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Goro Matsumiya
Ryota Shirakura
Shuji Miyagawa
Hironori Izutani
Seizoh Nakata
Yasuhisa Shimazaki
Susumu Nakano
Hikaru Matsuda

Role of CD4$^+$ T cells in the rat to mouse cardiac xenotransplantation

G. Matsumiya (✉) · S. Miyagawa
H. Izutani · S. Nakata
Y. Shimazaki · S. Nakano
H. Matsuda
First Department of Surgery,
Osaka University Medical School,
2-2 Yamadaoka, Suita,
Osaka, 565, Japan

R. Shirakura
Division of Organ Transplantation,
Biomedical research center,
Osaka University Medical School,
Japan

Abstract T cell subsets involved in rejection of xenografts were analyzed using a rat to mouse cardiac xenotransplant model. Proliferating response and interleulin-2 (IL-2) production in recipients' spleen cells were almost completely abrogated by elimination of L3T4$^+$ T cells, but not by elimination of Lyt2.1$^+$ T cells. Cytotoxic T lymphocyte (CTL) activities were mediated by both L3T4$^+$ and Lyt2.1$^+$ T cells with the help of IL-2-producing L3T4$^+$ T cells. Administration of anti-L3T4 monoclonal antibody (mAb) into recipient mice resulted in a significant prolongation of graft survival (mean graft survival was 29.2 days). Moreover, anti-L3T4 mAb treatment plus thymectomy led to indefinite graft survival. Anti-rat endothelial cell (EC) antibody production in the grafted mice was remarkably suppressed by anti-L3T4 mAb treatment. In contrast, Lyt2.1 mAb treatment did not prolong the graft survival and did not suppress anti-EC antibody production. These results indicated the absolute requirement of L3T4$^+$ T cells in the rejection of rat to mouse cardiac xenografts.

Key words Concordant xenotransplant · Cardiac xenograft L3T4$^+$ T cell · Thymectomy Anti-endothelial cell antibody

Introduction

Allograft rejection results from interactions between T cells with distinct phenotypes, antigen specificities, and functional capabilities [1–3]. In xenograft rejection, however, the role of T cells and cell-cell interactions between T cell subsets have not been clearly defined [4, 5]. In particular, there have been few basic immunological studies elucidating the role of cellular immunities in rejection of vascular organ xenografts [4–6].

Some previous studies have reported the effects of anti-CD4 and/or anti-CD8 monoclonal antibody (mAb) treatment in nonvascular organ xenografts. Pierson et al. have achieved longer survival in discordant skin xenografts than in skin allografts with anti-CD4 mAb treatment [7]. The importance of CD4$^+$ T cells has also been demonstrated in islet xenografts in concordant and discordant species combinations [8, 9]. These reports, however, have not analyzed the precise immunological role of CD4$^+$ T cells involved in graft rejection mechanisms. Furthermore, these results could not be applied to vascular organ xenografts, as differences exist in the rejection mechanism between vascular and nonvascular organ transplantation. Nonvascular models have been shown to be resistant to humoral effectors and are more susceptible to cell-mediated rejection [10], although in-

duced antibodies elicited by xeno-antigens are primarily important in the rejection of vascular concordant xenografts [11–13].

The aim of this study was to investigate T cell responses, especially the role of the interaction of T cell subsets involved in both the helper and effector phase of rejection in concordant cardiac xenografts. As a model of concordant cardiac xenotransplantation, we used the inbred rat to mouse combination, because it provides more precise immunological information than the outbred models have done in previous studies.

Materials and methods

Animals

Male inbred Lewis rats (Oriental East Inc., Japan), 10–15 days old, were used as donors. Male C3H/HeN mice (Japan SLC. Inc., Japan), 8–10 weeks old, were used as recipients. They were maintained under specific pathogen-free conditions.

Cardiac transplantation

The donor rats and recipient mice were anesthetized with intraperitoneal chloral hydrate. Heterotopic cardiac transplants were placed in the abdomen of recipients, using a modification of the microvascular technique described by Corry et al. [14]. Rejection was defined as the complete cessation of the myocardial contraction, which was determined by daily palpation and confirmed by laparotomy when necessary.

Thymectomy

The recipient C3H/HeN mice were anesthetized by ether and thymectomized through a cervical incision.

Medium used for in vitro cell culturing

Culture medium used for cell culturing was RPMI 1640 supplemented with 10% fetal calf serum, 5×10^{-5} *M* 2-mercaptoethanol, 2 m*M* L-glutamine, 1 m*M* sodium pyruvate, 0.1 m*M* nonessential amino acids, 10 m*M* HEPES solution, 100 U/ml penicillin, and 0.1 mg/ml streptomycin.

Monoclonal antibodies

Anti-L3T4 (GK1.5) [15] and anti-Lyt2.1 mAb [16] (kindly presented by Dr. E. Nakayama, University of Okayama, Japan) were obtained from the ascitic fluid of hybridoma-bearing mice. The gamma globulin fraction of the ascitic fluid was obtained by ammonium sulfate precipitation. The titer of mAbs, determined by the antibody-mediated complement-dependent cytotoxicity assay, was greater than 1:10000. We injected 0.2 ml mAbs diluted 1:6 (anti-L3T4) or 1:8 (anti-Lyt2.1) intravenously on day −4 and day 0 of the cardiac transplantation for in vivo depletion of T cell subsets.

In vitro elimination of T cell subsets with mAb and complement (C)

Spleen cells (2×10^7/ml) were incubated at 4 °C for 30 min with × 200 diluted anti-L3T4 or anti-Lyt2.1 mAb. The cells were washed and incubated at 37 °C for 45 min with rabbit C diluted 1:8, which has a low toxicity for mouse lymphocytes (Cedarlane, Ontario, Canada). The efficacy of these elimination protocols was confirmed by flow cytometrical (FCM) analysis, which demonstrated that more than 99% of the respective $L3T4^+$ or $Lyt2.1^+$ T cell subset was eliminated (data not shown).

Mixed lymphocyte reaction (MLR)

We cultured 2×10^5 mouse responder spleen cells with 4×10^5 irradiated (2000 rad) stimulator mouse (syngeneic) or rat (xenogeneic) spleen cells in a total volume of 0.2 ml in triplicate wells. After 96 h of culture [^{3}H]TdR uptake was measured by the scintillation counter. Results are expressed as mean uptake in CPM ± SEM of four distinct experiments.

Assay for cytotoxic T lymphocyte (CTL) activity

We cultured 4×10^6 spleen cells with 1×10^6 irradiated (2000 rad) rat spleen cells in a 24-well culture plate in a volume of 2 ml. Effector cells generated after 96 h of culture were assayed on ^{51}Cr-labeled target cells (rat spleen cells cultured for 3 days with concanavalin A, 5 μg/ml). Percentage of specific lysis was calculated as follows: percentage of specific lysis = (experimental-spontaneous ^{51}Cr release)/(maximum-spontaneous ^{51}Cr release) × 100%. For analysis of the effect of exogeneous IL-2 addition, recombinant mouse IL-2 was added to the culture medium in a final concentration of 10 U/ml. Results are expressed as the mean percentage of specific lysis of four distinct experiments.

Interleukin-2 (IL-2) production and assay system for IL-2 activity

Culture supernatants from the MLR were harvested after 48 h of culture. The IL-2-dependent T cell line CTLL-2 (10^4/well) was cultured with 0.2 ml of the culture supernatant for 24 h. Proliferation of CTLL-2 was assessed by the uptake of [^{3}H]TdR.

Explantation of rat endothelial cells (ECs) and detection of anti-EC antibody by FCM

Rat coronary ECs were obtained using the method described by Piper et al. [17]. We incubated 1×10^6 ECs at 4 °C for 30 min with 0.05 ml diluted mouse serum. The cells were then washed three times and incubated with 0.01 ml FITC-conjugated goat anti-mouse Ig antibody (Organ Teknika, Durham, N.C.). After 30 min incubation at 4 °C, the cells were washed twice, and analyzed for fluorescence using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems).

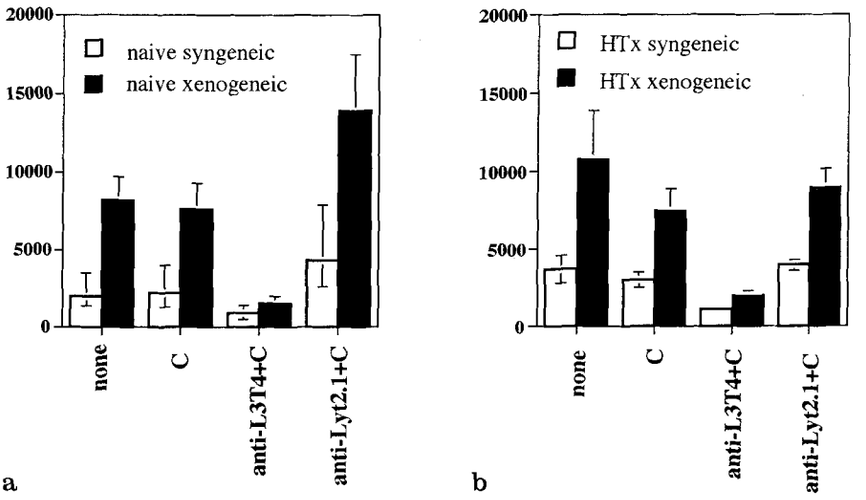

Fig. 1 a, b Proliferating response (MLR) of (**a**) naive or (**b**) xeno-heart-transplanted C3H/HeN mouse spleen cells depleted of T cell subsets. Spleen cells obtained at (**a**) pretransplant or (**b**) 6th posttransplant day were treated with anti-L3T4 or anti-Lyt2.1 mAb and complement (C), and cultured with irradiated syngeneic or xenogeneic (rat) spleen cells for 4 days. Proliferation was measured by [^{3}H]TdR uptake

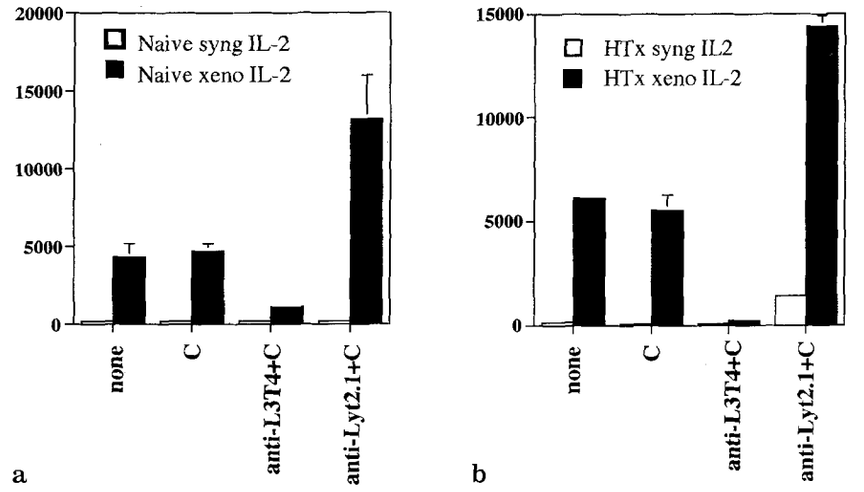

Fig. 2 a, b IL-2 production by (**a**) naive or (**b**) xeno-heart-transplanted C^{3}H/HeN mouse spleen cells in response to syngeneic or xenogeneic (rat) spleen cell stimulators. Responding cells were the same as used in Fig. 1 and cultured with irradiated spleen cell stimulators for 2 days. Culture supernatants were harvested and tested for stimulation of CTLL-2 proliferation

Results

Primary and secondary helper T cell responses

Results of proliferating response in naive and post-heart-transplanted mouse spleen cells harvested at rejection to xenogeneic rat spleen cells are demonstrated in Fig. 1. Depletion of L3T4$^+$ T cells from responder spleen cells led to almost complete abrogation of proliferating response in both the primary and secondary MLR. Lyt2.1$^+$ T cell depletion, however, resulted in no decreased proliferating response. IL-2 production was also dependent on L3T4$^+$ T cells in the primary and secondary response (Fig. 2).

T cell subsets involved in CTL activity

CTL activity to rat xeno-antigens was detected in mouse spleen cells at rejection. To determine the T cell subset involved in this response, effects of depletion of the T cell subsets were investigated. Depletion of L3T4$^+$ T cells from responder mouse spleen cells resulted in a significant reduction in CTL activity. In contrast, depletion of Lyt2.1$^+$ T cell subset had only a marginal suppressive effect (Fig. 3). The addition of exogeneous recombinant mouse IL-2 into the culture medium resulted in a recovery of the CTL activity in responders depleted of L3T4$^+$ T cells to the same level as that in the no treatment group (Fig. 3). These results indicated that both L3T4$^+$ and

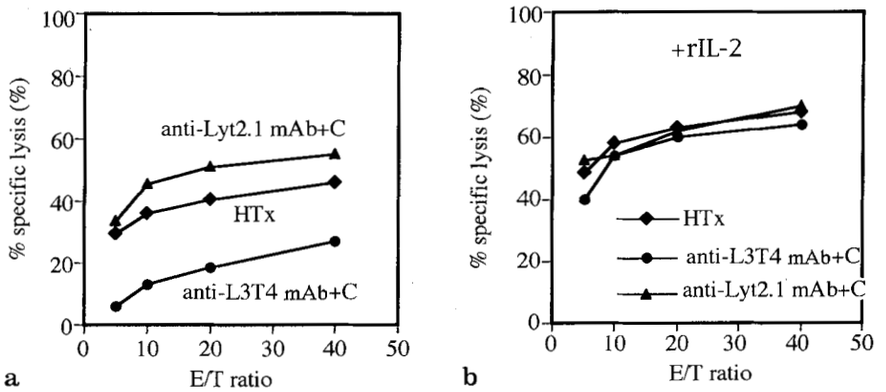

Fig. 3 **a** Anti-rat CTL activity in xeno-heart-transplanted C3H/HeN mice. Spleen cells from mice transplanted with rat hearts were untreated or treated with anti-L3T4 or anti-Lyt2.1 mAb and C. These cells were cultured with irradiated rat spleen cells, and effector cells generated after 4 days culture were assayed for ^{51}Cr-labeled target blast cells. The results are shown as the mean percentage specific lysis. **b** Effect of exogeneous IL-2 addition on anti-rat CTL activity. Responding spleen cells were the same as described above, and cultured in the medium with 10 U/ml recombinant mouse IL-2 added

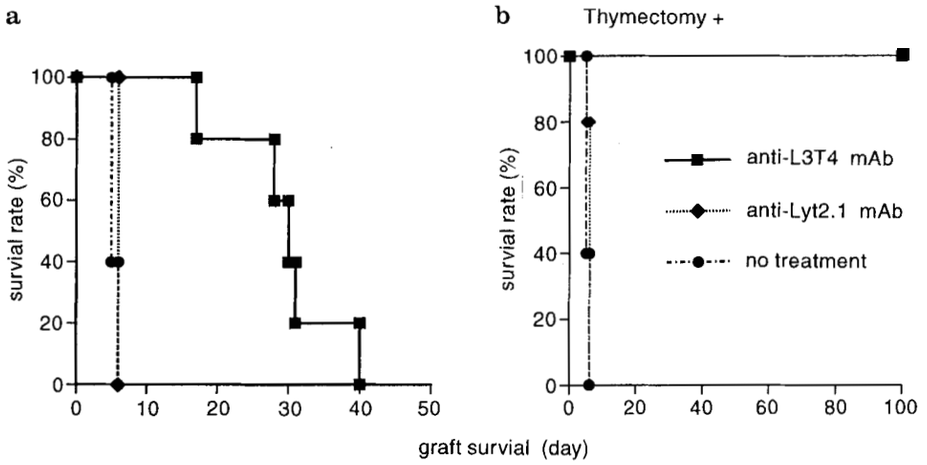

Fig. 4 **a** Graft survival of mAb-treated mice. Anti-L3T4 or anti-Lyt2.1 mAb were injected into recipient mice on day −4 and day 0 of transplantation. **b** Graft survival of thymectomized mice not treated or treated with anti-L3T4 or anti-Lyt2.1 mAb

Lyt2.1$^+$ T cells act as CTLs to rat xeno-antigens, although this reaction needs the IL-2-producing L3T4$^+$ T cell help.

Effect of in vivo administration of anti-L3T4 or anti-Lyt2.1 mAb and thymectomy

To confirm the results obtained from the in vitro experiments, a series of studies were performed, using anti-L3T4 or anti-Lyt2.1 mAb in vivo. Treatment with the mAbs eliminated more than 98% of each subclass of T cells. This eliminated state lasted for 2 weeks (data not shown). Anti-L3T4 mAb treatment resulted in a marked prolongation of the mean graft survival from 5.6 to 26.2 days. In contrast, anti-Lyt2.1 mAb treatment did not prolong the graft survival (Fig. 4a). To determine whether the rejection in the anti-L3T4 mAb treatment group was mediated by the L3T4$^+$ T cells repopulated from the recipient's thymus or other types of cells, the effect of thymectomy was investigated. The thymectomized recipients treated with anti-L3T4 mAb did not reject the rat hearts for more than 100 days, although those with anti-Lyt2.1 mAb treatment rejected as fast as those without mAb treatment (Fig. 4b). Production of anti-endothelial cell antibodies was suppressed in anti-L3T4 mAb treatment group but not in the anti-Lyt2.1 mAb treatment group (Fig. 5).

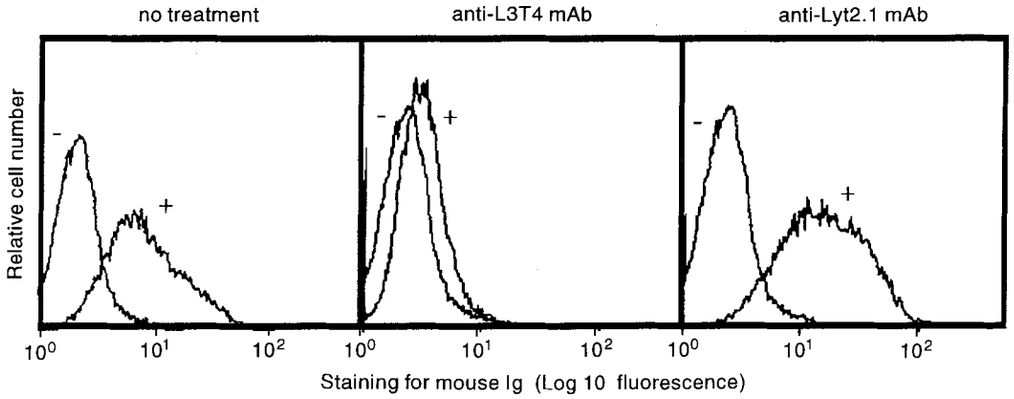

Fig. 5 Anti-rat endothelial cell antibody in serum of mice not treated or treated with anti-L3T4 or anti-Lyt2.1 mAb

Discussion

Many studies concerning allograft rejection in vivo have revealed interactions between helper and effector T cells of different phenotypes and antigen specificities [1–3]. Furthermore, several immune-manipulating methods of T cell responses to control allograft rejection have been demonstrated [18, 19]. In contrast, T cell responses in xenograft rejection in vivo remain unclear [4, 5].

Concordant xenografts were first defined as "first set cross-species grafts which are rejected at a tempo and with morphologic features similar to first-set allografts" [20]. However, recent reports have demonstrated that induced antibodies elicited by xeno-antigens are of primary importance in rejection of vascular concordant xenografts [8–10]. We have also demonstrated the essential role of anti-EC antibodies in the rat to mouse cardiac xenograft rejection [21], but the correlation with helper T cells that might mediate this antibody production remains unclear.

The role of cell-mediated graft rejection mechanisms such as CTLs, which play a central role in allograft rejection, are also obscure in concordant cardiac xenografts [4, 5]. In vitro analysis has shown the presence of CTL precursors to xeno-antigens although with less frequency than to alloantigens [22, 23]. Furthermore, several recent reports have demonstrated cellular infiltration in xenografts under suppressed humoral responses [12, 13]. These observations suggest the importance of effector T cells as the potential rejection mechanism secondary to humoral responses, but these effector T cells have not been well characterized.

In the present study, we demonstrated the absolute requirement of $L3T4^+$ T cells in the rejection of the rat to mouse cardiac xenograft, which was supported by the finding that anti-L3T4 mAb treatment plus thymectomy resulted in indefinite graft survival. Our results revealed two distinct functions of $L3T4^+$ T cells in mediating graft rejection.

First, only $L3T4^+$ T cells but not $Lyt2.1^+$ mediated the helper function both in xeno-antibody production and in the generation of CTLs in this xenograft rejection, although both $L3T4^+$ and $Lyt2.1^+$ T cells mediate these helper responses in allograft rejection [24]. Auchincloss et al. have shown that xeno-antigens are processed by self-APCs and presented together with class II MHC molecules as nominal antigens, which leads to the recognition of xeno-antigens by $CD4^+$ T cells in discordant monkey and mouse combinations [25]. We also showed that in the concordant rat to mouse cardiac xenograft model, non-MHC antigens on graft ECs cells act as strong immunogens (manuscript in preparation), which could explain the $L3T4^+$ T cell requirement in mediating helper function in this model.

Second, an important aspect of the $L3T4^+$ T cell function is their potential to mediate graft rejection as CTLs. Histological analysis of the rejected rat hearts showed scant cellular infiltration, which suggested the poor contribution of cellular effectors in the primary rejection in this model [21]. However, elevated CTL activity indicated the possible participation of CTLs in the secondary graft rejection. Our results showed that both $L3T4^+$ and $Lyt2.1^+$ T cells can act as CTLs under the IL-2-producing $L3T4^+$ T cell help, that is, $L3T4^+$ T cells not only mediate the production of CTLs but also act as CTLs. These results are compatible with the observation that mouse skin allograft with multiple dis-

parate minor histocompatibility antigens is rejected by $L3T4^+$ and $Lyt2.1^+$ CTLs [1–3].

In conclusion, $L3T4^+$ T cells are an essential factor in the rejection of rat to mouse cardiac xenografts. The indefinite graft survival by depletion of $L3T4^+$ T cells was obtained by the suppression of induced antibody production and CTL activation.

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