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Receptor-stimulated death pathway is opened by antigen in mature T cells

(programmed cell death/T-cell maturation/tolerance)

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ABSTRACT **Clonal deletion provides an important mechanism for the elimination of autoreactive T cells. Deletion is accomplished by programmed cell death directed by interaction of the T-cell receptor (TCR) of the developing thymocyte with major histocompatibility complex elements in the thymic environment. In this report we present evidence to support the hypothesis that the activation and the maturation state of the T cell may be important in coupling the TCR to the "death program." We show that coupling of the TCR to the death program is open during maturation but closed in naive mature T cells. However, during primary antigenic stimulation, coupling between the TCR and the death program is reopened, as demonstrated by the stimulation of the death of these cells by immobilized anti-TCR. Our results suggest that further examination of mature cells that are either resistant or sensitive to receptor-stimulated death may lead to the identification of the components of the death pathway and may provide clues to the regulation of their coupling to TCR signals.**

Death of specific cells plays a major role in shaping morphological and functional maturity in a variety of systems. It is active in tail resorption, digit and neuronal network formation, and clonal deletion of autoreactive T cells. A major question in T-cell biology is how the T-cell receptor (TCR) can stimulate both positive selection and death of autoreactive cells (clonal deletion). These seemingly antagonistic responses appear to be regulated during ontogeny by the interaction of the thymocyte–TCR complex (the clonally distinct chains of the TCR with its associated pan-T-cell–CD3 complex) with its environment (1–3). Based on these experiments (1–3), both positive selection and negative selection appear to require, in addition to TCR stimulation, an interaction of thymic stromal major histocompatibility complex class I or class II antigens with CD8 or CD4, respectively, on the surface of the developing thymocyte. This complex interaction in the developing thymocyte is, therefore, homologous to that required in the periphery for activating a functional (lytic, proliferative, or lymphokine secretion) T-cell response.

Deletion of autoreactive T cells apparently occurs because the signaling pathway between the TCR and the "death program" is open during development. Smith *et al.* (4) have observed that a monoclonal antibody (mAb) directed at the CD3 complex caused death of immature thymocytes in organ culture but stimulated proliferation of mature T cells. Thus, signals from the TCR may activate different genes depending on the maturation state of the cell. In this report, we demonstrate that the activation as well as the maturation state of the T cell may be important in coupling the TCR to the death program.

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Experiments from this laboratory with cytotoxic T-lymphocyte (CTL) clones (5), from Nau *et al.* (6) with $CD4^+$ clones, and from Ashwell *et al.* (7) with hybridomas indicated that the TCR could have a negative effect on proliferative responses of mature T cells. For the hybridomas, a high dose of antigen or anti-TCR antibodies clearly induced cell death and was capable of selective toxicity *in vivo*. This phenomenon, which results in the relatively rapid death of the responding cell, therefore, is clearly distinct from the "anergic" response of type 1 T helper cells lacking "costimulator" activity (8). In the hybridoma system and the $CD8^+$ system described below, death of these peripheral T cells is initiated by the TCR, and, since other events during differentiation appear to be homologous to the activation of peripheral cells, understanding TCR-stimulated death in peripheral T cells may be important both in maintaining the repertoire selected in the thymus and in understanding the process of clonal deletion in the thymus.

An interesting observation in the hybridoma system was that prior to initiating the death pathway, the antigen or anti-TCR altered the transit of the hybridomas through the cell cycle by creating a blockade at the G_1/S boundary (9). The observation that the cell cycle status of the responding T cell may influence the functional outcome may have important analogies within the thymus. Penit and colleagues (10, 11) have determined that immature thymocytes undergo at least one and possibly two periods of proliferation that culminate in selection. Similarly, the thymic injection experiments of Guidos *et al.* (12) indicate that the selected cells come from the blast-like subset of $CD4^+/CD8^+$ thymocytes. Thus regulation of the cell cycle within the thymus is poorly understood but may also play an important role in selection.

EXPERIMENTAL PROCEDURES

Mice. C57BL/6J, DBA/2J, and BALB/cJ (The Jackson Laboratory) or H-2^b mice expressing a transgenic TCR for H-$2L^d$ as described (2) were used in all experiments. The transgenic TCR originated from the 2C CTL line and is recognized by mAb 1B2 (13).

Cell Culture. RPMI 1640 medium-based medium supplemented with 5% (vol/vol) heat-inactivated fetal calf serum, 2 mM glutamine, 15 mM Hepes, nonessential amino acids (GIBCO, 100×), 1 mM sodium pyruvate, penicillin (2.5×10^4 units/liter), and streptomycin (25 mg/liter) was used in all experiments. Immulon I plates (Dynatech) were sterilized and coated with mAb purified from ascites fluid or tissue culture medium, and unbound sites were blocked with bovine serum albumin as described (14). Antibodies used were the anti-clonotype mAb 1B2 (anti-TCR) described above, the anti-CD3 mAb 2C11 (15), or the anti-CD8 mAb 53–6.72 (16). In some experiments, $CD8^+$ cells were enriched by nylon

Abbreviations: CTL, cytotoxic T lymphocyte; TCR, T-cell receptor; mAb, monoclonal antibody; IL-2, interleukin 2.

wool adsorption and elimination of residual B cells and CD4$^+$ cells with mAb J11d (17) and mAb RL.172 (18), respectively, plus normal rabbit serum (Low Tox M, Cedarlane Laboratories, Hornby, ON, Canada) as a source of complement.

Flow Cytometry. A Becton Dickinson FACS model 440 instrument was used for all experiments. Primary staining reagents were the anti-TCR and anti-CD3 mAbs, described above. Secondary staining reagents were fluorescein isothiocyanate-conjugated goat anti-rat or anti-mouse IgG (Southern Biotechnology, Birmingham, AL) or fluorescein isothiocyanate-conjugated goat anti-hamster (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Cell cycle analysis was performed by staining cells with propidium iodide after lysis in modified Krishan's reagent (19).

[^{3}H]Thymidine Incorporation. Triplicate cultures of 1–5 × 10^4 cells were incubated in 0.2 ml of medium containing recombinant interleukin 2 (IL-2; Cetus) at 10 units/ml for 3 days. The last 4 hr cells were pulse-labeled with [^{3}H]thymidine (1 μCi per well; 1 Ci = 37 GBq) before harvesting on glass-fiber filters.

EXPERIMENTAL RESULTS

Anti-TCR or Anti-CD3 Stimulated Proliferation of Naive Splenocytes but Blocked Proliferation of Activated Splenocytes. A comparison of immobilized anti-TCR or anti-CD3 on a cloned CTL line or transgenic splenocytes previously activated by antigen (mixed lymphocyte culture) demonstrated these mAbs have a strong negative effect on proliferation in response to IL-2 alone. In contrast, naive splenocytes were stimulated to proliferate under nearly identical conditions (Fig. 1 *A–C*). Even the highest doses of mAb were unable to produce a negative response in the naive populations.

One potential explanation for the failure to observe the inhibitory effects of anti-TCR or -CD3 on naive cell proliferation could be that they are more refractory to TCR stimulation and require much higher levels of mAb to produce the same effect. We had found (14) that coimmobilization of anti-TCR and anti-CD8 could increase the sensitivity of naive cells to anti-TCR by as much as 10,000-fold, as indicated by a parallel shift (both maximal and half-maximal concentrations) in the concentration–effect relationship of anti-TCR and proliferation. In Fig. 1 *D* and *E*, we have taken advantage of this observation to demonstrate that this increased sensitivity to TCR by immobilized anti-CD8 increased the sensitivity of previously activated cells to anti-TCR inhibition of proliferation but still was unable to produce a negative effect on naive splenocytes. These data suggest that a qualitative rather than a quantitative difference in responsiveness exists between naive and activated splenocytes.

Anti-TCR or Anti-CD3 Stimulated Death of Antigen-Activated Splenocytes. The mechanism of the block in proliferation of activated splenocytes was further investigated by assessing the viability of cells after incubation on plates coated with anti-CD3 or anti-TCR (1B2). Fig. 2 demonstrates that anti-CD3 caused death in both transgenic and conventional CD8$^+$ splenocytes. In contrast, the anti-TCR specific for the transgenic T cells selectively caused death in the transgenic population. In other experiments, this effect was demonstrated to be specific for anti-TCR or anti-CD3 because anti-CD8 had no effect on cell viability (data not shown).

An important question was whether the lytic effect of the anti-TCR or anti-CD3 was selective for the stimulated population or was the result of nonselective toxic products released by TCR stimulation. This was tested in a mixing experiment in which transgenic CD8$^+$ cells were mixed with a conventional CTL clone. Both populations were equally sensitive to this concentration of immobilized anti-CD3 (Fig. 1 *A* and *B*). However, when the cells were mixed on immobilized 1B2 (specific for the transgenic clonotype), the transgenic cells were selectively depleted (compare Fig. 3 *B* and *C*), as determined by fluorescence-activated cell sorter analysis of cells stained for CD3 and TCR. This experiment confirms that anti-TCR- or anti-CD3-stimulated death is a homologous process rather than the result of nonspecific lysis of bystanders by activated CTLs.

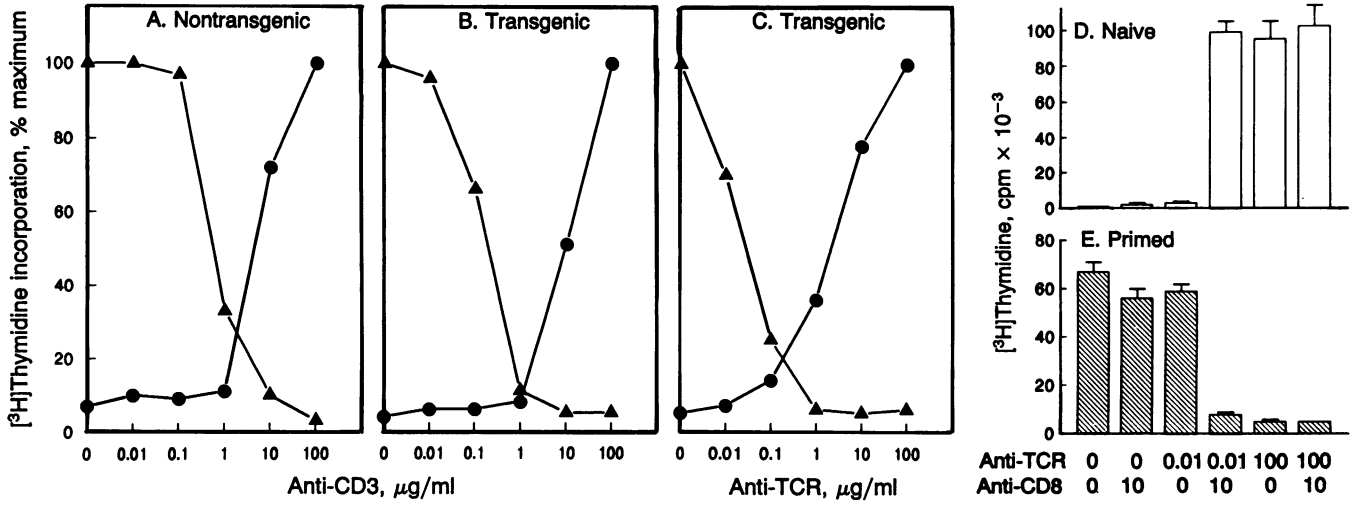

FIG. 1. (*A–C*) Stimulation and inhibition of proliferation of naive and primed cells, respectively, by similar concentrations of anti-TCR (*C*) or anti-CD3 (*A* and *B*). Primed cells (▲) were either a C57BL/6 anti-DBA/2 clone (CTL 3) (*A*) or 2C transgenic cells (*B* and *C*) stimulated 10–12 days earlier with irradiated spleen cells plus IL-2 (10 units/ml). Naive cells (●) were CD8$^+$ splenocytes from either nontransgenic (*A*) or 2C transgenic (*B* and *C*) H-2^b mice purified by removal of nylon wool adherent cells and treatment with anti-CD4 (RL.172) and J11d plus complement. Cells were incubated for 3 days in the presence of IL-2 (10 units/ml) on wells precoated with the indicated concentrations of anti-CD3 (2C-11) or anti-transgenic TCR (1B2). The cells were pulse-labeled with [^{3}H]thymidine for the last 4 hr of culture and incorporated radioactivity was determined. Wells precoated with 1B2 had no effect on the activity of either primed or resting nontransgenic cells (data not shown). (*D* and *E*) Wells were coated with the indicated concentrations of mAb (in μg/ml), and naive (*D*) or antigen plus IL-2-stimulated (*E*) CD8$^+$ cells from transgenic mice (prepared as in *A–C*) were incubated for 3 days with IL-2 (10 units/ml). [^{3}H]Thymidine was added to the cultures for the last 4 hr. Results are expressed as the mean ± SEM of triplicate cultures.

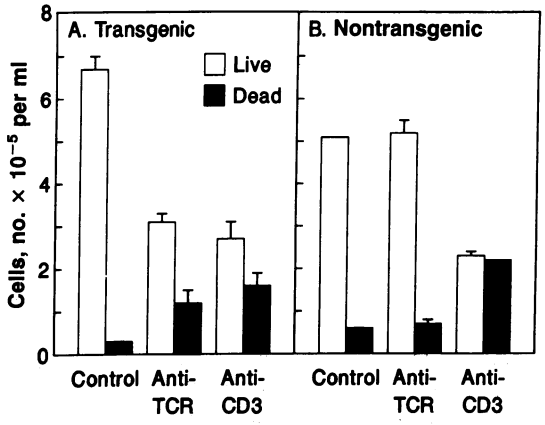

FIG. 2. Immobilized anti-TCR or anti-CD3 antibodies cause death of activated $CD8^+$ T cells. Spleen cells from H-2^b transgenic or conventional mice were stimulated with irradiated allogeneic splenocytes (BALB/c) in culture for 7 days. $CD8^+$ cells were enriched as in Fig. 1 and the cells were incubated in the presence of IL-2 (10 units/ml; 5×10^5 cells per ml) for 24 hr on control wells or wells coated with anti-TCR or anti-CD3. Cells were harvested, and live and dead cells were determined by trypan blue exclusion.

DNA Fragmentation in Anti-TCR Death. One of the characteristics of natural or programmed cell death as originally described independently by Sanderson (20) and by Don *et al.* (21) is the "boiling" of the cell and fragmentation of cellular organelles including the nucleus. This results in fragmentation of the DNA into nucleosomal-sized fragments (≈180-base-pair repeating units). This phenomenon has been observed in a variety of systems including thymocytes lysed by glucocorticoids (22), target cells lysed by CTLs (23, 24), and thymic organ cultures treated with anti-CD3. Examination of DNA extracted from antigen-activated splenocytes (Fig. 4*A*) early in the time course of TCR-stimulated death (various experiments indicate the $t_{1/2}$ of death to be 18–24 hr, data not shown) confirmed that DNA fragmentation was associated with TCR-stimulated death as well.

Cells Accumulated at the G_1/S Interface of the Cell Cycle. One of the most striking phenomena of TCR-stimulated death in the hybridoma system is the accumulation of cells at the G_1/S boundary of the cell cycle. Fig. 4*B* demonstrates that antigen-activated $CD8^+$ cells also accumulated with a DNA content associated with the G_1/S boundary of the cell cycle. In this experiment, control and anti-TCR stimulated cells were analyzed for DNA content per cell by fluorescence-activated cell sorter after staining with propidium iodide. Mercep *et al.* (25) have demonstrated that the G_1/S blockade can occur in the absence of death in the hybridoma system. However, there is no evidence to indicate that TCR-stimulated death can occur in the absence of a G_1/S blockade. Thus it is possible that specific points of the cell cycle are essential for either the production of TCR-stimulated toxins or the targets of such toxins. These experiments further highlight the potential importance of cell cycle regulation during thymic development for the process of selection to occur.

Antigen but Not Anti-TCR Primed Splenocytes for Subsequent TCR-Stimulated Death. The experiments above raised several questions. The cell cycle experiments raised the question of whether activation and entry into cycle were sufficient to produce sensitivity to TCR-stimulated death. In addition, immobilized anti-TCR produced positive and negative effects on naive and activated splenocytes, respectively. Similar amounts of immobilized anti-TCR have been used in limiting dilution experiments (14). Thus it was difficult to imagine how toxic concentrations of immobilized anti-TCR could allow the development of clones.

We resolved these issues by examining the sensitivity to TCR-stimulated death of splenocytes initially activated by either antigen or anti-TCR. In both cases, cells were harvested after 4 days of stimulation, allowed to recover overnight in the absence of antigen or anti-TCR, and retested for sensitivity to immobilized anti-TCR. The results (Fig. 5) demonstrate that antigen-activated cells became sensitive to TCR-stimulated death within a few days of antigen exposure. In contrast, anti-TCR primed cells remained resistant to TCR-stimulated death.

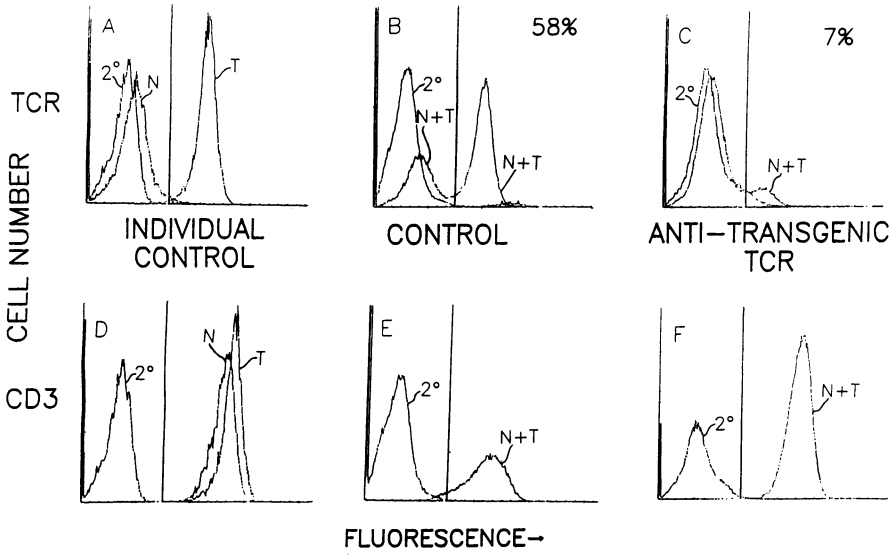

FIG. 3. Death stimulated by immobilized anti-TCR is specific. Primed $CD8^+$ transgenic cells (T histograms) and the nontransgenic clone CTL 3 (N histograms) were incubated separately (*A* and *D*) or in a 1:1 mixture in the presence of IL-2 on control wells (*B* and *E*) or on 1B2-coated wells (*C* and *F*). After 30 hr the cells were harvested, recovery was determined, and the cells were plated in new uncoated wells for 16 hr before staining with the anti-TCR 1B2 (*A–C*) or anti-CD3 (*D–F*). After the 30 hr of 1B2 stimulation, the recovery of transgenic cells incubated alone on 1B2-coated plates was reduced to 20% of the similar cells on control (bovine serum albumin coated) wells. Recovery of CTL 3 was the same on both control and 1B2-coated wells. The broad CD3 peak in the control mixed population (*E*) is the sum of the conventional and transgenic levels of CD3 expression (*D*). The histograms labeled 2° refer to cells incubated with the secondary staining reagent alone.

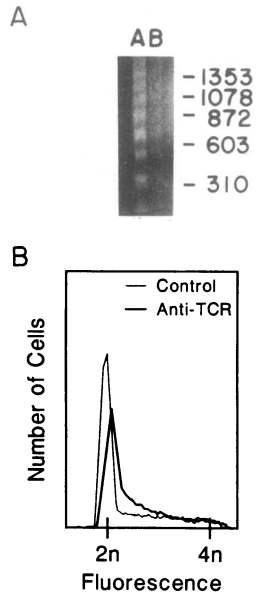

FIG. 4. Anti-TCR-stimulated death produces DNA fragmentation and accumulation of cells at the G_1/S boundary of the cell cycle. (*A*) Transgenic splenocytes were primed with allogeneic splenocytes, harvested, and cultured for 12 hr (with IL-2) on control or anti-TCR-coated wells. Cells were harvested, viability was determined by trypan blue (37% and 10% dead for anti-TCR and control populations, respectively), and DNA was extracted and separated on an agarose gel. Lanes: A, anti-TCR; B, control. The gel was stained with ethidium bromide (1 μg/ml). The positions of ϕX174 phage DNA fragments digested by *Hae* III are indicated in bases. (*B*) Activated transgenic splenocytes were incubated (with IL-2) on control or anti-TCR-coated wells for 15 hr. Cells were harvested, and viable cells were isolated by Ficoll/Hypaque (d = 1.077) and stained for DNA content with propidium iodide. DNA content of control (thin line) and anti-TCR (thick line) cells was determined on a FACS model 440 (Becton Dickinson).

Trivial explanations that we have considered for this finding were as follows: (*i*) TCR modulation during anti-TCR priming; (*ii*) a TCR signaling defect in the anti-TCR-primed population; and (*iii*) selective expansion of a subset of cells by either priming regimen. However, none of these trivial explanations appear to be valid. Both primary populations expressed similar levels of TCR by fluorescence-activated cell sorter analysis (data not shown). Similarly, a signaling defect in the anti-TCR-primed population seems unlikely because they assumed the characteristic blast-like morphology after the subsequent anti-TCR stimulation but not in control wells. Finally, parallel limiting dilution experiments have demonstrated identical precursor frequencies with either antigen or immobilized anti-TCR (data not shown). Therefore, these results indicate (*i*) that activation and entry into cycle alone is not sufficient to produce sensitivity to TCR-stimulated death and (*ii*) that antigen but not anti-TCR priming can convert the TCR-stimulated-death-resistant naive splenocyte to a sensitive phenotype.

DISCUSSION

Several transformed T-cell lines respond to TCR stimulation by a cell cycle blockade at the G_1/S boundary and slowing cell growth (ref. 26 and J.H.R., unpublished observations). However, a large fraction of only the $CD4^+$ hybridomas (7) and the antigen-stimulated splenocytes described in the present report are able to complete the death response. Thus TCR-stimulated death requires not only signaling by the TCR but also a cell primed to respond to those signals. The data herein indicate that mature as well as immature thymocytes

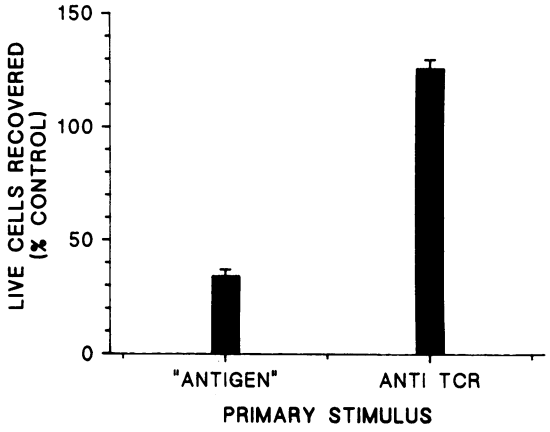

FIG. 5. Resting transgenic splenocytes are sensitive to subsequent TCR-stimulated death after antigen activation but not after activation by TCR alone. Fresh transgenic splenocytes were activated by irradiated BALB/c splenocytes (with IL-2) as before or $CD8^+$ cells from the same spleen were enriched by nylon wool purification followed by anti-CD4 and J11d treatment plus complement. The $CD8^+$-enriched population was primed on wells coated with 1B2 (10 μg/ml). Both populations were stimulated for 4 days in the presence of IL-2 (10 units/ml), harvested, and incubated in uncoated wells for an additional 24 hr [also in medium containing IL-2 (10 units/ml)] before plating in IL-2-containing medium on control wells or wells coated with 1B2 (anti-TCR; 10 μg/ml). After 24 hr cells were harvested, viability was determined by trypan blue exclusion, and cells were stained for TCR expression with 1B2. Greater than 85% of both populations were $1B2^+$ with identical levels of expression (data not shown).

can have TCR signals coupled to the death response. The ability of the hybridomas to die in response to TCR stimulation may reflect an intrinsic property of the BW5147 parent or may reflect a property of the nontransformed fusion partner. In most cases, the fusion partner is an activated T cell. Thus the hybridoma may rescue and perpetuate the sensitive phenotype by constitutively expressing the elements necessary for coupling the TCR to the death pathway.

An important question is whether the death stimulated by immobilized anti-TCR or anti-CD3 is physiologically relevant. Certainly, immobilized anti-TCR or anti-CD3 is a nonphysiological stimulus. However, the data presented above indicate that it is a useful tool in determining if TCR coupling to the death pathway is present. Further, the data indicate that TCR coupling to the death pathway is not necessarily restricted to immature T cells.

Other experiments (J.H.R., unpublished data) indicate that although precursor frequencies vary somewhat from animal to animal, the ratio of the precursor frequencies for naive transgenic cells stimulated by antigen or anti-TCR is nearly one. Similarly, that precursor frequency remains relatively constant for antigen-primed cells when antigen is used as a secondary stimulus. In contrast, the precursor frequency of antigen-primed cells assayed with anti-TCR as a secondary stimulus falls >100-fold within 1 week and >10-fold within 4 days of antigen priming. Thus responsiveness to nominal antigen is not affected by the primary activation. However, studies with heteroclitic clones indicate that higher-avidity forms of antigen (as assessed by cold target inhibition) do inhibit the expansion of CTL clones (5, 27). Therefore, a secondary stimulus with higher-avidity antigen, such as anti-TCR, may also stimulate death in primary cells. This phenomenon could serve as a mechanism to limit affinity maturation of clones by mutation to higher-avidity specificities not originally selected in the thymus.

The results above indicate that the coupling of the TCR to an endogenous death pathway can be regulated by interaction

with other cells, even in terminally differentiated T cells. Given the increasing number of homologies between selection of developing thymocytes and antigen activation of peripheral cells, we would suggest that the conversion of naive splenocytes from TCR-stimulated death-resistant to -sensitive phenotype by antigen may reflect a homologous property of the thymic environment on developing thymocytes. Thus some interaction between a developing thymocyte and its environment may be required before it becomes sensitive to clonal deletion.

Neither the nature of the elements coupling the TCR to the death program nor the mechanism of death is identified here. However, Fig. 3 demonstrates that death is selective for cells of the same clone rather than the nonselective death of all potentially sensitive clones in the culture. Therefore, death is either the result of TCR-stimulated suicide or fratricide in which TCR stimulation is required to both activate and sensitize similar cells. The limiting dilution analysis discussed above argues against a fratricidal mechanism requiring cell contact. The availability of cell populations of the same clonotype expressing the sensitive and resistant phenotypes should assist in efforts to identify the elements responsible for death and the subsequent understanding of their regulation.

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