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Transgenic mice carrying the diphtheria toxin A chain gene under the control of the granzyme A promoter: Expected depletion of cytotoxic cells and unexpected depletion of CD8 T cells

(cell-mediated cytotoxicity/CD8 cells)

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ABSTRACT **We have generated transgenic mice bearing the diphtheria toxin A chain (DTA) gene under the control of granzyme A (GrA) promoter sequences (GrA-DTA). GrA is expressed in activated cytotoxic cells but not in their immediate progenitors. These GrA-DTA mice are deficient in cytotoxic functions, indicating that most cytotoxic cells express GrA *in vivo*. Surprisingly, one founder strain containing a multicopy GrA-DTA insert show a marked and selective deficiency in CD8$^+$ cells in peripheral lymphoid organs. This depletion was not observed in thymus, where the distribution of CD4$^+$ and CD8$^+$ cells is normal. Moreover, the emigration of T cells from thymus is normal, indicating that the depletion occurs in the periphery. GrA-DTA mice should be useful as models to dissect the role of cytotoxic cells in immune responses and as recipients of normal and neoplastic hematopoietic cells. The selective depletion of CD8$^+$ cells in one founder strain could have implications for postthymic T-cell development.**

Cytotoxic cells are implicated in immune responses against tumors, intracellular infections, self antigens, and transplants (1). Two major types of cytotoxic cells have been described: cytotoxic T lymphocytes (CTL) and natural killer (NK) cells (2, 3). These cells differ in their recognition properties, but they share most or all of the effector components for the killing process. Activated cytotoxic cells may have cytoplasmic granules that contain a pore-forming protein called perforin and a series of serine proteases called granzymes (4–6) and can lyse target cells by plasma membrane damage and DNA degradation. In addition, cytotoxic cells may express Fas ligand, which interacts with its receptor on target cells, triggering apoptosis (7–10).

It is important to test which functions are carried out by cytotoxic cells *in vivo*. Genetic disruption of granzyme B (11) or perforin (12) gives rise to animals with moderate to almost complete impairment in cytotoxicity. C3H/gld mice have a mutated Fas ligand (9), yet have granule-based cytotoxic cells. We have approached the *in vivo* functions of granule-based cytotoxicity by using a suicide gene to delete cells containing cytolytic granules.

Granzyme A (GrA) is restricted in its expression to cytotoxic cells. *In vivo*, most GrA-positive cells are activated CD8$^+$ and NK cells, with a small but significant fraction of CD4$^+$ cells. Unstimulated peripheral lymphoid organs contain few GrA-positive cells [which include CTL precursors (CTLp)] (13–18). Activation of GrA mRNA expression in T cells follows signal transduction events that involve the T-cell receptor and the interleukin 2 (IL-2) receptor (for a review, see ref. 19). Similarly, activation of IL-2 receptors on NK cells greatly augments their expression of GrA mRNA (20, 21).

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CTLp are derived from emigrant cells developed in the thymus (for a review, see ref. 22). The peripheral T-cell pool is thought to comprise recent thymic emigrants, mature T cells, memory T cells, and effector T cells.

We have made DNA constructs harboring the diphtheria toxin A chain (DTA) gene under the control of GrA genomic sequences. Tissue-specific and developmentally regulated expression of toxin genes has allowed the ablation of other selected cell populations (23–25). We describe here the successful generation of GrA-DTA transgenic founder mouse strains, their quantitative ablation of CTL and NK cells, the nature of surviving cytolytic clones, and the surprising depletion and/or absence of circulating CD8$^+$ T cells in one founder strain.

MATERIALS AND METHODS

Constructs and Generation of Transgenic Mice. An 18-kb genomic clone containing the coding sequences of GrA and 10 kb of upstream sequences was isolated (26). We used a DTA plasmid bearing a 795-bp *Bgl* II cassette containing a polylinker and human metallothionein II_A gene sequences, the DTA coding sequences, a stop codon, and the small intron from simian virus 40 (23). This cassette was inserted within a *Xho* II site located 28 bp downstream of the GrA TATA box sequences. The GrA-DTA construct is a 19-kb fragment containing 10 kb of GrA sequences carrying the putative control–promoter regions, the DTA cassette, and around 8 kb of GrA sequences containing the coding sequences, the natural termination codon, and polyadenylylation sites. Transgenic mice were generated in (C3H × C57BL/6)F_1 mouse eggs as described (27) and were identified by PCR analysis with DTA primers.

Northern Blot Analysis. Total RNA from freshly isolated or activated splenocytes was prepared by phenol extraction in the presence of guanidine thiocyanate and probed as described (28).

Flow Cytometric Analysis. Biotinylated anti-Thy-1.2 (53.2.1), phycoerythrin (PE)-conjugated anti-CD4 (GK1.5), and fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (GK1.5) and anti-CD8 (53.6.7) (29) were used on fluorescence-activated cell sorting (FACS) analysis as described (29). The cytometry analysis was carried out with a modified Beckton Dickinson FACScan or Flasher available through the Stanford Beckman Center FACS facility. All of the data analyses were done with the FACS-DESK software (Stanford University).

Cytotoxic Assays. Analysis of cytotoxic functions was done by standard ^{51}Cr release assays (30).

Precursor Frequency Analysis. Effector cells were primarily derived from freshly isolated splenocytes fractionated through

Abbreviations: GrA, granzyme A; DTA, diphtheria toxin A chain; CTL, cytotoxic T lymphocytes; CTLp, CTL precursors; NK, natural killer; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorting/sorter; IL-2, interleukin 2; ConA, concanavalin A; PE, phycoerythrin.

nylon wool columns and placed in 1:2 dilutions in 96-well plates. Cells were activated by incubation with irradiated BALB/c ($H\text{-}2^d$) splenocytes, and 6–10 days after activation cytotoxic assays were performed with $H\text{-}2^d$ P815 targets.

Thymic Migration. Analysis of thymic emigration following intrathymic injection of FITC was done as described (31). Animals were analyzed for $FITC^+$ emigrants stained with anti-Thy-1-, anti-CD4-, and anti-CD8-specific antibodies conjugated to biotin and then were incubated with avidin-Texas red.

RESULTS AND DISCUSSION

Transgenic Mice Generation. Fig. 1 shows the constructs designed for the generation of transgenic mice. Fourteen transgenic founders were obtained. All were healthy and indistinguishable from nontransgenic littermates. Four transgenic animals surprisingly had low levels of blood $CD8^+$ cells (see above), while the rest had normal levels of T cells, B cells, macrophages, and granulocytes (data not shown). Of the $CD8^-$ animals, three were males, one was female, and only two of them (T13.1 and T42.11) were fertile. T13.1, a female, gave rise to transgenic progeny of which all males were sterile, while the females were fertile. Although T42.11, a male, could breed normally, most of his transgene-positive male progeny were sterile. The association between sterility and the GrA-DTA transgene it is not yet clear.

Depletion of Cytotoxic Potential. Cytotoxic assays were carried out with NK cells as well as activated splenic T cells. GrA-DTA animals showed a reduction in NK cell cytotoxicity by a factor of 5–10 compared with normal animals (Fig. 2*A*). Splenic T cells activated in mixed lymphocyte cultures against BALB/c ($H\text{-}2^d$) cells and tested on day 6 in ^{51}Cr-release assays against $H\text{-}2^d$ P815 cells show that activated T13.1 T cells are severely depleted in cytolytic activity (Fig. 2*B*). This result was repeated with T cells activated with concanavalin A (Con A) and IL-2, as shown in Fig. 2*C*; both $CD4^+$ and $CD8^+$ cells from normal mice contained CTLs, and all T13.1 cell populations were depleted of cytotoxicity in a lectin-dependent killing assay. Similar tests using transgenic mice derived from founders with normal $CD8^+$ levels showed cytotoxic activities comparable to control animals (data not shown). The finding that transgenic mice quantitatively delete cells with NK- and T-cell cytolytic activities constitutes direct evidence for the role of GrA-expressing cells in cytotoxicity *in vivo*, a proposition that had been challenged by several reports (32, 33).

It is important to note that in none of the assays using cells from T13.1 and T42.11 mice was the killing activity ablated completely, and continuous *in vitro* culture of these cells in the presence of IL-2 selected for cells with normal killing abilities. These cells are derived from precursors that apparently escaped the DTA selection. As a population these cytolytic cells express the endogenous GrA gene but do not express detectable transcripts for the DTA transgene (Fig. 3). The inability to detect RNA encoding DTA is not due to inability of the probe to hybridize because the same probe could efficiently hybridize the DTA sequences in the DNA constructs. This inactivation phenomenon, characterized by the loss of expression of the transgene, is similar to that reported for elastase promoter–DTA transgenic mice (23).

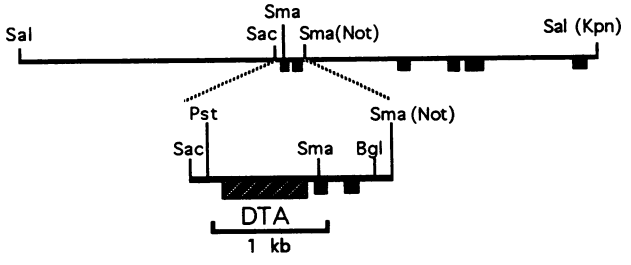

FIG. 1. GrA-DTA construct. A 795-bp DTA cassette (dashed box) was inserted within a 19-kb genomic clone containing all of the GrA coding sequences (closed boxes) and 10 kb of 5′ sequences carrying the putative control–promoter regions. The DTA fragment was placed in a *Xho* II site located 28 bp upstream of the GrA TATA box sequences.

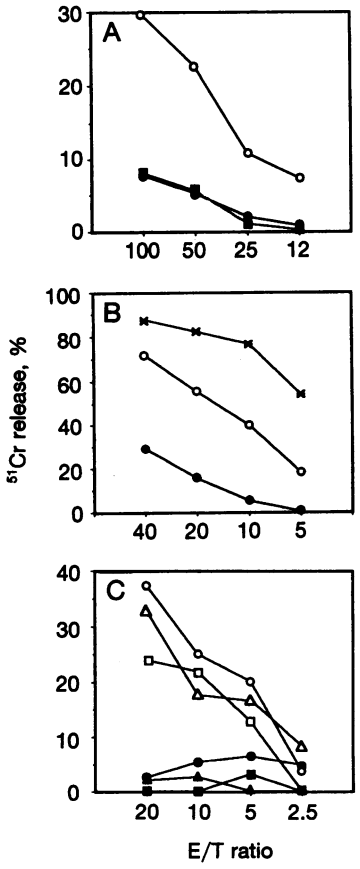

FIG. 2. Cytotoxic activity in GrA-DTA transgenic mice. (*A*) Nylon wood nonadherent splenocytes were tested for NK activity by their ability to lyse ^{51}CR-labeled YAC-1 target cells at various effector/target cell (E/T) ratios. ○, C57BL/6 control; ●, T13.1 transgenic mice; ■, T42.11 transgenic mice. (*B*) Nylon wool nonadherent splenocytes were activated with a ×10 excess of irradiated (4000 R) BALB/c ($H\text{-}2^d$) splenocytes for 6 days and were placed in a cytotoxic assay with P815 ($H\text{-}2^d$) cells as targets. AR-1 is a cytotoxic T-cell clone specific for $H\text{-}2^d$ class I determinants. ○, C57BL/6 control; ●, T13.1 transgenic; ×, AR-1. (*C*) Total-spleen, $CD4^+$, and $CD8^+$ sorted cells were polyclonally activated with 2 μg of Con A plus 100 units of recombinant IL-2 per ml for 6 days and were tested in a lectin-induced Cr-release assays in the presence of 2 μg of Con A per ml with P815 cells as targets. ○, CD8-control; △, CD4-control; □, total spleen-control; ●, CD8-T13.1; ▲, total spleen-T13.1. All of the assays were carried out for 4 hr at 37°C. The results are represented as the percent of total specific ^{51}Cr that was released in a 4-hr assay at the indicated E/T ratios.

Depletion of the $CD8^+$ Peripheral T-Cell Compartment in DTA Transgenic Mice. Four of 14 founders bearing DTA constructs contained low levels of T, mostly $CD8^+$ cells in peripheral lymphoid sites (Fig. 4). The percent and absolute number of $CD8^+$ cells were lower by a factor of 3–10 than in normal controls. $CD4^+$ T-cell levels also decreased to a lesser extent. The CD8-depletion phenotype is more dramatic in the T13.1 subline than in the T42.11 subline. The number of transgene copies, assessed by Southern blot analysis, is at least 10 times higher in the T13.1 mouse than in the T42.11 mouse (data not shown).

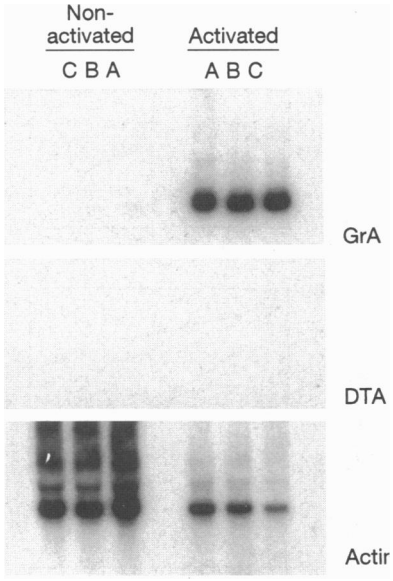

FIG. 3. GrA and DTA transcription in GrA-DTA transgenic mice. Shown is a Northern blot of total RNA isolated from splenocytes activated for 14 days with Con A plus IL-2 and from nonactivated splenocytes from nontransgenic (lanes A), T13.1 (lanes B), T42.11 (lanes C) mice. The same blot was hybridized sequentially to a GrA cDNA probe (*Top*), a DTA probe from the original DTA cassette (*Middle*), and a β-actin probe (*Bottom*). Five times more RNA was loaded for the nonactivated cells (50 μg) in comparison to activated cells (10 μg).

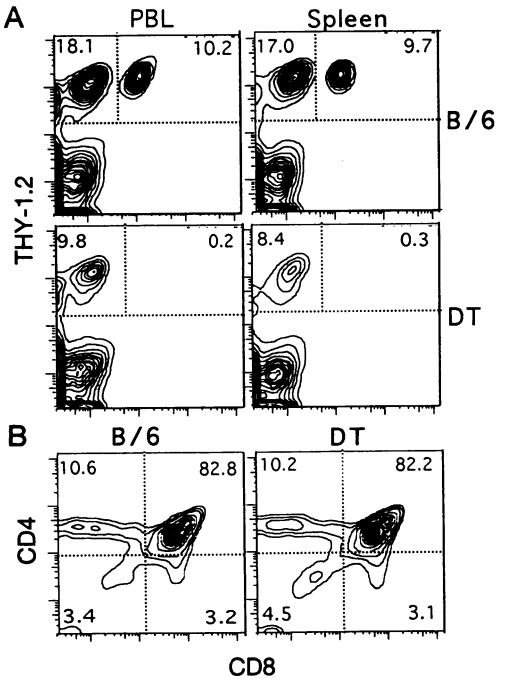

FIG. 5. T-cell compartment of T13.1 transgenic and T13.1 × T42.11 doubly transgenic mice. (*A*) Freshly isolated cells from peripheral blood and spleen were stained with biotinylated anti-Thy-1.2 antibodies followed by fluoresceinated anti-CD8 antibodies plus avidin-Texas red. (*B*) Thymic cells from T13.1 × T42.11 double transgenics and nontransgenic mice were stained with anti-CD4 antibodies coupled to PE and fluoresceinated anti-CD8 antibodies. The cells were analyzed by flow cytometry as described. The percentage of relevant populations of cells is indicated. PBL, peripheral blood lymphocytes.

In contrast to the $CD8^+$ cell levels in periphery, the thymuses of transgenic mice have a normal distribution of $CD4^+$ and $CD8^+$ cells, indicating that the generation of these thymic populations is unaltered (Fig. 4). Moreover, the CD3 distribution in thymocytes was also normal, indicating that thymic maturation processes in general are normal (data not shown).

The most likely explanation is that the depletion of the $CD8^+$ cell compartment was the result of a postthymic event. The incomplete nature of $CD8^+$-cell and CTL depletion could have resulted from the survival and expansion of a rare subset of cells that had inactivated the transgene, or the events that cause postthymic depletion of $CD8^+$ cells by GrA-DTA activation spare a minor but significant fraction of T cells.

Crosses Between Two Independent GrA-DTA Transgenic Lines Result in Mice Profoundly Depleted of Cytolytic and $CD8^+$ Cells. Each GrA-DTA transgenic mouse contains cytolytic precursors that can express high levels of GrA message under continuous *in vitro* stimulation, indicating that those cells escaped DTA-negative selection. If this leakage is the

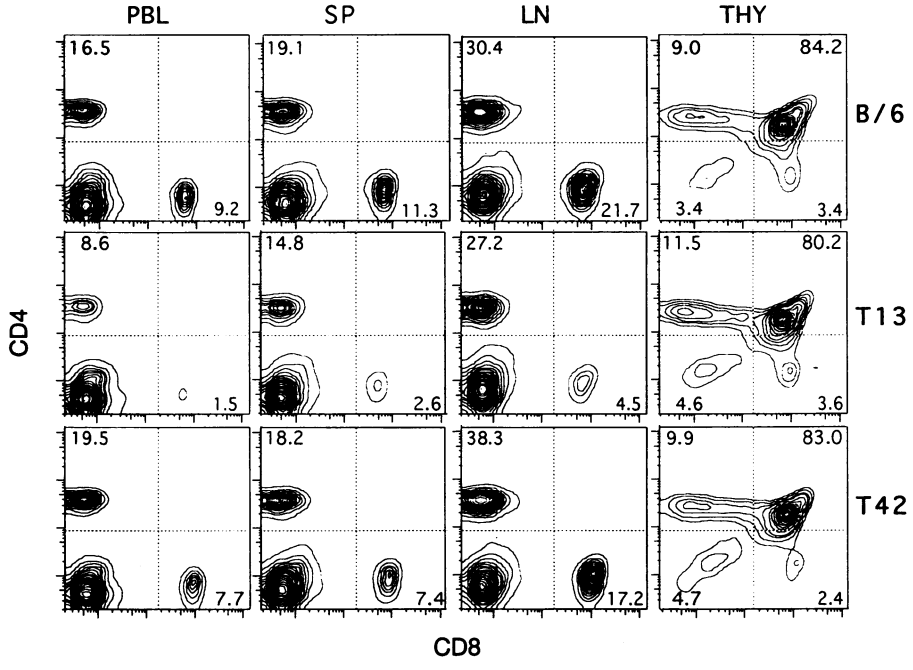

FIG. 4. CD4 and CD8 expression in normal and transgenic mice. Freshly isolated cells from thymus (Thy) and peripheral lymphoid organs from 3-month-old animals were stained with PE-conjugated anti-CD4 and FITC-conjugated CD8 antibodies and were analyzed by flow cytometry as described in *Materials and Methods*. The percentage of cells of a given phenotype is indicated. PBL, peripheral blood lymphocytes; SP, spleen; LN, lymph nodes; B/6, C57BL/6 mice; T13, T13.1 mice; T42, T42.11 mice.

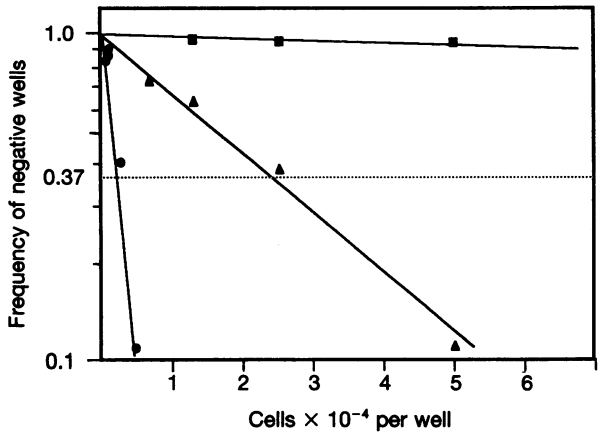

FIG. 6. Cytotoxic cell precursor frequency in transgenic mice. Nylon wool nonadherent splenocytes from normal (●), T13.1 transgenic (▲), and T13.1 × 42.11 (■) doubly transgenic mice were plated in a limiting dilution assay in the presence of a 5–10 times excess of irradiated BALB/c splenocytes for 6 days. Cytotoxic cells were tested as described. The frequency of cytotoxic cell precursors was 1 of 2100, 1 of 36,000, and 1 of 260,000, respectively.

result of transgene inactivation in rare clonogenic precursors dependent on single genetic events, it should follow that the probability of two or three such events at two or three independent GrA-DTA insertion sites should be very small. Unfortunately, the GrA-DTA transgenic mouse lines are all affected with partial (T42.11) or complete (T13.1) male sterility. The few T42.11 males that were fertile were used to generate T13.1 × T42.11 double transgenics. The $CD8^+$ T-cell profile in blood and spleen of these mice is depicted in Fig. 5*A*. Up to 40% of the offspring contained only rare or no $CD8^+$ cells in their peripheral lymphoid organs. Most strikingly, the analysis of thymocytes from these mice showed a normal distribution of $CD4^+$ and $CD8^+$ cells (Fig. 5*B*).

To quantify the effect of the GrA-DTA transgene in CTL generation, we calculated the allogenic CTL precursor frequency by limiting dilution analysis. The precursor frequency of cytotoxic cells in spleen is lower by a factor of ≈200 in double transgenics than in normal mice (Fig. 6). If we consider the obtained frequencies in conjunction with the low number of $CD8^+$ cells in the periphery, it is clear that the potential to generate cytotoxic cells in spleens in these mice is almost null (Table 1).

Apart from the granule-mediated cytotoxic pathway, at least one other cytolytic pathway exists (34). It involves the recognition of fas on the surface of the target cells by the fas ligand in the surface of effector cells. This recognition triggers the death of targets by induction of apoptosis (7). The almost complete loss of cytotoxic cells in (T13.1 × T42.11)F_1 mice implies that GrA cytolytic cells could include fas ligand-positive cells, but a direct demonstration of that speculation is not yet at hand.

Table 1. Cytotoxic T-cell precursor analysis

Mice	T cells*, no. (%)	Precursor frequency‡	Precursors per spleen, no.
Control	1.5×10^7 (100)	2.1×10^3	7100
T13	8.3×10^6 (55)	3.6×10^4	230
T13×42	5.0×10^6 (33)	2.6×10^5	19

*Values are derived from the assumptions that in a normal spleen there are 1.3×10^8 cells and that 4.5×10^7 are T cells. The numbers in parentheses correspond to the percentage of T cells in each case, compared with 100% in normal control mice. These numbers are derived by the sum of $CD8^+$ cells plus $CD4^+$ cells from Fig. 5.
‡Values obtained from limiting dilution analysis (Fig. 6).

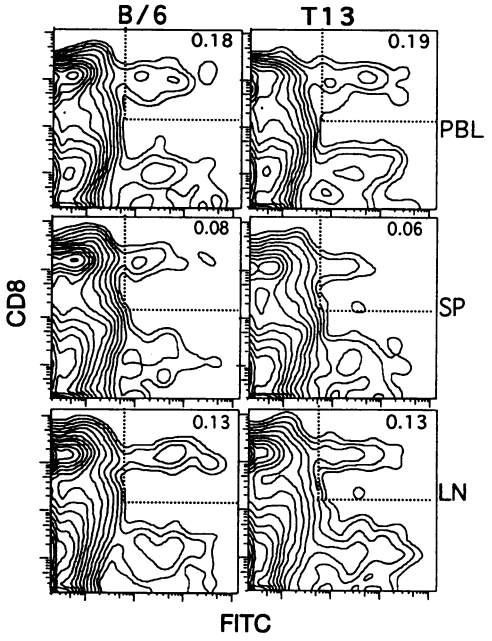

FIG. 7. Thymic emigration studies in T13.1 (T13) transgenic mice. Thymi from normal and transgenic animals were labeled *in situ* by intrathymic injection of FITC. Twelve hours after injection, the animals were sacrificed and cell suspensions from peripheral organs were stained with biotinylated anti-CD8 antibody followed by avidin-Texas red. Two-color flow cytometry analysis was done to define the emigration of $CD8^+$ T cells from thymus. The percentage of $CD8^+$ emigrants is boxed. B/6, C57BL/6 mice; PBL, peripheral blood lymphocytes; SP, spleen; LN, lymph nodes.

Thymic $CD8^+$ T Cells Emigrate Normally from GrA-DTA Mouse Thymuses. The pattern of CD8 expression in periphery and thymus could be indicative of an active process of postthymic depletion. However, it is possible that an impairment in thymic cell emigration could be responsible for the observed phenotype. We analyzed the emigration of cells from thymus to periphery, labeling thymocytes with FITC by intrathymic injection and tracking the appearance of early emigrants in the periphery (31). The same proportion of $Thy1.2^+$ cells were seeded from thymus to the periphery in T13.1 and normal animals during a 12-h period (Fig. 7). Analysis of the specific $CD4^+$ and $CD8^+$ cell compartments also indicated a normal early emigration of both subsets from the thymus. The rate of thymic emigration was normal and falls in the range previously reported of ≈2×10^6 emigrants in 24 hr (31). These observations confirm that the loss of $CD8^+$ cells occurs after thymic emigration.

Evidence for Postthymic Events Affecting Recent Thymic Emigrants. The extent of deletion of the $CD8^+$ population does not agree with the expression of GrA found in a normal mouse, in which about 1% of the $CD8^+$ splenocytes express GrA (14). The thymi in the transgenic animals showed an apparently normal distribution of cells expressing CD8, CD4, and CD3 markers, indicating that the deletion is a postthymic event. By *in situ* hybridization studies it has been shown that around 1.5% of thymic cells may express GrA mRNA (16). The signal was concentrated in the $CD8^-$ $CD4^-$ double-negative population and to a lesser extent in the $CD8^+$ $CD4^-$ population. In a recent report by Ebnet *et al.* (35), GrA activity was restricted by *in situ* enzymatic assay to the $CD8^+$ $CD3^+$ HSA^- population—the most mature stage of $CD8^+$ cells. We could not find a major quantitative difference between the $CD8^+$ $CD3^+$ HSA^- population of transgenics vs. normal mice (data not shown), probably because the fraction of the $CD8^+$ $CD3^+$ HSA^- population expressing GrA activity is low (≈1%), and such a difference could escape the limit of our FACS analysis.

It would be surprising if the low percentage of $CD8^+$ cells in GrA-DTA mice is due to toxin suicide after antigenic stimulation, because the extensive diversity of the T-cell repertoire would require a wide diversity of antigenic stimulations after thymic emigration.

Speculations. We propose that after exiting the thymus, emigrants undergo a process of functional commitment, resulting in an opening of the GrA genomic region to inducibility by transcription factors. For most $CD8^+$ and some $CD4^+$ cells, this activation could include the transient expression of activation molecules but not the full set of events leading to effector CTL generation. The high toxicity of DTA in multicopy GrA-DTA mice could serve as a sensitive indicator of such changes by deleting these cells.

The possibility of postthymic T-cell maturation has been proposed to account for discrepancies in the maintenance of the peripheral T-cell pool, when one considers the output from the thymus and the half-life of peripheral T lymphocytes (reviewed in ref. 36). In that view, early thymic emigrants replenish a postthymic precursor compartment, which undergoes an antigen-independent proliferation in peripheral lymphoid organs. Alternatively, precursors with long half-lives could maintain the peripheral T-cell pool without the need of active cell division. This last possibility has been validated by labeling dividing cells *in vitro* with BrdUrd (37, 38). If lack of cell division were the hallmark of postthymic T cells, the activation of the GrA would not be dependent on division. It is also possible that the depletion of $CD8^+$ cells in the periphery of GrA-DTA mice could be the result of transient thymic stimulation or lineage commitment prior to thymic migration, as proposed by some investigators (39) and supported by the recent findings of Ebnet *et al.* (35). Those studies suggest that early emigrants behave as recently activated cells. If so, according to the data in Fig. 7, they do not express or do not respond to lethal concentrations of DTA in the immediate postmigration stage.

Whether these animals are qualitatively defective in their ability to eliminate tumor cells or virally infected cells or both should be determined. If the animals are defective, they could provide reservoirs to grow cells or microorganisms that are hard to propagate in mice with normal cytotoxic functions and are difficult to study in less selectively immunodeficient mice (e.g., severe combined immunodeficient or *nu/nu* mice).

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