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Immunology

Class II-positive hematopoietic cells cannot mediate positive selection of CD4+ T lymphocytes in class II-deficient mice

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ABSTRACT **Generation of immunocompetent α/β T-cell receptor-positive T cells from CD4+CD8+ thymocytes depends upon their interaction with thymic major histocompatibility complex (MHC) molecules. This process of positive selection provides mature T cells that can recognize antigens in the context of self-MHC proteins. Previous studies investigating haplotype restriction in thymic and bone-marrow chimeras concluded that radioresistant thymic cortical epithelium directs the positive selection of thymocytes. There is controversy, however, as to whether intra- or extrathymic radiosensitive bone marrow-derived macrophage and dendritic cells also can mediate positive selection. To determine whether CD4+ T cells can be positively selected by hematopoietic cells, we generated chimeric animals expressing MHC class II molecules on either bone marrow-derived or thymic stromal cells by using a recently produced strain of MHC class II-deficient mice. CD4+ T cells developed only when class II MHC molecules were expressed on radioresistant thymic cells. In contrast to what recently has been observed for the selection of CD8+ T lymphocytes, MHC class II-positive bone marrow-derived cells were unable to mediate the selection of CD4+ T cells when the thymic epithelium lacked MHC class II expression. These data suggest that CD4+ and CD8+ T cells may be generated by overlapping, but not identical, mechanisms.**

A pivotal role for radioresistant thymic elements in T-cell selection first was demonstrated by Zinkernagel *et al.* (1, 2) and Bevan and Fink (3, 4) using $F_1 \rightarrow P$ and $P \rightarrow F_1$ bone marrow and thymic chimeras. In these systems, virus-specific cytotoxic lymphocytes were generated only against infected targets sharing the same major histocompatibility complex (MHC) determinants as the thymic stroma but not radiosensitive hematopoietic cells. Subsequent studies, however, have suggested that restriction of cytotoxic T lymphocytes (5, 6) and, to a lesser degree, of T helper cells (7, 8) to host MHC is preferential but not absolute. This raised the possibility that T cells also could be educated by bone marrow-derived cells resident in the thymus, a possibility lent support by the studies of Longo *et al.* (9–11). Such education need not be intrathymic. The existence of an extrathymic pathway of selection for cytotoxic lymphocytes has been demonstrated in experiments analyzing positive selection in nude mice (12–15) and in MHC-disparate bone marrow chimeras (16, 17). A recent report using athymic → severe combined immunodeficiency mouse bone marrow chimeras also was consistent with the presence of extrathymic selection of MHC class I-restricted α/β T cells by cells of hematopoietic lineage (18). Moreover, in chimeric mice generated from a β_2-microglobulin-deficient strain, functional CD8+ T cells developed, albeit inefficiently, when hematopoietic cells, but not thymic epithelium, expressed MHC class I molecules (19).

The studies cited above, based largely upon patterns of MHC restriction, suggest a greater stringency in the requirements for selection of CD4+ versus CD8+ T cells. With a different experimental approach, positive selection of Vβ6+ (I-E-restricted) cells was examined in I-E transgenic animals where thymic I-E expression was compartmentalized (20, 21). Positive selection occurred when I-E expression was limited to thymic cortical cells; however, mice expressing I-E exclusively in the medulla generated small, but probably significant, numbers of Vβ6+ cells, as compared to the I-E-negative control, suggesting a role for noncortical elements in mediating positive selection. Only a small percentage of CD4+ T cells could be examined in this system, however, because Vβ6+ cells constitute a minority of the T-cell repertoire and the majority of the CD4 response is I-A and not I-E restricted.

To study the positive selection of CD4+ cells we took advantage of a recently derived strain of MHC class II-deficient mice (22) to produce bone marrow and thymic chimeras with tissue-selective expression of MHC class II. In both models, development of CD4-single-positive T cells occurred only when the thymic epithelium expressed MHC class II molecules—class II-expressing hematopoietic cells could not mediate and were not necessary for the positive selection of CD4+ T cells.

MATERIALS AND METHODS

Mice. The production and characterization of MHC class II-deficient mice have been described (22). The animals used in these experiments were 6–10-week-old third- and fourth-generation intercrosses between 129/Sv × C57BL/6 founders. BALB/c and C57BL/6 nu/nu mice were purchased from Taconic Laboratories; C57BL/10 nu/nu mice were from A. Singer (National Institutes of Health, Bethesda, MD). All animals were housed initially in microisolator cages (Allentown Caging, Allentown, NJ) in a specific pathogen-free facility and subsequently in autoclaved microisolator cages and provided with autoclaved food and water. When housed under either of these conditions, MHC class II-deficient mice appear healthy.

Production of Bone Marrow Chimeras. The protocol used has been described (23). In brief, recipient animals were dosed with 950 rads (1 rad = 0.01 Gy) of γ radiation delivered by a cesium source. Donor animals were anesthetized with ether and exsanguinated; their bone marrow then was harvested. Depletion of T cells from the bone marrow was achieved by incubating cells at 4°C with anti-Thy 1.2 (13-4-9) and anti-CD4 (GK1.5) monoclonal antibodies, followed by lysis at 37°C in a 1:3 dilution of guinea pig complement (Cedarlane Laboratories, Hornby, ON). Two to five hours

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Abbreviations: MHC, major histocompatibility complex; TCR, T-cell receptor.

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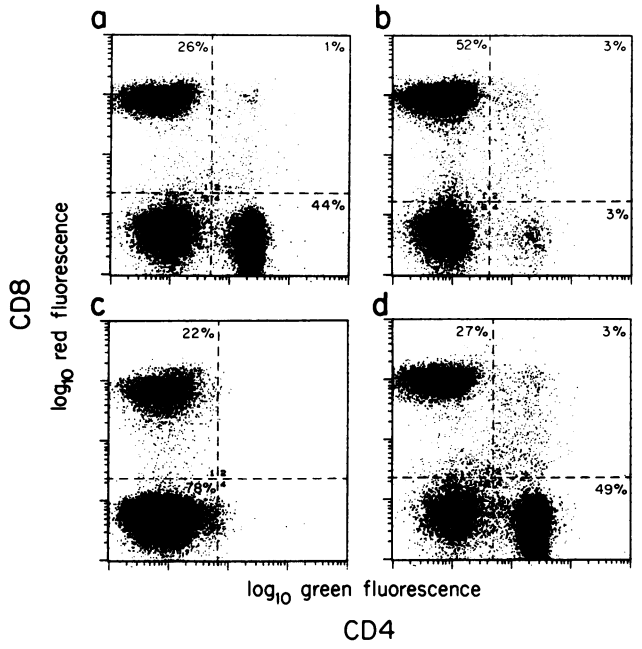

FIG. 1. Cytofluorometric profiles of CD4 and CD8 expression in chimeric animals indicate that CD4+ T cells do not develop unless the thymic stroma expresses MHC class II molecules. Cells were stained with CD4-fluorescein isothiocyanate and CD8-phycoerythrin as described; 20,000 events were recorded. (*a*) Lymph node cells from an irradiated control animal given class II-deficient bone marrow. (*b*) Lymph node cells from an irradiated class II-deficient animal given control bone marrow. (*c*) Peripheral blood leukocytes from a nu/nu recipient of a class II-deficient thymus. (*d*) Lymph node cells from a normal control.

after irradiation, recipients were reconstituted with the i.v. injection of 5–20 million T-cell-depleted bone marrow cells.

Production of Thymic Chimeras. Thymuses from neonatal (<24 hr old) animals were removed, irradiated (as above) with 2000 rads, and implanted under the left kidney capsule in anesthetized nu/nu recipients (8).

Flow Cytometric Analysis. Cell preparation and staining for cytofluorometric analysis were as described (22). Approximately 1×10^6 cells were incubated at 4°C, in Hanks' balanced salt solution/0.2% bovine serum albumin/0.1% sodium azide, with various fluorochrome-conjugated antibodies, washed twice, fixed with 2% paraformaldehyde, and analyzed by using FACStar Plus (Becton Dickinson) hardware and software. Fluorescein isothiocyanate-conjugated anti-CD4 and anti-α/β T-cell receptor (TCR), and phycoerythrin-conjugated anti-CD8α were purchased from Pharmingen.

Immunohistochemistry. Five-micrometer cryostat sections, cut from snap-frozen thymi, were air-dried, fixed in acetone, and incubated with culture supernatant from the hybridoma B21-2 (TIB 229, American Type Culture Collection), specific for I-$A^{b,d}$. After washing, biotinylated mouse F(ab')$_2$ anti-rat IgG (Jackson ImmunoResearch) was added, followed by the addition of streptavidin-alkaline phosphatase (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Histomark red (Kirkegaard and Perry) was used, per manufacturer's instructions, as the developing reagent (24).

RESULTS AND DISCUSSION

Two types of chimeric animals were produced. (*i*) T-cell-depleted bone marrow from either class II-deficient or control littermates was used to repopulate irradiated control or class II-negative recipients. (*ii*) nu/nu recipient animals received an irradiated thymus transplant from either a class II-deficient donor or from a control littermate. After >12 weeks, cells from the peripheral blood, spleen, and lymph node from both types of chimeric animals were analyzed for CD4 and CD8 expression.

In bone marrow chimeras where the recipient was class II+ (class II+ thymus) and the donor of the bone marrow was class II−, peripheral CD4+ and CD8+ T cells were present at normal levels (Fig. 1 *a* and *d*). In contrast, the irradiated class II-deficient animals reconstituted with class II-expressing bone marrow cells did not generate a significant CD4+ T-cell population (1–5%) but did generate normal or increased numbers of CD8+ cells (Fig. 1*b*). The latter cell population served as a control for the integrity of the thymic stroma. Consistent with these results, the nu/nu animals transplanted with class II-deficient thymi (Fig. 1*c*) also did not generate CD4+ cells above what was observed in unmanipulated class II-deficient animals (refs. 22 and 25; data not shown), whereas the nude recipients of a control thymus did (data not shown). Further characterization revealed that the resulting T-cell populations of the chimeras expressed $\alpha\beta$ TCR at normal levels (data not shown). Thus, by surface antigen criteria, mature CD4+ T cells developed in an animal in which thymic radioresistant elements, and not lymphohematopoietic elements, expressed MHC class II molecules. In a chimera lacking class II expression on radioresistant thymic cells, but expressing class II on radiosensitive bone marrow-derived cells, there was no increase in CD4+ T cells compared to the class II-deficient mice.

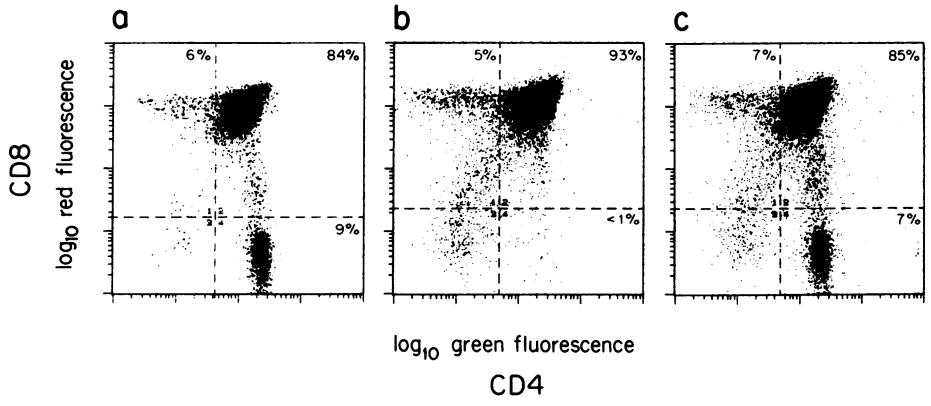

FIG. 2. In the absence of class II MHC expression by thymic epithelium, CD4+CD8+ thymocytes are unable to differentiate into CD4+CD8− cells. Twenty thousand thymocytes stained with CD4-fluorescein isothiocyanate and CD8-phycoerythrin were analyzed. Cells were from irradiated control reconstituted with class II-deficient bone marrow (*a*), irradiated class II-deficient animal reconstituted with control bone marrow (*b*), and normal control (*c*).

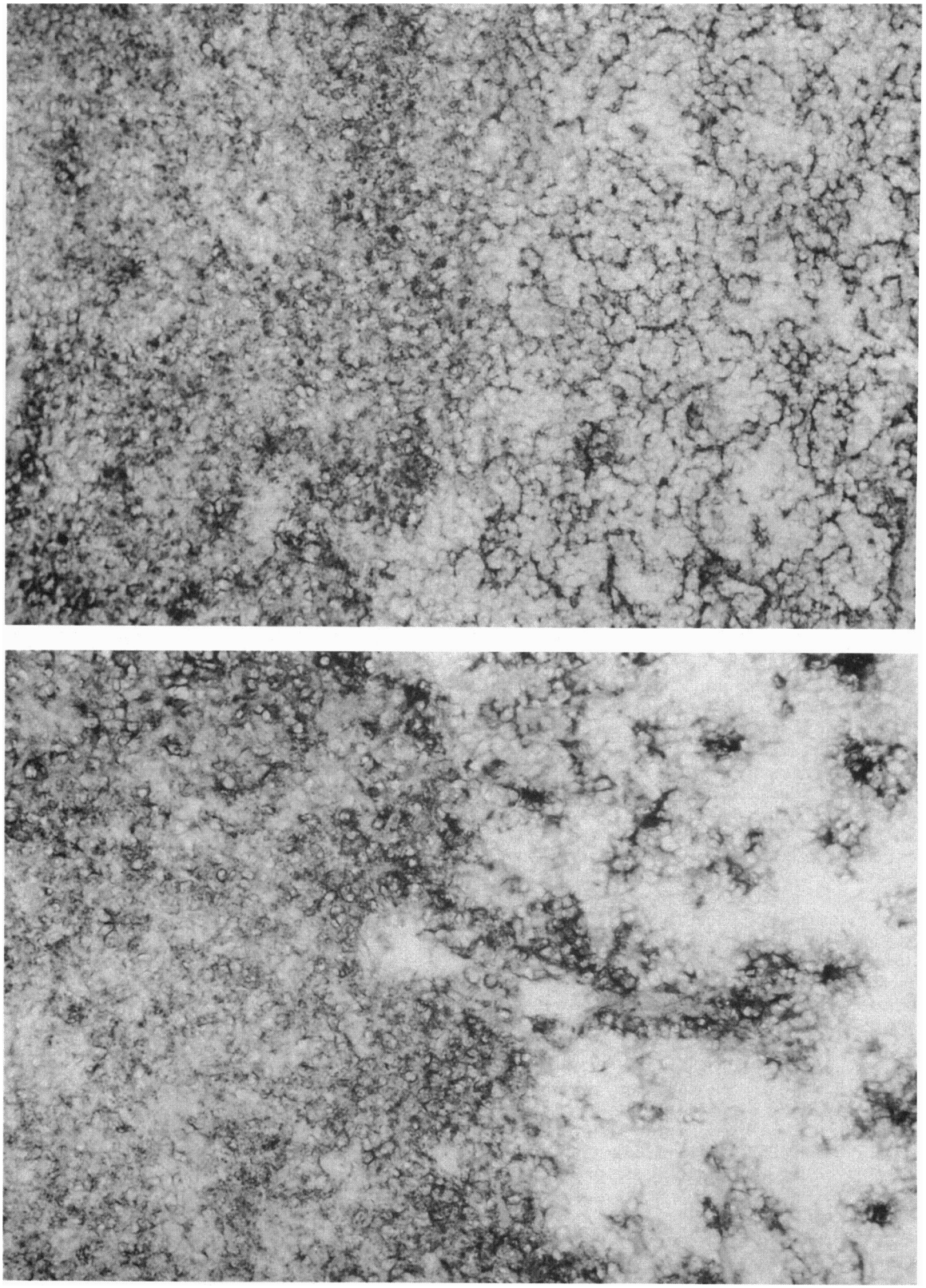

FIG. 3. Hematopoietic cells repopulate the thymus of an irradiated bone-marrow transplant recipient. Frozen thymic sections from a normal control (*Upper*) and a class II-deficient animal 6 mo after irradiation and reconstitution with BALB/c bone marrow (*Lower*) were incubated with B21-2 hybridoma supernatant (αI-$A^{b,d}$) and developed as described in text. Positively staining cells express MHC class II molecules. In each photomicrograph the cortex is at right, and the medulla is at left. (×315.)

The pattern of CD4 and CD8 expression on thymocytes from bone marrow chimeras was compared to normal control animals. As anticipated, considering the above findings, animals expressing MHC class II molecules on thymic radioresistant but not hematopoietic elements manifested a pattern of thymic development indistinguishable from control (Fig. 2 *a* and *c*). Maturation of $CD4^+CD8^+$ cells to the single-positive stage seemed unaffected by the lack of class

II expression on bone marrow-derived cells. Additionally, TCR α/β levels mirrored control (data not shown). Determination of CD4/CD8 expression on thymocytes from mice in which bone marrow-derived but not radioresistant cells expressed class II MHC molecules revealed that, as in class II-deficient mice (refs. 22 and 25; data not shown), there was a normal $CD4^-CD8^+$ population, few or no $CD4^+CD8^-$ cells (1% or less), and a slightly increased $CD4^+CD8^+$ population (Fig. 2*b*). Thus, MHC class II-positive hematopoietic-derived cells were incapable of mediating the progression from the double-positive to the CD4-single-positive stage.

Because a possible explanation for the failure of hematopoietic cells to mediate positive selection was that they did not repopulate the thymus, an immunohistochemical determination of MHC class II expression was performed. The thymus from a normal control animal (Fig. 3 *Upper*) had a fine reticular pattern of class II expression in the cortex, characteristic of the epithelial matrix. In the medulla, staining was more diffuse, with interspersed regions of intensely staining class II-positive, macrophage-like cells. The staining of a thymus from an irradiated control animal reconstituted with class II-deficient bone marrow (data not shown) was qualitatively similar, with respect to MHC class II expression, to the normal control. Therefore, it is possible that class II-positive hematopoietic-derived cells survived irradiation. There was virtually no background staining in the class II-deficient animal (ref. 22 and data not shown). The thymus from an irradiated class II-deficient animal repopulated with class II-positive bone marrow, however, had a distinctive pattern of class II expression (Fig. 3 *Lower*). There was no cortical reticular staining, consistent with the thymic stroma being class II deficient. There were areas of marked MHC class II expression primarily in the medulla and also scattered throughout the cortex, demonstrating that class II-positive hematopoietic donor-derived cells, indeed, were able to populate the recipient thymus.

To ensure that chimerism truly was achieved, chimeric animals were generated using MHC class I-disparate ($H\text{-}2^d$ vs. $H\text{-}2^b$) donors and recipients, and the haplotype of the regenerated T cells was determined to confirm donor origin. To allow for the maximal establishment of the chimeric state, animals were examined up to 6 mo after their creation. From 50% to 90% of lymphoid cells were of donor MHC origin. Other means used to prove the chimeric nature of the animals included cytofluorometric evaluation of peripheral lymphoid cell class II surface expression and Southern blot analysis of endogenous versus mutated class II alleles.

These experiments demonstrate that class II-expressing bone marrow-derived cells, in a thymic environment where radioresistant elements are class II deficient, cannot induce the maturation of $CD4^+$ T cells. These data support the pivotal role ascribed to thymic epithelium, and perhaps other radioresistant cell types, in T-cell selection (1–4, 26–28). In addition, our findings extend those of a previous investigation of $CD4^+$ T-cell development in mice expressing both TCR and I-E transgenes (29). Although that report demonstrated that TCR transgenic $CD4^+$ lymphocytes developed only when their MHC ligand was expressed on thymic cortical epithelium, the finding is intrinsically limited to one particular TCR and might not be applicable to T-cell development, in general. In the present study, the dependence of $CD4^+$ T-cell maturation on class II MHC expression by thymic cortical epithelium held true for an entire range of TCRs.

The origin, restriction specificity, and function of the small number of $CD4^+$ T cells observed in the irradiated class II-deficient animals reconstituted with normal bone marrow have yet to be determined. A similarly sparse population of $CD4^+$ T cells is present in both unmanipulated class II deficient (22, 25) and MHC-deficient mice [made by crossing class II-deficient mice with β_2-microglobulin-deficient mice (M.J.G., H.A., L.H.G., unpublished observations)]. Thus, it is unlikely that the few $CD4^+$ T cells in the chimeras are positively selected either by MHC class II-expressing hematopoietic cells or by class I MHC molecules. Elucidation of the means by which these $CD4^+$ T cells mature may reveal another pathway of T-cell development.

Recently, Bix and Raulet (19) described the generation of functional $CD8^+$ T cells in a chimera expressing MHC class I molecules on hematopoietic but not on thymic epithelial cells. Although the authors emphasized that selection of $CD8^+$ T cells in such chimeras was inefficient, their findings compared to ours make it likely that $CD4^+$ and $CD8^+$ α/β TCR T cells can differentiate by subtly different pathways. This hypothesis is consistent with observations that in thymic and bone marrow chimeras, T-helper functions were stringently restricted to the MHC of the thymic stroma, whereas CTL could be restricted to the MHC expressed by hematopoietic elements (15, 16, 30). An explanation for these differential requirements for selection is that maturation of $CD4^+$ T cells is more dependent upon interactions with MHC/peptide and/or costimulatory molecules specifically expressed by thymic cortical epithelium (31, 32).

From a clinical perspective, the findings in this report reinforce the necessity for HLA matching in allogeneic bone-marrow transplantation, not only to prevent graft-versus-host disease but also to enable the recipient to achieve immunocompetence. Otherwise, $CD4^+$ T cells restricted to the HLA type of the recipient thymus will be unable to interact with the antigen-presenting cells of donor haplotype.

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