REGULATION OF IN VITRO CYTOTOXIC
T LYMPHOCYTE GENERATION*

I. Evidence that Killer Cell Precursors
Differentiate to Effector Cells in Two Steps

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Two distinct T helper cell (T_h)[1] populations are required to induce B cells to produce optimal amounts of antibody (1–6). The hypothesis that these two T_h have different effects on B cells, i.e., that T_h1 is mainly responsible for quantitative effects, whereas T_h2 determines the qualitative aspects of antibody formation is supported by the work of Rohrer and his associates (manuscript in preparation) in their studies on the effects of T_h on the activity of MOPC-315 myeloma cells. They have shown that the two T_h affect the myeloma cells in different ways. The first T_h causes an increase in proliferation of the myeloma cells and the second an increase in their secretion of paraprotein. The T_h for proliferation expresses a different phenotype (Lyt-1^+2^-; Qa-1^-) than the T_h for secretion (Lyt-1^+2^-; Qa-1^+). These data show that there are at least two steps in the differentiation of B cells.

The generation of alloantigen-specific cytotoxic T lymphocyte effector cells (CTL) is similar to the induction of antibody forming cells, in that T cell help is required for optimal induction of CTL from CTL precursors (CTL-P) (7). We have asked whether CTL might also be similar to B cells in the existence of two steps in the development of CTL, perhaps influenced by a distinct T_h at each step. To do this we have inhibited the induction of CTL in allogeneic mixed leukocyte culture (MLC) by treating the cultures with the drug pyrilamine (8) or by using heat-inactivated stimulator cells (9). We have found that either of these treatments can block the generation of CTL while allowing CTL-P to differentiate from a naïve state (CTL-PN) into an activated state (CTL-PA). CTL-PA can be distinguished from CTL-PN by the fact that the former can develop into CTL in the face of levels of T cell-mediated suppression, which inhibit the production of CTL from CTL-PN. Thus, the generation of CTL is a two-step process. Step 1, which is the induction of CTL-PN to CTL-PA, is sensitive to the

[1] *Abbreviations used in this paper*: BSS, phosphate-buffered balanced salt solution; C', complement; CTL, cytotoxic T lymphocytes; CTL-P, CTL precursor; CTL-PA, activated CTL-P; CTL-PN, naïve CTL-P; FBS, fetal bovine serum; MLC, mixed leukocyte culture; PEC, thioglycolate-induced peritoneal exudate cells; T_h, T helper lymphocytes; T_s, suppressor T lymphocytes; T_sN, nonspecific T_s; T_sS, alloantigen-specific T_s.

effects of in vitro generated suppressor T cells (T_s), but is not inhibited by addition to culture of pyrilamine or heat inactivation of stimulator cells. On the other hand, step 2, the differentiation of CTL-PA to CTL, can be blocked by pyrilamine, but not by the same suppressor cells that inhibit the transition from CTL-PN to CTL-PA. The CTL-PA to CTL step and, possibly the CTL-PN to CTL-PA step, require T_h activity.

Materials and Methods

Animals. Male C57BL/6 (B6) (H-2^b, Thy-1.2$^+$, Lyt-1.2$^+$,2.2$^+$,3.2$^+$) BALB/c (H-2^d), DBA/2 (H-2^d), CBA (H-2^k), C3H/He (H-2^k,Thy-1.2$^+$) and AKR/J (H-2^k,Thy-1.1$^+$) mice, 6-8 wk of age were obtained from The Jackson Laboratory, Bar Harbor, ME. B6.PLThy1^a mice (H-2^b,Thy-1.1$^+$ Lyt-1.2$^+$,2.2$^+$,3.2$^+$) are Thy-1 congenic B6 mice bred by Dr. Donal Murphy of the Yale Medical School or in the Cancer Center breeding colonies at Tufts University. All mice were rested for at least 1 wk in our animal facilities before use.

In Vitro Induction of CTL. Induction and assay of CTL were performed as described previously (10). Briefly, single-cell suspensions of spleen cells were washed three times in phosphate-buffered balanced salt solution (BSS) containing 5% fetal bovine serum (BSS-FBS). CTL were generated in 2 ml MLC in 24-well tissue culture plates (76-033-05, Linbro Scientific Co., Hamden, CT). 5 × 10^6 viable responding spleen cells were incubated with 5 × 10^5 allogeneic stimulator spleen cells in modified Mishell-Dutton plating medium containing 10% FBS (11, 12), in an atmosphere of 5% CO_2 in humidified air at 37°C. Stimulators were prepared by incubating 5 × 10^7 viable splenic lymphoid cells in 1 ml of BSS-FBS containing 30 µg of mitomycin C (Sigma Chemical Co., St. Louis, MO) for 1 h at 37°C.

The cells were then washed three times in BSS-FBS, diluted in plating medium, and added to culture wells. After 4-6 d, cells were harvested by repeated agitation using a pasteur pipette. Before transfer or assessment of CTL activity, cells were washed once, unless pyrilamine (*vide infra*) had been added to the culture, in which case they were washed three times.

Pyrilamine Maleate. Pyrilamine, a histamine$_1$ receptor antagonist (Sigma Chemical Co.), was added to 4-6-d MLC at the time of culture initiation. Addition of 1 × 10^{-4} M pyrilamine generally resulted in a decrease in viable cell yields from the usual 60-70% to 15-30% of the cells originally placed in culture.

Assay of CTL Function. CTL were assayed on the basis of MLC yield equivalents. That is, three or more wells per group, incubated as described above, were harvested, washed, pooled, counted, and diluted in BSS-FBS so that the viable cell concentration was usually 8 × 10^6 to 1 × 10^7 per ml for the highest concentration of the effectors tested (i.e., a 40:1-50:1 effector/target cell ratio). Threefold and ninefold dilutions were also tested in most cases, using quadruplicate microwells per dilution, per group. In certain instances, equal numbers of effectors treated in different ways were compared for cytotoxicity. 100 µl of each effector cell suspension was added to 100 µl containing 2 × 10^4 ^{51}CrNa$_2$CrO$_4$ (New England Nuclear, Boston, MA) -labeled target cells (see below), in 96-well flat-bottomed tissue culture plates (3040, Falcon Labware, Oxnard, CA). Cells were sedimented by centrifugation of the trays, which were then incubated for 4 h at 37°C. Subsequently, a 100-µl supernatant sample was obtained from each well and assayed for gamma counts. Target cells for CTL assay included cultured DBA/2 mastocytoma P815 (H-2^d) cells, or, alternatively, 3- or 4-d thioglycolate-induced peritoneal exudate cells (PEC) from CBA or DBA/2 mice. The latter cells were depleted of erythrocytes by hypotonic lysis before ^{51}Cr labeling.

Analysis of Data. Cytotoxicity assays were always performed in quadruplicate. Release of ^{51}Cr into the supernatant by killer cells was compared with release from target cells incubated either with medium (0% or spontaneous release) or with 2% Triton X-100 (Amersham Corp., Arlington Heights, IL) (100% release). Spontaneous (0%) release usually varied from 10% to 25% of Triton X-100 controls. When significant cytotoxicity (that is, ≥20% release) was detected, the standard deviation (SD) was generally ≤10% of the mean release. Because multiple effector dilutions were tested for cytotoxicity, it was possible, in each experiment presented, to compare groups under conditions in which killing was linear function of the effector cell number. Only such data are presented.

Induction and Assessment of Allosuppressor Cells and CTL-PA. CTL-PA and T_s were prepared

similarly to CTL. Briefly, 5×10^6 male B6 or B6.PLThy1[a] (H-2[b]) responder spleen cells were cultured with 5×10^6 mitomycin C-treated male allogeneic (H-2[d]) stimulator spleen cells for 4–6 d in the presence or absence of 10^{-4} M pyrilamine. In some cases, stimulator cells were not mitomycin C treated, but were heat inactivated at 45°C for 60 min and 2×10^7 (preheating viable cell number; usually, 50%–60% of the cells were dead after this treatment) were incubated with 2×10^7 viable responder cells in 10-ml flask cultures (3050, Costar, Data Packaging, Cambridge, MA) according to method of Burton et al. (9). The putative suppressor cells and CTL-PA were then washed and transferred to a 5-d second MLC in which B6 or B6.PLThy1[a] spleen cells were responders. The use of Thy-1-different congenic B6 responder cells in the first and second cultures allowed us to determine, at assay, the MLC from which CTL were derived. This was accomplished by treating cells harvested from second cultures with the appropriate anti-Thy-1 serum and rabbit complement (C') before CTL assay. The percentage suppression of CTL generation was calculated by the formula: percent suppression $= 100 -$ (percent ^{51}Cr release with suppressors ÷ percent ^{51}Cr release with no suppressors). For example, to estimate the relative activity of B6- vs. B6.PLThy1[a]-derived CTL in a mixture of the two cell types, anti-Thy-1.2 serum + C'-resistant percent ^{51}Cr release (i.e., B6.PLThy1[a] derived) was subtracted from pooled normal mouse serum (NMS) + C'-resistant percent ^{51}Cr release (B6 and B6.PLThy1[a] derived).

Antiserum Treatment of Lymphoid Cells. Fresh spleen cells or spleen cells harvested from MLC were washed three times. They were then treated at 0°C for 30 min with NMS, allogeneic anti-Thy-1.2 serum (AKR/J-anti-C3H/He serum, kindly provided by Dr. Charles Janeway of Yale University), diluted 1:40 in BSS-FBS or with rat-anti-mouse Thy-1.2 hybridoma culture supernatant (kindly provided by Dr. Michael Iverson of Yale University) at a 1:50 dilution of stock. Cells were suspended at a 1×10^7 per ml maximum concentration in the appropriate dilution of the desired anti-Thy-1 reagent. The tubes were incubated for 30 min at 37°C and the cells were washed and resuspended in 1 ml of rabbit C' (prescreened for low toxicity and depending on the lot, diluted from 1:10 to 1:16 as indicated by titration) and incubated 35 min at 37°C with shaking. Subsequently, cells were washed twice, counted for viable cell yield, and assayed for either CTL-P or effector function, or for suppressive capability. It is noteworthy that both anti-Thy-1 reagents used were completely specific for Thy-1.2 antigen-bearing cells, in that neither reagent, even when used at five times the final concentrations described above, had any effect on either the viability or CTL precursor or effector function of B6.PLThy-1[a] cells.

To deplete Lyt-1^{+} (Ly-1 and Ly-123) T lymphocytes, cells were incubated under conditions similar to those described above. A 1:1,000 dilution of the anti-Lyt-1.2 hybridoma reagent (C3H/Lilly-anti-I-SL-57 clone 22-10.3) kills 68% of anti-immunoglobulin plate-purified T cells, whereas a 1:1,000 dilution of the anti-Lyt-2.2 hybridoma reagent (C3H/β-anti-ERLD clone 41-3) kills 50% of T cells (M. Iverson, personal communication). Both reagents were generously supplied by Dr. F. W. Shen of the Memorial Sloan-Kettering Cancer Center, New York).

Preparation of Nylon-nonadherent, Splenic T Cell-enriched Populations. Nylon wool (Leuko-pak, Fenwal Laboratories, Div. Travenol Laboratories, Deerfield, IL) was prepared, placed into plastic disposable syringes, autoclaved, and used by the method of Julius et al. (13). After elution of nonadherent spleen cells from the column, they were concentrated by centrifugation and applied, similarly, to a second nylon wool column. Subsequently eluted cells ("twice-passed nylon wool nonadherent cells") were >95% viable.

Preparation of Glass-nonadherent Spleen Cells. 1×10^8–1.5×10^8 spleen cells were added to 100 $\times$ 15 mm Pyrex petri dishes in 15 ml BSS-FBS. They were incubated at 37°C for 1 h, after which the nonadherent cells were resuspended by gentle swirling and were collected by pipetting all fluid. The procedure was repeated in an identical manner using fresh BSS-FBS and fresh petri dishes, yielding "twice-treated glass-nonadherent cells".

Results

Effect of Pyrilamine on CTL Induction. We have previously evaluated the effect of histamine on the generation of CTL (10). During these experiments we discovered that the histamine$_1$ receptor antagonist, pyrilamine, severely inhibited CTL genera-

tion when added at the start of a 4- or 5-d MLC at a 10^{-4} M concentration. Table I exemplifies the results of such experiments. More than 95% inhibition of CTL generation was routinely obtained with a 10^{-4} M concentration of pyrilamine, associated with a 50–75% decrease in viable cell yield compared with control cultures incubated without pyrilamine. The relationship of inhibition of CTL generation to the "toxic" effects of the drug is not clear, but is not relevant to the studies that follow. $10^{-4.5}$ M or 10^{-5} M (Table I), and as low as 10^{-6} M (data not presented) pyrilamine were also inhibitory of CTL generation in a concentration-dependent manner. The profound inhibitory effect of 10^{-4} M pyrilamine on CTL generation (used in all further studies) persisted through at least 144 h of culture (data not presented).

A Thy-1 Congenic System for Study of Suppressor Cells and CTL-P.   In other studies (8) we have presented data indicating that pyrilamine inhibits not only CTL generation in MLC, but also the generation of nonspecific suppressor T cells (T_sN), whereas allospecific suppressor T cell induction (T_sS) in MLC is not inhibited. Thus, B6-anti-DBA/2 suppressors (H-2^b-anti-H-2^d), raised in the presence of pyrilamine for 4–6 d, could inhibit CTL generation upon transfer to fresh, new B6-anti-DBA/2 cultures under conditions in which they had no suppressive effect on fresh B6-anti-C3H cultures (H-2^b-anti-H-2^k).

In order to simultaneously study T_s and CTL-P functions, we have used a Thy-1 congenic B6 mouse, two-culture system. In the typical protocol, responder cells in the first MLC are from B6 (Thy-1.2^+) mice, whereas responder cells in the second (assay) MLC are from congenic B6.PLThy1^a (Thy-1.1^+) mice. Treatment with anti-Thy-1.2 serum plus C' of cells harvested after the second MLC then leaves only CTL generated in the second MLC (i.e., Thy-1.1^+ killers). We can reverse the Thy-1 phenotype of first and second culture responders, and the system works equally well. Therefore, suppression can be determined accurately by comparing anti-Thy-1.2-resistant CTL activity in suppressed MLC with anti-Thy-1.2-resistant CTL activity in control MLC. An example of results obtained with this system is portrayed in Table II. Spleen cells of B6 or B6.PLThy1^a mice were stimulated with DBA/2 cells in vitro. 5 d later the

TABLE I

Pyrilamine Inhibition of Cytotoxic T Cell Generation and Viable Cell Yields from MLC*

Pyrilamine concentration (molarity)	Percent ^{51}Cr release (± SD) at culture dilutions		Effector/ target cell ratio undiluted	Percent viable cell yield per culture
	1:3	Undiluted		
0	45.2 (3.4)	56.7 (2.8)	45:1	48
10^{-5}	32.8 (3.0)	48.6 (1.7)	39:1	41
$10^{-4.5}$	19.5 (2.5)	38.9 (2.5)	30:1	32
10^{-4}	−0.1 (1.2)	1.3 (1.7)	12:1	15

* 5×10^6 B6 spleen cells were incubated with 5×10^5 mitomycin C-treated DBA/2 spleen cells for 5 d in the presence or absence of pyrilamine. Cells from triplicate cultures per group were then washed three times, pooled and diluted equally, and tested for ability to kill ^{51}Cr-labeled P815 target cells in quadruplicate assay cultures per group. The percent release due to B6 responder cells cultured alone has been subtracted.

TABLE II

*Thy-1 Congenic B6 Mouse Spleen Cell Two-culture System for Evaluation of Suppression and of the Culture from Which CTL Arise**

		Percent ^{51}Cr release (± SD)	
		Serum treatment before CTL assay	
Cells from first MLC	Fresh responders in second MLC	NMS + C'	Anti-Thy-1.2 + C'
None	Thy-1.1+	45.3 (5.3)	40.4 (2.9)
None	Thy-1.2+	38.2 (4.2)	−3.6 (1.5)
Thy 1.1+	Thy-1.2+	51.3 (5.7)	43.5 (2.8)
Thy 1.2+	Thy-1.2+	24.5 (3.0)	−4.8 (3.3)
Thy 1.2+	Thy-1.1+	25.5 (2.5)	2.8 (2.0)

* First MLC were established in triplicate cultures per group with 5 × 10⁶ B6 (Thy-1.2+) or B6.PLThy1ª (Thy-1.1+) responders and 5 × 10⁶ mitomycin C-treated DBA/2 stimulators. 5 d later 5 × 10⁶ viable cells were transferred to similar cultures as indicated. After an additional 5 d, cells harvested from three wells of each group were treated with NMS + C' or with anti-Thy-1.2 serum + C'. The residual cells were diluted equally in each case, so that the results given are using culture-treatment yield equivalent numbers of effectors. 2 × 10⁴ ⁵¹Cr-labeled DBA/2 PEC targets were mixed with effectors in quadruplicate cultures per group. MLC without transferred cells contained effectors at a 45:1 effector/target cell ratio. Similar results were obtained, in the same experiment, using 15:1 and 5:1 effector/target cell ratios.

cells were washed and transferred to fresh MLC. Cells from such MLC were harvested 5 d later and were treated with either NMS plus C' or an anti-Thy-1.2 serum plus C', and were then evaluated for ability to kill target cells. Treatment of B6-derived CTL (to which no suppressors had been added) with anti-Thy-1.2 serum plus C' completely prevented killing, whereas similar treatment had no significant effect on killing by B6.PLThy1ª CTL. When sufficient (usually ≥1 × 10⁵-2 × 10⁵) Thy-1.1+ suppressor cells were transferred to Thy-1.2+ second cultures, treatment with anti-Thy-1.2 serum plus C' after the second culture had little or no influence on effector function. Thus, in this situation the CTL detected were all derived from the first culture, showing that the second culture's capacity to generate CTL was severely suppressed. This conclusion was supported by the finding that when Thy-1.2+ suppressor cells were added to cultures in which the responder cells were either Thy-1.1+ or Thy-1.2+, all CTL function detected was susceptible to anti-Thy-1.2 serum plus C'. These data show that the Thy-1 congenic responder cell system can be used to evaluate suppression of CTL generation, as well as to examine the source and function of in vitro-induced CTL.

Effect of Pyrilamine on T_s and CTL-P Activities. We have used the Thy-1 congenic responder cell system to evaluate the effects of pyrilamine on T_s generation and CTL-P activation. In the experiment portrayed in Table III, B6-anti-DBA/2 MLC were established in the presence or absence of 10^{-4} M pyrilamine. Harvested cells were subsequently transferred to fresh second MLC consisting of B6.PLThy1ª responders and DBA/2 stimulators. Once again, it can be seen that CTL that developed in control MLC (Thy-1.1+) without transferred cells were not affected by treatment with anti-Thy-1.2 serum plus C' before assay. However, when B6-anti-DBA/2 MLC-

TABLE III

*Ability of Pyrilamine-treated MLC-derived Cells to Suppress CTL Generation and to Differentiate to CTL Effectors upon Transfer to Fresh MLC**

Cells from first MLC	Fresh responders in second MLC	Percent ^{51}Cr Release (± SD)		Percent suppression of second MLC
		Serum treatment before assay		
		NMS + C'	Anti-Thy-1.2 + C'	
None	Thy-1.1$^+$	47.4 (5.5)	47.7 (4.2)	Standard
Thy-1.2$^+$	Thy-1.1$^+$	52.8 (3.2)	2.1 (2.8)	96
Thy-1.2$^+$ + P‡	Thy-1.1$^+$	31.8 (4.0)	−3.1 (1.4)	106

* B6-anti-DBA/2 (Thy-1.2$^+$) cultures were established as in legend to Table I. After 5 d cells were harvested from triplicate cultures, pooled, washed three times, and 2 × 10^5 cells were transferred to six fresh B6.PLThy-1^a (Thy-1.1$^+$)-anti- DBA/2 second MLC each, as indicated. After 5 additional d cells in these cultures were pooled and divided into two equal aliquots, which were treated with either NMS + C' or anti-Thy-1.2 serum + C'. Cells were then washed and diluted to an equal volume per group. Given is the percent ^{51}Cr release for a 22:1 effector/target cell ratio in control cultures (no suppressors), using 2 × 10^4 DBA/2 PEC as targets.
‡ Pyrilamine, 10^{-4} M.

derived cells were transferred to second MLC, essentially all CTL activity was anti-Thy-1.2 serum sensitive. Thus, there was virtually total suppression of CTL generation from fresh CTL-PN in the second MLC, and all CTL activity detected arose from the first MLC cells. In the presence of pyrilamine no CTL generation occurs. Even so, when these cells were transferred, total suppression of CTL generation in the second MLC occurred, and first culture-derived CTL arose. These data show that the Thy-1-congenic system allows quantitation of transferred CTL-P activity. As demonstrated in Table III (1 of >10 such experiments), the quantity of pyrilamine-treated first culture-derived CTL activity was decreased compared with untreated cells. This decrease varied from one-third of, to equal to the CTL-function developed in second cultures by non-pyrilamine-treated, transferred cells. When pyrilamine-treated MLC-derived cells were transferred to fresh MLC which were also treated with pyrilamine, no CTL activity arose from either culture in three of three experiments (data not presented). This shows that the failure of CTL-PA to differentiate to CTL in pyrilamine-treated cultures is not due to deteriorating culture conditions. These results demonstrate that (a) CTL-P do not differentiate to CTL in pyrilamine-treated cultures; (b) once removed from pyrilamine-treated cultures and placed in fresh MLC, CTL-P can differentiate to CTL; and (c) the latter differentiation to CTL is resistant to the same suppressive influences that are capable of completely blocking CTL generation by fresh unprimed cells. Therefore, CTL generation occurs in two steps. The first step, a change from CTL-PN to CTL-PA, is suppressor cell sensitive and pyrilamine resistant, whereas the second step, CTL-PA to CTL, is suppressor cell resistant and pyrilamine sensitive.

Effect of Heat Treatment of Stimulator Cells on Suppressor Cell and CTL-P Activation. Recently, Burton et al. (9) have shown that heat inactivation at 45°C for 60 min of allogeneic cells used as MLC stimulators allows suppressor T cell activation, whereas

TABLE IV

*Effect of Heat-inactivated Stimulator Cells on Induction of CTL Activity**

Additions to B6 responders	Percent ^{51}Cr release ($\pm$ SD) at culture dilutions				Effector/ target cell ratio un- diluted
	1:27	1:9	1:3	Undiluted	
Experiment 1 6-d culture					
None	ND‡	0.1 (3.3)	0.2 (4.6)	1.2 (3.8)	ND
BALB/c-HS	ND	1.5 (3.0)	12.8 (1.6)	31.1 (6.4)	25:1
BALB/c-Mito	ND	31.5 (4.6)	53.6 (4.6)	68.4 (5.5)	42:1
Experiment 2 4-d culture					
None	1.2 (4.5)	−0.7 (3.7)	2.3 (3.1)	−0.9 (1.2)	32:1
DBA/2-HS	0.6 (2.7)	2.6 (2.6)	6.0 (2.8)	15.2 (2.0)	36:1
DBA/2-mito	17.2 (4.7)	33.7 (5.9)	49.1 (2.5)	48.8 (2.3)	57:1
DBA/2-mito + P	−1.2 (2.1)	−1.5 (2.9)	−1.9 (4.1)	0.2 (3.0)	15:1
DBA/2-HS + P	−1.9 (3.8)	−1.7 (2.4)	−2.5 (3.7)	1.3 (1.7)	9:1

* 2 × 10^7 responder spleen cells, with or without 2 × 10^7 DBA/2 or BALB/c stimulator spleen cells, either heated at 45°C for 1 h (heat stimulator [HS]) or treated with mitomycin C (mito), were incubated in 10 ml plating medium, with or without 10^{-4} M pyrilamine (P), for 4 or 6 d. Harvested cells were washed three times, counted, and diluted to 0.8 ml (undiluted) each. Therefore, cells were assayed for cytotoxicity vs. P815 target cells (2 × 10^4/well) on a culture-yield equivalent basis. Given are the results of quadruplicate CTL assay cultures per group.
‡ Not done.

TABLE V

*Effect of Heating Stimulator Cells and Addition of Pyrilamine to MLC on CTL-P and T_s Activation**

Cells from first MLC		Cell number trans- ferred	Fresh responders in second MLC	Percent ^{51}Cr release ($\pm$ SD)	
				Treatment before CTL assay	
Respond- ers	Conditions			NMS + C′	Anti-Thy- 1.2 + C′
—	—	0	Thy-1.1$^+$	49.8 (3.2)	48.7 (2.3)
Thy-1.2$^+$	HS No P	9 × 10^5	Thy-1.1$^+$	81.4 (5.4)	21.5 (2.5)
		3 × 10^5		71.4 (3.4)	7.4 (2.8)
		1 × 10^5		39.5 (1.1)	4.9 (3.0)
Thy-1.2$^+$	HS P	9 × 10^5	Thy-1.1$^+$	3.6 (3.1)	1.3 (1.8)
		3 × 10^5		5.5 (3.0)	−0.3 (3.1)
		1 × 10^5		12.6 (3.1)	8.3 (0.8)

* 2 × 10^7 B6 (Thy-1.2$^+$) responding spleen cells were incubated for 6 d in the presence or absence of 10^{-4} P, with 2 × 10^7 BALB/c stimulator cells which had been heated at 45°C for 1 h (HS). The suppressors were washed three times and were transferred to fresh triplicate second MLC of 5 × 10^6 B6.PLThy-1^a (Thy-1.1$^+$) responders and 5 × 10^5 mitomycin C-treated BALB/c stimulators. After 5 d, cultures were harvested, pooled, and divided into two equal aliquots, one of which was treated with NMS + C′ and the other with rat anti-mouse Thy-1.2 hybridoma supernatant + C′. Given are the results obtained from quadruplicate CTL assay cultures per group.

CTL effector generation in inhibited. As in our earlier studies (8), this also gave evidence that CTL effector function per se is not required in T_s populations for suppression of CTL induction to occur. We have repeated these experiments in our system and in several experiments have corroborated a $\geq90\%$ reduction in the quantity of CTL effector induction, through at least 144 h culture, in MLC stimulated by heated allogeneic cells (e.g., see Table IV). When examined in our Thy-1 congenic system, we have found that, as in pyrilamine-treated MLC-generated cells, potent T_s-insensitive CTL-PA activity remains in these cultures. This conclusion stems from the results of two consecutive experiments (one of which is portrayed in Table V), in which first MLC-derived cells became CTL effectors following transfer to a second, fresh MLC. The addition of pyrilamine to cultures containing heated stimulator cells prevented detectable generation of CTL-PA in the first culture, whereas T_s function persisted (Table V). This result is particularly important because it shows that CTL-PA (i.e., activated cells capable of differentiating to CTL) are not required for suppression of CTL-PN. Whether the effects of combining pyrilamine treatment and heat-inactivated stimulator cells are merely additive at the same site of action or act on two different sites on the pathway to differentiation is unknown.

Cellular Requirements for CTL-PA Differentiation to CTL.   Following transfer of pyrilamine-treated or heated stimulator-induced MLC-derived cells to fresh cultures, differentiation of first culture CTL-PA to CTL occurs. The next question asked was, what cellular elements contributed by the second MLC are required to cause CTL-PA differentiation to CTL? We found that merely exposing CTL-PA to fresh stimulator cells is not sufficient (Table VI, experiment 1). As shown in Table VII, fresh responder cells alone are likewise insufficient, but if fresh stimulator cells also are added, CTL-PA differentiate to CTL in a stimulator cell dose-dependent manner. These stimulator cells need not be identical to those used to prime the CTL-PA (Table VI, experiment 2). Therefore, step 2 in CTL-P to CTL differentiation requires a nonspecific signal provided by a fresh MLC.

Responder Cell Sets Required for CTL-PA Differentiation to CTL.   We next explored the nature of second MLC responder cell populations necessary for CTL-PA to CTL differentiation. In experiments presented in Table VIII, B6.PLThy1^a (Thy-1.1$^+$) responder cells were used in the first MLC and B6 (Thy-1.2$^+$) in the second, the reverse of most previous experiments. Therefore, in this case, treatment with anti-Thy-1.2 antibodies + C' after the second MLC left only first MLC-derived CTL. As shown in experiment 1, twice-passed nylon-nonadherent spleen cells (depleted of B cells and accessory cells, and highly enriched for T cells) failed to generate a primary CTL response upon exposure to alloantigen, yet supported a decreased (compared with untreated responder cells) but significant level of CTL-PA to CTL differentiation. Twice-treated glass-nonadherent cells (depleted of accessory cells), produced less than one third the primary CTL response of untreated responder cells and supported a similarly decreased amount of CTL-PA to CTL differentiation as twice-passed nylon nonadherent cells. On the other hand, as demonstrated in experiment 2, anti-Thy-1.2-treated cells (depleted of T cells) failed to make either a primary CTL response or to support CTL-PA differentiation to CTL. These data show that responding populations probably supply at least two important cellular constituents necessary for CTL-PA differentiation to CTL: T cells and glass and nylon wool-adherent accessory cells.

TABLE VI

*Reculture of MLC-derived CTL-PA: Requirement for Fresh Responder vs. Stimulator Cells for Differentiation of CTL-PA to CTL**

| Cells from first MLC | Cell number transferred | Fresh cells second culture | | Percent ^{51}Cr release ($\pm$ SD) | |
| | | | | PEC target cells | |
		Stimulators	Re- spon- ders	DBA/2	CBA
		5×10^5	5×10^6		
Experiment 1					
—	—	DBA/2	B6	22.7 (3.6)	−1.4 (1.8)
B6 α DBA/2	5×10^6	0	0	12.2 (0.6)	ND‡
	5×10^6	DBA/2	0	14.3 (2.4)	ND
B6 α DBA/2 + P§	5×10^6	0	0	−1.4 (3.1)	ND
	5×10^6	DBA/2	0	2.1 (1.8)	ND
Experiment 2					
—	—	CBA	B6	9.7 (1.2)	45.5 (6.1)
B6 α DBA/2	1×10^6	0	0	3.0 (3.6)	ND
	1×10^6	CBA	B6	28.5 (2.7)	ND
	2.5×10^5	CBA	B6	25.6 (4.6)	ND
B6 α DBA/2 + P	1×10^6	0	0	1.7 (3.2)	ND
	1×10^6	CBA	B6	19.2 (2.9)	ND
	2.5×10^5	CBA	B6	7.4 (3.3)	ND

* B6-anti-DBA/2 spleen cell MLC, established in triplicate with or without 10^{-4} M pyrilamine, were harvested after 5 d and were transferred in triplicate as shown to fresh culture medium with or without fresh responders and/or mitomycin C-treated stimulators. 5 d later, cells were harvested, diluted equally, and tested for cytotoxicity in a culture yield-equivalent manner.
‡ Not done.
§ 10^{-4} M pyrilamine.

Discussion

These studies have demonstrated that the generation of allospecific CTL occurs in two steps. In the first step CTL-PN become activated, and in the second step CTL-PA differentiate to functional effector cells. This analysis was facilitated by (a) the use of the histamine$_1$ antagonist, pyrilamine, or heat-inactivated stimulators to block CTL generation but not suppressor generation, and (b) a system involving transfer of MLC-derived cells into a second MLC, containing Thy-1 congenic responders and homologous stimulators. Pyrilamine-treated or heated stimulator-induced, MLC-derived cells, which contain few or no detectable CTL, were discovered to differentiate into CTL upon transfer to a second, fresh MLC. In addition, CTL generation in the second MLC was suppressed completely when as few as 1 to 2 × 10⁵ cells, containing both suppressor and CTL-PA activities, were transferred per 5 × 10⁶ fresh responders. The presence of either detectable CTL-PA or CTL was not necessary to the occurrence of T$_s$ activity (Table V).

An uninteresting explanation for these data is that the already activated cells derived from the first MLC simply "crowd" the second MLC in some nonspecific manner, not related to suppressor T cells. The result is that the second MLC responding cell population cannot differentiate optimally into CTL. However, in a preliminary manuscript (8) and in the second paper in this series (A. Schwartz, S. L.

TABLE VII

*Requirement for Stimulator Cells to Support Differentiation of CTL-PA to CTL**

Pyrilamine-treated Thy-1.2⁺ cells from first MLC	Second culture stimulators × 10⁻⁵ added to 5 × 10⁶ Thy-1.1⁺ responders	Percent ^{51}Cr release (± SD)	
		Serum treatment before CTL assay	
		NMS + C'	Anti-Thy-1.2 + C'
−	0	−1.2 (2.1)	ND‡
−	5	37.3 (1.2)	41.6 (3.2)
+	0	−0.3 (2.1)	−0.2 (3.6)
+	1.25	11.6 (2.7)	−3.5 (2.8)
+	5	14.8 (1.3)	−0.7 (2.6)
+	20	25.4 (3.8)	−0.6 (2.0)
+	80	34.6 (2.0)	2.6 (1.3)

* B6 (Thy-1.2⁺)-anti-DBA/2 MLC were established in the presence or absence of 10^{-4} M pyrilamine for 5 d. Harvested cells were then washed and 2 × 10⁵ cells were transferred to six fresh second MLC/group, of B6.PLThy-1ᵃ (Thy-1.1⁺) responders and DBA/2 stimulators. After 5 d culture, harvested cells were divided into two equal aliquots, which were treated with either NMS + C' or anti-Thy-1.2 serum + C'. Aliquots were then washed, diluted equally, and tested in quadruplicate for ability to kill 2 × 10⁴ P815 target cells, at a 37:1 effector/target cell ratio.
‡ Not done.

TABLE VIII

*Responder Cell Populations Required for Differentiation of CTL-PA to CTL**

Cells from first MLC		Number of cells transferred	Fresh responder cells in second MLC		Percent ^{51}Cr release (± SD) at culture dilution			
					Treatment of effectors before CTL assay			
					1:3		Undiluted	
Responders	Conditions		Phenotype	Pre-treatment‡	Medium + C'	Anti-Thy-1.2 + C'	Medium + C'	Anti-Thy-1.2 + C'
Experiment 1								
—	—	0	Thy-1.2⁺	None	46.1 (4.6)	4.7 (3.9)	64.8 (7.1)	17.7 (4.2)
Thy-1.1⁺	+ P	5 × 10⁵	Thy-1.2⁺	None	19.2 (3.6)	19.0 (4.9)	39.8 (5.6)	36.9 (2.2)
—	—	0	Thy-1.2⁺	Nylon	−0.4 (1.7)	−0.5 (0.6)	−1.5 (4.1)	1.2 (4.3)
Thy-1.1⁺	+ P	5 × 10⁵	Thy-1.2⁺	Nylon	2.0 (5.2)	4.1 (3.7)	12.4 (4.5)	9.4 (4.8)
—	—	0	Thy-1.2⁺	Glass	10.9 (1.1)	−3.8 (3.6)	27.0 (2.3)	0.2 (4.0)
Thy-1.1⁺	+ P	5 × 10⁵	Thy-1.2⁺	Glass	3.1 (4.1)	3.2 (6.3)	13.8 (1.7)	11.6 (4.3)
Experiment 2								
—	—	0	Thy-1.2⁺	Medium + C'	27.7 (2.1)	−1.2 (2.2)	40.6 (2.0)	0.5 (1.9)
Thy-1.1⁺	+ P	5 × 10⁶	Thy-1.2⁺	Medium + C'	6.7 (2.0)	3.5 (4.2)	13.3 (1.0)	11.1 (5.7)
—	—	0	Thy-1.2⁺	Anti-Thy-1.2 + C'	0.5 (3.6)	0.8 (2.1)	−1.8 (1.3)	4.0 (2.1)
Thy-1.1⁺	+ P	5 × 10⁵	Thy-1.2⁺	Anti-Thy-1.2 + C'	−0.1 (2.9)	−2.8 (3.2)	3.5 (2.3)	4.5 (4.7)

* First and second anti-DBA/2 MLC were established, and transfers and assays were performed as described in the legend of Table II, except that first MLC responder cells were B6.PLThy1ᵃ (Thy-1.1⁺) and second culture responder cells were B6 (Thy-1.2⁺). Target cells were P815.
‡ Second culture responder spleen cells were treated before culture by twice depleting nylon wool adherent cells (23% yield), by twice depleting glass adherent cells (70% yield), or by incubating with either medium or with anti-Thy-1.2 hybridoma reagent + C' (65% yield compared to medium + C' treatment). 5 × 10⁶ of each type of responder cell were added to 5 × 10⁵ stimulator cells.

Sutton, and R. K. Gershon, submitted for publication), we have shown that under the conditions used in the current study (*a*) suppression is completely removed by treatment with anti-Thy-1 antibody plus complement and (*b*) alloantigen specificity can be demonstrated for these T_s (e.g., pyrilamine-treated, H-2ᵇ-anti-H-2ᵈ MLC-derived cells could suppress CTL generation by responding H-2ᵇ cells stimulated by

$H-2^d$ antigens, whereas, simultaneously, they had little or no effect on CTL generation by $H-2^b$ cells stimulated by $H-2^k$ antigens). Therefore, the above trivial explanation for our results is ruled out. This leaves us with the conclusion that the CTL-PN to CTL-PA step is pyrilamine-resistant, can occur when heated stimulator cells are used and is suppressor T cell-sensitive, while the CTL-PA to CTL step is pyrilamine-sensitive, cannot occur when heated stimulator cells are used and is suppressor T cell resistant (see Fig. 1). Our data also show that the differentiation step requires the interaction of CTL-PA with a nonspecific signal from fresh, alloantigen-induced, responding T cells and accessory cells.

Findings not presented here imply that the effects of pyrilamine studied might be primarily due to its local anesthetic capability, and not its histamine$_1$ receptor antagonist function, although the true mechanism of its action remains unknown, and is irrelevant to the message of this paper.

T_h activity is required for optimal induction of CTL (7). The antigens required for stimulation of T_h for CTL responses are sensitive to heat inactivation (14), whereas CTL-P are triggered to become T_h-responsive (14) and T_s are induced (9) under identical conditions. We have found that as in pyrilamine-treated MLC, heated stimulator-induced CTL-PN become CTL-PA, which are T_s-insensitive. By also adding pyrilamine to such cultures, differentiation to CTL subsequent to transfer is prevented (Table V). This implies either that pyrilamine is blocking a different component in CTL-P activation from that prevented by use of heated stimulators, or that the block in CTL-P differentiation is the same, but is made more complete by combined treatment with pyrilamine.

Studies from several laboratories have shown that at least two different T_h interact in the production of an optimal B cell response (1-6). Rohrer et al. (manuscript in preparation), have recently found that two different $Lyt-1^+23^-$ T_h populations are needed for the proliferation and then the secretion of antibody by malignant B cells clones. These T_h are $Qa1^-$ and $Qa1^+$, respectively. In addition, Eichmann et al. (6) have shown, by limiting dilution analysis, that one of two B cell-inducing T_h populations in their system, is present in large numbers, is probably a T_h precursor cell and is sensitive to the effects of suppressor cells. In contrast, a second T_h exists which appears more mature, is present in smaller numbers and is resistant to suppression. Similarly, Green et al. (16), using an intermediate culture system, have subdivided Ly-1 T_h subsets by virtue of their different sensitivities to suppression.

More cogent to our studies are the findings of Goronzy et al. (15), demonstrating
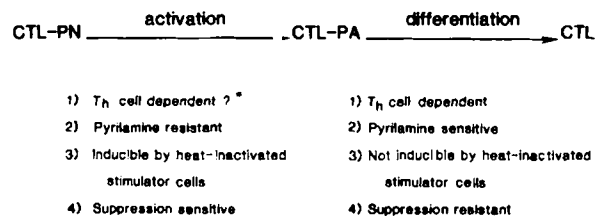

Fig. 1. Two steps in the differentiation of CTL-P to CTL. The apparent inability of CTL-PN to be induced to CTL-PA in the presence of suppressor cells that do not prevent CTL-PA from becoming CTL effector cells could be due either to qualitative or quantitative differences in their sensitivity to suppression. (*) In preliminary experiments the CTL-PN to CTL-PA step was found to depend on the presence of $Lyt-1^+$ cells for optimal induction (data not presented).

that in polyclonally activated T cell populations, two different CTL-P are present, one of which is frequent, sensitive to suppression, and probably less mature (Lyt-1^+23^+), and a second, which is rare, resistant to suppression, and phenotypically more mature (Lyt-1^-23^+). These and other findings might be interpreted to indicate that their two populations might be primary (naïve) and secondary (activated or "memory") precursors respectively. Because the CTL-PN and CTL-PA activities we have described here have differential functional characteristics similar to these frequent and rare CTL-P, respectively, is it possible that we have demonstrated an arrest in CTL production that allows memory to be activated in the absence of prior demonstrable effector function?

That the CTL-PN to CTL-PA step is alloantigen specific, whereas CTL differentiation is not, is inferred from experiments showing that the culture into which the CTL-PA are transferred does not have to contain stimulators bearing the same alloantigen as in the first MLC, although first culture antigen specificity is maintained (Table VI). The second culture appears to provide a non-antigen-specific lymphokine, such as interleukin 2, which is responsible for CTL-PA to CTL differentiation. The latter lymphokine is produced in the presence of adherent cells (17) by antigen-activated or polyclonally activated Lyt-1^+23^- cells and can cause only previously antigen-stimulated or polyclonally activated CTL-P (18–20) or Lyt-1^+23^+ thymocyte CTL-P (21) to differentiate to CTL. In accord with this possibility, we have found that pyrilamine-treated CTL-PA fail to become effectors until 2–3 d after transfer to the second MLC (data not presented), and that fresh T cells, adherent cells, and antigen are required for optimal differentiation of CTL-PA to CTL (present study).

One explanation for our data is that a different T_h is required for activation of CTL-PN to CTL-PA than that required for the CTL-PA to CTL step and that the dissimilar pyrilamine, heated stimulator, and suppressor T cell sensitivities of the two T_h are actually what are being measured. The CTL-PA to CTL step is clearly T_h dependent. The CTL-PN to CTL-PA step is currently under investigation. In preliminary studies (data not presented) we have found that the CTL-PN to CTL-PA step may require the presence of Lyt-1^+ cells for optimal induction. This being the case, another analogy exists between CTL and B cells, in that Keller and colleagues (22) have described two T_h for antibody formation, the second and late acting of which is antigen specific in its ability to be triggered but non-antigen specific in its delivery of a helper signal.

The data presented here provide additional evidence that the cellular interactions involved in regulation of CTL generation and of B cell function are similar, and that the steps in the development of effector cells in these two major arms of the immune system utilize conserved activating and differentiative events. Many of the unanswered questions raised here should be approachable by using the described methodology as an investigative tool.

Summary

The differentiation of cytotoxic T lymphocyte precursor cells (CTL-P) into CTL effector cells is a two-step process. In the first step, naïve CTL-P (CTL-PN) become activated (CTL-PA) but do not yet have the capacity to kill target cells. CTL-PA can be distinguished from CTL-PN because the former are far less sensitive than the latter to the effects of in vitro-generated suppressor cells. Thus, the addition of suppressor T

cells (T_s) to a fresh MLC can totally inhibit the production of CTL from CTL-PN, whereas the same T_s only minimally affect the generation of CTL from CTL-PA. It is not known whether these T_s act directly on CTL-PN or on a helper cell needed for activation to CTL-PA. The production of CTL-PA can take place in allogeneic mixed leukocyte cultures (MLC) treated with the drug pyrilamine, or when heat-inactivated stimulator cells are used. Each of these treatments inhibits the differentiation of CTL-PA to CTL. However, if pyrilamine is removed, a nonspecific MLC-derived signal can induce these CTL-PA to become CTL, even in the presence of significant numbers of T_s. This two step process of differentiation of CTL-P to CTL may be analogous to the way naïve B cells become antibody-producing cells.

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