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Cellular and peptide requirements for *in vitro* clonal deletion of immature thymocytes

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ABSTRACT **Thymocytes from DO10 T-cell-receptor transgenic mice undergo apoptosis, or programmed cell death, when chicken ovalbumin-(323–339) peptide is administered *in vivo*. Using DO10 mice thymocytes, we have now developed a simple *in vitro* model system that recapitulates the *in vivo* clonal-deletion process. When transgenic thymocytes were cocultured with fibroblasts, B cells, or thymic nurse cell lines (all bearing I-A^d) in the presence of chicken ovalbumin-(323–339), deletion of the transgenic TCR$^+$CD4$^+$CD8$^+$ thymocytes was seen within 8–20 hr. Thymocytes designed to bear I-A^d on their surface could mediate the deletion themselves. Thus, thymocyte clonal deletion entirely depends on the stage at which the thymocytes are vulnerable to the onset of apoptosis, rather than on the nature of the peptide antigen-presenting cells. Furthermore, thymic nurse cell line TNC-R3.1 could cause deletion, strongly suggesting that some thymic epithelial/stromal components are potentially capable of participating in negative selection. In all cases examined, little deletion could be induced at a peptide concentration <10 nM, thus defining the minimum amount of peptide antigen required for negative selection. The peptide-dependent *in vitro* negative-selection system will allow further dissection of the molecular and cellular processes involved in clonal deletion due to apoptosis in the thymus.**

Clonal deletion or negative selection of self-reactive T cells during thymic development plays a critical role in inducing immunological self-tolerance (1–3). With transgenic mice bearing T-cell receptor (TCR) of defined peptide antigen specificity, self-reactive T cells have been shown to undergo apoptosis, or programmed cell death, during the process of clonal deletion in the thymus (4). Thymocytes can thus be triggered to undergo apoptosis upon engagement of the TCR with the peptides presented in the context of the appropriate major histocompatibility complex (MHC) molecules. The exact nature of the cell–cell interactions or the signaling pathways that precipitate programmed cell death in the developing thymocytes is unknown (5–9). Tolerance induction is thought to largely depend on the antigen-presenting capability of the bone-marrow-derived cells like macrophages and dendritic cells, whereas the role of thymic epithelial/stromal cells is controversial (10–12). However, little is known about the amount and form of antigen required to delete the developing thymocytes. We have recently developed a TCR transgenic mouse, DO10, that has a TCR that recognizes a chicken ovalbumin (cOVA) peptide-(323–339) in the context of a class II MHC molecule, I-A^d (4). When cOVA-(323–339) was injected i.p. into these mice, the developing, immature thymocytes underwent apoptosis in an antigen-specific manner. Using these mice, we now report on the development of a simple *in vitro* thymocyte clonal-deletion system that can be used to elucidate the cellular and peptide-antigen requirements for inducing clonal deletion.

EXPERIMENTAL PROCEDURES

Mice. TCR transgenic mice (DO10) bearing TCR from a hybridoma, DO11.10, with specificity for cOVA-(323–339) and I-A^d have been backcrossed to BALB/c mice and are maintained in our own animal facility. KA mice were created by the microinjection of I-A^d genes (α and β) driven by class I MHC K^b promoter into mouse embryos and maintained in the H-2^d background (13).

Cells. RT2.3.3.HD6, I-A^d-transfected L-cell fibroblasts, was provided by R. Germain (National Institutes of Health, Bethesda, MD) (14). TNC-R3.1, a thymic nurse cell line from a AKR/J (H-2^k) mouse that shows properties similar to thymic nurse cells, was transfected with genomic I-A^d genes (α and β) linked to class I MHC K^b promoter and selected for their cell-surface expression (15). A20 and TA3, B-cell lymphoma lines, were obtained from E. Unanue and T. Braciale (Washington University, St. Louis).

Peptides. Peptides were made by using a model 430 peptide synthesizer (Applied Biosystems) and purified by HPLC as described (16). Peptide sequences are as follows: cOVA-(323–339), ISQAVHAAHAEINEAGR, and cOVA-(324–334), SQAVTAAHAEI (which also has a histidine to threonine change at position 328).

Flow Cytometry. Lyt-2 fluorescein isothiocyanate (Becton Dickinson), L3T4–phycoerythrin (Becton Dickinson), and biotinylated KJ1-26 (anticlonotypic TCR monoclonal antibody) plus streptavidin-Red613 (GIBCO/BRL) were used. Three-color flow cytometric analysis was done with FACScan (Becton Dickinson). Dead cells were gated out by staining with propidium iodide at 5 μg/ml (Sigma) in the FL3 channel, whereas RT2.3.3.HD6, A20, TA3, and TNC-R3.1 cells were easily gated out from the thymocytes by using forward- and side-scatter channels. For experiments with T-cell-depleted splenic antigen-presenting cells (APCs), Thy-1.2–phycoerythrin (PharMingen, San Diego) was used instead of CD4–phycoerythrin to exclude splenic APCs from the thymocytes by gating on FL2 channel.

***In Vitro* Deletion Assay.** For the adherent APCs (L-cell, RT2.3.3.HD6, and TNC-R3.1), 4×10^6 thymocytes were cultured with APCs confluently covering the bottom of a 6-well flat-bottomed tissue culture plate (Costar) in 3 ml of RPMI 1640 medium/10% fetal calf serum containing the designated amount of appropriate peptides for the indicated durations. Thymocytes and APCs were harvested by extensive pipetting in 1.2 mM EDTA/phosphate-buffered saline. Similarly, 1×10^5 nonadherent APCs (A20, TA3, and T-cell-depleted splenic APCs) and 1×10^6 thymocytes were cultured in a 96-well round-bottomed tissue-culture plate (Costar) in 0.2

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Abbreviations: APC, antigen-presenting cell; DP, double-positive; TCR, T-cell receptor; cOVA, chicken ovalbumin; MHC, major histocompatibility complex.

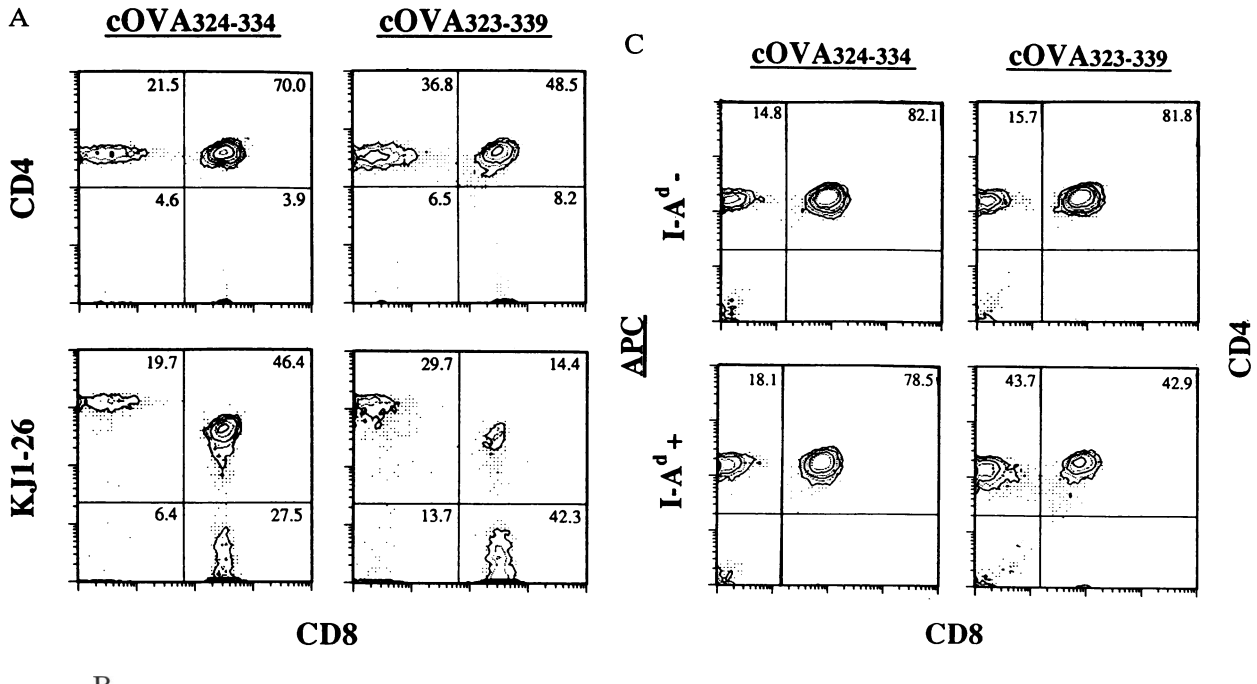
ml of identical medium. The harvested cells were counted, stained, and analyzed by FACScan as above. Typical background was 25–30% spontaneous deaths in a 20-hr assay.

Detection of DNA Fragmentation and EM. Thymocytes and A20 cells were cultured as described, with cOVA-(324–334) or cOVA-(323–339) at 1 μM. Cells were harvested after 5 hr because DNA fragmentation can be seen earlier than the decrease of the $CD4^+CD8^+$ population. Fragmented DNA was prepared from 5×10^5 thymocytes by the method of Newell *et al.* (17). Samples were separated by electrophoresis on a 1.5% agarose gel containing ethidium bromide at 0.5 μg/ml. For EM, thymocytes and A20 cells were cultured, as described, with cOVA-(324–334) or cOVA-(323–339) at 1 μM for 20 hr. Culture medium was aspirated and immediately replaced by an isotonic 3% (wt/vol) glutaraldehyde solution. Electron micrographs were taken as described (4).

RESULTS AND DISCUSSION

cOVA-(323–339) Deletes $TCR^+CD4^+CD8^+$ Immature Thymocytes *in Vitro*. T cells from the DO10 TCR transgenic mice respond to cOVA-(323–339) when presented in the context of $I\text{-}A^d$ and bear the TCR clonotypic marker recognized by the monoclonal antibody KJ1-26 (18, 19). Upon *in vivo* administration of cOVA-(323–339), KJ1-26^+ $CD4^+CD8^+$ double-positive (DP) immature thymocytes are preferentially deleted. Because cOVA-(324–334), which binds to $I\text{-}A^d$ but cannot stimulate the transgenic TCR, does not cause deletion, apoptosis among the DP thymocytes requires the recognition of both the $I\text{-}A^d$ and cOVA-(323–339) by the KJ1-26^+ TCR (4, 20, 21). Previous *in vivo* studies showed that apoptotic cells were distributed extensively and uniformly throughout the thymic cortex (4). Because these thymocytes themselves are $I\text{-}A^d$ negative and the macrophage/dendritic cells reside predominantly in the thymic medulla, the possibility that other cells in the thymic cortex may cause clonal deletion by presenting the cOVA-(323–339) peptide to the cortical thymocytes was raised. One such candidate would be the cortical epithelial/stromal cells that are known to be class I and II MHC positive. To test which cells can provide the necessary trigger to initiate apoptosis among immature thymocytes, an *in vitro* clonal-deletion system was developed.

Initially, DO10 thymocytes were incubated with T-cell-depleted splenocytes from BALB/c ($H\text{-}2^d$) mice with the cOVA peptides. The cells were harvested after 15- to 20-hr incubation with 1 μM cOVA-(323–339); ≈70–75% of DP thymocytes were deleted, whereas the $TCR^{hi}CD4^+CD8^-$ (single-positive) cells were unaffected. Clonal deletion was not seen when either the cOVA-(323–339) or the APCs were absent (data not shown). To explore whether other cell types could induce clonal deletion, an $I\text{-}A^d$-expressing L-cell fibroblast transfectant, RT2.3.3.HD6, was used to present peptides (14). Deletion of the KJ1-26^+ DP cells was seen upon incubation with cOVA-(323–339) (Fig. 1*A*). No such deletion was seen with cOVA-(324–334) (Fig. 1*A*) or with influenza nucleoprotein peptide, which does not bind to $I\text{-}A^d$ [A/nt/60/68-(345–360), data not shown]. Relatively, the percentage of $CD4^+CD8^-$ and KJ1-26^- DP cells increased with cOVA-(322–339), but the actual cell numbers remained unchanged (Fig. 1*B*). (Some variation in percentage of the latter population occurred with age, but these cells always survive



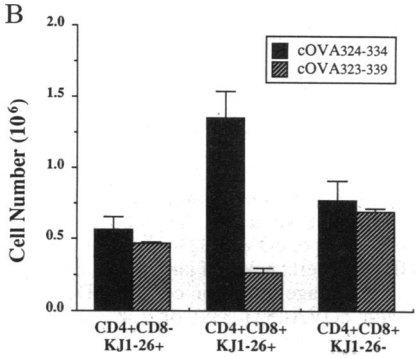


FIG. 1. *In vitro* clonal deletion of the $CD4^+CD8^+$KJ1-26^+ thymocytes from DO10 transgenic mice by cOVA-(323–339) at 1 μM peptide concentration for 20 hr. (*A*) Three-color flow cytometric analysis of transgenic thymocytes cultured with either cOVA-(324–334) or cOVA-(323–339) on $I\text{-}A^d$-transfected L-cell (RT2.3.3.HD6). (*B*) Absolute cell numbers of KJ1-$26^+CD4^+CD8^-$, KJ1-26^+CD4^+-$CD8^+$, and KJ1-$26^-CD4^+CD8^+$ thymocytes with cOVA-(324–334) (black bar) or cOVA-(323–339) (hatched bar). Results represent means and SDs of the calculated cell numbers ($n = 3$). (*C*) Two-color flow cytometric analysis of thymocytes cultured with either cOVA-(324–334) or cOVA-(323–339) on either wild-type L-cell ($I\text{-}A^{d-}$) or $I\text{-}A^d$-transfected L-cell ($I\text{-}A^{d+}$).

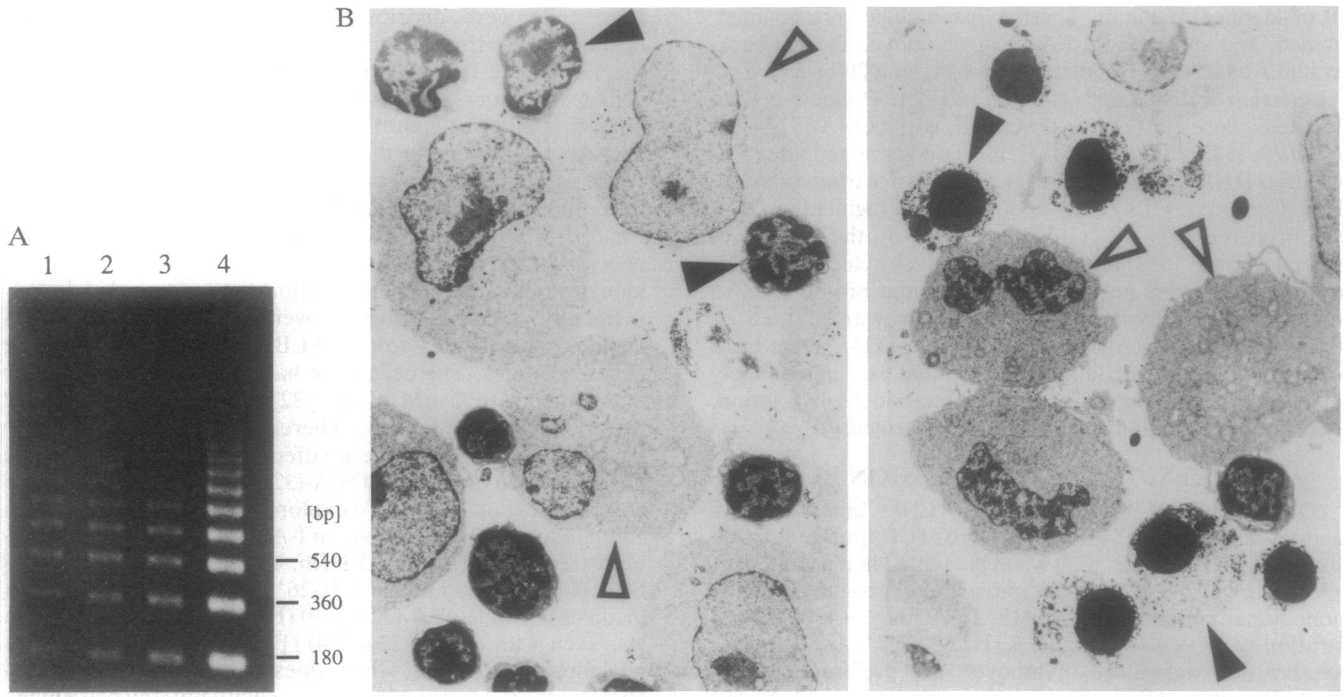


FIG. 2. Apoptosis of transgenic thymocytes in *in vitro* clonal-deletion system. (*A*) DNA fragmentation induced in thymocytes by peptide-antigen addition. Thymocytes of a nontransgenic littermate (lanes 1 and 2) and a transgenic mouse (lanes 3 and 4) were cultured with cOVA-(324–334) (lanes 1 and 3) or cOVA-(323–339) (lanes 2 and 4). (*B*) Electron micrographs of cultured thymocytes with cOVA-(324–334) (*Left*) or cOVA-(323–339) (*Right*). Large cells with plenty of cytoplasm and low-density nuclei (◁) are A20 cells; small cells with small cytoplasm and medium/high-density nuclei (◀) are thymocytes.

preferentially.) Furthermore, the appropriate I-A^{d+} fibroblasts were necessary for deletion to occur (Fig. 1*C*). These data suggest that not enough endogenous cells functioned in our thymic preparations (for example, macrophages and dendritic cells) to present the peptide antigens and cause deletion unless exogenous APCs were introduced. Although the effect of peptide-induced clonal deletion was most pronounced after 15–20 hr, initial effects could be detected as early as 5–8 hr. Similar results were obtained when other cells, such as A20 cells, were used as APCs (see below). To confirm that clonal deletion was actually accompanied by the presence of apoptotic cells, DNA-fragmentation analysis as well as EM was done. Fig. 2 *A* and *B* shows findings consistent with extensive apoptosis. Taken together, these results strongly suggest that the *in vitro* experiments recapitulate the *in vivo* clonal deletion.

Dose–Response Analysis Reveals the Minimum Amount of Peptide Necessary for Clonal Deletion. In DO10 transgenic mice, the i.p. administration once a day for 3 days of cOVA-(323–339) (250 μl of 100 μM solution) was sufficient for clonal

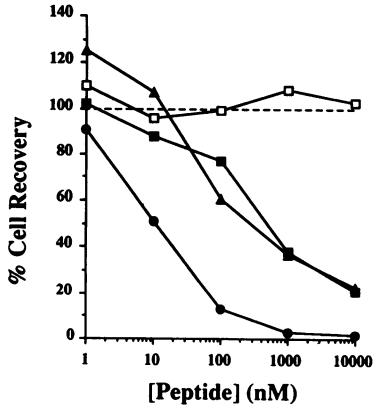


FIG. 3. Dose–response relationship in induction of clonal deletion of transgenic thymocytes with different cell types as APCs. Transgenic thymocytes were cultured with the indicated concentrations of either cOVA-(324–334) or cOVA-(323–339) with either the RT2.3.3.HD6 (□ ■), A20 (●), or T-cell depleted spleen cells (▲). Open and closed symbols denote KJ1-$26^+CD4^+CD8^-$ and KJ1-$26^+CD4^+CD8^+$, respectively. Percentage of cell recovery was defined as follows: [cell number with cOVA-(323–339)/cell number with cOVA-(324–334)] × 100. All experiments were done in triplicates, and the mean of cell number in triplicates was used for calculating percentage of cell recovery. SDs of cell numbers were usually <15% of means.

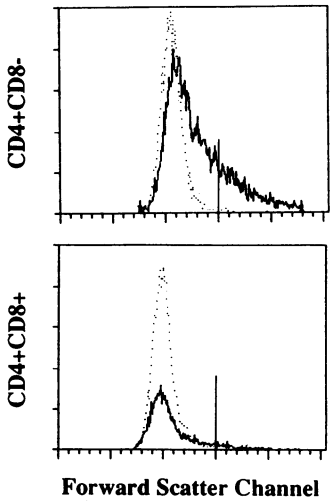


FIG. 4. Appearance of blastic cells in *in vitro* deletion culture system. Data in Fig. 1 were gated on $CD4^+CD8^-$ (*Upper*) or $CD4^+CD8^+$ (*Lower*) thymocytes. Thymocytes were cultured with cOVA-(324–334) (· · ·) or cOVA-(323–339) (—). Percentage of blastic cells was defined as percentage of large-sized cell over 150 forward-scatter units. Percentage of blastic cells in $CD4^+CD8^-$ thymocytes was 22.5% with cOVA-(323–339) or 1.4% with cOVA-(324–334) peptide.

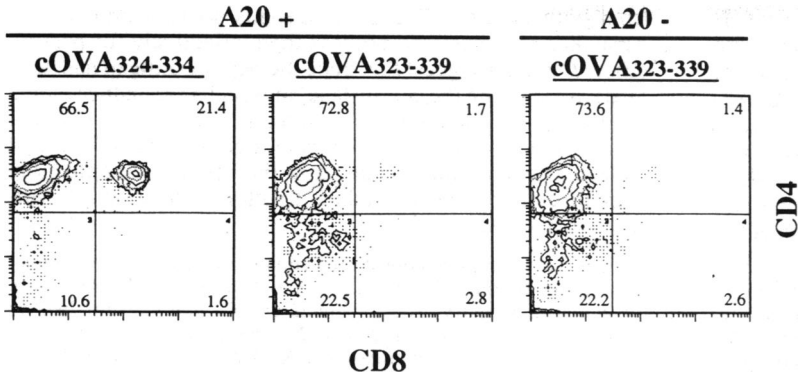


FIG. 5. *In vitro* clonal deletion with thymocytes from TCR and I-A^d class II MHC double-transgenic mice (DO10 × KA50). Thymocytes were cultured as described by using cOVA-(324–334) with A20 cells (*Left*), cOVA-(323–339) with A20 cells (*Middle*), or cOVA-(323–339) without A20 cells (*Right*). Peptide concentration was 1 μM.

deletion of the immature KJ1-26$^+$ DP thymocytes (4). To determine the peptide concentration required to induce apoptosis *in vitro*, dose–response curves were determined with RT2.3.3.HD6, T-cell-depleted splenocytes, and B-cell lymphoma line A20 as APCs. When thymocytes were cultured with RT2.3.3.HD6 or splenic APCs, ≈50% of the DP cells were deleted at several hundred nanomolar range (Fig. 3). No effect of peptide was seen at <10 nM, thus setting the lower limit of peptide concentration necessary to precipitate clonal deletion by these APCs. In contrast, 10 nM of cOVA-(323–339) was clearly sufficient to cause deletion with A20 cells (Fig. 3). However, once peptide concentration was lowered to 1 nM, even A20 cells could not elicit clonal deletion. Although the immature DP cells were deleted, the number of single-positive cells showed little change with RT2.3.3.HD6 (Fig. 3) or with other APCs (data not shown). Rather, single-positive cells appeared to be activated under these conditions by blastogenesis (Fig. 4) and [^{3}H]thymidine-uptake assays (31).

To see whether thymocytes themselves can present and mediate apoptosis, we crossed the DO10 mice to KA mice. In the KA mice, the transgenic I-A^d is expressed on thymocytes because the I-A^d genes are transcribed by MHC class I K^b promoter (13). In F$_1$ (DO10 × KA) mice, we found an increased percentage of KJ1-26$^+$CD4$^+$CD8$^-$ cells, probably due to the distinctive expression pattern of the I-A^d transgene (Fig. 5 and unpublished data). Although in F$_1$ mice (DO10 × KA), the percentage of DP cells is lower than in DO10 mice, almost complete deletion of these DP cells was seen with A20 cells and cOVA-(323–339). Furthermore, a similar extent of deletion was seen when A20 cells were absent (Fig. 5). These results imply that T cells themselves can mediate apoptosis, characterized by DNA fragmentation, when the peptide antigens that can interact with the specific TCRs are present on the T-cell surfaces (data not shown).

Although specific comparisons of the differential effectiveness as inducers of clonal deletion cannot be fully ascertained due to many potential differences—such as cell size, expression level of I-A^d, nature of the various adhesion molecules among others—B cells were consistently more effective than other APCs. A similar instance of the B cells being more effective than L-cells has been recently described by using T-cell activation as an assay (21). We suggest, then, that a peptide concentration of at least 10 nM is required in the thymus before an individual can be tolerized against such antigens by clonal deletion. Thus, for immunological tolerance to develop against antigens that may be found extrathymically or circulating in the serum, free peptides must be available at >10 nM threshold (22). Alternatively, specialized APCs may exist in the thymus that can function to specifically focus proteins and peptide antigens, leading to clonal deletion of the self-reactive thymocytes despite lower nominal concentrations *in vivo*.

TNC-R3.1, a Thymic Nurse Cell Line, Can Also Present Peptides and Mediate Clonal Deletion *in Vitro*. In contrast to the general agreement that the radio-resistant portion of the thymus is crucial in mediating positive selection that results in MHC restriction, the role of thymocytes in inducing tolerance has been controversial (10). Having shown that a variety of different cell types, including thymocytes themselves, can mediate clonal deletion, we asked whether a thymic nurse cell line, TNC-R3.1, can function similarly. TNC-R3.1 shows properties close to those of thymic nurse cells that are considered important in thymocyte differentiation (15). Specifically, TNC-R3.1 cells (which express only

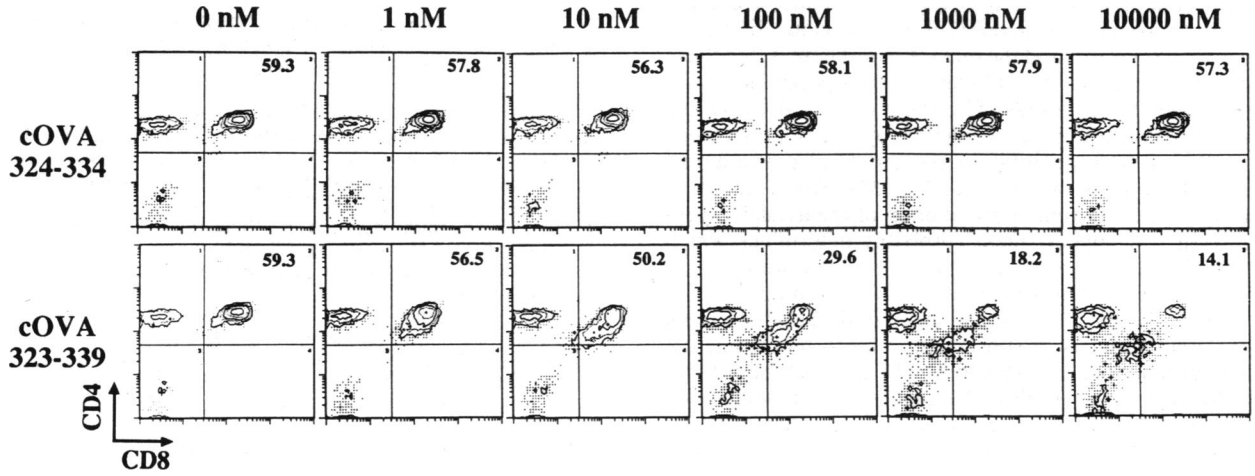


FIG. 6. Dose–response relationship in the induction of clonal deletion of transgenic thymocytes with I-A^d-transfected TNC-R3.1 cells. Expression pattern of CD8/CD4 of live thymocytes after 20-hr culture with indicated concentrations of either cOVA-(324–334) (*Upper*) or cOVA-(323–339) (*Lower*) peptide.

class I MHC molecules constitutively, whereas class II molecules are γ interferon inducible) form a complex with $CD4^+CD8^+$ and $CD4^-CD8^-$ thymocytes and can induce the differentiation of $CD4^+CD8^+$ thymocytes into $CD4^-CD8^+$-TCR^+ mature T cells. To test whether this particular thymic nurse cell line can mediate clonal deletion, we stably transfected I-A^d genes (α and β) linked to K^b class I MHC promoter into TNC-R3.1 cells (data not shown). When these cells were cocultured with DO10 thymocytes, deletion of the KJ1-26^+ DP cells was seen in a dose-dependent manner quite similar to that of A20 cells (Fig. 6). As before, this deletion depended on cOVA-(323–339) and I-A^d because only transfected cells could induce deletion (data not shown). We conclude that TNC-R3.1 cells can induce clonal deletion of the susceptible thymocytes.

Cellular and Peptide Requirements for Clonal Deletion. Thus far, we have shown that fibroblasts, splenic APCs, B-cell lymphoma cells, a thymic nurse cell line, as well as I-A^d thymocytes themselves, all can induce apoptosis, or programmed cell death of immature thymocytes. If generalized, the data strongly suggest that the cellular origin of the APCs are probably not of paramount importance in inducing clonal deletion as long as these cells express the relevant cell-surface MHC molecules complexed with the appropriate peptides. However, some variations in the dose–response curves, which may have some biological relevance in relation to the differential role of the distinct cell types involved in tolerance induction, were seen (23). What appears more critical about thymocyte clonal deletion is the stage at which thymocytes are susceptible to such a process (24–28). As shown (4) in the *in vivo* peptide-administration experiments with the DO10 TCR transgenic mice, the KJ1-26^+ DP thymocytes are the most sensitive to antigen-induced programmed cell death.

Although it is tempting to conclude that all the different thymic epithelial/stromal cells in the thymus can induce clonal deletion, it is probable that at least some of these cells can do so. Even though TNC-R3.1 cells have features, suggestive of similarity to the thymic nurse cells, that might facilitate positive selection, we cannot unequivocally conclude that the thymic epithelial/stromal cells responsible for positive selection *in vivo* are also capable of clonal deletion, although we favor such a possibility. Previous studies using radiation-induced bone-marrow chimera and thymus grafts treated with deoxyguanosine showed that thymic epithelial/stromal cells are weakly, but at least partly, tolerogenic. For example, 25% of $V\beta17^+$ cells was deleted when I-E class II MHC molecules were predominantly expressed by the thymic epithelium (29). By contrast, $\approx$70% of $V\beta11^+$ T cells was depleted under a very stringent system in which host-type bone-marrow-derived cells were thoroughly removed (10). One obvious difference between these systems and the one we report here is that these studies rely on the analysis of heterogeneous populations of T cells with potentially various TCR affinities. It is also possible that the deletion efficiency mediated by the superantigens differs, at least in a quantitative manner, from conventional peptide antigens (for review, see ref. 30). However, most importantly, the differential distribution of the available peptide antigens (or superantigens) probably determines which cells can delete the individual thymocytes. Because our *in vitro* model system relies on peptide-induced apoptosis, we have effectively bypassed the influence of differential access to the protein antigens or subsequent antigen-processing capabilities of individual non-T and T cells that may be present in the thymus. Thus, *in vivo*, the capacity of individual cell types to mediate thymocyte deletion may depend heavily on (*i*) the capability of the cells to have access to protein antigens from both exogenous and endogenous sources and (*ii*) the capability of the cells to process them effectively for presentation. Our data suggest that once the peptide antigen has been made available on the cell surface, most cells can induce clonal deletion of the immature thymocytes. The *in vitro* system described here should help in further defining the cellular and molecular events that accompany the onset of apoptosis in immature thymocytes, leading to clonal deletion of self-reactive T cells.

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