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Early detection of acute allograft rejection in rat heart transplantation: flowcytometric monitoring of interleukin 2 receptor expression on CD8 positive lymphocytes.

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Abstract

To assess the usefulness of flowcytometric monitoring in the early detection of acute allograft rejection, we studied surface markers of graft infiltrating lymphocytes, coronary sinus blood lymphocytes and peripheral blood lymphocytes after rat heart transplantation. Fisher rats served as donors and Lewis rats as recipients. Among recipients that received no immunosuppression, grafts were removed 2 days after transplantation (Ongoing Rejection Group: n = 7) and on the day of terminal rejection (Rejection Group: n = 7). The Immunosuppression Group (n = 7) was treated with cyclosporine A at a dose of 3 mg/kg/day intramuscularly for 14 days. The following two color analyses were studied: OX8 (anti-CD8) with OX39 (anti-interleukin 2 receptor; IL2R), W3/25 (anti-CD4) with OX39, W3/25 with OX8. Histological grading demonstrated no significant difference between the Ongoing Rejection Group and the Immunosuppression Group, which showed mild rejection (1.29 +/- 0.27 versus 1.14 +/- 0.24). The proportion of CD8(+)IL2R(+) graft infiltrating lymphocytes showed a more significant increase in the Ongoing Rejection Group than in the Immunosuppression Group (32.1 +/- 3.05 versus 20.6 +/- 9.02; $p < 0.01$). The proportion of CD8(+) IL2R(+) coronary sinus blood lymphocytes also showed significant increase in the Ongoing Rejection Group compared with the Immunosuppression Group (4.63 +/- 1.91 versus 2.52 +/- 1.60; $p < 0.05$). These results suggest that this technique can detect acute allograft rejection earlier than endomyocardial biopsy, before the phase in which histological findings become evident.

KEYWORDS: acute rejection, heart transplantation, flowcytometry, immunologic monitoring, interleukin 2 receptor

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Early Detection of Acute Allograft Rejection in Rat Heart Transplantation: Flowcytometric Monitoring of Interleukin 2 Receptor Expression on CD8 Positive Lymphocytes

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To assess the usefulness of flowcytometric monitoring in the early detection of acute allograft rejection, we studied surface markers of graft infiltrating lymphocytes, coronary sinus blood lymphocytes and peripheral blood lymphocytes after rat heart transplantation. Fisher rats served as donors and Lewis rats as recipients. Among recipients that received no immunosuppression, grafts were removed 2 days after transplantation (Ongoing Rejection Group: n = 7) and on the day of terminal rejection (Rejection Group: n = 7). The Immunosuppression Group (n = 7) was treated with cyclosporine A at a dose of 3 mg/kg/day intramuscularly for 14 days. The following two color analyses were studied: OX8 (anti-CD8) with OX39 (anti-interleukin 2 receptor; IL2R), W3/25 (anti-CD4) with OX39, W3/25 with OX8. Histological grading demonstrated no significant difference between the Ongoing Rejection Group and the Immunosuppression Group, which showed mild rejection (1.29 ± 0.27 *versus* 1.14 ± 0.24). The proportion of CD8(+)IL2R(+) graft infiltrating lymphocytes showed a more significant increase in the Ongoing Rejection Group than in the Immunosuppression Group (32.1 ± 3.05 *versus* 20.6 ± 9.02; $p < 0.01$). The proportion of CD8 (+) IL2R(+) coronary sinus blood lymphocytes also showed significant increase in the Ongoing Rejection Group compared with the Immunosuppression Group (4.63 ± 1.91 *versus* 2.52 ± 1.60; $p < 0.05$). These results suggest that this technique can detect acute allograft rejection earlier than endomyocardial biopsy, before the phase in which histological findings become evident.

Key words : **acute rejection, heart transplantation, flowcytometry, immunologic monitoring, interleukin 2 receptor**

Heart transplantation has become accepted as a viable therapeutic option for patients with end-stage heart disease. The Registry of the International Society for Heart and Lung Transplantation (ISHLT) demonstrated a significant improvement in survival at 1 year from 72.8 % to 80 % (1981 through 1985 *versus* 1986 through 1990) and at 5 years from 58 % to 70 % (1). But acute allograft rejection is a leading cause of graft failure in heart transplant recipients. And early detection of acute allograft rejection still remains the most challenging aspect of heart transplantation.

In the present study, we examined flowcytometric monitoring of various surface markers of graft infiltrating lymphocytes (GIL), peripheral blood lymphocytes (PBL) and coronary sinus blood lymphocytes (CSL) which was drained from the coronary sinus of heart allografts. Results were then compared with histological grading of acute rejection.

The purpose of this study was to evaluate the usefulness of flowcytometric monitoring for early detection of acute allograft rejection in heart transplantation.

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Materials and Methods

Animals. Adult male Lewis (LEW) ($RT1^l$) and Fisher (F344) ($RT1^{lv1}$) inbred rats were obtained from Charles River Co. (Atsugi, Japan). They 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 the National Institute of Health (NIH Publication No. 86-23 revised 1985).

Heterotopic heart transplantation. Fisher rats served as donors and Lewis rats as recipients. Twenty-one heart allografts were transplanted intraabdominally using a standard microsurgical technique as Ono & Lindsey described (2). 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 into the inferior vena cava. Afterward the cardioplegic solution was injected from the descending aorta and cardiac arrest was accomplished. The ascending aorta and pulmonary artery were transected and the three vena cavae and left atrium were ligated and divided, completing the procurement of the graft heart. The graft heart was then immersed in cold saline. The recipient Lewis rat was anesthetized by ether inhalation. A 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. Aortoaortic and pulmonocaval end to side anastomosis were performed with 8-0 polypropylene (Prolene; Ethicon Inc., Somerville, NJ) continuous sutures (3).

Ischemic times averaged approximately 60 min. Allografts were followed by daily palpation and cessation of pulsation was regarded as graft rejection.

Experimental groups. Transplanted rats were divided into 3 groups as follows. Rejection Group ($n = 7$) received no immunosuppression as a control, in which grafts were removed on the day of graft rejection. Ongoing Rejection Group ($n = 7$) received no immunosuppression and were killed 2 days after transplantation. Immunosuppression Group ($n = 7$) was treated with Cyclosporine A at a dose of 3 mg/kg/day. The immunosuppressants were injected intramuscularly in alternative legs daily for 14 days.

All rats were killed by terminal ether anesthesia and the removed grafts were examined for graft rejection and surface markers of lymphocytes.

Histological examination. The basal half of the graft hearts were fixed with 10 % neutral buffered formalin and stained with hematoxylin and eosin for microscopic examination. Graft rejection was graded according to the ISHLT Standardized Grading System (4).

PBL preparation. PBL was prepared from heparinized blood using Lympholyte-R (Cedarlane Laboratories Limited, Hornby, Ontario, Canada). Blood was overlaid on Lympholyte-R and centrifuged at 2,200 rpm for 30 min in room temperature. The layer of lymphocytes was carefully aspirated. Lymphocytes were suspended in RPMI 1640 culture medium (Gibco laboratories, Grand Island, NY) containing 1 % fetal calf serum and 0.1 % NaN_2 (3).

CSL preparation. In this heterotopic intraabdominal heart transplantation model, only coronary sinus blood was drained into the right atrium of the heart allograft. Heparinized coronary sinus blood was obtained from a puncture in the right atrium of the beating heart allograft. The rejected heart allografts were so severely damaged that right atrial blood could not aspirated. Therefore, CSL evaluation was not carried out in the Rejection Group. CSL preparation was the same as PBL preparation.

GIL preparation. The apical half of graft hearts were minced by a tissue homogenizer and cells were released according to the method described by Totterman *et al.* (5). The tissue homogenate was incubated with 3 ml of digestion medium (20 mM Hepes Buffer from Sigma Chemical Co., St. Louis, MO., 136 mM NaCl, 4.7 mM KCl, 0.65 mM $MgSO_4$, 1.2 mM $CaCl_2$, pH 7.45) containing collagenase (Worthington Biochemical Co., Freehold, NJ) 2 mg/ml, DNAse (Sigma Chemical Co., St. Louis, MO) 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 ($100\,\mu m^2$) to remove aggregates and overlaid on Lympholyte-R. After centrifugation at 2,200 rpm for 30 min, cells in the lymphocytic layer were carefully aspirated and suspended in the same medium as PBL (3). In general, approximately 10,000 lymphocytes were obtained per one g of allograft in the Rejection Group and approximately 1,000 to 2,000 lymphocytes were obtained per one g of allograft in the Ongoing Rejection Group and the Inmunosuppression Group.

Pretreatment of cells with antibodies. The lymphocyte suspension of PBL, CSL and GIL were incubated with the optimal concentration of monoclonal antibodies (Table 1) at 4 °C for 30 min in a dark incubation chamber. The following two color analyses were studied: W3/25 (CD4) anti-helper T lymphocyte antibody conjugated with fluorescein isothionate (FITC) and OX8 (CD8) anti-suppressor/cytotoxic T lymphocyte antibody conjugated with phycoerythrin (PE), W3/25-FITC and OX39 anti-interleukin 2 receptor (IL2R) conjugated with PE, OX8-FITC and OX39-PE. All monoclonal antibodies were obtained from Serotec Ltd. (Oxford, England).

Flowcytometric analysis. Lymphocytic surface marker analysis was done using EPICS, model 753 (Coulter Electronic Co., Hialeah, Fla.). An argon laser (200 mw, 488 nm) was used for excitation of FITC and PE. The cells were gated optimally by forward scatter (cell size) and lateral scatter (granularity) for

Table 1 Panel of monoclonal antibodies (Ab) used in this study

Monoclonal Ab	CD designation	Cell specificity
W3/25	CD4	T helper/inducer
OX8	CD8	T suppressor/cytotoxic
OX39	CD25	Interleukin 2 receptor (IL2R)

lymphocytes. Appropriate filters, 560nm short-pass filter for FITC and 590nm long-pass filter for PE, were used in this analysis. Quadrant analysis was performed and the percentage of CD4, CD8 and IL2R expressing lymphocytes (CD4(+)Ly, CD8(+)Ly and IL2R(+)Ly), the percentage of CD4 and CD8 lymphocytes coexpressing IL2R (CD4(+)IL2R(+)Ly and CD8(+)IL2R(+)Ly) and the CD4:CD8 ratio were calculated.

Statistical analysis. All values are expressed as mean ± standard deviation. Significant differences were calculated with ANOVA test, followed by Student's t test. A p value of less than 0.05 was considered significant.

Results

Graft survival. All Lewis rats gained weight appropriately and appeared healthy throughout the study. All grafts of the Rejection Group was rejected and mean graft survival was 18.6 ± 2.9 days. Allografts of the Ongoing Rejection Group and the Immunosuppression Group demonstrated excellent contraction until the day of removal.

Histological grading. Graft rejection of the Rejection Group was severe, compatible with ISHLT Grade 4. The Ongoing Rejection Group and the Immunosuppression Group showed mild rejection (grades 1A or 1B of ISHLT standardized grading system) and there was no significant difference between the two groups (Table 2).

Flowcytometric Analysis

Number of lymphocytes analyzed. Approximately 5,000 lymphocytes were analyzed in CSL and PBL analyses. In GIL analyses, at least 1,000 lymphocytes were analyzed.

GIL surface markers. The results are shown in Table 3. The proportions of CD8(+)IL2R(+)Ly in the Rejection Group were significantly higher than those in the Ongoing Rejection Group ($p < 0.01$) and the Immunosuppression Group ($p < 0.001$), and those in the Ongoing Rejection Group were significantly higher than the Immunosuppression Group ($p < 0.01$) (Fig. 1). IL2R(+)Ly in the Rejection Group ($p < 0.05$) and the Ongoing Rejection Group ($p < 0.05$) were significantly higher than in the Immunosuppression Group. But there

Table 2 Grading of graft rejection

Group	No.	Immunosuppression	Graft rejection
Rejection	7	No treatment	4.00 ± 0.00*
Ongoing	7	No treatment	1.29 ± 0.27
CsA	7	CsA 3mg/kg/day i.m.	1.14 ± 0.24

Ongoing: Ongoing Rejection Group CsA: Immunosuppression Group
* $p < 0.001$ *vs* both Ongoing and CsA

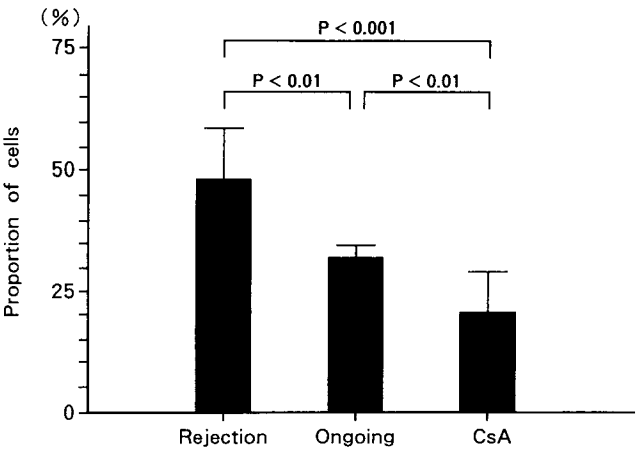

Fig. 1 Proportion of CD8 lymphocytes coexpressing interleukin 2 receptor among graft infiltrating lymphocytes
Ongoing: Ongoing Rejection Group CsA: Immunosuppression Group

Table 3 Flowcytometric analysis of graft infiltrating lymphocytes

Group	Proportion of surface markers-positive cells (%)					CD4:CD8 ratio
	CD4	CD8	IL2R	CD4 (+) IL2R (+)	CD8 (+) IL2R (+)	
Rejection	55.3 ± 19.9	52.2 ± 15.1	63.9 ± 16.3	35.9 ± 14.5	48.9 ± 10.1**	1.05 ± 0.24
Ongoing	49.3 ± 17.9	52.0 ± 11.5	65.0 ± 15.5	26.8 ± 5.74	32.1 ± 3.05***	0.95 ± 0.30
CsA	47.0 ± 12.5	39.8 ± 12.7	43.1 ± 22.0*	26.9 ± 11.4	20.6 ± 9.02	1.24 ± 0.34

Ongoing: Ongoing Rejection Group CsA: Immunosuppression Group IL2R: anti-interleukin 2 receptor.
* $p < 0.05$ *vs* both Rejection and Ongoing ** $p < 0.01$ *vs* Ongoing and $p < 0.001$ *vs* CsA *** $p < 0.01$ *vs* CsA

was no significant difference between the Rejection Group and the Ongoing Rejection Group (Fig. 2). The proportions of CD4(+)Ly, CD8(+)Ly and CD4(+)IL2R(+)Ly and CD4:CD8 ratio showed no statistically significant difference among the three groups.

CSL surface markers. The results are shown in Table 4. The proportions of CD8(+)IL2R(+)Ly in the Ongoing Rejection Group were significantly higher than in the Immunosuppression Group ($p < 0.05$) (Fig. 3). The proportions of CD4(+)Ly, CD8(+)Ly, IL2R(+)Ly and CD4(+)IL2R(+)Ly and CD4:CD8 ratio showed no statistically significant difference between the two groups.

PBL surface markers. In this analysis, none of these markers showed a statistically significant difference

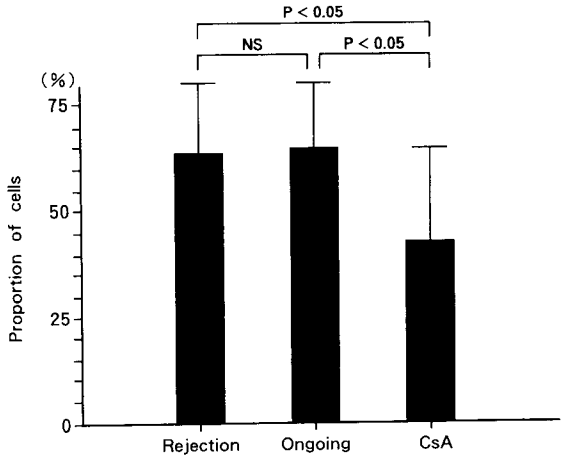

Fig. 2 Proportion of interleukin 2 receptor expressing lymphocytes among graft infiltrating lymphocytes
Ongoing: Ongoing Rejection Group CsA: Immunosuppression Group

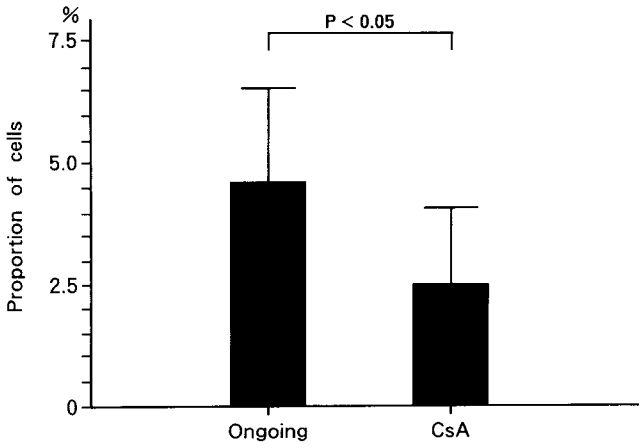

Fig. 3 Proportion of CD8 lymphocytes coexpressing interleukin 2 receptor among coronary sinus blood lymphocytes
Ongoing: Ongoing Rejection Group CsA: Immunosuppression Group

Table 4 Flowcytometric analysis of coronary sinus blood lymphocytes

Group	Proportion of surface markers-positive cells (%)					CD4:CD8 ratio
	CD4	CD8	IL2R	CD4 (+) IL2R (+)	CD8 (+) IL2R (+)	
Ongoing	58.4 ± 13.2	35.8 ± 12.9	3.48 ± 2.36	1.62 ± 0.65	4.63 ± 1.91*	1.74 ± 0.43
CsA	63.3 ± 12.0	33.8 ± 5.87	2.40 ± 1.67	1.86 ± 1.82	2.52 ± 1.60	1.88 ± 0.26

Ongoing, CsA, IL2R: See Table 3. $*p < 0.05$ *vs* CsA

Table 5 Flowcytometric analysis of peripheral blood lymphocytes

Group	Proportion of surface markers-positive cells (%)					CD4:CD8 ratio
	CD4	CD8	IL2R	CD4 (+) IL2R (+)	CD8 (+) IL2R (+)	
Rejection	50.2 ± 15.5	23.4 ± 4.96	4.32 ± 2.24	2.98 ± 1.82	4.54 ± 1.91	2.11 ± 0.41
Ongoing	62.0 ± 10.6	35.4 ± 12.6	3.64 ± 2.37	2.69 ± 2.65	4.02 ± 1.62	1.89 ± 0.47
CsA	58.2 ± 14.1	33.0 ± 8.97	2.47 ± 1.83	1.64 ± 1.33	2.55 ± 1.83	1.81 ± 0.31

Ongoing, CsA, IL2R: See Table 3. All values showed no significance.

among the three groups (Table 5).

Discussion

Acute rejection is a T cell mediated event which causes a sudden graft dysfunction which can be successfully reversed by prompt immunosuppressive therapy if detected early. Endomyocardial biopsy is now the standard technique for diagnosis of acute allograft rejection in heart transplantation. Unfortunately, the localized sampling of myocardial tissue, one of the major disadvantages of this technique, permits the possibility of the false grading of endomyocardial biopsy. If the endomyocardial biopsy specimen shows even mild rejection, subsequent severe rejection and graft dysfunction can occur within a few days (6, 7). This invasive biopsy procedure is associated with considerable discomfort for the patient. Therefore, the monitoring of patients for early signs of acute rejection and less invasive methods are being sought and would represent a major advantage.

Over the past several years, the monitoring of CD4 and CD8 T cell subsets to assess the immunological status of transplanted recipients have been reported. Early studies indicated that elevated CD4:CD8 ratios of peripheral blood lymphocytes were associated with allograft rejection (8, 9). Subsequent studies, however, found that lymphocyte subpopulation studies were not useful to predict rejection or to establish the degree of immunosuppression (6, 10, 11). These apparently conflicting data underscore the need for a more detailed analysis of lymphocytes from patients experiencing acute rejection.

During graft rejection, helper T lymphocytes secrete interleukin 2 (IL2), a growth factor essential to the activation of cytotoxic T cells. In order to respond to IL2, cytotoxic T cells must first express cell surface receptors for IL2. Therefore, expression of IL2R is one of the earliest indications of T cell activation.

Previously, we reported that flowcytometric analysis of heart allograft infiltrating lymphocytes reflects the rejection process more accurately and quantitatively than the same analysis of peripheral blood lymphocytes (3). The analysis is highly effective for monitoring the intragraft event. And we also reported that CD8 suppressor/cytotoxic lymphocytes might be the key to understanding allograft rejection (3, 12).

Farge *et al.* (13) reported that acute rejection of Fisher heart allografts transplanted into Lewis rats progressed in ongoing fashion with no immunosuppression and they also reported myocyte damage was observed at 3 days after transplantation. Since myocyte damage was not observed at 2 days after transplantation in our preliminary study, we decided that grafts should be removed at that point in the Ongoing Rejection Group.

In this study, histological gradings of acute rejection in the Ongoing Rejection Group and the Immunosuppression Group were mild rejections, which demonstrated no significant difference. The proportions of CD8(+) IL2R(+) graft infiltrating lymphocytes in the Ongoing Rejection Group were significantly higher than in the Immunosuppression Group. In contrast, CD4(+) IL2R(+) graft infiltrating lymphocytes demonstrated no significant difference between the two groups. This result suggests that monitoring of IL2R expression on CD8 positive graft infiltrating lymphocytes can be effective in early detection of acute rejection of heart allograft before histological changes become evident.

The endothelial cells express major histocompatibility complex class I and class II antigen induced by interferon-gamma. In contrast, myocytes express only class I antigen on the membrane after stimulation with interferon-gamma (14-17). Major histocompatibility complex class I serves as restriction elements for the CD8 subset of T lymphocytes, whereas class II serve as restriction elements for the CD4 subset of T lymphocytes (18). This fact suggests a possible explanation of the results of present study. In the case of ongoing rejection, even if the histological findings demonstrates mild rejection, those lymphocytes infiltrating into allografts are already activated and preparing to proliferate and attack myocytes. In the case of non-ongoing rejection, those infiltrating lymphocytes are neither activated to proliferate nor to attack myocytes.

We also studied coronary sinus monitoring. Coles *et al.* reported that the appearance in the peripheral blood of cells bearing the IL2R did not correlate with endomyocardial biopsy findings (19). The coronary sinus drains myocardial blood into the right atrium. Because lymphatic vessels are rarely developed within the myocardial wall, all cells participating in immunologic process pass through the coronary sinus (7). Holzinger *et al.* reported that amount of IL2R-bearing lymphocytes obtained from the coronary sinus of the patient presenting mild rejection is significantly higher than from the patient presenting no signs of rejection (7). In this study, the Ongoing Rejec-

tion Group demonstrated a significantly higher proportion of CD8(+)IL2R(+) coronary sinus blood lymphocytes than the Immunosuppression Group. But there were no significant differences between the two groups in the proportions of IL2R(+)Ly or CD4(+)IL2R(+)Ly obtained from the coronary sinus.

Holzinger *et al.* reported that only a small amount of cells responsible for graft destruction remain in the myocardium, and that all the other chemotactically attracted cells, activated by various cytokines, appear in the blood of the coronary sinus (7). In our present study, the method of graft ifiltrating lymphocyte monitoring seems to be as reliable as the monitoring coronary sinus blood lymphocytes. Our data suggests that most activated T lymphocytes seem to remain in the myocardium and smaller number of lymphocytes than expected are drained into the coronary sinus.

Flowcytometric monitoring of IL2R expression on CD8 positive graft infiltrating lymphocytes and coronary sinus blood lymphocytes proved to be more useful for the early detection of acute allograft rejection than histological diagnosis by endomyocardial biopsy. Clinical evaluation and further investigation of newly developed immunosuppressants such as FK506, and of the NK cell and other T cell subsets are required. We believe these flowcytometric analyses may result in pre-clinical diagnosis of acute rejection of transplanted hearts which have an impact on long-term allograft survival. It is also necessary to continue to pursue more reliable and less invasive method for monitoring acute allograft rejection.

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