CYTOTOXIC T LYMPHOCYTES REACTIVE AGAINST A SYNGENEIC MURINE TUMOR AND THEIR SPECIFIC SUPPRESSOR T CELLS ARE BOTH ELICITED BY IN VITRO ALLOSENSITIZATION

By BENNY LESHEM and ELI KEDAR

From the Lautenberg Center for General and Tumor Immunology, The Hebrew University-Hadassah Medical School, Jerusalem 91010, Israel

The induction of cytotoxic response against syngeneic or autologous murine or human tumor cells by pool allosensitization in bulk MLC was demonstrated by Bach et al. (1, 2). Subsequently, we showed the existence of murine cytotoxic cell precursors that can be activated to react with syngeneic tumor cells by single-donor allosensitization in bulk MLC, applying the limiting dilution cultures (LDC)[1] technique (3, 4). We also showed that a high frequency (1/20) of cytotoxic cells can be elicited in the same manner even against syngeneic, weakly immunogeneic (MT-2, mammary carcinoma in C3H mice; M109, lung carcinoma in BALB/c mice) or nonimmunogenic (PIR-2, x-irradiation–induced lymphoma in B6 mice) tumors (5, 6). We postulated that shared or crossreactive antigenic determinants between the allogeneic leukocytes and the syngeneic tumor cells lead to the elicitation of the antisyngeneic tumor cytotoxic (ASTC) cells. Similar findings were reported by others (7–11). Recently, we have demonstrated (12) that the ASTC precursors are phenotypically "classical" CTL precursors (i.e., Thy-1$^+$; Lyt-2$^+$; L3T4$^-$; asialo GM1$^-$) rather than classical NK-type lymphokine-activated killer (LAK) cells that might have arisen in the presence of the high concentration of IL-2 (>250 U/ml) in the LDC.

In our MLC-LDC system, in which the syngeneic target cells were the syngeneic PIR-2 cells, no ASTC response could be demonstrated by directly testing lymphocytes sensitized in bulk MLC (5, 6). The high ASTC frequency, as well as the antiallotarget response, were demonstrated in LDC wells plated with a low number of MLC-derived responding cells (<200/well). As the number of responding cells plated per well increased beyond a critical number (usually 500/well), a gradual decrease of ASTC response was observed. No anti-PIR-2 cytotoxicity could be detected when ≥5,000 MLC-derived cells were plated per well in LDC (3–5), whereas the percentage of cytotoxically positive wells against the allotarget, as well as the level of antiallocytotoxicity of the individual wells, remained at maximum.

[1] *Abbreviations used in this paper:* ASTC, antisyngeneic tumor cytotoxicity; LAK, lymphokine-activated killer; LDC, limiting dilution culture; PLL, poly-L-lysine.

Based on the above findings, we speculated (3–5) that suppressor cells, able to block the cytotoxic response against the syngeneic tumor target, are activated in bulk MLC. When the MLC-sensitized cells are then plated at low numbers in the LDC wells, the suppressor cells are diluted out, thereby allowing the manifestation of ASTC response, whereas LDC wells plated with high numbers (>500) of cells, as in bulk MLC, contain suppressor cells in sufficient numbers to cause the decrease of ASTC response (3–5). The same interpretation of similar phenomena was suggested by others (13–16).

In this study, we directly demonstrate the existence of radioresistant, Thy-1$^+$, Lyt-2$^+$, L3T4$^-$ suppressor cells that specifically suppress, in a noncytolytic mechanism, the cytotoxic response against syngeneic tumor target cells at the effector phase. Based on data reported here and in other studies (3–5, 11), showing that the same B6 T cell clone can lyse both the syngeneic tumor and the allogeneic target cells, we assume that a single MLC-derived CTL may exhibit two killing activities: one directed against the allotarget and the other against the syngeneic target cells. Only the antisyngeneic response is suppressed by CD8$^+$ suppressor cells, in an as yet unknown mechanism.

Materials and Methods

Mice. Female C57BL/6 (B6, H-2^b), BALB/c (H-2^d), C3H (H-2^k), and strain A (H-2K^k; D^d) mice, aged 6–12 wk, were used.

Medium. DME (Biological Industries, Beth Haemek, Israel) was supplemented with 10% FCS, 1 mM sodium pyruvate, 10 mM Hepes buffer, 0.5 mM asparagine-HCl, 0.03 mM folic acid, 0.5 mM L-aspartic acid, 5 × 10^{-5} M 2-ME, 2 mM glutamine, and antibiotics (complete DME).

Tumor Lines. PIR-2 thymoma was induced in a B6 mouse by fractionated x-irradiation (4× 170 cGy) (16). To avoid prolonged maintenance in vitro, frozen aliquots of PIR-2 were thawed for use every 8–12 wk. The PIR-2 tumor was repeatedly shown to be nonimmunogenic and to lack membrane gp-70 expression (5, 17). As allotarget, we used P815, a mastocytoma of DBA/2 (H-2^d) origin. The tumor cell lines were carried in vitro in complete DME.

Mixed Leukocyte Culture (MLC). Responding spleen cells and irradiated (30 Gy) stimulating splenocytes (1.25 × 10^6/ml each) were cocultured in 20 ml of complete DME, in 25-cm^2 flasks (Nunc, Roskilde, Denmark) for 5 d at 37°C, in a 10% CO$_2$ air incubator.

Limiting Dilution Culture (LDC). Freshly obtained splenocytes or lymphocytes harvested from 5-d MLC were plated in LDC as previously described (5, 18). Briefly, graded numbers (10–50,000) of responding cells were cultured for 8–9 d in round-bottomed 96-well plates (Nunc) in 16–24 replicates, in the presence of 10^6/well of irradiated (30 Gy) allogeneic splenocytes and human rIL-2 (250 U/ml, Cetus Corp., Emeryville, CA) in a final volume of 0.2 ml complete DME. In some experiments (secondary LDC), the responding cells were obtained from selected LDC wells.

Estimate of Cytotoxic Cell Frequency. Lymphocytes harvested from individual LDC wells (50-μl aliquots) were transferred to conical-bottomed 96-well plates (Nunc) and tested in a 4-h ^{51}Cr release assay as described previously (5). Frequency (*f*) of cytotoxic cell precursors and correlation coefficient of regression analysis for Poisson's distribution (r^2) were calculated as described elsewhere (5, 18). In all the experiments described herein, r^2 was ≥0.9.

Assessment of Cytotoxic Activity. Lymphocytes harvested from MLC or pooled from LDC wells (effector cells) were tested for cytotoxicity in a 4-h ^{51}Cr release assay, in conical-bottomed 96-well plates, at four E/T cell ratios as described previously (5). Lytic units (LU) were calculated from the cytotoxicity measured at the four E/T cell ratios (19). 1 LU was defined as the number of effector cells necessary to cause 30% target cell lysis.

Mixing Experiments. Effector cells were admixed with putative suppressor cells in either one of the following protocols: (*a*) the putative suppressor cells (2 × 10^3 or 10^5) were added

to wells containing the 50-μl aliquots transferred from the individual LDC wells; or (b) effector cells were pooled from LDC wells, counted, and then admixed with putative suppressor cells at a 1:1 cell ratio; the cell mixtures were further diluted to the desired E/T ratios. In both protocols, ^{51}Cr-labeled target cells (2 × 10^3/well) were added to the cell mixtures, and ^{51}Cr release was measured after a 4-h incubation. In protocol a, the apparent CTL frequency was determined, and in protocol b, LU/10^6 were calculated.

Preparation of Lymphoblasts.   3 × 10^7 spleen cells were cultured at 37°C for 2 d with either 3 μg/ml Con A (Bio-Makor, Rehovot, Israel), 10 μg/ml PHA-P (Difco Laboratories Inc., Detroit, MI) or 50 μg/ml LPS (Difco Laboratories Inc.) in 10 ml complete DME (25-cm^2 flasks, Nunc).

Cell Depletion.   Spleen cells (10^7/ml) were incubated for 45 min at 4°C with either of the following antibodies: monoclonal anti-Thy 1.2, monoclonal anti-Lyt-2.2 (both from Cedarlane, Hornby, Canada) (final dilution 1:250), anti-asialo GM1 antiserum (Wako Chemicals, Osaka, Japan) (final dilution 1:200), or monoclonal anti-L3T4 antibody (1:50 dilution of an acites fluid from sublethally irradiated BALB/c mice injected intraperitoneally with the GK1.5 hybridoma cell line). Antibody-treated cells were then pelleted and resuspended to the original volume with Low-Tox rabbit complement (C'; Cedarlane) (final dilution 1:10). After a 45-min incubation at 37°C, cells were washed three times before further use. All dilutions were made in RPMI 1640 medium containing Hepes buffer, antibiotics, and 0.3% BSA.

Adsorption to Monolayers.   Cell monolayers were prepared as described earlier (20). Briefly, tissue culture petri dishes (5 cm, Nunc) were incubated with 2 ml poly-L-lysine (PLL; mol wt >20,000; Sigma Chemical Co., St. Louis, MO) (0.1 mg/ml in PBS) for 30 min at 37°C. Excess of the PLL was washed with PBS. LPS-induced lymphoblasts (3 × 10^7 viable cells/dish) were incubated on the PLL-coated dishes for 30 min at 37°C. The nonadherent cells were aspirated and the monolayer was rinsed with HBSS-10% FCS. For adsorption, cells harvested from 5-d bulk MLC (5–7 × 10^6 in 2 ml HBSS-10% FCS) were overlayed on the lymphoblast monolayers. The petri dishes were then centrifuged for 2 min at 300 rpm and incubated for 30 min at 37°C. The nonadherent cells were collected by gentle agitation.

Results

Lysis of Syngeneic and Allogeneic Targets Is Mediated by the Same Cell Clone.   To reaffirm our initial observation that the same cytotoxic cell clone that lysed the syngeneic tumor PIR-2 also killed the corresponding allotarget (3, 5), B6 a/BALB MLC-sensitized lymphocytes were plated in LDC at 0.1 (Fig. 1 *A*) or at 0.2 (*B*) cells/well (clone probability of 95 and 90%, respectively; plating efficiency of 50 and 33%, respectively). Cytotoxic activity of the cloned cells harvested from the LDC wells was tested against PIR-2 and P815 target cells. As seen in Fig. 1, none of the clones could lyse PIR-2 only, and 17–30% of them lysed the allogeneic target P815 only. The majority (50–60%) were able to lyse both allogeneic and the syngeneic PIR-2. As was demonstrated previously (5, 12), both target cells were lysed by Thy-1$^+$, Lyt-2$^+$, L3T4$^-$, asialo GM1$^-$ effector cells. The data suggest that the same CTL clones that lyse the syngeneic target PIR-2 also lyse the allogeneic target P815.

Suppression of ASTC Response in LDC.   The observation indicating the existence of anti-ASTC suppressor cells in the PIR-2 cells system is depicted in Fig. 2. Lymphocytes derived from bulk B6 a/BALB MLC were plated in LDC at 10–50,000 cells/well with 10^6 irradiated BALB/c splenocytes as stimulating cells and 250 U/ml of IL-2 (B6 a/BALB LDC). Cytotoxic activity of cells harvested from individual LDC wells against syngeneic (PIR-2) and allogeneic (P815) target cells was tested 8 d later. As seen in Fig. 2, when responding cells were plated at 10–200 cells/well (low cell-number LDC), the percentage of wells cytotoxically positive against both P815 and PIR-2 increased concomitantly with the increase of the number of cells
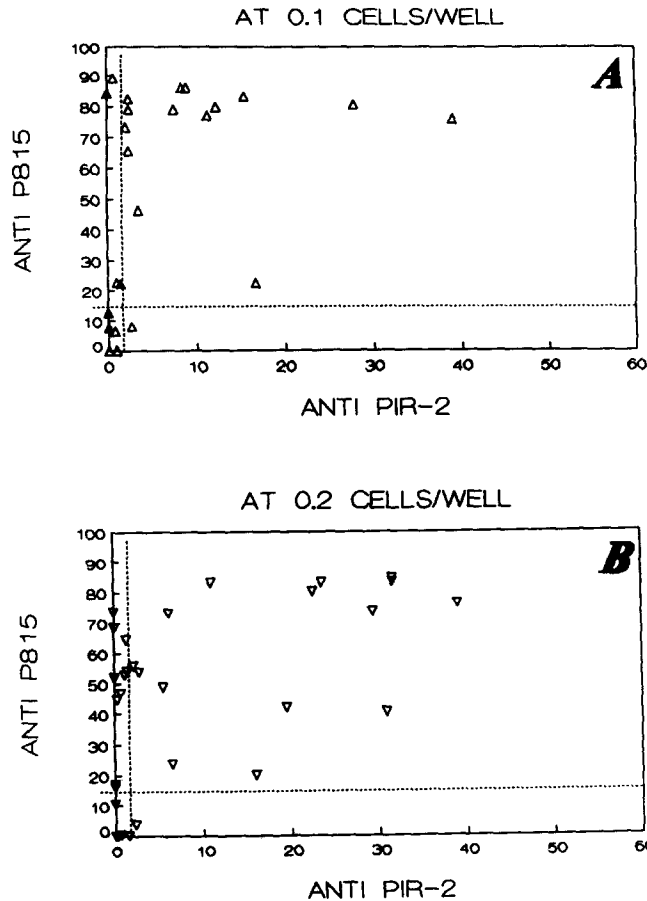
FIGURE 1.   Lysis of syngeneic tumor and allogeneic target cells is mediated by the same CTL. Lymphocytes derived from B6 a/BALB bulk MLC were plated in LDC wells, at 0.1 (*A*) and 0.2 (*B*) cells/well, with 10^6 irradiated BALB/c splenocytes and 50 U IL-2 per well, and were cultured for 9 d. Cytotoxicity against the allogeneic target P815 and the syngeneic tumor PIR-2 was tested on cells harvested from individual wells.

originally plated in the wells. The frequencies of CTL (CTL-f) against P815 and against PIR-2, as determined from the LDC curves at this range, were 1/18 and 1/36, respectively. In contrast, when the number of cells plated increased to ≥ 500/well (high cell–number LDC), the percentage of cytotoxically positive wells against the syngeneic target PIR-2 markedly decreased, yet the cytotoxicity of cells from the same LDC wells against the allogeneic target P815 remained at maximal level even when 50,000 cells were plated per well.

The described decrease in anti-PIR-2 response might be due to selective death of the double-reactive anti-PIR-2 anti-P815 CTL (50–60% of the cells; Fig. 1), but not of the specific anti-P815 CTL (17–30%), in the high cell–number LDC wells. To test if this was the case, B6 a/BALB LDC was set up, plating 50 and 50,000 MLC-derived cells/well (500 wells each). Cells harvested from the individual wells were tested for cytotoxicity against both PIR-2 and P815 target cells. The remaining lymphocytes were then pooled in two separate groups as follows: cells from wells originally plated at 50 cells/well that were cytotoxically positive against both PIR-2 and P815 (PIR$^+$ P815$^+$; Table I, group *a*), and cells from wells originally plated at 50,000
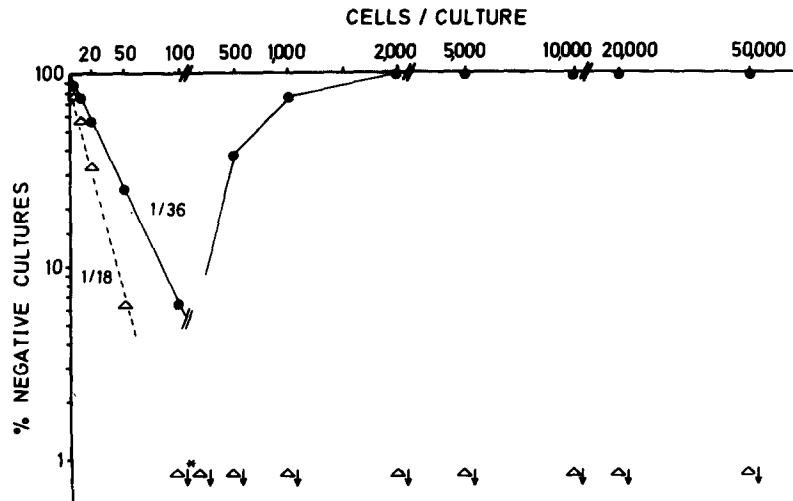
FIGURE 2. Elicitation and suppression of cytotoxicity against the syngeneic tumor PIR-2 in allogeneic LDC. B6 a/BALB MLC cells were plated in allogeneic LDC. Cytotoxicity (4-h ^{51}Cr release assay) was assayed after an 8-d incubation against the allogeneic (P815; △) and syngeneic (PIR-2; ●) target cells. (*) 0% negative wells (all wells are cytotoxic).

cells/well that were cytotoxically positive only against the allogeneic target (PIR-2$^-$ P815$^+$; Table I, group b). The pooled cells were then replated in LDC conditions with IL-2 and BALB/c stimulating cells. Table I shows that although the cells of group b appeared initially to be devoid of anti-PIR-2 cytotoxic cell activity (PIR-2$^-$,P815$^+$), the frequency of anti-PIR-2 CTL among these cells, as detected by the secondary LDC, was high and similar to that found among the PIR-2$^+$,P815$^+$ cells of group a. Thus, the data indicate that the lack of anti-PIR-2 activity in the high cell number PIR$^-$ P815$^+$ LDC wells was not due to selective death of anti-PIR-2 CTL.

We hypothesized that suppressor cells, able to block the response only against the syngeneic target cells PIR-2, are induced during allosensitization in bulk MLC. In

TABLE I

Existence of Anti-PIR-2 CTL in High Cell-number PIR-2$^-$ LDC Wells

Group	No. of cells plated per well in primary LDC	Cytotoxic activity in primary LDC	Anti-PIR-2 frequency
a	50	PIR-2$^+$ P815$^+$	1/32
b	50,000	PIR-2$^-$ P815$^+$	1/15

Cells harvested from allogeneic LDC wells originally plated at 50 cells/well that were cytotoxic against both P815 and PIR-2 (PIR-2$^+$ P815$^+$) (group a), and at 50,000 cells/well that were cytotoxic against P815 only (PIR-2$^-$ P815$^+$), were replated in allogeneic LDC conditions. 4-h ^{51}Cr release was measured after incubation of 9 d. CTL frequency was calculated as described in Materials and Methods.

the low cell–number LDC wells, the suppressor cells are diluted out, allowing the manifestation of the anti-PIR-2 cytotoxicity. When >500 MLC-derived lymphocytes are plated per well (high cell–number LDC), enough suppressor cells are present in the wells to block the cytotoxic response against PIR-2. Our finding that high cell–number LDC wells contain high frequency of anti-PIR-2 CTL (Table I) also suggests that the described suppression takes place at the effector phase and not at the induction of anti-PIR-2 CTL.

Suppression of ASTC Response in Mixing Experiments. The notion of suppressor cells inhibiting the ASTC response was substantiated in mixing experiments. In a preliminary experiment (data not shown), we found that cells harvested from high cell–number (50,000 cells/well), PIR-2⁻,P815⁺ LDC wells were suppressive. This conclusion was based on the finding that mixing these cells (1:1 ratio) with lymphocytes harvested from low cell–number (50 cells/well), PIR-2⁺ P815⁺ LDC wells resulted in a 75% decrease in anti-PIR-2 cytotoxicity, while freshly obtained B6 spleen cells were not significantly suppressive.

Since bulk MLC-derived lymphocytes do not exhibit any anti-PIR-2 cytotoxicity, in spite of the high anti-PIR-2 CTL-f among them (as evidenced by LDC analysis of low cell–number wells), we assumed that these cells could be used as a source of suppressor cells. Suppression of ASTC response by MLC-derived lymphocytes, in two mixing experiments, is described in Table II. Lymphocytes from various sources were added (10^5 cells/well) to 50 μl aliquots from B6 a/BALB LDC wells (protocol *a* of the mixing experiments in Materials and Methods). Only cells harvested from

TABLE II
Specificity of Suppression of ASTC Response by B6 a/BALB MLC-derived Lymphocytes

Exp.	Cells added to LDC plates	Anti-PIR-2 CTL frequency
1	None	1/72
	B6 (fresh)	1/210
	B6 (incubated)	1/80
	B6 a/B6 MLC	1/91
	B6 a/BALB MLC	1/890
	B6 a/C3H MLC	1/190
	B6 LAK cells*	1/130
	Supernatant from B6 a/BALB MLC	1/77
2	None	1/35
	B6 (fresh)	1/29
	B6 a/BALB MLC	1/2230
	B6 Con A blasts	1/52
	B6 PHA blasts	1/35
	B6 LPS blasts	1/29

B6 lymphocytes (10^5/well) or supernatant (0.1 ml) were added to 50-μl aliquots from B6 a/BALB LDC wells. 30 min later, ^{51}Cr-labeled PIR-2 target cells were added to the wells. ^{51}Cr release was measured after a 4-h incubation, and CTL frequency was calculated as described in Materials and Methods.
* B6 splenocytes were incubated for 3 d with 1,000 U/ml IL-2.

a B6 a/BALB MLC were able to significantly suppress anti-PIR-2 cytotoxicity. In contrast, freshly obtained B6 splenocytes, cultured unsensitized B6 cells, lymphocytes from B6 a/B6 MLC, B6 a/C3H MLC, and B6 LAK cells (induced by 1,000 U/ml IL-2), mitogen (Con A, PHA, and LPS)-stimulated B6 splenocytes, and a supernatant (50% vol/vol) from B6 a/BALB MLC, did not significantly suppress anti-PIR-2 cytotoxicity.

Phenotype of the Suppressor Cells. To phenotype the suppressor cells, lymphocytes from B6 a/BALB MLC were pretreated with either anti-Thy-1.2, anti-Lyt-2.2, or anti-L3T4 mAbs and C'. The mAB + C'-treated cells (and cells treated with C' alone, as control), were then added to aliquots from LDC wells as detailed in the previous experiment. Pretreatment with either anti-Thy-1.2 or anti-Lyt-2.2 + C', but not with anti-L3T4 + C', abrogated the anti-ASTC suppressive capacity of B6 a/BALB MLC cells (Table III). Thus, the anti-ASTC suppressor cells are of the CD8 type.

Specificity of Suppression. To determine whether or not the suppressor cells block only the response against the syngeneic PIR-2 target cells, LDC was set up in suboptimal conditions (i.e., 50 U/ml of IL-2 instead of the standard 250 U/ml, see reference 12). We used here the suboptimal conditions, because in these conditions, even low numbers (2 × 10^3/well) of B6 a/BALB MLC-derived suppressor cells could suppress the anti PIR-2 cytotoxicity without significant anti-P815 cytotoxicity (<5%). The use of larger numbers of cells (10^5/well), necessary to suppress the anti-PIR-2 cytotoxicity elicited in LDC in optimal conditions, caused by themselves 100% of P815 lysis. Cells from bulk MLC (2 × 10^3/well) were added to 50-μl aliquots from the suboptimal LDC wells, as in the previous two experiments. Table IV shows a 10-fold decrease of the anti-PIR-2 frequency caused by the addition of as few as 2 × 10^3 B6 a/BALB MLC-derived cells, while they did not affect the anti-P815 CTL frequency. Under these experimental conditions, no significant lysis of P815 target cells, directly caused by 2 × 10^3 B6 a/BALB MLC cells, was observed (data not shown).

To further characterize the suppressor cells, anti-PIR-2 effector cells were pooled from wells of low cell-number B6 a/BALB LDC (anti-PIR-2 CTL frequency, 1/27;

TABLE III
Phenotype of the Anti-ASTC Suppressor Cells

Cells added to LDC wells	Pretreatment of added cells	Anti-PIR-2 frequency
None	–	1/72
B6 a/BALB MLC	–	1/860
B6 a/BALB MLC	C'	1/890
B6 a/BALB MLC	Anti-Thy-1.2 + C'	1/150
B6 a/BALB MLC	Anti-Lyt-2.2 + C'	1/180
B6 a/BALB MLC	Anti-L3T4 + C'	1/950

B6 a/BALB MLC cells, pretreated as indicated, were added (10^5/well) to 50-μl aliquots from individual B6 a/BALB LDC wells. 30 min later, ^{51}Cr-labeled PIR-2 target cells were added to the wells. [51]Cr release was measured after a 4-h incubation, and CTL frequency was calculated as described in Materials and Methods.

Table IV

Suppression of ASTC and not of Antiallogeneic Cytotoxic Response Elicited in LDC

Cells added to LDC plates	Anti-PIR-2 CTL frequency	Anti-P815 CTL frequency
None	1/350	1/25
B6 (fresh)	1/270	1/30
B6 (incubated)	1/700	1/35
B6 a/BALB MLC	1/3420	1/30
B6 a/C3H MLC	1/900	1/20
Supernatant from B6 a/BALB MLC	1/290	1/35

B6 lymphocytes (2×10^3/well) or supernatant (0.1 ml) were added to 50-μl aliquots from B6 a/BALB LDC wells (set in suboptimal conditions, i.e., with 50 U of IL-2/ml). 30 min later, ^{51}Cr-labeled PIR-2 target cells were added to the wells. ^{51}Cr release was measured after a 4-h incubation, and CTL frequency was calculated as described in Materials and Methods.

100 cells/well) and of low cell–number B6 a/C3H LDC (anti-PIR-2 CTL frequency, 1/360; 500 cells/well). The pooled anti-PIR-2 effector cells were mixed with cells (1:1 ratio), supernatants, or IL-2, as detailed in Table V. Cytotoxicity against PIR-2 was then tested at four E/T cell ratios, and LU/10^6 cells (defined at 30% lysis) for each mixture were calculated. Results similar to those depicted in Table V were found in two to five repeated experiments. As seen in Table V, cells derived from B6 a/BALB MLC (group 4) and from high cell–number (50,000 cells/well) PIR-2$^-$P815$^+$ B6 a/BALB LDC wells (group 12), but not from B6 a/C3H MLC (group 5), blocked the anti-PIR-2 cytotoxicity of CTL derived from B6 a/BALB LDC (group 1); reciprocally, anti-PIR-2 cytotoxicity of CTL derived from B6 a/C3H LDC (group 1) was suppressed by cells derived from B6 a/C3H MLC (group 5), but not from B6 a/BALB MLC (group 4). Moreover, the suppressive activity of the MLC-derived suppressor cells could be adsorbed only on lymphoblast monolayers of the same haplotype that had been used as stimulating cells in the bulk MLC. The suppressive capacity of lymphocytes derived from B6 a/BALB MLC was adsorbed on BALB/c but not C3H monolayers (groups 6 and 7), and that of B6 a/C3H MLC was adsorbed only on C3H monolayers (groups 8 and 9).

As in Table III, here too the MLC-derived suppressor cell activity was abrogated by pretreatment with anti-Thy-1.2 or anti-Lyt-2.2 (groups 14 and 15, respectively), but not with anti-L3T4 mAb + C' (group 16). Their activity was not affected by 30 Gy x-irradiation (group 10). Removal of NK-LAK cell precursors (by anti-asialo GM1 antibody + C') from B6 splenocytes, before allosensitization, did not affect the suppressive capacity of the MLC-derived lymphocytes (group 11), indicating that the suppressor cells are not of NK-LAK cell lineage (21). IL-2 (250 U/ml, group 18) and B6 a/BALB MLC supernatant (group 17) did not change the ASTC activity.

The suppression of anti-PIR-2 activity is not mediated by lysis of the anti-PIR-2 cytotoxic cells, since B6 a/BALB MLC lymphocytes did not lyse ^{51}Cr-labeled anti-PIR-2 cytotoxic cells (pooled from low cell–number LDC wells). Similarly, the MLC-derived lymphocytes were not lysed by the anti-PIR-2, LDC-derived effector cells (data not shown).

TABLE V

Phenotype and Specificity of Suppressor Cells as Determined by Mixing Experiments

Group	Cells added	Pretreatment of added cells	Anti-PIR-2 cytotoxicity of cells derived from:	
			B6 a/BALB LDC	B6 a/C3H LDC
			$LU/10^6$	
1	None		85	93
2	Fresh B6		118	95
3	MLC, B6 a/B6		88	118
4	MLC, B6 a/BALB		31	112
5	MLC, B6 a/C3H		109	7
6	MLC, B6 a/BALB	Adsorption on BALB*	79	ND
7	MLC, B6 a/BALB	Adsorption on C3H*	21	ND
8	MLC, B6 a/C3H	Adsorption on BALB*	ND	10
9	MLC, B6 a/C3H	Adsorption on C3H*	ND	71
10	MLC, B6 a/BALB	Irradiation (30Gy)	33	ND
11	MLC, B6 a/BALB	Anti-ASGM1 + C'‡	27	ND
12	LDC, B6 a/BALB 50,000 cells/well§		22	ND
13	MLC, B6 a/BALB	C'	37	ND
14	MLC, B6 a/BALB	Anti-Thy-1.2 + C'	91	ND
15	MLC, B6 a/BALB	Anti-Lyt-2.2 + C'	109	ND
16	MLC, B6 a/BALB	Anti-L3T4 + C'	35	ND
17	Supernatant of B6 a/BALB (0.1 ml)		101	ND
18	IL-2 (250 U/ml)		91	ND

Cells were harvested from anti-PIR-2^{+} wells of B6 a/BALB or B6 a/C3H LDC (plated originally with 100 and 500 cells/well, respectively). The cells were mixed at a 1:1 ratio with cells from various sources, with or without pretreatment, as described in the table. The mixtures were then diluted (four threefold dilutions) and 2×10^3 ^{51}Cr-labeled PIR-2 cells were added per well. ^{51}Cr release was measured after a 4-h incubation. LU were calculated at 30% lysis.

* MLC-derived lymphocytes were first incubated on monolayers of either BALB/c- or C3H LPS-induced lymphoblasts, and the nonadhering cells were admixed with anti-PIR-2 effector cells.

‡ B6 splenocytes were treated to remove NK-LAK precursors before MLC.

§ Lymphocytes were harvested from LDC wells originally plated at 50,000 cells/well and were not cytotoxic against PIR-2.

Discussion

The paradox that in spite of the relatively high frequency of CTL with ASTC activity detected by LDC among splenocytes freshly obtained from normal or tumor-bearing mice or among lymphocytes derived from allogeneic bulk MLC, no ASTC activity was mediated by these cell populations (3–6, 11), led us to hypothesize (3–5) that ASTC activity might be blocked by suppressor cells that coexist with the ASTC CTL.

The data presented in Fig. 2 support this hypothesis: when small numbers of responding cells were plated in LDC wells (low cell–number LDC), the putative suppressor cells were presumably diluted out and full manifestation of ASTC response could be observed. The number of putative suppressor cells present in wells plated with high numbers of responding cells (high cell–number LDC) sufficed to block the ASTC response. The fact that decreasing the number of cells plated per well

caused the dilution out of the suppressor cells before that of the anti PIR-2 cytotoxic cells suggests that the frequency of the former cells is lower than that of the latter.

Since no decrease in the anti-P815 cytotoxicity was observed in high cell–number LDC wells (Fig. 2), we ruled out the possibility that the decrease in ASTC activity observed in these wells was the result of overall cell death (due to overcrowding). We also ruled out the possibility that selective death of ASTC CTL or ASTC cell "suicide" (lysis of B6 antisyngeneic CTL by their identical counterparts) in high cell density conditions account for the decrease in ASTC activity. Such mechanisms should have resulted in a decrease in the relative number (or frequency) of ASTC CTL in high cell–number LDC wells. However, as seen in Table I, the ASTC frequency among cells harvested from high cell–number LDC wells, apparently devoid of anti-PIR-2 cytotoxicity (PIR-2$^-$), was in fact similar to that found among cells harvested from low cell–number, PIR-2$^+$ LDC wells.

An alternative explanation for the decrease in the anti-PIR-2 response in wells plated with high cell numbers is that the anti-PIR-2 cytotoxic cells are more sensitive to IL-2 concentration in the LDC wells than the specific antiallogeneic CTL. However, we have previously shown (12) that the anti-PIR-2 effector cells are not of the NK/LAK lineage, but rather "classical" CTL. Moreover, even a fivefold increase in the IL-2 concentration in the LDC did not change the anti-PIR-2 response. These findings and the fact that the anti-PIR-2 LDC curves fit to the single-hit Poisson's distribution indicate that IL-2 concentration is not the limiting factor that could cause the decrease in the anti-PIR-2 reactivity in the high cell–number wells.

The fact that a high ASTC CTL frequency was found even among lymphocytes harvested from PIR-2$^-$ high cell–number LDC wells (Table I) also indicates that the suggested suppression is not operative at the level of induction of ASTC cells but rather on their cytotoxic activity at the effector phase; suppression of the induction of ASTC would have resulted in a reduced frequency of ASTC CTL among cells harvested from PIR-2$^-$ high cell–number LDC wells. The existence of suppressor cells regulating autologous cytotoxicity at the effector phase has been recently reported in cancer patients (22). In addition, Joly et al. (16) recently reported the existence of CD8$^+$ class I–nonrestricted suppressor cells that suppress, at the effector phase, the cytotoxicity against autologous anti-HIV-infected target cells, as well as the activity of antiautologous HLA CTL.

In mixing experiments, we demonstrated that, indeed, cells harvested from high cell–number B6 a/BALB LDC wells (Table V), and from B6 a/BALB MLC-derived lymphocytes (Tables II–V), both suppressed the ASTC activity of cells harvested from low cell–number B6 a/BALB LDC. The fact that supernatant from B6 a/BALB MLC did not suppress this activity (Tables II, IV, and V) suggests that the suppression is mediated by cells rather than by soluble factor(s). The anti-ASTC suppressor cells, as the ASTC cells themselves (5), were phenotyped as CD8$^+$ cells, i.e. Thy-1$^+$, Lyt-2$^+$, L3T4$^-$, and asialo GM1$^-$ cells (Tables III and V), and are radioresistant. IL-2-elicited LAK cells were not suppressive (Table II).

The need for specifically allosensitized MLC-derived cells in the suppression of ASTC response is demonstrated in Tables II, IV, and V: B6 splenocytes that were allosensitized against BALB/c (but not against C3H) splenocytes suppressed the ASTC response elicited in B6 a/BALB LDC. In addition, the suppressive activity of MLC-derived cells could be specifically adsorbed only on monolayers of lymphoblasts de-

rived from the same haplotype used to stimulate the responding cells (Table V). In preliminary experiments (data not shown), we also found that lymphocytes derived from bulk MLC of B6 anti-strain A (H-2K^{k}D^d) suppressed the anti-PIR-2 CTL activity induced in B6 a/BALB LDC as efficiently as lymphocytes derived from bulk MLC of B6 anti-BALB/c (H-2K^{d}D^d). Moreover, B6 a/BALB MLC-induced suppressor cells could be adsorbed on monolayers of BALB/c and of strain A, but not on B6 or C3H (H-2^k) lymphoblasts. Interestingly, BALB/c and strain A splenocytes were 10-fold more effective in eliciting anti-PIR-2 CTL than splenocytes from C3H mice (data not shown). Although additional experiments are needed to investigate the H-2 restriction pattern of the elicitation of ASTC cells and their suppressors, we have preliminary indications linking the H-2D region to the induction of these activities in our experimental system.

The specificity of the suppressor cells was further demonstrated by the ability of MLC-derived lymphocytes to suppress the anti-PIR-2, but not the anti-P815 cytotoxicity (Table IV). Suppression of anti-PIR-2 and not anti-P815 cytotoxicity is also evident in high cell–number LDC (Fig. 2). The exact mechanism of the suppression of ASTC response is yet to be determined. However, we were able to demonstrate that although the suppression is mediated by CTL-like CD8$^+$ cells, is does not appear to be caused by the lysis of the anti-PIR-2 effector cells themselves. The effective ratio of suppressor/effector cell ratios that resulted in CTL suppression was hard to estimate. Although in the mixing experiments a 1:1 ratio was used, it should be noted that both "suppressor" cells (from bulk MLC) and "effector" cells (from PIR-2$^+$ LDC wells) were mixed populations in which we could not estimate the frequency of the suppressor cells.

The observation that the same CTL clone could lyse both syngeneic and allogeneic target cells (5, 6, 11, and Fig. 1), and our data (23), showing that the antiallogeneic cytotoxicity elicited in LDC could be blocked by mAbs directed against H-2K products (MHC-restricted killing) whereas ASTC response could not, led us to conclude that the Thy-1$^+$, Lyt-2$^+$, L3T4$^-$ cells induced by allosensitization exhibit at least two distinct cytotoxic activities: one is the classical, MHC-restricted CTL activity aimed specifically against the allogeneic H-2 determinants, and the other is aimed against the syngeneic tumor PIR-2 and most likely against other syngeneic target cells. Only the latter activity can be blocked by suppressor cells elicited by allosensitization. The above preliminary observation also suggests that: (a) the target antigens on PIR-2 recognized by the ASTC cells are probably not crossreactive with the H-2 K/D^d determinants on the BALB/c stimulating cells; and (b) the PIR-2$^+$, P815$^+$ CTL recognize their target cells via two distinct receptors present on the same CTL.

That two different activities could be exhibited by the same T cell clone was already shown by others: helper (IL-2 production) and cytotoxic activities (24); helper and suppressive activities (25), and cytotoxic activity against two distinct target cells (26–28). In addition, MHC nonrestricted killing mediated by "classical" CTL and loss of antigenic specificity were demonstrated in cases where classical, allogeneically elicited CTL were carried in vitro with a high concentration of IL-2 only, in the absence of the alloantigen (29, 30). Cytotoxic activity against syngeneic target cells exhibited by allogeneic CTL was also reported (31).

Since we (3–6) and others (7–11, 15, 28, 32, 33) were able to demonstrate the exis-

tence of autoreactive (or ASTC) clones among normal murine and human lympho-cytes, one might speculate that the function of the suppression described here is to prevent autoimmune responses that might be elicited as a consequence of normal or abnormal activity of the immune system. Our findings are in accordance with the veto cell hypothesis, as described by Miller (34, 35) and by others (36–38): the veto cells, with the phenotype and specificity of CTL, block, by a noncytolytic mech-anism, the cytotoxic activity aimed at syngeneic target cells. Additional preliminary data (Leshem and Bach, unpublished results) showed that even cloned ASTC popu-lations, when replated in allogeneic LDC, exhibited a decrease in ASTC response in the high cell–number LDC wells, the same that characterizes the suppression of ASTC response in allogeneic LDC using uncloned populations, as shown here (Fig. 2). Based on this observation and assuming that the cloned ASTC population had not undergone any change during their growth, we are tempted to speculate that the ASTC cells themselves might be the suppressive cells.

We showed that allosensitization elicits cytotoxic response against syngeneic normal lymphoblasts as well as ASTC response (3, 4), and that syngeneic (H-2^b) cells block the cytotoxicity against PIR-2 (Leshem et al., unpublished results). We therefore believe that the ASTC response induced by allostimulation, as described here, is representative of responses against normal self antigens. ASTC cells (6) as well as autoreactive cells (15, 32) were shown to exist among splenocytes of normal mice, but their activity may normally not be manifested because it is specifically suppressed. It may be that under certain conditions, as in low cell–number LDC where sup-pressor cells are diluted out, or in autoimmune diseases that are often accompanied by a decrease in suppressor cell activity (39–42), the anti-self response is manifested. Indeed, suppression of L3T4$^+$ autoreactive cells by Lyt-2$^+$ suppressor cells was re-cently demonstrated, both in vitro and in vivo (43). In addition, CD8$^+$ suppressor cells that block, at the effector phase, the cytotoxicity against autologous HIV-infected cells, as well as the activity of antiautologous HLA CTL, were also identified (16).

Experiments in our laboratory are undertaken to elucidate the mechanism and the MHC restriction patterns of the elicitation of ASTC response and its suppres-sion and the relevance of these phenomena in vivo.

Summary

Sensitization of C57BL/6 (B6, H-2^b) splenocytes against normal BALB/c (H-2^d) leukocytes (B6 a/BALB) in bulk MLC induced CTL reactive against the syngeneic (H-2^b) nonimmunogenic lymphoma PIR-2, in addition to the CTL directed against the corresponding (H-2^d) allotargets. However, MLC-derived lymphocytes did not directly exhibit anti-PIR-2 cytotoxicity in spite of the high anti-PIR-2 CTL frequency (up to 1/20) among them, as demonstrated by the limiting dilution culture (LDC) technique. The present study was undertaken to resolve this contradiction. We found that anti-PIR-2 cytotoxicity could be detected only when B6 a/BALB MLC-derived responding cells were plated in LDC at low numbers (<200) of cells/well. In con-trast, increasing the number of the plated cells to 500–5,000 resulted in a gradual decrease in the percentage of wells cytotoxically reactive against PIR-2, whereas the percentage of wells exhibiting cytotoxicity against the allotargets remained unchanged (100%). This decrease of anti-PIR-2 cytotoxicity in LDC and the lack of anti-PIR-2

reactivity among MLC-derived lymphocytes were shown by mixing experiments to result from the activity of radioresistant Thy-1$^+$, Lyt-2$^+$, L3T4$^-$ suppressor cells, blocking the anti-PIR-2 cytotoxicity at the effector phase. The suppression was specific as indicated by the following observations: (a) freshly obtained B6 splenocytes, cultured unsensitized B6 splenocytes, mitogen-induced B6 lymphoblasts, B6 LAK cells, or B6 a/B6 MLC-derived lymphocytes were not suppressive; (b) anti-PIR-2 cytotoxicity elicited in B6 a/BALB LDC was suppressed only by lymphocytes derived from B6 a/BALB MLC and not from B6 a/C3H (H-2^k) MLC; and (c) B6 a/BALB MLC-induced suppressor cells could be adsorbed on monolayers of BALB/c but not of C3H lymphoblasts. Since both syngeneic tumor and allogeneic target cells were lysed by the same clonal cell population but only the antisyngeneic activity was suppressed, we suggest that a single CTL can exhibit two cytotoxic activities that are differentially affected by the described suppressor cells. This mode of suppression may play a role in controlling autoimmune reactivity.

References

1. Bach, M. L., F. H. Bach, and J. M. Zarling. 1978. Pool priming: a means of generating T lymphocytes cytotoxic to tumor or virus infected cells. *Lancet.* i:20.

2. Zarling, J. M., H. I. Robins, P. C. Raich, F. H. Bach, and M. L. Bach. 1978. Generation of cytotoxic T lymphocytes to autologous human leukemia cells by sensitization to pooled allogeneic normal cells. *Nature (Lond.).* 274:269.

3. Kuperman, O. J., B. Leshem, and F. H. Bach. 1981. Elicitation of response against syngeneic tumor by allosensitization: limiting dilution analysis. *In* Mechanisms of Lymphocyte Activation. K. Resch and H. Kirchner, editors. Elsevier Science Publishers B. V., Amsterdam. 446–449.

4. Bach, F. H., B. Leshem, O. J. Kuperman, H. Bunzendahl, B. J. Alter, and J. M. Zarling. 1982. Allosensitization to obtain anti-tumor immunity. *In* The Potential Role of T Cells in Cancer Therapy. A. Fefer and A. Goldstein, editors. Raven Press Ltd., New York. 7–19.

5. Leshem, B., B. Gotsman, and E. Kedar. 1984. In vitro elicitation of cytotoxic response against a nonimmunogenic murine tumor by allosensitization. *Cancer Immunol. Immunother.* 17:117.

6. Leshem, B., B. Gotsman, Z. Lebendiker, and E. Kedar. 1985. Induction of cytotoxic response against syngeneic, weakly immunogenic murine tumors by in vitro allosensitization. *Transplant. Proc.* 17:896.

7. Parmiani, G., M.-L. Sensi, G. Carbone, M. Colombo, M. Pierotti, D. Ballinari, J. Hilgers, and J. Hilkens. 1982. Cross-reaction between tumor cells and allogeneic normal tissues. Inhibition of a syngeneic lymphoma outgrowth in H-2 and non-H-2 alloimmune BALB/c mice. *Int. J. Cancer.* 29:323.

8. Vanky, F., T. Gorsky, Y. Gorsky, M.-G. Masucci, and E. Klein. 1982. Lysis of tumor biopsy cells by autologous T lymphocytes activated in mixed cultures and propagated with T cell growth factor. *J. Exp. Med.* 155:83.

9. Sensi, M.-L., M. Parenza, and G. Parmiani. 1983. Alloreactivity and tumor antigens: generation of syngeneic antilymphoma killer lymphocytes by alloimmunization of mice

with normal cells. *J. Natl. Cancer Inst.* 70:291.

10. Mazumder, A., E. A. Grimm, and S. A. Rosenberg. 1983. Lysis of fresh human solid tumors by autologous lymphocytes activated in vitro by allosensitization. *Cancer Immunol. Immunother.* 15:1.

11. Sensi, M.-L., C. G. Orosz, and F. H. Bach. 1984. Alloantigen-induced cytotoxicity against tumor cells: analysis at the clonal level. *J. Immunol.* 132:3218.

12. Leshem, B., E. Epstein, and E. Kedar. 1989. Allosensitization in IL-2 containing limiting dilution cultures generates cytotoxic T lymphocytes rather than LAK cells reactive with a syngeneic nonimmunogenic murine tumor. *Immunol. Lett.* 20:53.

13. Coreley, R. B., B. Kindred, and I. Lefkovits. 1978. Positive and negative allogeneic effects mediated by MLR-primed lymphocytes: quantitation by limiting dilution analysis. *J. Immunol.* 121:1082.

14. Goronzy, J., U. Schaefer, K. Eichman, and M. M. Simon. 1981. Quantitative studies on T cell diversity. II. Determination of the frequencies and Lyt phenotypes of two types of precursor cells for alloreactive cytotoxic T cells in polyclonally and specifically activated T cells. *J. Exp. Med.* 153:857.

15. Rozenkrantz, K., B. Dupont, and N. Flomenberg. 1985. Generation and regulation of autocytotoxicity in mixed lymphocyte cultures: evidence for active suppression of autocytotoxic cells. *Proc. Natl. Acad. Sci. USA.* 82:4508.

16. Joly, P., J.-M. Guillon, C. Mayaud, F. Plata, I. Theodorou, M. Denis, P. Debre, and B. Autran. 1989. Cell-mediated suppression of HIV-specific cytotoxic T lymphocytes. *J. Immunol.* 143:2193.

17. Yefenof, E., R. Tchakirov, and E. Kedar. 1980. In vitro generation of cytotoxic lymphocytes against radiation and radiation leukemia virus-induced tumors. *Cancer Immunol. Immunother.* 8:171.

18. MacDonald, H. R., J. C. Cerottini, J. E. Ryser, J. L. Maryanski, C. Taswell, M. B. Widmer, and K. T. Brunner. 1980. Quantitation and cloning of cytolytic T lymphocytes and their precursors. *Immunol. Rev.* 51:93.

19. Cerottini, J. C., and K. T. Brunner. 1974. Cell-mediated cytotoxicity, allograft rejection, and tumor immunity. *Adv. Immunol.* 18:67.

20. Kedar, E., M. O. deLandazuri, B. Bonavida, and J. L. Fahey. 1974. Cellular immunoadsorbents: an improved technique for specific depletion of cytotoxic (T) lymphoid cells. *J. Immunol. Methods.* 5:97.

21. Herberman, R. B., et al. 1987. Lymphokine-activated killer cell activity. Characteristics of effector cells and their progenitors in blood and spleen. *Immunol. Today.* 8:178.

22. Eura, M., T. Maehara, T. Ikawa, and T. Ishikawa. 1988. Suppressor cells in the effector phase of autologous cytotoxic reactions in cancer patients. *Cancer Immunol. Immunother.* 27:147.

23. Leshem, B., E. Epstein, and E. Kedar. 1989. Murine cytotoxic T lymphocytes exhibiting two distinct cytotoxic activities. *Proc. 2nd International Conference on Graft Infiltrating Cells, Pittsburgh, PA.* V2 (Abstr.)

24. Roopenian, D. C., M. B. Widmer, C. G. Orosz, and F. H. Bach. 1983. Helper cell independent cytolytic T lymphocytes specific for a minor histocompatibility antigen. *J. Immunol.* 130:542.

25. Kotani, H., H. Mitsuya, E. Benson, S. P. James, and W. Strober. 1988. Activation and function of an autoreactive T cell clone with dual immunoregulatory activity. *J. Immunol.* 140:4167.

26. Ben-Nun, A., Z. Lando, M. E. Dorf, and S. J. Burakoff. 1983. Analysis of crossreactive antigen-specific T cell clones. Specific recognition of two major histocompatibility complex (MHC) and two non-MHC antigens by a single clone. *J. Exp. Med.* 157:2147.

27. Sheil, J. M., M. J. Bevan, and L. Lefrancois. 1987. Characterization of dual-reactive

H-2K^b-restricted anti vesicular stomatitus virus and alloreactive cytotoxic T cells. *J. Immunol.* 138:3654.

28. Altman, A., and D. H. Katz. 1980. Existence of T cells manifesting self-reactivity indistinguishable from alloreactivity. *J. Immunol.* 125:1536.

29. Brooks, C. G., D. L. Urdal, and C. S. Henney. 1983. Lymphokine-driven differentiation of cytotoxic T-cell clones into cells with NK-like specificity: correlation with display of membrane macromolecules. *Immunol. Rev.* 72:43.

30. Shau, H., J. D. Gray, and S. H. Golub. 1988. Studies on cytotoxicity generated in human mixed lymphocyte culture. IV. Interleukin 2 alone or from mixed lymphocyte culture yields natural killer-like cytotoxic cells distinct from allospecific cytotoxic T lymphocytes. *Cancer Immunol. Immunother.* 27:255.

31. Duke, R. C. 1989. Self recognition by T cells. I. Bystander killing of target cells bearing syngeneic MHC antigens. *J. Exp. Med.* 170:59.

32. Reimann, J., and R. G. Miller. 1983. Generation of autoreactive cytotoxic lymphocytes under limiting dilution conditions. *J. Immunol.* 131:2128.

33. Martin, A., T. H. Eiermann, L. Tricas, and S. F. Goldmann. 1987. MHC class II restricted self-recognition after allostimulation: clonal analysis. *Tissue Antigens.* 30:1.

34. Miller, R. G. 1980. An immunological suppressor cell inactivating cytotoxic T-lymphocyte precursor cells recognizing it. *Nature (Lond.).* 287:544.

35. Miller, R. G. 1986. The veto phenomenon and T-cell regulation. *Immunol. Today.* 7:112.

36. Fink, P. J., H.-G. Rammensee, J. D. Benedetto, W. D. Staerz, L. Lefrancois, and M. J. Bevan. 1984. Studies on the mechanism of suppression of primary cytotoxic responses by cloned cytotoxic T lymphocytes. *J. Immunol.* 133:1769.

37. Rammensee, H.-G., M. J. Bevan, and P. J. Fink. 1985. Antigen specific suppression of T-cell responses: the veto concept. *Immunol. Today.* 6:41.

38. Ogasawara, M., K. Iwabuchi, R. A. Good, and K. Onoe. 1988. Bone marrow cells from allogeneic bone marrow chimeras inhibit the generation of cytotoxic lymphocyte response against both donor and recipient cells. *J. Immunol.* 141:3306.

39. Theofilopoulos, A. N., and F. J. Dixon. 1981. Etiopathogenesis of murine SLE. *Immunol. Rev.* 55:179.

40. Sakaguchi, S., K. Fukumara, K. Kuribayashi, and T. Masuda. 1985. Organ-specific autoimmune disease induced in mice by elimination of T cell subset. I. Evidence for the active participation of T cells in natural self-tolerance: deficit of a T cell subset as a possible cause of autoimmune disease. *J. Exp. Med.* 161:72.

41. Cohen, I. R. 1986. Regulation of autoimmune disease: Physiological and therapeutic. *Immunol. Rev.* 94:5.

42. Ellerman, K. E., J. M. Powers, and S. W. Brostoff. 1988. A suppressor T-lymphocyte for autoimmune encephalomyelitis. *Nature (Lond.).* 331:265.

43. Nagarkatti, P. S., M. Nagarkatti, L. W. Mann, L. A. Jones, and A. M. Kaplan. 1988. Characterization of an endogenous Lyt2$^+$ T-suppressor cell population regulating autoreactive T cells in vitro and in vivo. *Cell. Immunol.* 112:64.