

GENERATION OF CYTOTOXIC T LYMPHOCYTES IN VITRO

III. Velocity Sedimentation Studies of the Differentiation and Fate of Effector Cells in Long-Term Mixed Leukocyte Cultures*

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The cell-mediated immune response to primary allogeneic stimulation is characterized by the appearance of cytotoxic T lymphocytes (CTL)¹ (see reference 1 for review). Recently, the existence of anamnestic CTL responses to allografts has been suggested by studies in which the ability of normal and alloimmune spleen cells to respond to relevant alloantigens both in vivo (2, 3) and in vitro (4) was quantitatively compared. In other studies, physical characterization of CTL in the spleens of mice undergoing tumor allograft rejection revealed significant differences in size and density between effector cells detected early or late after immunization (5–9), the former being predominantly large and of low density, whereas the latter were mainly small and dense. In view of these observations, two important questions regarding the differentiation and ultimate fate of CTL can be raised: (a) Do the changes in physical properties of CTL with time reflect the differentiation of a single cell lineage, or alternatively the parallel development of several cell lineages? (b) Are the cells capable of mounting an anamnestic response to alloantigens derived from CTL, or are CTL truly end cells capable of no further differentiation?

Until individual CTL can be identified and isolated, no definitive answer to these questions is possible. However, with the concomitant development of in vitro systems for the generation of CTL (10–12) and quantitative assays for the estimation of their relative frequency (1, 12), indirect approaches to the study of the differentiation and fate of CTL are now feasible. In a previous paper of this series (13), we presented evidence that an anamnestic response to alloantigens could be demonstrated in long-term mixed leukocyte cultures (MLC). In this

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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; s, sedimentation velocity.

report, physical separation of various MLC populations on the basis of their sedimentation velocity at 1 *g*, a parameter that depends primarily on cell size (14, 15), is utilized to characterize the lineage of CTL in MLC and, at the same time, to investigate the origin of those cells responding to secondary allogeneic stimulation. The results of these studies strongly support the hypothesis that large, highly cytotoxic CTL differentiate in MLC into small T lymphocytes which gradually lose their cytotoxic activity. Moreover, it would seem most likely that these small lymphocytes function as long-lived "memory" cells, rapidly regaining their functional activity and proliferating upon re-exposure to specific alloantigens.

Materials and Methods

Mice. Adult female mice of the inbred strains C57BL/6 and DBA/2 were used in all experiments. They were supplied by the animal colony at the Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland. Breeding pairs were originally obtained from the Jackson Laboratories, Bar Harbor, Maine.

Mixed Leukocyte Cultures. Spleen cell suspensions were prepared as described previously (16), and finally suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Flow Laboratories Ltd.), 5×10^{-5} M 2-mercaptoethanol, and additional amino acids (4). Primary mixed leukocyte cultures were established by mixing 25×10^6 viable (i.e., trypan blue excluding) C57BL/6 spleen cells with an equal number of irradiated (1,000 rads) DBA/2 spleen cells in 20 ml of the above medium in 30-ml tissue culture flasks (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). The flasks were incubated upright at 37°C in a humidified atmosphere of 5% CO₂ in air for various periods of time as indicated. Secondary cultures were made by mixing 0.4×10^6 viable MLC cells with an equal number of unprimed irradiated (1,000 rads) DBA/2 spleen cells in plastic test tubes as described previously (13).

Cytotoxic Assay. P-815-X2 mastocytoma cells (which are syngeneic to DBA/2 mice) were maintained in culture and used as target cells in all experiments. Before the cytotoxic test, they were labeled with ⁵¹Cr as described previously (4), washed three times, and resuspended in DMEM supplemented with 5% FBS and 10 mM *N*-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (Hepes), pH 7.2.

For the cytotoxic assay, various numbers of viable MLC cells were then incubated with 10×10^3 ⁵¹Cr-labeled P-815 (DBA/2) target cells in 0.4 ml of DMEM supplemented with 5% FBS and 10 mM Hepes for 3 h at 37°C in a shaking water bath (4). At the end of this incubation, 0.6 ml of cold phosphate-buffered saline was added to each tube. The tubes were then centrifuged at 500 *g* for 5 min and 0.5 ml of the supernate was decanted and counted in a well-type scintillation counter (Nuclear-Chicago Inc., Des Plaines, Ill.). Specific cytotoxicity was calculated and normalized as described previously (4).

Velocity Sedimentation Cell Separation at 1 *g*. The technique has been described previously (14). In this instance, a glass sedimentation chamber of 11 cm diameter was employed (Glasapparatebau, Weil, Germany). Cells to be separated were harvested from MLC, washed once in DMEM and resuspended in DMEM supplemented with 3% (vol/vol) calf serum at a concentration of $0.5-2 \times 10^6$ cells/ml. 30 ml of this suspension were then applied to a buffered step gradient consisting of 7%-30% calf serum in DMEM, and allowed to sediment for 4 h at 4°C. Fractions of 15 ml were collected, concentrated to 1 ml by centrifugation, and either tested for cytotoxicity or recultured as indicated in the text. Sedimentation velocity (*s*) is arbitrarily expressed in mm/h as described previously (14).

Quantitation of CTL Activity. Activity of CTL is expressed in terms of lytic units (LU) according to the definition described previously (4). Briefly, 1 LU is defined as the absolute number of cells required to lyse 50% of 10×10^3 ⁵¹Cr-labeled P-815 target cells in 3 h under the conditions of the standard cytotoxic assay. In this context, LU per 10^6 cells and LU per culture can be regarded as relative measures of the frequency and absolute number of CTL in various cell populations, respectively.

Results

Characterization of CTL in MLC by Velocity Sedimentation. During the course of tumor allograft rejection in mice, the distribution in sedimentation velocity of splenic CTL changes with time, suggesting that a transition from large to small CTL may occur (7–9). Since CTL could be detected for several weeks in MLC under appropriate culture conditions (13), it was of interest to investigate the physical properties of CTL induced in vitro. For this purpose, multiple cultures of 25×10^6 C57BL/6 spleen cells and 25×10^6 irradiated DBA/2 spleen cells were established. On day 4 and again on day 14, approximately 50×10^6 cells were recovered, washed, and separated by velocity sedimentation at 1 g (14). Fractions were collected and assessed for viability, and the specific cytotoxicity of each fraction was assayed on ^{51}Cr -labeled P-815 (DBA/2) target cells at several lymphocyte:target cell ratios.

From these values, lytic units (LU) per fraction were determined. The results are presented in Fig. 1. It can be seen that CTL on day 4 of a primary MLC are predominantly medium to large-sized cells. 95% of the recovered LU were found in fractions with a sedimentation velocity (s) of >4 mm/h whereas only 50% of the total cells were found in these fractions. The maximum enrichment, expressed in this case as LU/ 10^6 cells, corresponded to cells sedimenting at 6–7 mm/h, and reached a value of approximately 2.5 times that of the unfractionated control cells (i.e., 184 vs. 71 LU/ 10^6 cells). By day 14, a dramatic change in the sedimentation distribution of CTL had occurred. Essentially all of the cytotoxic activity (91% of the recovered LU) sedimented in the region of the gradient occupied by small lymphocytes (i.e., $s < 4$ mm/h), and the maximum (twofold) enrichment was observed at a sedimentation velocity of 3.5 mm/h (8 as compared to 4 LU/ 10^6 cells in the unfractionated control).

It should be noted in these experiments that both cell recovery and relative CTL frequency decreased dramatically in MLC between day 4 and day 14. In particular, approximately 15% of the input viable cells were present in day 14 cultures (compared to 45% on day 4), and LU/ 10^6 cells decreased from 71 to 4 during this time period. Hence, in absolute terms, 50-fold fewer LU were present in day 14 MLC as compared to day 4. In view of this loss of absolute CTL activity, the sedimentation profiles are insufficient in themselves to establish that a size transition of CTL had taken place.

Evidence for Size Progression of CTL. As suggested above, the observed change with time in the sedimentation velocity distribution of in vitro generated CTL could represent a size progression of the sensitized cells. Alternatively, the presence of small CTL in day 14 MLC may reflect differential survival or the late appearance of an independent population of small CTL. To distinguish between these alternatives, day 4 MLC cells were separated by velocity sedimentation into three pooled fractions as indicated in Table I. The relative CTL activity of each fraction and of an unseparated control was assayed on P-815 (DBA/2) target cells, and an equal number of cells from each pool was recultured with irradiated syngeneic (C57BL/6) spleen cells, which were included to optimize the culture conditions. The cytotoxic activity of these latter cultures was again assessed on day 8 and on day 14. The results of this experiment, summarized in Table I,

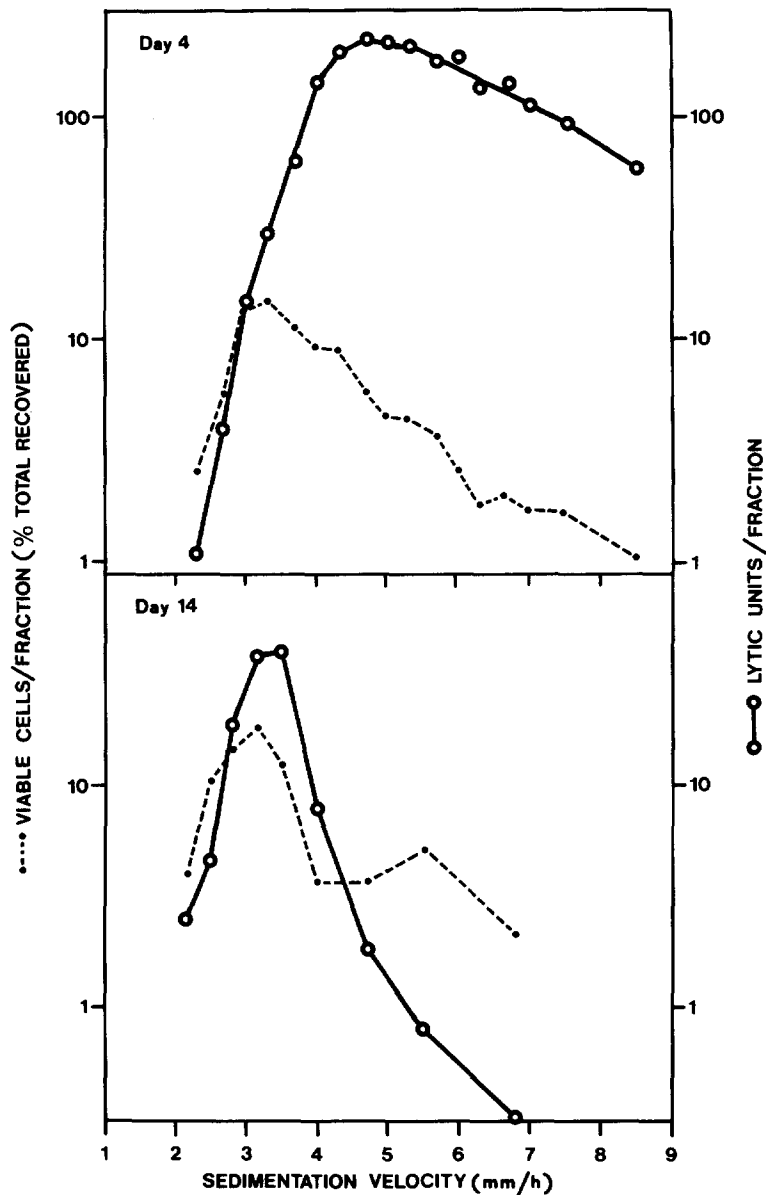


FIG. 1. Kinetic analysis of CTL in MLC by velocity sedimentation. Equal numbers of C57BL/6 spleen cells and irradiated DBA/2 spleen cells were cultured. On day 4 and again on day 14, 50×10^6 viable recovered MLC cells were separated by velocity sedimentation at 1 *g*, and each fraction was assayed for cytotoxicity on ^{51}Cr -labeled P-815 (DBA/2) target cells at several lymphocyte:target cell ratios. The distribution of viable cells (●---●) is compared to that of LU (O—O).

indicate that fraction A, which was composed predominantly of large cells on day 4, contained the majority of the per-cell and 87–96% of the total CTL activity recovered on day 8 and day 14. In particular, this large cell fraction was two- to fourfold enriched in LU/ 10^6 cells as compared to an unseparated control, while

TABLE I
Cytotoxic Activity of MLC Cells Fractionated on Day 4 and Recultured with Irradiated Syngeneic Cells*

Fraction	Cell pool		LU/10 ⁶ cells‡ (% total recovered LU)		
	s(mm/h)	Recovered cells (% total)	Day 4	Day 8	Day 14
Unseparated			36	11	1.2
A	>5.2	31	80 (95)	19 (87)	5.4 (97)
B	3.8-5.1	32	3.3 (4)	2.3 (12)	0.2 (3)
C	2.7-3.7	36	0.4 (1)	<0.2 (<1)	<0.1 (<1)

* Primary cultures of 25×10^6 C57BL/6 spleen cells and 25×10^6 irradiated DBA/2 spleen cells were established. On day 4, 50×10^6 recovered MLC cells were separated by velocity sedimentation at 1 g and pooled as indicated. Pooled fractions were assayed for cytotoxicity on ⁵¹Cr-labeled P-815 (DBA/2) target cells at the time of separation and following reculture with equal numbers of irradiated syngeneic (C57BL/6) spleen cells for 4 or 10 days respectively.

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

the activity of the small lymphocyte fraction (fraction C) declined from extremely low to undetectable values with time. No evidence for the late appearance of CTL in the small cell fractions was obtained, and the data are thus quantitatively consistent with the possibility that essentially all CTL found on day 14 are derived from cells which were initially large. Since the majority of CTL in undisturbed cultures are small at 14 days (Fig. 1), this suggests that a size progression of CTL occurs with time.

Characterization of Cells Responsive to Secondary Alloantigenic Stimulus. Since a rapid appearance of high levels of CTL activity could be demonstrated following secondary antigenic stimulation in long-term MLC (13), it was of considerable interest to investigate whether residual CTL were responsible for this activity. To this end, day 14 MLC cells were separated by velocity sedimentation at unit gravity and divided into three pooled fractions corresponding to those described in the previous section. Each pool (in addition to an unfractionated control) was tested immediately for cytotoxicity on ⁵¹Cr-labeled P-815 (DBA/2) target cells. As can be seen in Table II, the vast majority of the CTL activity (83%) was found in the two smaller fractions (fractions B and C), although the large cell pool (fraction A) did have detectable activity in this experiment. To evaluate the relative ability of cells in each fraction to respond to a second alloantigenic stimulus, 0.4×10^6 cells were incubated with an equal number of irradiated allogeneic (DBA/2) spleen cells for 3 additional days, at which time LU/10⁶ cells and per original fraction were again determined. The increase in LU/culture, which should reflect the relative content of CTL progenitors in a particular fraction, is also shown in Table II. It can be seen that fractions B and C accounted for essentially the entire increase in CTL observed following secondary allogeneic stimulation in vitro, increasing about 40-fold in terms of LU/10⁶ cells and approximately 100-fold in terms of LU/original fraction. In contrast, LU/10⁶ cells and per culture for the large cell

TABLE II
*Effect of Reexposure to Alloantigens on the Cytotoxic Activity of
 Fractionated day 14 MLC Cells**

Cell pool		LU/10 ⁶ cells‡ (LU/fraction)		
Fraction	s(mm/h)	Recovered cells (% total)	Residual (Day 14)	After restimulation (Day 17)
Unseparated			3.8	180
A	>5.0	33	2.0 (5)	4 (10)
B	3.7-4.9	28	5.8 (13)	186 (1,060)
C	2.8-3.6	39	3.7 (12)	172 (1,110)

* Primary cultures of 25×10^6 C57BL/6 spleen cells and 25×10^6 irradiated DBA/2 spleen cells were established. On day 14, 50×10^6 recovered MLC cells were separated by velocity sedimentation at 1 g and pooled as indicated. Pooled fractions were assayed for cytotoxicity on ⁵¹Cr-labeled P-815 (DBA/2) target cells at the time of separation and following reculture for 3 days with equal numbers of irradiated allogeneic (DBA/2) spleen cells.

‡ LU were calculated from the dose-response curve obtained with each fraction.

population increased only by twofold under the same culture conditions, thereby accounting for <1% of the net increase in LU observed. No increase in cytotoxicity was observed in parallel control cultures in which cells from each fraction were incubated with irradiated syngeneic (C57BL/6) spleen cells (data not shown). Thus the majority of the cells responsive to a second allogeneic stimulus in day 14 MLC were found in the same fractions which contained the residual CTL activity.

Size Progression of Cells Responsive to Secondary Stimulation. In view of the size change previously documented for CTL, we further investigated whether residual CTL progenitors (i.e., those small cells present in day 14 MLC which had the capacity to be restimulated by alloantigen) were themselves derived from larger cells. To approach this question, day 4 MLC cells were again separated into three cell pools comparable to those described in the previous experiments. Each of these cell pools was tested for cytotoxic activity on P-815 (DBA/2) target cells, and then recultured with irradiated syngeneic (C57BL/6) spleen cells until day 14. 0.4×10^6 cells from each group were then cultured with irradiated DBA/2 spleen cells for an additional 1 or 4 days, at which time the cytotoxic activity was again determined. The results of this experiment, summarized in Table III, indicate that 94% of the CTL activity generated at the peak of the secondary stimulation came from progenitor cells which were initially large on day 4 (fraction A); furthermore, the same proportion of the total cytotoxic activity was associated with these same large cells on day 4 of the primary response (i.e., 95%). Hence, the results are quantitatively consistent with the hypothesis that all progenitors of CTL in day 14 MLC were in fact derived from primary CTL.

Evidence for Enlargement of CTL after Reexposure to Alloantigens. The distribution in sedimentation velocity of those CTL generated following re-exposure of day 14 MLC cells to alloantigens was also investigated. During the

TABLE III
*Cytotoxic Activity of Fractionated Day 4 MLC Cells Following Reculture and Subsequent Reexposure to Alloantigens**

Cell pool			LU/fraction‡ (% total recovered LU)			
Fraction	s(mm/h)	Recovered cells (% total)	Day 4	Day 14	Day 15	Day 18
A	>5.2	32	860 (95)	9 (98)	104 (98)	1,150 (94)
B	3.8-5.1	31	36 (4)	0.2 (2)	2 (2)	67 (5)
C	2.7-3.7	37	5 (1)	0.1 (<1)	1 (<1)	<2 (<1)

* 50×10^6 day 4 primary MLC cells were fractionated by velocity sedimentation at 1 g. Pooled fractions were assayed for cytotoxicity on ^{51}Cr -labeled P-815 (DBA/2) target cells immediately after separation and following reculture with irradiated syngeneic (C57BL/6) spleen cells until day 14. At that time, aliquots of recovered cells from each pool were cultured with equal numbers of irradiated allogeneic (DBA/2) spleen cells and cytotoxicity was again assayed 1 (day 15) and 4 (day 18) days after restimulation.

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

primary MLC response, it has been established that the medium to large sized CTL found on day 4-5 develop from small T-lymphocyte precursors (7). In order to establish whether an analogous size transformation occurred following secondary alloantigenic stimulation in vitro, day 14 MLC cells were again separated by velocity sedimentation and the cytotoxic activity of each fraction was determined. The resulting CTL distribution (Fig. 2 a) was essentially similar to that found previously under comparable conditions (cf. Fig. 1). A pool of the small lymphocyte fractions containing the majority of the CTL activity ($2.6 < s < 3.8$ mm/h) was then cultured with an equal number of irradiated allogeneic spleen cells for 2 days. At that time, the surviving cells were again separated by velocity sedimentation and the CTL activity of each fraction was determined. The results of this experiment (Fig. 2 b) revealed several interesting features. Firstly, the distribution of viable nucleated cells was extremely heterogeneous (cf. Fig. 2 a), with 70-80% of the recovered cells sedimenting more rapidly than the input small lymphocytes from which they were derived. Furthermore, the CTL activity had increased dramatically (about 13-fold) and was concentrated in the rapidly sedimenting fractions, such that only 3% of the recovered activity remained associated with cells of the size of the input small lymphocytes. Hence this double separation experiment provided strong direct evidence that residual alloantigen responsive small lymphocytes in day 14 MLC could rapidly differentiate into large, highly cytotoxic CTL when re-exposed to the appropriate alloantigens.

Discussion

The development of CTL after allogeneic stimulation in the mouse is at the present time the best characterized model system available for the study of T-cell-mediated immunity. The ability to generate CTL in defined in vitro

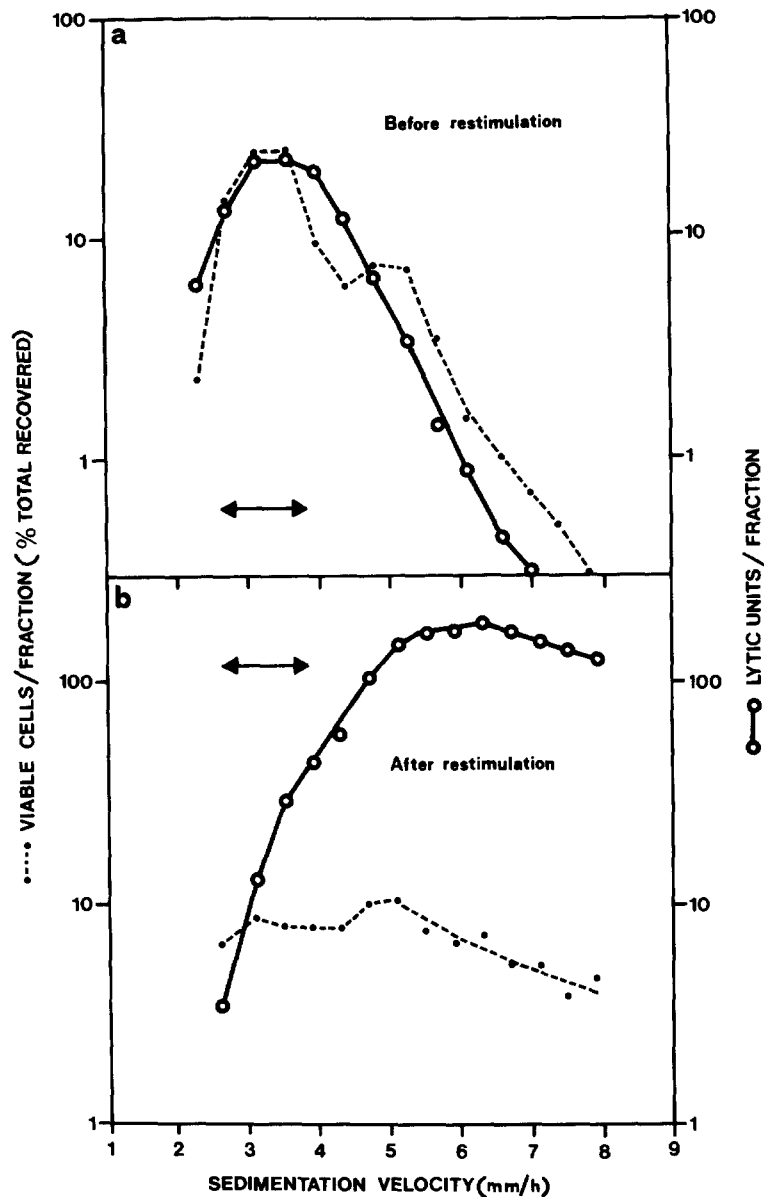


FIG. 2. Change in sedimentation properties of CTL following reexposure to alloantigens. Equal numbers of C57BL/6 spleen cells and irradiated DBA/2 spleen cells were cultured for 14 days. At that time, 50×10^6 recovered MLC cells were separated by 1 g velocity sedimentation, and each fraction was assayed for cytotoxicity on ^{51}Cr -labeled P-815 (DBA/2) target cells. In Fig. 2 a, the distribution of viable cells (●---●) is compared to that of LU (O—O). In addition, a pool of small lymphocyte fractions encompassing the range of sedimentation velocity indicated by the arrow was reexposed to equal numbers of irradiated DBA/2 spleen cells. After 2 further days, the recovered cells from these secondary cultures were again separated by 1 g velocity sedimentation, and the cytotoxic activity of each fraction was similarly determined (Fig. 2 b). For purposes of comparison, the distributions of viable cells (●---●) and of LU (O—O) for this latter separation are expressed relative to the same total number of fractionated cells as in Fig 2 a.

systems, notably the MLC, has provided a basis whereby the inductive and efferent aspects of T-lymphocyte function can be studied and compared to earlier *in vivo* findings. By extending this *in vitro* model to encompass the detection and restimulation of CTL after prolonged periods of time in culture, as described here and in a previous report (13), it was hoped that an approach to the more general study of T-cell differentiation could be made.

From this report, several preliminary conclusions with respect to the differentiation of CTL can be made. Studies employing velocity sedimentation at unit gravity as a means of separating cells primarily on the basis of size demonstrated that CTL in day 14 MLC were much smaller than those found in day 4 MLC. These results confirm and extend previous studies of the size distribution of *in vitro* generated CTL (7-8, 17) and are compatible with *in vivo* studies in which CTL found in the spleens of mice rejecting a tumor allograft were large at early times, and much smaller at later times (7-9). In addition, by means of the isolation and reculture of MLC cells of different sizes, it was possible to demonstrate quantitatively that CTL activity remained associated with the cell population which was large at the peak of the response. Hence these results provide direct evidence that a progression from large to small effector cells is taking place. Clearly, the possibility that small CTL are derived from cells which are large on day 4 but not cytotoxic cannot be excluded by these studies. Methods for the selective identification and/or removal of CTL are needed to test this alternative.

An important question raised by the demonstration that CTL activity can be rapidly and specifically regenerated upon re-exposure to relevant alloantigens (13) is whether or not the residual effector CTL are the progenitors of the secondary CTL activity, that is, whether CTL at the single cell level can be multiply stimulated by alloantigens. Since individual CTL cannot be identified in the day 14 population, and since the absolute frequency of CTL is not known, no formal proof of this hypothesis can be made. Nevertheless, several similarities in the physical properties of effector and progenitor cells can be demonstrated. The presence of the majority of CTL progenitor activity in day 14 MLC in those small lymphocyte fractions which also contained the majority of residual CTL activity following velocity sedimentation cell separation suggests that the two cannot be readily dissociated. Furthermore, restimulation on day 14 of cell fractions isolated on the basis of size on day 4 indicated that secondary CTL progenitors, like the residual CTL, belong to a class of cells which are initially large but become small. In other experiments,² residual CTL and secondary CTL progenitors in day 14 MLC were found to have a comparable sensitivity to limiting doses of anti- θ serum plus complement, thereby suggesting that they exhibit a similar concentration of θ -antigen on their surface. Taken together, these data suggest that there is a good correlation between residual CTL and CTL progenitors in day 14 MLC. It remains possible, however, that CTL progenitors in day 14 MLC derive from an independent T-cell lineage which concomitantly undergoes enlargement and subsequent reversion to small lymphocyte status.

By combining the most straightforward interpretation of the above data with

² MacDonald, H. R. Unpublished observation.

previous results, a tentative model for the differentiation pathway of CTL may be envisaged. Upon the initial interaction with alloantigen, large blastlike CTL arise via the proliferation and/or differentiation of small T-lymphocyte precursor cells present in normal lymphoid tissue (7). These large CTL are highly cytotoxic but appear to be insensitive to further allogeneic stimulation (13). As a function of time, these CTL (or some fraction thereof) revert to small T lymphocytes which gradually lose their cytotoxic activity. Preliminary experiments suggest that this size transition may be largely independent of DNA synthesis and cell mitosis.³ When these small lymphocytes are then re-exposed to the original stimulating alloantigens, they respond by rapid proliferation and differentiation to yield a population of large, highly cytotoxic CTL. In contrast to what has been postulated for the primary response (18) there is preliminary evidence that differentiation and proliferation can be dissociated during the initial 24 h of the secondary response to alloantigens, that is, that reappearance of cytotoxic activity can occur even when DNA synthesis is completely inhibited.³ Since the sensitivity of current methods is insufficient to detect CTL at a comparably early stage of the primary response, further studies are needed to establish whether this represents a true qualitative difference between normal and alloimmune cells.

Summary

Separation of cells by velocity sedimentation at unit gravity was utilized to investigate the physical properties of cytotoxic thymus-derived lymphocytes (CTL) generated in long-term mixed leukocyte cultures (MLC). In kinetic studies, CTL were found almost exclusively in the large cell fractions at the peak of the response on day 4, whereas the majority of CTL in day 14 MLC had the sedimentation properties of small lymphocytes. Reculture until day 14 of cells fractionated on the basis of size on day 4 indicated that the small CTL were derived exclusively from cells which had been large on day 4. Re-exposure of day 14 MLC cells to the original stimulating alloantigens resulted in significant cell proliferation and rapid regeneration of CTL activity. Cell fractionation experiments demonstrated that the cells in the day 14 MLC population which responded to the secondary allogeneic stimulus were small T lymphocytes, and that these cells rapidly developed into large, highly cytotoxic CTL following stimulation. Moreover, by restimulating on day 14 fractions which were selected on the basis of size on day 4, it was found that the responding small lymphocytes were themselves the progeny of cells which were large at the peak of the response. Since CTL and CTL progenitors showed concomitant changes in physical properties with time, the possibility exists that they belong to the same cell lineage, and hence that CTL can differentiate into cells which are no longer cytotoxic, but capable of mounting an anamnestic response.

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³ MacDonald, H. R., J.-C. Cerottini, and K. T. Brunner. Manuscript in preparation.

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