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Contribution of Fas ligand to cardiac allograft rejection

（同種心移植拒絶反応におけるFasリガンドの関与）

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Contribution of Fas ligand to cardiac allograft rejection

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Abstract

Effector mechanisms for allograft injury remain unclear. In the present study, we verified the contribution of Fas and Fas ligand (FasL) to cardiac allograft rejection by utilizing the Fas-deficient *lpr* or FasL-deficient *gld* mice as the donor or recipient. Cardiac myocytes prepared from normal mice, but not those from *lpr* mice, constitutively expressed Fas and were susceptible to FasL-mediated lysis. Survival of cardiac allografts was substantially prolonged when *gld* or *lpr* mice were used as the recipient. In contrast, cardiac allografts from *lpr* mice were normally rejected without a delay. Histological examination of the grafts in the *gld* or *lpr* recipients demonstrated a lesser cellular infiltration and much milder myocyte damage. Proliferative response and cytotoxic T lymphocyte induction against the donor-type alloantigens were not impaired in the *gld* or *lpr* recipients. These results indicate a substantial contribution of FasL to cardiac allograft rejection, independent of Fas in the grafts. This raises a possibility that FasL may be more generally involved in tissue damage associated with various diseases than expected from the expression of Fas in the target organs.

Introduction

It has been generally agreed that T cells play a central role in allograft rejection. Extensive cellular infiltration, consisting of $CD4^+$ or $CD8^+$ T cells and macrophages, is commonly observed in the allografts undergoing acute rejection. However, the effector mechanisms that lead to tissue damage and graft dysfunction remain unclear. It has been supposed that cytotoxic T lymphocytes (CTL) play a critical role since potent allo-specific CTL can be readily generated during rejection. Recently, two predominant mechanisms for CTL cytotoxicity have been revealed, that are mediated by perforin and granzymes stored in cytoplasmic granules or FasL expressed on activated CTL (1–4). Perforin and granzymes have been implicated in cardiac allograft rejection since expression of these molecules could be demonstrated in the grafts undergoing rejection (5). However, Schulz *et al.* recently demonstrated that cardiac allografts were normally rejected in perforin-deficient mice generated by gene targeting (6). This formally excluded perforin as a crucial effector molecule in cardiac allograft rejection. On the other hand, the heart has been reported to express Fas mRNA (7) but the involvement of Fas and its ligand (FasL) in cardiac allograft rejection remains to be verified. In this context, we examined the expression and function of Fas on cardiac myocytes and the involvement of Fas and FasL by using Fas-deficient *lpr* or FasL-deficient *gld* mice as the donor or recipient. We obtained inconsistent results that survival of cardiac allografts was prolonged in the FasL-deficient recipients but the Fas-deficient allografts were normally rejected. The mechanism apparently dependent on FasL but not on Fas in the graft is discussed.

Methods

Mice

Five-week-old male BALB/c mice (H-2^d) and C3H/He (C3H) +/+ mice (H-2^k) were purchased from Charles River (Atsugi, Japan). Five-week-old male C3H *lpr/lpr* (*lpr*) or *gld/gld* (*gld*) mice and C57BL/6 (B6) +/+ or *lpr* mice (H-2^b) were purchased from SLC (Shizuoka, Japan). B6 *gld* mice, originally obtained from Jackson Laboratory (Bar Harbor, ME), were maintained in our animal facilities. Timed pregnant BALB/c and MRL *lpr* mice were purchased from Charles River.

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Preparation of cardiac myocytes

Fetal cardiac myocytes were prepared as described previously (8) with minor modifications. In brief, the hearts were aseptically removed from 16-day-old fetal mice, minced in PBS and digested with 0.05% trypsin–EDTA. The isolated myocytes were washed and cultured in DMEM containing 10% FCS with or without 200 U/ml of murine IFN-γ (Genzyme, Cambridge, MA). After 24 h, the cells were subjected to immunofluorescence and cytotoxic assay.

Immunofluorescence

The cultured myocytes were collected by brief trypsinization and stained with phycoerythrin-labeled anti-mouse Fas mAb (Jo2) or FITC-labeled anti-mouse ICAM-1 mAb (3E2), both of which were obtained from PharMingen (San Diego, CA). After washing with PBS, the cells were analyzed on a FACScan (Becton Dickinson, San Jose, CA) and data were processed by using the CELLQuest program.

Preparation of mouse FasL (mFasL) transfectants

mFasL cDNA was prepared by RT-PCR from total RNA of ionomycin/PMA-activated B6 spleen cells by using an oligonucleotide corresponding to the first six codons as the 5′ primer and that corresponding to the last six codons as the 3′ primer, according to the published sequence (9). The 5′ and 3′ primers were tagged with a *Xho*I or a *Not*I site respectively. After *Xho*I and *Not*I digestion, the PCR product of 850 bp was subcloned into pBluescript II SK(+) (Strategene, La Jolla, CA) and nucleotide sequence was confirmed by sequencing. The 850 bp cDNA was then transferred into the *Xho*I and *Not*I sites of the BCMGSneo expression vector (10) (kindly provided by Dr H. Karasuyama, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). For generating stable transfectants, mFasL/BCMGSneo was transfected into a Fas$^-$ mouse T lymphoma L5178Y (kindly provided by Dr S. Yonehara, Kyoto University, Kyoto, Japan) by electroporation (290 V, 960 μF) using a Gene Pulser (BioRad, Hercules, CA) as described previously (11). After selection with 0.4 mg/ml G418 and cloning by limiting dilution, a transfectant (mFasL/L5178Y) expressing a high level of mouse FasL mRNA was selected by Northern hybridization.

Cytotoxic assay

Cytotoxic activity of the mouse FasL transfectant against Fas$^-$ L5178Y and its mouse Fas transfectant (mFas/L5178Y) (12) was estimated by a standard 4 h ^{51}Cr-release assay as described previously (12). Since the incorporation of ^{51}Cr by cardiac myocytes was minimal, we estimated the cytotoxicity against these cells by measuring the cell viability by a fluorimetric assay using the dye alamar Blue™, which gives similar results to the conventional MTT assay as described previously (11). The cardiac myocytes (4×10^4) were cultured with or without mFasL/L5178Y or L5178Y cells (1×10^5) in the presence or absence of an anti-Fas mAb (Jo2, 10 μg/ml) in 200 μl of 10% FCS/DMEM. After 20 h, non-adherent cells were removed, and 100 μl of the medium and 10 μl of alamar Blue (Alamar Biosciences, Sacremento, CA) were added. After a further 4 h incubation, fluorescence of the reduced alamar Blue was measured on a Titertek Fluoroskan II (Labosystems Japan, Tokyo, Japan) at 590 nm by excitation at 544 nm. Percent viability was calculated by defining the value with no treatment as 100%.

Heart transplantation

Abdominal heterotopic heart transplantation was performed as described previously (13). The rejection was defined by complete cessation of palpable heartbeats, and confirmed by laparotomy and histological examination. Statistical analysis was performed using the Wilcoxon/Mann–Whitney rank sum test. $P < 0.05$ was considered significant.

Histology

The allografts were removed for histological examination at 4–7 days after the transplantation. For hematoxylin & eosin staining, the grafts were fixed in 10% formalin and embedded in paraffin. Sections (5 μm) were stained with hematoxylin & eosin by a standard procedure. For immunohistochemical staining, the grafts were immediately frozen in OCT compound. Cryostat sections were fixed with acetone and stained with anti-mouse CD4 (RM4-5; PharMingen), anti-mouse CD8 (53-6.7; PharMingen) or anti-mouse macrophage (Mac-3; Serotec, Oxford, UK) mAb by using the Vectastain ABC kit (Vector, Burlingame, CA) or FITC-labeled goat anti-rat IgG antibody (Caltag, San Francisco, CA) according to the manufacturer's instruction.

Mixed lymphocyte reaction

Splenic T cells were prepared from the recipients at day 7 after the heart transplantation by passing the splenocytes through a nylon wool column. These cells (2.5×10^5) were co-cultured with irradiated (2000 rad) BALB/c splenocytes (2.5×10^5) in RPMI 1640 medium supplemented with 5 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 1 mM sodium pyruvate and 10% FCS in 96-well flat-bottomed microtiter plates. Proliferative response was estimated by pulsing the cultures with 1 μCi [^{3}H]thymidine for the last 18 h. Thymidine uptake was assessed as described previously (14).

CTL induction and assay

Splenic T cells (5×10^6) from the recipients were co-cultured with irradiated BALB/c splenocytes (5×10^6) in 2 ml of the culture medium for 5 days. CTL activity generated in the cultures was tested against P815 (H-2^d) target cells at the indicated E/T ratios by a standard 4 h ^{51}Cr-release assay. Percent specific cytotoxicity was calculated as described previously (12). For generating alloreactive CTL *in vivo*, C3H+/+, *gld* or *lpr* mice were i.p. injected with 1×10^7 P815 cells. At 7 days after the second injection, peritoneal cells were collected and their cytotoxic activity was tested against ^{51}Cr-labeled P815 target cells.

Results

Functional Expression of Fas on cardiac myocytes

We first examined the surface expression of Fas on murine cardiac myocytes. Fetal cardiac myocytes prepared from

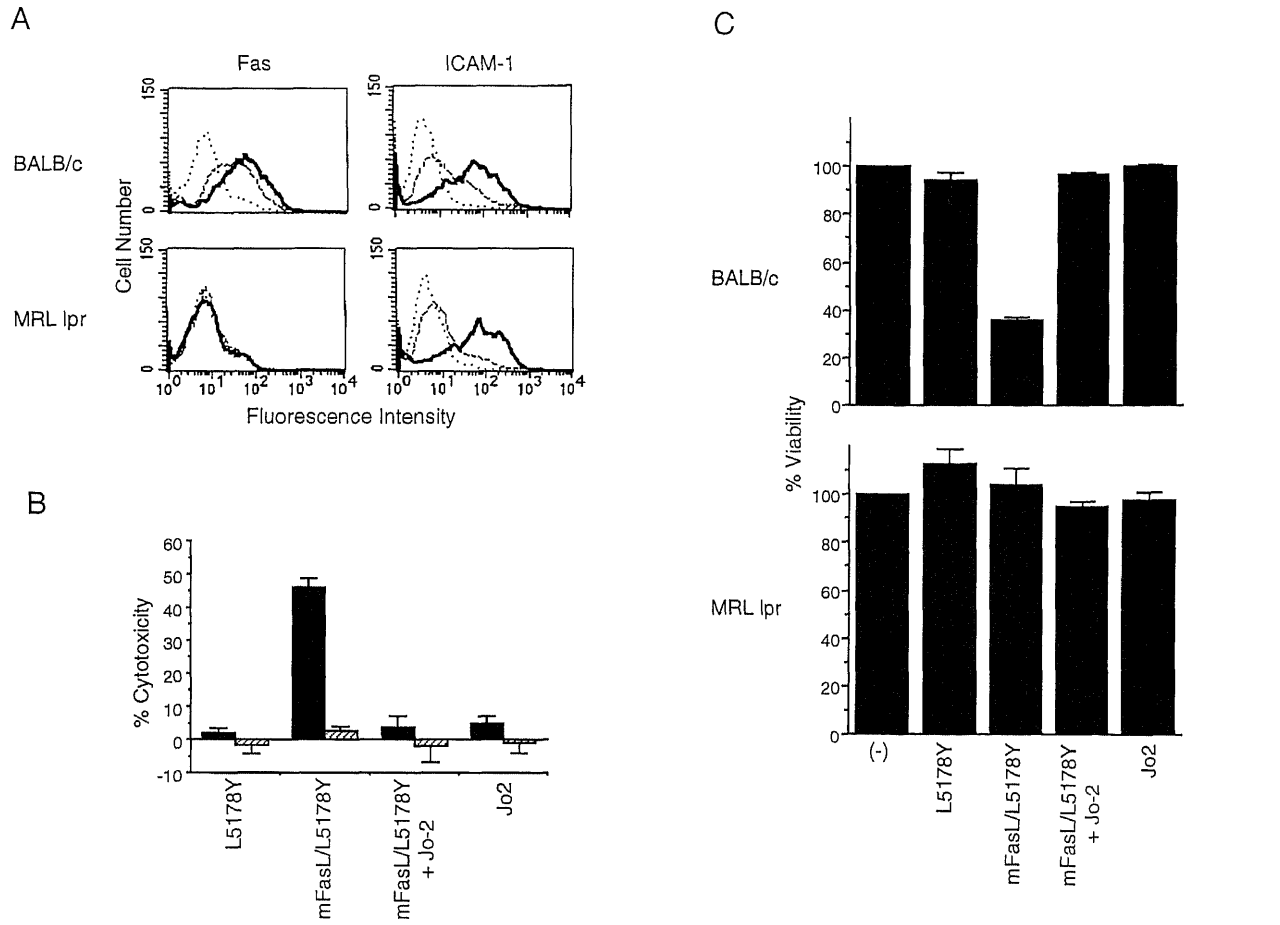

Fig. 1. Functional expression of Fas on cardiac myocytes. (A) Surface expression of Fas. Cardiac myocytes prepared from BALB/c or MRL *lpr* fetal mice were stained with anti-Fas or anti-ICAM-1 mAb before (broken lines) and after (solid lines) IFN-γ treatment. Dotted lines indicate background staining with control mAb. (B) Fas-dependent cytotoxic activity of the FasL transfectant. Cytotoxic activity of L5178Y or mFasL/L5178Y was tested against Fas$^-$L5178Y (hatched bars) or mFas/L5178Y (closed bars) target cells by 4 h ^{51}Cr-release assay at an E/T ratio of 25 in the presence or absence of anti-Fas mAb (Jo2) at 10 μg/ml. (C) FasL-mediated cytotoxicity against cardiac myocytes. BALB/c or MRL *lpr* myocytes were co-cultured with L5178Y or mFasL/L5178Y cells in the presence or absence of anti-Fas mAb (Jo2) for 20 h. After removing non-adherent cells, residual viable myocytes were measured by the alamar Blue method. Percent viability was calculated by defining the value with no treatment as 100%. Data represent mean ± SD of triplicate samples. Similar results were obtained in three independent experiments.

normal mice (BALB/c) constitutively expressed Fas as estimated by staining with an anti-Fas mAb (Fig. 1A). The expression of Fas was slightly increased by the IFN-γ treatment. In contrast, no significant expression of Fas was detectable on cardiac myocytes from *lpr* mice (MRL *lpr*) even after the IFN-γ treatment, which increased the ICAM-1 expression on both normal and *lpr* myocytes. We next examined the susceptibility of these cells to anti-Fas mAb or mouse FasL transfectant which exhibited Fas-dependent cytotoxic activity against the mouse Fas transfectant (mFas/L5178Y) (Fig. 1B). Cardiac myocytes from normal mice, but not those from *lpr* mice, were susceptible to the FasL transfectant (Fig. 1C). The anti-Fas mAb (Jo2), which has been reported to lyse thymocytes and Fas transfectants (15,16), was not directly cytotoxic against the myocytes but blocked the FasL-mediated cytotoxicity. A similar blocking property of this mAb on FasL-mediated cytotoxicity in the short-term assay was also observed when mFas/L5178Y was used as the target (Fig. 1B) and has been demonstrated by others (3). This seems to be due to a weaker agonistic activity of this mAb than FasL. These results indicated that cardiac myocytes of normal mice, but not those of *lpr* mice, express functional Fas that can mediate FasL cytotoxicity.

Cardiac allograft survival in gld mice

This functional expression of Fas on cardiac myocytes prompted us to examine the contribution of Fas/FasL-mediated cytotoxicity to cardiac allograft rejection. We first utilized FasL-deficient *gld* mice as recipients for estimating the requirement of FasL. Survival of fully allogeneic BALB/c (H-2^d) allografts was significantly prolonged in *gld* mice of both C3H (H-2^k) and B6 (H-2^b) backgrounds as compared with that in wild-type (+/+) mice (Fig. 2A and B). Histological examination of the BALB/c allografts in C3H+/+ or *gld* recipients at day 7 after transplantation indicated a lesser cellular infiltration and much milder myocyte damage in the

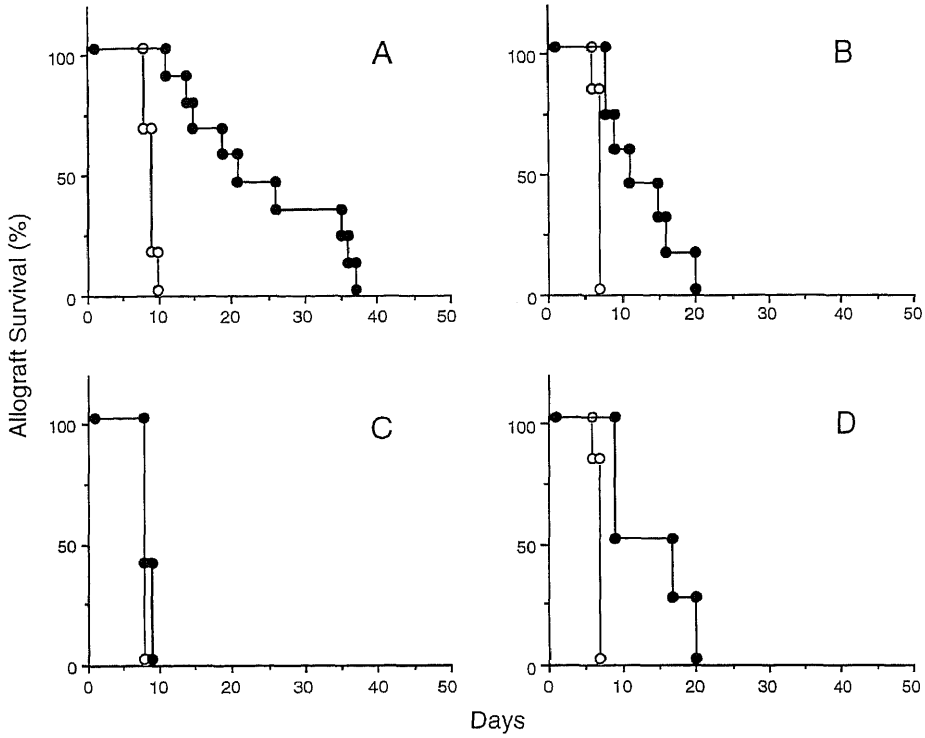

Fig. 2. Cardiac allograft survival. (A) BALB/c hearts were transplanted into C3H+/+ (open circles, $n = 6$) or C3H *gld* (closed circles, $n = 9$) recipients. $P < 0.01$. (B) BALB/c hearts were transplanted into B6+/+ (open circles, $n = 6$) or B6 *gld* (closed circles, $n = 7$) recipients. $P < 0.01$. (C) C3H+/+ (open circles, $n = 4$) or C3H *lpr* (closed circles, $n = 5$) hearts were transplanted into BALB/c recipients. Not significant. (D) BALB/c hearts were transplanted into B6+/+ (open circles, $n = 6$) or B6 *lpr* (closed circles, $n = 4$) recipients. $P < 0.01$.

gld recipients (Fig. 3A and E). Immunohistochemical staining with anti-CD4, anti-CD8 or anti-macrophage mAb showed that the infiltration of all these phenotypes was decreased in the *gld* recipients (Fig. 3B–D and F–H). These results suggested a substantial contribution of FasL to the cellular infiltration and myocyte damage during acute rejection.

Rejection of lpr allografts or in lpr recipients

We next verified the contribution of Fas expressed in the cardiac allografts by utilizing the Fas-deficient *lpr* mice as the donor. Unexpectedly, no significant prolongation of the *lpr* allograft survival was observed (Fig. 2C). In order to estimate the possible contribution of Fas in the recipient side, we next utilized *lpr* mice as the recipient. Interestingly, allograft survival was also prolonged in the *lpr* recipients (Fig. 2D) to a similar extent to the case in the *gld* recipients (Fig. 2B). Histological examination of the graft in B6+/+ or *lpr* recipients at day 5 after transplantation indicated a lesser cellular infiltration and milder myocyte damage in the *lpr* recipients (Fig. 3I and J). These results suggest that Fas in the recipient, but not that in the graft, plays some role in the cardiac allograft rejection.

Immune responses of gld recipients to the donor alloantigens

One possible explanation for the prolonged allograft survival in both *gld* and *lpr* recipients might be a hyporesponsiveness of these mice to the donor alloantigens. It has been known that abnormal lymphocytes of the Thy-1$^+$B220$^+$CD4$^-$CD8$^-$ phenotype accumulate with aging in both *lpr* and *gld* mice, which are hyporesponsive to antigenic and mitogenic stimulations (17). Although a substantial accumulation of the Thy-1$^+$B220$^+$ cells in the *gld* and *lpr* recipients was not observed during our experimental periods (5–10 weeks of the age) (data not shown), we confirmed whether the T cell responses against the donor alloantigens were intact. The magnitude of proliferative response of splenic T cells from the *gld* recipients against the donor-type alloantigens was comparable to the wild-type recipients (Fig. 4A). *In vitro* CTL induction from the *gld* recipients was rather higher than the wild-type recipients (Fig. 4B). Moreover, alloreactive CTL induction *in vivo* in the *lpr* and *gld* mice was also comparable to that in the wild-type mice (Fig. 4C). These results indicate that the prolonged allograft survival in the *gld* and *lpr* recipients was not due to their hyporesponsiveness to the donor alloantigens.

Discussion

In the present study, contribution of the Fas/FasL interaction to cardiac allograft rejection was verified by utilizing the Fas-deficient *lpr* or FasL-deficient *gld* mice as the recipient or donor.

While we here observed that the survival of cardiac allografts transplanted in *gld* recipients was prolonged as compared to that in control mice, Larsen *et al.* (18) recently reported that the *gld* recipients rejected cardiac allografts at a similar

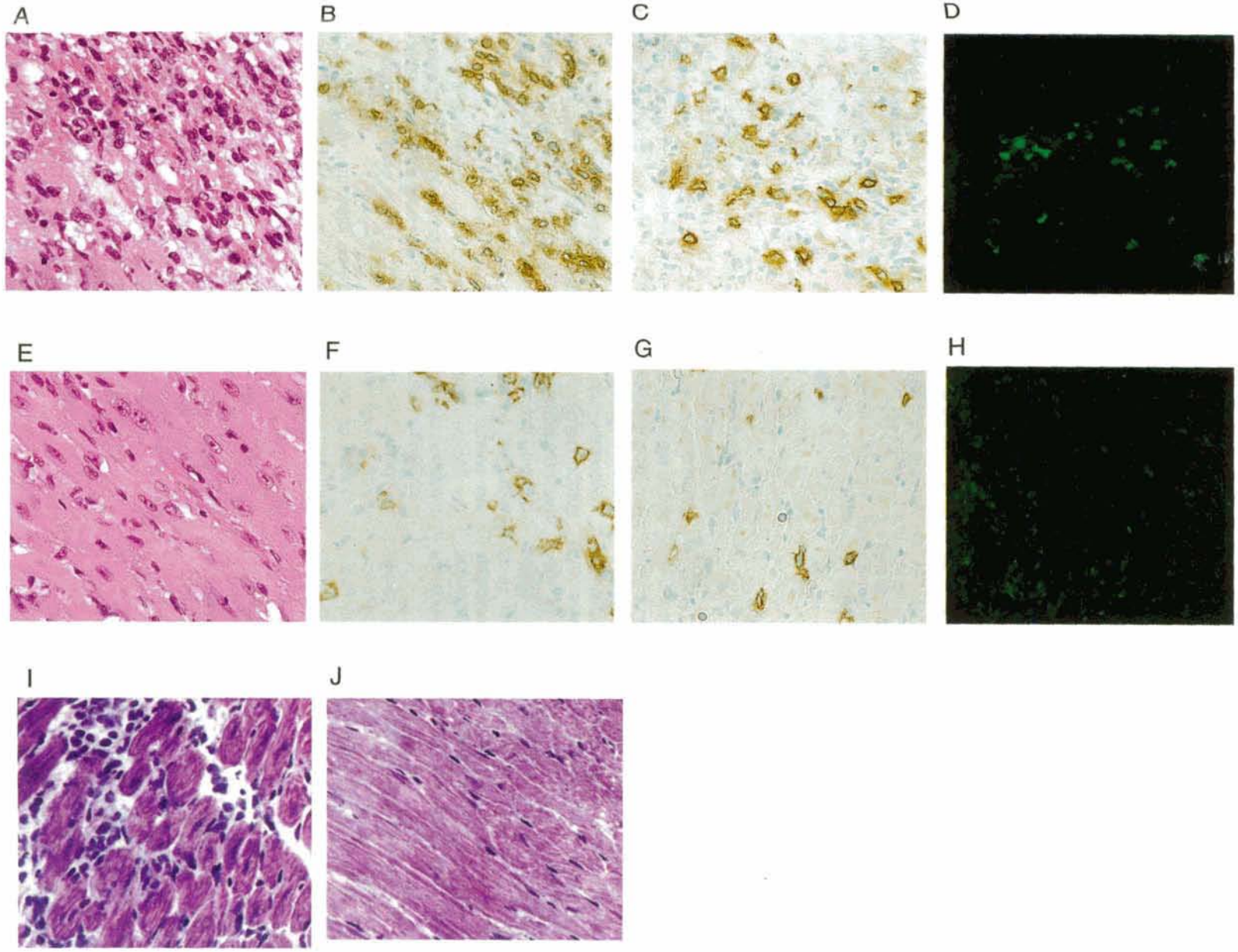

Fig. 3. Histology of allografts. BALB/c cardiac allografts were removed from C3H+/+ (A–D), C3H *gld* (E–H), B6+/+ (I) or B6 *lpr* (J) recipients at 5 days (I and J) or 7 days (A–H) after the transplantation. Formalin-fixed paraffin sections were stained with hematoxylin & eosin (A, E, I and J). Cryostat sections were stained with anti-CD8 (B and F), anti-CD4 (C and G) or Mac-3 (D and H) mAb. Data are representative of five animals examined.

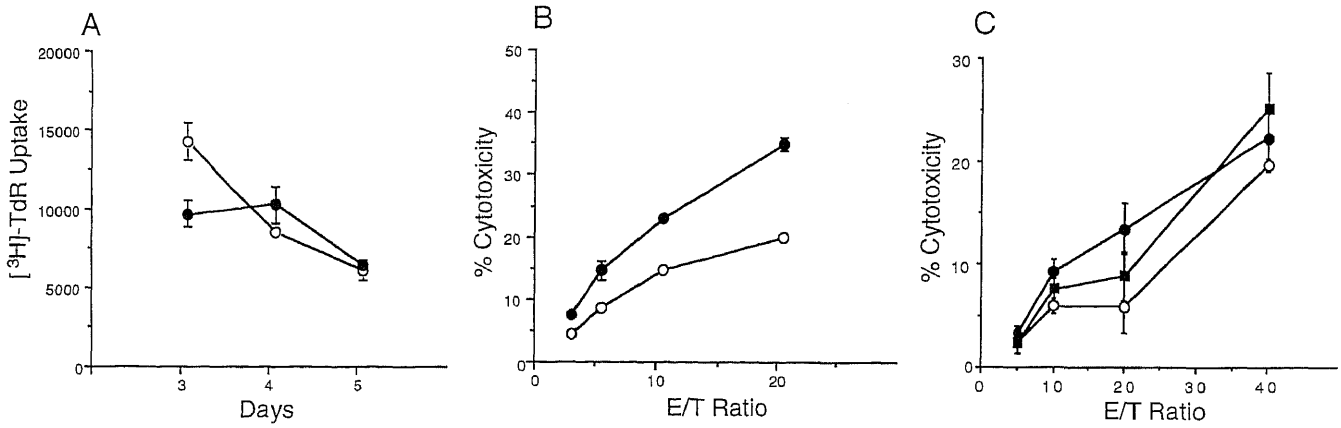

Fig. 4. Proliferative response and CTL induction against donor-type alloantigens. Splenic T cells from C3H+/+ (open circles) or C3H *gld* (closed circles) recipients were prepared at 7 days after BALB/c heart transplantation and co-cultured with irradiated BALB/c spleen cells. After 3–5 days, [^{3}H]thymidine uptake was estimated during the last 18 h of culture (A). CTL induction was estimated after culture for 5 days by 4 h ^{51}Cr-release assay against P815 target cells at the indicated E/T ratios (B). In (C), *in vivo* CTL were induced by two i.p. injections of P815 cells into C3H+/+ (open circles), *gld* (closed circles) or *lpr* (closed squares) mice and cytotoxic activity of the peritoneal cells was tested against P815 target cells by 4 h ^{51}Cr-release assay at the indicated E/T ratios. Data represent mean ± SD of triplicate samples. Similar results were obtained in three independent experiments.

tempo to control mice by using the same strain combination as we used. Their C3H *gld* recipients (n = 3) rejected BALB/c cardiac grafts in 12–22 days [median survival time (MST) = 16 days] while ours did in 10–36 days (MST = 20 days). We think that their and our allograft survival data in *gld* recipients were almost consistent. However, the allograft survival in control C3H mice described in their report was exceptionally longer (up to 26 days) than that we indicated here and that reported by others previously (13,19). Moreover, they themselves described a shorter survival of BALB/c cardiac grafts in C3H+/+ recipients (MST = 12) in another study (20). Because most cardiac allografts in our *gld* recipients survived longer than in the control they reported, we believe that the cardiac allograft rejection in *gld* recipients is substantially delayed.

In this study, we obtained apparently inconsistent results indicating a substantial contribution of FasL but no contribution of Fas in the graft. This mechanism which is apparently FasL-dependent but independent of Fas in the graft might be open to several possible explanations.

First, this discrepancy might be an artifact resulting from a leakiness of the Fas deficiency in *lpr* mice. The defective expression of Fas in *lpr* mice has been revealed to result from a transcriptional defect caused by a retrotransposon insertion into the *fas* gene (21,22). This defect in transcription is not absolute and a substantial level of intact Fas mRNA expression has been reported to be demonstrable (23). On the other hand, the FasL deficiency in *gld* mice has been revealed to result from a single amino acid substitution that abrogates FasL activity to trigger Fas-mediated apoptosis (9). Therefore, normal rejection of the *lpr* allografts might be explained by a low but functionally adequate level of Fas expression in the graft. However, this seems not the case in the present study since Fas was not detectable on the *lpr* myocytes and these cells were totally resistant to the FasL-mediated cytotoxicity. Alternatively, Fas protein expression on myocytes *in vivo* may be lower than that observed *in vitro* although abundant Fas mRNA expression has been reported (7). Thus, it is possible that myocytes are resistant to the FasL-mediated cytotoxicity *in vivo*. Consistent with this notion, no apparent damage in the heart has been demonstrated in the mice administered with an anti-Fas mAb (15).

Second, FasL might have another receptor than Fas as tumor necrosis factor has two receptors (24). This possibility has not been properly tested since the FasL has been cloned by using soluble Fas as the probe (25) but a reciprocal trial with FasL as the probe has not been performed. However, this seems also not the case in the present study since the Fas-deficient *lpr* myocytes were resistant to the FasL-mediated cytotoxicity and that against normal myocytes was completely blocked by anti-Fas mAb, indicating that no receptor other than Fas can mediate FasL cytotoxicity on cardiac myocytes.

In this context, we noticed an alternative explanation that might be responsible for the FasL-dependent mechanism which needs Fas in the recipient but not in the graft. We tested this possibility by utilizing *lpr* mice as the recipients, and found that allograft survival was also prolonged to a similar extent to the *gld* recipient and that the cellular infiltration was also reduced. This suggests that Fas in the recipient side is responsible for the FasL-mediated mechanism.

Recently, ligation of Fas by some mAb has been reported to augment the proliferation of human peripheral blood T cells stimulated by anti-CD3 mAb, suggesting that Fas-mediated signal may act co-stimulatory in a certain situation (26,27). Therefore, FasL produced by alloreactive T cells may predominantly act on themselves to enhance the primary responses.

Alternatively, Fas on the recipient-derived APC might be the main target of FasL. It has been reported that macrophages are susceptible to Fas-mediated apoptosis (28). It has been also reported that Fas-mediated signaling activated the IL-1β converting enzyme (29) and that apoptotic macro-

phages released IL-1β (30), which is a potent proinflammatory cytokine initiating local inflammation. Therefore, FasL produced by alloreactive T cells may act on the recipient-derived macrophages in the grafts to initiate inflammatory tissue damage. In addition, a recent report (31) demonstrated that ligation of Fas on epithelial cells induced production of IL-8, which represented another function mediated by Fas besides the induction of apoptosis. Thus, it is possible that FasL may act on recipient-derived infiltrating cells to produce some cytokines. Consistent with this notion, cellular infiltrates into the grafts were greatly decreased in the *gld* or *lpr* recipients.

We recently found that locally produced FasL by the transfectants induces cellular infiltration leading to graft rejection even if the grafted cells do not express Fas (32). This FasL-mediated graft rejection was also observed in T cell-deficient mice and mediated by inflammatory cells. A similar FasL-mediated mechanism may be involved in the tissue damage associated with allograft rejection and also in various T cell-mediated inflammatory diseases even if the target organ lacks Fas.

In the present study, we demonstrated some contribution of FasL to allograft rejection, which was directed to the recipient side but not to the graft side. The cardiac allograft rejection was somewhat delayed but consistently occurred in the Fas- or FasL-deficient recipients. Also considering a recent observation that the *lpr*-derived cardiac allografts were normally rejected even in the perforin-deficient recipients (6), neither perforin nor FasL appear to play a crucial role in the cardiac allograft rejection. Therefore, delayed-type hypersensitivity-like inflammatory responses, which are mediated by alloreactive T cells and macrophages and where FasL would exhibit an enhancing effect, may play a more important role than the CTL-mediated cytotoxicity as the predominant effector mechanism that leads to graft injury.

Acknowledgements

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Abbreviations

B6	C57BL/6
CTL	cytotoxic T lymphocyte
FasL	Fas ligand
mFas	mouse Fas
MST	median survival time

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