

**Mechanism of the Rejection of Major Histocompatibility Complex Class I-disparate Murine Skin Grafts: Rejection Can Be Mediated by CD4<sup>+</sup> Cells Activated by Allo-class I + II Antigen in CD8<sup>+</sup> Cell-depleted Hosts**

By Eiji Kobayashi,\* Kazuhiro Kawai,† Yoshinori Ikarashi,§ and Michio Fujiwara§

From the \*Department of Surgery, Omiya Medical Center, Jichi Medical School, Omiya 330; the †Department of Dermatology, Niigata University School of Medicine, Niigata 951; and the §Animal Center for Biomedical Research, Faculty of Medicine, the University of Tokyo, Tokyo 113, Japan

**Summary**

In the preceding article, we analyzed the immunohistochemical rejection mechanism of major histocompatibility complex (MHC) class I (H-2K)-disparate murine skin grafts, and showed that only CD8<sup>+</sup> cells infiltrated at the site of the epithelial tissue of MHC class I-disparate graft. We also showed that perfect survival of MHC class I-disparate grafts were attained in thymectomized recipients treated with anti-Lyt-2 monoclonal antibody. In this report, we showed that these long-surviving allo-class I grafts were rejected in the absence of CD8<sup>+</sup> cells by stimulation with allo-MHC class I + II-disparate graft as the second stimulation. Furthermore, it was immunohistochemically revealed that under that condition, a large number of CD4<sup>+</sup> cells infiltrated into the epithelial tissue of these long-surviving class I grafts, which were going to be rejected 2-5 d after the transplantation of a second graft with MHC class I + II difference. This result directly shows that CD4<sup>+</sup> cells are able to become effectors for the rejection of allo-MHC class I (H-2K) skin graft.

Mature peripheral T cells are classified into CD4<sup>+</sup> or CD8<sup>+</sup> subsets according to the difference in cell surface marker (1, 2). Many recent investigators have clarified the mode of action of these T cell subsets in terms of reactivities to allogeneic MHC class I or II antigens. CD8<sup>+</sup> cells react predominantly with allo-MHC class I antigens, and CD4<sup>+</sup> cells react selectively with allo-MHC class II antigens (3, 4). There are also many reports showing that CD4<sup>+</sup> T cells recognize allo-MHC class I antigen in association with self-Ia structure, and help the generation of CD8<sup>+</sup> cytotoxic T cells (Tc) in the in vitro system (5, 6). A similar mechanism is considered to be involved in the rejection of skin graft. In preceding articles (7-9), we immunohistochemically analyzed the rejection mechanism of murine skin graft different solely in MHC class I (H-2K) antigen, and showed that only CD8<sup>+</sup> T cells infiltrated the epithelial tissue of the graft. Furthermore, it was shown that CD4<sup>+</sup> cells were not involved in the rejection of allo-MHC class I (H-2K)-disparate skin graft in thymectomized recipients treated with anti-Lyt-2 mAb (10). On the contrary, there is a report suggesting that CD4<sup>+</sup> cells may be able to mediate the rejection of skin graft with MHC class I difference (11). In this article, we

examine whether or not CD4<sup>+</sup> T cells are able to react in vivo with allo-MHC class I antigen under the experimental condition in which CD8<sup>+</sup> T cells are totally depleted from the recipient. It is shown that CD4<sup>+</sup> T cells do mediate the rejection of allo-MHC class I-disparate graft. The mechanism of such a phenomenon is discussed.

**Materials and Methods**

*Mice.* Female C57BL/6 (B6) mice, either thymectomized or untreated, were used as skin graft recipients. Male B6.C-H-2<sup>bm1</sup> (bm1) mice, which carry mutant gene at the H-2K locus, and B6.C-H-2<sup>bm12</sup> (bm12) mice with mutation at I-A region, were used to obtain F<sub>1</sub> hybrids between B6 mice. These strains of mice were originally derived from The Jackson Laboratory (Bar Harbor, ME). When thymectomized recipients were used, the operation was done at 6-7 wk of age and received the graft 4-6 wk later. All mice used in the transplantation experiments were female to avoid the involvement of H-Y antigen.

*Administration of mAb.* Anti-Lyt-2 (2.43) and/or anti-L3T4 (GK1.5) mAb were administered intraperitoneally at a dose of 0.5 mg on days -4, 0, 7, 14, and 21 (day 0=day of skin grafting). This protocol was shown to be sufficient to deplete Lyt-2<sup>+</sup>

(CD8<sup>+</sup>) and/or L3T4<sup>+</sup> (CD4<sup>+</sup>) cells from the host for at least 50 d (10).

**Skin Grafting.** Doubly transplanted recipients were used for the experiments. In the first grafting, couple of full-thickness skin pieces were taken from the dorsal part of a donor and transplanted to a pair of graft beds prepared at the dorsal part of each recipient on day 0, as previously described (7–10). Recipients were 8–12-wk-old when the first grafting was done. Second graft was implanted on day 28 to another dorsal part near the long-surviving first grafts.

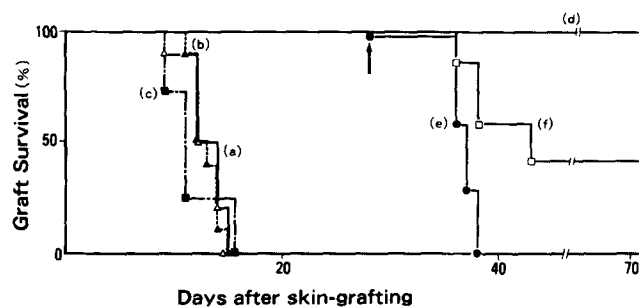
**Biopsy, Histological, and Immunohistochemical Studies.** The first implanted grafts were observed every day through transparent plaster which was removed on day 7. One of the paired grafts on each recipient was biopsied on days 7, 9, 11, and 13. In thymectomized B6 recipients treated with anti-Lyt-2 mAb (B6 × bm1)F<sub>1</sub> skin grafts survived. The second graft was implanted on these recipients on the 28th day after the first grafting. One of the long-surviving paired grafts was biopsied on days 35, 37, 39, and 41, and both the counterpart of the long-surviving graft and the second graft were observed to record the survival. The biopsied grafts were used for histological and immunohistochemical studies as previously described (7–10), to examine subsets of T cells infiltrating at the site of graft. Sampling of one of the paired grafts did not affect the survival of the other graft (7).

**Flow Cytometric Analyses.** The degree of depletion of CD8<sup>+</sup> cells and the presence of L3T4<sup>+</sup> (CD4<sup>+</sup>) cells in the spleen of various recipients were examined by flow cytometry. After spleen cell suspension was treated with hemolytic buffer, 10<sup>6</sup> cells were reacted with appropriately diluted FITC-conjugated anti-Lyt-2 (53–6.7) or anti-L3T4 (GK1.5) mAb. The stained cells were fixed with 1% paraformaldehyde and their fluorescence intensities were analyzed by FACScan<sup>®</sup> (Becton Dickinson & Co., Mountain View, CA).

## Results and Discussion

**Survival of MHC Class I-disparate Grafts.** Survival of MHC class I-disparate [(B6 × bm1)F<sub>1</sub>] grafts was followed, and the results obtained are summarized in Fig. 1. The grafts to thymectomized B6 recipients were rejected at 11.5 ± 2.5 d (mean ± SD of four mice), almost at the same time as those to untreated intact B6 recipients. The grafts to euthymic mice treated with anti-Lyt-2 mAb were also rejected at the same time as those to untreated control. The spleen cells of the euthymic recipients treated with anti-Lyt-2 mAb were tested on day 18 to confirm the effect of anti-Lyt-2 mAb treatment. Flow cytometric patterns of the spleen cells showed almost complete depletion (to a background level of <0.6–1.2%) of CD8<sup>+</sup> cells from the spleen of anti-Lyt-2 mAb-treated mice, whereas 10.6–14.1% of CD8<sup>+</sup> cells were detected in the spleen of untreated mice in several experiments. As was reported by others (12, 13), long survival of MHC class I-disparate skin graft was observed only in the group of thymectomized recipients treated with anti-Lyt-2 mAb. In our experiment, completely accepted graft survived throughout the observation period of 70 d.

These results suggest that newly recruited CD8<sup>+</sup> cells from thymus become effectors to allo-MHC class I grafts in anti-Lyt-2 mAb-treated host as previously described (10). From our immunohistochemical studies (10), we conclude that CD4<sup>+</sup> cells are not involved in the rejection of MHC

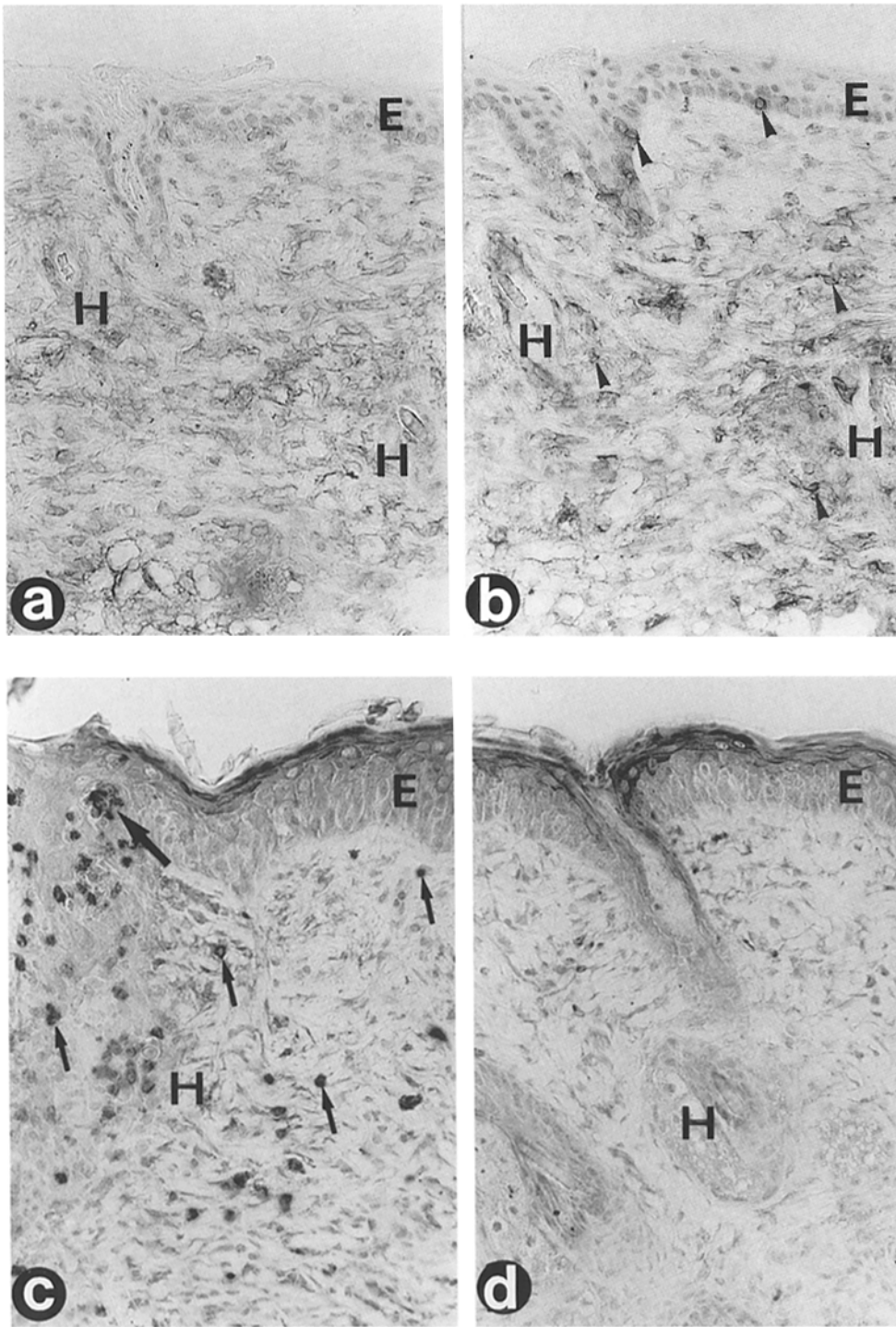


**Survival of MHC class I-disparate murine skin grafts**

**Figure 1.** Survival of MHC class I-disparate skin grafts. Survival of (B6 × bm1)F<sub>1</sub> grafts on (a) intact B6 recipients ( $n = 10$ ); (b) euthymic recipients treated with anti-Lyt-2 mAb ( $n = 10$ ); (c) thymectomized recipients ( $n = 4$ ); and (d) thymectomized recipients treated with anti-Lyt-2 mAb ( $n = 4$ ). The grafts of (a–c), were rejected almost at the same time. Long survival of MHC class I-disparate grafts was observed only in (d). (e) Survival of (bm1 × bm12)F<sub>1</sub> grafts implanted at day 28 on thymectomized and Lyt-2 mAb-treated recipients which carried (B6 × bm1)F<sub>1</sub> skin grafts ( $n = 7$ ). (f) Survival of long-standing allo-class I different grafts after the second allo-class I + II-different grafting on day 28. The second grafts of the group (e) were rejected at  $8.9 \pm 0.9$  d after the second grafting, and four of seven of (f) were also rejected 2–5 d later after rejection of the second graft.

class I-disparate skin grafts in our experimental system. On the contrary, CD4<sup>+</sup> cells have been shown by others to have CTL activity against allo-MHC class I antigen in in vitro culture (14, 15) and to mediate skin graft rejection against different amounts of transgenic H-2K<sup>b</sup> antigen (16). To examine possible involvement of CD4<sup>+</sup> cells in the rejection of allo-MHC class I grafts on the recipients depleted of CD8<sup>+</sup> cells, second graft was implanted to the thymectomized recipients which had been treated with anti-Lyt-2 mAb and which had carried long-surviving class I-disparate grafts. When allo-class I graft was used as the second graft, both the first and second grafts survived for more than 70 d after the first grafting ( $n = 4$ ). When allo-class II [(B6 × bm12)F<sub>1</sub>] graft was implanted as second graft, long-surviving class I first grafts were not affected but class II-disparate second grafts were rejected at  $9.5 \pm 1.0$  d (mean ± SD of four mice tested) after the grafting. We have confirmed that effector for the rejection of MHC class II-disparate skin graft was CD4<sup>+</sup> T cells, so that treatment of the thymectomized hosts with anti-L3T4 mAb allowed long survival of the graft (8). When class I + II-different [(bm1 × bm12)F<sub>1</sub>] grafts were used as the second grafts, they were rejected at  $10.8 \pm 3.0$  d, and four of seven long-surviving class I-different grafts were rejected 2–5 d later after the second graft rejection (Fig. 1 f). In another experiment, we confirmed that MHC class I + II-different skin grafts were completely taken by the recipients ( $n = 5$ ) thymectomized and treated with both anti-L3T4 and anti-Lyt-2 mAbs. This fact indicates that graft rejection in our experimental system was effected by either CD4<sup>+</sup>, CD8<sup>+</sup> cells, or both.

There are reports showing that CD4<sup>+</sup> cells reacting with



**Figure 2.** Immunohistochemical findings of T cell subsets in MHC class I-disparate [(B6 × bm1)F<sub>1</sub>] graft. (a and c) CD4<sup>+</sup> cells and (b and d) CD8<sup>+</sup> cells detected by avidin-biotin-peroxidase complex staining method (original magnification ×150). (a and b) the graft of a euthymic B6 recipient treated with anti-Lyt-2 mAb, whose counterpart was rejected 2 d after the biopsy. Many of CD8<sup>+</sup> cells (arrowhead) are seen in the epidermis (E) and hair follicles (H). No CD4<sup>+</sup> cells are infiltrating the epithelial tissue of the graft. (c and d) The long-standing class I-disparate graft on thymectomized and anti-Lyt-2 treated B6 recipient 7 d after the class I + II-disparate [(bm1 × bm12)F<sub>1</sub>] skin graft. The counterpart of the biopsied graft was rejected 2 d after sampling. No CD8<sup>+</sup> cells are detected in the epithelial tissue. On the contrary, many CD4<sup>+</sup> cells (arrow) are detected in E and H of the graft, showing focal satellite cell necrosis (large arrow).

class I alloantigens presented in the context of MHC class II antigens help the activation of CD8<sup>+</sup> T<sub>c</sub> which act as effectors for the rejection of MHC class I-disparate grafts (17, 18). Thus far, there are no reports directly showing that CD4<sup>+</sup> T cells per se mediated MHC class I graft rejection. In this report, we showed that CD4<sup>+</sup> cells activated by the stimulation with allo-class I + II antigen induced the rejection

of allo-MHC class I graft without the participation of CD8<sup>+</sup> cells. Depletion of CD8<sup>+</sup> cells in thymectomized mice treated with anti-Lyt-2 mAb was confirmed by FACScan<sup>®</sup> on day 50 after the first grafting. In a preceding article (10), we showed that CD8<sup>+</sup> cells were immunohistochemically detected at the site of allo-MHC class I-different graft on anti-Lyt-2-treated hosts, even if this subset of

spleen cells was undetectable by flow cytometric analyses and mixed lymphocyte reaction against bm1 stimulator. Considering these previous results, the following immunohistochemical examinations were performed.

**Immunohistological Surveys of MHC Class I-disparate Grafts.** Immunohistochemical examination of the MHC class I-disparate skin grafts of both intact and thymectomized recipients showed that only CD8<sup>+</sup> but no CD4<sup>+</sup> were infiltrating at the site of the epithelial tissues of the grafts, as previously reported (7, 10). Even if euthymic recipients were administered with anti-Lyt-2 mAb, the allo-MHC class I grafts were rejected at the same time as those to untreated recipients. In these cases, a large number of CD8<sup>+</sup> cells appeared in the epidermis of the grafts, and some of them were infiltrating into the epithelial tissue, but no CD4<sup>+</sup> cells were detected in situ (Fig. 2, a and b).

When the recipient of long-surviving (B6 × bm1)F<sub>1</sub> grafts was implanted with the same graft or (B6 × bm12)F<sub>1</sub> skin graft, the first graft was not affected, and no T cells infiltrated into the graft (data not shown). However, when (bm1 × bm12)F<sub>1</sub> skin graft was implanted as the second graft, the long-standing (B6 × bm1)F<sub>1</sub> grafts (four of seven cases) were rejected 2–5 d after rejection of the second graft. Immunohistochemical examination revealed that in the rejected cases a large number of CD4<sup>+</sup> cells were detected at the site of the biopsied long-standing allo-class I graft and infiltrated the epidermis and hair tissue (Fig. 2 c). On the contrary, few CD8<sup>+</sup> cells in the dermis and none in the epithelial tissue were detected (Fig. 2 d). In the biopsied samples of unrejected cases (three of seven) with long-standing MHC class I-disparate grafts, no T cell infiltration was observed (data not shown). The reasons why long-standing class I-disparate grafts were not rejected in all cases after implantation

of the second allo-class I + II grafts remain unclear. Clone size of H-2K<sup>bm1</sup> antigen-reactive CD4<sup>+</sup> T cells might be small, and stimulation of such T cells by allo-class I + II grafts might be at a threshold level.

The previous immunohistochemical results showed that CD4<sup>+</sup> cells did not infiltrate the epithelial tissues of allo-MHC class I graft, but CD8<sup>+</sup> cells did (7, 9, 10). On the contrary, the present experiment demonstrated that a large number of CD4<sup>+</sup> cells were detected in the epithelial tissue of the MHC class I-disparate graft in the absence of CD8<sup>+</sup> cells. These CD4<sup>+</sup> cells might be generated by recognizing allo-MHC class I antigen in association with self-Ia antigen (19) and become effectors for the rejection of class I-bearing cells. In that process, CD4<sup>+</sup> cells recognizing allo-MHC class II antigen might help the activation of CD4<sup>+</sup> cells reacting with allo-MHC class I antigen in MHC class I + II-different graft. Thus far, the involvement of CD4<sup>+</sup> cells in immunological reactions to H-2K<sup>bm1</sup> antigen has not been reported. In previous extensive studies, we could not obtain T cell clones with CD4<sup>+</sup> phenotype which responded to H-2K<sup>bm1</sup> (20). A recent report by Rosenberg et al. (21) showed that MHC class I-disparate (H-2D) skin graft was rejected in CD8-depleted recipients. They stated that CD8<sup>+</sup> cells resistant to anti-CD8 mAb mediated the graft rejection. However, involvement of CD8<sup>+</sup> cells at the site of grafted skin was not examined in their experiments. Our report directly indicates that CD4<sup>+</sup> cells are able to become effectors for the rejection of allo-MHC class I (H-2K) skin graft using immunohistochemical analyses of the graft site. Such immune responses might occur under a particular condition where CD8<sup>+</sup> T cells preferentially responding to MHC class I antigen are absent from the host. Characterization of such CD4<sup>+</sup> T cells is a target for further studies.

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Address correspondence to Dr. Michio Fujiwara, Animal Center for Biomedical Research, Faculty of Medicine, the University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113, Japan.

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