

# FUNCTIONAL HETEROGENEITY IN ALLOSPECIFIC CYTOTOXIC T LYMPHOCYTE CLONES

## I. CTL Clones Express Strong Anti-Self Suppressive Activity

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It is now routinely possible to maintain cytotoxic T lymphocytes (CTL)<sup>1</sup> as long-term clonal populations in tissue culture (1, 2). Although such CTL clones have been widely considered to be functionally stable cell lines, an alternative possibility is that they represent a differentiating cell system continuously constrained by environmental influences to produce CTL of the appropriate specificity. Maintenance of cloned CTL requires not only the continuous presence of growth factors but also of the appropriate stimulator cells. Deviation from standard culture conditions might lead to aberrations from the normal differentiation program. Indeed, such deviations can lead to the rapid appearance of relatively nonspecific, natural killer cell (NK)-like cytolytic effector cells (3, 4), and long-term culture under standard conditions can lead to the appearance of new specificities (5).

There is much evidence that self-reactivity is a normal aspect of T cell differentiation (see Discussion and references 6 and 7). Thus, if CTL lines, as suggested above, represent differentiating cell systems, the maintenance of stable cytolytic specificity might depend critically on the presence of a mechanism for controlling the development of self-reactivity. We describe here five CTL clones, maintained under standard culture conditions, which appear to possess such a mechanism: when cells from any of these clones were added to a mixed lymphocyte culture (MLC), they could prevent the development of cytotoxic activity against their own H-2 antigens; i.e., from the point of view of the added CTL, an anti-self response was prevented. This activity within a CTL clone is in many respects similar to a phenomenon we have previously described as "veto" activity (8-12).

### Materials and Methods

*Mice.* The inbred strains C57BL/6J<sub>Oci</sub> (B6, H-2<sup>b</sup>), BALB.B (H-2<sup>b</sup>; breeding pairs kindly provided by Dr. P. F. Halloran, Mt. Sinai Hospital, Toronto), AKR/J (AKR, H-

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<sup>1</sup> *Abbreviations used in this paper:* Con A, concanavalin A; CTL, cytotoxic T lymphocyte; FCS, fetal calf serum; 2-ME, 2-mercaptoethanol; MHC, major histocompatibility complex; MLC/MLR, mixed lymphocyte culture/reaction; NK, natural killer cell.

<sup>2k</sup>), C3H/HeJ (C3H, H-2<sup>k</sup>), and C57BL/6-H-2<sup>k</sup>Oci (B6.K, AKR donor of H-2; breeding pairs kindly provided by Dr. E. A. Boyse, Memorial Sloan-Kettering Cancer Center, NY) were bred at the Ontario Cancer Institute. CBA (H-2<sup>k</sup>), A.SW/Sn (H-2<sup>s</sup>), BALB/c (H-2<sup>d</sup>), DBA/2 (H-2<sup>d</sup>), and the three congenic strains, C57BL/10 (B10, H-2<sup>b</sup>), B10.D2 (H-2<sup>d</sup>), and B10.BR (H-2<sup>k</sup>), were purchased from The Jackson Laboratory, Bar Harbor, ME. 8–12-wk-old mice were used for all mixed lymphocyte reactions (MLR).

**CTL Clones.** The five CTL clones studied in the present work were derived from secondary MLR cultures of B6 spleen cells stimulated with irradiated BALB/c (4A2, 4B3), DBA/2 (8D5), or C3H (8F4, 8C5) spleen cells by methods previously described (13). CTL were cloned at limiting dilution in the presence of irradiated allostimulator cells and 30% supernatant from secondary MLR cultures. The cloned CTL were maintained by weekly reculturing of 10<sup>5</sup> CTL with 10<sup>7</sup> fresh, irradiated (1,800 rad) stimulator cells in 10-ml cultures after removal of dead cells on Lympholyte (Cedarlane Laboratories, Hornby, Ontario).

**MLR Cultures.** Cultures were set up in replicates of six in 96-well, V-bottom microtiter trays (Linbro Chemical Co., New Haven, CT). Responder lymph node cells, 3 × 10<sup>4</sup>/culture, and irradiated (1,800 rad) stimulator cells (3 × 10<sup>5</sup>/culture), were cultured in a total volume of 200 μl Hepes-buffered medium containing 10% FCS, 5 × 10<sup>-5</sup> 2-ME, streptomycin, and penicillin. In experiments assessing suppressive activity of CTL clones, 100, 300, or 1,000 CTL were added to individual MLR cultures on day 1 of culture unless otherwise indicated. In some experiments (Fig. 2, Table V), MLR cultures were instead set up in 12 × 75 mm plastic tubes (Fisher Scientific Co., Pittsburgh, PA) using 3 × 10<sup>5</sup> responder cells and 1.5 × 10<sup>6</sup> irradiated stimulator cells in 1.5 ml of medium as above, but also containing 25% supernatant from secondary MLR cultures.

**Cytotoxicity Assay.** Cytotoxic activity of MLR cultures was determined after 5 d of culture in a 4 h (6 h for experiments of Table V) <sup>51</sup>Cr-release assay. Target cells were usually 2,000 concanavalin A (Con A)-induced spleen cell blasts from the MLR stimulator strain but, in some experiments, <sup>51</sup>Cr-labeled RBL5 (H-2<sup>b</sup>) tumor cells maintained in vitro were used, and in one series (Table V), responder cells from MLR cultures were used. In all cases, target cells, 5 × 10<sup>6</sup> cells in 0.2 ml fetal calf serum (FCS), were labeled with 300 μCi sodium [<sup>51</sup>Cr] chromate (<sup>51</sup>Cr) for 1–2 h at 37°C. Cells were then washed four times before the cytotoxicity assay. Spontaneous <sup>51</sup>Cr release was determined from target cells added to cultures containing only irradiated stimulator spleen cells and 100–1,000 CTL cells. Total releasable <sup>51</sup>Cr was determined from acetic acid-treated target cells. Under these conditions, total releasable counts were 2–5 cpm/target cell and spontaneous release was 10–25% of the total release. Specific <sup>51</sup>Cr release was calculated as: (observed release – spontaneous release)/(total release – spontaneous release). The relative cytotoxic activity of a group containing CTL cells was expressed as the percentage of the response observed in a control group without added CTL cells.

**Fluorescence Microscopy.** Cells cultured in MLR for 5 d were centrifuged on Lympholyte and incubated at 4°C for 45 min with an alloanti-H-2<sup>b</sup> serum at a dilution of 1:50 (see Results). In the presence of complement this antiserum killed 50% of RBL-5 tumor cells (H-2<sup>b</sup>) at a dilution of 1:500. The cells were washed and stained at 4°C for 30 min with a fluorescein-labeled goat anti-rat-Ig serum at a dilution of 1:20. The cells were examined in a Leitz Ortholux fluorescence microscope using incident UV illumination.

**Antibody and Complement Treatment.** Monoclonal anti-H-2<sup>b</sup> antibody was obtained from culture supernatants of hybridoma clone 20-8-4S from the American Type Culture Collection (Rockville, MD). By complement-dependent lysis this antibody reacts strongly with both the K and D end of the H-2<sup>b</sup> haplotype and cross-reacts with H-2K<sup>d</sup>, H-2<sup>r</sup>, and H-2<sup>s</sup> (14). Culture supernatant from 20-8-4S cells grown at 10<sup>6</sup> cells/ml for 24 h was used at a dilution of 1:10. Cells from B10.BR anti-B6 MLR cultures set up in plastic tubes with or without added 4B3 CTL were centrifuged at 200 g, and 0.5 ml of anti-H-2<sup>b</sup> antibody was added to the cell pellet. The cells were incubated at 4°C for 1 h, centrifuged, and resuspended in 1 ml of diluted (1:8) rabbit complement (LoTox; Cedarlane Laboratories) and incubated for another 60 min at 37°C. The cells were then centrifuged and fresh culture medium including 25% secondary MLR supernatant and, when MLR cultures

were <72 h old, fresh irradiated stimulator cells, were added to the pellet. This treatment killed 100% of 4B3 CTL cells or RBL-5 (H-2<sup>b</sup>) tumor cells.

## Results

*Anti-Self Suppressive Activity Is a Normal Property of Cloned Allospecific CTL.* We first show that adding a cloned CTL line to an MLR can reduce the level of cytotoxic activity developed in the MLR when the CTL are syngeneic to the stimulator cells of the MLR, even though the added CTL cannot recognize either the responder or stimulator cells of the MLR. In one experiment (Fig. 1), four clones derived from B6 mice (H-2<sup>b</sup>) were tested. Clones 4A2, 4B3, and 8D5 are specific for the D end of the H-2<sup>d</sup> haplotype; clone 8F4 is specific for the K end of the H-2<sup>k</sup> haplotype. Cells from the four CTL clones were added on day 1 of MLR culture and cytotoxic activity was measured on day 5. The added CTL clearly inhibited the development of cytotoxic activity generated against syngeneic stimulator cells, but had no effect when added to cultures containing syngeneic responder cells. In all of the cultures, the added CTL could recognize neither responder nor stimulator. Rather, suppression was always specific for the (self) H-2 antigen of the added CTL so that, from the point of view of the added CTL, a response against self was suppressed. As few as 300 added CTL cells were capable of inhibiting CTL generation by >50%. Irradiation (1,200 rad) of the added CTL (clone 4B3) immediately before the addition on day 1 of an MLR culture abolished anti-self suppression (data not shown).

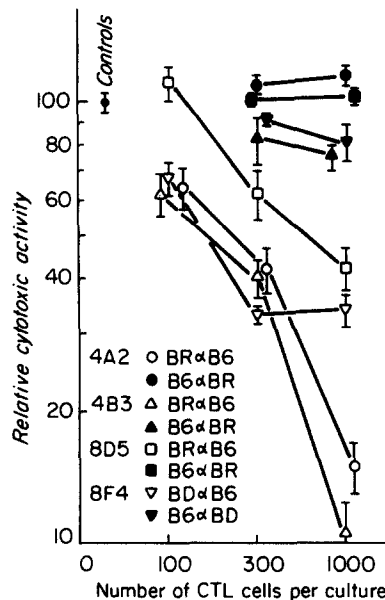


FIGURE 1. Specific suppression of CTL generation in H-2<sup>k</sup> anti-H-2<sup>b</sup> MLR (CTL lines 4A2, 4B3, 8D5) and H-2<sup>d</sup> anti-H-2<sup>b</sup> MLR (CTL line 8F4). Each data point represents mean cytotoxic activity obtained from replicates of six MLR cultures. Bars represent 2 SEM. Specific release values obtained from control MLR cultures were: B10.BR anti-B6 (BR α B6), 59.7 ± 3.0; B6 anti-B10.BR (B6 α BR), 35.2 ± 3.2; B10.D2 anti-B6 (BD α B6), 29.0 ± 0.9; B6 anti-B10.D2 (B6 α BD), 43.1 ± 2.0. Spontaneous release was <25% maximum release here and elsewhere.

Table I shows the results of 51 separate titrations of clones 4A2, 4B3, or 8D5, all of which are B6 (H-2<sup>b</sup>) and directed against H-2<sup>d</sup> (D end). All titrations were performed as in Fig. 1. All CTL clones were tested at the same point of their growth cycle, i.e., 5–6 d after dilution and reculture. This point corresponds to just past the time they have reached the plateau phase of their growth curve. Almost all (23/24) titrations containing syngeneic stimulator cells (B10.BR anti-B6 and A-SW anti-B6) were significantly ( $P < 0.05$ ) suppressed by 1,000 added CTL. The two titrations performed with H-2-identical, but otherwise allogeneic, stimulator cells (B10.BR anti-BALB.B) were significantly suppressed by 1,000 added CTL. Few (3/19) cultures containing syngeneic responder cells (B6 anti-B10.BR, B6 anti-A.SW, B6 anti-B10.D2) were significantly suppressed by 1,000 added CTL. There was no suppression observed in titrations in which both responder and stimulator cells were H-2 different from the added CTL. These results clearly demonstrate the specificity of the suppression for syngeneic or H-2-identical stimulator cells although, in a few cases, suppression of cultures containing syngeneic responder cells was noted. The specificity of the suppression implies that it is not caused by competition for growth factor between the added CTL and the MLR responder cells.

*MLR Are Most Sensitive to Anti-Self Suppressive Activity at Intermediate Culture Times.* CTL from the 4B3 cloned line were added to and removed from B10.BR anti-B6 MLR cultures at different times after culture initiation (Fig. 2). Suppression was almost total when the 4B3 CTL were present from 0 to 72 h of culture and was minimal when the 4B3 CTL were present only from 72 to 96 h of culture. Having the CTL present from 24 to 48 h or from 48 to 72 h produced much more suppression than having the CTL present from 0 to 24 h. Similar results were obtained with a second CTL line, 4A2, except that the presence of CTL from 48 to 72 h was much less effective than for 4B3. We conclude that

TABLE I  
*Production of Suppression by Added Cloned CTL in Different MLR Combinations*

MLR combination	CTL added		
	1,000	300	100
B10.BR anti-B6	21/22	19/22	7/18
A.SW anti-B6	2/2	2/2	2/2
B10.BR anti-BALB.B	2/2	1/2	2/2
B6 anti-B10.BR	3/12	1/12	0/10
B6 anti-A.SW	0/2	0/2	0/2
B6 anti-B10.D2	0/5	0/5	0/5
B10.BR anti-A.SW	0/4	0/4	0/4
B10.BR anti-B10.D2	0/2	0/2	0/2

CTL were from 4A2, 4B3, or 8D5 cloned CTL, all of B6 origin and directed against the D end of H-2<sup>d</sup>. They were added on day 1 of MLR culture. Entries are the number of MLR titrations out of total tested with a significantly lower ( $P < 0.05$  as assessed by the Wilcoxon rank sum test) cytotoxic activity on day 5 than in control cultures.

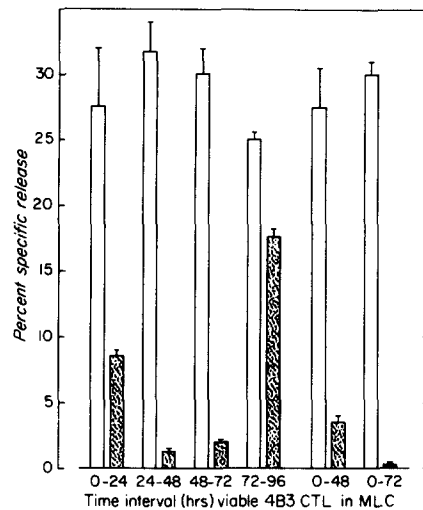


FIGURE 2. Kinetic analysis of anti-self suppressive activity. B10.BR anti-B6 MLR cultures were set up with  $3 \times 10^5$  responder and  $1.5 \times 10^6$  stimulator cells with (stippled bars) or without (open bars)  $10^4$  4B3 cloned CTL line cells (B6 origin, anti-H-2<sup>d</sup> specificity). These were added and removed at the times indicated using a strongly cytotoxic anti-H-2<sup>b</sup> monoclonal antibody plus complement as described in Materials and Methods. On day 5 (120 h) each MLR culture was split into three replicates and assayed for cytotoxic activity against B6 Con A blasts. Mean percentage of <sup>51</sup>Cr release  $\pm$  SD are shown.

the responder cells in the MLR became most sensitive to suppression at some time after the first day of culture and became much less sensitive to suppression after the second or third day of culture.

*Anti-Self Suppressive Activity Is Not Due to "Cold" Target Inhibition in the Cytotoxicity Assay.* A trivial explanation for the results shown in Fig. 1 is that the added CTL proliferate during the MLR culture and act as "cold" target inhibitors in the subsequent cytotoxicity assay. The fact that suppression was still observed upon removal of the added CTL after 24, 48, or 72 h of culture (Fig. 2) would appear to eliminate this possibility. Nevertheless, we decided to make more direct tests. In the first design, CTL were cultured with irradiated syngeneic spleen cells in the medium used for MLR cultures. After 4 d of incubation, they were added to day 5 anti-H-2<sup>b</sup> MLR cultures 2 h before the cytotoxicity assay and the results compared with the cytotoxicity found in cultures that did or did not have CTL added on day 1 of culture. As shown in Table II (Exp. 1), no inhibition of the specific <sup>51</sup>Cr release was seen in MLR cultures to which such cultured CTL cells were added. In the second design (Exp. 2), the effect on cytotoxic activity of mixing suppressed (CTL-containing) and unsuppressed cultures just before assay was measured. Again, no inhibition in specific <sup>51</sup>Cr release was seen. In the third design (Exp. 3), we attempted to inhibit the cytotoxic activity in an anti-H-2<sup>b</sup> MLR with normal cold CTL cells. Only a slight reduction in specific <sup>51</sup>Cr release was observed after addition of as many as  $10^4$  CTL cells to the MLR cultures before the assay. This greatly exceeded the number of CTL found on day 5 in a culture to which 4A2 CTL were added on day 1: when  $10^3$  were added on day 1,  $\sim 4 \times 10^3$  were seen on day 5 (Table III) (detected by fluorescence

TABLE II  
*CTL-induced Anti-Self Suppression in MLR Cultures Is Not Inhibited by the Presence of the Admixed CTL Cells*

	Cytotoxic activity in H-2 <sup>k</sup> anti-H-2 <sup>b</sup> MLR cultures*			
	Exp. 1 Clone 4B3	Exp. 2		Exp. 3 Clone 4B3
		Clone 4A2	Clone 8D5	
Control MLR	85 ± 9	50 ± 3	50 ± 3	91 ± 15
Plus 1,000 CTL <sup>‡</sup>	11 + 1 <sup>§</sup>	24 + 4 <sup>§</sup>	27 + 6 <sup>§</sup>	11 + 2 <sup>§</sup>
Mixing of control MLR before cytotoxicity assay with:				
Cultured CTL <sup>†</sup>	77 ± 9	47 ± 4	49 ± 2	ND
2 × 10 <sup>5</sup> cold CTL <sup>‡</sup>	ND	ND	ND	88 ± 9
10 <sup>4</sup> CTL	ND	ND	ND	68 ± 4

\* Data represent mean specific <sup>51</sup>Cr release values from six replicate cultures ± SEM.

<sup>‡</sup> 10<sup>5</sup> CTL were added to the MLR culture at day 1 of culture.

<sup>§</sup> Indicates a significant difference from control cultures without added CTL cells. (*P* < 0.05 according to Wilcoxon rank sum test).

<sup>†</sup> Exp. 1: CTL were obtained from a 5-d culture of 1,000 CTL cells stimulated with 40% MLR supernatant and irradiated, syngeneic spleen cells. Exp. 2: CTL cells obtained from 5-d CTL-suppressed MLR cultures.

<sup>‡</sup> CTL were freshly obtained 4B3 cells.

TABLE III  
*Cell Numbers, Frequency of H-2<sup>b</sup>-positive 4A2 CTL Cells, and Specific Killing in Day-5 MLR Cultures to Which CTL Cells Were Added at Day 1 of Culture*

Type of MLR culture	Number of viable cells per MLR culture (×10 <sup>3</sup> )		Percent H-2 <sup>b</sup> -positive cells		Specific <sup>51</sup> Cr release	
	0	1,000*	0	1,000*	0	
					0	1,000*
H-2 <sup>k</sup> anti-H-2 <sup>b</sup>	39 ± 5 <sup>‡</sup>	35 ± 6	0	9 <sup>§</sup>	43 ± 6	10 ± 3 <sup>†</sup>
H-2 <sup>k</sup> anti-H-2 <sup>a</sup>	39 ± 5	27 ± 8	0	1 <sup>§</sup>	40 ± 3	66 ± 9

\* Number of 4A2 CTL cells added on day 1 of MLR culture.

<sup>‡</sup> Data represent means of six replicate cultures ± SD.

<sup>§</sup> Indirect fluorescence microscopy on pooled cells from six cultures. 200 cells were counted from each sample.

<sup>†</sup> Significantly suppressed (*P* < 0.01, rank sum test).

using anti-H-2<sup>b</sup> alloantiserum, which stains all 4A2 CTL cells). From these data we conclude that the anti-self suppressive activity mediated by CTL cells is not simply due to cold target cell inhibition at the time of assay.

*Relationship Between Cytotoxic Activity and Anti-Self Suppressive Activity.* We first tested whether cytotoxic activity and anti-self suppressive activity were correlated by comparing the two activities for individual CTL clones on different points of their growth curves. CTL clones 4A2, 4B3, 8C5, 8D5, and 8F4 were assayed for allospecific cytotoxicity and anti-self suppressive activity when cultured under exponential (days 3–4 after reculture) and stationary (days 8–10 after reculture) growth conditions. All stationary CTL cultures showed poor specific killing as compared with exponentially growing cultures. Fig. 3 shows the cytotoxicity results for clone 4A2, which are typical for all the clones. The variation in anti-

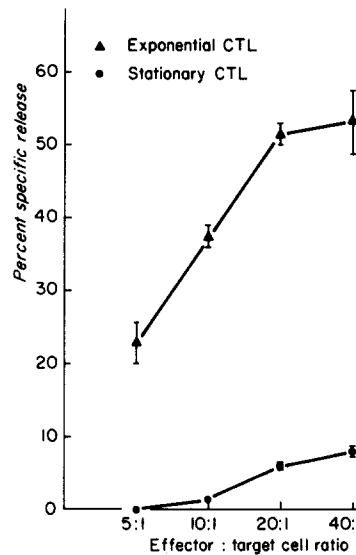


FIGURE 3. Specific killing of B10.D2 spleen blast cells by exponential and stationary phase 4A2 CTL cells. Each point is the mean of three replicate assay cultures. Bars are 2 SD.

self suppressive activity, however, was different from clone to clone (Fig. 4). The activity of the stationary 4B3, 8D5, and 8F4 CTL clones was lower than that of exponentially growing cultures. However, suppressive activity of the 4A2 CTL clone (three separate experiments shown in Fig. 4), and the 8C5 clone was not influenced by the proliferative stage of the CTL culture. These results suggest that cytotoxic activity and anti-self suppressive activity are not necessarily correlated.

It is possible that cytotoxic activity and anti-self suppressive activity are due to independent divergent subclones within the cloned CTL populations. To test for this, six subclones were generated from the 4A2 and the 8D5 CTL clones by limiting dilution at 0.3 cells/well and were tested as soon as possible after establishment. All six subclones showed comparable levels of specific anti-H-2<sup>d</sup> killing in comparison with each other and with the parental line (Table IV). All six subclones also possessed anti-self suppressive activity, definitely establishing that both activities develop within the same clone. However, they varied widely in their anti-self suppressive activity (Fig. 5) when each was tested by adding 300 CTL to a B10.BR(H-2<sup>k</sup>) anti-B6(H-2<sup>b</sup>) MLR. No inhibition was seen when the same subclones were tested in the same way in a B6 anti-B10.BR MLR (Fig. 5).

*Responder Cells from a 2-d MLR Can Be Killed by CTL Syngeneic to the Stimulator Cells.* We next examined whether CTL lines could directly kill cells from different types of MLR. Table V shows results for line 4B3 (H-2<sup>b</sup> anti-H-2<sup>d</sup>). When cells taken from B10.BR anti-B6 MLR cultures after 24, 48, or 72 h of culture were killed, there was a clear maximum of activity against MLR cells taken after 48 h of culture. In contrast, cells taken from B10.BR anti-B10.D2 MLR cultures were not killed at any time point, suggesting that the specificity of killing is for MLR blasts capable of recognizing the added CTL. The rate at which cells taken from B10.D2 anti-B10.BR MLR cultures, which should be

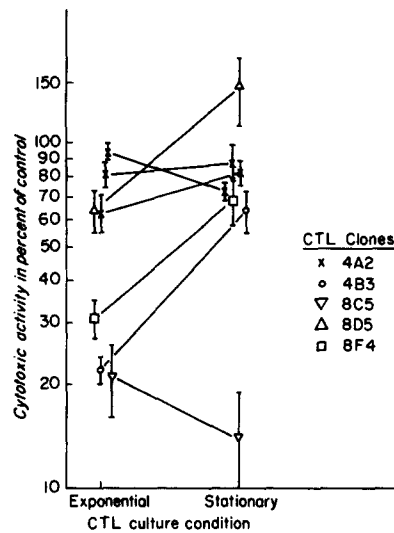


FIGURE 4. Comparison of inhibitory activities of CTL clones in exponential and stationary growth phases. 300 cells from stationary or exponentially growing CTL clones were added either to B10.BR anti-B6 (H-2<sup>k</sup> anti-H-2<sup>b</sup>) MLR cultures at day 1 of culture (clones 4A2, 4B3, and 8D5, which are B6 anti-H-2<sup>d</sup>) or to B10.D2 anti-B6 (H-2<sup>d</sup> anti-H-2<sup>b</sup>) MLR cultures (clones 8C5 and 8F4, which are B6 anti-H-2<sup>k</sup>). Control cytotoxic activity was: B10.BR anti-B6, 59.8% (mean of four experiments); B10.D2 anti-B6, 66.7% (mean of three experiments). Each point represents the mean cytotoxic activity of six replicate cultures. Bars are 2 SEM.

TABLE IV  
Cytotoxic Activity of the Six Subclones Depicted in Fig. 4

Subclones	Effector/ target ratio	Target cells		
		H-2 <sup>b</sup> (B6)	H-2 <sup>d</sup> (B10.D2)	H-2 <sup>k</sup> (B10.BR)
4A2/1	13:1	0*	64.1 ± 7.5	0
4A2/2	13:1	1.0 ± 0.2	70.2 ± 8.2	10.9 ± 1.2
4A2/3	13:1	3.6 ± 0.5	38.0 ± 8.2	8.3 ± 1.3
8D5/3	10:1	0	58.1 ± 9.8	ND <sup>‡</sup>
8D5/4	10:1	0	56.7 ± 2.2	ND
8D5/5	10:1	0	52.3 ± 1.3	ND

\* Data are means of specific <sup>51</sup>Cr release of triplicate cultures ± SD.

<sup>‡</sup> Not done.

directly recognized and killed by the added CTL, were in fact killed reached a peak against cells taken after 72 h of culture. Results identical to those of Table V were obtained with a second CTL line (4A2, also H-2<sup>b</sup> anti-H-2<sup>d</sup>). In particular, there was the same difference in kinetics with maximum killing of cells from B10.BR anti-B6 MLR after 48 h of culture and, of B10.D2 anti-B10.BR, after 72 h of culture.

*Extent of Suppression of H-2<sup>k</sup> Anti-H-2<sup>b</sup> MLR Responses Varies with the Responding Strain.* The degree of CTL-mediated suppression of H-2<sup>k</sup> responder cells was dependent upon the background of the H-2<sup>k</sup> mouse strain. The suppressive effect of two of the subclones (A2/1 and D5/4) used in Fig. 5 was studied in an



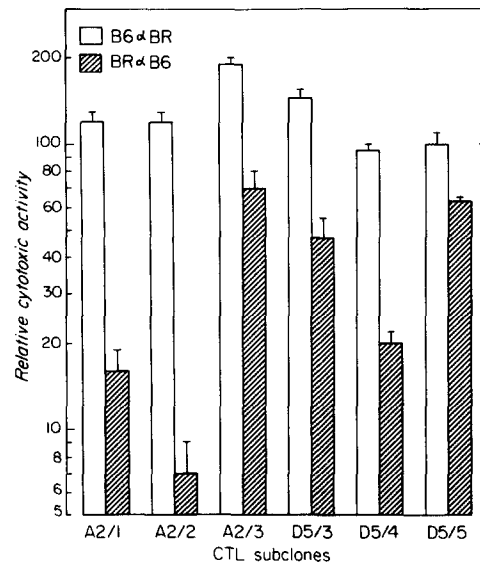


FIGURE 5. Specific suppression of CTL generation in a H-2<sup>k</sup> anti-H-2<sup>b</sup> MLR (B10.BR anti-B6) after addition of 300 CTL subclone cells per culture. CTL cells were obtained from six subclones derived from the 4A2 and 8D5 CTL lines. Each column represents the mean cytotoxic activity obtained from replicates of six MLR cultures. Error flags represent SEM. Specific <sup>51</sup>Cr-release values obtained in the control B6 anti-B10.BR MLR and the B10.BR anti-B6 MLR were 29.0 ± 1.7 and 23.6 ± 1.3, respectively.

TABLE V  
*Ability of Cells from a Cloned CTL Line to Kill Responder Cells  
Generated in Various Types of MLR*

Hours of MLR culture	MLR used as source of target cells		
	B10.BR anti-B6	B10.BR anti- B10.D2	B10.D2 anti- B10.BR
24	8.6 ± 0.8	1.2 ± 0.1	8.4 ± 0.8
48	21.7 ± 1.2	2.4 ± 0.2	16.5 ± 0.5
72	7.0 ± 0.5	-1.2 ± 0.1	33.2 ± 2.5
96	1.8 ± 0.3	-1.3 ± 0.2	5.8 ± 2.1
120	0.3	ND*	ND

Data are percentage of <sup>51</sup>Cr release in a 6-h <sup>51</sup>Cr-release assay using cells from the cloned CTL line 4B3 (H-2<sup>b</sup> anti-H-2<sup>d</sup>) as effector cells at an effector-to-target cell ratio of 25:1. Target cells were viable cells from the respective MLR, collected at the time indicated, separated from dead (and stimulator) cells on a Lympholyte gradient, and labeled with <sup>51</sup>Cr.

\* Not done.

H-2<sup>k</sup> anti-H-2<sup>b</sup> MLR using five different H-2<sup>k</sup> strains as responders. The experiment was performed twice with similar results. Fig. 6 illustrates the results from one of the experiments with the A2/1 subclone. B10.BR anti-B6 MLR reactivity was more strongly suppressed by A2/1 CTL cells than were any of the other MLR combinations depicted in Fig. 6. The CBA anti-B6 MLR was only weakly suppressed and the B6.K anti-B6 MLR was not suppressed at all by the subclone.

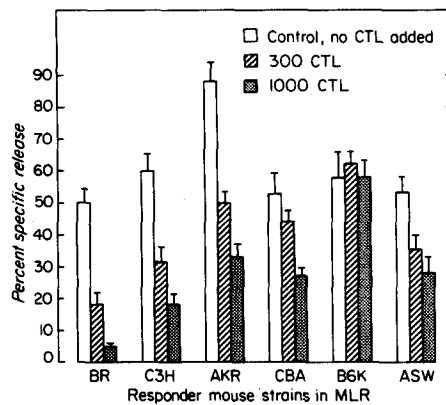


FIGURE 6. Cytotoxic activity of MLR cultures using different H-2<sup>k</sup> strains as responders in an anti-B6 (H-2<sup>b</sup>) response and with addition of 0, 300, or 1,000 A2/1 CTL subclone cells (B6 origin) at day 1 of MLR culture. A.SW (H-2<sup>s</sup>) anti-H-2<sup>b</sup> is included as an unrelated control. Each column represents the mean specific <sup>51</sup>Cr release of six replicate MLR cultures. Bars represent SEM.

The suppression obtained with the D5/4 subclone showed nearly the same pattern of inhibitory activity, the only difference being that the CBA anti-B6 MLR was not suppressed at all, whereas a slight inhibition was obtained in the B6.K anti-B6 MLR culture (data not shown). As indicated from the data in Fig. 6, the degree of CTL-induced suppression was not related to the actual strength of the MLR reactivity. Thus, both the strong AKR anti-B6 and the moderate C3H anti-B6 MLR reactivity were equally inhibited by both CTL subclones.

### Discussion

The present study describes five long-term CTL clones that can suppress the generation of cytotoxic T lymphocytes against stimulator cells syngeneic or H-2-identical to the strain of origin of the CTL line. From the point of view of the CTL line, a response against self is being suppressed. In most cases such "anti-self" suppression was specific, i.e., no suppression was observed when CTL cells were added to MLR cultures in which the CTL and responder cells were syngeneic, or in which the CTL were H-2-different from both stimulator and responder cells. The suppression did not appear to be affected by the cytotoxic specificity or correlated with the cytotoxic activity of the added CTL clone. In three out of the five CTL clones studied, as few as 100 CTL cells added at day 1 of an MLR culture containing  $3 \times 10^4$  responder cells and  $3 \times 10^5$  stimulator cells syngeneic to the added CTL were routinely capable of producing "anti-self" suppression. Suppression appeared to occur early in the MLR culture, most strongly between 24 and 48 h of culture, and is most easily explained by assuming that a CTL precursor from the responder population is inactivated by a cell in the CTL clone on recognizing an H-2 determinant on the cell in the CTL clone early in the MLR culture.

The mechanism by which CTL clones inhibit the development of cytotoxic lymphocytes against their (self) H-2 haplotype is unknown at present but there are a number of arguments suggesting it is not directly associated with their

cytotoxic potential. Comparing CTL clones in stationary and exponential growth phases, it was found that cytotoxic activity was always greatly reduced in the stationary phase, whereas anti-self suppressive activity showed only moderate or no decline. A problem with this argument is that cytotoxic activity is measured immediately after removing the CTL from culture, whereas anti-self suppressive activity of the CTL may manifest itself only 1–2 d after the CTL are added to the MLR, allowing substantial time for possible differentiation. The observation that freshly established CTL subclones all showed similar levels of cytotoxic activity but varied in their anti-self suppressive activity is perhaps stronger evidence for disassociating the two activities. Attempts to disassociate the two activities on the basis of differential expression of Thy-1 or Lyt-2, as assessed by fluorescence-activated cell sorting, have been unsuccessful (unpublished).

Anti-self suppressive activity might involve direct killing of the cytotoxic lymphocyte precursor cell, even if this does not take place by the conventional killing process. In short-term *in vitro* cytotoxicity assays, it has been clearly demonstrated that killing by CTL is unidirectional; i.e., CTL can only kill target cells that they recognize specifically (15, 16). The suppression we observed would require killing in the other direction. Thus, CTL precursors (not known to have cytotoxic potential) would recognize and form conjugates with the “suppressor” CTL (which share H-2 with the stimulator cells) and then be killed. Such back-killing might occur in such a conjugate by conventional mechanisms over a long period of time (much longer than the incubation time of the standard *in vitro* cytotoxicity test) and we have in fact seen low levels of killing explicable in this way in a short-term (6 h) cytotoxicity assay; i.e., MLR blasts potentially capable of recognizing the added CTL were killed (Table V). MLR blasts directly recognizable by the added CTL could also be killed, but there was a significant difference in blast sensitivity to lysis as a function of culture time, suggesting there may be a difference in the processes involved. It is not clear whether the direct back-killing seen in Table V is of biological significance. Very large numbers of CTL (25:1 CTL/target ratio) had to be added to obtain lysis compared with the rather low number of CTL (1:50 to 1:500 CTL/responder ratio) required to obtain significant suppression of the development of cytotoxicity in an MLR.

Differences outside the H-2-KD MHC loci may influence the outcome of anti-self suppression. Thus, the anti-self suppressive capacity of two individual CTL subclones varied with different responder H-2<sup>k</sup> mouse strains. In this context, B10.BR mice routinely more strongly suppressed than the other strains tested, are Qa1<sup>+</sup>, Qa2<sup>-</sup>, Tla<sup>+</sup>, whereas the other H-2<sup>k</sup> responder mouse strains tested are Qa1<sup>-</sup>, Qa2<sup>-</sup>, Tla<sup>-</sup> (17). The B6 (strain of origin of the CTL clone) is Qa1<sup>-</sup>, Qa2<sup>+</sup>, Tla<sup>-</sup>. Thus, the B10.BR responder cells are the only H-2<sup>k</sup>-positive cells in our panel that might recognize the Qa1 and Tla products of the stimulator (and CTL clone) cell populations. In this context, it is interesting to notice that the Qa/Tla antigens recently have been considered to act as specific target cell recognition structures for autologous NK cell killing (18). Likewise, the weak or absent CTL-mediated anti-self/suppressor activity in the CBA or B6.K anti-B6 MLR cultures might suggest that differences outside the responder cell H-2

MHC locus influence whether or not responder cells are sensitive to CTL-induced suppression.

Whether all of the cells in a CTL clone possess anti-self suppressive activity, or whether this activity is limited to a subset of cells at a certain stage of differentiation within the clone, cannot be directly answered from our present data. All five CTL lines tested, initially selected, and then maintained on the basis of their cytotoxic activity, possessed anti-self suppressive activity. Six subclones established from two of the parental lines retained both cytotoxic activity and anti-self suppressive activity, although the relative levels of the two varied from subclone to subclone (Fig. 5). Our hypothesis is that the CTL clones are maintained by progenitor cells that are at different stages in the T cell differentiation pathway and that these different progenitors can give rise to differing levels of both suppressive activity and conventional cytotoxic activity within the same clone. Recently, we have shown that, on appropriate manipulation of the tissue culture conditions, some CTL clones can give rise to killer cells specific for target cells bearing self MHC (19). The relationship between these anti-self killer cells and the suppressive activity described in this manuscript is presently under investigation.

We have previously described (8–12) a type of anti-self suppressive activity (“veto” activity) which is detectable in an MLR in exactly the manner described here and is indistinguishable in its specificity of action (i.e., specific for a response against MHC-identical stimulator cells) and kinetics of action (i.e., maximum suppressive activity within the first 24–48 h of culture (12)). The suppressive activity was shown to be a property of spleen and bone marrow cells from athymic nude mice, bone marrow, thymus, and fetal liver of euthymic mice, and lymphoid colonies grown from bone marrow and fetal liver cells. It was argued that this anti-self suppressive activity might play an important role in establishing self tolerance during T cell ontogeny, particularly if the repertoire develops from cells initially reactive against self MHC (6), as suggested by recent work from this laboratory (7). In these previous studies, “anti-self” suppression appeared to be mediated by immature cells of the T cell lineage. In contrast, the anti-self suppressive activity described here is clearly mediated by relatively mature T cells and whether it is directly related to our previous results is presently unclear. However, recent studies by others (20–22) have described suppressor cells with similar specificity properties and mode of action, but which appear to play a role in the regulation of an ongoing response and to carry markers characteristic of more mature T cells. Using this approach, it was demonstrated recently that suppression mediated by normal spleen cells may also occur *in vivo* (21, 22). Taken together, these previous data and our present results suggest that there are T lineage cells at all stages of development which have the innate ability to inactivate other T cells capable of recognizing them and that this ability may play an important role in the maintenance of self tolerance.

Note that the veto phenomenon can be distinguished from other regulatory mechanisms by the direction in which recognition takes place (8). In more conventional forms of regulation, e.g. antiidiotypic regulation, the regulator cell recognizes the idio type of the cell to be regulated. In regulation by veto, the cell recognizing an antigen on the surface of the veto cell is inactivated, i.e., the

regulator regulates on being recognized. In models of antiidiotypic regulation of an anti-self response, one must devise mechanisms whereby one develops a repertoire of receptors capable of controlling cells carrying receptors directed against self and not cells with other receptors. Regulation by veto has the advantage that the veto cell need only carry the antigen (self or otherwise) against which responses are to be regulated. In conclusion, we feel that sufficient evidence has now accumulated so that one must seriously consider the veto phenomenon as a general mechanism of T cell regulation at all levels of ontogeny.

### Summary

Five out of five allo-specific cytotoxic T lymphocyte (CTL) clones tested strongly suppressed the development of CTLs directed against the H-2 haplotype of the CTL clone and independent of the H-2 specificity recognized by the CTL clone. This was shown by including 100–1,000 cells from the five clones in one-way mixed lymphocyte reaction (MLR) cultures in which the stimulator cells were of the same H-2 type as the CTL cells. When these cultures were assayed for cytotoxicity against the stimulator cell haplotype, the cytotoxic activity was decreased in a CTL cell dose-dependent manner by 50 to more than 90%. Suppression was usually not observed in MLR cultures where the CTL-H-2 type was identical with the responder cells or was different from both the responder or stimulator cells. Suppression was demonstrated not to be due to “cold” target inhibition at the time of cytotoxicity assay. Even if the added CTL were completely removed after 48–72 h of culture, significant suppression was obtained. Suppressive ability did not appear to be correlated with the level of allo-specific cytotoxic activity present in the CTL clones, but might involve direct killing of MLR precursor cells by cells in the added CTL clones. The suppression observed here, which is anti-self from the point of view of the added CTL clone, appears to be triggered by precursor cells in the MLR responder population recognizing MHC determinants on cells from the added CTL clone. This peculiar type of suppression, in which the regulator regulates on being recognized, has been christened the veto phenomenon and may play a role in maintenance of self tolerance.

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*Note added in proof:* Since this paper went to press, Fink et al. (23, 24) have published results in substantial agreement with those presented here.

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