Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 3913–3917, May 1993
Immunology

Mice lacking major histocompatibility complex class I and class II molecules

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Communicated by Elkan Blout, January 19, 1993 (received for review October 14, 1992)

ABSTRACT **Mice lacking major histocompatibility complex (MHC) antigens were generated by mating β_2-microglobulin-deficient, and therefore class I-deficient, animals with MHC class II-deficient animals. When housed under sterile conditions, the resulting MHC-deficient mice appear healthy, survive for many months, and breed successfully. Phenotypically, MHC-deficient mice are depleted of $CD4^+$ and $CD8^+$ T cells in peripheral lymphoid organs due to a lack of appropriate restricting elements. In contrast, the B-cell compartment of these animals appears intact, and MHC-deficient mice can mount specific antibody responses when challenged with a T-independent antigen. Spleen cells from MHC-deficient animals are poor stimulators and responders in a mixed lymphocyte reaction. Despite their relatively weak cellular immune responses *in vitro*, MHC-deficient mice reject allogeneic skin grafts with little delay, and grafts from MHC-deficient animals are rapidly rejected by normal allogeneic recipients. Taken together, these results emphasize the plasticity of the immune system and suggest that MHC-deficient mice may be useful for examining compensatory mechanisms in severely immunocompromised animals.**

Our understanding of the development and function of cells in the immune system has been aided by the use of mice that carry spontaneously arising mutations (1). For example, mutations such as *nu, scid, xid,* and *bg* have resulted in animals that lack functional populations of B lymphocytes, T lymphocytes, and/or natural killer cells. However, the occurrence of these mutant animals is rare, and the nature of the genetic defect responsible for the observed phenotypic change is often not known. With the advent of gene targeting in embryonic stem cells, it is now possible to inactivate specific genes in the immune system to examine their function (2, 3).

One important set of molecules in the immune system is the major histocompatibility complex (MHC) antigens. Gene targeting has been used to generate mutant mice that lack the cell surface expression of MHC class I (4, 5) and class II (6, 7) antigens. These novel strains have been used to reexamine the maturation of individual T-cell populations and their roles in various immune responses. Thus, although several lines of evidence had suggested that MHC molecules are important for the development of mature T lymphocytes, the phenotypic analysis of these mutant animals confirmed that β_2-microglobulin (β_2m)-deficient, and therefore class I-deficient, mice lack $CD8^+$ cytolytic cells (4, 5), whereas class II-deficient mice lack $CD4^+$ helper cells (6, 7). Recently, these mice have been used to define more clearly the role of $CD4^+$ and $CD8^+$ cells in the response to a number of viral and protozoan infections (8–12). Moreover, several reports have demonstrated that β_2m-deficient cells are highly susceptible to lysis by natural killer cells from MHC matched normal mice (13–15), thereby providing evidence for the "missing-self" hypothesis.

The availability of mutant mice that, individually, do not express MHC class I or class II molecules provided the opportunity to attempt to create a murine model of total MHC deficiency. Given the importance of MHC molecules in the initiation and maintenance of immune responses, such MHC-deficient mice might be expected to be severely immunocompromised, if they could be created at all. Indeed, humans who are afflicted with bare lymphocyte syndrome fail to express MHC molecules and die at an early age unless provided with bone marrow transplants (16). In this report, we present the phenotypic and functional characterization of mice that lack the cell surface expression of MHC class I and class II molecules. Although our results show that MHC-deficient mice do have many of the expected immune deficiencies, they also suggest that there must be compensatory mechanisms to account for the surprising immunocompetence of these animals.

MATERIALS AND METHODS

Mice. The genotypic and phenotypic analyses of β_2m-deficient (4) and class II-deficient mice (6) have been described. Animals of these two strains on the background of 129/Sv were mated, and subsequently intercrossed, to produce mice homozygous for the $\beta_2 m$ and class II mutant alleles (MHC-deficient mice). Routinely, animals homozygous for the disrupted $\beta_2 m$ allele and heterozygous for the disrupted $A_\beta{}^b$ allele were mated to mice heterozygous for the disrupted $\beta_2 m$ allele and homozygous for the disrupted $A_\beta{}^b$ allele, thereby generating litters that contain control, class I-deficient, class II-deficient, and MHC-deficient animals. All animals were maintained in autoclaved microisolator cages and provided with autoclaved food and water. When housed under these conditions, MHC-deficient animals and their littermates appear healthy. BALB/cBy mice were purchased from The Jackson Laboratory.

Flow Cytometric Analysis. Flow cytometry was performed as described (6). Approximately 1×10^6 cells were stained with hybridoma supernatant containing MHC class I- or class

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Abbreviations: MHC, major histocompatibility complex; β_2m, β_2-microglobulin; mAb, monoclonal antibody; TNP, trinitrophenol; MLR, mixed lymphocyte reaction.

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II-specific monoclonal antibodies (mAbs). mAbs were 3-83P (*H-2K*^{b}), 28-14-8S (*H-2D*^{b}), 25-9-17S (*I-A*$_\beta$^{b}), and 1E9 (*I-A*$_\alpha$^{b}). Cells were washed once in Hanks' balanced salt solution, 3% fetal calf serum, and 0.1% sodium azide and then incubated with a fluorescein-conjugated F(ab')$_2$ fragment of goat antibody to mouse IgG (γ-chain-specific). Cells were then washed twice again, fixed in 2% paraformaldehyde, and analyzed by flow cytometry. For directly conjugated antibodies, cells were preincubated with purified anti-FcγIIR antibody (2.4G2) for 5 min at 4°C. Fluorescein-conjugated anti-CD4 and anti-IgM and phycoerythrin-conjugated anti-CD8 and anti-B220 were from Pharmingen (San Diego).

Immunization and ELISA Analysis. Animals were immunized i.p. with 100 μg of trinitrophenol (TNP)-conjugated Ficoll in phosphate-buffered saline. Animals were bled 3 days prior to immunization and 8 and 16 days post-immunization. Serum was prepared and stored at 4°C. TNP-specific antibody responses were measured by coating flat-well microtiter plates overnight at 4°C with TNP-conjugated bovine serum albumin (25 μg/ml) in Tris-buffered saline (TBS). After washing twice with TBS, wells were blocked with 2% goat serum in TBS and then washed twice again with TBS. Serial 5-fold dilutions of the mouse sera, ranging from 1:100 to 1:12,500 in blocking serum, were then incubated for 2 hr at room temperature. Plates were washed five times with TBS prior to adding alkaline phosphatase-conjugated goat anti-mouse isotype-specific antibodies (1:750 in blocking serum; Southern Biotechnology Associates, Birmingham, AL). After seven washes with TBS, the assays were developed with 0.1 M diethanolamine, pH 9.8/0.5 mM $MgCl_2$/1 mg of *p*-nitrophenyl phosphate per ml (Sigma) between 40 min and 2 hr and measured for absorbance at 405 nm in a Molecular Devices (Palo Alto, CA) microplate reader. Semilogarithmic plots of the averages of duplicate samples from two MHC-deficient and two control mice were analyzed to ensure linearity in the assay.

Mixed Lymphocyte Reaction (MLR). Responder spleen cells (2×10^5) and irradiated (2000 rad; 1 rad = 0.01 Gy) stimulator spleen cells (4×10^5) were added in triplicate to U-bottom wells in a final volume of 200 μl of RPMI 1640 medium supplemented with 20 mM Hepes, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 50 μM 2-mercaptoethanol, 10% fetal calf serum, and 100 μg of gentamicin per ml. The cultures were pulsed with 1 μCi of [^{3}H]thymidine (6.7 Ci/mmol; 1 Ci = 37 GBq) for the last 8–14 hr of a 3- to 4-day culture period. The samples were harvested onto glass fiber filters and [^{3}H]thymidine uptake was measured by β scintillation counting (30 sec per sample). Results are expressed as mean cpm ± SEM. In some experiments, spleen cells were depleted of CD4$^+$ or CD8$^+$ cells. Spleen cells (50×10^6 cells per ml) were incubated for 30 min on ice with medium alone or a 1:4 dilution of ascites fluid containing mAb GK1.5 (anti-CD4) or 2.43 (anti-CD8). Cells were washed once, resuspended at 40×10^6 cells per ml, and then incubated with a 1:5 dilution of rabbit complement (C-Six Diagnostic, Mequon, WI) for 30 min at 37°C. The cells were then washed twice and counted.

Skin Grafting. Skin grafting was performed as described by Billingham and Medawar (17). Mice were anesthetized with chloral hydrate supplemented with ether and engrafted with donor trunk skin placed onto the thoracic area. Grafts were held in place for 7–9 days with vaseline gauze and plaster bandages. Rejection was scored when >90% destruction of the tissue had occurred.

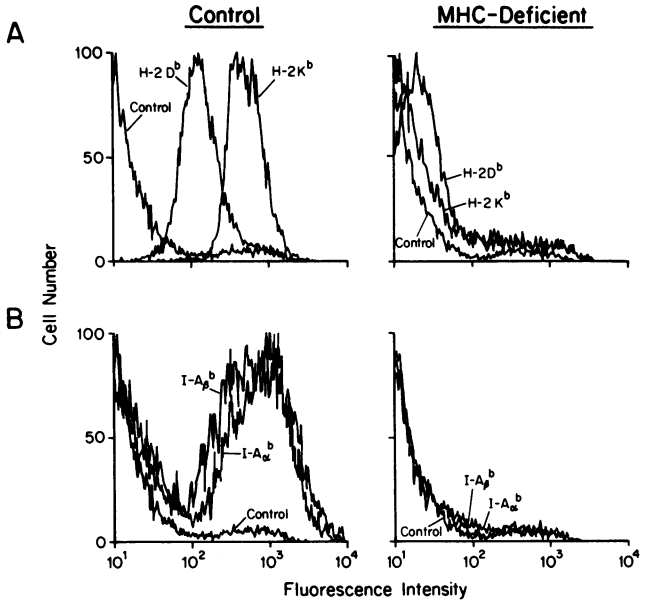

FIG. 1. Flow cytometric analysis of MHC class I and class II expression in the periphery of MHC-deficient animals. Single cell suspensions were prepared from spleens of control and MHC-deficient animals and 1×10^6 cells were stained with hybridoma supernatants containing class I-specific (*A*) or class II-specific (*B*) antibodies, followed by a fluorescein-conjugated F(ab')$_2$ fragment of goat antibody to mouse IgG. mAbs were 3-83P (*H-2K*^{b}), 28-14-8S (*H-2D*^{b}), 25-9-17S (*I-A*$_\beta$^{b}), and 1E9 (*I-A*$_\alpha$^{b}). Control shows background staining with the secondary reagent alone. Flow cytometric analysis was performed four separate times with one control and one MHC-deficient animal. Essentially identical results were obtained in each experiment, although the levels of *H-2K*^{b} and *H-2D*^{b} expressed by MHC-deficient mice were somewhat variable but never greater than that shown in *A*.

RESULTS AND DISCUSSION

To generate an inbred strain of mice that lacks expression of MHC class I and class II molecules, 129/Sv mice harboring

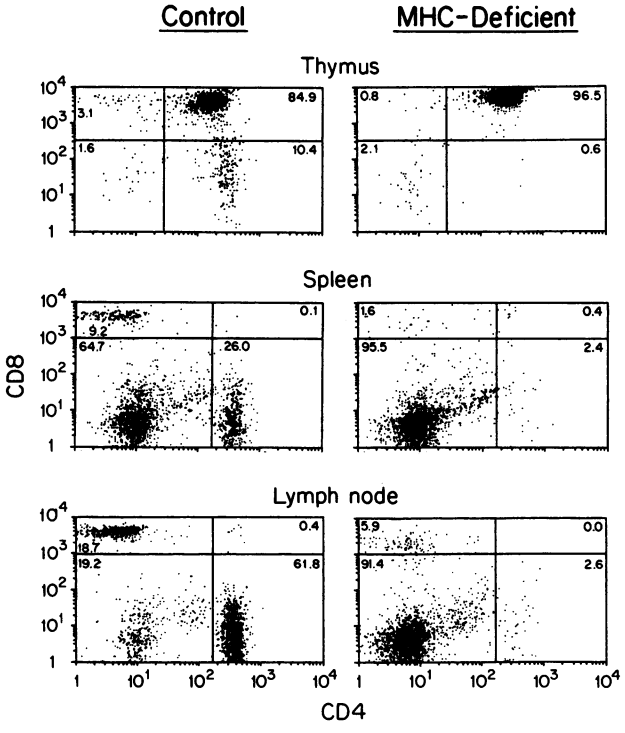

FIG. 2. Flow cytometric analysis of T-cell subsets in the lymphoid organs of MHC-deficient animals. Single cell suspensions were prepared from thymi, spleens, and lymph nodes of control and MHC-deficient animals and analyzed as in Fig. 1. Approximately 10,000 events were recorded for each analysis.

a gene disruption at the $\beta_2 m$ locus were mated to animals carrying a similar mutation in the $A_\beta{}^b$ gene. Southern blot analysis of DNA from mice born of an intercross between animals heterozygous for gene disruptions at both loci revealed that doubly homozygous offspring were present at the expected Mendelian frequency. When housed under specific pathogen-free conditions and provided with sterile food and water, MHC-deficient mice appear healthy.

To confirm that mice homozygous for gene disruptions at both loci do not express MHC molecules, flow cytometry was performed on spleen cells using several class I and class II mAbs. As shown in Fig. 1*A*, flow cytometry with mAbs for *H-2K*^{b} and *H-2D*^{b} revealed barely detectable staining on spleen cells from MHC-deficient animals. Low levels of class I heavy chain have been shown to be expressed at the cell surface in the absence of β_2m protein (18), and this phenomenon was also observed in the analysis of β_2m-deficient mice (4, 19). Staining with mAbs to either $A_\alpha{}^b$ or $A_\beta{}^b$ failed to reveal specific staining, again consistent with that previously observed with class II-deficient mice (6, 7). Thus, mice homozygous for mutations at the $\beta_2 m$ and $A_\beta{}^b$ loci are essentially devoid of MHC class I and class II molecules.

We next examined the phenotype of T-cell subsets present in the lymphoid organs of MHC-deficient animals. Flow cytometric analysis of thymocytes from MHC-deficient mice using mAbs specific for CD4 and CD8 revealed the presence of normal numbers of double-positive cells, suggesting that MHC molecules are not required for thymocytes to enter the CD4/CD8 developmental pathway (Fig. 2). In contrast, there was a virtual absence of single-positive CD4$^+$ and CD8$^+$ cells. Furthermore, these two populations were significantly depleted in the spleens and lymph nodes of MHC-deficient animals. The small number of single-positive cells seen in these peripheral lymphoid organs (1–5%) is similar to that seen for CD8$^+$ cells in β_2m-deficient mice (4, 5) and CD4$^+$ in class II-deficient animals (6, 7). At present, it is unclear whether the small number of these CD4$^+$ and/or CD8$^+$ T cells in the periphery is related to the small amounts of free class I heavy chain expressed on the surface of MHC-deficient cells. Although the spleens from MHC-deficient animals are essentially devoid of mature T cells as defined by markers for CD4 and CD8 (Fig. 2) as well as CD3 and $\alpha\beta$ T-cell receptor (data not shown), their cellularity is approximately twice that of littermate controls. When examined for the presence of cells displaying other surface markers, spleens from MHC-deficient animals have normal numbers of natural killer cells and $\gamma\delta$ T cells but greatly increased numbers of B cells, macrophages, and granulocytes (data not shown).

Class II-deficient mice have normal numbers of mature B lymphocytes but are unable to mount antibody responses to T-dependent antigens (6, 7). Flow cytometric analysis of spleen cells from MHC-deficient animals shows that mature B220$^+$ IgM$^+$ B cells also develop normally in an environment devoid of MHC class I and class II molecules (Fig. 3*A*). Furthermore, TNP-specific antibody responses of all isotypes can be elicited in MHC-deficient animals following

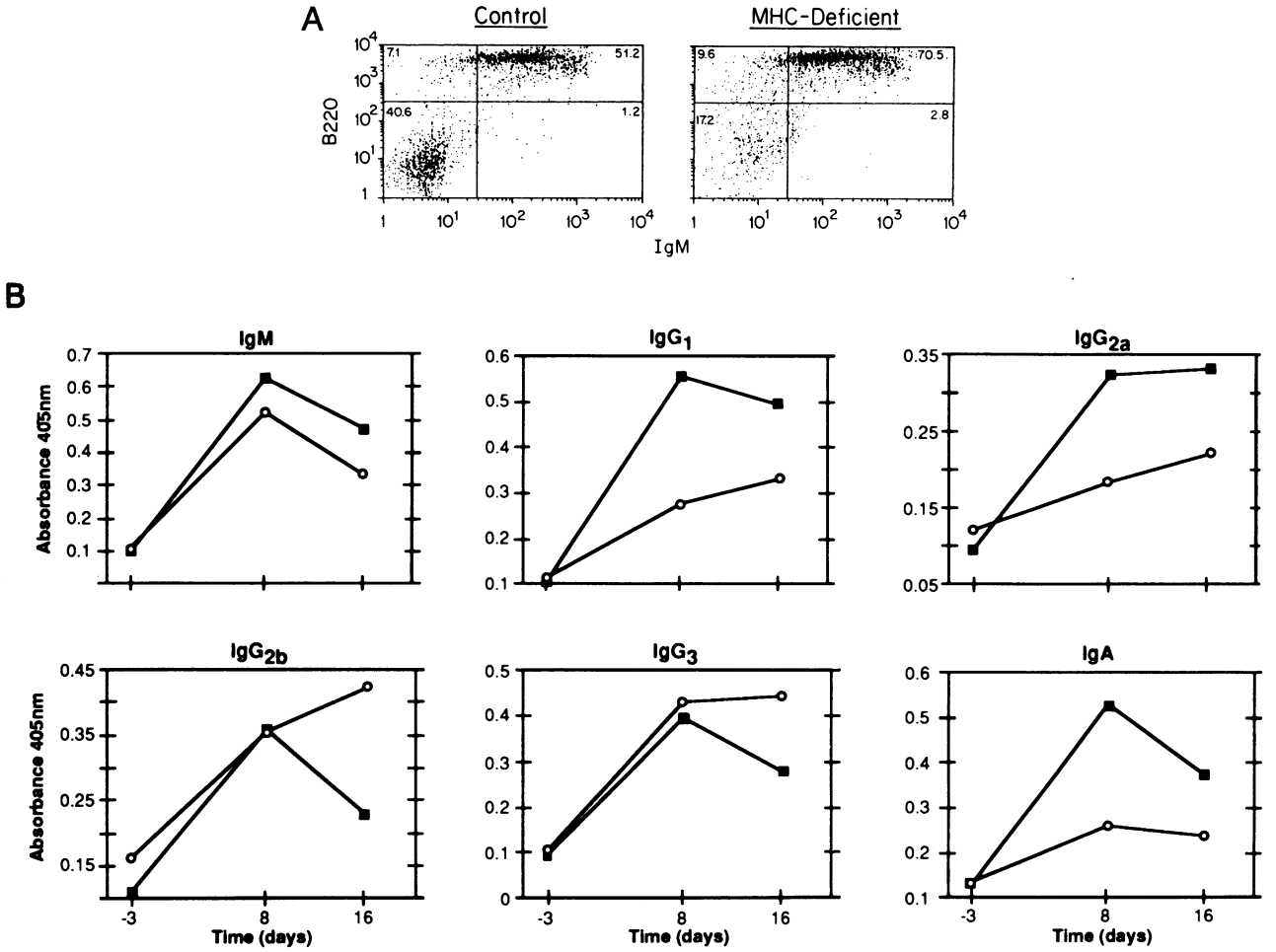

FIG. 3. Phenotype and function of B cells in MHC-deficient animals. (*A*) Single cell suspensions were prepared from spleens of control and MHC-deficient animals and analyzed by flow cytometry as in Fig. 1. Approximately 10,000 events were recorded for each analysis. (*B*) Control (○) and MHC-deficient (■) animals were bled at the indicated days prior- and post-immunization with TNP-Ficoll. Sera from two animals in each group were analyzed for the presence of isotype-specific, TNP-specific antibody by ELISA. Absorbance values at each time point were averaged and did not differ by >5%.

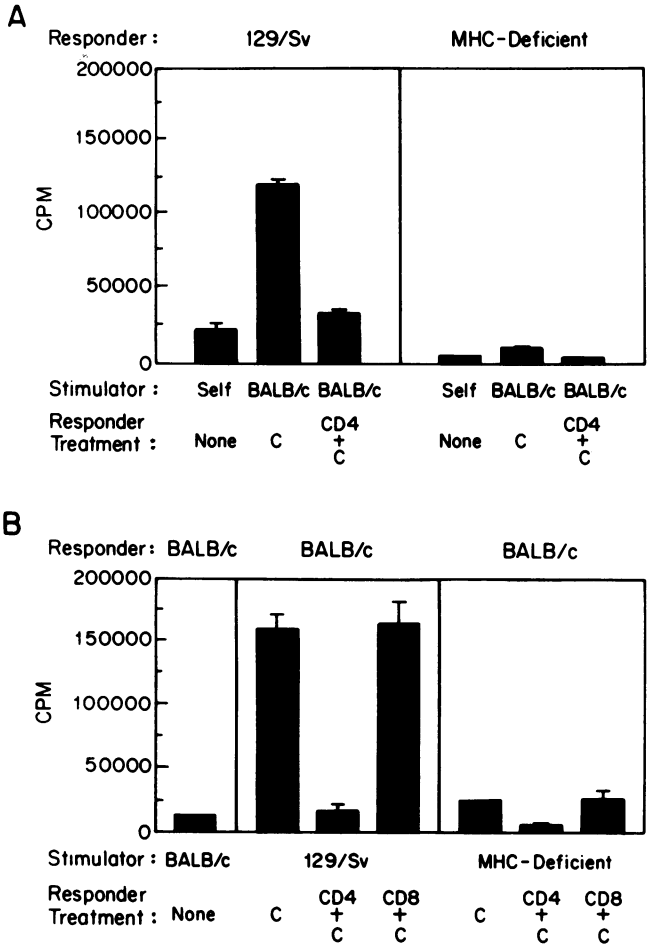

FIG. 4. MLR using MHC-deficient spleen cells as responder and stimulator. (*A*) Proliferative response of MHC-deficient and control 129/Sv spleen cells to BALB/c (*H-2*^{d}) stimulator cells. (*B*) Proliferative response of BALB/c spleen cells to MHC-deficient and control 129/Sv stimulator cells. The responder populations were depleted of CD4$^+$ or CD8$^+$ cells where indicated. C, complement.

immunization with the T-independent antigen TNP-Ficoll (Fig. 3*B*). In addition, B cells from MHC-deficient animals can be induced to proliferate following stimulation with the mitogen lipopolysaccharide (data not shown). Thus, despite their failure to express MHC molecules at the cell surface, the development and function of B cells in MHC-deficient animals appear normal.

To assess the integrity of the cellular immune response in MHC-deficient mice, we next examined the capacity of MHC-deficient cells to serve as responder and stimulator populations in a MLR. As shown in Fig. 4*A*, normal 129/Sv (*H-2*^{b}) spleen cells are induced to proliferate when cultured with allogeneic (*H-2*^{d}) stimulator cells. In contrast, spleen cells from MHC-deficient 129/Sv mice demonstrate only marginal levels of proliferation to the same allogeneic stimulator cells. This proliferation is completely abolished when the MHC-deficient responding population is pretreated with CD4 mAb plus complement, suggesting that the responding population is contained within, or dependent on, the small numbers of CD4$^+$ cells found in the periphery of MHC-deficient animals. Given that the peripheral lymphoid organs of MHC-deficient mice are significantly depleted of potentially alloreactive T cells (Fig. 2), it is not unexpected that spleen cells from these mice demonstrate low levels of proliferation when stimulated in a MLR.

When MHC-deficient spleen cells are used as stimulator cells in a MLR (Fig. 4*B*), they stimulate very low levels of proliferation of allogeneic responder cells relative to normal 129/Sv stimulator cells. This proliferation is also due to CD4$^+$ cells as pretreatment of the responder population with CD4, but not CD8, mAb plus complement abrogates the response. This result was surprising since MHC-deficient cells do not express the class II antigens for which allogeneic CD4$^+$ cells might be specific. Preliminary experiments suggest that this proliferative response is due to indirect recognition of 129/Sv minor antigens on antigen-presenting cells in the responder population as the proliferative response is blocked by *I-A*^{d}, but not *I-A*^{b}, mAb (data not shown). Thus, compared to MHC-expressing cells, MHC-deficient spleen cells are significantly less efficient at stimulating and responding in a MLR.

Numerous experiments have indicated that either CD4$^+$ or CD8$^+$ T cells can mediate rejection of MHC-disparate skin grafts but that depletion of both T-cell populations leads to prolonged graft survival (20, 21). Given that MHC-deficient mice are depleted of CD4$^+$ and CD8$^+$ T cells, we expected that they would allow prolonged survival of MHC-disparate grafts. As shown in Table 1, MHC-deficient mice reject allogeneic skin grafts almost as rapidly as do normal 129/Sv animals (mean survival time of 13.0 days versus 9.5 days). Though it is possible that this rapid rejection is mediated by the residual 1–5% CD4$^+$ and/or CD8$^+$ T cells present in MHC-deficient mice, we believe this to be unlikely given that spleen cells from MHC-deficient animals demonstrate very low levels of proliferation when stimulated with allogeneic cells *in vitro* (Fig. 4*A*). Thus, although the mechanism by which MHC-deficient mice reject skin grafts is not yet known, these animals may use novel mechanisms to mediate *in vivo* immune responses.

When MHC-deficient skin is engrafted onto normal allogeneic animals (Table 1), the grafts are rejected almost as rapidly as grafts from allogeneic MHC-expressing mice (11.4 days versus 9.5 days). Rapid rejection also occurs when MHC-deficient skin is engrafted onto β_2m-deficient mice (Table 1). This result suggests that rapid graft rejection is not due to reexpression of MHC class I antigens on the donor graft as a result of the presence of recipient β_2m and that rejection is not dependent on CD8$^+$ cells. That the rapid rejection of MHC-deficient skin grafts by normal mice is dependent on CD4$^+$ cells is suggested by the observation that prolonged graft survival occurs when MHC-deficient skin is engrafted onto either normal mice depleted of CD4$^+$ cells by mAb treatment *in vivo* or class II-deficient mice, which lack CD4$^+$ cells (data not shown). Finally, all animals that rejected MHC-deficient skin grafts rejected secondary allografts faster, thereby demonstrating immunological memory (data not shown).

Table 1. Skin graft survival times using MHC-deficient mice as donors and recipients

Donor → recipient control group	MST, days	*n*	Donor → recipient experimental group	MST, days	*n*
BALB/c → 129/Sv	9.5	2	BALB/c → MHC-deficient	13.0	3
129/Sv → BALB/c	9.5	4	MHC-deficient → BALB/c	11.4	5
129/Sv → C57BL/6	11.0	4	MHC-deficient → β_2m-deficient	12.0	3

Survival times represent the mean survival time (MST). *n*, Number of animals in each group. The β_2m-deficient mice used in these studies were on the background of C57BL/6.

Although the above data suggest that the rapid rejection of MHC-deficient skin grafts is immune mediated, the mechanism of this rejection is unclear at present. One potential explanation is the expression of low levels of class I molecules. Whereas MHC-deficient mice do not express detectable levels of class II molecules, they do express very low levels of free class I heavy chain at the cell surface (Fig. 1). Recently, it has been demonstrated that the conformation of free class I heavy chain is functionally intact, as β_2m-deficient target cells can be lysed by alloreactive cytolytic T cells (19). However, β_2m-deficient cells are lysed 50-fold less efficiently than MHC class I-expressing cells, suggesting that the ability of the free class I heavy chain to present antigenic peptides is significantly impaired. Thus, it would be remarkable if the low levels of free class I heavy chain expressed by MHC-deficient mice can alone account for the rapid rejection of their skin grafts. Another possibility is that effector cells in the recipient might be primed by indirect recognition of donor antigens on recipient antigen-presenting cells. However, the effector cells generated by such a mechanism would not be expected to recognize donor antigens on cells in the graft as these cells express such low levels of MHC antigens and probably cannot present immunogenic peptides. Thus, although the mechanism by which these grafts are rejected remains to be elucidated, it is possible that rejection of MHC-deficient grafts may involve a noncytolytic T-cell immune response.

Overall, the phenotype of MHC-deficient mice resembles the combination of that seen in β_2m-deficient and class II-deficient mice. In particular, these animals are almost completely devoid of MHC class I and class II molecules and of mature $CD4^+$ and $CD8^+$ T cells. Given these deficiencies, it is not unexpected that spleen cells from MHC-deficient mice exhibit poor cellular immune responses *in vitro*. When analyzed for their ability to mount immune responses *in vivo*, however, MHC-deficient mice were found to be surprisingly immunocompetent. Their B-cell compartment appears to develop normally, and antibody responses to T-independent antigens can be elicited. Furthermore, MHC-deficient mice reject allogeneic skin grafts almost as rapidly as do normal MHC-expressing animals. Thus, our results suggest that some compensatory immune responses may not require MHC antigens, and MHC-deficient mice should therefore serve as useful tools for investigating new pathways of immunoreactivity.

We thank Laurie Jackson-Grusby for help with the ELISA, Susan Shea for technical assistance, and John Douhan, III, for critical reading of the manuscript. This work was supported by National Institutes of Health Grants to H.A. (HL36372 and HL18646) and L.H.G. (AI21569) and by grants from the Mather Foundation and the William F. Milton Fund of Harvard University. M.J.G. is the recipient of an Arthritis Foundation Investigator Award and a Leukemia Society Special Fellowship.

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