Brief Definitive Report

Differential Involvement of CD4+ Cells in Mediating Skin Graft Rejection against Different Amounts of Transgenic H-2K^b Antigen

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Summary

Differential involvement of CD4+ cells in mediating class I–disparate skin graft rejection was investigated using quantitatively different K^b transgenic mice as donors under conditions in which CD8+ cells were blocked in vivo by administration of anti-CD8 monoclonal antibody (mAb). Tg.H-2K^b-1 and -2 are C3H transgenic mice with 14 and 4 copies, respectively, of the H-2K^b gene. Cell surface expression of K^b antigen and the K^b antigenicity of skin for eliciting graft rejection with homozygous and heterozygous transgenic mice were correlated with the copy number. In vivo administration of anti-Lyt-2.1 (CD8) mAb markedly prolonged survival of heterozygous and homozygous C3H Tg.H-2K^b-2 skin grafted onto C3H mice, but prolonged survival of heterozygous Tg.H-2K^b-1 skin grafts much less and did not prolong survival of homozygous Tg.H-2K^b-1 grafts. Administration of anti-L3T4 (CD4) mAb alone did not have any effect on skin graft rejection. Administration of anti-L3T4 (CD4) mAb with anti-Lyt-2.1 (CD8) mAb blocked rejection in all combinations. These findings indicate that a quantitative difference of class I antigen caused differential activation of CD4+ cells under conditions in which CD8+ cells were blocked.

From studies on the immune responses of C57BL/6 (B6) mice to grafts from B6.C-H-2^{bm1} (bm1) and B6.C-H-2^{bm12} (bm12) mutant mice (1), CD4+ cells are now thought to mediate rejection of MHC class II antigen, and CD8+ cells are thought to mediate rejection of MHC class I antigen. But evidence is accumulating that the ability to reject an allograft is not a unique feature of a given T cell phenotype (2). For example, CD4+ cells have been shown to mediate skin graft rejection of allogeneic class I antigen (3, 4) and minor H antigen, the recognition of which was shown to be restricted to class I (2). Moreover, the presence of CD4+ CTL reactive against MHC class I antigen has recently been demonstrated (5–7).

We showed previously that CD4+ cells were activated immediately for mediating skin graft rejection in some class I–disparate combinations, when CD8+ cells were blocked in vivo, but were activated less efficiently in other combinations, and that this activation was correlated with antibody production against donor MHC class I antigen (4). Thus, CD4+ cells were differentially activated in response to allelic MHC class I antigens by recognizing a difference between the donor and recipient in polymorphic determinants on class I molecules (8).

In this study, using transgenic C3H/He (C3H) mice into which different numbers of K^b genes were introduced, we found that activation of CD4+ cells for mediating skin graft rejection is also determined by the quantity of MHC class I antigen.

Materials and Methods

Mice. C57BL/6 (B6) mice were obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan). C3H/He (C3H) mice were purchased from Charles River Breeding Laboratories (Hino, Japan).

Transgenic Mice. Transgenic strains carrying the H-2K^b genes, Tg.H-2K^b-1 and -2, were described previously (9). Briefly, the DNA suspension was injected into the male pronuclei of fertilized eggs obtained from C3H mice by the method of Gordon et al. (10). The copy numbers of transgenes were determined by Southern blot analysis with a DNA preparation from the tail to be seven in Tg.H-2K^b-1 and two in Tg.H-2K^b-2 per haploid.

mAbs. Anti-L3T4 (CD4) mAb, a rat antibody of the γ2bκ Ig

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class, produced by hybridoma GK1.5, was kindly provided by Dr. F. Fitch, University of Chicago (Chicago, IL) (11). Anti-Lyt-2.1 (CD8) mAb has been described previously (12). These antibodies were used in the form of ascites from hybridoma-bearing mice.

Radioiodination. Cells were radioiodinated with $Na^{125}I$ (New England Nuclear, Boston, MA) by Iodo-gen (Pierce Chemical Co., Rockford, IL) (13). Cells were then treated with 3,3'-dithiobissulfosuccinimidylpropionate (DTSSP) (Pierce Chemical Co.) (20 μg/ml) for 20 min at 4°C for crosslinking. Cell lysates were prepared by incubating labeled cells in lysis buffer consisting of 1% NP-40, 5×10^{-5} M PMSF, and 0.05 M iodoacetamide in 0.1 M Tris.

Immunoprecipitation. Aliquots of radiolabeled cell extracts were incubated with mAb for 1 h at room temperature, and 100 μl of 20% Pansorbin (Calbiochem-Behring Corp., La Jolla, CA) was added to induce immunoprecipitation. Bound antigen was eluted by heating and was analyzed by SDS-PAGE (14).

Cytofluorometry Analysis. Cells (10^6) were incubated with biotinylated NU5-2 (anti-H-2K^b) mAb, and then with FITC-conjugated avidin. For double staining, FITC-conjugated anti-L3T4 (CD4) mAb (clone GK1.5) and PE-conjugated rat anti-Lyt-2 (CD8) mAb (clone 53-6.7) (Becton Dickinson & Co., Sunnyvale, CA) were used. Then the cells were washed, suspended in PBS, and analyzed in an Epics-C apparatus (Coulter Electronics Inc., Hialeah, FL).

Skin Grafting. Full-thickness skin was grafted, and rejection was defined as 50% necrosis.

Antibody Administration. Mice received injections of 0.2 ml of antibodies (ascites), diluted 1:8 with MEM, on days 0, 4, and 14, and then at weekly intervals until rejection.

Results

Expression of H-2K^b Antigen in Transgenic Tg.H-2K^b-1 and -2 Mice. The H-2K^b gene was introduced into fertilized eggs of C3H mice, and two strains of transgenic mice, Tg.H-2K^b-1 and -2, were established (9). Southern blot analysis showed that the Tg.H-2K^b-1 and -2 mice contained seven and two copies per haploid, respectively, integrated into a single chromosomal site (9). Expression of H-2K^b antigen on lymphoid cells from these mice was investigated by cytofluorometry using NU5-2 mAb, and compared with that on lymphoid cells from B6 mice (Fig. 1, *a–e*). The peak fluorescence modes of homozygous and heterozygous Tg.H-2K^b-1 were within ranges of 1.48–2.01 and 1.02–1.07 ($n = 3$), respectively, while those of homozygous and heterozygous Tg.H-2K^b-2 were within ranges of 0.89–0.96 and 0.60–0.72 ($n = 3$), respectively. In immunoprecipitation tests, NU5-2 mAb reacted with 45-kD (class I H chain) and 57-kD ($\beta_2$microglobulin-associated H chain) molecules on crosslinking in the lysates of surface-iodinated lymph node cells from B6, Tg.H-2K^b-1 and -2 mice, but not from C3H mice (Fig. 1 *f*).

Survival of Skin from Tg.H-2K^b-1 and -2 Mice Grafted onto C3H Mice. The effect of the quantity of donor H-2K^b antigen on rejection was investigated by skin grafting with combinations of Tg.H-2K^b-1 or -2 mice as donors, and C3H mice as recipients. The mean graft survivals (MGS) of skins from homozygous and heterozygous Tg.H-2K^b-1 mice grafted onto C3H mice were 13.9 ± 0.8 and 15.5 ± 1.2 d, respectively (Table 1). The MGS of grafts from homozygous and heterozygous C3H Tg.H-2K^b-2 mice grafted onto

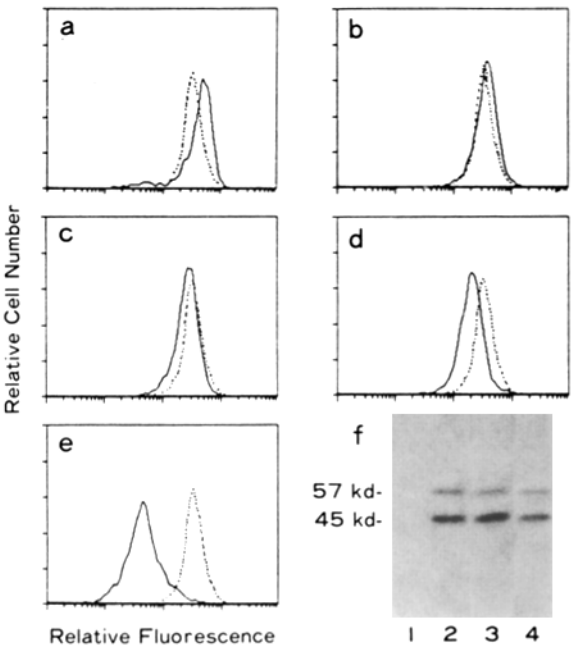

Figure 1. Expression of transgenic K^b antigen examined by cytofluorometry analysis (*a–e*) and immunoprecipitation (*f*). Lymph node cells from homozygous (*a*) and heterozygous (*b*) Tg.H-2K^b-1, and homozygous (*c*) and heterozygous (*d*) Tg.H-2K^b-2 mice were incubated with biotinylated NU5-2 (anti-K^b) mAb and then with FITC conjugated avidin. Dotted line represents control B6 lymph node cells. (*e*) Normal C3H lymph node cells. In *f*, molecules reactive with NU5-2 (anti-K^b) mAb were crosslinked by DTSSP and immunoprecipitated from NP-40 lysates of surface-iodinated lymph node cells from C3H (lane *1*), B6 (lane *2*), Tg.H-2K^b-1 (lane *3*), and -2 (lane *4*) mice.

C3H mice were 17.9 ± 1.2 and 19.8 ± 1.6 d, respectively. No rejection of skin grafts was observed with reciprocal combinations between Tg.H-2K^b-1 and -2 mice.

Effect of In Vivo Administration of Anti-L3T4 (CD4) and/or Anti-Lyt-2.1 (CD8) mAb on Transgenic H-2K^b-disparate Skin Graft Rejection. To investigate the responses of CD4$^+$ cells to quantitatively different amounts of transgenic H-2 K^b antigen, we examined the effect of in vivo administration of anti-L3T4 (CD4) and/or anti-Lyt-2.1 (CD8) mAb on skin graft rejection. Antibodies were injected on days 0, 4, and 14, and then at weekly intervals until rejection. Cytofluorometric analysis showed that CD4$^+$ and CD8$^+$ cells were selectively depleted for at least 50 d after in vivo treatment of C3H mice with anti-L3T4 (CD4) and anti-Lyt-2.1 (CD8) mAb, respectively (Fig. 2). Blocking of the functions of CD4$^+$ cells and CD8$^+$ cells in vivo by mAb treatment was examined by monitoring skin graft rejection in combinations of H-2^b mutant strains and B6.

A single injection of anti-L3T4 (CD4) mAb prolonged survival in B6 mice of bm12 skin (MGS, 32.8 ± 12.1 d [$p < 0.05$]; MGS in MEM-treated mice, 12.8 ± 1.2 d), but not bm1 skin (MGS, 12.0 ± 1.0 d; MGS in MEM-treated mice, 15.0 ± 0.9 d). On the other hand, anti-Lyt-2.1 (CD8) mAb prolonged the survival in B6 mice of bm1 skin (28.4 ± 9.9 d [$p < 0.05$]), but not bm12 skin (12.2 ± 0.8 d).

Table 1. *Effect of Transgenic K^b Antigen Quantity on Skin Graft Survival*

Donor	Recipient	Skin graft survival
		d
Tg.H-2K^b-1 (homozygous)	C3H	13.9 ± 0.8* (*n* = 10)
Tg.H-2K^b-1 (heterozygous)	C3H	15.5 ± 1.2 (*n* = 10)
Tg.H-2K^b-2 (homozygous)	C3H	17.9 ± 1.2 (*n* = 10)
Tg.H-2K^b-2 (heterozygous)	C3H	19.8 ± 1.6 (*n* = 9)
Tg.H-2K^b-1 (homozygous)	Tg.H-2K^b-2 (homozygous)	>200 × 5
Tg.H-2K^b-2 (homozygous)	Tg.H-2K^b-1 (homozygous)	>200 × 5

* Mean ± SD.

Administration of anti-L3T4 (CD4) mAb did not alter the survival of skin from either homozygous or heterozygous C3H Tg.H-2K^b-1 or -2 mice grafted onto C3H mice (Fig. 3). Administration of anti-Lyt-2.1 (CD8) mAb markedly prolonged the survival of grafts from heterozygous and homozygous Tg.H-2K^b-2 mice slightly, but not significantly, prolonged the survival of skin from heterozygous Tg.H-2K^b-1, but did not prolong survival of skin from homozygous Tg. H-2K^b-1. Administration of anti-L3T4 (CD4) mAb in addition to anti-Lyt-2.1 (CD8) mAb blocked rejection in all combinations.

Discussion

In this study, using transgenic mice, we showed that graft survival was determined by the quantity of donor K^b antigen. The expressions of K^b antigen in homozygous and heterozygous Tg.H-2K^b-1 and -2 mice depended on the number of copies introduced. A correlation of the quantity of transgenic K^b antigen expressed on the cell surface with in vivo antigenicity was demonstrated in skin graft rejection. The MGS of skin from homozygous and heterozygous Tg.H-2K^b-1, and homozygous and heterozygous Tg.H-2K^b-2, grafted onto C3H mice increased in this order in parallel with a decrease in the quantity of K^b antigen expressed. These results are consistent with previous findings that an increase in the MHC class I product appears to have a direct effect on T cell recognition and enhances susceptibility to lysis by cytotoxic T cells (15).

We also demonstrated that the mode of involvement of CD4$^+$ cells in mediating rejection was determined by the quantity of donor K^b antigen. Prolongation of graft survival by in vivo administration of anti-CD8 mAb was greatest with heterozygous Tg.H-2K^b-2 skin, and was not observed with homozygous Tg.H-2K^b-1 skin grafted onto C3H mice. Ad-

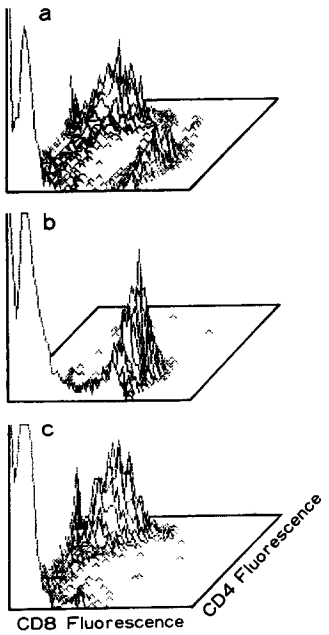

Figure 2. Three-dimensional cytofluorometric analysis of lymph node cells from C3H mice 21 d after injections of anti-L3T4 (CD4) mAb (*b*) or anti-Lyt-2.1 (CD8) mAb (*c*) on days 0, 4, and 14. (*a*) Untreated.

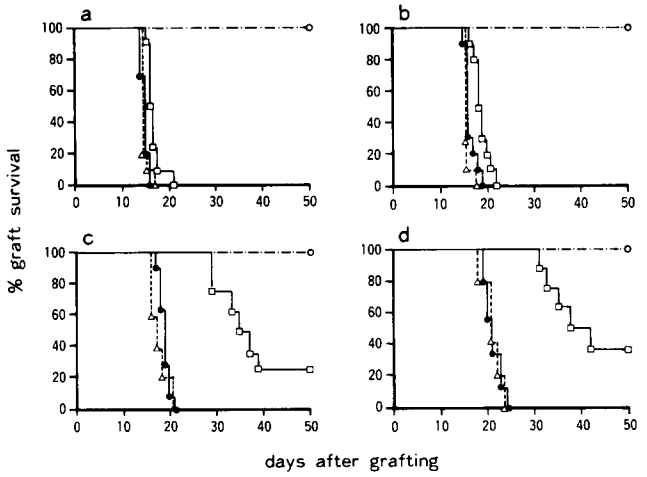

Figure 3. Effects of in vivo administration of MEM (control) (●), anti-L3T4 (CD4) mAb (△), anti-Lyt-2.1 (CD8) mAb (□), or both (○) on survival of skin from homozygous (*a*) and heterozygous (*b*) Tg.H-2K^b-1, homozygous (*c*) and heterozygous (*d*) Tg. H-2K^b-2 grafted onto C3H mice. No rejection was observed by a 200-d observation period with all five grafts examined.

ministration of anti-CD4 mAb with anti-CD8 mAb blocked rejection in all combinations tested, confirming the involvement of $CD4^+$ cells in rejection when $CD8^+$ cells were blocked. We previously demonstrated that in some strain combinations with a class I antigen difference, $CD4^+$ cells were capable of mediating immediate rejection of skin grafts when $CD8^+$ cells had been blocked by administration of anti-CD8 mAb, whereas, in other strain combinations, $CD4^+$ cells were much less effective (4). Thus, not only a qualitative difference based on allelic polymorphism, but also a quantitative difference of class I antigen caused differential activation of $CD4^+$ cells when $CD8^+$ cells were blocked.

The response of $CD4^+$ cells is generally thought to be restricted to class II antigen both in vitro and in vivo (1). However, recently, $CD4^+$ class I–restricted CTL were shown to be generated in MLC when the responder spleen cells were depleted of $CD8^+$ cells (5, 6), and cloned $CD4^+$ H-$2D^b$-restricted CTL specific for tumor antigen was isolated (7). Moreover, the involvement of $CD4^+$ cells in class I–disparate graft rejection was shown with skin (3, 4) and pancreatic islet cells (16). Singer and colleagues (2) demonstrated helper and effector $CD4^+$ T cells in skin graft rejection with a difference of minor H antigen, the recognition of which was shown to be restricted to class I. The mechanism by which $CD4^+$ cells cause class I–disparate graft rejection is unknown. $CD4^+$ cells themselves may operate as effector cells, or macrophages or other cell types may be involved as effector cells in $CD4^+$ cell–mediated rejection of class I–disparate grafts.

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