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A novel noninvasive method to detect rejection after heart transplantation

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Abstract

Prompt and accurate detection of rejection prior to pathological changes after organ transplantation is vital for monitoring rejections. Although biopsy remains the current gold standard for rejection diagnosis, it is an invasive method and cannot be repeated daily. Thus, noninvasive monitoring methods are needed. In this study, by introducing an IL-2 neutralizing monoclonal antibody (IL-2 N-mAb) and immunosuppressants into the culture with the presence of specific stimulators and activated lymphocytes, an activated lymphocyte-specific assay (ALSA) system was established to detect the specific activated lymphocytes. This assay demonstrated that the suppression in the ALSA test was closely related to the existence of specific activated lymphocytes. The ALSA test was applied to 47 heart graft recipients and the proliferation of activated lymphocytes from all rejection recipients proven by endomyocardial biopsies was found to be inhibited by spleen cells from the corresponding donors, suggesting that this suppression could reflect the existence of activated lymphocytes against donor antigens, and thus the rejection of a heart graft. The sensitivity of the ALSA test in these 47 heart graft recipients was 100%; however, the specificity was only 37.5%. It was also demonstrated that IL-2 N-mAb was indispensable, and the proper culture time courses and concentrations of stimulators were essential for the ALSA test. This preliminary study with 47 grafts revealed that the ALSA test was a promising noninvasive tool, which could be used *in vitro* to assist with the diagnosis of rejection post-heart transplantation.

Key words: Heart transplantation rejection; Activated lymphocytes; Noninvasive method; Primed lymphocyte typing; Interleukin-2 neutralization monoclonal antibody; ALSA test

Introduction

Organ transplantation is the last hope for patients with incurable organ failure. However, post-transplantation, the antigenicity difference between donor and recipient, such as the difference between the major histocompatibility antigen and the minor histocompatibility antigen, usually elicits host versus graft reaction, which is well recognized as a potentially lethal factor after solid organ transplantation (1). Thus, for the survival of the transplanted organs, it is vital to monitor rejection.

Since organ transplantation techniques were developed, researchers and surgeons have tried various approaches to monitor rejection. Among them, biopsy, which is the current gold standard for rejection diagnosis worldwide, is an invasive method that cannot be repeated daily. Furthermore, lesions caused by rejection only exist in certain parts of an organ, so

the biopsy samples may be beyond the foci and lead to a misdiagnosis. In addition, the discovery of pathological changes actually indicates that the organ has been already damaged. Therefore, it is essential to develop a convenient, sensitive and noninvasive diagnostic method to monitor rejection so that clear treatment thresholds could be adopted.

Specific activated lymphocytes targeting donor HLA antigens are mainly responsible for rejection (2-4). Based on this, noninvasive methods have been attempted to utilize the presence of the specific activated lymphocytes in the peripheral blood of the recipient to predict the rejection conveniently. The primed lymphocyte typing (PLT) test (also known as secondary mixed lymphocyte culture) was established in 1975 and provided rapid detection of the activated lymphocytes (5). In the PLT test, the lymphocytes that were activated

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by mixed lymphocyte culture (MLC) for 14 days, when cocultured with the specific antigen again, would demonstrate an accelerating secondary response (6,7). However, studies by Birkeland (8) and Sampson et al. (9) have revealed that when the PLT test was applied to transplantation rejection, the proliferation of activated lymphocytes in the PLT tests were either increased, inhibited, or slightly changed, rather than exhibiting a stable tendency. These phenomena could not be explained by the current secondary response theory. Seki's (10) study in 1983 suggested that this suppression in PLT when rejection occurred was specific for the donor, and unrelated to other factors. Then, the feasibility of using PLT to diagnose rejection of the transplant was definitely excluded. Since then, further reports have been rare and the mechanism of this suppression still remains unclear.

In the present study, an activated lymphocyte-specific assay (ALSA) was established to detect the presence of specific activated lymphocytes by restimulation with the specific antigen, which showed that the suppression in the ALSA test was closely related to the presence of specific activated lymphocytes targeting the specific antigen. A prospective study was also designed to identify the correlation between suppression and specific activated lymphocytes targeting donor antigens in the recipients of a heart graft. A preliminary analysis demonstrated that, when rejection occurred, the suppression in the ALSA test was strongly associated with the presence of activated lymphocytes. Thus, the noninvasive ALSA test had the potential to be used as a complementary method to detect transplant rejection.

Material and Methods

Materials and reagents

Methylprednisone and FK506 were purchased from Fujisawa Pharmaceutical (Japan). The RPMI 1640 culture medium and fetal calf serum (FCS) were purchased from Invitrogen (USA). The purified IL-2 neutralizing monoclonal antibody (IL-2 N-mAb) and the control monoclonal antibody (anti-staphylococcal enterotoxin D monoclonal antibody, anti-SED mAb) were gifts from Professor Boquan Jin, Department of Immunology, Fourth Military Medical University, China. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was purchased from Sigma-Aldrich (USA). The Ficoll separation solution was purchased from Biochrom (Germany). The round-profile 96-well plates were purchased from Greiner Bio-One (Germany). Mitomycin was purchased from Kyowa Hakko Kogyo (Japan). The immunosuppressants methylprednisolone and FK506 were purchased from Fujisawa Pharmaceutical.

Participants

Participants in this study included 6 unrelated healthy male volunteers aged 18 to 40 years (average: 32 years old), and 47 heart graft patients (between 2001 and 2010, 28 males and 19 females) aged 16 to 54 years (average: 42 years old). Another 3 healthy volunteers (1 male and 2

females, average age: 29 years) were also chosen to evaluate the antigen specificity of the ALSA test. After transplant, all recipients were monitored for rejection by endomyocardial biopsies performed at scheduled intervals: weekly during the 1st month, biweekly until the 3rd month, monthly until the 6th month, and every 3 months until the 12th month. The 1-year survival rate for these 47 recipients was 89.36% (42/47). Written informed consent was obtained from all participants or the persons responsible for them, as appropriate. The study was approved by the Ethics Committee of Fourth Military Medical University, China, and all patients remained under continuous medical supervision and assistance.

Establishment of the ALSA system

Peripheral blood mononuclear cells (PBMCs) were isolated from the 6 healthy volunteers using a Ficoll density gradient. PBMCs from 3 of the volunteers were treated with 25 µg/mL mitomycin in serum-free RPMI 1640 culture medium containing 10% FCS for 30 min, and then washed with normal saline to be used as stimulators. PBMCs from the other 3 volunteers without any treatment were used as responders. The stimulators and responders were both diluted to 2×10^6 cells/mL with RPMI 1640 culture medium containing 20% FCS. Parts of the stimulators were then used in primary one-way MLC (11), and the others were preserved in liquid nitrogen for future re-stimulation of the activated lymphocytes obtained from primary one-way MLC. Four days after primary one-way MLC, the primed lymphocytes were collected and used as responder cells in the secondary MLC. The corresponding stimulators and responders were adjusted to 2×10^6 cells/mL with RPMI 1640 culture medium containing 10% FCS. Experiments were grouped as follows: treatment group of the ALSA test: 100 µL responders, 100 µL stimulators and 120 pg IL-2 N-mAb; control group of the ALSA test: 100 µL responders, 120 pg IL-2 N-mAb and 100 µL RPMI 1640 culture medium containing 10% FCS; treatment group of the PLT test: 100 µL responders and 100 µL stimulators; control group of the PLT test: 100 µL responders and 100 µL RPMI 1640 culture medium containing 10% FCS.

The RPMI 1640 culture medium used in the ALSA test also contained the immunosuppressants methylprednisolone (5 µg/mL) and FK506 (0.125 ng/mL; Fujisawa Pharmaceutical). The inhibitory rate was calculated as follows: $1 - (\text{mean value of absorbance of the treatment group at 550 nm} / \text{mean value of absorbance of the control group at 550 nm}) \times 100\%$.

Endomyocardial biopsy

Cardiac allograft biopsies were collected as part of routine clinical care and routinely stained with hematoxylin and eosin. Rejection was assessed according to the grading system for the histological diagnosis of rejection based on endomyocardial biopsy (12).

Clinical tests and donor antigen-specific experiments of the ALSA system

During heart transplantation, donor spleen cells were

collected and stored in liquid nitrogen. PBMCs separated from heart graft patients were used as responders, and the corresponding frozen-stored donor spleen cells treated with mitomycin were used as the stimulators in ALSA tests. One-to-one ALSA tests were performed to examine the proliferation changes of responders.

Influence of IL-2 N-mAb, concentrations of stimulators, and cell culture time courses on the ALSA system

PBMCs isolated from a heart graft rejection patient at grade 1 proven by biopsy were used as responders, and the corresponding frozen-stored specific donor spleen cells treated with mitomycin were used as stimulators in the ALSA test. Experiments were grouped as described above. To investigate the role of IL-2 N-mAb in the ALSA test, IL-2 N-mAb was replaced with anti-SED mAb. To reveal the influence of the concentration of stimulators on ALSA, the stimulators were diluted into a series of concentration gradients. To determine the effect of cell culture time course on ALSA, the MTT solution was added to each well at different time points, as indicated.

Detection of lymphocyte proliferation

After 20-24 h culturing in ALSA tests or 72 h in routine PLT tests, the lymphocyte proliferation in the above experiments was evaluated by the MTT assay (13). The absorbance at 550 nm was measured in each well with a microplate reader (Corona Electric, Japan).

Statistical analysis

The statistical significance of the difference in inhibitory rate between different rejection grades was analyzed using the Mann-Whitney test. Comparisons between group means were performed using an independent sample *t*-test. Data are reported as means \pm SEM. A value of $P < 0.05$ was regarded as significant.

Results

ALSA system

PBMCs isolated from the 6 healthy volunteers were used in a primary one-way MLC. The primed lymphocytes collected from the primary one-way MLC were used as responder cells in the secondary MLC tests. Experiments were grouped as described. As shown in Figure 1, in the PLT test, the proliferation of activated lymphocytes was usually enhanced by the restimulation of the stimulators. However, by adding IL-2 N-mAb and immunosuppressive agents, the proliferation of activated lymphocytes was dramatically inhibited by comparing the treatment group to the control

group when the corresponding spleen cells were used as stimulators, and this inhibitory effect seemed to be stable and repeatable (Figure 1). However, this suppression did not appear when the spleen cells from an unrelated volunteer were used as stimulators, indicating that this suppression was closely related to the correspondence of the specific responders and stimulators.

By comparing the control group between the ALSA experiment and the routine PLT experiment in Figure 1, it was obvious that IL-2 N-mAb and immunosuppressants contributed to the suppression since the only difference between these two tests was that the IL-2 N-mAb and immunosuppressants were introduced into the ALSA test. However, this inhibitory effect was milder than that observed between the treatment group and the control group in the ALSA test, indicating that the trigger of suppression was the specific antigen.

These data taken together suggest that the reaction system described here was indeed different from the routine PLT, and therefore we designated it to be an ALSA system. For this assay, the presence of the specific activated lymphocytes could be determined by re-stimulation with the corresponding antigen since the suppression was closely

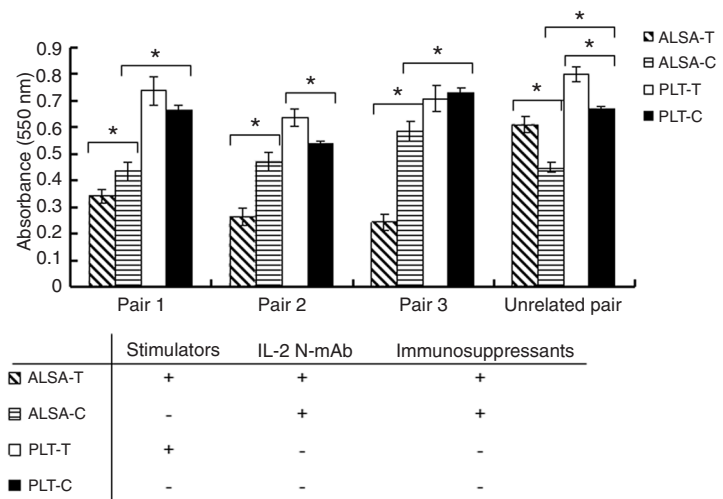


Figure 1. The proliferation of activated lymphocytes was significantly inhibited during the ALSA tests. PBMCs isolated from 3 healthy male volunteers and from another 3 healthy male volunteers were used as stimulators and responders, respectively, in a primary one-way MLC. Four days later, the activated lymphocytes collected from primary one-way MLC were used as responders in the ALSA tests and the routine PLT tests. MTT colorimetry was employed to evaluate the proliferation of activated lymphocytes. An independent sample *t*-test was performed to evaluate the difference between group means. The height of each column indicates the mean value of absorbance in each group at 550 nm and SEM; $N = 3$; $P < 0.05$, statistically significant. ALSA-T = treatment group of the ALSA test; ALSA-C = control group of the ALSA test; PLT-T = treatment group of the PLT test; PLT-C = control group of the PLT test. PBMCs = peripheral blood mononuclear cells; ALSA = activated lymphocyte-specific assay; PLT = primed lymphocyte typing; MLC = mixed lymphocyte culture; IL-2 N-mAb = IL-2 neutralizing monoclonal antibody; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

related to the correspondence of the activated lymphocytes and the antigen. Although the effect of IL-2 N-mAb and immunosuppressants were indispensable, the trigger of the suppression was the re-stimulation with the corresponding antigen.

Suppression in the ALSA test was associated with heart graft rejection

After organ transplantation, the specific activated lymphocytes targeting the donor also existed in the PBMCs of recipients when rejection occurred. Therefore, it was easy to assume that the ALSA test could be induced to reflect the rejection after organ transplantation.

According to the grading system for the histological diagnosis of rejection based on endomyocardial biopsy (12), the 47 patients were divided into grade 0 (N = 8), grade 1 (N = 14), grade 3A (N = 10) and grade 3B (N = 15) (data not shown). To determine whether the suppression in the ALSA test was associated with specific activated lymphocytes from the heart graft recipients, PBMCs isolated from 47 heart graft patients were used as responders, and the corresponding frozen-stored donor spleen cells treated with mitomycin were used as stimulators. According to the current immunological theory, proliferation of the cells in the treatment group should be increased in the routine PLT experiment. However, the results in Figure 2A showed that, in the routine PLT test, the proliferation of responders was increased, inhibited or little changed. In fact, this unstable tendency phenomenon had been reported previously (8,9) as well as the use of the routine PLT test to detect specific activated lymphocytes in clinic (10). In contrast, in the present ALSA test, the proliferation of responders from all rejection recipients (grade 1, grade 3A, and grade 3B) proven by endomyocardial biopsies was inhibited by donor spleen cells (Figure 2A), but no suppression was observed when the corresponding stimulators were replaced with spleen cells from unrelated volunteers (Figure 2A), suggesting that the suppression in ALSA tests depended on the re-stimulation of the corresponding antigen. However, the variation in suppression among patients suffering rejection was quite significant. This might be the result of the different constitutions of these patients. Suppression also occurred in 5 of the 8 non-rejection recipients (grade 0) proven by endomyocardial biopsies (Figure 2A). The sensitivity of the ALSA test was 100%; however, the specificity was only 37.5% in these 47 patients. There were 62.5% false positives. Among the 5 non-rejection recipients, one who suffered from significant arrhythmias after transplantation showed an inhibitory rate of 38% in the ALSA test. The biopsy showed less than grade 1 rejection according to diagnostic criteria (Figure 2B). After the routine methylprednisolone pulse therapy for acute rejection, arrhythmia symptoms disappeared. Since

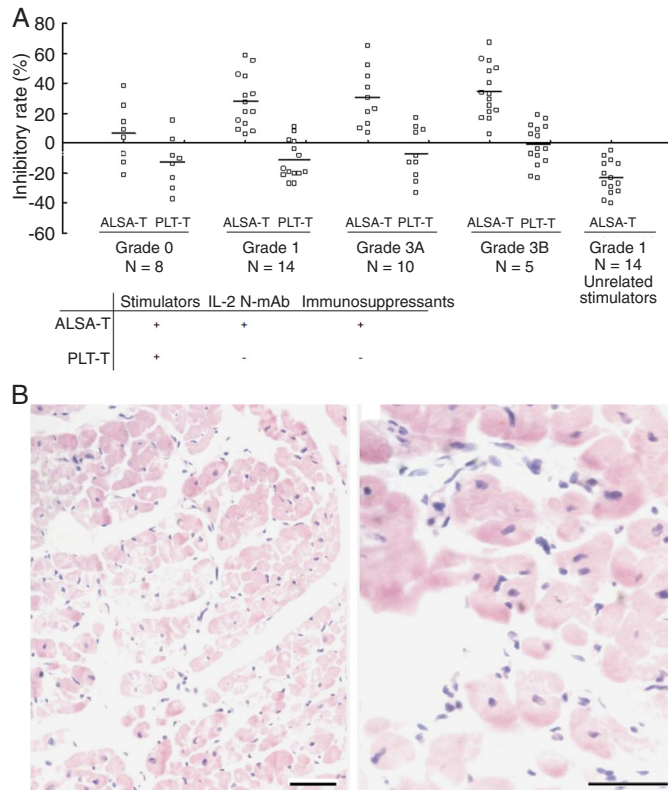


Figure 2. Suppression in the ALSA test was associated with rejection. **A**, The PBMCs isolated from 47 heart graft recipients were used as responders, and corresponding donor spleen cells were used as stimulators. In the ALSA test, the proliferation of responders from all rejection recipients was inhibited by stimulators. In contrast, the responders reflected an irregular alteration in the routine PLT test. MTT colorimetry was employed to evaluate the proliferation of activated lymphocytes. The statistical significance of the inhibitory rate was analyzed using the Mann-Whitney test. Positive values indicate suppression and negative values indicate enhancement. Horizontal bars represent the mean of the group. **B**, Representative endomyocardial biopsies from a non-rejection recipient with an inhibitory rate of 38% in the ALSA test. This recipient suffered from significant arrhythmias after transplantation, but the biopsy result was equivalent to less than grade 1 rejection according to diagnostic criteria. Hematoxylin and eosin stains. Bar = 50 μ m. For abbreviations, see legend to Figure 1.

the appearance of activated lymphocytes targeting donor antigens preceded the histological changes (3), and the biopsy samples might contain no foci, we speculated that the specific activated lymphocytes had appeared and rejection would be subsequently elicited in this non-rejection recipient, as well as in the other 4 non-rejection recipients. If so, the specificity of the ALSA test would be significantly higher than 37.5%. Because continuous drug therapy was given to these patients, it was difficult to ethically verify this speculation. In the present study, no significant ($P < 0.05$) difference in inhibitory rate was observed between different rejection grades (grade 1, grade 3A, and grade 3B; Figure 2A).

Taken together, these results show that the suppression

caused by the re-stimulation of the corresponding antigen was associated with the existence of specific activated lymphocytes in heart graft recipients, and could reflect the rejection of the heart graft in these 39 rejection cases.

IL-2 N-mAb was indispensable for the ALSA test

To evaluate the role of IL-2 N-mAb in the ALSA test, the anti-SED mAb was used instead of the IL-2 N-mAb in the ALSA test. PBMCs isolated from the 14 patients with heart graft rejection (grade 1) proven by endomyocardial biopsies were used as responders and the corresponding frozen-stored donor spleen cells treated with mitomycin were used as stimulators. When IL-2 N-mAb was replaced with anti-SED mAb, no obvious suppression was observed (Figure 3). Thus, it was concluded that IL-2 N-mAb was essential to the ALSA system but that the anti-SED mAb was not. Because IL-2 N-mAb in the ALSA test neutralized IL-2, it could also be inferred that IL-2 played a key role in the survival of the responders and the neutralization of IL-2 would have a great impact on the result of the ALSA test.

Effects of stimulator concentrations and culture time course on the ALSA system

To demonstrate the effect of stimulator cell concentration and culture time course on the ALSA system, a patient with grade 1 heart graft rejection proven by biopsy was chosen. The results of the ALSA test (Figure 4A) with different stimu-

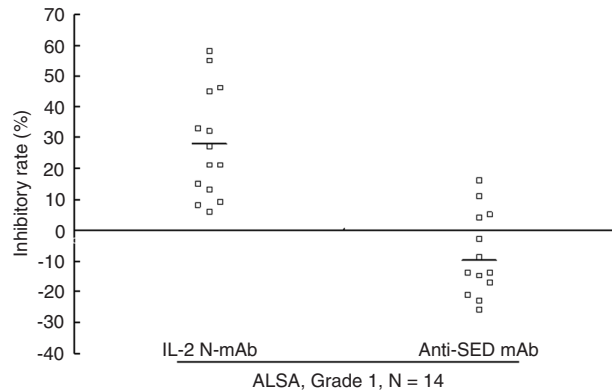


Figure 3. IL-2 N-mAb was essential for a positive ALSA test. PBMCs were isolated from patients with grade 1 heart graft rejection proven by biopsy. The specific stimulators were from the corresponding donor spleen. MTT colorimetry was employed to evaluate the proliferation of the responders. In the presence of IL-2 N-mAb, the responders were significantly suppressed by stimulators in the ALSA test. When IL-2 N-mAb was replaced with anti-SED mAb, no obvious suppression was observed. The statistical significance of the inhibitory rate was analyzed by the Mann-Whitney test. Positive values indicate suppression and negative values indicate enhancement. Horizontal bars represent the mean of the group. N = 14. Anti-SED mAb = anti-staphylococcal enterotoxin D monoclonal antibody. For other abbreviations, see legend to Figure 1.

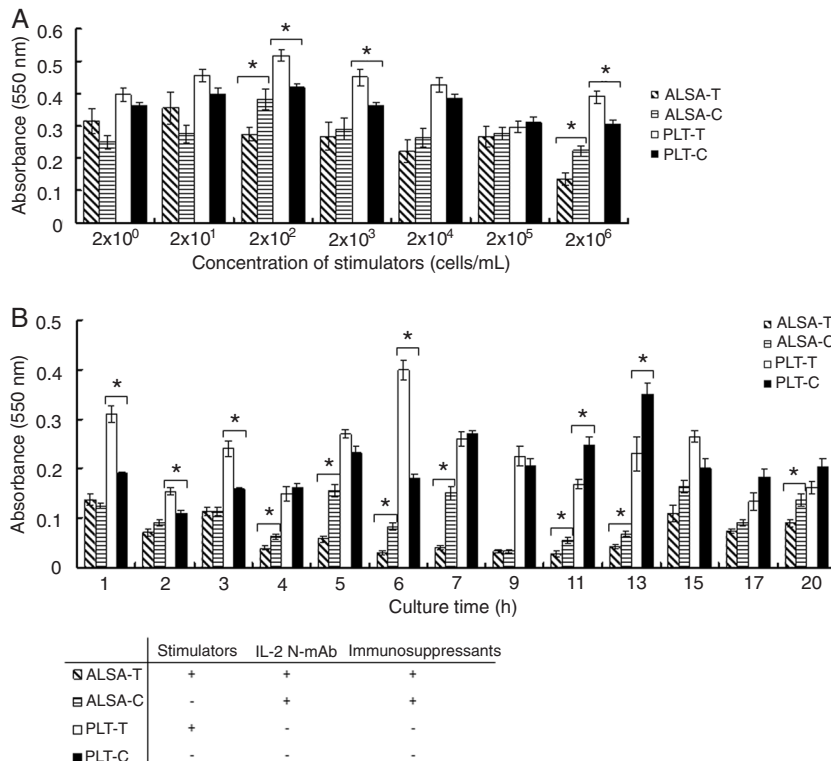


Figure 4. Effects of stimulator cell concentration and culture time on the ALSA test and the routine PLT test. PBMCs were isolated from a patient with grade 1 heart graft rejection proven by biopsy. Frozen-stored donor spleen cells were used as stimulators. MTT chromometry was employed to evaluate the proliferation of activated lymphocytes. Each histogram column indicates the mean \pm SEM value of triplicate wells in each group at 550 nm. *P < 0.05 compared to the corresponding control group (independent sample t-test). A, Effect of stimulator cell concentration on the ALSA test and the routine PLT test; B, effect of culture time on the ALSA test and the routine PLT test. For other abbreviations, see legend to Figure 1.

lator cell concentrations demonstrated that the suppression did not show a stably increasing or decreasing tendency with increasing concentration of stimulators. Significant inhibition only occurred when the stimulator cell concentration was 2×10^2 cells/mL or 2×10^6 cells/mL in the range of 2×10^0 to 2×10^6 cells/mL, with an inhibitory rate of 39 and 29%, respectively. Lenardo et al. (14) also showed that apoptosis of primed lymphocytes could be induced by too little or too much antigen re-stimulation. Thus, the proper concentration of stimulators was essential for the ALSA test.

To investigate the effect of cell culture time course on the ALSA system, the MTT solution was added to each well at different culture time points. The results in Figure 4B show that the inhibitory effect of specific stimulators against activated lymphocytes appeared steadily after 4 h culture in the ALSA test, and the highest inhibitory rate (74%) appeared at 7 h. The time when the suppression occurred might be related to the inherent cell cycle of the primed lymphocytes.

Discussion

It is vital to monitor the rejection reaction for successful solid organ transplantations. However, as a current gold standard for rejection diagnosis, biopsy is an invasive method that recipients are always reluctant to accept. Therefore, a number of methods have been explored as a noninvasive method for the diagnosis of allograft rejection. As cell death was generally accepted to be an important reason for the release of DNA into the plasma, Lo et al. (15) showed that donor-derived DNA sequences could be detected in the plasma of transplant recipients in 1998. Then, researchers attempted to use polymorphisms in the HLA region (16) and a Y chromosome-specific gene (17) to detect donor-derived DNA, in order to reflect rejection. However, these methods were either too complicated or were confined to gender-mismatched donor-recipient transplants. Recently, Snyder et al. (18) described a universal noninvasive detection of solid organ transplant rejection based on the detection of donor-derived DNA. Chemokines CXCL9 and CXCL10 (19), donor-specific antibodies (20), neutrophil gelatinase-associated lipocalin (21), and procalcitonin (22) were also used in assays designed to detect rejection.

In the present study, we developed the ALSA test to detect activated lymphocytes by re-stimulation with the corresponding antigen. It was demonstrated that the suppression in the ALSA test was closely related to the presence of specific activated lymphocytes. By introducing the ALSA test for the detection of heart graft rejection, it was also demonstrated that the suppression could reflect rejection since it was also associated with activated lymphocytes in heart graft recipients. Thus, the ALSA test had the potential to be used as a complementary method to diagnose transplant rejection. Regarding this test, it was speculated that, if the proliferation of activated lymphocytes isolated from the peripheral blood of a heart graft recipient was inhibited by donor spleen cells, re-

jection was occurring *in vivo*. Moreover, post-transplantation, activated lymphocytes targeting the donor appeared prior to the visible rejection, allowing rejection to be diagnosed earlier by the ALSA test.

This raised the question as to why the results obtained with the PLT and the present ALSA tests were different. Comparing the two tests, the culture time in primary one-way MLC was an important difference. Responders used in the PLT test were those that were still alive after 9-14 days primary one-way MLC, which had been proven to be memory cells. However, the responders used in the ALSA test were prepared by primary one-way MLC for 4 days and were still in the active period of cell division and proliferation. When re-stimulated with antigens, these active cells would undergo re-stimulation-induced cell death (RICD) (14,23). It was also shown that RICD occurred through the action of multiple biochemical pathways, including both death receptor signals (FAS-FASL) and pro-apoptotic Bcl-2 family proteins targeting the mitochondria, such as Bim (24-26). As the culture time increased, these cell membrane molecules also changed (27,28), thereby causing alterations in cell response properties (29,30). Therefore, different activation states of lymphocytes were likely to be the main reason causing different results when the PLT and the present ALSA test were compared.

Besides the culture time course in primary one-way MLC, the specific antigen and IL-2 N-mAb might also play vital roles in the ALSA test. The two apoptotic mechanisms of effector T cells were RICD and cytokine withdrawal-induced death (14,23). During the immune response, as antigens were successfully eliminated and T cell growth cytokines such as IL-2 dissipated, an intrinsic pathway of apoptosis caused a major contraction phase of the T cell response (24). As IL-2 was a cytokine essential for the growth and proliferation of activated T cells, it was not surprising that the proliferation of activated lymphocytes was inhibited when IL-2 was neutralized by the addition of IL-2 N-mAb in the ALSA test. At the peak of the immune response, re-stimulation through the T cell receptor also deletes effector T cells when antigens and IL-2 still remain abundant (24). In the ALSA test described here, the proliferation of activated lymphocytes was inhibited most intensively when the stimulator concentration was 2×10^6 mL or 2×10^2 mL, respectively (Figure 4A), which coincided with Lenardo's opinion that too much or too little antigen re-stimulation could induce apoptosis in activated lymphocytes (14). Thus, apoptosis of the activated lymphocytes in the ALSA system should be a result of the optimum environment with high-dose antigens and low IL-2 levels.

An ALSA test was developed to detect the presence of specific activated lymphocytes by adding IL-2 N-mAb and immunosuppressants to the reaction system in the presence of specific stimulators and responders. Although molecular mechanisms underlying this suppression are still poorly understood, the suppression in the ALSA test was also shown to be closely related to the presence of specific activated

lymphocytes, thus reflecting the rejection in these cases. Further studies with larger sample sizes regarding rejection episodes are required to verify whether the ALSA test could be used as a noninvasive method to promptly diagnose heart

graft rejection in clinical practice. The ALSA test was not particularly dependent on pathologies specifically related to the heart. Further studies are needed to determine if it could also be used for solid organ transplants in other tissues.

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