

Thy-1 Triggers Mouse Thymocyte Apoptosis Through a *bcl-2*-resistant Mechanism

By Anne-Odile Hueber, Graça Raposo, Michel Pierres,
and Hai-Tao He

From the Centre d'Immunologie Institut National de la Santé et de la Recherche Médicale-Centre National de la Recherche Scientifique de Marseille Luminy, 13288 Marseille Cedex 9, France

Summary

Programmed cell death plays an important role during thymocyte development, since a vast majority (97%) of mouse cortical thymocytes die in thymus, whereas only 3% of these cells are rescued from cell death and positively selected. Although it seems well established that thymocyte fate depends upon appropriate surface-expressed T cell receptor, little is known about the molecular mechanism(s) responsible for the massive thymocyte elimination that occurs in the thymus. We report here that Thy-1 is capable of triggering mouse thymocyte death in vitro through a *bcl-2*-resistant mechanism. We have previously shown that Thy-1 is involved in mouse thymocyte adhesion to thymic stroma through interaction with an epithelial cell ligand. To examine the Thy-1 signaling function in thymocytes, we have mimicked its interaction with stromal cells by culturing mouse thymocytes onto tissue culture plates coated with monoclonal antibodies (mAb) directed at distinct Thy-1 epitope regions. mAb recognizing determinants in a defined Thy-1 structural domain, but not others, were found to induce marked thymocyte apoptosis as evidenced by morphological and biochemical data. Use of a quantitative DNA dot blot assay indicated that Thy-1-mediated thymocyte apoptosis was not blocked by RNA or protein synthesis inhibitors, EGTA, or by cyclosporin A, and differed, therefore, from "activation-driven cell death". Moreover, Thy-1⁺-transfected, but not wild-type AKR1 (*Thy-1*^{-d}) thymoma cells underwent apoptosis after ligation with apoptosis-inducing, Thy-1-specific mAb. In contrast to thymocytes, the latter event was inhibitable by RNA and protein synthesis inhibitors, an indication that thymocytes, but not thymoma cells, contain the molecular components necessary for Thy-1-driven apoptosis. We further showed that Thy-1-triggered thymocyte death is a developmentally regulated process operative in fetal thymocytes from day 17 of gestation, but not in peripheral T cells. Indeed, the target of apoptosis by anti-Thy-1 was found to reside mainly within the CD4⁺8⁺3⁻ and CD4⁺8⁺3^{lo} double positive immature thymocyte subsets. Finally, it is of major interest that Thy-1-mediated apoptosis, which was found to be readily detectable in thymocytes from *bcl-2*-transgenic mice, represents a thus far unique experimental system for studying *bcl-2*-resistant thymocyte death mechanism(s).

Cell division and death regulate the generation of appropriate numbers and types of cells during development and maintain cellular homeostasis in adult animals. This naturally occurring cell death, which is referred to as programmed cell death (PCD)¹ (1, 2) depends upon both genetic and environmental factors (3) and usually occurs by apoptosis, a morphologically defined process including loss of cell volume, membrane blebbing, shrinkage and breakdown of nuclei (4),

and in many cases internucleosomal fragmentation of chromatin (5).

PCD is pivotal in thymic ontogeny. During T cell development, thymocyte precursors enter the thymus and mature in the vicinity of thymic stroma (6, 7). Before reaching the fully differentiated CD4⁺ or CD8⁺ single-positive (SP) phenotype, immature thymocytes undergo massive cell expansion (during which they rearrange their gene segments coding for the TCR) and stringent selection processes (8–10). Experimental evidence indicates that thymocytes recognizing self-antigen/MHC complexes or surface TCR-negative or -ignored thymocytes are eliminated (6, 11–14) by apoptosis (15, 16). In contrast, thymocytes recognizing self-MHC molecules are rescued from PCD (12, 17). Only 3% of cortical

¹ Abbreviations used in this paper: ADCD, activation-driven cell death; DEX, dexamethasone; DP, double-positive; Fd, fetal day; GPI, glycosylphosphatidylinositol; HSA, heat-stable antigen; PCD, programmed cell death; SP, single-positive.

thymocytes are positively selected and migrate to the periphery, whereas the great majority of thymocytes (97%) die in the thymus (6, 13).

In addition to thymocyte apoptosis depending upon the state of TCR engagement, defined external signals have been shown to promote thymocyte PCD. Thus, CD3-specific mAb can induce thymocyte apoptosis both *in vivo* (18) and in fetal thymic organ culture (19). Glucocorticoid-induced thymocyte death represents a classical model of apoptosis (14). It has been shown recently that mouse thymocytes could be killed by cytolytic T cells through a Fas/APO1-mediated mechanism (20). Several environmental signals appear, therefore, capable of triggering thymocyte apoptosis. However, the biological significance of these different thymocyte death pathways remains to be explored. It has been proposed that CD3-induced apoptosis could mimic the negative selection of autoreactive thymocytes (18, 19), whereas glucocorticoid-induced cell death is reminiscent of the elimination of nonselected immature thymocytes (14). Recent studies on the *bcl-2* protooncogene are of particular interest in this context. Originally isolated from the breakpoint of t(14;18) in human B cell follicular lymphoma cells, *bcl-2* encodes a protein that is located at the nuclear envelope, endoplasmic reticulum, and inner membrane of mitochondria (21, 22). Experiments using *bcl-2*-transfected cell lines and *bcl-2*-transgenic mice have shown that *bcl-2* behaves as a repressor of PCD in different tissues (2). In transgenic mice overexpressing *bcl-2* in the thymus, thymocytes become resistant to apoptosis induced by various stimuli such as anti-CD3 mAb, glucocorticoids, PMA, or radiation (23, 24). It is of marked interest that in such mice, no obvious alteration of either thymocyte homeostasis or negative selection has been observed (23, 24), an indication that *bcl-2*-resistant PCD mechanisms can be operative in these processes. In the same context, Nakayama et al. (25) have recently reported that Bcl-2 was dispensable for lymphocyte maturation.

Thy-1 is a surface glycoprotein of the Ig superfamily encoded by a well conserved gene (26, 27). This molecule is expressed in brain and hemopoietic stem cells of mammals (28, 29). It is also highly expressed in rodent thymocytes and used as a differentiation marker for mouse T lymphocytes (30). Several members of the Ig superfamily play important roles in cell-cell and cell-matrix recognition/adhesion processes (26), and their specific interactions with appropriate ligands have been shown to initiate signal transduction into cells (31). Thy-1 is anchored into the plasma membrane by a glycosylphosphatidylinositol (GPI) tail (27). GPI-linked proteins are known to interact with membrane lipids in a special way and to participate in microdomains likely related to morphologically defined structures, caveolae (32). Protein tyrosine kinases of the *src* family (p56^{lck}, p60^{lyn}, and p55^{hyn}) have been reported to be associated with these microdomains and to be coimmunoprecipitable with Thy-1 (33). Association of Thy-1 with the CD45 protein tyrosine phosphatase has also been detected (34). These findings make Thy-1, and more generally GPI-linked molecules, good candidates as signal-transducing molecules. Accordingly, Thy-1-specific mAb (35,

36), as well as mAb directed at other GPI-linked molecules (33), have been reported to be mitogenic for mature T cells.

Thy-1 surface expression peaks ($\sim 10^6$ molecules per cell) at the CD4⁺8⁺ double-positive (DP) stage of thymocyte development (27, 37). This expression is downregulated in mature SP thymocytes and in peripheral T cells (38). Mitogenic Thy-1-specific mAb can activate mouse SP TCR- α/β and TCR- γ/δ thymocyte subsets to proliferate (39, 40). Little is known, however, about the possible function of Thy-1 in immature thymocytes which represent 80–90% of thymocytes. We have recently shown that Thy-1 is capable of mediating mouse thymocyte adhesion to thymic stromal cells (41, 42). We report here that immobilized mAb, directed at determinants in a defined Thy-1 epitope region, assayed in an experimental system mimicking such a cellular interaction, can promote marked apoptosis in cultured immature DP thymocytes. This cell death pathway is developmentally regulated and occurs through a *bcl-2*-resistant mechanism.

Materials and Methods

Materials and Reagents. Swiss mice were maintained in our animal facilities and used at the age of 4–6 wk. Timed-pregnant Swiss mice were from Iffa-Credo (Les Ullis, France). E μ -*bcl-2* strain 36 transgenic mice were kindly donated by A. W. Harris (Walter and Eliza Hall Institute of Medical Research, Victoria, Australia). All reagents were from Sigma (Saint-Quentin-Fallavier, France), except Trypsin-EDTA (GIBCO-BRL, Cergy-Pontoise, France) and Dynabeads M-450 sheep anti-rat IgG (Biosys S.A., Compiègne, France). Dexamethasone (DEX) and PMA were prepared as 1 mM stock solutions in 95% ethanol and kept at -20°C . Cycloheximide and actinomycin D were prepared as 1 mM or 1 mg/ml stock solutions in H₂O, respectively, and kept at 4°C .

Cells. AKR1 (*Thy-1^d*) thymoma cells and the derivative TFX435 Thy-1.1⁺ transfected cells were kindly provided by R. Hyman (The Salk Institute, San Diego, CA). All cells were grown in DMEM supplemented with 1 mM sodium pyruvate and 5% FCS in a humidified 10% CO₂ atmosphere, except as otherwise indicated. Single thymocyte suspensions were prepared by teasing thymuses in culture medium (DMEM plus 10% FCS). Spontaneous thymocyte apoptosis, as measured by DNA fragmentation at the end of the culture period (24 h), was comprised between 5 and 10%. T cell-enriched splenocytes were obtained by the nylon-wool method.

mAb. The main characteristics of the mAb used in this study are listed in Table 1. Both the KT16 and G7 mAb were kindly donated by M. Millrain (Clinical Research Centre, Harrow, UK). mAb were purified from ascitic fluids and stored in PBS at -20°C . mAb coating of microtiter plates (37°C , overnight) was performed at a concentration of 25 $\mu\text{g}/\text{ml}$ in PBS, unless otherwise indicated. The wells were washed twice with DMEM and once with complete culture medium before use.

Cortisone Treatment. 5-wk-old mice were injected intraperitoneally with 2.5 mg/mice of hydrocortisone acetate (Roussel, Paris, France) at 48 and 24 h before the experiment day.

DNA Dot Blot Assay for Measuring Cell Death-associated DNA Fragmentation. This original DNA fragmentation assay, which will be detailed elsewhere (Hueber, A.-O., M. Pierres, and H.-T. He, manuscript in preparation) has been adapted from those previously described (43, 44). The assay relies on the fact that fiberglass can

only trap intact chromatin through vacuum aspiration, but not DNA fragments from apoptotic cells (43) or RNA. Briefly, at the end of the culture, cells were harvested onto fiberglass filters by vacuum aspiration. After extensive washings, the filters were sequentially incubated in ethanol and in 3X SSC, 0.1% SDS for 30 min, respectively, and saturated for another 30-min period in 5X Denhart's solution diluted in 89 mM Tris, 89 mM boric acid, and 2 mM EDTA, pH 8.4. The filters were stained with ethidium bromide (5 µg/ml in the same solution) for 15 min and washed further with ethanol to eliminate nonspecific binding. Fluorescent filters were photographed and digitized by The Imager® (Appligene, Illkirch, France). The quantitation of digitized spots was made using the NIH image version 1.42 software system. Percent fluorescence was defined as 100 × the fluorescence intensity ratio between treated and untreated cells. The values were expressed as the means (± SE) of four to six identical assays.

Agarose Gel Electrophoresis of DNA. Cultured thymocytes in microtiter plates were centrifuged for 10 min at 1,200 g. Cell pellets were mixed with 30 µl of 10 mM EDTA, 50 mM Tris-HCl, pH 8.0, 0.5% sodium lauryl sarkosinate buffer, and samples were incubated for 1 h at 50°C. RNase A (10 µl of a 0.5 µg/ml solution) was added and samples were incubated further for 1 h at 50°C. The temperature was increased to 70°C before adding 10 µl of 10 mM EDTA, pH 8.0, 1% low-melting temperature agarose, 0.25% bromophenol blue, and 40% sucrose. Samples (20 µl) were loaded into wells of a 1.5% agarose gel and run at 10 V overnight in 89 mM Tris, 89 mM boric acid, and 2 mM EDTA, pH 8.4. Ethidium bromide (0.5 µg/ml) was added to the gel before electrophoresis. DNA was visualized and photographed by The Imager®.

Electron Microscopy. Thymocytes (10⁶), cultured in 24-well plates, were centrifuged at 1,200 g for 10 min and the medium

was replaced by Trypsin-EDTA for 15 min at 37°C. The culture plates were assembled with empty plates, turned upside down, and centrifuged at 2,000 g for 15 min at 4°C. The solution of Trypsin-EDTA was discarded and replaced by cold PBS. Thymocytes were then transferred in Eppendorf tubes and centrifuged at 2,000 g for 15 min at 4°C. Pelleted cells were fixed with 2% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4) at 4°C for 2 h. After several washes in the same buffer, cell pellets were postfixed with 2% OsO₄ in the same buffer for 45 min, and dehydrated in 30, 50, 70, 95, and 100% ethanol, followed by two incubations in 100% propylene oxide for 10 min. Specimens were embedded in epon 812 according to standard procedures. Ultra-thin sections were cut with an ultramicrotome (LKB-Ultratome III, Bromma, Sweden) and examined on an electron microscope (model H-600; Hitachi Ltd., Tokyo, Japan) after counterstaining with uranyl acetate and lead citrate.

Immunomagnetic Cell Depletion. Thymocytes from day 18 fetal mice were incubated with CD3-specific mAb KT3 diluted in culture medium for 30 min at 4°C. After washes, the Dynabeads sheep anti-rat IgG was added to the cell suspension, and the mixture was rotated for 30 min at 4°C. Thymocytes with attached beads were then removed, using a Dynal Magnetic Particle Concentrator (Biosys S.A., Compiègne, France).

Results

Immobilized Thy-1-specific mAb Induce Mouse Thymocyte Apoptosis. As an approach to analyze Thy-1-mediated signaling, mouse thymocytes were cultured in microtiter plates coated with mAb directed at different Thy-1 determinants (36, 45). It was observed that more than 50% of the thymo-

Table 1. mAbs Used in this Study

mAb	Specificity	IgH isotype	Reference
KT16	Thy-1A*	rat IgG _{2c}	45
G7	Thy-1A	rat IgG _{2c}	45
H194-539	Thy-1A	rat IgM	58
H129-93	Thy-1.2A	rat IgG _{2c}	36
H140-150	Thy-1B	rat IgG _{2b}	36
H154-177	Thy-1C	rat IgG _{2b}	36
H154-200	Thy-1C	rat IgG _{2b}	36
H155-124	Thy-1C	rat IgG _{2b}	36
145.2C11	CD3	hamster IgG	59
KT3	CD3	rat IgG _{2a}	60
H129-19	CD4	rat IgG _{2a}	61
H35-17	CD8	rat IgG _{2b}	62
H194-112	CD26	rat IgG _{2a}	63
H193-16	CD45	rat IgG _{2a}	42
H35-89	LFA-1α (CD11a)	rat IgG _{2b}	62
H194-563	HSA	rat IgG _{2b}	64
49-h4.3	ThB	rat IgG _{2c}	65

* Specificity for Thy-1 epitope regions (A, B, and C).

cytes cultured for 24 h on Thy-1-specific mAb KT16-coated plates displayed specific morphological changes including reduction of cell volume, condensation of chromatin, and flocculation of the cytoplasm, as visualized by electron microscopy (Fig. 1, *a* and *b*). These KT16-induced morphological modifications (Fig. 1 *d*) were not observed with a control mAb (Fig. 1 *e*) and resembled the features of DEX-induced apoptotic cell death (4, and Fig. 1 *f*). Indeed, cells lost surface microvilli, displayed membrane blebbing and a nucleus condensed into a uniform mass located at one edge of the cell. Several stages of Thy-1-mediated apoptosis occurring in a same culture are shown in Fig. 1, *g-j*. Enhanced internucleosomal DNA fragmentation was observed in thymo-

cytes cultured on Thy-1A-specific mAb KT16, but not on mAb H194-563 or H154-200 (directed at the heat-stable antigen (HSA) or Thy-1C, respectively, see Table 1) (Fig. 2).

Measurement of Thymocyte Apoptosis by Means of DNA Dot Blot Assay. We developed a DNA fragmentation assay in order to quantify apoptosis of nonproliferating cultured thymocytes. This assay took advantage of the observation (43, 44) that harvesting cells onto glassfiber filters permitted the separation of intact chromatin (retained on the filter) from DNA fragments of apoptotic cells (been washed through). Indeed, quantitation of apoptosis could be deduced from the measurement of DNA on the filter, a reflection of the number of remaining viable cells in the culture. As can be seen in

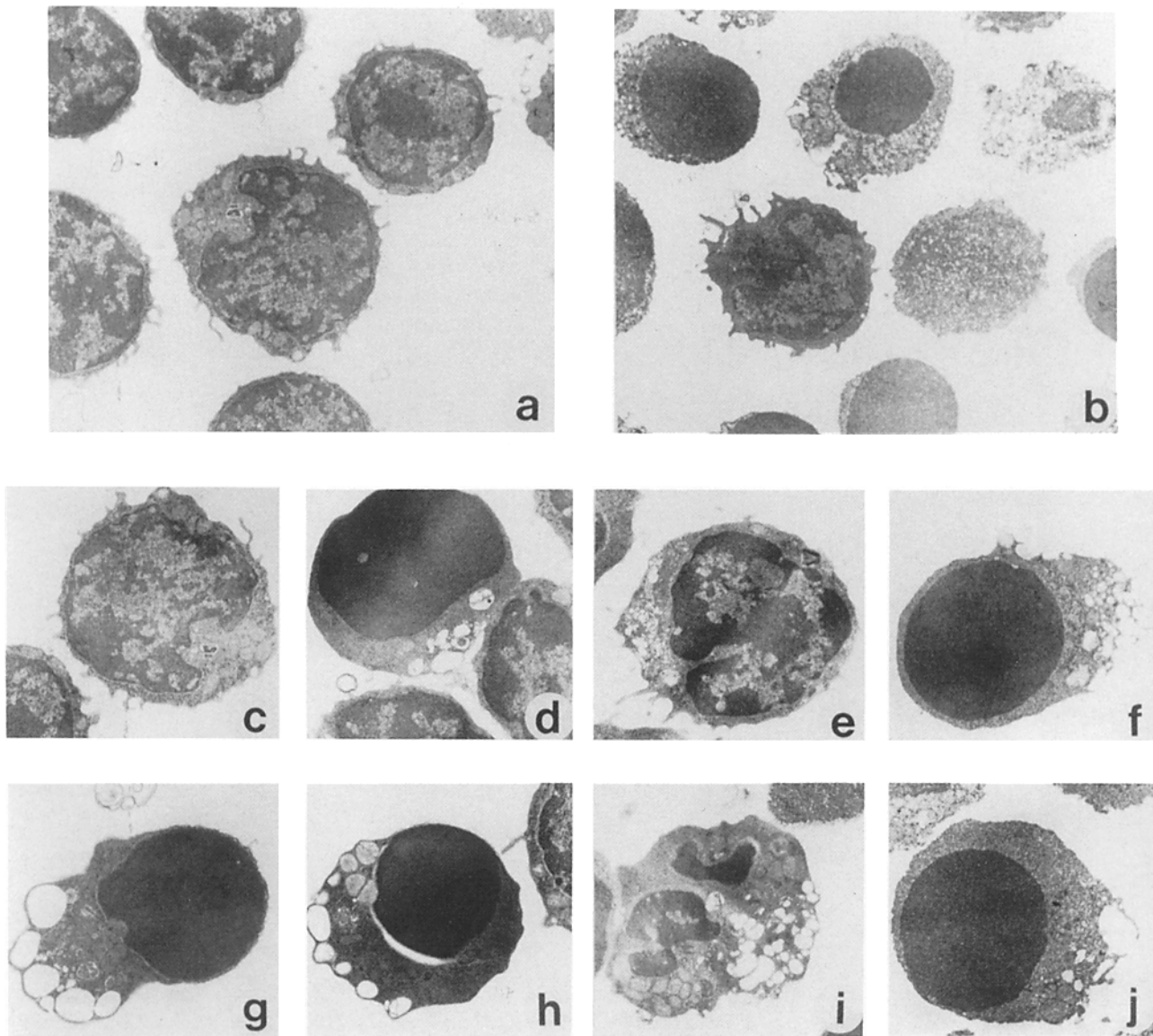


Figure 1. Electron microscopic examination of Thy-1-mediated thymocyte apoptosis. Thymocytes cultured in the absence (*a*) or presence (*b*) of plastic-bound Thy-1-specific mAb KT16, for 24 h at 37°C. Thymocyte morphology in control culture (*c*); in the presence of KT16 (Thy-1-specific mAb) (*d*); H194-563 (HSA-specific mAb) (*e*); or after treatment with DEX (*f*). (*g-j*) Different stages of Thy-1-mediated apoptosis including that induced by KT16 mAb (*g*, *i*, and *j*) and by H194-539 mAb, an IgM recognizing like the KT16 Thy-1A epitope region (*h*). Bar (*a* and *b*) 1.14 μm ; (*c-f*) 1.2 μm ; (*g* and *h*) 1 μm ; (*i* and *j*) 1.2 μm .

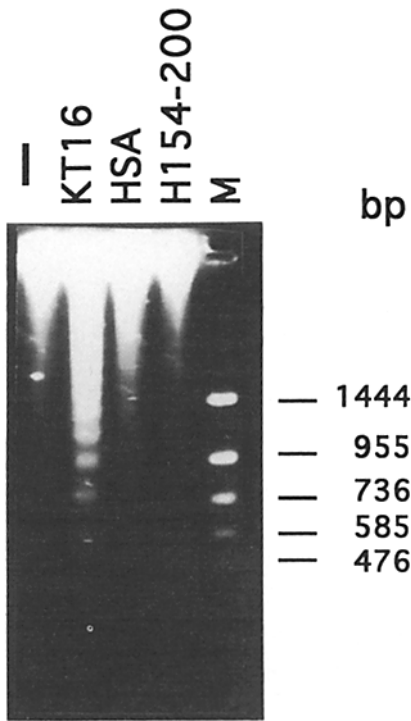


Figure 2. Interchromosomal DNA fragmentation of thymocytes upon culture on plastic-immobilized KT16 mAb. Thymocytes (10^5) were cultured in the presence or absence of mAb for 24 h at 37°C. Cell lysates were prepared as described in Materials and Methods. Fragmented DNA

Fig. 3 A, a linear relationship was observed between DNA fluorescent staining and the number of living thymocytes. Use of this DNA dot blot assay revealed, in accordance with previous studies, that DEX (10^{-7} M) induced specific death of most thymocytes (>80%) after 24 h of culture. Moreover, immobilized mAb KT16 but not a control HSA-specific mAb triggered apoptosis of more than 50% thymocytes (Fig. 3 B). The dose-response curve of thymocyte apoptosis on plates coated with increasing amounts of mAb KT16 (Fig. 4) indicated that Thy-1-mediated death was maximal at a coating concentration of 10 μ g/ml.

Apoptosis Is Induced by mAb Directed at Determinants in a Defined Thy-1 Structural Domain. To ascertain the specificity of thymocyte apoptosis induction by KT16 mAb, we tested several Thy-1-specific mAb (all of the IgG isotype) directed at determinants in spatially distinct Thy-1 epitope regions (i.e., A, B, and C) (Table 1). After 24 h of culture, thymocyte apoptosis, as measured by DNA fragmentation, was assayed as described above. It was observed that Thy-1B (H140-150)- and Thy-1C (H154-177, H154-200, and H155-124)-reactive

and intact chromatin were resolved on a 1.5% agarose gel and stained with ethidium bromide. Lanes (-) Untreated cells; (KT16) cells incubated with KT16 mAb; (HSA) cells incubated with HSA-specific mAb H194-563; (H154-200) cells incubated with H154-200 mAb directed at a Thy-1 determinant distinct from that recognized by KT16 mAb; and (M) molecular weight markers.

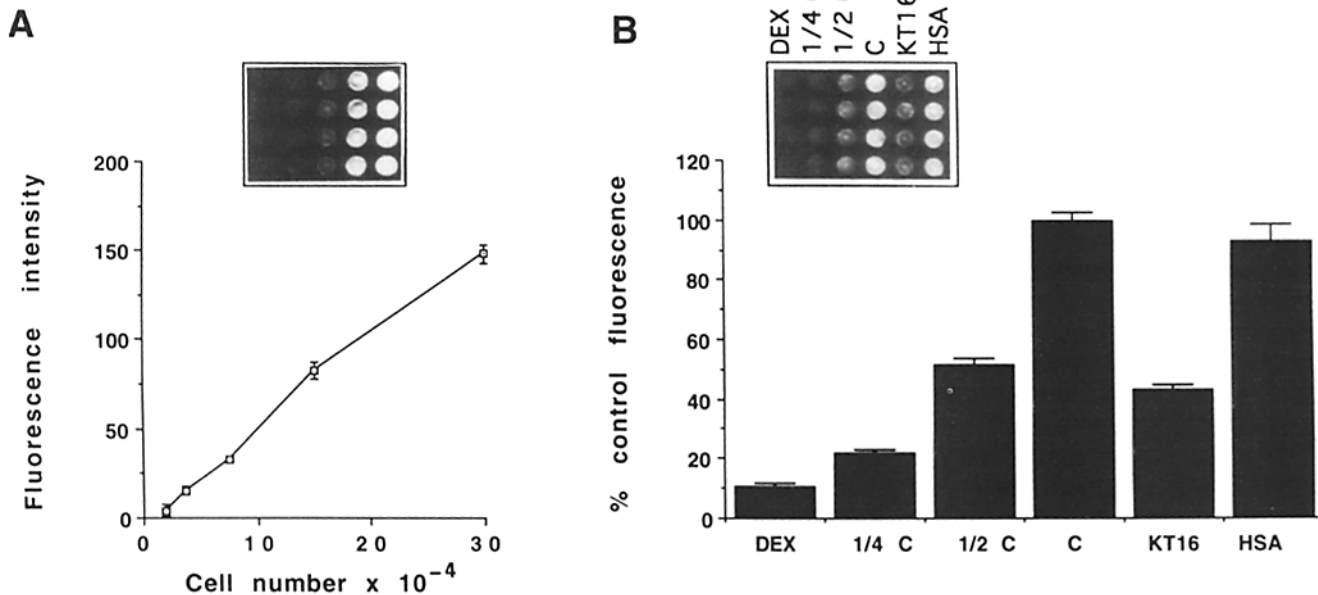


Figure 3. Thy-1-mediated thymocyte apoptosis as measured by DNA dot blot assay. (A) The linear relationship between the number of living cells harvested and DNA staining on the filter. Increasing amounts of freshly collected thymocytes were distributed in microtiter plates and analyzed as described in Materials and Methods. Fluorescent intensity (arbitrary units) of the filter spots (inset) was measured by the NIH image version 1.42 software program. (B) Thymocyte apoptosis quantified by the reduction of living cell number (i.e., DNA staining) at the end of culture. Thymocytes (1.5×10^3) from 4–6-wk-old Swiss mice were cultured in DMEM supplemented with 10% FCS, in the absence (lane C) or presence of immobilized mAb (coating concentration, 25 μ g/ml) KT16, H194-563 (anti-HSA), or DEX (10^{-7} M). After 24 h at 37°C, thymocytes were analyzed by DNA dot blot assay as in A. In lanes 1/2C and 1/4C, 7.5×10^4 and 3.75×10^4 thymocytes were cultured, respectively. The results were expressed as percent fluorescence of untreated cells.

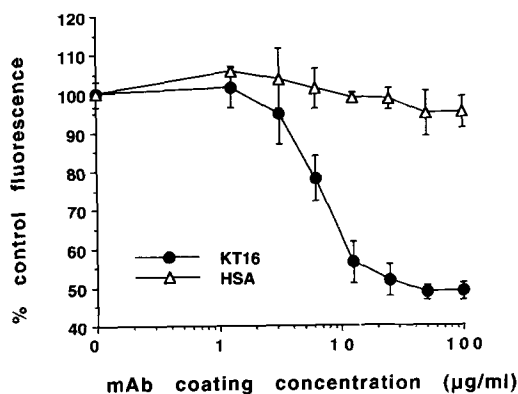


Figure 4. KT16 mAb promotes thymocyte apoptosis in a dose-dependent manner. KT16 and a control mAb (H194-563, anti-HSA) were coated to the plates at increasing concentrations in PBS. Thymocytes (1.5×10^5) were assayed for apoptosis as described in the legend to Fig. 3 B. The results were expressed as percent fluorescence of untreated cells.

mAb (36) were unable to promote thymocyte apoptosis (even on plates coated with 100 µg/ml mAb solution, data not shown), regardless of their mitogenic capacity (Fig. 5 A). Two mAbs (KT16 and G7), as well as the IgM H194-539 (see Fig. 1), all of which recognize Thy-1 epitope region A, induced marked thymocyte apoptosis, whereas another mAb H129-93 directed at a Thy-1 polymorphic determinant in the same epitope region was less efficient in this assay. We also tested the effects of several mAb directed at thymocyte surface proteins, including signaling molecules such as CD3, CD4, CD8, CD26, and CD45, as well as HSA and ThB, both of which represent abundant GPI-anchored thymocyte molecules. In addition to KT16, only one mAb (145.2C11, CD3 specific) was found to induce thymocyte death (Fig. 5 B). However, as already reported (46), the *in vitro*-induced, 145.2C11-mediated thymocyte death was much lower than that obtained *in vivo*. These observations further confirmed the specificity of KT16-inducing apoptosis. It should be emphasized, however, that such results did not rule out the role

Table 2. *Thy-1-mediated Thymocyte Apoptosis Is Not Blocked by Cycloheximide, Actinomycin D, or EGTA*

Treatment [§]	Percent fluorescence*		
	Anti-Thy-1 [‡]		
	KT16	H154-200	DEX
Control	50.4 ± 3.4	85.5 ± 2.6	11.6 ± 0.8
Cycloheximide	47.6 ± 2.3	85.7 ± 6.2	78.6 ± 5.5
Actinomycin D	45.8 ± 3.3	98.4 ± 5.4	90.0 ± 3.6
EGTA	49.2 ± 6.7	97.7 ± 5.3	11.3 ± 4.3

* Results were expressed as percent fluorescence (means from three separate experiments ± SEM) of untreated cells, in the absence or presence of inhibitors, respectively.

‡ mAb coating was performed at a concentration of 25 µg/ml in PBS.
 § Cycloheximide and actinomycin D concentrations were 10 µg/ml and 2 µg/ml, respectively. EGTA was added (3.2 mM) in the presence of 1.8 mM MgCl₂. No toxic effect was detected in the presence of these reagents, at the concentrations indicated.

|| DEX was added at a final concentration of 10⁻⁷ M.

of these signaling molecules (other than CD3) in thymocyte apoptosis. In further experiments, two mAb (KT16 and H154-200) with similar affinity for thymocytes as determined by a centrifuge-based adhesion assay (41) (data not shown), were used as prototypic apoptosis-inducer or control reagents, respectively.

Thy-1-mediated Apoptosis Is Not Inhibited by RNA or Protein Synthesis Inhibitors and Differs from ADCD. Inhibition of macromolecule synthesis has been reported to prevent apoptosis in some cases (3, 14). Data summarized in Table 2 indicate that the translation inhibitor cycloheximide or the transcription inhibitor actinomycin D were unable to modify KT16-induced thymocyte apoptosis. In contrast, both compounds were capable of completely reversing DEX-induced

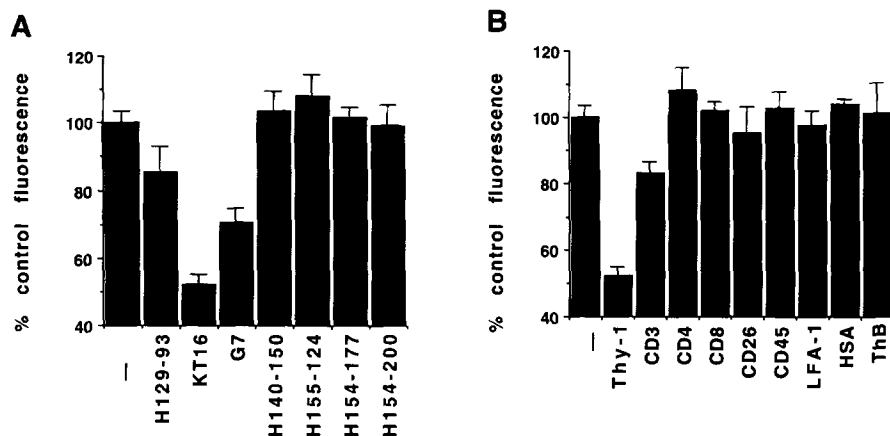


Figure 5. Thymocyte apoptosis is induced by mAb directed at determinants in a defined Thy-1 structural domain. mAb-induced thymocyte apoptosis was tested as described in the legend to Fig. 3 B; see Table 1 for the characteristics of the mAb used. (A) mAb directed at determinants in a structural domain of Thy-1 (i.e., epitope region A), but not those directed at other Thy-1 domains (i.e., epitope regions B and C) were efficient at triggering thymocyte apoptosis. (B) A variety of mAb specific for signaling molecules including CD3, CD4, CD8, CD26, CD45, and LFA-1, as well as those specific for other GPI-anchored molecules (e.g., HSA and ThB) were analyzed for thymocyte apoptosis induction, as compared with Thy-1-specific mAb KT16. The results were expressed as percent fluorescence of untreated cells (-).

thymocyte death. These observations indicated that macromolecules putatively at play in the Thy-1-dependent apoptosis pathway were already present before KT16-mediated thymocyte triggering.

Previous studies have shown that mAb directed at GPI-anchored molecules, such as Thy-1 and Ly-6, are capable of activating mature T cells to proliferate in a manner similar to antigen- or anti-TCR/CD3 mAb-driven T cell activation (33). The same stimuli have also been found to elicit T hybridoma or nontransformed T cell death (47, 48). This ADCD is of apoptotic nature and can be prevented by RNA and protein synthesis inhibitors, and the two compounds EGTA and cyclosporin A (47-49). Interestingly, all these reagents did not affect Thy-1-mediated thymocyte death (Table 2, and data not shown), which appears, therefore, distinct from ADCD. Furthermore, anti-Thy-1 mAb-induced ADCD has been reported to be operating in mature, but not in immature T cells (48).

Thy-1-specific mAb Promotes Death of Thy-1⁺-transfected AKR1 (Thy-1^{-d}) Thymoma Cells which Can Be Blocked by RNA and Protein Synthesis Inhibitors. We next tested the ability of KT16 mAb to elicit death signals in TFX435 cells, a Thy-1.1⁺-transfectant of mouse AKR1 (Thy-1^{-d}) thymoma cells which fail to express Thy-1, because of a subchromosomal deletion encompassing the Thy-1 gene (50). These AKR1 cells display the DP immature phenotype (data not shown). As can be seen in Fig. 6 A, KT16 mAb specifically induced apoptosis of 50% of the TFX435, but not of the wild-type AKR1 (Thy-1^{-d}) cells, after 24 h of culture in the presence of 0.5% FCS, during which these cells were not dividing. In contrast to that observed in thymocytes, KT16-induced TFX435 cell death could be completely inhibited by macromolecule synthesis inhibitors (Fig. 6 B).

Developmental Regulation of Thy-1-mediated Mouse T Cell Death. Since PCD is known to be regulated during development, we next explored whether Thy-1-mediated T cell apoptosis could also be developmentally controlled. We first

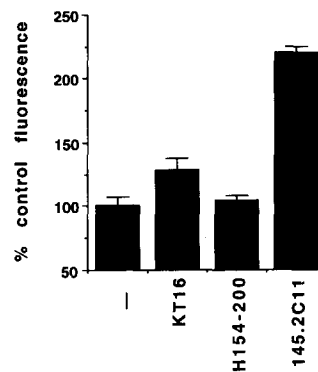


Figure 7. Mature peripheral T cells are refractory to apoptosis induction by Thy-1-specific mAb. T cell-enriched splenocytes (2×10^5 cells/well) were cultured for 48 h at 37°C, in the presence or absence of mAb. DNA of cultured cells was measured as described in the legend to Fig. 3 B. Thy-1-specific mAb KT16, H154-200, and the CD3-specific mAb 145.2C11 were tested. The results were expressed as percent fluorescence of untreated cells (-).

tested the effect of immobilized anti-Thy-1 mAb on peripheral T cells. Nylon wool-enriched splenic T cells were cultured for 48 h under the conditions described above, and analyzed by DNA dot blot assay (Fig. 7). The proliferative responses of these cells to CD3-specific mAb resulted in strongly enhanced DNA staining. KT16 mAb promoted a very weak cell proliferation (rather than cell death), whereas the nonmitogenic, Thy-1-specific mAb H154-200 was devoid of any effect. We then examined whether Thy-1-dependent thymocyte death could be regulated during intrathymic T cell development. Fetal and neonatal thymocytes were assayed for their sensitivity to KT16 mAb. It was observed that mouse thymocytes, although refractory to Thy-1 mAb-induced apoptosis up to fetal day (Fd) 16, became sensitive to this cell death induction from Fd 17 (Fig. 8). Interestingly, DEX-induced thymocyte apoptosis displayed a similar regulation to that observed with KT16 mAb.

Thy-1 Triggers Apoptosis of DP Immature Thymocytes. To further assess the potential physiological role of Thy-1-triggered apoptosis in T cell development, we carried out experiments in order to phenotype the target cells for anti-Thy-1-induced apoptosis. It was observed that thymocytes obtained

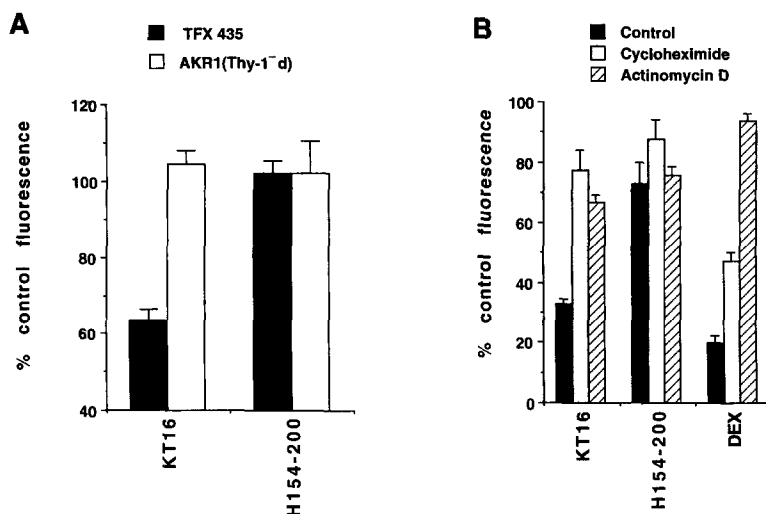


Figure 6. KT16 mAb triggers apoptosis of transfected AKR1 (Thy-1^{-d}) thymoma cells expressing the Thy-1 gene. (A) Thy-1-transfected (TFX435) and wild-type AKR1 (Thy-1^{-d}) thymoma cells (5×10^4) were cultured in the presence of immobilized KT16 mAb or control mAb H154-200 at 37°C for 24 h. FCS concentration in the medium was reduced to 0.5% to maintain good viability in nonproliferating culture cells. Cell death was assayed as described in the legend to Fig. 3 B. The results were expressed as percent fluorescence of untreated TFX435, or wild-type AKR1 (Thy-1^{-d}) cells, respectively. (B) Thy-1-dependent apoptosis of TFX435 is inhibitable by cycloheximide and actinomycin D. Thy-1-triggered apoptosis was tested with KT16 mAb, mAb H154-200 and DEX were used as controls. The inhibitors were found to be non-toxic at the concentrations used in these experiments (i.e., 1 and 0.2 $\mu\text{g/ml}$ for cycloheximide and actinomycin D, respectively). The results were expressed as percent fluorescence of untreated TFX435 cells, cultured in the absence or presence of inhibitors, respectively.

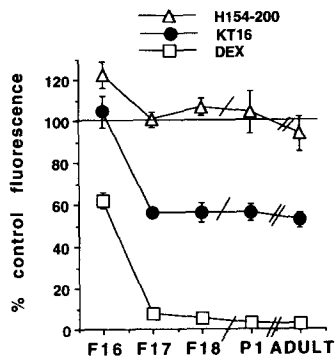


Figure 8. Intrathymic regulation of Thy-1-dependent apoptosis. Thymocytes from Fd 16 (F16), 17 (F17), 18 (F18), postnatal day 1 (P1), and adult (ADULT) NMR1 mice were collected and apoptosis induced by KT16 mAb, and DEX were assayed as described in the legend to Fig. 3 B. mAb H154-200 was used as control. The results were expressed as percent fluorescence of untreated thymocytes from mice of corresponding ages, respectively.

from hydrocortisone-treated mice, in which the DP immature cells were eliminated, became resistant to KT16-mediated cell death (Fig. 9 A). This result indicated that DP, but not double negative or SP thymocytes, were the main target for Thy-1-dependent apoptosis, an observation consistent with the regulation during thymic ontogeny described above. We further evaluated the sensitivity to apoptosis of DP $CD4^+8^+3^-$ and $CD4^+8^+3^0$ thymocyte subsets by performing immunomagnetic separation before cell death assay. We favored this experimental design rather than the simultaneous flow cytometry phenotyping techniques after apoptosis induction because thymocyte recovery (without trypsinization) from Thy-1-specific mAb-coated plates caused substantial cell damage. Depletion from Fd 18 thymocytes of the $CD4^+8^+3^0$ subset by anti-CD3 mAb, which makes up to ~20% of total thymocytes at this age (51 and data not shown), reduced KT16-mediated apoptosis from 50 to 35% (Fig. 9 B). Altogether, these results, along with the observation that Thy-1-triggered apoptosis of Fd 17 thymocytes in which the DP cells are mainly negative for CD3 surface expression (51), indicated that apoptosis induction by anti-Thy-1 mAb occurred primarily in both the $CD4^+8^+3^-$ and $CD4^+8^+3^0$ DP thymocyte subsets.

Thy-1 Triggers Thymocyte Apoptosis through a bcl-2-resistant Mechanism. Overexpression of the *bcl-2* protooncogene is known to protect cells from apoptosis induced by a variety

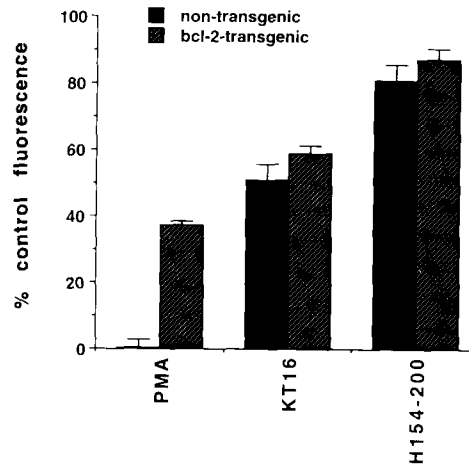


Figure 10. Thy-1 triggers thymocyte apoptosis through a *bcl-2*-resistant mechanism. Thymocytes from *bcl-2*-transgenic and nontransgenic littermate mice were assayed for Thy-1-mediated apoptosis (by the KT16 mAb), as described in the legend to Fig. 3 B. mAb H154-200 were used as control. Note the significant protection from PMA-induced cell death in *bcl-2*-transgenic mice. The results were expressed as percent fluorescence of untreated thymocytes from *bcl-2*-transgenic or nontransgenic littermate mice, respectively.

of stimuli (21). In $E\mu$ -*bcl-2* transgenic mice that carry a human *bcl-2* cDNA under the control of the 5' *Igh* enhancer and overexpressing the transgene within the lymphoid cell compartment, a marked inhibition of thymocyte apoptosis induced by a series of compounds including CD3-specific mAb, glucocorticoids, or PMA has been observed (24). We analyzed, therefore, the Thy-1-mediated thymocyte apoptosis in such $E\mu$ -*bcl-2*-transgenic mice. Data summarized in Fig. 10 show that thymocytes from either such transgenic mice or their nontransgenic littermates were similarly sensitive to apoptosis induction by KT16 mAb. Conversely, in accordance with previous results (24), marked survival enhancement was detected in thymocytes from $E\mu$ -*bcl-2*-transgenic mice after PMA treatment since the survival was <1% in normal thymocytes, but about 40% in transgenic thymocytes.

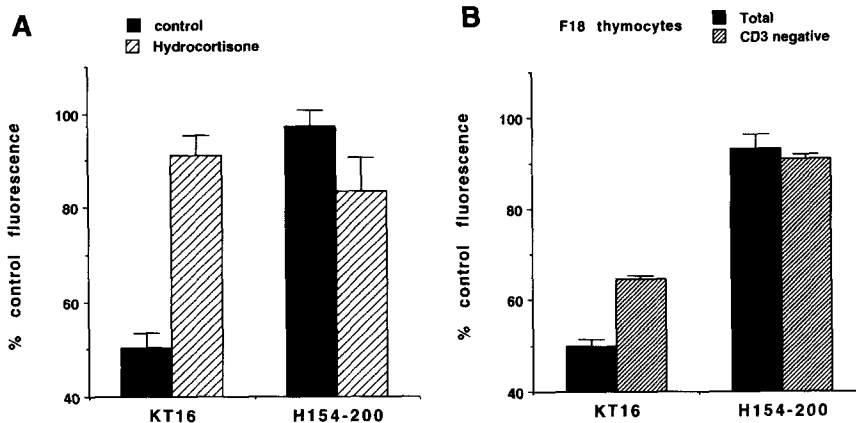


Figure 9. Thy-1 triggers apoptosis of DP immature thymocytes. (A) Thymocytes from control and hydrocortisone-treated mice were assayed for KT16 mAb-induced apoptosis, as described in the legend to Fig. 3 B. mAb H154-200 was used as control. The results were expressed as percent fluorescence of untreated thymocytes. (B) Thymocytes from day 18 fetal mice were submitted to depletion with Dynabeads coated with or without CD3-specific mAb KT3. The remaining cells corresponding to $CD3^-$ and total thymocytes, respectively, were assayed for KT16-mediated apoptosis, as described in the legend to Fig. 3 B. mAb H154-200 was used as control. The results were expressed as percent fluorescence of untreated thymocytes.

Discussion

In this study, we aimed at analyzing the signaling events in mouse thymocytes resulting from solid-phase bound mAb-mediated Thy-1 ligation. We have shown that Thy-1 could trigger marked thymocyte apoptosis through a *bcl-2*-resistant and developmentally regulated mechanism. A similar experimental approach has permitted a functional analysis of CD40-dependent B cell activation (52), before the cDNA cloning of its ligand (53). Solid phase-bound, neural cell adhesion molecule (NCAM)-specific mAb have also been successfully used to examine the role of NCAM in contact-dependent inhibition of fibroblastic cell growth (54). It is of interest that in both CD40 and NCAM studies, soluble mAb were devoid of signaling effects. In this study, we also found that soluble Thy-1-specific mAb could promote only very weak thymocyte death (data not shown). This observation is reminiscent of the failure of soluble anti-Thy-1 mAb to promote significant thymocyte death both in vitro (46) and in fetal thymic organ cultures (19). The reason for this difference is obscure especially because soluble Thy-1-specific mAb cross-linked by a secondary anti-Ig reagent failed to induce thymocyte death (data not shown). Indeed, soluble Thy-1-specific mAb (in particular those recognizing epitope region A) have been reported to induce both Thy-1 clustering and T cell aggregation, even in the absence of cross-linker (45). It is possible that such aggregation allows additional signaling in thymocytes which would alter Thy-1-dependent apoptosis induction. It is noteworthy, however, that soluble Thy-1-specific mAb have been shown to potentiate CD3-driven thymocyte apoptosis when used as Thy-1-CD3 heterocross-linked mAb (46).

mAb (i.e., KT16, H194-539, and G7) directed at determinants in a particular Thy-1 structural domain were found to be potent thymocyte apoptosis inducers. These findings indicate that the sole interaction of any immobilized mAb with Thy-1 is not sufficient, per se, for promoting thymocyte death, which rather depends on their ability to bind a Thy-1 active domain.

Early studies have shown that mAb directed at GPI-anchored molecules (e.g., Thy-1 and Ly-6) are capable of activating T cells to proliferate in a CD3-dependent fashion (33). In addition, the same reagents have been found to induce ADCD in both transformed T hybridoma and nontransformed T cell lines (47, 48). Although ADCD can be prevented by RNA or the protein synthesis inhibitors, EGTA or cyclosporin A (47-49), we found Thy-1-dependent thymocyte apoptosis to be insensitive to such compounds. These two types of cell death appear, therefore, unrelated to each other, a notion consistent with the observation that Thy-1-dependent ADCD is exclusively observed in mature T cells (48).

An important observation in this study bears on the developmental regulation of Thy-1-mediated cell death. The earliest cortical DP thymocytes are detectable at Fd 16 and subsequently enter the selection processes (6, 13, 51). The nonselected CD4⁺8⁺3⁻ and CD4⁺8⁺3^{lo} DP thymocytes, which represent the surface TCR-negative and -ignored cell

populations, respectively, undergo a 3.5-d end stage (13). Our results indicate that Thy-1-triggered fetal thymocyte apoptosis becomes operative at this period and that the main targets of this apoptosis reside within both the CD4⁺CD8⁺3⁻ and CD4⁺8⁺3^{lo} DP thymocyte subsets. It is tempting to speculate, therefore, that Thy-1-mediated apoptosis is involved in PCD of the nonselected thymocytes during thymic maturation. Our ongoing analysis of Thy-1 stromal ligand distribution in thymus, may give important clues in this respect. The molecular mechanism of the intrathymic regulation for Thy-1-dependent apoptosis is presently unknown. Indeed, the variation of Thy-1 surface expression in thymocytes (i.e., very high from day 17, and slightly reduced at day 16 of gestation) (37) represents a parameter to be considered in this context. However, the fact that both DEX- and Thy-1-triggered thymocyte apoptosis were similarly regulated during development suggests that other intracellular control events could be at play. Later on, in mature T cells, some regulatory mechanisms, distal to the initial signaling events, could repress the death program, since such T cells are resistant to Thy-1-, CD3-, or glucocorticoid-induced apoptosis.

The removal by apoptosis of nonselected DP cortical thymocytes is likely to be of major physiological importance by making, for instance, appropriate room for efficient selection processes (14). It is generally assumed that death of such thymocytes occurs independently of external stimuli, a contention supported by the observation that thymocytes die spontaneously *ex vivo*. However, recent studies on the survival gene *bcl-2* have challenged this argument. Expression of Bcl-2 protein can protect cells from PCD in different tissues (2, 21). In transgenic mice overexpressing *bcl-2* in the thymus, the life span of thymocytes in culture is markedly increased. T lymphoid cell homeostasis remains however unaffected in these transgenic mice, since the whole T cell number as well as the major T cell subsets were found to be normal both in the thymus and the periphery (23, 24). Although external stimuli like anti-CD3 mAb or glucocorticoids are known as apoptosis inducers capable of triggering massive thymocyte death both in vivo and in vitro (14, 18, 19), their role in thymic PCD was also questioned by the fact that thymocytes from *bcl-2*-transgenic mice resisted efficiently to death induced by such factors (23, 24). These findings suggest that other cell death pathways can be responsible for the *bcl-2*-resistant thymocyte elimination. The ability of Thy-1 to trigger thymocyte apoptosis in *bcl-2*-transgenic mice is of interest in such a context, and also a strong indication that this apoptosis does not result from an acceleration of the "spontaneous" thymocyte death, as *bcl-2*-transgenic thymocytes show remarkable long life spans in vitro.

We suggest that several internal and external death program components are acting to accurately eliminate the large number of nonselected thymocytes. Our data showing that Thy-1-induced apoptosis proceeds in the absence of new macromolecule synthesis suggests that immature DP thymocytes are programmed to die in response to an external stimulus. In contrast, apoptosis induction in AKR1 thymoma cells could

depend on the activation of a "suicide gene", a process inhibitable by cycloheximide or actinomycin D. Hence, signaling through Thy-1 can also induce cell death in cells that do not already contain all the factors required for apoptosis.

It could be envisioned that Thy-1-triggered apoptosis is also involved in the negative selection of self-reactive thymocytes. Engagement of CD8 to MHC class I molecules (55,

56) and LFA-1 to intercellular adhesion molecule 1 (57) have been found to cooperate with TCR/CD3 in driving autoreactive T cell elimination in some experimental systems. Thy-1, like CD8 and LFA-1, could hence modulate, through its adhesive and signaling functions, both the overall affinity of the interactions between thymocytes and thymic stromal cells and the transduction mechanisms involved in thymocyte PCD.

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Address correspondence to Dr. Hai-Tao He, Centre d'Immunologie INSERM-CNRS de Marseille Luminy, Case 906, 13288 Marseille Cedex 9, France.

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