Ligand-stimulated signaling events in immature CD4$^+$CD8$^+$ thymocytes expressing competent T-cell receptor complexes

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ABSTRACT During thymic selection of the developing T-cell repertoire, the fate of individual CD4$^+$CD8$^+$ thymocytes is determined by the specificity of the T-cell antigen receptors (TCRs) they express. Paradoxically, most CD4$^+$CD8$^+$ thymocytes express few TCR molecules, and those they express are essentially incapable of transducing intracellular signals as measured by intracellular calcium mobilization. However, both TCR number and calcium-signaling capability are significantly induced in CD4$^+$CD8$^+$ thymocytes when the cells are released from intrathymic inhibitory signals that are mediated by their CD4 molecules. Here, the response to ligand engagement of TCR on "induced" CD4$^+$CD8$^+$ thymocytes that have been released from CD4-mediated inhibition was examined and was found to result in internalization of surface TCR complexes and rephosphorylation of ζ chains of the TCR complex. In addition, a proportion of induced CD4$^+$CD8$^+$ thymocytes were found to fragment their DNA upon ligand engagement. Thus, this study describes early events in immature CD4$^+$CD8$^+$ thymocytes resulting from TCR-mediated signals.

In the thymus, developing CD4$^+$CD8$^+$ thymocytes encounter Ia$^+$ thymic epithelial cells that engage their CD4 molecules and induce intracellular signals that contribute to the low surface T-cell antigen receptor (TCR) phenotype of most immature CD4$^+$CD8$^+$ thymocytes (1). In CD4$^+$CD8$^+$ thymocytes, CD4-mediated signals induce the retention and degradation of newly synthesized TCR complexes within the endoplasmic reticulum (2) and induce tyrosine phosphorylation of ζ chains of the TCR complex (TCR-ζ chains) (3). CD4$^+$CD8$^+$ thymocytes can be experimentally released from intrathymic CD4-mediated inhibitory signals either by *in vivo* injection of anti-CD4 monoclonal antibodies (mAbs) that physically disrupt intrathymic CD4–ligand interactions (4) or by physically removing CD4$^+$CD8$^+$ thymocytes from the thymus and placing them in short-term single-cell suspension culture at 37°C (1, 5). Release from CD4-mediated inhibition results in increased TCR expression and in decreased phosphorylation of TCR-ζ chains, both of which are correlated with a marked improvement in TCR signaling in CD4$^+$CD8$^+$ thymocytes as measured by intracellular calcium mobilization upon TCR$\alpha\beta$ crosslinking (1). Thus, in marked contrast to "uninduced" CD4$^+$CD8$^+$ thymocytes that have not yet been released from CD4-mediated inhibition, "induced" CD4$^+$CD8$^+$ thymocytes express increased numbers of surface TCR complexes, have dephosphorylated TCR-ζ chains, and are highly competent to transduce TCR signals mobilizing intracellular calcium (1). Because little is known about the response of CD4$^+$CD8$^+$ thymocytes to TCR-mediated signals, we performed the present study to examine the conse-

quences of ligand engagement for induced CD4$^+$CD8$^+$ thymocytes expressing competent TCR complexes.

MATERIALS AND METHODS

Animals. C57BL/6, DBA/2, and C3H/HeJ mice were obtained from The Jackson Laboratory. (B6 × SJL)F$_1$ mice were bred in our own colony. TCR-Vβ8.2 transgenic mice (6) were generously provided by H. von Boehmer (Basel). Mice were used at 6–8 weeks of age.

Cell Preparation and Cell Cultures. CD4$^+$CD8$^+$ thymocytes were isolated prior to culture by adherence to plastic plates coated with anti-CD8 mAb and were >96% CD4$^+$CD8$^+$ as described (3). B-cell lines used were LK35.2 (IA$^{d/k}$IE$^{d/k}$) (7), M12.4.1 (IA^{d}IE^d), M12.C3 (Ia$^-$ variant of M12.4.1), and M12.A2 (IA$^-$IE^d variant of M12.4.1) (8); L-cell lines used were DCEK (IE^k transfectant) (9) and DAP.3 (untransfected parental line) (9); thymoma cell lines were BW5147 and R1.1 (10). Induction cultures of CD4$^+$CD8$^+$ thymocytes consisted of purified CD4$^+$CD8$^+$ thymocytes cultured for 4–8 h at 5 × 10^6 per ml in 2-ml single-cell suspension cultures in RPMI 1640 supplemented with 10% (vol/vol) fetal calf serum. At the end of the induction, graded numbers of stimulator cell lines were added to the cultures for an additional 8 h.

At the end of the stimulation, thymocytes were assessed by flow cytometry for surface expression of TCR molecules using mAbs specific for CD3ε (145-2C11) (11), TCR-Vβ8 (F23.1) (12), TCR-Vβ6 (RR4-7) (13), or TCR-Vβ17a (KJ23a) (14). Viability of thymocytes at the end of culture was >85% by three criteria: trypan blue exclusion, propidium iodide exclusion, and forward light scatter. To exclude all stimulator cells from the flow cytometry analysis, TCR profiles were obtained by electronic gating of Thy1.2$^+$ cells after staining the harvested cells in green with anti-TCR mAb and in red with anti-Thy1.2 mAb (30-H12) (15). Fluorescence data were collected on 50,000 viable Thy1.2$^+$ cells as determined by forward light scatter intensity and propidium iodide exclusion. Fluorescence intensity was quantitated in fluorescence units (FU), such that FU = cell frequency × median intensity. Median intensity was calculated by converting median logarithmic channel numbers to linear units using an empirically derived calibration curve for each 3-decade logarithmic amplifier used and subtracting negative control antibody fluorescence from experimental antibody fluorescence. To quantitatively compare TCR expression on thymocytes from various response cultures, results are expressed as relative FU; TCR surface levels of CD4$^+$CD8$^+$ thymocytes cultured

Abbreviations: FU, fluorescence units; mAb, monoclonal antibody; TCR, T-cell antigen receptor; MHC, major histocompatibility complex; SEB, staphylococcal enterotoxin B.
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alone at 4°C and 37°C were normalized to 0% and 100%, respectively. Relative FU = 100 × [(change in FU of thymocytes cultured with stimulator cells)/(change in FU of thymocytes cultured alone)].

Immunoblotting. For anti-phosphotyrosine immunoblotting, cultured CD4⁺CD8⁺ thymocytes were solubilized in 0.5% Triton X-100 lysis buffer with protease and phosphatase inhibitors. After immunoprecipitation with anti-CD3 mAb (145-2C11) or anti-TCR-Vβ8 mAb (F23.1), SDS/13% PAGE under reducing conditions, and electrotransfer, phosphorylated TCR-ζ was detected by immunoblotting with anti-phosphotyrosine antibodies as described (3).
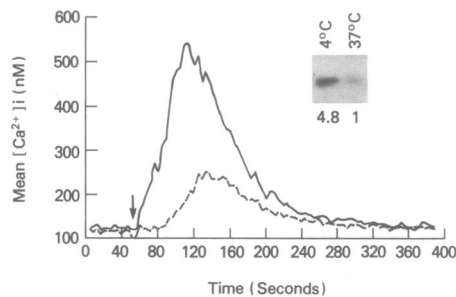
For anti-TCRα immunoblotting, Nonidet P-40 (NP-40) lysates of stimulated CD4⁺CD8⁺ thymocytes (2 × 10⁶ per lane) were applied to SDS/12.5% PAGE under nonreducing conditions and then subjected to electrotransfer, immunoblotting with anti-TCRα mAb (H28-710), and visualization with the use of ¹²⁵I-labeled protein A as described (1).

Calcium Mobilization. Indo-1 Ca²⁺ measurements were done as described (16). Cells were incubated with a saturating amount of biotin-conjugated H57-597 anti-TCRβ mAb (17) at 4°C, then warmed to 37°C for 10 min prior to analysis.

DNA Fragmentation. Harvested cells were washed and resuspended in 10 ml of digestion solution consisting of 0.1 M NaCl, 1 mM EDTA, 50 mM Tris·HCl (pH 7.5), 0.5% SDS, proteinase K at 100 μg/ml, and RNase A at 100 μg/ml. Digestion was performed at 55°C for 8 h. Genomic DNA was extracted with an equal volume of equilibrated phenol, phenol/chloroform, and then equilibrated chloroform. DNA was precipitated with 66% ethanol, washed with 80% ethanol, dried, and then dissolved in a buffer containing 10 mM Tris·HCl and 1 mM EDTA (pH 8.0).
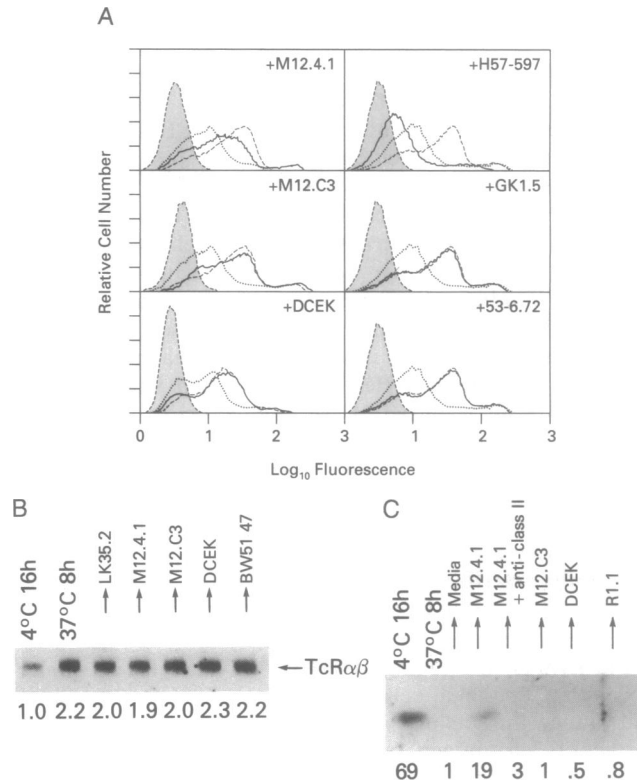
RESULTS AND DISCUSSION

To induce CD4⁺CD8⁺ thymocytes, thymocytes were first cultured alone in single-cell suspension at 37°C for 4–8 hr. Control thymocytes were cultured in parallel at 4°C at which

temperature their TCR phenotype does not change and resembles that of freshly explanted thymocytes (1). During the 37°C induction culture, TCR-ζ chains on CD4⁺CD8⁺ thymocytes dephosphorylate, and surface TCR complexes become competent to transduce signals mobilizing intracellular calcium (Fig. 1), as described (1). In the present study, we wished to examine the ability of CD4⁺CD8⁺ thymocytes to respond to ligand after they had been induced to express functional TCR complexes. Since CD4⁺CD8⁺ thymocytes do not appear to proliferate or secrete lymphokines in response

FIG. 1. Induction of TCR on immature CD4⁺CD8⁺ thymocytes in 37°C suspension culture results in TCR-ζ dephosphorylation and improved TCR signaling competence. C57BL/6 CD4⁺CD8⁺ thymocytes were cultured for 4 h in single-cell suspension at either 4°C (dashed line) or 37°C (solid line). After 4 h in culture, the relative competence of their TCR molecules was assessed by their ability to mobilize calcium upon TCR crosslinking. After harvesting, the cells were loaded with indo-1. Biotinylated anti-TCRαβ mAb (H57-597) (17) was then added to the cells, which was crosslinked by addition of 25 μg of avidin at the indicated time (arrow). As a positive control, the two thymocyte cell populations were equivalent in their mobilization of intracellular calcium in response to the guanine nucleotide-binding regulatory protein activator aluminum fluoride (data not shown). (*Inset*) In parallel with calcium flux measurements, the phosphorylation status of TCR-ζ was determined by immunoprecipitating solubilized TCR complexes from the same cell populations (3 × 10⁷ cells per lane) with anti-CD3ε mAb (145-2C11) and then immunoblotting the electrophoresed precipitates with anti-phosphotyrosine antibodies, which were then visualized by ¹²⁵I-labeled protein A. Relative band intensities were determined by a densitometer and are listed under each band.

FIG. 2. Response of CD4⁺CD8⁺ thymocytes, after induction, to various MHC-bearing stimulator cells. (*A*) DBA/2 (*H-2^d*) thymocytes that had been precultured for 8 h at 37°C were then stimulated for an additional 8 h with 3 × 10⁶ stimulator B cells from the cell line M12.4.1 (IA^dIE^d; *Top Left*) or M12.C3 (Ia⁻ variant of M12.4.1; *Middle Left*). C3H (*H-2^k*) thymocytes, following their 8-h preculture, were stimulated for an additional 8 h with the IE^k transfectant L-cell line DCEK (*Bottom Left*). Surface TCR expression was assessed by flow cytometry using a fluorescein-conjugated mAb to CD3ε (145-2C11). After their 8-h preculture, C57BL/6 thymocytes were stimulated for an additional 8 h with mAb specific for TCRαβ (H57-597; *Top Right*), CD4 (GK1.5; *Middle Right*) (18), or CD8 (53-6.72; *Bottom Right*) (15). CD3 staining was performed after the cells had been precultured alone for 8 h at 4°C (dotted line) or 37°C (dashed line), or had been precultured alone for 8 h at 37°C and then stimulated at 37°C for an additional 8 h (solid line). Without stimulator cells, surface CD3 staining did not change between 8 and 16 h in culture (data not shown). Shaded areas represent negative control staining with a mAb to human CD3 (Leu4), which does not specifically bind to murine thymocytes. (*B* and *C*) Purified C57BL/6 CD4⁺CD8⁺ thymocytes were precultured at 37°C for 8 h followed by an additional 8 h with the indicated stimulator cell lines. BW5147 and R1.1 are Ia⁻ thymoma cell lines. Where indicated anti-IA^d/IE^d mAb (M5-114; 20 μg/ml) (19) was added to M12.4.1 stimulator cell cultures. As a baseline control, CD4⁺CD8⁺ thymocytes were also cultured alone at 4°C for 16 h. (*B*) Total cellular levels of TCRαβ were determined by immunoblotting cell lysates with mAb to TCRα (H28-710) (20). (*C*) TCR-ζ phosphorylation levels were determined by immunoblotting CD3 precipitates with anti-phosphotyrosine antibody. Relative band intensities were determined by a densitometer and are listed under each lane.

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to antigen, we assessed responsiveness in CD4⁺CD8⁺ thymocytes by changes in TCR expression and by changes in TCR-ζ phosphorylation.

CD4⁺CD8⁺ thymocytes were first placed in 37°C induction cultures for 8 h and then subsequently cocultured with various major histocompatibility complex (MHC)-bearing stimulator cell lines for an additional 8 h, after which time TCR expression and TCR-ζ phosphorylation were assessed. During the initial 37°C induction cultures, TCR surface levels increased on CD4⁺CD8⁺ thymocytes (Fig. 2A), total cellular levels of TCR increased (Fig. 2B, compare lanes 1 and 2), and TCR-ζ phosphorylation significantly decreased (Fig. 2C, compare lanes 1 and 2). In the absence of stimulator cells, TCR expression by induced CD4⁺CD8⁺ thymocytes was unchanged during an additional 8 h of culture (data not shown). However, TCR expression by induced CD4⁺CD8⁺ thymocytes changed significantly in response to subsequent coculture with some, but not all, MHC-bearing stimulator cells. Following coculture with M12.4.1 cells, an Ia⁺ B-cell line, induced CD4⁺CD8⁺ thymocytes had markedly reduced surface levels of TCR (as determined by flow cytometry) but unchanged total cellular quantities of TCR (as determined by immunoblotting) (Fig. 2 A and B), indicating that induced CD4⁺CD8⁺ thymocytes had responded to these Ia⁺ B-cell stimulators by internalizing surface TCR complexes. The amount of TCR internalized in response to stimulation with Ia⁺ B cells was dose-dependent; it decreased with decreased numbers of stimulator B cells (compare Figs. 2A and 3A in which different numbers of M12.4.1 stimulators were used). In contrast, induced CD4⁺CD8⁺ thymocytes were not stimulated to internalize surface TCR complexes in response to M12.C3 cells, an Ia⁻ variant B-cell line of M12.4.1 that expresses equivalent amounts of MHC class I (Fig. 2A), suggesting that TCR internalization is predominantly stimulated by engagement of B-cell Ia determinants. Indeed, all Ia⁺ B cells so far examined have stimulated CD4⁺CD8⁺ thymocytes to internalize surface TCR complexes, regardless of whether the B-cell Ia determinants were syngeneic or allogeneic to the responding thymocytes. We have used different strain combinations of thymocytes and stimulator cells throughout this study to illustrate this point. In all cases, the stimulation of CD4⁺CD8⁺ thymocytes was blocked by the addition to culture of specific anti-Ia mAbs (data not shown). However, expression of Ia determinants does not appear to be sufficient to stimulate CD4⁺CD8⁺ thymocytes to internalize surface TCR complexes, as Ia⁺ L-cell transfectants such as DCEK failed to be stimulatory (Fig. 2A), presumably because L cells lack either necessary adhesion molecules or appropriate self-peptides that are present on B cells. In addition to reduced numbers of surface TCR complexes, the response of induced CD4⁺CD8⁺ thymocytes to stimulatory Ia⁺ B cells also resulted in tyrosine rephosphorylation of TCR-ζ chains that had been dephosphorylated in the initial induction cultures (Fig. 2C). As was the case with TCR internalization, B-cell stimulation of TCR-ζ rephosphorylation was blocked by anti-Ia mAb and did not occur in response to either Ia⁺ L cells (DCEK) or Ia⁻ cell lines (M12.C3 or R1.1) (Fig. 2C). Thus, after induction, CD4⁺CD8⁺ thymocytes responded to Ia⁺ B-cell stimulators by rephosphorylating TCR-ζ and by internalizing cell surface TCR complexes.

CD4⁺CD8⁺ thymocytes have on their surface two molecules with potential specificity for Ia (i.e., CD4 and TCR) and which could have been engaged by Ia⁺ B-cell stimulators. Indeed, engagement of either CD4 or TCR molecules on induced CD4⁺CD8⁺ thymocytes by specific mAbs has previously been reported to stimulate TCR-ζ rephosphorylation (3). In contrast, only engagement of TCR molecules by mAbs
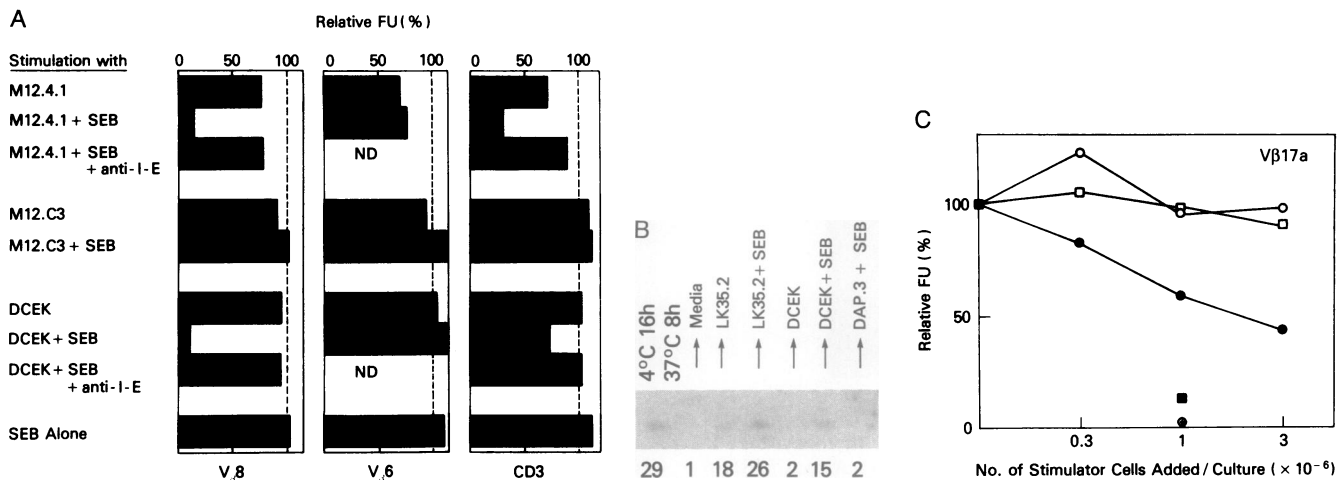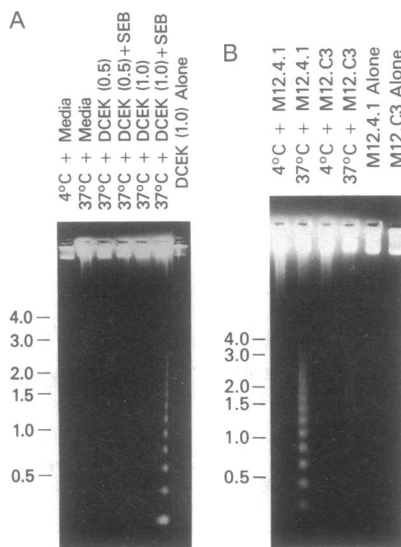

FIG. 3.   TCR-Vβ-specific down-modulation of surface TCR complexes in response to SEB and IE alloantigens. (A) Young adult DBA/2 (H-2ᵈ) thymocytes (1 × 10⁷) that had been precultured for 8 h at 37°C to allow maximal TCR induction were then stimulated for an additional 8 h with SEB (10 μg/ml) in the presence or absence of 1 × 10⁶ stimulator cells. At the end of the stimulation cultures, thymocytes were assessed for surface expression of specific TCR-Vβ molecules using mAb to TCR-Vβ8 (F23.1), TCR-Vβ6 (RR4-7), or CD3ε (145-2C11). To permit quantitative comparisons among all the groups, results are expressed as relative FU, with TCR surface levels of CD4⁺CD8⁺ thymocytes cultured alone at 4°C and 37°C normalized to 0% and 100%, respectively. Where indicated, mAb to I-Eᵏ,ᵈ (14-4-4s, 50 μg/ml) (23) was added to the stimulation culture. ND, not done. (B) The amount of phospho-ζ associated with TCR-Vβ8⁺ C57BL/6 CD4⁺CD8⁺ thymocytes after 8 h of preculture and 8 h of stimulation with SEB in the presence of various stimulator cell lines was assessed by immunoprecipitating solubilized TCR-Vβ8 complexes with mAbs to Vβ8 (F23.1) and then immunoblotting the electrophoresed precipitates (1 × 10⁸ cell equivalents per lane) with anti-phosphotyrosine antibody followed by ¹²⁵I-labeled protein A. LK35.2 is an MHC class II⁺ B-cell line, DCEK is an I-Eᵏ-transfected L-cell line, and DAP.3 is the parental L-cell line of DCEK. Relative band intensities were determined by a densitometer and are listed under each lane. (C) TCR-Vβ17a surface expression was assessed on young adult (B6 × SJL)F₁ CD4⁺CD8⁺ thymocytes (1 × 10⁷) that had been precultured for 8 h at 37°C to allow maximal TCR induction and then stimulated for 8 h with graded numbers of cells of the IA⁻IE⁺ L-cell line DCEK (□), the IA⁻IE⁺ B-cell line M12.A2 (●), and the Ia⁻ B-cell line M12.C3 (○). At the end of the stimulation cultures, thymocytes were assessed for surface expression of TCR-Vβ17a by using a mAb to TCR-Vβ17a (KJ23a). To permit comparisons among all the groups, results are expressed as relative FU, with TCR surface levels of TCR-Vβ17a⁺ CD4⁺CD8⁺ thymocytes cultured alone at 4°C and 37°C normalized to 0% and 100%, respectively. As positive controls, the TCR-Vβ17a expression levels of the same thymocyte populations were also determined following stimulation with SEB (10 μg/ml) presented by either 1 × 10⁶ M12.A2 B cells (⊙) or 1 × 10⁶ DCEK L cells (■).
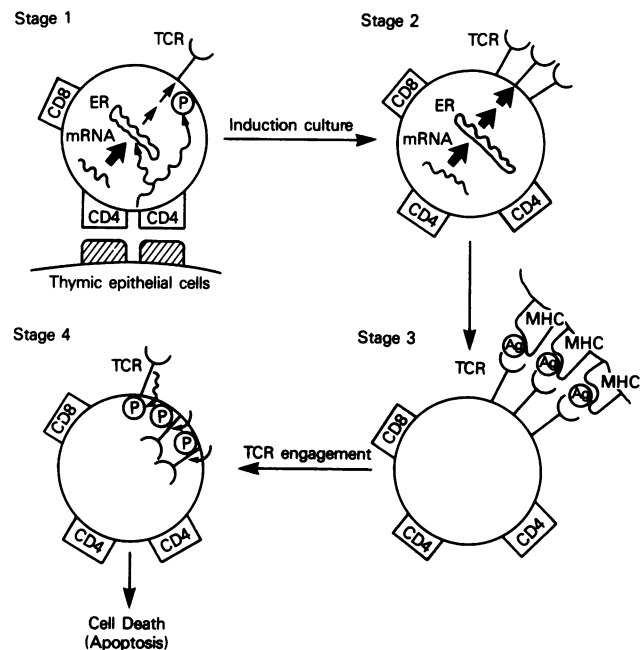
stimulated induced CD4+CD8+ thymocytes to reduce surface TCR complexes (Fig. 2A), suggesting that TCR engagement results in reduced numbers of surface TCR complexes on induced CD4+CD8+ thymocytes but that CD4 engagement does not. To more directly examine the response of induced CD4+CD8+ thymocytes to TCR-mediated signals, we stimulated induced CD4+CD8+ thymocytes with the superantigen staphylococcal enterotoxin B (SEB) which, in the presence of IE+ presenting cells, selectively engages TCR-Vβ8 and -Vβ17a molecules but does not engage TCR-Vβ6 molecules (21, 22). Thymocytes were placed in 37°C induction cultures for 8 h, then cocultured with SEB in the presence of relatively low numbers (to minimize the effect of stimulator cells alone) of various MHC stimulator cell lines for an additional 8 h, and assessed for surface TCR levels (Fig. 3A). SEB had no effect on TCR expression by TCR-Vβ6+ CD4+CD8+ thymocytes (Fig. 3A, *Middle*). However, SEB did induce a dramatic reduction in TCR surface levels on TCR-Vβ8+ CD4+CD8+ thymocytes, but only in the presence of IE+ M12.4.1 B cells or IE+ DCEK L cells (Fig. 3A, *Left*), and its effect was blocked by anti-IE mAb. It is noteworthy that for presentation of an exogenous IE-dependent antigen such as SEB, the L-cell line DCEK was as effective as M12.4.1 B cells. The SEB-induced reduction in TCR-Vβ8 surface levels was a result of TCR internalization by the responding thymocytes, as their total cellular levels of TCR were unchanged upon immunoblotting with mAbs to TCR-Vβ8 (data not shown). To determine if SEB also stimulated intracellular signals in

responding CD4+CD8+ thymocytes, we assessed TCR-ζ rephosphorylation in induced Vβ8+ CD4+CD8+ thymocytes that had been stimulated with or without SEB in the presence of various IE+ cells. As before, stimulation with an Ia+ B-cell line (LK35.2) was itself sufficient to induce significant TCR-ζ rephosphorylation, whereas stimulation with the IE+ L-cell line DCEK was not (Fig. 3B). However, significantly increased TCR-ζ rephosphorylation was induced in Vβ8+ CD4+CD8+ thymocytes by SEB presented by either IE+ LK35.2 B cells or IE+ DCEK L cells, but not by control IE− DAP.3 L cells (Fig. 3B). Thus, in the presence of IE+ stimulator cells, engagement of TCR-Vβ8 molecules by SEB induced the generation of intracellular signals in CD4+CD8+ thymocytes that resulted in tyrosine kinase activation, TCR-ζ rephosphorylation, and TCR internalization.

The responsiveness of induced CD4+CD8+ thymocytes to TCR-mediated signals was further verified by examining the IE-specific responses of TCR-Vβ17a+ thymocytes. Mature TCR-Vβ17a+ T cells react against IE+ B cells but not against IE+ L cells, presumably because L cells lack either the nominal B-cell antigen or adhesion molecule that is necessary for engagement of these receptors (24, 25). Similarly, we found that induced Vβ17a+ CD4+CD8+ thymocytes responded by TCR internalization to the IA−IE+ B-cell line

FIG. 5. Schematic summary of events in CD4+CD8+ thymocytes. Stage 1: In the thymus, CD4-mediated signals are induced in immature CD4+CD8+ thymocytes by interaction with thymic epithelial cells, causing (i) retention and degradation of newly synthesized TCR chains within the endoplasmic reticulum (ER), resulting in low surface TCR levels, (ii) phosphorylation of TCR-ζ chains, and (iii) the inability of surface TCR complexes to transduce signals mobilizing intracellular calcium, as described (1). Stage 2: In 37°C induction cultures, CD4+CD8+ thymocytes are separated from thymic epithelial cells and so are released from CD4-mediated inhibitory signals, resulting in (i) increased release of newly synthesized TCR chains from the endoplasmic reticulum with resultant increase in surface TCR levels, (ii) dephosphorylation of TCR-ζ chains, and (iii) increased competence of surface TCR complexes to transduce signals mobilizing intracellular calcium, as described (1). Stage 3: Competent TCR complexes on "induced" CD4+CD8+ thymocytes with ligand-bearing stimulator cells are engaged. Stage 4: Induced CD4+CD8+ thymocytes respond to TCR engagement of ligand by internalizing surface TCR molecules, resulting in decreased surface TCR levels, and rephosphorylation of TCR-ζ chains. A proportion of responding CD4+CD8+ thymocytes degrade their genomic DNA, presumably reflecting the induction of apoptosis. Ag, antigen.

FIG. 4. DNA fragmentation in induced CD4+CD8+ thymocytes following TCR engagement. Purified CD4+CD8+ thymocytes (1 × 10⁷) from TCR-Vβ8.2 transgenic mice (A) or from normal C57BL/6 mice (B) that had been precultured in the absence of stimulator cells for 2 h at either 4°C or 37°C were then cocultured with various stimulator cells for 4 h. (A) DNA fragmentation was assessed in purified CD4+CD8+ thymocytes from TCR-Vβ8.2 transgenic mice cultured alone at 4°C and 37°C or cocultured with graded numbers of DCEK (0.5 × 10⁶–1.0 × 10⁶) IEᵏ L cells in the presence or absence of SEB (10 μg/ml). (B) DNA fragmentation was assessed in purified CD4+CD8+ thymocytes from normal C57BL/6 mice cocultured with 1 × 10⁶ B cells from either the Ia+ cell line M12.4.1 or the Ia− variant cell line M12.C3. Genomic DNA from cultured cells was prepared and loaded onto each lane of an 0.8% agarose gel containing ethidium bromide. Control DNA from stimulator cells cultured for 4 h at 37°C without thymocytes was also run. The amount of DNA in each sample, which includes DNA from both responder and stimulator cells, was determined by a spectrophotometer: (A) Lanes 1–5, 9 μg; lane 6, 0.9 μg. (B) Lanes 1–4, 5 μg; lanes 5 and 6, 2.2 μg. Electrophoresis was done at 10 V for 16 h. The gel was photographed using ultraviolet light illumination. The position of molecular size markers (in kilobase pairs) is indicated on the left.

M12.A2 but failed to respond to the IA$^-$IE$^+$ L-cell line DCEK (Fig. 3C). It should be noted that both IA$^-$IE$^+$ M12.A2 B cells and IA$^-$IE$^+$ DCEK L cells were able to present SEB to Vβ17a$^+$ CD4$^+$CD8$^+$ thymocytes (Fig. 3C), indicating that both IE$^+$ cell populations were competent to engage Vβ17a$^+$ CD4$^+$CD8$^+$ thymocytes when they possessed the appropriate antigen.

Finally, since one outcome of TCR signaling in early thymocytes *in vivo* is clonal deletion by induction of programmed cell death (26, 27), we examined whether any of the CD4$^+$CD8$^+$ thymocytes responding *in vitro* in this study had been stimulated to fragment their genomic DNA. First, we assessed DNA fragmentation in CD4$^+$CD8$^+$ thymocytes responding to SEB superantigen (Fig. 4A). To increase the number of CD4$^+$CD8$^+$ thymocytes expressing SEB-reactive TCR, we isolated CD4$^+$CD8$^+$ thymocytes from mice expressing the TCR-Vβ8.2 transgene since such TCR are capable of engaging the SEB superantigen. Indeed, DNA fragmentation was observed in transgenic CD4$^+$CD8$^+$ thymocytes from 37°C cultures upon stimulation with the IE$^+$ L-cell line DCEK (1 × 10^6 per culture) plus SEB (10 μg/ml), but it was not observed upon stimulation with DCEK cells alone (Fig. 4A). Second, we assessed CD4$^+$CD8$^+$ thymocyte populations from normal mice for DNA fragmentation in response to Ia$^+$ B-cell stimulators (Fig. 4B). DNA fragmentation was observed in induced CD4$^+$CD8$^+$ thymocytes in response to Ia$^+$ M12.4.1 B cells but not in response to Ia$^-$ M12.C3 cells (Fig. 4B). Thus, these results indicate that a fraction of induced CD4$^+$CD8$^+$ thymocytes (perhaps those that were most differentiated) degraded their DNA in response to ligand engagement of TCR.

In the thymus, CD4$^+$CD8$^+$ thymocytes arise from CD4$^-$CD8$^-$ precursor cells, and only those CD4$^+$CD8$^+$ cells expressing appropriate TCR specificities are positively selected to further differentiate into CD4$^+$ and/or CD8$^+$ "single-positive" thymocytes. Most CD4$^+$CD8$^+$ thymocytes are retained and die in the thymus, either because they have not been positively selected or because they have been negatively selected as a consequence of TCR-mediated signals stimulated by antigens expressed in the thymus. Thus, TCR-mediated signals in developing CD4$^+$CD8$^+$ thymocytes have at least two alternative outcomes: (*i*) positive selection for further maturation into single-positive thymocytes with concomitant shutoff of either CD4 or CD8 expression, and (*ii*) negative selection by elimination of antigen-reactive thymocyte clones. These TCR-mediated selection events are thought to occur only in the small subset of CD4$^+$CD8$^+$ thymocytes that express intermediate surface levels of TCR (28), which are comparable to those observed on CD4$^+$CD8$^+$ thymocytes in the present study after *in vitro* induction culture. Thus, placing CD4$^+$CD8$^+$ thymocytes in single-cell suspension culture experimentally induces TCR expression levels that are otherwise found in only a tiny fraction of CD4$^+$CD8$^+$ thymocytes undergoing selection, making such cells more amenable to study and experimentation (Fig. 5). This *in vitro* model has made it possible to assess the responses of such CD4$^+$CD8$^+$ thymocytes to ligand-induced signals.

In their response to ligand-bearing stimulator cells, *in vitro*-induced CD4$^+$CD8$^+$ thymocytes behaved as if they were "preselection" thymocytes in that they did not distinguish between stimulator cells expressing syngeneic and allogeneic MHC determinants. One way in which induced CD4$^+$CD8$^+$ thymocytes responded to ligand-bearing stimulator cells was to reduce surface TCR expression, indicating that low surface expression of TCR may not itself be a definitive marker of preselection CD4$^+$CD8$^+$ thymocytes as low surface TCR expression also was a consequence of ligand engagement (Fig. 5, compare stages 1 and 4). Another way in which some induced CD4$^+$CD8$^+$ thymocytes responded to

ligand-bearing stimulator cells was to degrade their genomic DNA, an event closely associated with negative selection and clonal deletion (26, 27). We do not yet know if other CD4$^+$CD8$^+$ thymocytes in these response cultures might have been positively selected. It is not known what distinguishes TCR signals leading to positive versus negative selection.

In summary, the present study demonstrates that CD4$^+$CD8$^+$ thymocytes, upon release from CD4-mediated inhibition in *in vitro* suspension culture, express increased numbers of surface TCR complexes that are competent to mobilize intracellular calcium and that are stimulated by ligand to transduce signals causing (*i*) internalization of surface TCR complexes, (*ii*) rephosphorylation of TCR-ζ chains, and (*iii*) (in some fraction of responding CD4$^+$CD8$^+$ thymocytes) degradation of genomic DNA.

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