GENERATION OF CYTOTOXIC T LYMPHOCYTES IN VITRO

II. Effect of Repeated Exposure to Alloantigens on the Cytotoxic Activity of Long-Term Mixed Leukocyte Cultures*

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In recent years, many studies have dealt with the generation of cytotoxic thymus-derived lymphocytes (CTL)[1] in response to an allogeneic stimulus either in vivo or in vitro (see reference 1 for review). However, very few attempts have been made to determine the ultimate fate of these effector cells. Previous studies of the physical characteristics of splenic CTL in mice undergoing tumor allograft rejection suggested that a transition from large, low density CTL to small dense CTL may occur with time (2, 3). In other experiments, an anamnestic CTL response was suggested by the demonstration of earlier appearance and higher peak levels of CTL activity in the spleens of mice injected twice with allogeneic tumor cells (4). Furthermore, in the preceding paper (5), we showed that the cell-mediated cytotoxic response in mixed leukocyte cultures (MLC) was increased when alloimmune spleen cells were used as responding cells.

Each of these findings is consistent with the possibility that CTL may differentiate into putative "memory" cells capable of mediating a specific secondary response to alloantigens. However, since the initial interaction with alloantigen in each of these model systems takes place in vivo, where cellular migration may account for the presence or absence of CTL in any tissue at any given time, it is extremely difficult to conclude that any such lymphocyte differentiation pathway exists. To critically evaluate this hypothesis then it is necessary to generate putative "memory" CT under defined in vitro conditions.

At the present time, the primary generation of CTL in vitro, and particularly in MLC, is well documented (6, 7), but culture conditions have not in general been adequate to support long-term survival of CTL. In this report, we will describe how an improvement in tissue culture conditions (notably the inclusion of 2-mercaptoethanol (2-ME) in the culture medium) has allowed us to

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[1] *Abbreviations used in this paper:* CTL, cytotoxic T lymphocytes; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LU, lytic units; MLC, mixed leukocyte culture; 2-ME, 2-mercaptoethanol.

investigate CTL in MLC on a longitudinal basis over extended periods of time. Furthermore, we will show that reexposure of long-term MLC cells to the original stimulating alloantigens results in a rapid and immunologically specific reappearance of high levels of CTL activity. Evidence is presented that this ability to respond to a second allogeneic challenge is a property which is acquired during the course of the MLC reaction and is absent from control unstimulated cultured cells. Finally, the reappearance of CTL activity following each of as many as four sequential alloantigenic stimulations of the same initial cell population over a period of 64 days will be documented, and the relevance of these findings to memory in T-cell-mediated immunity will be discussed.

Materials and Methods

Mice.—Adult female mice of the inbred strains C57BL/6 and DBA/2 were supplied by the animal colony maintained at the Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland. The breeding pairs for this colony were originally obtained from the Jackson Laboratories, Bar Harbor, Maine.

Antisera.—Antiserum to theta alloantigen of C3H mice (anti-θ) was raised and tested as described previously (8); it had a cytotoxic titer of 1:2000 when measured on C3H thymocytes.

Target Cells.—P-815-X2 mastocytoma cells were maintained in vitro and also in ascitic form in DBA/2 mice (9). Cultured cells to be used as target cells in the cytotoxic assay were labeled with ^{51}Cr as described previously (5), and suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS, Flow Laboratories, Inc., Rockville, Md.), and 10 mM *N*-Hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (Hepes) pH 7.2.

Mixed Leukocyte Cultures.—Spleen cell suspensions were prepared as described previously (10), and finally suspended in an enriched DMEM (5) supplemented with 5% FBS and 5×10^{-5} M 2-ME (MLC medium). Primary MLC were established by mixing either 5×10^6 or 25×10^6 viable C57BL/6 responding spleen cells with an equal number of irradiated (1,000 rads) DBA/2 stimulating spleen cells (5). Secondary cultures were established by mixing 0.4×10^6 viable MLC cells with an equal number of irradiated (1,000 rads) DBA/2 normal spleen cells in a final vol of 0.8 ml of MLC medium in 12 × 75 mm round-bottomed tubes (no. 2054, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). These cultures were incubated upright at 37°C in a humidified atmosphere of 5% CO_2 in air for varying periods of time as described in the text.

Cytotoxic Assay.—For the cytotoxic assay, various numbers of viable MLC cells were incubated with 10×10^3 ^{51}Cr-labeled target cells in 0.4 ml DMEM-5% FBS-10 mM Hepes for 3 h at 37°C, in a shaking water bath (5). To terminate the assay, 0.6 ml cold phosphate-buffered saline was added to each tube. After centrifugation at 500 *g* for 5 min, 0.5 ml of the supernatant fluid was removed for counting in a well type scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.). Specific cytotoxicity and lytic units (LU) were calculated and normalized according to the method described in the preceding paper (5).

RESULTS

Presence of CTL in Long-Term Primary MLC.—In the course of studies investigating the generation of CTL in MLC, we found that the inclusion of appropriate concentrations of 2-ME in the culture medium resulted in both higher cell recovery and greater per-cell cytotoxic activity at the peak of the response on day 5 (5). In view of this finding, it was of interest to investigate the survival

of CTL over extended culture periods in the presence of 2-ME. For these experiments, a series of MLC cultures containing equal numbers of C57BL/6 spleen cells and irradiated (1,000 rads) DBA/2 spleen cells were established in tissue culture flasks. At various times thereafter, cultures were harvested and the number of viable nucleated cells determined by trypan blue exclusion. Percent specific ^{51}Cr release was then determined at various ratios of viable nucleated cells to target cells in a standard 3-h assay using 10×10^3 ^{51}Cr-labeled P-815 (DBA/2) target cells. The results of these determinations are shown in Table I.

TABLE I

*Survival and Regeneration of Cytotoxic T Lymphocytes in MLC**

Day of culture	Recovery of viable cells (% input)	Specific ^{51}Cr release						LU/10^6 cells§	LU/culture
		0.2:1‡	0.6:1	2:1	6:1	20:1	60:1		
		%	%	%	%	%	%		
4	44	7	26	57	88	97	100	67	147
6	41	6	19	42	88	99	100	51	105
8	38	6	14	34	69	95	100	32	61
12	29	1	6	13	36	79	100	11	16
15	19	1	3	9	27	70	95	8.3	7.9
19	10	0	0	6	13	29	70	3.0	1.5
19‖	10	—	1	8	18	43	73	4.0	2.0
19¶	18	—	31	58	81	98	100	71	64

* 5×10^6 C57BL/6 spleen cells were cultured with an equal number of irradiated DBA/2 spleen cells. At the indicated times, cytotoxicity was assayed on ^{51}Cr-labeled P-815 (DBA/2) target cells.

‡ Lymphocyte:target cell ratio.

§ LU calculated from dose-response curves for individual populations.

‖ 2×10^6 irradiated syngeneic (C57BL/6) spleen cells added on day 15.

¶ 2×10^6 irradiated allogeneic (DBA/2) spleen cells added on day 15.

To facilitate quantitative comparison, these results are also expressed in terms of LU. As discussed previously (1, 5), LU/10^6 viable cells can be regarded as a relative measure of the CTL frequency in any given cell population. In the time-course of the MLC depicted in Table I, LU/10^6 cells reached a maximum of 67 on day 4 and declined progressively to a value of 3 on day 19, the last day assayed. Since the number of viable cells per culture (expressed as a percentage of the input) decreased from a maximum of 44% on day 4 to 10% on day 19, the number of LU/culture declined from 147 at the peak to 1.5 on day 19. To test whether the cytotoxic cells in long-term MLC were T cells, as is the case for the effector cells obtained at the peak of the response (2, 11–12), day 14 MLC cells were incubated with anti-θ serum and complement as described previously (8). It was found that this treatment completely abolished the cytotoxic activity of the cells (data not shown), suggesting that surviving cytotoxic cells were thymus-derived.

Cytotoxic Activity of MLC Cells Reexposed to Alloantigens.—Since CTL could be detected for as long as 3 wk under our modified culture conditions, it was feasible to investigate the effect of readdition of irradiated stimulating spleen cells on the cytotoxic activity of MLC populations. In a preliminary experiment (Table I), it was found that direct addition of irradiated stimulating (DBA/2) spleen cells to MLC on day 15 (a time at which LU/culture were 19-fold reduced from the maximum) resulted in a dramatic increase in cytotoxic activity when measured 4 days later. In particular, LU/10^6 cells reached a value of 71 (compared to 67 at the peak of the response on day 4), and LU/culture attained almost 50% of the earlier maximum value. In control cultures where irradiated syngeneic (C57BL/6) spleen cells were added, no significant increase in cytotoxicity was observed (Table I).

Subsequent experiments established that a greater increase in cytotoxic activity could be achieved when 0.4×10^6 MLC cells were incubated with an equal number of irradiated allogeneic spleen cells in small plastic tubes. Using this latter protocol, the effect of reexposure to alloantigens on MLC cells harvested at various times during the MLC reaction was investigated. For this purpose, MLC cultures were harvested after 4, 14, 20, 28, or 41 days. The surviving cells were enumerated and tested immediately for their cytotoxic activity, and 0.4×10^6 cells from each group were also incubated for a further 4 days with an equal number of irradiated DBA/2 spleen cells. Cytotoxic activity was again assessed following the secondary culture to determine the effect of the antigenic reexposure. As shown in Table II, reexposure of day 4 MLC cells to relevant alloantigens had no significant effect on viable cell number or cytotoxic activity measured 4 days later. Beginning at day 14, however, all populations tested increased dramatically in cell number and cytotoxic activity when exposed to irradiated allogeneic cells. LU/10^6 cells were comparable in all restimulated cultures; on a per culture basis, however, it appeared that day 14

TABLE II

*Cytotoxic Activity of MLC Cells Reexposed to Alloantigens**

Day of reexposure	Recovery of viable cells (% input)		LU/10^6 cells§	
	Before restimulation	After restimulation‡	Before restimulation	After restimulation
4	45	28	33	25
14	12	30	3.0	100
20	8	22	2.2	120
28	3	5	0.5	118
41	1	2	<0.2	53

* 25×10^6 C57BL/6 spleen cells cultured with an equal number of irradiated DBA/2 spleen cells until day indicated.

‡ 4 days after culturing 0.4×10^6 surviving MLC cells with an equal number of irradiated DBA/2 spleen cells.

§ Cytotoxicity was assayed on ^{51}Cr-labeled P-815 (DBA/2) target cells, and LU were calculated from the dose-response curves for individual populations.

cells were more efficiently stimulated than those harvested at later times. Even day 41 MLC cells (in which no residual cytotoxicity was detectable) increased twofold in viable cell number upon stimulation, and reached levels of LU/10^6 cells only twofold lower than in earlier cultures. The T-cell nature of those cytotoxic cells formed after reexposure of day 14 and 20 MLC cells to alloantigens was demonstrated by further experiments showing that treatment with anti-θ serum and complement abolished their cytotoxic activity (data not shown). Hence it would appear from these findings that the ability to regenerate increased CTL activity upon reexposure to the original alloantigenic stimulus is a property of a MLC cell population which takes longer than 4 days to appear and can be relatively long-lived, at least in vitro. In addition, the effector cells generated in secondary cultures were found to be as specific as CTL generated in vivo and in primary MLC (for references, see 1) as demonstrated by their lack of cytotoxic activity on syngeneic target cells.

Kinetics of Regeneration of CTL Activity.—The demonstration of regeneration of functional CTL in long-term MLC prompted the investigation of the kinetics of this phenomenon. To this end, primary MLC were assayed for LU/10^6 cells and viable cell recovery on day 5 (the peak of the response), and subsequently left until day 14. At that time, aliquots of 0.4×10^6 MLC cells were cultured with equal numbers of irradiated DBA/2 spleen cells or with irradiated C57BL/6 spleen cells as a control. After varying periods of time, cultures were harvested and quantitatively assayed for cytotoxicity on P-815 (DBA/2) target cells. As can be seen in Fig. 1, greatly enhanced cytotoxicity was already demonstrable 24 h after specific restimulation (i.e., day 15). LU/10^6 cells remained essentially constant from day 15 until day 18, but declined significantly by the 20th day. Viable cell counts for the same cultures revealed no increase in cell number during the first 24 h, but a constant increase from that time until day 18, after which it declined. In control cultures incubated with irradiated C57BL/6 spleen cells, a progressive decrease in both cell number and cytotoxic activity was observed throughout the time-course of the experiment. A summary of these data, in which cytotoxic activity is expressed as LU per initial primary culture of 5×10^6 cells, and viable cell recovery is normalized accordingly, is shown in Fig. 2. The 10-fold increase in LU/culture with no accompanying increase in viable cell number during the first 24 h after reexposure to alloantigens is very striking. Subsequently, the parallel increase in LU and viable cell number suggests that no selective enrichment or depletion of CTL is taking place. It is noteworthy that, in this experiment, absolute cell number increased sevenfold and LU/culture reached a level threefold higher than was observed at the peak of the primary response on day 5.

Survival of Unstimulated CTL Progenitors in Long-Term Culture.—In order to rule out the possibility that the response of day 14 MLC cells to a second stimulation with the same alloantigens could be accounted for simply by the survival of unstimulated CTL progenitors, C57BL/6 spleen cells were cultured with

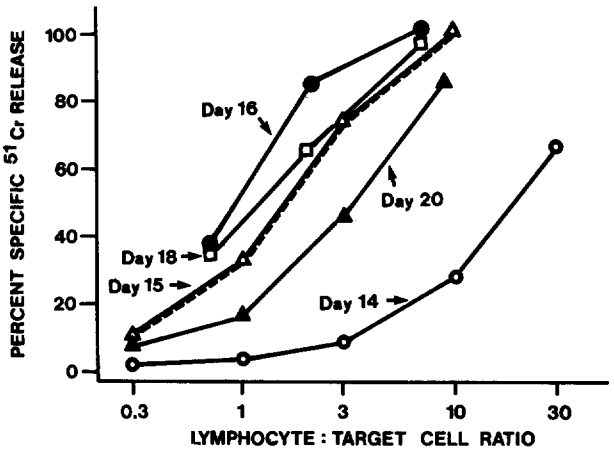

FIG. 1. Cytotoxic activity of MLC cells following reexposure to alloantigens. Equal numbers of C57BL/6 spleen cells and irradiated DBA/2 spleen cells were cultured for 14 days. At that time, the cultures were harvested, and multiple cultures of 0.4×10^6 viable recovered cells and 0.4×10^6 irradiated DBA/2 spleen cells were established. Cytotoxicity was assayed on ^{51}Cr-labeled P-815 (DBA/2) target cells on day 14 (○—○), day 15 (△—△), day 16 (●—●), day 18 (□—□), and day 20 (▲—▲). For comparison, the cytotoxic activity of the same MLC population on day 5 is indicated (----).

irradiated spleen cells either syngeneic (C57BL/6) or allogeneic (DBA/2) to the responding cells for 14 days. These cultures were then harvested and 0.4×10^6 viable cells of each were established in secondary cultures with equal numbers of irradiated DBA/2 spleen cells for an additional 4 days. The cytotoxic activity of these latter (day 18) cultures was determined, and the LU/10^6 cells and LU/culture were calculated. These results are presented in Table III. It can be seen that day 14 control syngeneic C57BL/6 cultures were still able to respond to DBA/2 alloantigens as measured by the generation of CTL, but only to about 1% the extent of the allogeneic cultures (in terms of LU produced per 5×10^6 C57BL/6 cells initially cultured). Hence, it appears that the vast majority of the CTL detected in secondary cultures are derived from cells which have previously interacted with alloantigens.

Fate of CTL Upon Repeated Antigenic Stimulation.—Since CTL could be rapidly regenerated following a secondary exposure to alloantigen in long-term MLC, we investigated the more general question of their potential to respond to repeated antigenic stimulation in vitro. To this end, primary (1°) MLC cultures of C57BL/6 spleen cells and irradiated DBA/2 spleen cells, established on day 0, were assessed for viable cell number and LU/10^6 cells on day 4 and subsequently left until day 20. At that time, secondary (2°) cultures were established by incubating 0.4×10^6 viable recovered cells with the same number of irradiated stimulating (DBA/2) spleen cells. For this and all subsequent restimula-

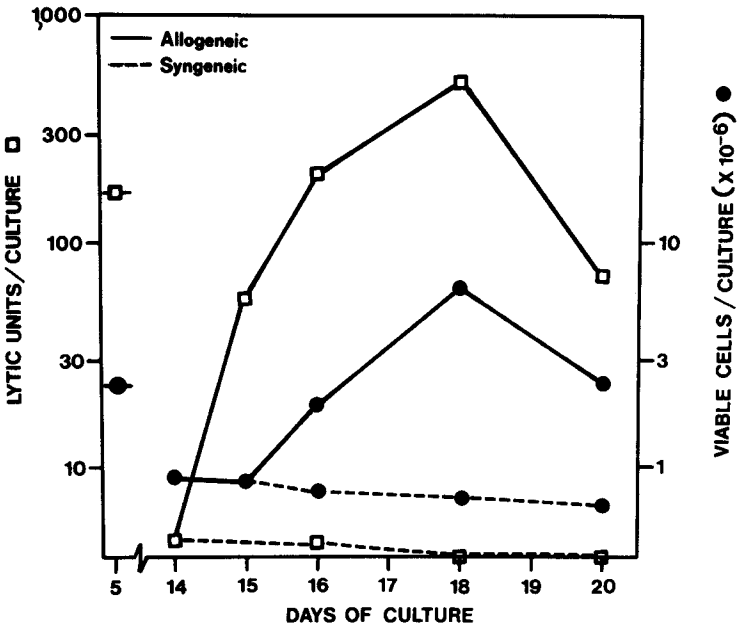

FIG. 2. Kinetics of regeneration of CTL activity. Equal numbers of C57BL/6 spleen cells and irradiated DBA/2 spleen cells were cultured for 14 days. These cultures were harvested, and secondary cultures were established with 0.4×10^6 viable recovered cells and equal numbers of either irradiated allogeneic (——) or irradiated syngeneic (----) spleen cells. On the days indicated, viable cells were enumerated (●), and cytotoxicity was assayed on ^{51}Cr-labeled P-815 (DBA/2) target cells. LU/5 $\times 10^6$ cells originally cultured (□) were calculated from dose-response curves as described in the text.

tions, irradiated syngeneic (C67BL/6) spleen cells were added to parallel cultures as controls. Cytotoxic activity and cell numbers were again determined 4 days later (day 24) and the restimulated secondary cultures were then left for a further 16 days. At day 40, a tertiary (3°) MLC was set up and tested in the same manner, and at day 60, the surviving cells from this MLC were tested for a fourth time (4°). The results of this experiment are summarized in Fig. 3 and Table IV.

In Fig. 3, the relative cytotoxicity of the cells recovered from the four sequential antigenic stimulations of the same initial cell population is compared at several lymphocyte:target cell ratios. As was found previously (Fig. 1) 2° MLC cells were slightly more cytotoxic on a per-cell basis than those found at the peak of the 1° response. Interestingly, viable cells recovered from 3° and 4° MLC were more cytotoxic than those observed after 2° MLC. No increase in cytotoxic activity was observed in control cultures incubated with irradiated C57BL/6 spleen cells (data not shown).

In order to more quantitatively assess CTL generation in MLC under these conditions, the number of LU present in individual cultures before and after

TABLE III

*Survival of CTL Progenitors in Syngeneic and Allogeneic Leukocyte Cultures**

Primary culture	Recovery of viable cells (% input)		LU/culture‡ (LU/10^6 cells)	
	Day 14	Day 18	Day 14	Day 18
Syngeneic	6	4	0 (0)	2.2 (10)
Allogeneic	11	30	2.8 (5)	164 (109)

* 5×10^6 C57BL/6 spleen cells were cultured with an equal number of irradiated syngeneic (C57BL/6) or allogeneic (DBA/2) spleen cells for 14 days. At that time, viable cell recovery was determined and cytotoxicity was assayed on ^{51}Cr-labeled P-815 (DBA/2) target cells. In addition, 0.4×10^6 viable cells from each group were cultured with an equal number of irradiated DBA/2 spleen cells for a further 4 days (i.e., until day 18), and cytotoxicity was similarly determined.

‡ LU calculated from the dose-response curves for individual populations.

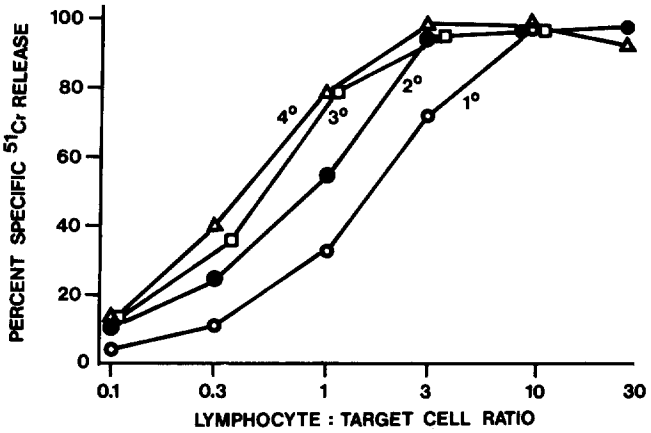

FIG. 3. Cytotoxic activity of MLC populations following repeated alloantigenic stimulation. 1° MLC were established by culturing equal numbers of normal C57BL/6 spleen cells and irradiated DBA/2 spleen cells. On day 20, 0.4×10^6 surviving cells were mixed with an equal number of irradiated DBA/2 spleen cells to establish 2° MLC. These cultures were left until day 40, when 3° MLC were established in the same fashion. On day 60, 4° MLC were set up with 0.4×10^6 surviving cells from 3° MLC. Cytoxicity was assayed on ^{51}Cr-labeled P-815 (DBA/2) target cells on the fourth day after each stimulation. 1° (○—○); 2° (●—●); 3° (□—□); 4° (△—△).

each antigenic stimulation was calculated (Table IV). For the purpose of this comparison, viable cell recoveries and LU are all expressed relative to an initial culture of 5×10^6 responding spleen cells. It can be seen that, beginning with the 2° culture, the number of viable cells recovered at 20-day intervals in the restimulated cultures decreased only slightly throughout the remainder of the experiment. Combined with the increasing values of LU/10^6 cells following each stimulation, this leads to comparable values of LU/culture (132–200) for the

TABLE IV

Quantitation of CTL Following Repeated Antigenic Stimulation in MLC

Designation of MLC	Day of stimulation*	Recovery of viable cells (% input)		LU/culture§ (LU/10^6 cells)	
		Before stimuation	After stimulation‡	Before stimulation	After stimulation‡
1°	0	—	42	—	137 (65)
2°	20	8	22	1.6 (4)	132 (120)
3°	40	5.4	20	1.4 (5)	190 (190)
4°	60	3.6	16	<0.2 (<1)	200 (250)

* Sequential stimulations of the same cell population as described in the text.

‡ 4 days after incubation of 5×10^6 normal (1°) or 0.4×10^6 MLC-derived (2°, 3°, 4°) C57BL/6 spleen cells with an equal number of irradiated DBA/2 spleen cells.

§ Cytotoxicity was assayed on ^{51}Cr-labeled P-815 (DBA/2) target cells, and LU were calculated from dose-response curves of individual cell populations.

four successive stimulations. In other words, the cytotoxic activity of the responding cells was quantitatively conserved.

DISCUSSION

The experiments described in this report provide evidence that, under appropriate culture conditions, functional CTL can be initiated and maintained for relatively long periods of time in MLC. Reexposure of surviving MLC cells to the original stimulating alloantigens after 14–41 days of culture results in significant cell proliferation and rapid regeneration of immunologically specific CTL. Furthermore, such proliferation and augmentation of CTL activity can be demonstrated following each of four successive stimulations of the same original cell population at 20-day intervals. By quantitative analysis, it could be shown that the maximum relative number of CTL, determined as LU/culture, was approximately the same following each stimulation. Clearly, these data raise interesting questions regarding the existence of memory in allograft responses and concerning the fate and longevity of the effector cells of cell-mediated immunity.

In general, attempts to demonstrate anamnestic responses in allograft immunity have provided equivocal results. Studies comparing the reactivity of normal and alloimmune lymphoid cells, as assessed by a graft-vs. host assay in vivo (13) or by a proliferative assay in vitro (14) were unable to detect significant differences between the two cell populations. On the other hand, skin grafts appear to be rejected more rapidly by specifically alloimmune hosts (15), and a more rapid and quantitatively greater generation of specific CTL has been observed in the spleens of mice twice immunized with allogeneic tumor cells (4). More recently, using improved culture conditions identical to those described in this report, we have found that the generation of CTL in MLC may be increased approximately fivefold when the responding spleen cells are taken

from alloimmune mice (5). However, since the primary response to alloantigens appears to involve a relatively large number of cells (13, 14), it is difficult in each of these model systems to compare the secondary response in the absence of a strong concomitant primary response.

The generation of high levels of specific CTL upon reexposure to alloantigens in a completely in vitro system, as described in this report, may provide a better basis for the study of allograft memory. In this regard, several observations suggest that a true secondary response is being measured. Restimulated cultures attain a slightly greater relative (per cell) and absolute (per culture) activity compared to the peak of the primary response. Furthermore, the rapid activation of CTL to an optimally cytotoxic state within 24 h of reexposure to the stimulating antigen in the absence of a corresponding increase in cell numbers is preliminary evidence for a qualitatively different phenomenon than is observed following primary antigenic challenge. An accelerated proliferative response following reexposure of long-term MLC cells to alloantigens has also been observed (16).

In addition, since the capacity of the surviving cells in unstimulated spleen cell cultures to generate CTL upon exposure to alloantigens is almost 100-fold reduced as compared to those in stimulated MLC cultures, it seems likely that the vast majority of CTL activity in the in vitro secondary response is derived from cells which have already interacted with antigen. This contrasts with the in vivo situation in which any strong selection in favor of specifically alloantigen-sensitized cells has proved extremely difficult to demonstrate.

The ability to detect CTL activity after long periods of time in culture as described here and elsewhere (16) implies that CTL can be relatively long-lived in vitro. 41-day old MLC cultures in which CTL were no longer detectable were found to give rise to almost optimal levels of CTL activity following restimulation, suggesting that some cells with specific reactivity were still present at that time. Furthermore, repeated stimulation experiments suggested that optimal CTL activity could be retained during four successive stimulations over a period of more than 60 days in vitro. Because individual CTL cannot as yet be detected and isolated, it cannot necessarily be concluded that the same cell lineage responded to the multiple stimulations in these tests. However, since each exposure to alloantigen induced extensive cell proliferation as well as an increase in CTL activity, it seems unlikely that CTL are "end cells", incapable of further antigen-induced reactivity. Cell separation experiments to be described in a subsequent report[2] support the hypothesis that CTL progenitors present in long-term MLC are indeed the progeny of CTL generated during the primary in vitro response.

An interesting finding from these experiments was the inability of day 4 MLC cells to respond to an allogeneic stimulus by further proliferation or increased

[2] MacDonald, H. R., J.-C. Cerottini, and K. T. Brunner. Manuscript in preparation.

CTL activity. Since the relative CTL frequency and absolute cell number in restimulated day 14 cultures greatly surpassed those of restimulated day 4 cultures under comparable conditions, it is unlikely that day 4 cells failed to respond because of limiting tissue culture conditions; alternatively, it may be that the ability to respond to alloantigens is a property which is dependent on the state of differentiation of CTL. If so, the inability to induce further proliferative (graft-vs.-host or MLC) responses in MLC cells harvested at the peak of the response (17) or in thoracic duct cells activated in vivo to histocompatibility antigens (18) cannot be taken as evidence that CTL are end cells. Further experiments in which alloantigen-activated cells are tested at later times are needed to clarify this point.

The capacity of the surviving cells in primary MLC to regain full functional CTL activity after a 24-h exposure to cell-bound alloantigen may be useful in studies of lymphocyte activation. To date, such studies have concentrated on mitogen-stimulated cells in order to obtain sufficiently high transformation frequencies for biochemical analysis. In our studies, the rapid appearance of functional cytotoxicity within 24 h is accompanied by the morphological transformation of a large proportion of the small lymphocyte population.[3] Although it is at present impossible to estimate what proportion of these transformed cells are functional CTL, it is nevertheless clear that antigen-specific activation of a large proportion of the cells takes place.

Finally, the ability to generate defined T-lymphocyte subpopulations which exhibit restricted antigenic specificity may be useful in studies of the potential interrelationship between various T-cell functions. To this end, studies are presently in progress to investigate the ability of such subpopulations to participate in cell-mediated and humoral immune reactions in vivo.

SUMMARY

Mouse cytotoxic T lymphocytes (CTL) were generated in unidirectional mixed leukocyte cultures (MLC) using normal C57BL/6 spleen cells as responding cells and irradiated DBA/2 spleen cells as stimulating cells. Cytotoxicity was assayed on ^{51}Cr-labeled P-815 (DBA/2) target cells, and the relative frequency of CTL in individual cell populations was estimated from dose-response curves. Upon inclusion of 2-mercaptoethanol in the culture medium, it was found that significant CTL activity could be detected for as long as 3 wk in primary MLC. Reexposure of MLC cells to the original stimulating alloantigens after 14–41 days in culture resulted in significant cell proliferation and rapid regeneration of high levels of immunologically specific cytotoxicity. CTL activity in these secondary cultures increased dramatically within the first 24 h and reached higher peak levels than those found at the peak of the primary response. Furthermore, proliferation and reappearance of CTL activity could be

[3] MacDonald, H. R., and B. Sordat. Unpublished observation.

demonstrated following each of as many as four sequential alloantigenic stimulations of the same initial cell population at 20-day intervals. Interestingly, cells recovered from MLC at the peak of the primary response on day 4 were insensitive to further allogeneic stimulation. Taken together, these results are consistent with the hypothesis that CTL differentiate in MLC to become long-lived memory cells which gradually lose their cytotoxic activity. Upon reexposure to specific alloantigen, such memory CTL rapidly regain their functional activity and proliferate to generate an expanded CTL population.

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