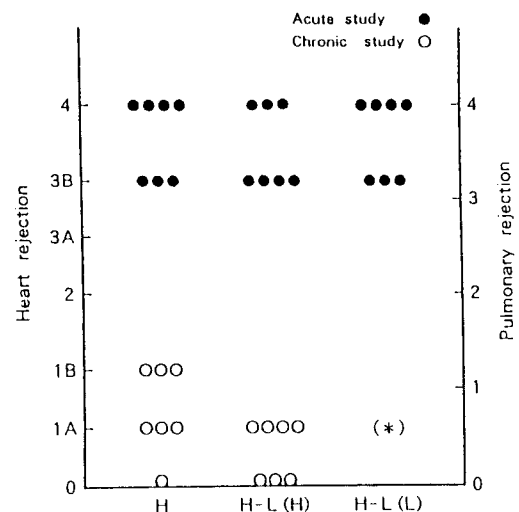


Fig. 1 Rejection grade of the grafts. Graft rejection was graded according to ISHT standardized grading system at acute study (●) and chronic study (○). (*) Chronic lung rejection in lung grafts of heart-lung allograft showed chronic airway rejection and chronic vascular rejection.

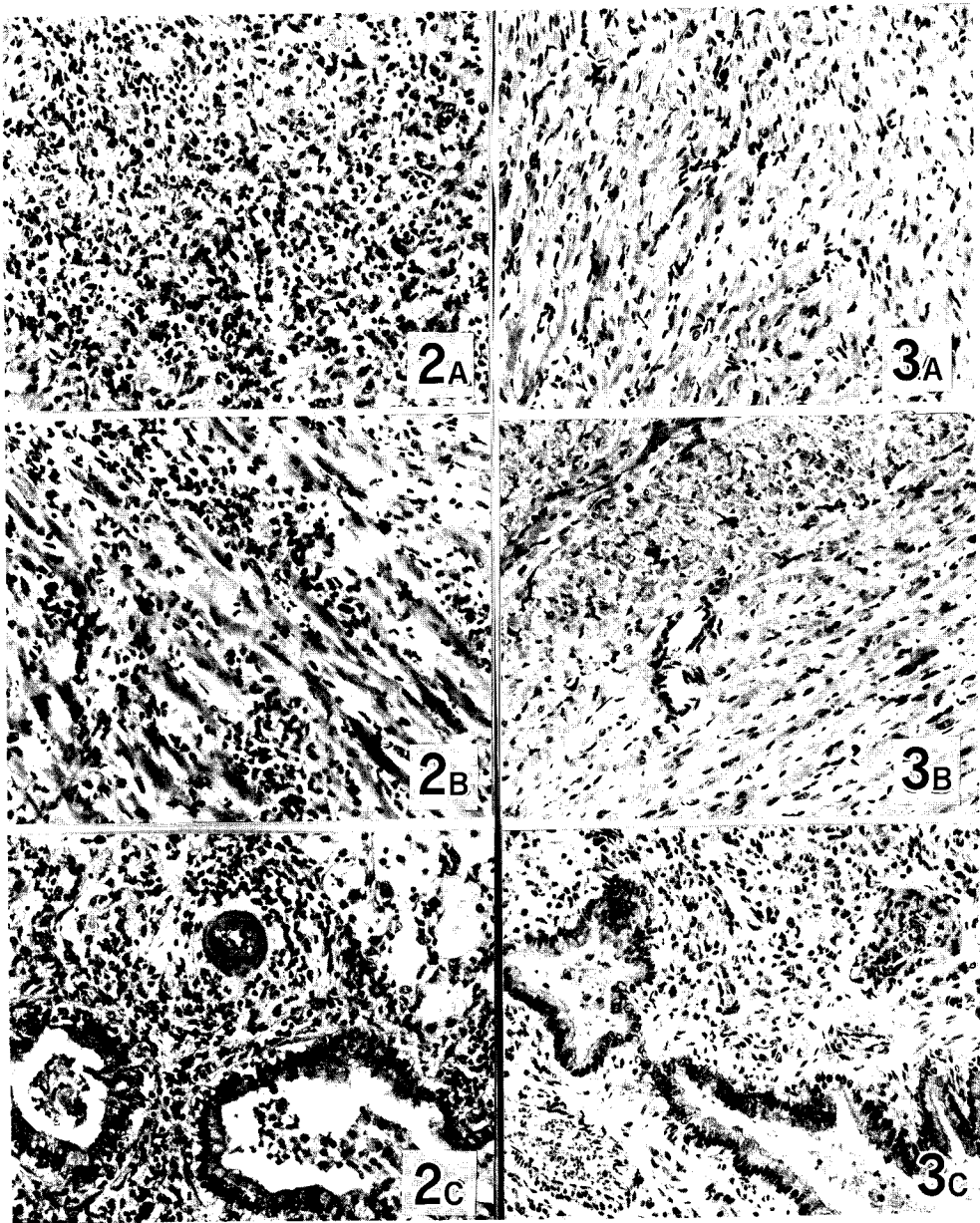


Fig. 2 Acute rejection. **A:** Heart graft of heart transplantation (HT). Myocyte damage, edema and an aggressive polymorphous inflammatory infiltrate are shown. Grade 4 (HE × 100) ; **B:** Heart graft of heart-lung transplantation (H-L T). Diffuse inflammatory process with focal myocyte damage and edema are shown. Grade 3B (HE × 100) ; **C:** Lung graft of H-L T. Diffuse perivascular, interstitial and peribronchiolar infiltrate round cells and alveolar pneumocyte damage are shown. (HE × 100)

Fig. 3 Chronic rejection. **A:** Heart graft of HT. Diffuse perivascular and interstitial lymphocyte infiltrate without myocyte damage are shown. Grade 1B (HE × 100) ; **B:** Heart graft of H-L T. Focal perivascular and sparse infiltrate of lymphocytes without myocyte damage are shown. Grade 1A (HE × 100); **C:** Lung graft of H-L T. The lumen of the airway is obliterated and the wall of arteries and arterioles are thickening. (HE × 100)

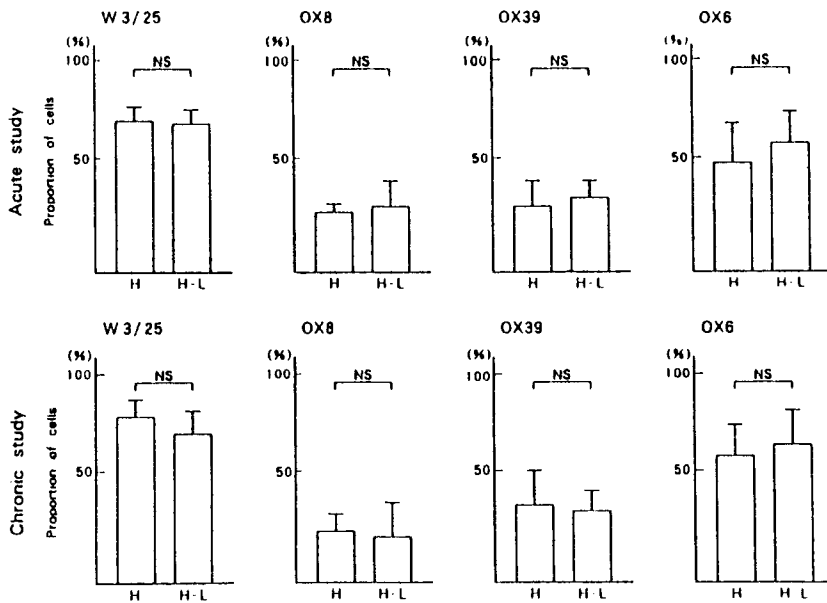


Fig. 4 Flowcytometric analysis of peripheral lymphocyte (PBL) subsets. H: PBL of heart allograft group. H-L: PBL of heart-lung allograft group.

Acute study

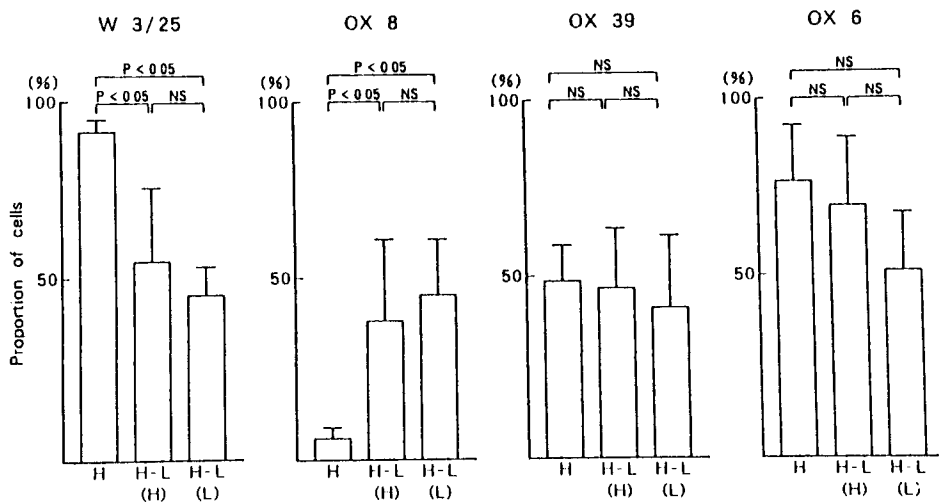


Fig. 5 Flowcytometric analysis of graft infiltrating lymphocyte (GIL) subsets in acute study. H : GIL in heart grafts of heart allograft group. H-L (H): GIL in heart grafts of heart-lung allograft group. H-L (L): GIL in lung grafts of heart-lung allograft group.

Chronic study

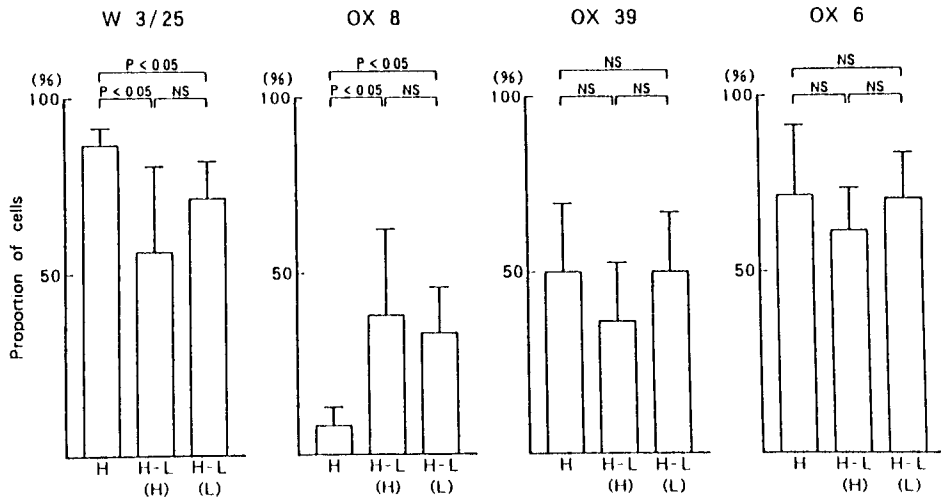


Fig. 6 Flowcytometric analysis of graft infiltrating lymphocyte (GIL) subsets in chronic study. H: GIL in heart grafts of heart allograft group. H-L (H): GIL in heart grafts of heart-lung allograft group. H-L (L): GIL in lung grafts of heart-lung allograft group.

higher in heart grafts ($91.8 \pm 3.9\%$) of the heart allograft group than either heart grafts ($55.2 \pm 19.7\%$) or lung grafts ($45.7 \pm 8.2\%$) of the heart-lung allograft group ($P < 0.05$). Heart grafts and lung grafts of the heart-lung allograft did not differ significantly from each other. OX 8 positive cell proportion in GIL, suppressor/cytotoxic T lymphocyte subpopulations, in both heart grafts ($37.8 \pm 25.3\%$) and lung grafts ($44.2 \pm 16.2\%$) of the heart-lung allograft group were significantly higher than in heart grafts ($6.1 \pm 3.5\%$) of the heart allograft group ($P < 0.05$). The proportion of GIL positive for OX 39 interleukin 2 receptor and for OX 6 MHC class II antigen were not statistically different among heart grafts of heart allograft, heart grafts of the heart-lung allograft, and lung grafts of the heart-lung allograft (Fig. 5). In the chronic study, W 3/25 and OX 8 positive cell proportion differed significantly from their counterparts in grafts in the acute study.

W 3/25 positive cell proportion in GIL of heart grafts ($86.1 \pm 4.5\%$) of the heart allograft group was higher than in heart grafts ($58.6 \pm$

21.9%) and lung grafts ($62.5 \pm 12.4\%$) of the heart-lung allograft group ($P < 0.05$). OX 8 positive cell proportion in GIL of heart grafts ($38.1 \pm 24.6\%$) and lung grafts ($34.9 \pm 19.9\%$) of the heart-lung allograft group was higher than in heart grafts ($7.7 \pm 3.8\%$) of the heart allograft group ($P < 0.05$). OX 39 and OX 6 positive cell population did not differ significantly among the graft types (6).

Discussion

Preliminary laboratory experience and early clinical experience with combined heart-lung transplantation led Reitz *et al.* to suggest that allograft rejection in the combined graft would manifest in the heart and lung at approximately the same time and with similar severity (11, 12). The simultaneous appearance of heart and lung rejection has been accepted as the working hypothesis in the management of patients after combined heart-lung transplantation (12, 13). Consequently the diagnosis of rejection in patients

after heart-lung transplantation has been dependent on the detection of heart allograft rejection by endomyocardial biopsy. Although right ventricular endomyocardial biopsy remains the fundamental clinical tool for diagnosis of rejection, it has recently become clear that isolated pulmonary rejection can occur in the heart-lung transplantation patient (14). Furthermore, clinical observations and recent retrospective studies have suggested that cardiac rejection occurs less frequently in heart-lung transplant recipients than in heart transplant recipients (1, 2, 15) and that the lung allograft may be more vigorously rejected than the heart allograft (16-18). This observation has considerable clinical significance and investigations of the possible mechanisms explaining this phenomenon are worth while in view of the potential therapeutic importance.

In our study, the greater prolongation of heart survival in heart-lung allografts than in heart allografts, may be attributed to heart rejection following aggressive lung rejection. In order to detail this rejection process, we evaluated surface markers of GIL by flowcytometric analysis, which directly represents intragraft immune response. Our study shows that suppressor/cytotoxic T lymphocyte proportion in GIL of heart and lung tissues in heart-lung allograft were significantly higher than heart.

The heart lacks local lymphoid tissue while lung has the bronchus-associated lymphoid tissue (BALT). We speculate that the BALT of lung grafts may influence the rejection process possibly as a stimulant of inducing suppressor T cells. Regarding the interaction between the recipient rejection process and donor BALT, Prop *et al.* (19-22) stated that the lung is rejected more aggressively than the heart in his experience with heart-lung transplantation in the rat model and that the BALT of the lung intensifies the immune response.

BALT lymphocytes have an essential role in the stimulation of the rejection response. Grafted lymphocytes disseminate into the recipients lymphoid organs. In other work, Prop *et al.* (23)

showed that pretreatment of lung donor rat with irradiation can prevent this accelerated rejection of the lung. This donor pretreatment with radiation results in diminution of the donor BALT. In the lung graft, the BALT is the initial site of infiltration by recipient lymphocytes, thereafter, BALT lymphocytes disseminate from the graft and induce a similar response systemically in the recipient's lymphoid tissue. Those rats were reported to have well tolerated and functioning grafts when they entered the graft induced immunocompromised condition.

Baldwin *et al.* (2) speculated that when the lung allograft is presented to the recipient in the setting of the pretreatment with Cyclosporine, a helper T-cell inhibitor, the lung may play the role of a relatively powerful stimulant of suppressor cells. Yuuki *et al.* (24) have investigated the cellular basis of tolerance induction in nude mice grafted with allogeneic thymus by assessing the presence of Mls-reactive T cells. The spleen cells from nude mice grafted with AKR/J mice thymus showed a significantly decreased level of primary cytotoxic T cell response. Aune *et al.* (25) studied activation and growth of normal T lymphocytes in long-term culture and stated that CD 8⁺ T cells began to express suppressor cell activity in the absence of detectable cytotoxic activity.

We observed higher proportion of suppressor/cytotoxic T lymphocytes in GIL of heart-lung allograft compared with heart allograft. These results lead us to suggest that suppressor T lymphocyte activity is the key to an explanation of the different rates of rejection of heart allograft and heart-lung allograft as observed in clinical experience. Further studies using intrathoracic models and investigations of BALT in heart-lung allograft rejection are required.

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