

The Selective Ablation of Interleukin 2-producing Cells Isolated from Transgenic Mice

By Lori-ann E. Minasi,* Yumiko Kamogawa,*[†] Simon Carding,[§] Kim Bottomly,*[†] and Richard A. Flavell*[†]

From the *Section of Immunobiology, [†]Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, Connecticut 06510; and the [§]Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19106

Summary

To better understand the requirement for interleukin 2 (IL-2) in specific immune responses, we have established the use of cell ablation to selectively eliminate T cells that produce IL-2. To accomplish this we have generated transgenic mice that express the herpes simplex virus 1-thymidine kinase (HSV-TK) gene under the transcriptional control of the murine IL-2 promoter that renders IL-2-producing cells sensitive to the cytotoxic effects of the antiviral drug ganciclovir (GANC). HSV-TK activity was specifically expressed in activated T cells from transgenic mice. When CD4 T cells from transgenic mice were stimulated with the superantigen staphylococcal enterotoxin A (SEA) in the presence of GANC, proliferation and IL-2 production were almost completely inhibited and the activated CD4⁺Vβ3⁺ T cell population, eliminated. Proliferation was not restored by adding IL-2, showing that most proliferating cells are not bystander cells. In contrast, the proliferative response to concanavalin A (Con A) was only partially inhibited by treatment of CD4 T cells with GANC, although the efficiency of eliminating IL-2-producing cells was shown to be comparable with that achieved using SEA. This suggests that a portion of the proliferative response to Con A occurs via an alternative pathway not requiring IL-2 synthesis and release.

The proliferation of clones of antigen-specific cells is one of the most important events that occurs during a primary immune response. Since the frequency of antigen-specific precursor T cells is low in peripheral lymphoid organs or in blood, the clonal expansion of antigen-reactive effector cells is critical for the expression of a specific immune response. Such clonal expansion is accomplished by tightly regulating the production of specific lymphokines that can function as growth factors, and by regulating the expression of the receptors for these lymphokines (1).

Antigen-induced T cell proliferation is regulated primarily through the actions of IL-2 on its specific cell surface receptor. IL-2 is a lymphocytotropic hormone released from antigen-triggered T lymphocytes within hours of activation. IL-2 mediates a switch in T cells from G1 into the proliferative phases of the cell cycle. Once T cells are stimulated to secrete IL-2, the lymphokine can interact with the same cell from which it was produced (an autocrine effect) or with any other cell that expresses IL-2 receptors (a paracrine effect), leading to clonal expansion (2, 3).

Although IL-2 remains a most important factor regulating T cell growth, there are clearly IL-2-independent mechanisms that can also regulate T cell proliferation (4–8). In addition, murine T helper cells can be divided into subsets based on the pattern of cytokines secreted in response to antigen or

mitogen stimulation (9). Cells of the Th1 subset produce IL-2, IFN-γ, and lymphotoxin, whereas Th2 cells secrete IL-4, IL-5, and IL-6. The different cytokines released by Th1 and Th2 clones lead to markedly different functions (10–12). Whereas Th1 clones require IL-2 as an autocrine growth factor, the growth of Th2 clones involves the autocrine secretion of IL-4, as well as the cofactor IL-1 (13, 14).

The dichotomy between T helper cells with regard to cytokine production poses the question of lineage relationship between T cells that secrete different cytokines, specifically IL-2, IL-4, and IFN-γ. Although models exist to explain the origins of the distinct functional subsets (15), it is unknown whether a given subset is derived from the other or if there is a common precursor cell.

To analyze the role of IL-2-producing T cells in T cell development and in a given immune response, we generated transgenic mice which allow the selective ablation of IL-2-producing T cells in vitro. Borrelli et al. (16) showed that the synthesis of HSV-thymidine kinase (TK)¹ in transgenic mice could be used to ablate cells that express this product. We chose this approach because it offers several advantages

¹ Abbreviations used in this paper: GANC, ganciclovir; HSV-TK, herpes simplex virus 1-thymidine kinase; SEA, staphylococcal enterotoxin A.

over studies that merely decrease the amount of IL-2 available, such as those that employ anti-IL-2 antibodies (17, 18) or IL-2-deficient mice (19). First, our approach allows us to determine the proportion of cells that are proliferating in a given immune response that are actually making IL-2 or, alternatively, are bystander cells that require IL-2 for cell growth but are not making IL-2. Second, the transgenic mice we have generated are ideal for studying precursor-product relationships between different cytokine-producing T cells. Finally, the ability to eliminate cells that produce IL-2 enables us to examine the function of these cells in a given immune response, as well as during development. Since our ability to selectively ablate IL-2-producing T cells is based on a toxic phenotype that is cell specific and inducible, these cells can be eliminated during different stages of development or of an immune response (i.e., during priming or during a recall response). More importantly, an inducible toxic phenotype allows us to study the function of a cell without interfering with the development of that cell.

We establish here that ablation is specific for cells that are proliferating in response to mitogenic stimulation, and that the cells that are not activated or do not produce IL-2 are not eliminated. This new approach also reveals an interesting difference between the requirement for IL-2-producing cells in the proliferation induced by Con A versus staphylococcal enterotoxin A (SEA) stimulation.

Materials and Methods

Gene Constructs and Transgenic Mice. The plasmid pATgMIL2-A containing the murine IL-2 gene was obtained from Drs. Erik Remaut and Walter Fiers (University of Ghent, Belgium) (20). The 2.7-kb IL-2 promoter was isolated by EcoRI-Pst restriction digestion of pATgMIL2-A. This fragment contains the TATA box and the DNase hypersensitive site located at -275 that have been shown to be necessary for transcription (21). The IL-2 promoter was linked to a 1.5-kb BglII-HindIII fragment of the HSV-1-TK gene (22) via a Pst-BglII linker to generate the construct pIL-2TK.

The recombinant DNA pIL-2TK was injected into C57BL/6 × SJL F₂ fertilized mouse eggs by the Yale Transgenic Facility. Five positive founder lines were established. The founder lines were back-crossed onto C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) to obtain progeny for these studies.

Cell Lines. The TK⁺ cell line was a generous gift of Dr. Wilma Summers (Yale University, New Haven, CT) and has been described (23).

Antibodies, Cytokines, and Reagents. Supernatants from the anti-Thy-1 hybridoma Y19 (24) and the mAb 212.A1 (anti-I-Ab) (25) were generated at Yale University. The antibodies T1B105 (anti-CD8), T1B146 (anti-B220), and HB191 (anti-NK cell) were obtained from the American Type Culture Collection (Rockville, MD). PE-conjugated anti-CD4 mAb was purchased from Becton Dickinson & Co. (Mountain View, CA) and FITC-conjugated anti-Vβ3 mAb (KJ25) was obtained from Pharmingen (San Diego, CA). Recombinant murine IL-2 (rIL-2) was purchased from Boehringer Mannheim Corp. (Indianapolis, IN), and had a sp act ≥10⁶ U/mg. SEA was obtained from Upjohn (Kalamazoo, MI) and ganciclovir (GANC) was a gift from Syntex (Palo Alto, CA). Tetrahydrouridine (THU) was a generous gift from Dr. William Summers (Yale University, New Haven, CT).

HSV-TK Assay. Spleen cells were plated at 10⁷ cells/well in 24-well plates (Costar Corp., Cambridge, MA) in 2 ml of EHAA medium with 10% FCS. T cells were obtained by depletion with the anti-I-Ab antibody 212.A1. B cells were obtained as described below. T cells were plated in the presence or absence of 2.5 μg/ml Con A (Boehringer Mannheim Corp.) for 42 h. B cells were stimulated with 50 μg/ml LPS (Sigma Chemical Co., St. Louis, MO). Cells were harvested and lysed in 20 μl TK assay buffer (see below) containing 0.1% Triton. Cell extracts from the HSV-TK⁺ cell line were used as a positive control for the assay.

The standard assay for TK was obtained from Summers and Summers (23) and modified slightly. The assay included 0.1 M sodium phosphate, pH 6., 0.01 M ATP, 0.01 M magnesium acetate, 0.025 M NaF. The substrate ¹²⁵I-deoxycytidine (dC) (100 μCi/0.4 ml; New England Nuclear, Boston, MA) was used at 0.5 μCi per 50 μl reaction. The dC deaminase inhibitor THU was added to a final concentration of 50 μg/ml. 10 μl cell extract was used in each reaction and the assay was run at 37°C. Incorporation of labeled substrate was determined by absorption of 10 μl of the reaction to DEAE paper disks that were washed in water 3 × 15 min and in ethanol 15 min, air-dried, and counted in a liquid scintillation counter (model LS2800; Beckman Instruments, Inc., Fullerton, CA).

Cell Growth Assay. The cytotoxicity of the drug GANC was determined by using a cell growth assay. To obtain CD4-enriched T cells, total spleen cells were incubated for 60 min at 4°C with an antibody cocktail containing T1B105 (anti-CD8), T1B146 (anti-B220), HB191 (anti-NK cell), and 212.A1 (anti-I-Ab). Cultures were subsequently incubated with magnetic anti-mouse IgG (Collaborative Research, Inc., Bedford, MA) for 20 min at 4°C and then passaged over a magnet three times. To obtain CD8-depleted cultures, the same procedure was followed except that T1B105 was the only antibody used. On average, 70% enrichment of CD4 cells was obtained.

To deplete cultures of T cells for generating APCs, total spleen cells were resuspended directly in supernatant from a Y19 (anti-Thy1) hybridoma and incubated for 60 min at 4°C. The cells were then treated with rabbit complement (Low-Tox-M; Cedarlane Laboratories Ltd., Hornby, ON, Canada) at 37°C for 30 min. To obtain syngeneic APCs, complement-lysed cultures were irradiated (2,500 rad).

CD4 cells from transgenic and control mice were cultured in a volume of 200 μl EHAA medium with 10% FCS in flat-bottomed 96-well plates (Costar Corp.). Where indicated, 10⁴ APCs were added to each well. Unless otherwise indicated, GANC was used at 10 μM. To measure proliferation for SEA (100 ng/ml)-stimulated cells, 1 μCi [³H]thymidine was added on day 6 of culture and cultures were harvested on day 7 using a harvester (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Cells treated with Con A (2.5 μg/ml) were pulsed on day 3 and harvested on day 4.

Cytokine Assay. The CTLL-2 cell line was obtained from Dr. F. Fitch (University of Chicago, Chicago, IL). CTLL cells (5 × 10⁵) were incubated with dilutions of test supernatants or control recombinant lymphokines in 96-well plates for 42 h, pulsed with 1 μCi [³H]thymidine, and harvested the next day.

Flow Cytometry. Spleen cells were treated with Gey's medium to lyse RBCs and subsequently enriched for CD4 cells. CD4-enriched cultures (10⁶ cells/well) and APC (10⁵) were plated in 24-well plates and stimulated with SEA (100 ng/ml) ± 20 μM GANC. Cells were harvested on days 4 and 6 and stained (2 × 10⁶ cells/sample) using PE-conjugated anti-CD4 mAb and FITC-conjugated anti-Vβ3 (KJ25) mAb in PBS with 1% FCS. The staining was performed at 4°C for 15 min in 96-well plates. The

samples were washed twice and analyzed on a FACS® Analyzer 1 (Becton Dickinson & Co.).

In Situ Hybridization. The IL-2 probe, kindly provided by Dr. V. Paetkau (University of Alberta, Alberta, Canada), corresponded to a full-length cDNA clone (26). In situ hybridization was carried out on cytocentrifuge preparations of mitogen-stimulated CD4 T cells using ³⁵S-labeled RNA sense or anti-sense transcripts essentially as described (26, 27). Before hybridization, stored slides were rehydrated, acetylated, and prehybridized at 50°C for 2 h. The slides were then dehydrated and hybridized at 50°C for 16 h. The hybridized slides were washed and dehydrated, and autoradiography was performed as described (26).

Results

Spleen Cells Isolated from Transgenic Mice Express HSV-TK Activity. To establish transgenic mice that allow the selective ablation of IL-2-producing T cells, the HSV-TK gene was placed downstream of the murine IL-2 promoter to direct expression of the HSV-TK gene (Fig. 1). The treatment of cells that express HSV-TK with an antiviral agent such as GANC results in cell death (16). Five positive founder lines were identified using Southern blot analysis.

To determine the level of HSV-TK activity expressed, TK assays specific for the viral enzyme were carried out. In the presence of T cell activation and subsequent IL-2 produc-

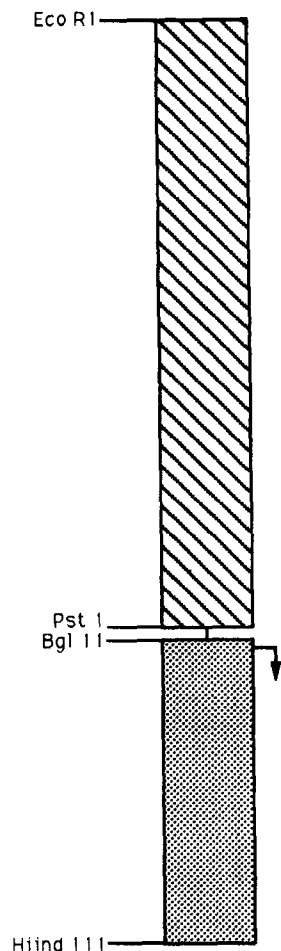


Figure 1. IL-2-TK construct used to generate transgenic mice. The 2.7-kb murine IL-2 promoter (hatched box) was fused to the 1.5-kb coding sequence (stippled box) of the HSV-1-TK gene by a PstI-BglII linker.

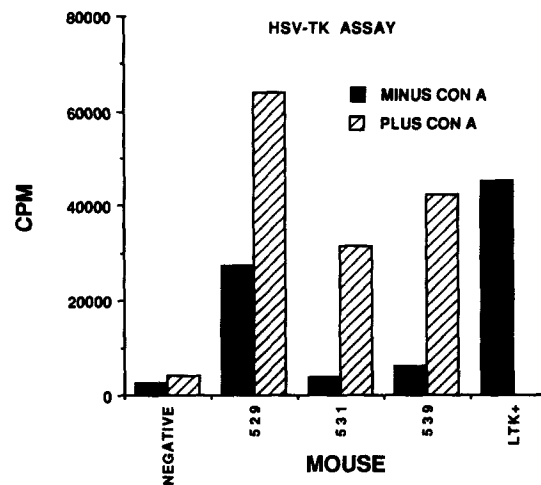


Figure 2. HSV-TK activity present in spleen cells isolated from transgenic mice. Spleen cells were plated at 10^7 cells/well in the presence or absence of 2.5 μ g/ml Con A. Cells were cultured for 42 h, harvested, and lysed in TK assay buffer containing 0.1% Triton. Each reaction consisted of 10 μ l of cell extract, 0.1 M sodium phosphate (pH 6), 0.01 M ATP, 0.01 M magnesium acetate, 0.025 M NaF, 50 μ g/ml THU, and 0.5 μ Ci ¹²⁵I-deoxycytidine in a total volume of 50 μ l. The incorporation of the substrate ¹²⁵I-deoxycytidine was determined after 3 h of incubation at 37°C by adsorption of 10 μ l of the reaction to DEAE paper and counting on a liquid scintillation counter. 10^6 TK⁺ cells were used as a positive control.

tion, the HSV-TK gene should be expressed. Total spleen cells were isolated from each transgenic line and cultured for 42 h in the presence or absence of Con A; cell extracts were assayed for HSV-TK activity (Fig. 2). An HSV-TK positive cell line was used as a positive control for the assay. The transgenic lines 529, 531, and 539 show substantial HSV-TK activity in the presence of Con A as compared with the B6 control. In the 529 pedigree (which has the highest copy number), expression of HSV-TK appears to be at least partly constitutive since HSV-TK activity can be detected in the absence of Con A stimulation. Two other founder lines, 536 and 726, had low to zero HSV-TK activity (data not shown).

Cell-specific Expression of HSV-TK Activity. To determine whether the transgene was expressed specifically in T cells, total spleen cells were isolated from the 539 pedigree and separated into T and B cells. T cells were cultured in the presence or absence of Con A and B cells were incubated with or without LPS. Significant TK activity was only present in cell extracts prepared from T cells stimulated with Con A (Fig. 3), not in transgenic B cells or B6 control cells. The B and T cells were activated by LPS and Con A, respectively, since they proliferated in response to the specific stimulus (data not shown).

Specific Killing of T Cells Isolated from Transgenic Mice Using GANC. To evaluate the efficacy of our system, the toxicity of GANC to T cells expressing the HSV-TK gene was tested in vitro. For GANC to kill T cells, the IL-2 promoter must be activated to allow HSV-TK expression. Though it is not clear to what extent chromosomal damage must occur to result in cell death, toxicity is very likely dependent on both GANC concentration and length of treatment. For these

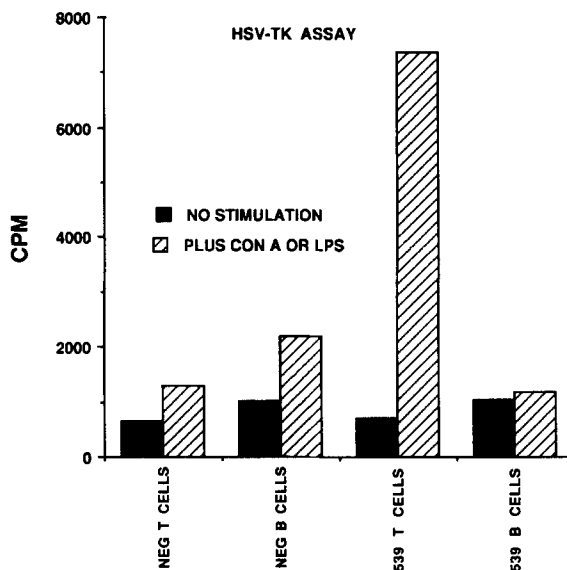


Figure 3. HSV-TK activity in T and B cells. Total T and B cells were obtained as described in Materials and Methods. 10^7 T or B cells were cultured with 2.5 $\mu\text{g/ml}$ Con A or 50 $\mu\text{g/ml}$ LPS, respectively, for 42 h. Cells were harvested and lysed in assay buffer and 50- μl reactions were run as described in the legend to Fig. 2. The incorporation of the substrate ^{125}I -deoxycytidine was determined after 3 h of incubation at 37°C by absorption of 10 μl of the reaction to DEAE paper and counting on a liquid scintillation counter. 10^6 TK⁺ cells were used as a positive control.

reasons, SEA was chosen for T cell activation. SEA is a superantigen which, when combined with MHC class II molecules, is a powerful stimulator of T cells via the V β element of the TCR (28). When unprimed splenic T cells are incubated in the presence of SEA, maximum proliferation is accompanied by a large production of IL-2 (data not shown). GANC treatment suppressed proliferation to SEA of CD4 T cells from transgenic lines 529, 531, and 539 (Fig. 4) showing that the proliferating cell has been ablated. Proliferation of T cells from the control B6 mouse is not inhibited in the presence of the drug. The transgenic lines 539 and 529 were chosen for their high HSV-TK activity and sensitivity to GANC. However, since the 529 pedigree shows some constitutive HSV-TK activity (Fig. 2), most experiments in this study were performed using the 539 mice.

Titration of GANC Concentration. To determine the optimum concentration of GANC, CD4-enriched T cells derived from 539 transgenics and control mice were cultured in the presence of SEA and various concentrations of GANC (Fig. 5). Suppression was partial at 1 μM GANC but increased to 89% at 10 μM GANC in CD4 cells from the transgenic mouse. An insignificant level of nonspecific inhibition (4%) of cells from the control mouse also occurred at 10 μM but increased significantly at 50–100 μM GANC. Similar results were observed with total spleen cells (data not shown). A concentration of 10 μM GANC was therefore selected for this study (unless otherwise noted).

Titration of Cell Number. The inhibitory effect of GANC on the ability of CD4 T cells to proliferate in response to

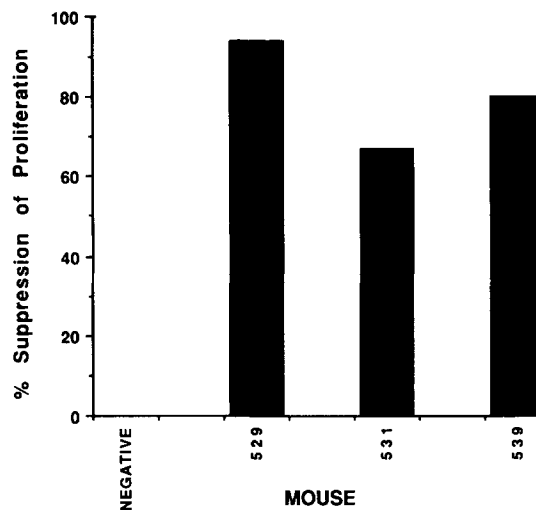


Figure 4. Suppression of proliferation in the presence of GANC. Spleen cells were depleted of CD8 cells and plated at 2.5×10^4 cells/well. Cells were activated using 100 ng/ml SEA in the presence or absence of 10 μM GANC. Cells were pulsed with [^3H]thymidine (1 μCi) on day 6 and incorporation was measured on day 7. Samples were run in duplicate and the maximum average cpm obtained was 189,634.

SEA is titratable with cell number (Fig. 6). The suppression of proliferation ranged from 85% using 5×10^4 cells to 98% using 1.25×10^4 cells. These results further demonstrate the importance of IL-2-producing cells in the proliferative response to SEA.

Elimination of IL-2 Production by the Ablation of TK-Expressing Cells. As an additional estimate of the efficiency of elimination of IL-2-producing cells, we stimulated IL-2-TK T cells with SEA plus or minus GANC and determined the amount of IL-2 produced as a function of input cell number (Fig.

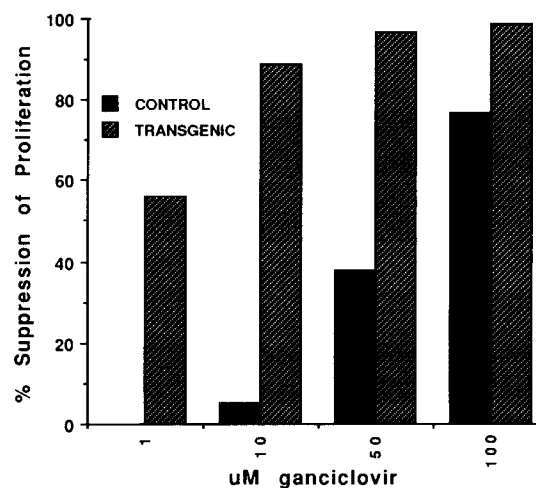


Figure 5. Titration of GANC concentration. 5×10^4 CD4-enriched spleen cells were cultured in the presence of 100 ng/ml SEA and various concentrations of GANC. 10^4 APC were added to each well and cells were pulsed with [^3H]thymidine on day 6 and harvested on day 7. Samples were run in duplicate and the maximum average cpm obtained was 89,352.

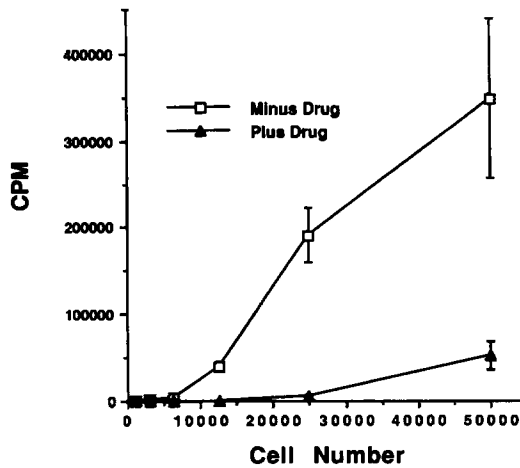


Figure 6. Suppression of proliferation is titratable with cell number. Various numbers of CD4-enriched spleen cells were cultured with 100 ng/ml SEA and 10^4 APC in the presence or absence of $10 \mu\text{M}$ GANC. Cells were pulsed with $[^3\text{H}]$ thymidine ($1 \mu\text{Ci}$) on day 6 and incorporation was measured on day 7. Samples were run in triplicate and the SE is given. The maximum average cpm obtained was 347,305.

7). The presence of GANC in the culture medium reduced the IL-2 secreted by about 80–90%. The efficiency of the IL-2-TK system is therefore ~ 80 –90%, whether measured by elimination of proliferation (Fig. 6), IL-2 production (Fig. 7), or the elimination of cells expressing IL-2 mRNA measured by in situ hybridization (see Table 2).

V β 3 T Cells Activated by SEA Are Specifically Killed by GANC. T cells bearing V β 3 receptors proliferate specifically in the presence of SEA (29). Since the strain of mouse used in this study is V β 3 $^+$, T cells were analyzed by FACS[®] to determine if GANC treatment of SEA-stimulated cells resulted in a loss of this T cell population. CD4-enriched T cells were stimulated with SEA in the presence or absence of $20 \mu\text{M}$

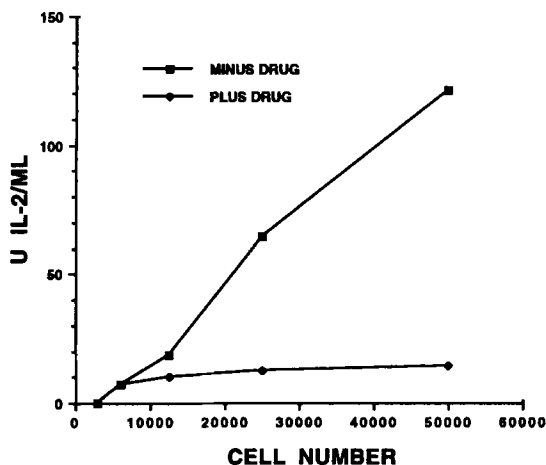


Figure 7. IL-2 production of CD4 T cells treated with GANC. Various numbers of CD4-enriched spleen cells were cultured with 100 ng/ml SEA and 10^4 APC in the presence or absence of $10 \mu\text{M}$ GANC. Culture supernatants were collected on day 3 and assayed for the presence of IL-2 by the induction of CTLL-2 proliferation.

Table 1. Effect of GANC on the V β 3 $^+$ T Cell Population

		B6		Transgenic	
		- Drug	+ Drug	- Drug	+ Drug
Day 4	CD4 $^+$ V β 3 $^-$	13.4	12.2	11.7	4.9
	CD4 $^+$ V β 3 $^+$	5.6	6.9	5.3	1.0
	CD4 $^-$ V β 3 $^-$	71.9	70.5	70.5	86.2
	CD4 $^-$ V β 3 $^+$	9.1	10.5	12.6	8.0
Day 6	CD4 $^+$ V β 3 $^-$	8.4	9.0	12.1	5.0
	CD4 $^+$ V β 3 $^+$	5.7	4.9	7.3	4.1
	CD4 $^-$ V β 3 $^-$	57.3	61.3	53.3	60.2
	CD4 $^-$ V β 3 $^+$	28.6	24.7	27.3	30.7

Shown are the percent cells present in different populations before and after GANC treatment of SEA-stimulated cultures. Values were determined by FACS[®] analysis. The percentage of CD4 $^+$ V β 3 $^+$ and CD4 $^-$ V β 3 $^+$ cells present in the starting population were 0.52 and 5.92, respectively.

GANC, harvested on days 4 and 6, and double stained for CD4 and V β 3 expression (Table 1, Fig. 8). Using this scheme, the cells were divided into CD4 $^+$ V β 3 $^+$, CD4 $^+$ V β 3 $^-$, CD4 $^-$ V β 3 $^-$, and CD4 $^-$ V β 3 $^+$ populations.

As expected, the percentage of CD4 $^+$ V β 3 $^+$ T cells increases substantially following SEA stimulation, as compared with the starting population. The CD4 $^-$ V β 3 $^+$ population also increases after stimulation with SEA, particularly on day 6 (these cells are most likely CD8 cells that are positive for V β 3 expression). Whereas GANC treatment has no effect on cells isolated from the B6 control mouse, there is a significant decrease in the number of V β 3 $^+$ T cells in samples from the transgenic mouse. On day 4, all V β 3 $^+$ T cells are significantly reduced whereas predominantly CD4 $^+$ V β 3 $^+$ cells are affected on day 6. There are more V β 3 $^+$ cells on day 6 as compared with day 4 (Fig. 8). This may represent an enrichment of a small population of V β 3 $^+$ cells which initially escaped killing by GANC. As expected, there is no effect on CD4 $^-$ V β 3 $^-$ T cells. These cells are most likely B cells that do not express HSK-TK activity. Whereas there is a selective ablation of V β 3 $^+$ T cells, there is also killing of CD4 $^+$ V β 3 $^-$ cells. The non-V β 3 CD4 cells that are eliminated are most likely other CD4 cells that respond to SEA such as V β 11 T cells (29). It is interesting to note that although we generally see an 80–90% suppression of proliferation in cell growth assays, this is not reflected in the decrease seen in the CD4 T cell population. A possible explanation is that these residual cells are CD4 cells that are not synthesizing IL-2 and are therefore not affected by GANC. It is also interesting that CD4 $^+$ V β 3 $^+$ T cells are preferentially eliminated on day 4, whereas a greater percentage of CD4 $^+$ V β 3 $^-$ cells are ablated on day 6. It is likely that the V β 3 $^+$ population responds to SEA before other V β -expressing T cells.

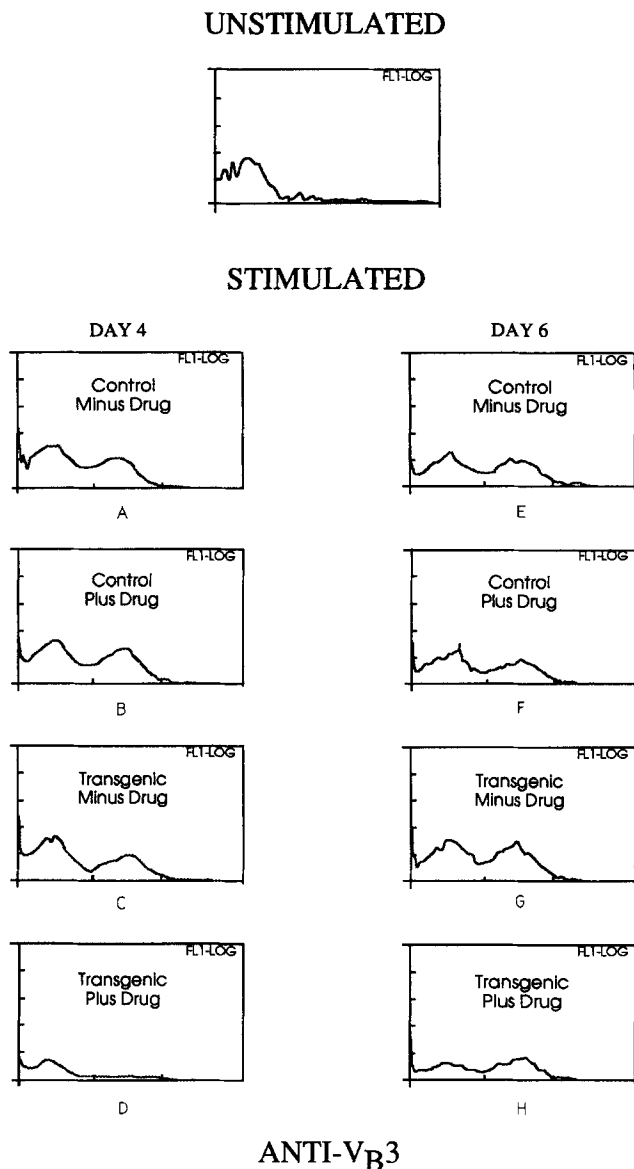


Figure 8. FACS[®] analysis of V β 3 expression of CD4 cells. 10^6 CD4-enriched cells were cultured with 10^5 APC and 100 ng/ml SEA in the presence and absence of 20 μ M GANC. Cells were harvested on days 4 and 6 and double stained using a PE-conjugated anti-CD4 mAb and a FITC-conjugated anti-V β 3 mAb. Samples were analyzed on a Becton Dickinson FACS[®] Analyzer 1. The CD4⁺ cells were gated on and the FACS[®] plot is only of these cells.

IL-2-producing Cells Are Required for a Proliferative Response to SEA but Are only Partially Required for a Response to Con A. To investigate the requirement of IL-2-producing cells in response to mitogens other than SEA, CD4 T cells from the transgenic mouse were stimulated with Con A. Fig. 9 compares the proliferative response of CD4 cells treated with GANC when stimulated with either SEA or Con A. GANC reduced proliferation of transgenic CD4 cells to SEA by 83%. When CD4 cells were stimulated with Con A, proliferation was suppressed by only 35% in cells from the transgenic mouse and 9% in cells from a control mouse (Fig. 9 B).

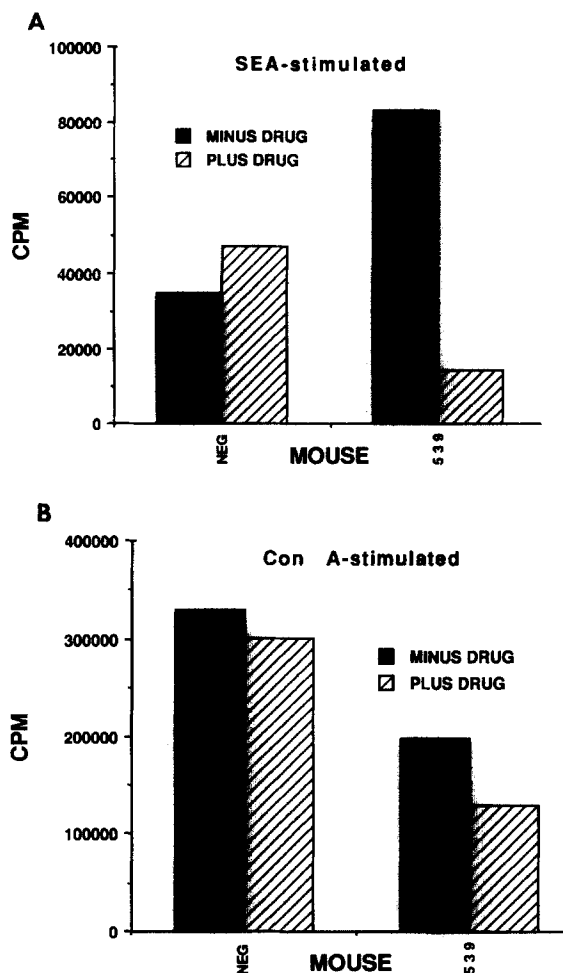


Figure 9. Suppression of proliferation in T cells activated with SEA and Con A. (A) 5×10^4 CD4-enriched spleen cells were cultured with 100 ng/ml SEA and 10^4 APC in the presence or absence of 10 μ M GANC. Cells were pulsed with [³H]thymidine (1 μ Ci) on day 6 and incorporation was measured on day 7. Samples were run in duplicate and the maximum average cpm obtained was 83,154. (B) 2×10^5 CD4-enriched spleen cells were cultured with 2.5 μ g/ml Con A and 10^4 APC in the presence or absence of 10 μ M GANC. Cells were pulsed with [³H]thymidine (1 μ Ci) on day 3 and incorporation was measured on day 4. Samples were run in duplicate and the maximum average cpm obtained was 329,883.

The difference in the effect of GANC on proliferation when cells are stimulated with Con A as compared with SEA could reflect differences in the mechanisms by which cells are activated by the two mitogens, or merely differences in the efficiency of killing in the two systems. The proliferation to Con A could involve IL-2-independent mechanisms; such cells would be insensitive to the effects of GANC. To test this hypothesis, cells were hybridized in situ after activation with either Con A or SEA in the presence or absence of GANC. Cells were analyzed for IL-2 mRNA expression after primary stimulation and after restimulation with the same mitogen. It is striking that the reduction in the proportion of cells expressing IL-2 mRNA in the primary cultures is nearly identical for both Con A- and SEA-activated cells (Table

Table 2. Frequency of CD4 T Cells Positive for IL-2 mRNA

	Percent cells expressing IL-2 mRNA	
	Primary	Restimulated
Con A-GANC	35 (15)	8 (24)
Con A plus GANC	9 (10)	4 (10)
SEA-GANC	19 (15)	10 (16)
SEA plus GANC	4 (9)	5 (10)

5×10^4 CD4 cells were stimulated with either Con A or SEA in the presence or absence of GANC. Cultures were either harvested on day 3 (primary cultures) or washed and restimulated on day 5 with the same mitogen and harvested on day 6 (restimulated cultures). Cyto centrifuge preparations were made and analyzed by in situ hybridization for IL-2 mRNA production. The numbers in parentheses refer to the average number of grains per mRNA⁺ cell. Grain counts per cell were recorded for 1,000 cells from 10 microscope fields per slide. Cells were considered positive when overlaid with greater than five grains.

2). Since the efficiency of eliminating cells that produce IL-2 is the same for both mitogens, the difference in the effect of GANC on proliferation is not a result of less efficient killing of Con A-treated cultures as compared with SEA. These results are consistent with there being an IL-2-independent mechanism of proliferation for Con A which is insensitive to drug treatment.

Upon restimulation, a small number of cells are capable of IL-2 expression following GANC treatment for both Con A and SEA (Table 2). These residual cells appear to be qualitatively different from cells not treated with drug since they have a lower grain count and therefore presumably lower IL-2 mRNA content. These cells may not be susceptible to GANC because they produce less IL-2 and are consequently unable to proliferate, which is necessary for killing with GANC. Consistent with this is the fact that proliferation of SEA-stimulated cells is significantly inhibited by GANC.

The Proliferating T Cell Is the Same Cell Making IL-2. In the present study, cells that produce IL-2 are ablated and, as a consequence, proliferation is reduced. If bystander cells which require IL-2 for proliferation, but which do not produce it, are still present in the culture, these cells could proliferate in response to IL-2 since they will not be killed by GANC. To test this, 5 U/ml (5 ng/ml) rIL-2 was added to CD4 cells stimulated with SEA in the presence or absence of GANC. Fig. 10 compares the results of proliferation assays carried out using cells from a control (Fig. 10, A and B) or a transgenic (Fig. 10, C and D) mouse incubated with or without added IL-2. In the absence of rIL-2, proliferation was suppressed in the transgenic mouse from 67% using 5×10^4 cells to 89% using 1.25×10^4 cells (Fig. 10 C). When rIL-2 was present during the incubation period, proliferation was suppressed from 86% using 1.25×10^4 cells to 91% using 2.5×10^4 or 5×10^4 cells (Fig. 10 D). The same failure to restore proliferation was obtained when 10 U/ml (IL-2) was added to GANC-treated CD4 cells (data

not shown). Therefore, the addition of rIL-2 to CD4 T cells treated with GANC does not restore the proliferative response to SEA.

Discussion

IL-2 has been shown to be the major growth factor for T cells and plays a key role in the clonal expansion of antigen-specific T cells. There are indications, however, that IL-2 is not the sole T cell growth factor and that IL-2-independent mechanisms may exist for T cell proliferation.

We have established the use of cell ablation to analyze the requirement for IL-2-producing cells in the proliferative responses to mitogens. Treatment with GANC is highly effective (~80–90%) in the removal of IL-2-producing cells, whether measured by suppression of T cell proliferation or IL-2 production, or the elimination of cells expressing IL-2 mRNA detected by in situ hybridization. The results presented here demonstrate that IL-2-producing T cells are required for a proliferative response to the superantigen SEA. We have also shown that proliferation to SEA is not restored when rIL-2 is added to cultures in which IL-2-producing cells have been eliminated. These results indicate that the majority of T cells that proliferate in response to SEA are making IL-2 and are not bystander cells which require IL-2 for cell growth. A less likely explanation which we have not excluded, however, is that the IL-2-producing cells provide something other than IL-2, which is required for proliferation by bystander cells.

The inability of rIL-2 to restore the proliferative response to SEA differs from results reported by Schorle et al. (19). When IL-2 was added to Con A-stimulated splenocytes isolated from mice that were unable to make IL-2 as a consequence of a homozygous null mutation at the IL-2 locus, proliferation was restored to wild-type levels. However, in these IL-2-deficient mice, the cells that respond to IL-2 are still present and able to proliferate when exogenous IL-2 is added. In the study described here, the cells that require IL-2 for proliferation to SEA have been ablated.

The ablation method used in this study only partially inhibits the proliferative response to Con A, whereas it nearly abolishes the response to SEA. Using in situ hybridization, we have shown that the efficiency of killing of cells that produce IL-2 is the same for Con A and SEA, despite the different effect on T cell proliferation. This suggests that a portion of the proliferative response to Con A occurs via an alternative pathway not requiring IL-2 expression and release. These results are consistent with those obtained by Schorle et al. (19) using IL-2-deficient mice. Although splenocytes isolated from such mice show a marked decrease in their ability to respond to Con A, there is still a residual proliferative response that is not inhibited by the absence of IL-2. Our results also demonstrate that residual cells capable of IL-2 expression are present after drug treatment when either mitogen is used for stimulation. However, the lower grain count present in these cells compared with cells not treated with the drug suggests that these cells may be unable to proliferate because of a reduced production of IL-2, which renders them insensitive to GANC.

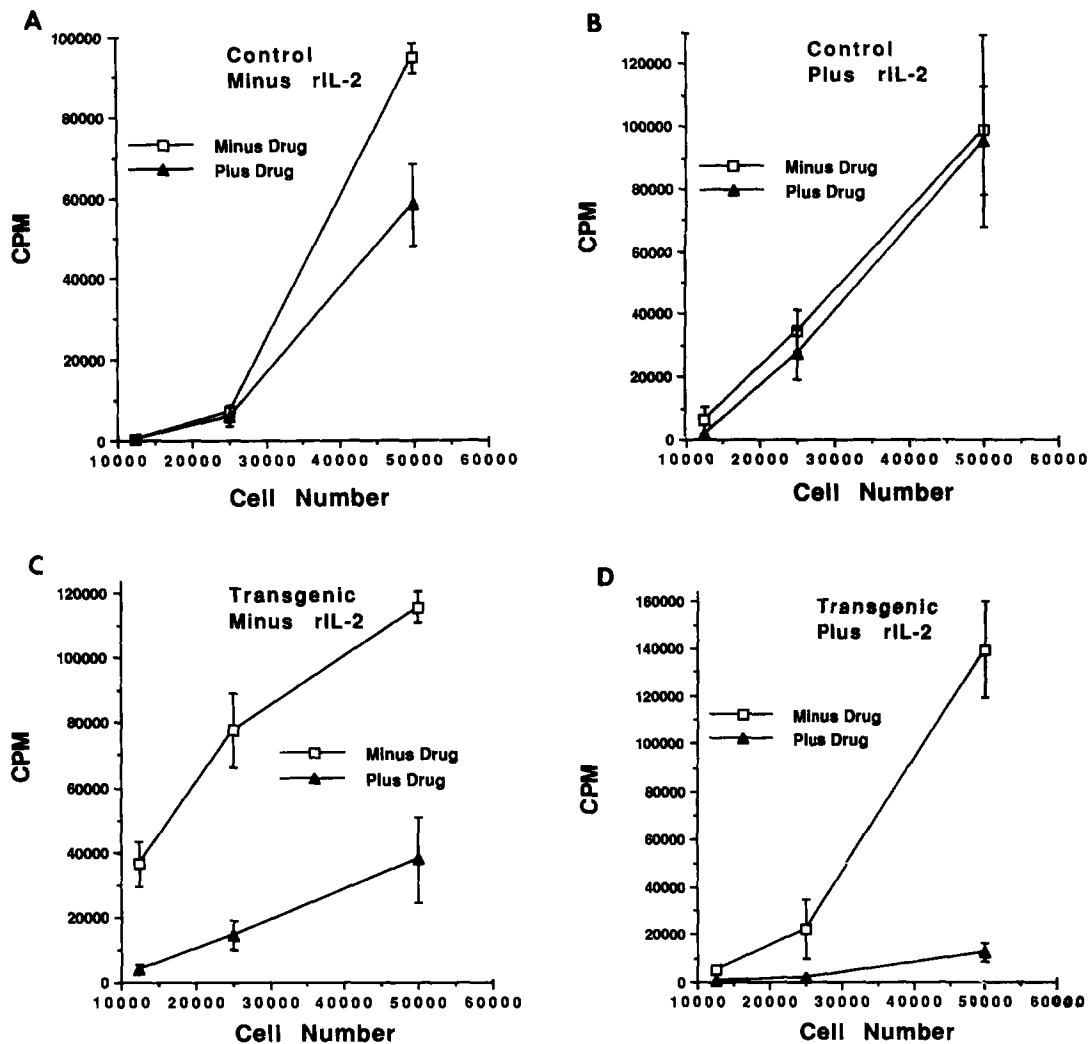


Figure 10. The addition of rIL-2 does not restore the proliferative response to SEA. Various numbers of CD4-enriched spleen cells were cultured with 100 ng/ml SEA and 10^4 APC in the presence or absence of $10 \mu\text{M}$ GANC. 5 U/ml rIL-2 was added to half the samples (B and D) at the start of culture. Cells were pulsed with $[^3\text{H}]$ thymidine ($1 \mu\text{Ci}$) on day 6 and incorporation was measured on day 7. Samples were run in triplicate and the SE is given. The maximum average cpm obtained were 94,619 (A), 98,350 (B), 115,628 (C), and 139,392 (D).

It is important that the ablation of T cells be specific for those cells that are activated and express IL-2. We have shown that HSV-TK activity is present in activated T cells but absent in B cells. More importantly, we have shown that the $V\beta 3^+$ population of T cells that are activated in the presence of SEA are selectively ablated whereas $CD4^+V\beta 3^-$ cells remain a viable population. In addition, unlike the response to SEA, the proliferative response to Con A is only partially inhibited by drug treatment of stimulated cells. This provides additional evidence that we are specifically eliminating activated T cells which are producing IL-2 and not all acti-

vated T cells. The transgenic mice that we have generated will therefore be a powerful tool for studying the requirement of IL-2 in specific immune responses, as well as during fetal development. We have preliminary data that shows the requirement of IL-2-producing cells for an in vitro recall response to KLH (Kamogawa, Y., unpublished results) and for the expression of IL-2 in the normal development of T cells (Carding, S., unpublished results). Finally, the selective ablation of IL-2-producing T cells provides a unique method for studying the lineage relationship between subsets of CD4 T cells.

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R. A. Flavell is an investigator; K. Bottomly is an associate investigator; Y. Kamogawa is an associate; and L. Minasi was formerly an associate of the Howard Hughes Medical Institute.

Address correspondence to Dr. Richard A. Flavell, Section of Immunology, Yale University School of Medicine, P.O. Box Box 3333, New Haven, CT 06510.

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