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Control of humoral and cellular immunity-mediated accelerated heart allograft rejection in sensitized rats by low dose FK 506 and splenectomy

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Abstract. ACI heart grafts are rejected, at an accelerated pace, in Lewis (LEW) rats sensitized by donor-type blood admixed with immunoadjuvant (adjuvant complete Freund, ACF) 7 days earlier. In an in vitro study, the anti-ACI cytotoxic antibody titers in the serum increased from 1:4 in nonsensitized rats to 1:128 in sensitized rats; the spontaneous blastogenesis in spleen cells was higher in sensitized rats than in nonsensitized rats; spleen cells from sensitized rats showed a strong proliferative response against donor strain stimulator cells compared with the control; the cytotoxic T cell activity of spleen cells from sensitized rats was higher than that of spleen cells from nonsensitized rats. Treatment with low dose FK506 in combination with splenectomy (Spx) synergistically prolonged the heart allograft survival in this sensitized rat model. In conclusion: (1) Both humoral and cellular responses against the donor antigen appear in the serum and in the spleen of rats sensitized by donor-type blood admixed with immunoadjuvant ACF. (2) A low dose of FK506 together with Spx appears to control this sensitization through different mechanisms, resulting in a prolongation of heart allograft survival.

Key words: Heart allograft survival – Humoral and cellular immunity – FK506 – Splenectomy

Control of allograft rejection in the sensitized recipient remains a challenge in both experimental and clinical transplantation [4]. In clinical studies, it has been shown that the various strategies used to overcome sensitization are mostly empirical. One major problem is the lack of understanding of the immunological mechanisms underlying the rejection of this type of transplant [3]. We have previously reported that the Lewis (LEW) rat, sensitized by donor-type blood admixed with immunoadjuvant (adjuvant complete Freund ACF), could reject an ACI heart allograft in an accelerated fashion and that FK506 could significantly overcome this rejection, although the dose of FK506 needed for such an effect was higher than that in the nonsensitized recipient rat [2].

Our present study was divided into two parts. In the first part, we tried to confirm that both humoral and cellular immunity play important roles in rejection in the sensitized recipient rat. In the second part, after having shown that both the humoral and cellular response could cause accelerated allograft rejection, we tested the effect of low dose FK506 in combination with splenectomy (Spx) on heart allograft survival in this sensitized rat model.

Materials and methods

Animals. Male Lewis rats (RT11) weighing 200–250 g were used as recipients and male ACI rats (RT1a) weighing 150–200 g were used as donors. They were obtained from commercial sources (LEW: charles River, Japan; ACI: Hoshino Experiment Animals, Japan) and kept under specific pathogen-free conditions in our animal facility.

Donor-specific sensitization. One milliliter of donor-type blood admixed with 0.1 ml of the immunostimulating agent ACF was administered subcutaneously 7 days prior to heart transplantation and in the in vitro studies.

Preparation of spleen cells. Lymphocytes were obtained from the rat spleen. The spleen was isolated and minced, and the red cells were then lysed with buffered hypotonic TRIS-ammonium chloride (0.83%, pH 7.21). The cells were washed twice with PRMI 1640, then suspended in RPMI complete medium containing 10% fetal calf serum (FCS), 30 m*M* HEPES, 2.5 m*M* L-glutamine, and 5 μg/ml gentamicin, for the in vitro assay.

Complement-dependent cytotoxicity assay. Prior to transplantation, all LEW rats were tested for serum titers of anti-ACI cytotoxic antibodies by CDC assay, as previously described [8]. The cytotoxic antibody titer was read as positive when more than 50% of donor T lymphocytes were killed compared with the negative control.

Spontaneous blastogenesis assay. Spontaneous blastogenesis (SB) was performed in triplicate by adding 1 μCr of tritiated thymidine (^{3}H-TdR) to 100 μl of 1.0×10^6/ml spleen cells, obtained from non-

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Table 1. Humoral and cellular immunity in nonsensitized or sensitized rats

Group	CDC[a]	SB[b] (cpm) (n = 3)	MLR[c] (cpm) (n = 3)	CML[d] (% killing) (n = 3)	Heart allograft survival[e]	
					Length (days)	mean ± SD
Nonsensitized rats	1:4	5337 ± 1439	55659 ± 2012	0.39 ± 1.0	5, 6 (3), 7 (2)	6.1 ± 0.7
Sensitized rats	1:128	17229 ± 755	10278 ± 642	8.4 ± 1.8	2 (4), 3 (2), 4, 5	2.8 ± 1.1

[a] Serum titers of anti-ACI cytotoxic antibodies
[b] Spontaneous blastogenesis in spleen cells
[c] Spleen cells were used as responder cells and irradiated ACI spleen cells as stimulator cells
[d] Responder cells were cocultured with irradiated ACI stimulator cells, which served as effector cells; target cells were prepared by culturing ACI spleen cells with concanavalin A for 2 days
[e] Nonsensitized and sensitized LEW rats were used as recipient and ACI rats as donor

sensitized or sensitized recipient rats. The mixture was incubated for 8 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. The cells were harvested onto glassfiber filter paper with an automatic harvester, and ^{3}H-TdR incorporation was measured in a beta-scintillation counter (1205 Beta Plate).

Mixed lymphocyte reaction (MLR). One-way MLR was performed, using spleen cells from nonsensitized or sensitized LEW rats as responder cells and from ACI rats was stimulator cells. The 0.5×10^6/ml responder cells were co-cultured with 1.0×10^6/ml 2000-rad-irradiated stimulator cells in RPMI 1640 complete medium. The cells are incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 4 days and then treated with an 16–20-h tritiated thymidine pulse. The cells were harvested, and the ^{3}H-TdR incorporation was measured as described.

Cell-mediated lympholysis (CML). Spleen cells from sensitized LEW rats as responder cells were co-cultured with 2000-rad-irradiated ACI stimulator cells in RPMI 1640 complete medium at 37 °C in a humidified atmosphere of 5% CO_2 for 6 days. Splenic responder cells from nonsensitized LEW rats were used as the control. After incubation, the cells were harvested and used as effector cells for CML. The target cells were prepared by culturing stimulator cells with 50 µg/ml concanavalin A (ConA) for 2 days. The 4.0×10^6/ml effector cells were cultured with 1.0×10^5/ml ^{51}Cr-labeled target cells, for 4 h at 37 °C in a humidified atmosphere of 5% CO_2. A fixed volume of supernatant was collected from each well after centrifugation of 1500 g for 10 min, and the ^{51}Cr release was counted in a gamma-counter (Aloka. JDC-752). The percentage cytotoxicity was calculated according to the following formula:

$$\%\,\text{Cytotoxicity} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximum release} - \text{Spontaneous release}} \times 100$$

Heterotopic heart transplantation and splenectomy. Heart transplantation was performed using the modified technique of Ono and Lindsey [7]. The heart allograft survival was determined by daily palpation. Rejection was considered complete at the time of cessation of a palpable heartbeat and confirmed by histological examination. Spx was done at the time of grafting.

Immunosuppressive agent. FK506 was supplied by Fujisawa Pharmaceutical (Osaka, Japan). The drug was dissolved in physiological saline and administered intramuscularly from day 0 to day 10 after transplantation.

Statistical analysis. The statistical significance of differences between the untreated and experimental groups was ascertained using Student's t-test.

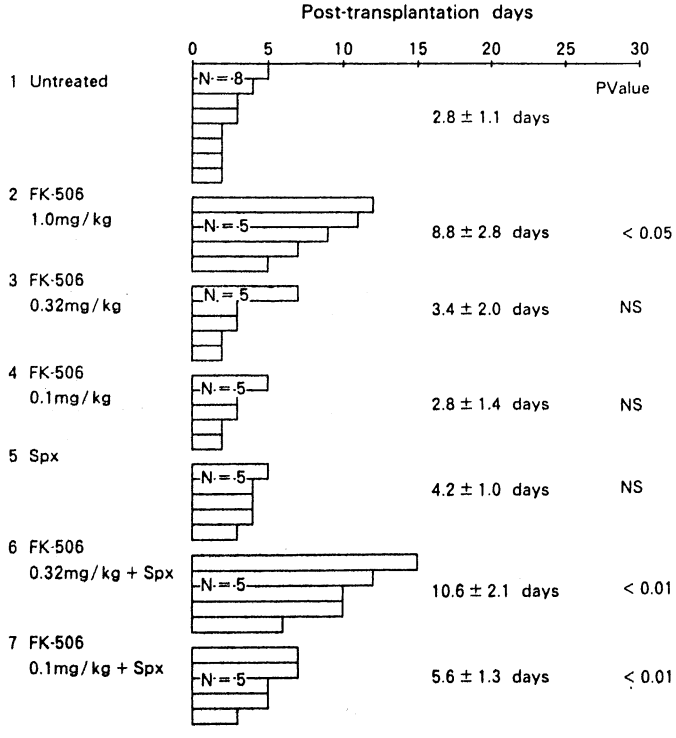

Fig. 1. Survival of heart allografts in sensitized recipient rats. ACI heart grafts were transplanted into sensitized LEW recipient rats. Splenectomy (Spx) was done at the time of grafting. FK506 was administered intramuscularly from day 0 to day 10 after transplantation. A P value < 0.05 was considered to indicate significant difference between the untreated control group and the experimental group

Results

Humoral and cellular immunity

Table 1 illustrates the results of antibody titers, SB, MLR, CML, and heart allograft survival in nonsensitized or sensitized LEW rats on day 7 after immunization with donor-type blood admixed with immunoadjuvant ACF. The positive serum antibody titers increased from 1:4 in nonsensitized rats to 1:128 in sensitized rats. SB was the stable measure of the nonspecific immune status of the ungrafted animal. A significant difference was seen in this assay between nonsensitized and sensitized rats. In the MLR, responder cells from sensitized LEW rats showed a higher proliferative response against ACI rat stimulator cells compared with nonsensitized ones. The CML activity was 0.3 ± 1.0% in spleen cells from nonsensitized LEW rats but 8.4 ± 1.8% in spleen cells from sensitized ones. The average ACI heart graft survival in the nonsensitized LEW control group was 6.1 ± 0.7 days; however, sensitized LEW recipient rats showed a mean graft survival time of 2.8 ± 1.1 days. These results suggest that subcuta-

neously injected donor-type blood admixed with immunoadjuvant ACF to the recipient rat 7 days prior to transplantation and in the in vitro assay could increase both humoral and cellular immunity against donor antigen, resulting in sensitization.

Effect of low dose FK506 and Spx on heart allograft survival in sensitized rat

Graft survival results are shown in Fig. 1. Untreated controls showed rejection in 2.8 ± 1.1 days. FK506 alone could significantly prolong ACI heart graft survival in the sensitized recipient rats in a dose-dependent manner. The lowest effective dose for overcoming sensitization was 1.0 gm/kg daily. Spx alone showed a prolongation of heart allograft survival to 4.2 ± 1.0 days on the average. On the other hand, the combinations of FK506 0.1 mg/kg daily plus Spx and FK506 0.32 mg/kg daily plus Spx prolonged heart allograft survival to 5.6 ± 1.3 days and 10.6 ± 2.1 days, respectively. These results indicate that the low dose of FK506, in combination with Spx, could synergistically prolong the heart allograft survival in sensitized recipient rats.

Discussion

These studies demonstrate that the importance of both humoral and cellular responses against donor antigen which appear in the serum and spleen of rats sensitized by subcutaneously injected donor-type blood admixed with immunoadjuvant ACF. The role of humoral activity in sensitized recipients is emphasized by a progressive increase of serum CDC titers, and the cellular component is reflected by strong SB, MLR, and CML responses in the spleen. The appearance of these humoral and cellular responses are associated with a hyperacute-like rejection of donor heart allografts in the ACI to LEW rat combination [5, 6]. However, as the allografts in this experiment fail in 2.8 ± 1.1 days, the term accelerated rejection may be more suitable for this kind of allograft outcome.

The mechanisms of accelerated rejection, induced by donor-type blood admixed with immunoadjuvant ACF, are not clear, but it may be assumed that a strong histocompatibility mismatching in the rat combination and a strong stimulation of donor antigen with immunoadjuvant could cause (1) cytotoxic antibody formation and (2) nonspecific lymphocyte, T lymphocyte, and cytotoxic T lymphocyte activation in the recipient rat. These cytotoxic antibodies and activated lymphocytes may contribute to the accelerated allograft destruction in this sensitized rat model [1, 5, 6].

The data obtained in this study show that FK506 could significantly prolong ACI heart graft survival in sensitized LEW recipient rats in a dose-dependent manner, with a dosage of 1.0 mg/kg daily, as compared with the control. However, the dosage of FK506 needed for such an effect is higher than that in nonsensitized recipient rats. Spx could also prolong the heart allograft survival in this sensitized rat model, but it proved to be insufficient for sustaining a graft survival for more than 4.2 days. This may be due to the fact that the Spx can control the cytotoxic antibody production to a certain degree but failed to overcome another barrier, cell-mediated rejection, in the sensitized situation.

An important finding in the present study was that combined treatment with a low dose of FK506 plus Spx could synergistically prolong the heart allograft survival in sensitized recipient rats. Although FK506 by itself, at a high dose, could overcome sensitization, this can be considered toxic to the recipients. A combination of low dose FK506 with Spx, as an adjunct therapy, appears to diminish the toxic effect of FK506 and induce an synergistic effect of heart allograft survival in this sensitized rat model. These results indicate that the low dose of FK506 combined with Spx could not only control the cytotoxic antibody formation but also overcome cellular immunity-mediated rejection, resulting in a 3.7-fold prolongation of heart allograft survival in sensitized recipient rats.

In conclusion, the accelerated rejection of a heart allograft in sensitized rats correlates with the host's humoral and cellular response. It can be controlled by a low dose of FK506 together with Spx, through different effector mechanisms. This combination approach may become an effective strategy for overcoming sensitization in clinical transplantation.

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