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Immunology

Tolerance induction by thymic medullary epithelium

(deletion/anergy/H-2 antigens/T lymphocytes)

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ABSTRACT To study the role of thymic medullary epithelium in tolerance induction, the third and fourth branchial clefts of embryos from Eμ-K^b transgenic mice, which express the major histocompatibility complex class I antigen H-2K^b exclusively on medullary thymic epithelium, were grafted to athymic nude mice. The grafts differentiated into tissue that morphologically resembled normal thymus. These grafts expressed the H-2K^b antigen appropriately and gave rise to a functional T cell repertoire. *In vivo* tolerance to H-2K^b disparate skin grafts was invariably found in mice expressing H-2K^b in the medulla or in both medulla and cortex of C57BL/6 branchial cleft-grafted controls. In marked contrast, *in vitro* cytotoxicity assays demonstrated reactivity toward H-2K^b in the presence of interleukin 2, and limiting-dilution analyses showed similar frequencies of cytolytic T cell precursors reactive to H-2K^b and to third-party stimulators. Medullary epithelium can, therefore, induce split tolerance, in which *in vivo* tolerance is accompanied by strong *in vitro* responses in the presence of interleukin 2.

T lymphocyte maturation in the thymus is governed by cells of at least two different lineages: thymic epithelial cells and bone marrow-derived macrophages and dendritic cells. Differentiating T cells are subjected to rigorous positive and negative selection tests. The former ensures that T cells with some degree of binding avidity for polymorphic regions of major histocompatibility complex (MHC) molecules displayed on thymic cortical epithelial cells are selected for survival. This selection is the basis for the phenomenon of MHC restriction, but it also allows the differentiation of cells with receptors having high affinity for self-peptides presented in the context of MHC molecules. Negative selection must, therefore, operate, at least on such high-affinity anti-self T cells, to prevent self-reactivity and induce self-tolerance (1). Anti-self censorship is clearly a function of the thymic dendritic cells or macrophages that are rich in class I and II molecules and situated predominantly at the cortico–medullary junction (2).

Studies on the role of thymic epithelium in toleragenesis have given conflicting results. In some models, the epithelium has induced complete or near-complete tolerance toward MHC class II antigens (3–5) and minor histocompatibility antigens *in vivo* and *in vitro* (6). For minor lymphocyte-stimulating (Mls) antigens, the results have been contradictory in different models: Ramsdell *et al.* (7) and Roberts *et al.* (8), using irradiated thymus grafts, have reported tolerance induction by a nondeletional mechanism, whereas Webb and Sprent (3) did not observe any tolerance with thymus tissue depleted of bone marrow-derived cells by treatment with 2-deoxyguanosine. Responses toward MHC class I antigens have varied depending on the experimental system. Grafting class I disparate 2-deoxyguanosine-treated, day-14, embryonic thymus tissue did not diminish the specific anti-class I cytolytic T lymphocyte (CTL) response in the presence of exogenous interleukin 2 (IL-2) (3, 6, 9). Recently a more refined approach was used to study the role of thymic epithelium in toleragenesis. Third and fourth branchial clefts from day-10 embryos (E10), taken before colonization of the thymic anlage by bone marrow-derived cells, were grafted to athymic nude mice. Interestingly, despite significant *in vitro* CTL responses, these mice were specifically tolerant of donor-type skin grafts (10).

Having established the toleragenic potential of MHC antigens expressed on thymic epithelial cells, it is necessary to determine which epithelial component, cortical or medullary, plays a role in tolerance induction. We, therefore, used a transgenic mouse model in which a subset of medullary thymic epithelial cells expressed the MHC class I antigen H-2K^b (K^b). In these "Eμ-K^b" transgenic mice the K^b antigen was also expressed on subsets of T and B lymphocytes (11). By grafting E10 branchial clefts from Eμ-K^b embryos onto athymic nude mice, we could determine the toleragenic potential of the K^b molecules expressed only on medullary epithelial cells. These grafts induced a state of split tolerance similar to that seen with expression of K^b in both cortex and medulla: specific *in vivo* tolerance to K^b-bearing skin grafts was accompanied by strong *in vitro* anti-K^b CTL responses in the presence of IL-2.

MATERIALS AND METHODS

Mice. Inbred mouse strains were obtained from the specific pathogen-free breeding facilities of the Walter and Eliza Hall Institute for Medical Research. The development and characterization of the Eμ-K^b transgenic mice have been published (11). Homozygous mice on a B10.BR background were obtained by appropriate breeding.

Medium. Culture medium was modified RPMI 1640 medium, pH 7.2, buffered with Hepes and supplemented with sodium pyruvate (0.11 g/liter), penicillin (50,000 units/ml), streptomycin (25 mg/ml), 2-mercaptoethanol (10^{-4} M), and 10% fetal calf serum. CTL assays were done in the absence of Hepes.

Branchial Cleft Grafts and Skin Grafts. A modification of the technique originally described by Khazaal *et al.* (12) was used. The third and fourth branchial clefts were excised from day-10 embryos (E10), and three to four branchial clefts were grafted on a small piece of nitrocellulose membrane under the kidney capsule of young adult CBA nude recipients. Twelve to 16 wk after branchial cleft grafting, these mice were simultaneously grafted with tail skin from C57BL/6, BALB/c, bml, and CBA mice by using the method of Billingham and Medawar (13).

Immunohistology. Immunoperoxidase histology with the anti-H-2K^b monoclonal antibody (mAb) 28.8.6s (14) and the anti-H-2K^k mAb A112F (15), was done on sequential cryostat

Abbreviations: K^b, H-2K^b; CTL, cytolytic T lymphocytes; CTLp, CTL precursors; IL-2, interleukin 2; mrIL-2, murine recombinant IL-2; MHC, major histocompatibility complex; mAb, monoclonal antibody; FACS, fluorescence-activated cell sorter.

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sections as described (11). The sections were not counterstained.

Con A Stimulation, CTL Assays, and Limiting-Dilution Analysis. Responder splenocytes (2×10^5) from branchial cleft grafted or control euthymic CBA or CBA × bml mice were cultured for 48 hr in a vol of 200 μl containing Con A at 5 μg/ml. For the final 8 hr 1 μCi of [^{3}H]thymidine (1 Ci = 37 GBq) was added. Con A stimulation was calculated relative to the euthymic control included in every assay. For CTL assays 5×10^6 responder splenocytes from branchial cleft-grafted or control euthymic mice were stimulated with 5×10^6 irradiated (1500 rad; 1 rad = 0.01 Gy) splenocytes in the presence of 20 international units of murine recombinant IL-2 (mrIL-2, provided by M. Howard, DNAX) per ml. After 5 days, cytotoxicity was determined on ^{51}Cr-labeled Con A blasts previously stimulated with Con A at 2.5 μg/ml for 2 days. In every experiment cytotoxicity was tested on the specific target as well as on a syngeneic CBA target to check for nonspecific killing. For limiting-dilution analysis, limiting numbers of responder lymph node cells were stimulated with 5×10^5 irradiated (1500 rad) stimulator splenocytes in the presence of mrIL-2 at 20 international units per ml. For each cell concentration, cells were plated in a vol of 200 μl into 32 wells in round-bottom microtiter trays. Seven days later, the wells were split, and cytotoxicity was determined on either specific or control CBA ^{51}Cr-labeled Con A blasts as described above. Clones were scored positive when specific lysis was >8% and there was no significant cross-reactivity on the syngeneic target. CTL precursor (CTLp) frequency was determined by using the zero order term of the Poisson probability distribution. To correct for differences in reconstitution, all frequencies were expressed relative to the number of input CD8$^+$ cells as determined by flow cytometry.

Flow Cytometry and Peripheral Blood Reconstitution Analysis. Analysis by the fluorescence-activated cell sorter (FACS) was done on a FACScan (Becton Dickinson) as described (11) with fluorescein isothiocyanate-labeled mAb 53.1 (anti-CD8; ref. 16) and phycoerythrin-labeled GK1.5 (anti-CD4; ref. 17). To determine the kinetics of reconstitution after branchial cleft grafting, 50 mice were bled from the retroorbital plexus at different time intervals, and the number of nucleated cells was determined on a Coulter Counter. Absolute numbers of CD4$^+$ and CD8$^+$ cells per ml of peripheral blood were calculated from the FACS profiles of the respective samples.

RESULTS

Three to four branchial clefts, derived from various donors, were grafted to athymic nude mice. Of 33 grafted mice, 20 (61%) survived with successful grafts, as judged by morphological and functional criteria. To determine the kinetics of T cell reconstitution in these mice the absolute numbers of CD4$^+$ and CD8$^+$ cells in peripheral blood were determined by counting nucleated cells on a Coulter counter and then analyzing the percentages of CD4$^+$ and CD8$^+$ cells by flow cytometry. T cells were detectable at 4–6 wk after grafting, and their numbers plateaued at 16 wk (Fig. 1). Compared to euthymic CBA controls, absolute numbers of peripheral blood T cells of branchial cleft-grafted nudes reached 12–73% (mean 34%) of CD4$^+$ and 12–49% (mean 25%) of CD8$^+$ cells. There were no significant differences in the kinetics of T cell reconstitution between allogeneic and syngeneic branchial cleft-grafted nudes (data not shown).

Histological analysis of mice sacrificed 8–25 wk after branchial cleft grafting revealed well-developed thymic tissue with clearly demarcated cortex and medulla, as well as cartilage and immature bone originating from the branchial cleft mesenchyme (Fig. 2). Mice with graft failures had tissues consisting exclusively of mesenchymal structures

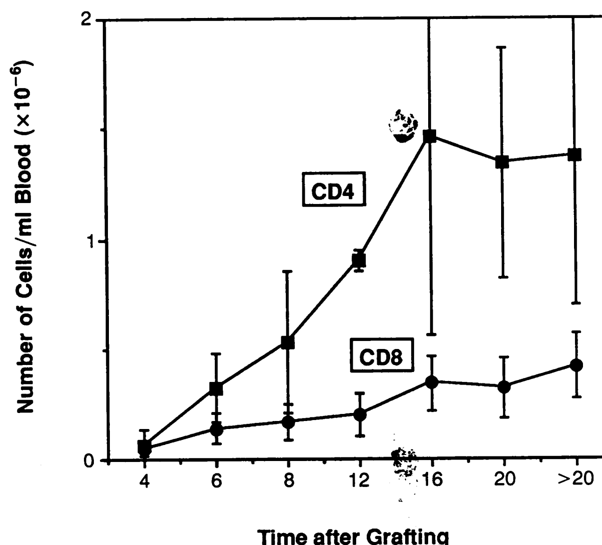

FIG. 1. T cell reconstitution in branchial cleft-grafted CBA nude mice. Peripheral blood leukocytes from 50 mice were taken at different time points after grafting, counted on a Coulter Counter, and analyzed for CD4 and CD8 expression by flow cytometry. The absolute numbers in euthymic CBA mice (n = 5) were 3.84 ± 0.83 × 10^6 CD4$^+$ cells per ml and 1.47 ± 0.46 × 10^6 CD8$^+$ cells per ml, and in CBA/nunu over 16 wk (n = 5) the numbers were 0.063 ± 0.028 × 10^6 CD4$^+$ cells per ml and 0.109 ± 0.051 × 10^6 CD8$^+$ cells per ml. ■, CD4$^+$ cells; ●, CD8$^+$ cells. Vertical bars represent SDs.

(cartilage, immature bone) and either died or showed no T cell reconstitution by FACS analysis (data not shown).

To study transgene expression in thymus tissue derived from branchial clefts grafted to young adult CBA nude mice, donor mice of the following strains were used: Eμ-K^b transgenic mice on the B10.BR background, (C57BL/6 × B10.BR)F$_1$ mice or B10.BR mice. Expression of the transgene relative to endogenous H-2K^k was studied by immunohistochemistry. Tissues, obtained 8 wk after engraftment, were stained for either H-2K^b or H-2K^k. As described (11) for Eμ-K^b transgenic mice, K^b expression in Eμ-K^b branchial cleft-grafted mice was found exclusively on a subpopulation of medullary epithelial cells. In (C57BL/6 × B10.BR)F$_1$ control grafts, on the other hand, K^b was expressed on medullary as well as cortical epithelium to a similar extent as the endogenous K^k antigen that showed equally strong immunoreactivity on mature thymocytes in the medulla (Fig. 2).

To determine the toleragenicity of K^b expressed on various thymus cell types, branchial clefts derived from (EμK^b × bml)F$_1$, (B10.BR × bml)F$_1$, and (B10.BR × C57BL/6)F$_1$ mice were grafted to CBA nude mice. These mice were challenged 12–16 wk later with tail skin from C57BL/6 mice because C57BL/6 and bml differ only at the H-2K locus. They were also challenged with skin from BALB/c, bml, and CBA donors. Fig. 3 shows that all recipients were immunocompetent because they rejected third-party BALB/c grafts within 11–21 days. Where the histocompatibility antigen was expressed on cortical as well as on medullary epithelium, grafts were accepted indefinitely—i.e., K^b but not K^{bml} skin grafts were accepted by (C57BL/6 × B10.BR)F$_1$ branchial cleft-grafted mice, and K^{bml} but not K^b skin grafts were accepted by (bml × B10.BR)F$_1$-grafted mice. More importantly, Eμ-K^b branchial cleft-grafted mice were tolerant to K^b skin, showing the ability of medullary thymic epithelium to induce tolerance *in vivo*.

Further studies analyzed the *in vitro* reactivity of T cells obtained from branchial cleft-grafted mice. Responsiveness of spleen cells to Con A ranged from 32–158% of euthymic controls (Table 1). When spleen cells from branchial cleft-

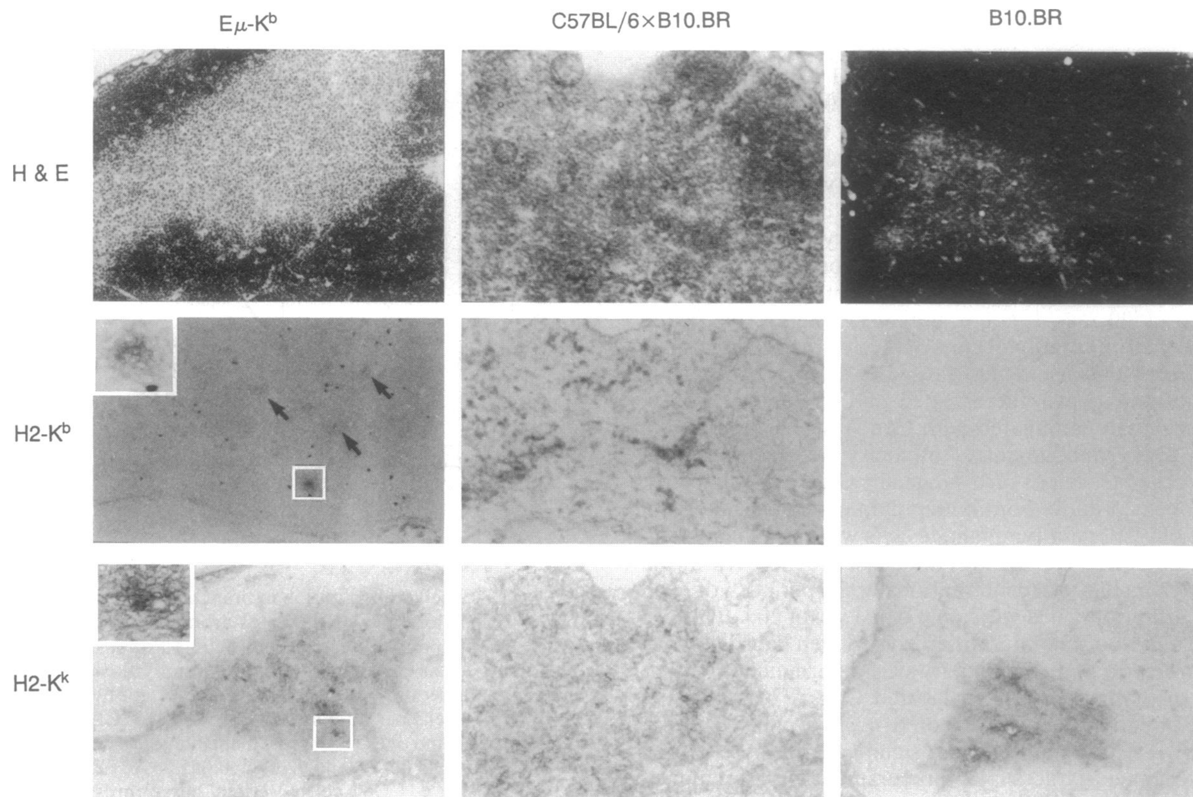

FIG. 2. Expression of K^b transgene and endogenous K^k antigen on medullary thymic epithelium in Eμ-K^b branchial cleft-grafted nudes compared with (C57BL/6 × B10.BR)F_1 mice or B10.BR mice controls. In sequential, not counterstained, sections of Eμ-K^b-grafted mice a subpopulation of medullary epithelial cells (arrows, *Inset*) stained for K^b, whereas in recipients of (C57BL/6 × B10.BR)F_1 grafts K^b expression was found on medullary and cortical epithelium. Endogenous K^k was detectable on thymic epithelium and mature medullary thymocytes in all grafts (hematoxylin/eosin and immunoperoxidase, ×250; *Insets* ×400).

grafted mice were stimulated *in vitro* with irradiated spleen cells from C57BL/6 or BALB/c donors in the presence of mrIL-2 at 20 international units per ml, CTL responses were, in general, weaker than those of euthymic controls. However, in individual mice the CTL activity was similar for K^b and for third-party antigens (Fig. 4). This result indicates that the observed skin graft tolerance toward K^b in (Eμ-k^b × bml)F_1 and (C57BL/6 × B10.BR)F_1 branchial cleft-grafted mice is accompanied by relatively strong CTL responses.

To determine whether the skin graft tolerance of K^b expressed on thymic epithelial cells was attributable to the deletion of a major population of K^b-reactive cells, limiting-dilution analyses were done. Lymph node T cells from branchial cleft-grafted or euthymic CBA or (CBA × bml)F_1 mice were stimulated with C57BL/6 or BALB/c stimulator cells under limiting-dilution conditions. On the day of assay, wells were split, and cytotoxicity was determined on specific targets as well as on syngeneic ^{51}Cr-labeled Con A blasts.

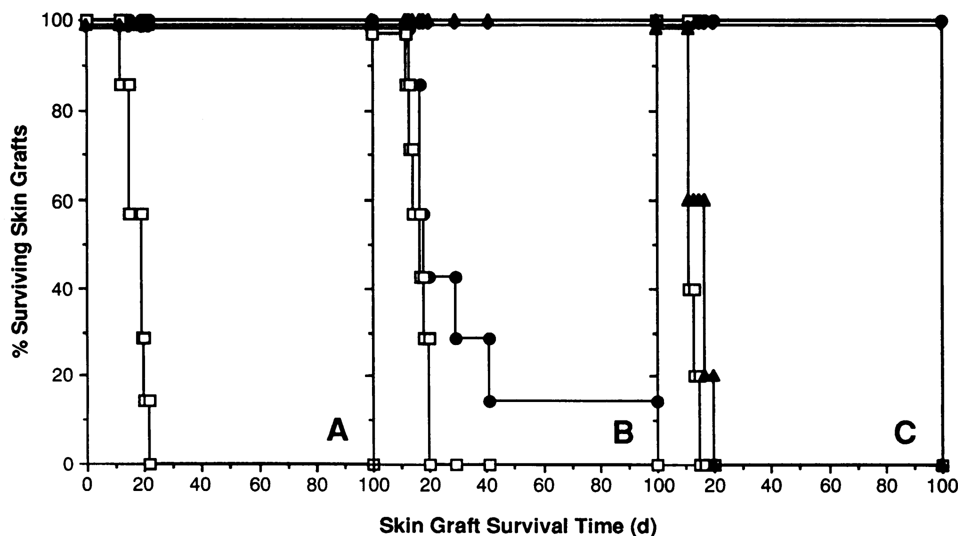

FIG. 3. Skin graft survival in Eμ-K^b branchial cleft-grafted nude mice. CBA nude mice grafted with (Eμ-K^b × bml)F_1 (*A; n* = 8), (bml × B10.BR)F_1 (*B; n* = 7), or (C57BL/6 × B10.BR)F_1 (*C; n* = 5) branchial clefts were grafted with C57BL/6, BALB/c, bml, and CBA tail skin. ●, C57BL/6 skin graft; □, BALB/c skin graft; ▲, bml skin graft; and ◆, CBA skin graft.

Table 1. Limiting-dilution analysis of CTL precursors from lymph nodes of branchial cleft-grafted mice

Graft donor	CD4 cells, %	CD8 cells, %	Relative Con A stimulation	CTLp frequency	
				C57BL/6 stimulators	BALB/c stimulators
CBA*	58	18	1	1/714	1/370
CBA × bml*	59	20	1	1/455	1/217
Eμ-Kb × bml					
TEG 32	26	5	0.76	1/3751	1/6250
TEG 38	40	6	1.13	1/2222	1/870
C57BL/6 × B10.BR					
TEG 151	51	7	1.58	1/556	1/769
TEG 153	47	6	1.14	1/171	1/333
bml × B10.BR					
TEG 80	24	8.5	0.33	1/1518	1/1420

CTLp frequencies, as determined against C57BL/6 and BALB/c Con A blasts, were corrected for the number of input $CD8^+$ cells. Relative Con A stimulation and FACS analysis were done as described. TEG, thymic epithelial graft; number following TEG refers to individual mice.
*Euthymic controls.

Killing in this system was highly specific with very few clones showing reactivity toward syngeneic targets or crossreacting on both specific and syngeneic targets (data not shown). Table 1 shows that frequencies varied considerably between mice. In individual mice, however, relative CTLp frequencies against specific and third-party targets were comparable. In general, the CTLp frequencies against third-party stimulators were very similar to the K^b frequencies in Eμ-K^b × bml, C57BL/6 × B10.BR, and bml × B10.BR branchial cleft-grafted mice (Table 1).

DISCUSSION

Results of this study indicate that medullary thymic epithelium can tolerize developing T cells toward allogeneic MHC class I antigens *in vivo*. These cells, however, retain the potential to mount strong *in vitro* responses toward targets expressing the same MHC molecules. This split-tolerance phenomenon has previously been observed with thymic epithelial grafts in which allogeneic class I molecules were expressed on cortical as well as on medullary epithelium. Our failure to show *in vitro* tolerance is, however, at variance with results obtained by Good *et al.* (18), who observed low specific CTLp frequencies after coculture of E10 branchial clefts with allogeneic fetal liver cells. The discrepancy between these results may be related to differences in the environment in which the branchial clefts developed.

It is unclear on which potentially reactive T cells tolerance is actually imposed by thymic epithelium. One possibility is that all K^b-specific T cells are inactivated homogeneously within the thymus and need special activation conditions (such as *in vitro* stimulation in the presence of IL-2) for reactivation. Another scenario, as proposed by Webb and Sprent (3), is that thymic epithelium tolerizes only high-affinity T cells. Emerging low-affinity, cytokine-dependent T cells would be unable to reject skin grafts while retaining the ability to mount strong *in vitro* CTL responses in the presence of IL-2.

Furthermore, it is not known whether thymic epithelial cells induce tolerance by clonally deleting self-reactive T cells, as do thymic antigen-presenting cells, or provide a qualitatively different signal inducing a state of T cell anergy,

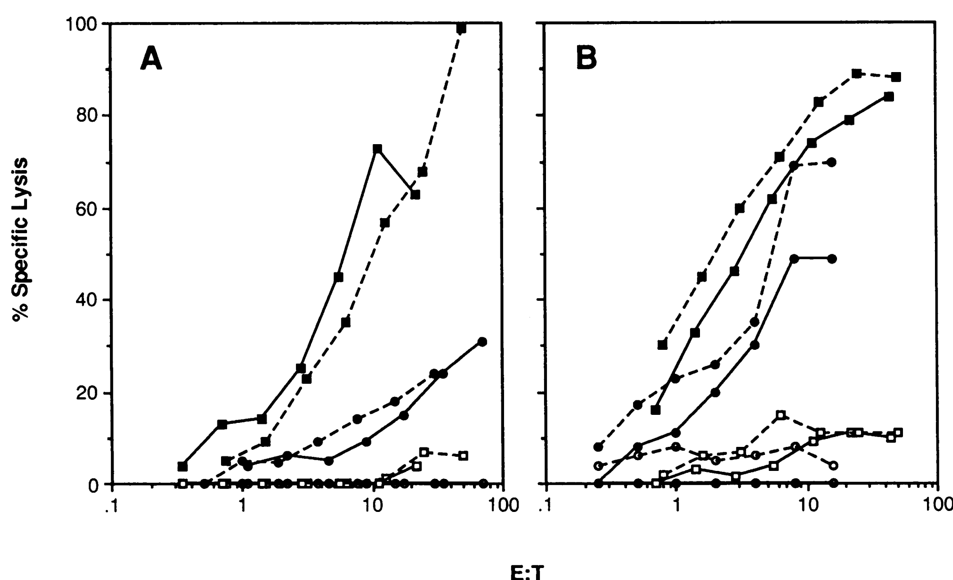

FIG. 4. CTL activity of Eμ-K^b (*A*) or B10.BR (*B*) branchial cleft-grafted nude mice compared with euthymic CBA mice. Responder spleen cells from either branchial cleft-grafted (circles) or euthymic (squares) mice were stimulated with irradiated (1500 rad) C57BL/6 (—) or BALB/c (---) stimulators with mrIL-2 at 20 international units per ml. Responding cells were tested on ^{51}Cr-labeled Con A blasts of C57BL/6 or BALB/c origin as specific targets (closed symbols), as well as syngeneic CBA control blasts (open symbols).

as reported by Ramsdell *et al.* and Roberts *et al.* (7, 8). It will, therefore, be very interesting to follow the fate of potentially reactive cells in a model where they constitute most of the T cell pool, as in T cell receptor transgenic mice with specificity for the H-2K^b antigen.

Preliminary results obtained in a transgenic model by Hämmerling *et al.* (19) suggest that K^b expressed on a subset of medullary thymic epithelial cells induces a state of anergy in K^b-specific T cells. In contrast to our results, these T cells showed no *in vitro* reactivity toward K^b targets. This difference might be accounted for by expression of the K^b transgene on separate cell populations within the medulla.

In conclusion, the results presented here confirm and extend previous findings on the toleragenicity of thymic epithelium. These results clearly show the ability of medullary thymic epithelium to induce *in vivo* tolerance toward an MHC class I antigen in spite of a high frequency of reactive T cells *in vitro*.

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