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Impact of donor MHC class I or class II antigen deficiency on first- and second-set rejection of mouse heart or liver allografts

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SUMMARY

The influence of donor major histocompatibility complex (MHC) class I- or class II-deficiency on the initiation of first- and second-set rejection of mouse heart and liver allografts was examined. C3H (H-2^k) mice received heterotopic cardiac or orthotopic liver grafts from unmodified B10 (H-2^b), B6 (H-2^b), b2m (H-2^b; class I deficient) or AB⁰ (H-2^b; class II deficient) donors. Organ survival was also investigated in C3H recipients that had been presensitized by a normal B10 skin graft 2-3 weeks before heart or liver transplantation. The absence of cell surface MHC class I or class II resulted in significant prolongation of primary cardiac allograft survival. Three of seven (43%) MHC class I-deficient, and two of five (40%) class II-deficient heart grafts were accepted indefinitely (survival time > 100 days). Thus both MHC class I and class II molecules appear to be important for the elicitation of first-set rejection in the heart allograft model. All liver allografts survived > 100 days in normal recipients. In C3H recipients that had been presensitized by a B10 skin graft, however, both heart and liver grafts from AB⁰ (class II deficient) donors underwent accelerated rejection (median survival time [MST] 3 and 4 days, respectively). In contrast, liver grafts from class I-deficient mice (b2m) were still accepted indefinitely by B10 skin-presensitized C3H recipients, whereas class I-deficient hearts survived significantly longer than those from class II-deficient or normal donors. These data demonstrate that the expression of donor MHC class I, and not class II is crucial in initiating second-set organ allograft rejection. *In vitro* monitoring revealed that at the time of organ transplant, both splenocytes and serum of the skin-presensitized animals displayed high cytotoxicity against AB⁰ (class II-deficient) but not against b2m (class I-deficient) targets.

INTRODUCTION

Major histocompatibility complex (MHC) antigens expressed on allografted tissue are considered to be the main stimuli inducing rejection. The role of MHC class I and class II antigens in the survival of vascularized allografts both in mice and rats has been studied using congenic and recombinant strains of experimental animals.¹⁻⁶ The results however, have been controversial, mainly because of the poor compatibility of the donor and recipient strains. In addition, the immunomodulatory function of soluble MHC class I proteins, has been uncertain.^{7,8} The recent availability of transgenic ('knockout') mice that do not express cell surface MHC class I^{9,10} or class II

molecules¹¹ provides the opportunity to delineate more clearly the roles of these MHC gene products in transplant responses, without changing the donor-recipient strain combination.¹²⁻¹⁴ Several studies have shown that skin grafts from MHC class I- or class II-deficient mice are rejected as rapidly as normal skin both by MHC-disparate hosts and by MHC-identical recipients differing only at non-MHC-loci.¹³⁻¹⁵ The tempo of immune responses to non-vascularized skin grafts however, is frequently different from that to vascularized organ allografts. In the present study therefore, we have examined the influence of donor MHC class I- or class II deficiency on first- and second-set rejection of mouse vascularized heart and liver transplants.

MATERIALS AND METHODS

Mice

Normal male mice of the C57BL/10J (B10; H-2^b), C57BL/6J (B6; H-2^b), C3H/HeJ (C3H; H-2^k) and BALB/cByJ (BALB/c; H-2^d) strains and MHC-deficient male animals were purchased from The Jackson Laboratory (Bar Harbor, ME). MHC class I-deficient C57BL/6J-b2m^{m1Uao} (b2m; H-2^b) mice are deficient in the expression of cell surface MHC class I antigens as the result of 'knockout' of the $\beta 2$ -microglobulin

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Abbreviations: AB⁰, C57BL/6J.AB⁰; B6, C57BL/6J; B10, C57BL/10J; b2m, C57BL/6J-b2m^{m1Uao}; CML, cell-mediated lymphocytotoxicity; mAb, monoclonal antibody; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; MST, median survival time.

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gene. MHC class II-deficient mice C57BL/6J-AB⁰ (AB⁰; H-2^b), contain a disrupted A^β gene necessary to form cell surface class II molecules. The mice were maintained in the specific pathogen-free facility of the University of Pittsburgh Medical Center, provided with Purina rodent chow and tap water *ad libitum*, and used at 10–12-weeks of age.

Surgical Procedures

All operations were performed under inhalation anaesthesia using methoxyflurane (Pitman–Moore, Atlanta, GA). Heterotopic cardiac transplantation to an abdominal site was performed as described previously.¹⁶ The function of the heart was monitored daily after transplantation by abdominal palpation. Total cessation of contraction was defined as rejection. Orthotopic liver transplantation, in which revascularization was with a combination of suture and cuff techniques, was performed as described in detail elsewhere.¹⁷ The hepatic artery was not reconstructed. To examine second-set rejection, a full thickness skin graft (8 mm²) from the donor (B10) tail was placed on the dorsal side of the recipient trunk, 2 to 3 weeks before whole-organ transplantation, as described previously.¹⁸

Mixed lymphocyte reaction (MLR)

Equal numbers (2 × 10⁵/well) of responder and γ-irradiated (20 Gy) stimulator splenocytes were suspended in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% v/v heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 5 × 10⁻⁵ M 2-mercaptoethanol, 100 U/ml penicillin, 100 mg/ml streptomycin, and 1 mM HEPES (Gibco). Aliquots (200 μl) were placed in triplicate, in U-bottomed 96-well microculture plates (Corning, Corning, NY). The cultures were maintained for 96 hr at 37° in 5% CO₂ in air. Each well was pulsed with 1 μCi [³H]TdR (NEN, Boston, MA) 18 hr before termination of the assay. The cultures were harvested onto glass fibre filters (Wallace Oy, Turku, Finland) and thymidine uptake was measured in a BetaplateTM 1205 liquid scintillation counter (Pharmacia LKB, Gaithersburg, MD). Results are expressed as the mean c.p.m. ± 1SD. Controls included responder or γ-irradiated stimulator cells incubated alone and responder cells incubated with γ-irradiated syngeneic stimulators.

Cell-mediated lymphocytotoxicity (CML) assay

Freshly isolated spleen cells obtained from C3H mice presensitized with B10 skin (2 weeks before the test) were used as effectors. Splenocytes from B10, B6, b2m or AB⁰ mice cultured for 48 hr in RPMI-1640 supplemented with 5–10 μg/ml Concanavalin A (Con A; Sigma, St. Louis, MO) were used as targets. Con A-activated BALB/c spleen cell targets were used as third-party controls. The target cells were labelled with 100 μCi Na₂ ⁵¹Cr O₄ (NEN), washed and plated at a concentration of 4 × 10³ cells/well in 96-well culture plates (Corning). Serial, twofold dilutions of effector cells were added to give effector:target (E:T) ratios of 100:1, 50:1 and 25:1 in a total volume of 200 μl/well. The percentage of specific ⁵¹Cr release was determined after incubating the plates for 4 hr at 37° in 10% CO₂ in air. An aliquot (100 μl) of supernatant was recovered from each well after centrifugation at 500 g for 1 min. Maximum ⁵¹Cr release was determined by osmotic lysis of the cells. The percentage-specific cytotoxicity was calculated using the following formula:

% cytotoxicity =

$$\frac{\text{experimental (c.p.m.)} - \text{spontaneous (c.p.m.)}}{\text{maximum (c.p.m.)} - \text{spontaneous (c.p.m.)}} \times 100$$

The results are expressed as means ± 1SD of percentage-specific ⁵¹Cr release in triplicate cultures.

Complement-dependent cytolytic antibody activity

Serum samples from normal (control) and B10 skin-sensitized C3H mice (2 weeks after skin grafting) were incubated at 56° for 30 min to inactivate complement. Thereafter, they were diluted serially in 96-well microtitre plates (Corning) in Hank's balanced salt solution (HBSS) containing 0.1% w/v bovine serum albumin (Sigma). Splenic target cells (5 × 10⁵) from C3H, B10, B6, b2m, AB⁰, or BALB/c mice were added to each well and the plates incubated for 1 hr at room temperature. The cells were then washed twice in HBSS and 100 μl of baby rabbit complement (Cedarlane, Hornby, Ontario, Canada) was added to each well. The plates were then incubated for 30 min at 37° in 5% CO₂ in air, washed twice with HBSS and incubated for an additional 3 hr in 100 ml HBSS supplemented with 10% FCS and 20 μl 3-[4,5-Dimethylthiazol-2-yl]-3, 5-diphenylformazan (MTT; Sigma). At the end of the incubation period, the cells were washed twice and 150 μl dimethyl sulfoxide (Sigma) was added to each well. MTT formazan was dissolved by shaking the plate vigorously, followed by centrifugation at 1180 g for 1 min. Optical density was measured at 550 nm using a kinetic microplate reader (Molecular Devices, Menlo Park, CA). Controls included blank wells and target cells alone.

Statistics

Median graft survival times between groups of transplanted animals were compared using the Kruskal–Wallis test, a non-parametric equivalent to the one-way analysis of variance. Pair-wise comparisons were performed by the Mann–Whitney *U*-test. A Bonferroni correction was used to adjust the overall significance of 0.05 by a factor of 1/3. Based on this correction, a *P* value < 0.02 for the pair-wise comparisons was considered significant.

RESULTS

The influence of MHC class I- or class II-deficiency on first- and second-set rejection of heart and liver allografts

The survival times of organ allografts from normal or MHC-deficient donors in unmodified hosts are shown in Table 1. Normal B10 or B6 heart grafts were rejected acutely by unmodified C3H recipients within 7–12 days (MST 8.5 and 11 days, respectively). The survival of hearts from MHC class I-deficient (b2m) or class II-deficient (AB⁰) mice, however, was prolonged significantly (MST 30 and 40 days, respectively). Three of seven (43%) of the class I-deficient, and two of five (40%) of class II-deficient heart grafts were accepted indefinitely (survival > 100 days) (Table 1). These results indicate that cell surface expression both of MHC class I and class II is important in the initiation of first-set heart allograft rejection. As reported previously,¹⁹ normal B10 and B6 liver grafts were accepted spontaneously in unmodified C3H recipients. With one exception, all grafts survived > 100 days. Not surprisingly, all liver grafts from class I- or class II-deficient mice also survived indefinitely in normal C3H recipients (Table 1).

Table 1. Organ allograft survival in normal C3H mice

Organ donor	Loci expressed	Graft survival (days) [MST*]	
		Heart	Liver
B10	MHC, non-MHC HA	7, 7, 7, 10, 10, 10 [8.5]	> 100, > 100, > 100, > 100 [> 100]
B6	MHC	11, 11, 11, 12, 12 [11]	58, > 100, > 100, > 100 [> 100]
b2m†	II	19, 20, 22, 30, > 100, > 100, > 100 [30]	> 100, > 100, > 100, > 100 [> 100]
Ab ⁰ ‡	I	25, 30, 40, > 100, > 100 [40]	> 100, > 100, > 100, > 100 [> 100]

*MST, median survival time (days).

†MHC class I deficient.

‡MHC class II deficient.

In presensitized C3H (H-2^k) mice, all normal (B10 and B6) and almost all (5/6) class II-deficient allografts (heart and liver) underwent accelerated acute rejection (Table 2). The survival of class I-deficient hearts in presensitized recipients, however, was prolonged significantly ($P < 0.01$) compared with that of hearts from normal or class II-deficient (AB⁰) mice. All liver grafts from class I knockout donors (b2m) were accepted indefinitely by presensitized recipients. In contrast, the MST of livers from class II-deficient mice was only 5 days, although one graft survived > 100 days. These data demonstrate the importance of donor MHC class I, but not class II expression in initiating second-set heart and liver allograft rejection.

Proliferative responses of splenocytes from skin-presensitized recipients to donor MHC class I or class II antigens

Four-day MLR responses of normal and B10 skin-presensitized C3H mice to C3H, B10, b2m (class I deficient), AB⁰ (class II deficient) or BALB/c (third party) cells are shown in Table 3. Marked increases (5–8-fold) in DNA synthesis were observed compared with controls (stimulated with syngeneic or third-party cells) when splenocytes from normal or presensitized C3H mice were cultured with γ -irradiated B10 cells. As expected, because MLR responses are known to be class II restricted, DNA synthesis by spleen cells from normal C3H mice in response to b2m (class I deficient) stimulators was significantly higher than that to AB⁰ (class II deficient) cells. However, the MLR responses of cells from skin-sensitized C3H mice to class I-deficient stimulators (b2m), although higher than responses to syngeneic cells, did not differ statistically

from those to MHC class II-deficient (AB⁰) spleen cells. Although determined at only one time point (day 4), these MLR data suggest that the proliferative responses of presensitized spleen cells are not class II restricted in the same way as primary T-cell responses. The inability, however, of *in vitro* assays such as the MLR to adequately reflect the *in vivo* immunologic status of allograft recipients has been observed previously both in humans^{20,21} and in mice.^{22,23}

Cell-mediated lymphocytotoxicity

Freshly isolated splenic effector cells from C3H mice sensitized 2-weeks previously with B10 skin were tested for cytotoxic activity against Con A-primed B10, b2m, AB⁰ and BALB/c target cells (Table 4). Cytolytic activity appeared to be directed against donor class I antigens, since a similar degree of cytotoxicity was mediated against AB⁰ (class II deficient) cells as against B10 cells and no specific killing was observed against b2m (class I deficient) targets. The accelerated rejection of B10 and AB⁰ liver and heart allografts observed in B10 skin-sensitized C3H recipients may therefore be explained, at least in part, by the generation of donor MHC class I-dependent cytotoxic T cells. The results further suggest that the prolonged survival of liver and heart allografts from b2m mutant (class I deficient) mice in B10 presensitized C3H recipients may be because of impairment of cytotoxic T-cell development.

Cytolytic antibody activity

Sera obtained from C3H mice sensitized 2 weeks previously by

Table 2. Organ allograft survival in C3H mice presensitized to B10 skin

Organ donor	Loci expressed	Graft survival (days) [MST*]	
		Heart	Liver
B10	MHC, non-MHC HA	3, 4, 4, 5, 5 [4]	4, 4, 5, 5, 5 [5]
B6	MHC	2, 3, 3, 3, 3 [3]	3, 3, 4, 4, 4 [4]
b2m	II	5, 7, 12, 13, 20 [12†]	> 100, > 100, > 100, > 100 [> 100]
AB ⁰	I	2, 2, 3, 4, 4, 7 [3.5]	4, 5, 5, 5, > 100 [5]

*MST, median survival time (days).

† $P < 0.01$ compared with other heart allografted groups.

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Table 3. Mixed lymphocyte reactivity of splenocytes from normal and presensitized* C3H mice

Responders	Stimulators: mean c.p.m. $\times 10^3 \pm$ ISD				
	C3H	B10	b2m	AB ⁰	BALB/c
Normal C3H	3.07 \pm 2.74	25.15 \pm 4.07	26.16 \pm 8.49†	5.55 \pm 0.48	3.05 \pm 0.41
Presensitized C3H	4.07 \pm 0.56	20.76 \pm 3.75	18.86 \pm 2.61	13.17 \pm 1.02	6.91 \pm 2.21

* B10 skin grafts were performed 2 weeks before MLR testing.

† $P < 0.01$ compared with the response of normal mice to AB⁰ cells.

Table 4. Cytotoxic activity of splenocytes from normal and B10 skin presensitized* C3H mice

Effectors	Target cells: % ⁵¹ Cr release				
	C3H	B10	b2m	AB ⁰	BALB/c
Normal C3H	7.9 \pm 1.4	6.8 \pm 0.8	4.6 \pm 2.6	7.7 \pm 1.9	6.1 \pm 0.5
Presensitized C3H	9.2 \pm 2.0	20.5 \pm 0.5	4.0 \pm 0.4	20.1 \pm 2.1	7.7 \pm 1.0

* B10 skin grafts were performed 2 weeks before CTL testing.

B10 skin grafting exhibited high titres of lytic activity against B10, B6, and AB⁰ (class II deficient) splcn cell targets (Fig. 1). Cytotoxic activity was maximal or near maximal up to 1:128 serum dilution, beyond which it was reduced progressively. In contrast, there was substantially lower cytotoxic antibody activity against b2m (class I deficient) and third-party (BALB/c) targets. These data indicate that presensitized recipients are able to generate specific complement-dependent cytolytic antibodies only against donor MHC class I antigens, and not against class II antigens. They further suggest that the accelerated rejection of AB⁰ (class II deficient) liver and heart

grafts in B10 skin-presensitized C3H mice may also be due to the cytolytic antibodies directed against donor class I antigens.

DISCUSSION

MHC gene products expressed on donor tissue are believed to be the molecules principally responsible for the induction of alloimmunity,²⁴ and that serve as the targets of the rejection response.²⁵ Although the specific roles of MHC class I and class II molecules in various immune reactions have been subject of intensive investigation, much remains to be clarified. In this study, we used MHC class I- or class II-deficient mice as donors of vascularized organ allografts to examine the contribution of these molecules to the initiation of first- and second-set rejection. The survival of primary heterotopic heart grafts from class II-deficient (AB⁰) or class I-deficient (b2m) mice was prolonged significantly in fully allogeneic C3H recipients, compared with that of grafts from normal mice sharing the same haplotype (B10 or B6). Some of the heart grafts from either class I- or class II-deficient donors survived indefinitely (> 100 days) (Table 1). These observations suggest that both MHC class I and class II molecules are important for first-set rejection of vascularized cardiac allografts. The influence of donor MHC class I or class II deficiency on first-set hepatic allograft rejection could not be determined because in mice, transplanted livers are accepted spontaneously.¹⁹

It is well recognized that graft rejection in recipients who have not been exposed previously to transplantation antigens is mediated mainly by effector T cells. In 'conventional' immune responses, T lymphocytes recognize foreign antigens as processed peptides presented in association with self-MHC molecules on antigen-presenting cells.²⁶ However, in allo-immune responses, T cells can also recognize foreign MHC molecules directly as intact structures on the surface of the foreign cells.^{24,27,28} These two pathways of T-cell allorecognition are referred to as 'indirect' and 'direct' respectively. The

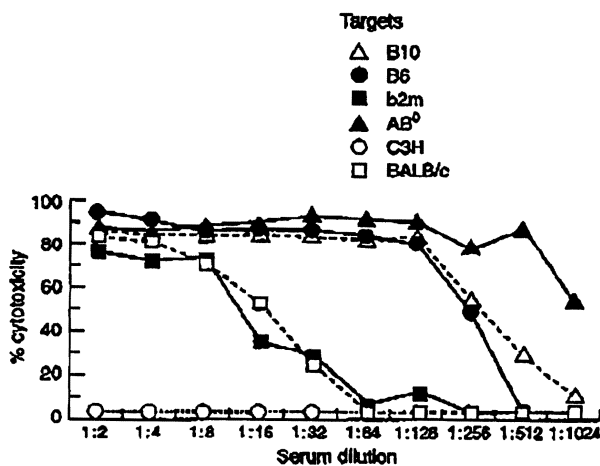


Figure 1. Complement-dependent lymphocytotoxic antibody titres in sera from C3H (H-2^k) mice sensitized by B10 (H-2^b) skin grafting 14 days previously. B10 (H-2^b), B6 (H-2^k), b2m (H-2^b, class I deficient), AB⁰ (H-2^b, class II deficient), C3H (syngeneic) or BALB/c (H-2^d; third party) targets were used. Results are mean values obtained from groups of six mice.

contributions of direct and indirect recognition to various effector mechanisms of allograft rejection remain unclear. Further insight is clearly important, however, as the two pathways are likely to have different requirements for activation and to be regulated by distinct mechanisms. Previously, it has been suggested that indirect recognition accounts for the absence of delay in rejection of MHC class II-deficient skin grafts in allogeneic mice.^{13,15} The prolonged survival of heart grafts from class II-deficient donors in this study suggests that direct rather than indirect antigen presentation may be more important in the initiation of vascularized graft rejection than in the acute rejection of skin allografts.

Second-set allograft rejection is mediated by complex cellular and humoral mechanisms. We reported previously that presensitization with skin from class II-deficient, but not from class I-deficient mice, induced subsequent accelerated rejection of both normal heart and liver grafts.¹⁴ This latter finding indicated that donor class I antigens were important in host sensitization. In the present study, we examined the role of donor class I and class II antigens in the initiation of second-set rejection of organ grafts in recipients presensitized to normal allogeneic skin. C3H (H-2^b) mice were presensitized with B10 (H-2^b) skin then transplanted with heart or liver grafts from b2m (class I-deficient) or AB⁰ (class II-deficient) donors 2-weeks later (to allow for 'full' sensitization).¹⁴ As expected, normal B10 and B6 heart and liver grafts underwent accelerated rejection in presensitized recipients. A similar pattern of accelerated rejection was observed in presensitized recipients of heart or liver grafts from AB⁰ (class II deficient) donors. In contrast, the survival of liver grafts from b2m (class I deficient) mice was not affected significantly by B10 skin sensitization (Table 2). Class I-deficient heart grafts were however, rejected earlier in sensitized than in normal recipients although the MST was longer than in the three other groups. These results could be explained by the trace expression of class I antigens in tissues of b2m mice.¹⁰ Taken together however, the data suggest that MHC class I, more than class II antigens play a key role both in recipient sensitization and in the initiation of 'second set' organ allograft rejection. An implication of these findings is that avoidance or minimization of class I mismatches could significantly reduce the potential for accelerated rejection in presensitized organ allograft recipients.

The four-day proliferative responses of splenocytes from skin-presensitized mice to b2m (class I deficient) and AB⁰ (class II deficient) cells did not differ significantly. Additional MLR time points (day 2 or day 3) would have been desirable to eliminate the possibility that the kinetics of the two responding cell populations (sensitized and non-sensitized) were different. In addition, it is conceivable that sensitized responders may react to stimulator allopeptides (e.g. from class I heavy chain expressed intracellularly) presented on responder APC present in the MLR cultures.

In vitro monitoring revealed that splenocytes and serum from skin-presensitized animals displayed higher cytotoxicity against AB⁰ (class II deficient) than b2m (class I deficient) cell targets. This suggests that both cell- and antibody-mediated responses account for second-set (accelerated) rejection of both mouse heart and liver allografts. It further appears that presensitized recipients generate both cytotoxic T cells and complement-dependent cytotoxic antibodies specific to donor MHC class I but not class II antigens. It has been observed that class I antigens expressed on donor tissue serve as targets both

for cytotoxic T cells and cytotoxic antibodies in presensitized human allograft recipients.^{29,30} The present findings obtained using class I and class II 'knockout' mice correspond well with other clinical data. Thus, a negative class I complement-dependent cytotoxic antibody cross-match has been a prerequisite for renal transplantation in many centres.³¹ However, in view of our present findings, lymphocyte-mediated cytotoxicity testing may require further evaluation as an additional test for donor-specific presensitization, as other authors have suggested previously.^{32,33}

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