Skin Graft Rejection by β_2-microglobulin-deficient Mice

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Summary

Mice homozygous for a β_2-microglobulin (β_2-m) gene disruption lack β_2-m protein and are deficient for functional major histocompatibility complex class I (MHC-I) molecules. The mutant mice have normal numbers of $CD4^+8^-$ T helper cells, but lack MHC-I-directed $CD4^-8^+$ cytotoxic T lymphocytes (CTLs). In this study we used the β_2-m mutant mice to study the importance of MHC-I-directed immunity in skin graft rejection. Our results indicate that MHC-I-directed $CD8^+$ CTLs are not essential in the rejection of allografts with whole MHC or multiple minor H differences. However, the absence of MHC-I-guided immunity profoundly reduces the ability of mutant mice to reject H-Y disparate grafts. In addition, we show that natural killer cells which vigorously reject MHC-I-deficient bone marrow grafts, are not effective in the destruction of MHC-I-deficient skin grafts.

Several crucial aspects of murine skin graft rejection remain unclear. One controversy concerns the cell surface marker expression and functional properties of the T cell populations effective in mediating graft rejection. Depending on the experimental model and the nature of the graft, a crucial role has been suggested for lymphokine-producing Th cells (1–3), CTLs, reference 4–7, or for a functional interaction between Th and CTLs (8–12). In addition, critical biological significance has been suggested for so called dual function T cells, namely Th cells with cytolytic function or IL-2–producing CTLs (13). Another, related issue is the relative importance of recognition of MHC-I[1] versus -II molecules by the effector T cells. Under normal circumstances, most cells in the skin only express MHC-I molecules. However, it has been documented that certain lymphokines e.g., IFN-γ, that are most likely produced by many antigen-stimulated Th host cells during skin graft rejection readily induce MHC-II expression on a number of defined cell types in vivo, including endothelia and keratinocytes (14–16). Finally, the presence and migration of highly immunogenic, MHC-II$^+$ "passenger" dendritic cells in the skin graft, may be of critical importance (17, 18), but controversy exists over whether these cells express MHC-I (19, 20).

Recently, we described the main characteristics of mutant mice homozygous for a β_2-microglobulin (β_2-m) gene disruption that were generated by homologous recombination in embryonic stem cells (21, 22). The mutant mice lack β_2-m protein and consequently express little if any functional MHC-I cell surface molecules (22). The mutant mice contain normal numbers of γ/δ T cells and MHC-II–guided α/β TCR^+ $CD4^+8^-$ cells, but are deficient for α/β TCR^+ $CD4^-8^+$ CTLs, because they lack expression of MHC-I molecules crucial for positive selection of $CD8^+$ CTLs in the thymus (22–25). Therefore, these mice lack both the MHC-I–directed CTLs, as well as the MHC-I restriction/target element. Radioresistant NK cells present in MHC-I$^+$ recipients vigorously reject bone marrow grafts from MHC-matched β_2-m mutant donors (26, 27). The mutant mice themselves are deficient for this NK cell activity, indicating that MHC-I molecules may be involved in the positive selection or tolerance induction of NK cells (26, 27). In the present study, we used the mutant mice to study the importance of MHC-I–guided T and NK cells in murine skin graft rejection.

Materials and Methods

Mice. Chimeric animals transmitting the disrupted β_2-m gene (21) of the D3 embryonic stem cell line (129/Sv strain-derived,

[1] *Abbreviations used in this paper:* β_2-m, β_2-microglobulin; CML, cell-mediated lymphocytotoxicity; MHC-I, major histocompatibility complex class I; MHC-II, major histocompatibility complex class II; MST, mean survival time; NMS, normal mouse serum; poly I.C., polyinosinic:polycytidylic acid.

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H-2^b) were bred with wild-type 129/Sv mice, and heterozygous mutant offspring were back-crossed three times with wild-type 129/Sv mice. Subsequently, heterozygous 129 mice were interbred to obtain heterozygous (+/−) and homozygous mutant (−/−) 129/Sv mice. In addition, (129/Sv × C57BL/6)F$_1$ +/− animals were back-crossed three times with C57BL/6 (H-2^b) and subsequently interbred to obtain mutant mice on a C57BL/6 background. Furthermore, (129/Sv × C57BL/6)F$_1$ mice were also back-crossed four times with B10.BR (H-2$^{k/k}$) mice and thereafter intercrossed to obtain heterozygous and mutant (H-2$^{k/k}$) mice.

The β_2-m genotype was determined by Southern blot analyses on tail DNA as described (21). B10.BR back-cross mice were selected for a H-2$^{k/k}$ genotype by PCR screening for the absence of the Ab^k gene and the presence of the Ea^k gene. The primers were 5′-cttccccaggggagtctccacatt-3′ (first intron) and 5′-cagcccaccacccagttctccaga-3′ (third exon) for the Ab^b gene and 5′-cctagcaacaggatgggtgtcagtct-3′ (first exon) and 5′-ctcaactaagtctgagtcatttt-3′ (second intron) for the Ea^b gene.

Skin Grafting. Skin grafting was performed according to the method of Billingham and Medawar (28). Full-thickness skin (∼1.5 cm in diameter) from the back or belly of a donor mouse (6–12-wk-old) was engrafted onto the right side of the thorax of a recipient mouse (2–4-mo-old). The graft was covered with gauze and plaster that were removed on day 9. Grafts were scored daily until rejection (defined as >80% of the grafted tissue), or until 120 d after transplantation.

H-Y Immunization. Female mice were primed by one intraperitoneal injection of 2,000 rad-irradiated 5 × 10^7 male spleen cells in 0.5 ml PBS, 4–5 wk before the in vitro generation of CTLs.

In Vitro Generation of H-Y or Allo-specific CTL. CTLs were generated with minor modifications as described (29). 10^8 spleen cells from naive or in vivo primed mice (3–5 wk after rejection of a skin graft) were cultured with 2,000 rad–irradiated stimulator spleen cells (10^8) in 80 ml culture medium for 5 d at 37°C in humidified air with 5% CO_2. The culture medium consisted of RPMI 1640 supplemented with 10% FCS, antibiotics and 2-ME (2 × 10^{-5} M). After 5 d in culture, viable cells were purified using a Lympholyte-M gradient (Cedarlane Labs., Ltd., Westbury, NY).

Cell-mediated Lymphocytotoxicity. Varying numbers of effectors cells were added to 5 × 10^3 $Na_2{}^{51}CrO_4$ (^{51}Cr)-labeled target cells in 0.2-ml culture medium in 96-well round-bottomed microtiter plates and incubated for 4 h at 37°C in humidified air with 5% CO_2, as described (29). Con A (0.7 μg/ml) or LPS (25 μg/ml)-induced lymphoblasts, the EL-4 (H-2^b) and YAC-1 (H-2^a) T cell lines, the P815 mastocytoma cell line (H-2^d), the R1.1(H-2^k), and R1E.TL8.X1(H-2^k, β_2-m-deficient variant, 30, 31) thymoma cell lines were used as targets. EL-4 is derived from a benzpyrene-induced T cell tumor of C57BL/6 mouse strain origin. YAC-1 is a T cell lymphoma induced by Moloney MuLV in the A/Sn strain. P815 cells are derived from a mastocytoma induced by benzpyrene in the DBA/2 strain. R1.1 cells were derived from a thymoma of C58 strain origin.

In the allo-specific CML, lysis was performed in RPMI 1640 supplemented with 10% FCS. In the H-Y–specific CML, the lysis was performed in AIM V® (serum-free lymphocyte) medium (Gibco Laboratories, Grand Island, NY). After incubation, the supernatant was collected with the Titertek Supernatant Collection system (Skatron Inc., Sterling, VA). The levels of cytotoxicity are expressed as percent specific ^{51}Cr-release in a 4-h assay (29).

SV40 Large T Immortalized Fibroblasts. 14-d-old embryonic 129 mouse strain fibroblasts were grown for 3 d in DMEM supplemented with 10% FCS, antibiotics, and L-glutamine (regular medium). Subsequently, cells were infected with a helper virus-free, cell-free supernatant of the ψ2 SV40-6 cells, containing a replication-defective recombinant retrovirus expressing the SV40 large T gene as described (32). Infected cells were selected by culture under low serum concentration (1%) for 2 wk. Uninfected control cultures rapidly died under these conditions. After selection, bulk cultures of infected, immortalized cells were maintained in regular medium. Before the NK cell assay, cells were washed twice with Hepes, and the cells were detached with Hepes supplemented with 1-mM EDTA. Thereafter, cells were washed in excess regular medium and labeled with ^{51}Cr, as described in the previous section.

NK Cell Assay. Single cell suspensions were obtained from the spleens of 6–12-wk-old (129 × C57BL/6)F$_1$ +/− mice injected intraperitoneally 24 h before with 150 μg polyinosinic:polycytidylic acid (poly I.C.) in PBS, as described (33). Erythrocytes were lysed by treatment with an isotonic NH_4Cl solution (29), and thereafter the majority of macrophages and B cells removed by incubation on 16-cm tissue culture dishes for 1.5 h at 37°C, as described (34). Varying numbers of the nonadherent effector cells and 5 × 10^3 $^{51}CrO_4$-labeled target cells were incubated for 5 h at 37°C in 0.2 ml of RPMI 1640 supplemented with 10% FCS, antibiotics, L-glutamine, and 2-ME (2 × 10^{-5} M).

Depletion of Effector Cell Populations. Viable effector cells (10^7/ml) were incubated for 1 h at 37°C with no antibody, NMS (1:100 dilution), anti-CD4 mAb (50% culture supernatant of hybridoma RL.172, [35]), anti-CD8α mAb (9YTS 169.4, 1:100 dilution of ascites fluid, [36]), or anti-NK1.1 mAb (PK 136, 1:100 dilution of ascites fluid, [37]) plus complement (1:10 low Tox-M rabbit complement, Cedarlane Labs., Ltd.). Subsequently, cells were washed with excess medium and the surviving cells were tested for cytolytic activity. No difference was observed in the cytolytic activity of cells nontreated or treated with NMS (1:100) and complement (data not shown). Effector cell numbers were not adjusted for the number of cells killed by the mAb plus C treatment.

Immunofluorescence Staining Analysis. Viable effector cells were washed in flow cytometry buffer (PBS containing 0.5% BSA and 0.01% NaN_3), and 5 × 10^5 cells were incubated with mAb in a volume of 25 μl. The following mAbs were used: PE-conjugated anti-CD4 (GK.1.5, Becton Dickinson & Co., Mountain View, CA [38]), FITC-conjugated anti-CD8α (53-6.7, Becton Dickinson & Co., [39]), biotin-labeled anti-TCR α/β (H57-597, [40]), FITC-labeled anti-CD3 (45-2C11, [41]), and control rat Ig2b FITC-labeled (Pharmingen, San Diego, CA). To stain cells with bound biotin-labeled antibodies, cells were washed and thereafter incubated with PE-streptavidin (Becton Dickinson & Co.). Cells (10^4) were analyzed on an Epics C (Coulter Corp. Cytometry, Hialeah, FL) in a two-color mode. Dead cells were excluded based on forward and 90°C light scatter characteristics.

Results

β_2-m Mutant Mice Rapidly Reject Whole MHC-disparate MHC Class I$^+$ Skin Grafts. To evaluate the importance of MHC-I-directed immunity, we used β_2-m mutant mice as donors and as recipients for engraftment of whole MHC and minor H antigens mismatched skin grafts. Normal BALB/c (H-2^d) mice readily rejected both −/− and +/− 129 (H-2^b) grafts with similar survival times (Table 1). In addition, both (129 × C57BL/6)F$_3$ −/− and +/− (H-2^b) mice rapidly rejected wild-type BALB/c (H-2^d) grafts. Furthermore, both 129 (H-2^b) −/− mutant and +/− control mice rapidly rejected skin grafts from normal B10.BR (H-2^k) donors (Table 1).

Table 1. *Rejection of MHC Disparate Grafts by Wild-Type and β_2-m Mutant Mice*

Donor			Host*			MST (mean ± SD)	Fraction rejecting
Strain	H-2	β_2-m‡	Strain	H-2	β_2-m		
						d	
129	b	–/–	BALB/c	d	+/+	12.7 ± 1.2	4/4
129	b	+/–	BALB/c	d	+/+	12.0 ± 0.8	5/5
BALB/c	d	+/+	(129 × C57BL/6)F3	b	–/–	9.4 ± 1.2	5/5
BALB/c	d	+/+	(129 × C57BL/6)F3	b	+/–	10.3 ± 0.4	3/3
B10.BR	k	+/+	129	b	–/–	14.2 ± 1.3	5/5
B10.BR	k	+/+	129	b	+/–	12.6 ± 0.4	3/3
129	b	+/–	129	b	–/–	13.7 ± 2.1	4/4
129§	b	+/–	129§	b	–/–	12.1 ± 2.1	6/6

* Male skin was transplanted to male recipients.
‡ β_2-m genotype: –/–, homozygous β_2-m gene mutant; +/–, heterozygous β_2-m mutant; +/+, wild-type.
§ Females.

To study the possible effector cells, we tested recipient mice after the rejection of the graft, for the presence of donor-specific CTLs. Spleen cells of recipient mice were restimulated in vitro with irradiated spleen cells of the strain used as skin donor. As expected, strong CTL activity was detected in cultures derived from naive or primed +/– recipients (Fig. 1). No CTL activity was detected in cultures from naive mutant mice (Fig. 1). In contrast, two of the five tested (129 × C57BL/6)F_3 mutant mice (H-2^b), that had rejected BALB/c (H-2^d) grafts, showed weak H-2^d-specific CTL activity, and in the other three cultures no CTL activity was seen (data not shown). When mutant 129 (H-2^b) mice that had rejected B10.BR (H-2^k) skin were tested, two of the four animals showed weak H-2^k-specific CTL responses (Fig. 1). The weak CTL activity was specific for MHC-I molecules of H-2^k because MHC-II$^-$ R1.1 (H-2^k) T cells were lysed, whereas EL-4 (H-2^b) and the variant R1E.TL$^-$ (H-2^k) target cells, which lack β_2-m protein synthesis and consequently MHC-I cell surface expression (30, 31), were not lysed (data not shown). The weak CTL activity was mediated by CD4$^-$8$^+$ cells because depletion of the effector cells by anti-CD4 mAb plus C had no effect (data not shown), but treatment with anti-CD8 mAb plus C eliminated the lysis of the R1.1 (H-2^k) target cells (Fig. 1). Flow cytometric analysis of the effector cells confirmed the presence of a low number of CD3$^+$, TCR-α/β^+ CD4$^-$8$^+$ cells (1–3%) in the mutant effector cells. In contrast, in the effector cells of naive mutant mice and of the two nonresponding primed mutant mice, no CD4$^-$8$^+$ cells were detected (data not shown). We conclude that the rejection of fully allogeneic skin grafts by mutant mice is accompanied by the generation of a low number of MHC-I–directed CD8$^+$ CTL$^-$ in some, but not in other recipients.

Mutant Mice Rapidly Reject "Syngeneic" MHC-I$^+$ Grafts. It was interesting to examine whether the mutant mice would be tolerant for the β_2-m protein and syngeneic MHC-class I molecules. For that reason, 129 mutant recipients were engrafted with sex-matched 129 strain +/– skin. Both female and male mutant hosts vigorously rejected these grafts in about 13 d (Table 1). To examine the role of self MHC-I–specific CTLs, we tested restimulated spleen cell cultures of four male

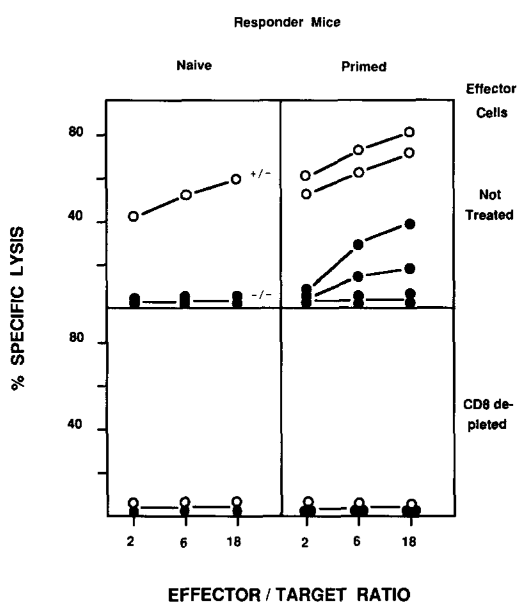

Figure 1. H-2^k-specific CTL responses of β_2-m mutant (●) and β_2-m heterozygous (○) 129 (H-2^b) mice after rejection of a +/+ B10.BR (H-2^k) skin graft. Spleen cells from mice 3–4 wk after the rejection of the graft or naive mice were stimulated for 5 d with B10.BR spleen cells, as described in Materials and Methods. Lysis by the effector cells was measured against R1.1 (H-2^k) T cell targets. No significant lysis was seen against β_2-m–deficient R1E.TL$^-$ (H-2^k) variant cells (data not shown). Each curve represents the CTL activity of an individual responder mouse.

Table 2. *Rejection of Skin Grafts with Multiple Minor H Differences in H-2^k Mice*

Donor			Host*		MST	
Strain		β_2-m‡	Strain	β_2-m	(mean ± SD)	Fraction rejecting
					d	
B10.BR BC§	donor 1	−/−	B10.BR	+/+	16.6 ± 4.2	5/5
B10.BR BC	donor 2	−/−	B10.BR	+/+	17.8 ± 2.0	6/6
B10.BR BC	donor 3	−/−	B10.BR	+/+	16.3 ± 3.8	3/3
B10.BR BC	donor 1	+/−	B10.BR	+/+	17.4 ± 3.6	5/5
B10.BR BC	donor 2	+/−	B10.BR	+/+	14.6 ± 2.5	6/6

* Male skin was grafted onto male recipients.
‡ β_2-m genotype (see Table 1 legend).
§ Derived from (129 × C57BL/6)F_1 mice back-crossed four generations onto a B10.BR (H-2^k) background.

recipients for the presence of CD8$^+$ CTLs reactive with EL-4 (H-2^b) target cells. Only one responder showed CD8$^+$ CTL activity, and in the other three effector cell populations, no CTL activity was seen (data not shown). Therefore, the generation of H-2^b-reactive CTLs is not essential for the rejection of syngeneic MHC-I$^+$ positive grafts.

Mutant Mice Readily Reject Skin Grafts with Multiple Minor H Differences. To study the importance of MHC-I in the rejection of skin grafts with multiple minor H differences, we performed skin grafting in both H-2^b and H-2^k mice. Mutant and +/− control skin derived from mice (H-2$^{k/k}$) back-crossed four generations onto a B10.BR background, were transplanted onto wild-type B10.BR (H-2^k) mice. As can be seen in Table 2, both mutant and control grafts were readily rejected, with similar mean survival times of the skin grafts.

Similarly, Table 3 shows that wild-type +/+ C57Bl/6 mice readily rejected both mutant and +/− skin 129 strain grafts (H-2^b, multiple minor H differences). It seems possible that cells of the 129 mutant skin graft absorbed some β_2-m protein from the C57BL/6 host, thereby acquiring some MHC-I surface expression and susceptibility to MHC-I–guided immunity (26, 42). To address this issue, we also grafted mutant skin onto mutant hosts with multiple minor H differences. In both directions, grafts were rapidly rejected by both mutant and control littermates after a similar latency (Table 3). These results indicate that in skin grafts with multiple minor H differences, the expression of MHC-I and MHC-I–guided CTLs are not essential for graft rejection.

MHC-1 Expression Profoundly Influences Rejection of H-Y–Disparate Grafts. A vast majority of female H-2^b mice readily reject H-Y disparate skin grafts which is generally accompanied by the generation of strong MHC-I–restricted H-Y–specific CTLs (43–45). We therefore examined inbred

Table 3. *Rejection of Skin Grafts with Multiple Minor H Differences in H-2^b Mice*

Donor		Host*		MST	
Strain	β_2-m‡	Strain	β_2-m	(mean ± SD)	Fraction rejecting
				d	
129	−/−	(129 × C57BL/6)F_1	+/−	>120	0/6
129	−/−	C57BL/6	+/+	14.0 ± 1.0	8/8
129	+/−	C57BL/6	+/+	12.5 ± 0.8	8/8
129	−/−	C57BL/6 BC§	−/−	13.0 ± 0.8	3/3
129	−/−	C57BL/6BC	+/−	13.0 ± 1.2	4/4
C57BL/6 BC	−/−	129	−/−	13.4 ± 0.4	5/5
C57BL/6 BC	−/−	129	+/−	13.3 ± 0.4	3/3

* Male skin was grafted onto male recipients.
‡ β_2-m genotype (see Table 1 legend).
§ Derived from (129 × C57BL/6)F_1 mice back-crossed three generations onto a C57BL/6 background.

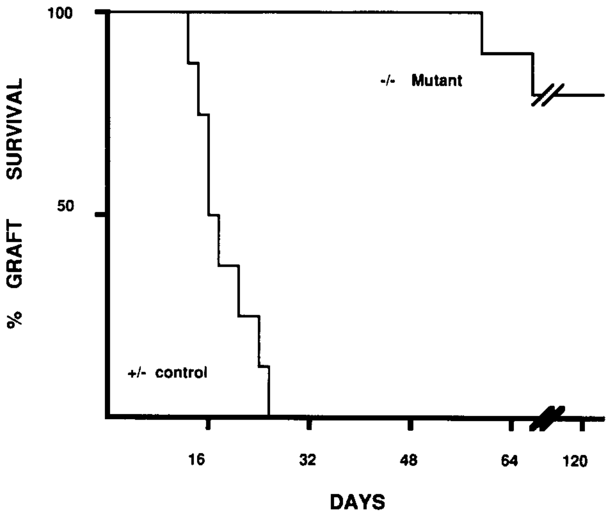

Figure 2. Survival of H-Y disparate skin grafts in inbred mutant and heterozygous control 129 (H-2^b) mice. +/− females (*F*) were engrafted with +/− male (*M*) (*n* = 8), and −/− F engrafted with −/− M (*n* = 11).

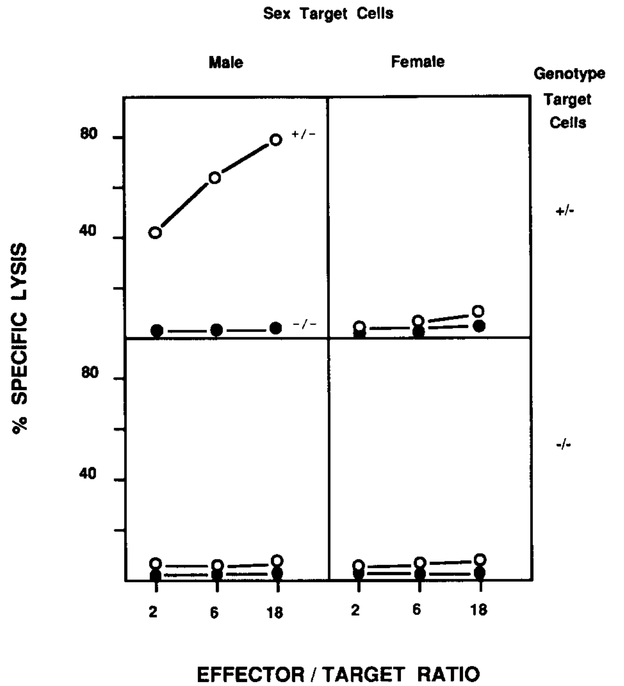

Figure 3. H-Y-specific CTL responses of an immunized β_2-m mutant (●) and an immunized heterozygous (○) 129 (H-2^b) female. Similar results were obtained with three other primed mutant and three +/− females (data not shown). Mutant females were immunized with mutant male cells and heterozygous females were immunized with heterozygous male cells as described in Materials and Methods. Spleen cells were restimulated in vitro with similar cells used for immunization. Effector cells were tested against con A-stimulated 129 mouse strain T cell blasts of indicated sex and β_2-m genotype. The CML was performed in AIMV serum-free medium.

129 (H-2^b) mutant and +/− control females for the rejection of male skin grafts. Control +/− females rapidly rejected male +/− grafts with a mean survival time of about 18 d (Fig. 2). In contrast, 9 of 11 mutant females accepted mutant male skin for at least 120 d (Fig. 2), and two other animals rejected it after a long latency. In these animals, a small residual graft was accepted, but because it consisted of <20% of the size of the original graft, they were scored as rejected. To examine the role of H-Y specific CTLs, we tested in vivo immunized mutant and +/− animals for the in vitro generation of H-Y-specific CTLs. To exclude the possible absorption by the target cells of β_2-m protein derived from the FCS in the regular culture medium, we performed the CML in serum-free medium. As expected, all four tested immunized +/− females, mounted strong H-Y-specific CTLs (Fig. 3). The CTLs possessed a CD4$^-$8$^+$ phenotype (data not shown). These H-Y-specific CTLs did not lyse mutant male target cells (Fig. 3). This result implies that in the absence of exogenous β_2-m protein, the MHC-I molecules cannot present H-Y antigen–derived peptides. Consistent with this conclusion, we could not detect any CTL activity, including H-Y specific, in cultures derived from four tested immunized mutant females (Fig. 3). In the latter experiment, no lysis was observed of con A-stimulated T cell blasts (Fig. 3), nor LPS-stimulated mutant male B cell blasts (data not shown). We therefore conclude that β_2-m mutant females do not generate MHC-I–guided H-Y specific CTLs and that this is not compensated for by MHC-II–restricted H-Y specific CTLs.

MHC-I$^+$ Recipients Fail to Reject MHC-I–deficient Skin Grafts. Previous studies have shown that radioresistant NK1.1$^+$ NK cells present in MHC-I$^+$ recipients vigorously reject haemopoietic grafts from MHC-matched β_2-m −/− donors (26, 27). NK cell–mediated rejection of skin grafts would severely complicate the interpretation of many of the above described experiments. In addition, we wanted to compare the biology of the NK cells rejecting MHC-deficient bone marrow grafts (26, 27) with those displaying the phenomenon of "hybrid resistance." In the latter situation, irradiated MHC-heterozygous mice often reject haemopoietic cells from one of the homozygous parental strain, but do accept nonlymphoid parental grafts, including skin grafts (46–49). To assess the possible role of NK cells in skin graft rejection, we performed skin grafting in similar combinations that lead to rejection of mutant bone marrow grafts, namely skin from 129 −/− donors to 129 +/− and (129 × C57BL/6)F$_1$ +/− (H-2^b) recipients. As can be seen in Table 4, all +/− hosts accepted the mutant graft over a period of at least 120 d. Therefore, NK cells are not effective in the rejection of MHC-I–deficient skin transplants.

Mutant Fibroblasts Are Not Highly Susceptible to Lysis by NK1.1$^+$ NK Cells. To study the difference between the rejection of mutant bone marrow grafts (26, 27) versus the acceptance of mutant skin grafts by +/− hosts (this study), we compared lymphoid and nonlymphoid mutant target cells with respect to NK cell–mediated lysis. As a source of nonlymphoid cells, we used immortalized, embryonic fibroblasts. No difference was seen in the level of NK cell mediated lysis of −/− and +/− fibroblasts (Fig. 4). Most effector cells did not express the NK1.1 cell marker because depletion with

Table 4. *MHC Class I⁺ Mice Fail to Reject β_2-m Mutant Skin Grafts*

Donor		Host*				
Strain	β_2-m‡	Strain	β_2-m	*n*	Antigenic Disparity	Survival time
						d
129§	+/−	129	+/−	14	None	>120
129	+/−	(129 × C57BL/6)F_1	+/−	6	None	>120
129	−/−	129	+/−	7	β_2-m	>120
129	−/−	(129 × C57BL/6)F_1	+/−	6	β_2-m	>120

* Male skin grafts were transplanted onto male recipients, except for the top group which included seven female grafts engrafted into female recipients.
‡ β_2-m genotype (see Table 1 legend).
§ Both 129 and (129 × C57BL/6)F_1 mice are of H-2^b type.

anti-NK1.1 mAb plus C only marginally affected target cell lysis. This is in contrast with the results obtained with −/− and +/− Con A-stimulated T cell blasts (Fig. 4 and reference 27). The NK cells efficiently lysed −/− targets, but did not lyse +/− targets. A similar difference was seen when −/− and +/− LPS-stimulated B cell blasts were compared (data not shown). Furthermore, anti-NK1.1 mAb plus C treatment of the effector cells completely abolished the lysis of the −/− con A targets (Fig. 4 and reference 27). We therefore conclude that the difference in fate between mutant bone marrow (rejected) versus skin (accepted) after engraftment into +/− hosts, can be explained by a difference in susceptibility to NK1.1⁺ NK cells between lymphoid (susceptible) and nonlymphoid (resistant) mutant cells.

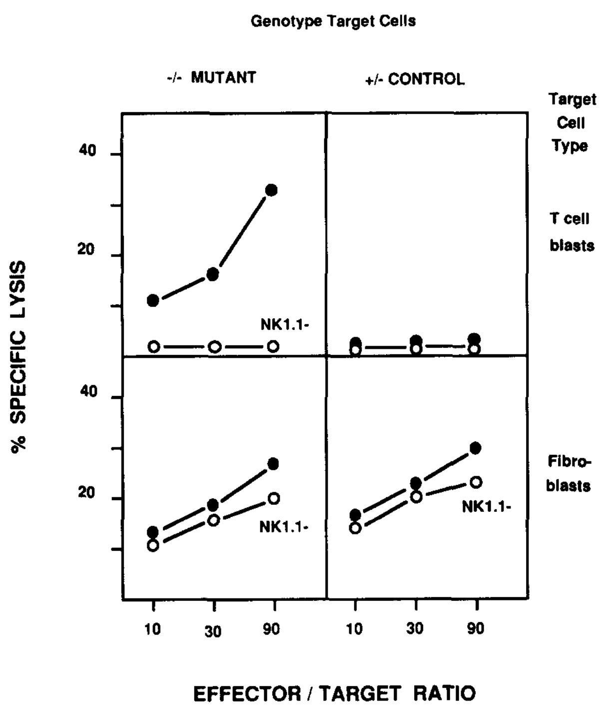

Figure 4. NK cell-mediated lysis of mutant and +/− T cell blasts and embryonic fibroblasts. T cell blasts were obtained from Con A-stimulated spleen cells from (129 × C57BL/6)F_3 mice. 129 mice derived, SV40 large T immortalized cultures of embryonic fibroblasts were obtained as described in Materials and Methods. Poly I.C.-stimulated spleen cells from (129 × C57BL/6)F_1 +/− mice were used as source of NK cells. No significant lysis was observed when unstimulated spleen cells were used as effector cells (data not shown). Enrichment of the NK cells and depletion of the effector cells by anti-NK-1.1 mAb plus C is described in Materials and Methods. Effector cells were treated with NMS plus C (●), or treated with anti-NK1.1 mAb plus C (○).

Discussion

The central issue addressed in this study is the need for MHC-I-directed immunity in skin graft rejection, using mutant mice that have normal subsets of TCR-α/β^+ $CD4^+8^-$ Th cells and γ/δ T cells, but that are deficient for mature $CD4^-8^+$ CTLs (22–24). The results suggest that MHC-I-directed CTLs are not essential in the rejection of strong antigenic grafts, such as whole MHC disparate grafts or grafts with multiple minor H differences. In contrast, our experiments indicate that MHC-I-guided immunity is pivotal in the rejection of H-Y disparate grafts.

After the engraftment of whole MHC disparate skin grafts, a low number of graft-specific CD8⁺ CTLs were detected in some of the recipient mutant mice, but no CTL activity or CD8⁺ T cells were seen in other recipients. Similar results were obtained with mutant mice grafted with syngeneic MHC-I⁺ grafts. No difference was seen in the survival time of the skin grafts in mice with or without CTL activity. In our view, this suggests that rejection of these grafts by the mutant mice is not dependent on MHC-I-directed CTL. The mechanism leading to the partial rescue of the mutant phenotype is not clear. One possibility is that β_2-m protein secreted by the skin graft or skin graft-derived lymphocytes, migrates to the thymus and, temporarily, partially restores the MHC-I-dependent process of positive selection of CD8⁺ CTLs. Otherwise, direct induction of thymus-independent CD8⁺ CTLs (50) by cells in the skin graft may occur. Mutant and control skin were both rapidly rejected by allogeneic wild-type hosts. In the presence of FCS-derived, exogenous β_2-m protein, mutant cells can serve as target cells for allo-specific CTLs, although about ninefold less susceptible than wild-type cells (22). This could be still sufficient for rapid skin graft destruction. Alternatively, our results are in concordance with studies showing that purified $CD4^+8^-$ T cells are able to reject fully allogeneic skin grafts (1–3, 11). After the engraftment of skin with multiple minor H differences, no differences were seen in the survival of mutant versus control grafts. Furthermore, reciprocal grafts between mutant mice with multiple minor H differences were rapidly rejected. This indicates that a strong Th cell response is sufficient for skin graft rejection. This conclusion is consistent with reported studies suggesting that purified $CD4^+8^-$ T cells can mediate rejection of skin grafts with multiple minor H differences (9, 11).

The β_2-m mutation has a profound impact on the ability of the mutant mice to reject H-Y disparate skin grafts. This strongly argues that in normal 129 mice, MHC-I–guided CTLs have a pivotal role in the destruction of H-Y disparate grafts. The same conclusion was made in studies showing that both Th and CTLs are required for the rejection of H-Y disparate grafts (11, 45). Wild-type H-Y-specific CTLs fail to lyse mutant male target cells and no H-Y-specific CTLs could be demonstrated in the mutant mice. These observations indicate that in the absence of endogenous β_2-m protein, the MHC-I molecules cannot present H-Y antigen–derived peptides. Because the mutant mice are deficient for both the MHC-I–guided CTLs (22, 24), as well as in the presentation of peptides by MHC-I, no H-Y–specific MHC-I–guided CTLs can be generated by the mutant mice. The failure to reject male grafts can be explained in several ways. It could be that H-Y-specific graft destruction can be mediated exclusively by $CD8^+$ MHC-I–guided CTLs. Alternatively, one might argue that Th cells do have the potential to mediate H-Y–specific graft rejection, but due to the weakness of the primary H-Y–specific Th cell response, no graft destruction occurs in the recipients. Yet perhaps, the strength of the MHC-II–restricted $CD4^+$ T cell response may not be crucial, but an essential component thereof, such as MHC-II–guided CTLs. Indeed, we could not detect any MHC-II–restricted H-Y–specific CTL activity in immunized mutant mice.

Our experiments indicate no significant role for allogeneic MHC-I–specific NK cells in skin graft rejection. This strongly contrasts with the finding that hemopoietic grafts from mutant mice are vigorously rejected by radioresistant $NK1.1^+$ NK cells present in normal MHC-matched recipients (26, 27). Our present study clearly demonstrates that this subset of NK cells is not effective in the rejection of mutant skin grafts. The rejection of mutant haemopoietic cells is consistent with the high susceptibility of mutant T and B cell blasts to $NK1.1^+$ NK cell–mediated lysis. In contrast, mutant fibroblasts seem to be refractory to lysis mediated by these NK cells. Our findings are in concordance with many studies involving $MHC\text{-}I^-$ tumor cells, in which an inverse correlation is often seen between MHC-I expression and NK cell–mediated lysis (for review see reference 48). However, in these studies a considerable number of exceptions have been reported, notably in the group of nonlymphoid tumors (48). Our results therefore suggest that β_2-m mutant fibroblasts may either lack the target antigen(s) recognized by NK cells, or may be devoid of some cell adhesion molecules crucial for NK-target cell interactions. Our skin graft experiment suggests that this may also be true for many cells in a mutant skin graft.

In summary, our experiments allow three main conclusions. First, NK cells highly efficient in the rejection of $MHC\text{-}I^-$ bone marrow grafts, are not effective in skin graft rejection. Second, $CD8^+$ CTLs are not essential in the rejection of strong antigenic skin grafts. This conclusion implies that MHC-II–guided T cells are sufficient to induce graft rejection, possibly by the generation of lymphokine-induced microvascular lesions, or direct lysis by MHC-II–directed CTLs. Finally, in grafts with a limited number of antigenic differences, e.g., the H-Y antigen, graft-specific CTLs may be pivotal for graft destruction. To explain the need for MHC-I–guided CTLs, we favor the hypothesis that the primary H-Y-specific Th cell response is too weak or limited in functional scope to induce graft rejection. This hypothesis predicts that immunized mutant mice will reject H-Y disparate grafts due to a boosted Th cell response, in the absence of any MHC-I–guided CTLs. This experiment and the adoptive transfer of H-Y-specific Th clones seems to be well suited to dissect the potential of distinct subsets of $CD4^+$ T cells in murine skin graft rejection.

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Note added in proof: Just as the corrected galley proofs of this paper arrived, we learned of the sudden and unexpected death of Maarten Zijlstra. Maarten was in the process of setting up his laboratory and developing his own exciting scientific program. With him we lost a good friend and colleague as well as an outstanding scientist. His integrity and dedication were an example to us all. His seminal contribution to immunology and biology in general will live on in the mouse model he created.

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