GENERATION OF CYTOTOXIC T LYMPHOCYTES IN VITRO
IV. Functional Activation of Memory Cells in the Absence of DNA Synthesis*

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The generation of mouse cytolytic T lymphocytes (CTL)[1] in mixed leukocyte cultures (MLC) is a well characterized model system in which both the afferent and efferent phases of T-cell-mediated immunity can be studied (1–3). Recently, the potential usefulness of this model has been extended by the demonstration that anamnestic CTL responses can occur under appropriate culture conditions. In particular, re-exposure of long-term MLC cells to the original stimulating alloantigens was found to result in significant proliferation and in rapid reappearance of high levels of CTL activity (4, 5).

Kinetic studies of the regeneration of CTL activity under these conditions revealed that cytotoxic activity increased significantly within the first 24 h with no detectable increase in viable cell number, but that activity and cell number increased in parallel thereafter (4). These results hence suggested the possibility that CTL activation may be dissociated from proliferation during the initial 24 h of alloantigenic re-exposure.

In this report, we have taken a more direct approach to the investigation of the relationship between differentiation and proliferation of restimulated CTL. By re-exposing long-term MLC cells to alloantigens during 24 h in the presence of cytosine arabinoside (ARA-C), a potent inhibitor of DNA synthesis, we have found that functional activation of CTL occurs even when DNA synthesis is completely blocked. Furthermore, morphological, immunological, and cell separation experiments indicate that this functional activation is accompanied by an enlargement of the responding small T lymphocytes.

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[1] *Abbreviations used in this paper:* ARA-C, cytosine arabinoside; CTL, cytolytic T lymphocytes; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; ^{125}IUdR, ^{125}I-labeled 5-iodo-2′-deoxyuridine; LU, lytic units; MLC, mixed leukocyte cultures; MTLA, mouse T-lymphocyte antigen; PBS, phosphate-buffered saline; s, sedimentation velocity; TdR, thymidine.

Materials and Methods

Mice. Adult female mice of the inbred strains C57BL/6 and DBA/2 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.

MLC. Spleen cell suspensions were prepared as described previously (6), and finally suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (Flow Laboratories Ltd., Irvine, Scotland), 5×10^{-5} M 2-mercaptoethanol, and additional amino acids (7). Primary MLC 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% CO_2 in air for various periods of time as indicated.

Secondary cultures were made by mixing 0.4×10^6 viable MLC cells harvested after 14 days of culture with an equal number of irradiated (1,000 rads) DBA/2 spleen cells in plastic test tubes as described previously (4). In the experiments involving inhibition of DNA synthesis, ARA-C (Serva, Heidelberg, West German) was added to the culture medium at a final concentration of 10 μg/ml.

Cytotoxic Assay. P-815 mastocytoma cells (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 ^{51}Cr as described previously (7), washed three times, and resuspended in DMEM supplemented with 5% FBS and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES), pH 7.2.

For the cytotoxic assay, various numbers of viable MLC cells were then incubated with 10×10^3 ^{51}Cr-labeled P-815 (DBA/2) target cells in 0.4 ml of DMEM supplemented with 5% FBA and 10 mM HEPES for 3 h at 37°C in a shaking water bath (7). At the end of this incubation, 0.6 ml of cold phosphate-buffered saline (PBS) was added to each tube. The tubes were then centrifuged at 500 *g* for 5 min and 0.5 ml of the supernate was collected and counted in a well-type scintillation counter (Nuclear-Chicago, Corp., Des Plaines, Ill.). Specific cytotoxicity was calculated and normalized as described previously (7).

Velocity Sedimentation cell Separation at 1 g. This technique has been described previously (8). For the present experiments, a glass sedimentation chamber of 11 cm diameter was employed (Glasapparatebau, Weil, West 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 supension 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 millimeters per hour as described elsewhere (8).

Quantitation of CTL Activity. Activity of CTL is expressed in terms of lytic units (LU) according to the definition described previously (7). Briefly, 1 LU is defined as the absolute number of cells required to lyse 50% of 10×10^3 ^{51}Cr-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.

Immunofluorescent Studies. Enumeration of lymphocytes bearing mouse T-lymphocyte antigen (MTLA) or surface immunoglobulin (Ig) was carried out as described previously (7).

Measurement of DNA Synthesis. DNA synthesis was measured as incorporation of ^{125}I-5-iodo-2'-deoxyuridine ($^{125}IUdR$) (The Radiochemical Centre, Amersham, England). Aliquots of 0.1 μCi $^{125}IUdR$ were added to each of triplicate cultures which were set up in parallel with those assayed for lytic activity. After a 24 h incubation period, cells were harvested, washed 4 times in PBS, and counted in a well-type scintillation counter. Results are expressed as mean counts per minute plus or minus the standard deviation. Machine background was subtracted in each case.

In some experiments, DNA synthesis was assessed by autoradiography. In such cases, cultures were incubated with 2 μCi of tritiated thymidine ([^3H]TdR) (New England Nuclear, Boston, Mass., 7 Ci/mmol) for 24 h. Cells harvested from these cultures were cytocentrifuged, air dried, fixed for

10 min with methanol and dipped in Kodak NTB_2 (dilution 1:3; Eastman Kodak Co., Rochester, N. Y.) or Ilford K2 (dilution 1:2; Ilford Ltd., Ilford, Essex, England) nuclear emulsions. Exposure times ranging between 2 and 7 days at 4°C were found to give satisfactory labeling intensity. Slides were developed with D19 Kodak developer, washed, fixed with Kodak Metafix, thoroughly washed in tap water, and finally counterstained with Giemsa. Labeled cells were defined as those having >10 grains concentrated over the nucleus. Lymphoid cells were classified as small-, medium-, or large-sized lymphoid cells according to the standardized system proposed by Cottier et al. (9).

Electron Microscope Studies. Cell suspensions ($5–10 \times 10^5$ cells) were centrifuged in conical tubes at 500 *g* for 5 min, and the supernate removed and replaced with a cold solution of 2.45% glutaraldehyde (TAAB Lab., Reading, England) in 0.08 M cacodylate buffer, pH 7.4. After 1–2 min, the pellet was gently removed with a fine glass rod from the tube wall, sectioned in small fragments, and further fixed for an additional 2- to 3-hr period at 4°C. The fragments were then washed in 0.1 M, pH 7.4, cacodylate buffer overnight and postfixed for 1 h with 1% cacodylate-buffered osmium tetroxide at room temperature.Embedding was performed in Durcupan (Fluka AG, Buchs, Switzerland) after dehydration in alcohol. Thin sections obtained with glass knives were counterstained with uranyl acetate and lead citrate and examined with an Hitachi HU-12 electron microscope, (Hitachi Ltd., Tokyo, Japan).

Results

Effect of ARA-C on the Regeneration of CTL Activity in MLC. In a previous study (4), it was observed that re-exposure of day 14 MLC cells to the original stimulating alloantigens resulted in rapid reappearance of CTL activity and in cell proliferation. In particular, it was found that CTL activity increased 10-fold during the first 24 h of the secondary culture, when no detectable increase in viable cell number was observed. In the following 3 days, a parallel increase in viable cell number and CTL activity occurred. In order to investigate the possibility that differentiation and proliferation could be dissociated during the secondary in vitro response to alloantigens, aliquots of day 14 MLC cells were restimulated with irradiated allogeneic (DBA/2) spleen cells in the presence or absence of 10 μg/ml ARA-C, a potent inhibitor of cellular DNA synthesis. In agreement with Röllinghoff et al. (10), control studies indicated that the ARA-C had no inhibitory effect on CTL activity when added at this concentration in the 3 h lytic assay. On days 1, 2, 3, and 6 after restimulation, surviving MLC cells were enumerated, and cytotoxicity was assayed on ^{51}Cr-labeled P-815 (DBA/2) target cells at several lymphocyte: target cell ratios. From these data, LU per culture were calculated.

The results of this experiment are summarized in Fig. 1. In the positive control cultures in which no ARA-C was present, the number of LU per culture increased 20-fold during the initial 24 h of culture, and continued to increase exponentially until day 3. In agreement with previous data (4), the viable cell number remained constant for the first 24 h and then increased in parallel with CTL activity. Interestingly, the presence of ARA-C had no effect on the increase in CTL activity observed in the initial 24 h of restimulation. Thereafter, CTL activity remained essentially unchanged for 24–48 h and then decreased rapidly, reaching levels at 6 days which were lower than control cultures which had not been restimulated. Viable cell numbers in the presence of ARA-C remained essentially constant for 48 h, and then decreased in parallel with decreasing CTL activity until day 6. These results thus indicated that the rapid appearance of CTL activity within 24 h of restimulation was not inhibited by ARA-C, but

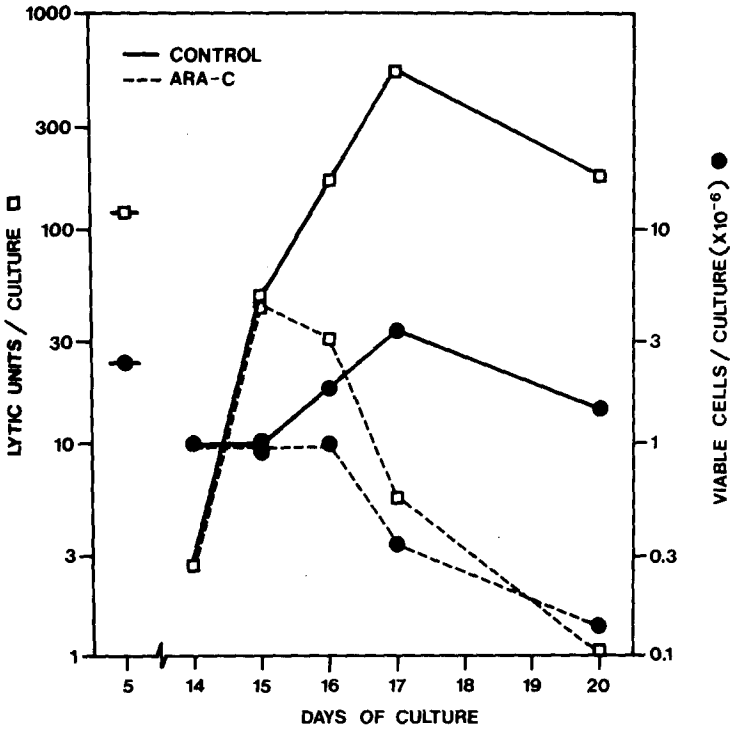

FIG. 1. Effect of ARA-C on the kinetics of generation of CTL activity in restimulated long-term MLC cells. Equal numbers of C57BL/6 spleen cells and irradiated DBA/2 spleen cells were cultured for 14 days. Aliquots of 0.4×10^6 viable cells recovered from these cultures were mixed with equal numbers of irradiated DBA/2 spleen cells and cultured in the presence (---) or absence (—) of ARA-C (10 μg/ml). On the days indicated, viable cells were enumberated (●), and cytotoxicity was assayed on ^{51}Cr-labeled P-815 (DBA/2) target cells. LU/5 × 10^6 cells originally cultured (□) were calculated from dose-response curves.

that the subsequent increase was sensitive to the effects of the drug. These findings were confirmed in four additional experiments in which the lytic activity of day 14 MLC cells after 24 h re-exposure to irradiated DBA/2 spleen cells in the presence or absence of 10 μg/ml ARA-C was compared. As shown in Table I, LU per culture in restimulated cultures increased 6-to 15-fold within 24 h by comparison with control unstimulated cultures. A similar increase in LU per culture was observed when ARA-C was present during the period of restimulation.

In order to verify that DNA synthesis was inhibited by ARA-C under the experimental conditions used, day 14 MLC populations restimulated during 24 h in the presence or absence of varying doses of ARA-C were tested for uptake of ^{125}IUdR and lytic activity. The result of one such experiment (Table II) demonstrated that concentrations of ARA-C ranging from 1 to 10 μg/ml were sufficient to inhibit ^{125}IUdR incorporation by 93–98% during a 24 h re-exposure to alloantigens. As was observed in all the previous experiments, the increase in LU per culture was unaffected by either of the doses of ARA-C tested. These experiments hence indicated that the reappearance of functional CTL activity in

TABLE I

*Effect of ARA-C on the 24 h Cytotoxic Response of day 14 MLC Cells**

Exp.	LU/culture‡		
	Unstimulated	Restimulated	
		Without ARA-C	With ARA-C
1	4.4	26	23
2	3.5	31	29
3	1.4	22	25
4	<1.0	17	13

* 25 × 10^6 C57BL/6 spleen cells were incubated with 25 × 10^6 irradiated (1,000 rads) DBA/2 spleen cells for 14 days. Aliquots of 0.4 × 10^6 surviving cells from these cultures were then incubated with 0.4 × 10^6 irradiated syngeneic C57BL/6 spleen cells (unstimulated) or allogeneic DBA/2 spleen cells (restimulated) for a further 24 h in the presence or absence of ARA-C (10 μg/ml).

‡ Cytotoxicity at 24 h after restimulation was assayed on ^{51}Cr-labeled P-815 (DBA/2) target cells, and LU were calculated from the dose-response curves for each population.

TABLE II

*Effect of ARA-C on DNA Synthesis and Cytotoxic Activity in Restimulated day 14 MLC**

Cell population	Dose of ARA-C	^{125}IUdR incorporation		LU/culture‡
	μg/ml	*cpm ± SD*	*% reduction*	
Unstimulated	None	3,374 ± 134	—	3.0
Unstimulated	10	251 ± 11	93	2.8
Stimulated	None	10,415 ± 335	—	18
Stimulated	1	687 ± 10	93	16
Stimulated	10	263 ± 14	98	16

* 25 × 10^6 C57BL/6 spleen cells were incubated with 25 × 10^6 irradiated (1,000 rads) DBA/2 spleen cells for 14 days. aliquots of 0.4 × 10^6 surviving cells from these cultures were then incubated with irradiated C57BL/6 (unstimulated) or DBA/2 (stimulated) spleen cells for a further 24 h in the presence or absence of ARA-C. DNA synthesis was assessed in triplicate samples by uptake of ^{125}IUdR.

‡ Cytotoxicity was assayed on ^{51}Cr-labeled P-815 target cells, and LU were calculated from dose-response curves.

restimulated cultures was independent of DNA synthesis.

Morphological, Immunological, and Autoradiographic Analysis of Restimulated Cells. In view of the observation that day 14 MLC cells could be functionally activated within 24 h of re-exposure to alloantigens in the apparent absence of DNA synthesis, it was of interest to investigate the morphology and the nature of the cells in such cultures. Since previous studies using 1 *g* velocity sedimentation to separate cells on the basis of size (15) had established that small lymphocytes were the cells which primarily responded to secondary allogeneic stimulus by the formation of CTL, a selected population of such cells was used as responder cells to simplify the morphological analysis. To this end, day 14 MLC cells were separated by velocity sedimentation at unit gravity, and

TABLE III

*Morphological and Autoradiographic analysis of day 14 MLC Cells Restimulated in the Presence or Absence of ARA-C**

Cell population	ARA-C	Cell type	% total lymphoid cells	
			Labeled	Unlabeled
Unstimulated	None	Small	0	94
		Medium	0.5	5
		Large	0.5	0
Stimulated	None	Small	0	21.7
		Medium	23.7	7.6
		Large	47.9	0.1
Stimulated	10 μg/ml	Small	0	38.2
		Medium	0	25.5
		Large	1.1	35.2

* Day 14 MLC cells were separated by 1 *g* velocity sedimentation and a pool of small-sized lymphocytes ($3.0 < s < 3.8$ mm/h) was selected. Aliquots of 0.4×10^6 cells from this pool were then cultured with a like number of irradiated C57BL/6 (unstimulated) or DBA/2 (stimulated) spleen cells with or without ARA-C for a further 24 h in the presence of 2 μCi[^{3}H]TdR. Cells from each group were then cytocentrifuged and processed for autoradiography.

the fractions containing the relatively slow sedimenting cells ($3.0 < s < 3.8$ mm/h) were pooled. Immunofluorescent studies indicated that 95% of the selected cells carried the MTLA antigen, a T-cell surface marker, whereas <1% were positive for surface Ig. The selected cell population was then restimulated with irradiated allogeneic spleen cells in the presence of low concentrations of [^{3}H]TdR and the number and size of the cells synthesizing DNA assessed by autoradiographic analysis. The results (Table III) demonstrated that, irrespective of the presence or absence of ARA-C, >60% of the cells present in cultures restimulated for 24 h were medium- and large-sized lymphoid cells. By contrast, unstimulated cultures contained almost exclusively small lymphocytes. As judged by immunofluorescence, >99% of the medium- and large-sized cells in restimulated cultures were MTLA positive. Autoradiographic analysis revealed that about 90% of the medium and large lymphoid cells were labeled in control restimulated cultures (Fig. 2), in contrast to <2% in restimulated cultures containing ARA-C. Small cells present in unstimulated or stimulated cultures were not labeled to any significant degree.

These studies demonstrated that a large proportion of the small-sized T lymphocytes in day 14 MLC enlarged and underwent DNA synthesis within 24 h after restimulation. Moreover, it was clear that the enlargement could be dissociated for DNA synthesis.

Velocity Sedimentation Analysis of Restimulated Cells. The results reported above indicated that two of the events triggered by restimulation of long-term MLC cell populations, namely formation of functional CTL and enlargment of small T lymphocytes, were independent of DNA synthesis. Although it was tempting to speculate that both events were linked, the data obtained were

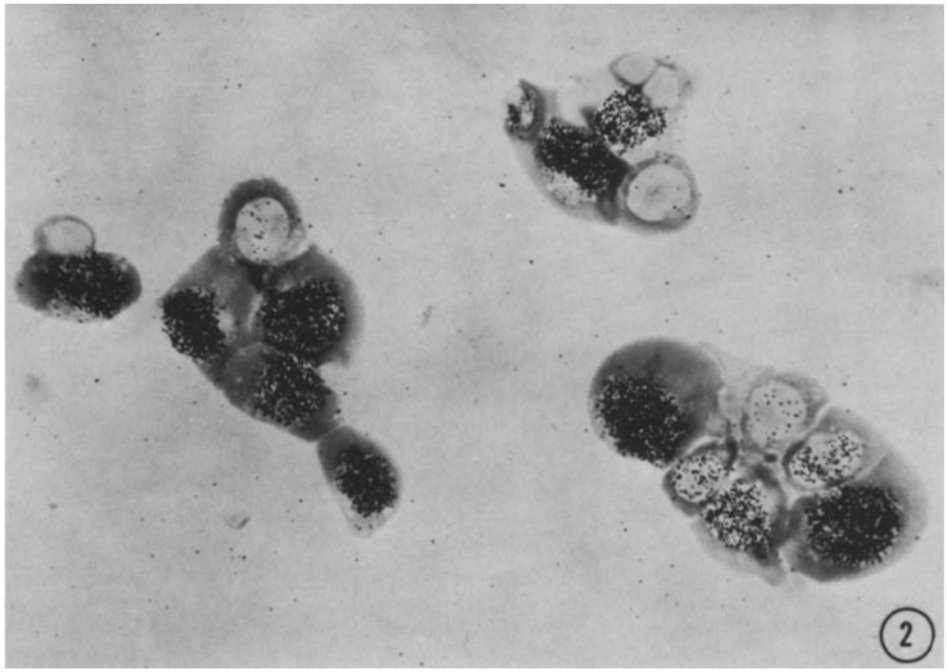

FIG. 2. Autoradiograph of a cytocentrifuged cell population obtained 24 h after restimulation of day 14 MLC cells with irradiated allogeneic spleen cells. [^{3}H] TdR was present during the 24 h incubation period. Most of the large lymphoid cells are labeled, whereas small lymphocytes and some medium-sized lymphocytes are not. Giemsa staining. × 1,000.

insufficient to provide direct proof of such association since the size distribution of CTL in the populations tested was unknown. In order to directly evaluate the association between CTL and the larger lymphoid T cells formed in the absence of DNA synthesis a pool of primarily small-sized lymphocytes ($3.0 < s < 3.8$ mm/h) was isolated from 14 day MLC cultures by 1 *g* velocity sedimentation. Multiple aliquots of 0.4×10^6 viable cells from this pool were then incubated with an equal number of irradiated allogeneic (DBA/2) spleen cells in the presence or absence of 10 μg/ml ARA-C. After 24 h, cells recovered from each of these 2 groups of cultures were pooled and separated in parallel by 1 *g* velocity sedimentation. For each separation, 13 individual fractions were collected, assessed for viable cell number, and assayed for cytotoxicity on ^{51}Cr-labeled P-815 (DBA/2) target cells. The distributions so obtained are shown in Fig. 3. It can be seen that the majority of the recovered viable cells obtained after restimulation for 24 h were more rapidly sedimenting than the input small lymphocytes selected from the day 14 MLC population, irrespective of the presence of ARA-C. Furthermore, the distribution of lytic activity in each case was likewise restricted to the rapidly sedimenting fractions, such that only 3–5% of the recovered LU were found in fractions corresponding to the input population. This experiment thus provided direct evidence that (*a*) small lymphocytes in day 14 MLC differentiated into large CTL within 24 h after restimulation, and (*b*) this functional activation could be dissociated from DNA synthesis.

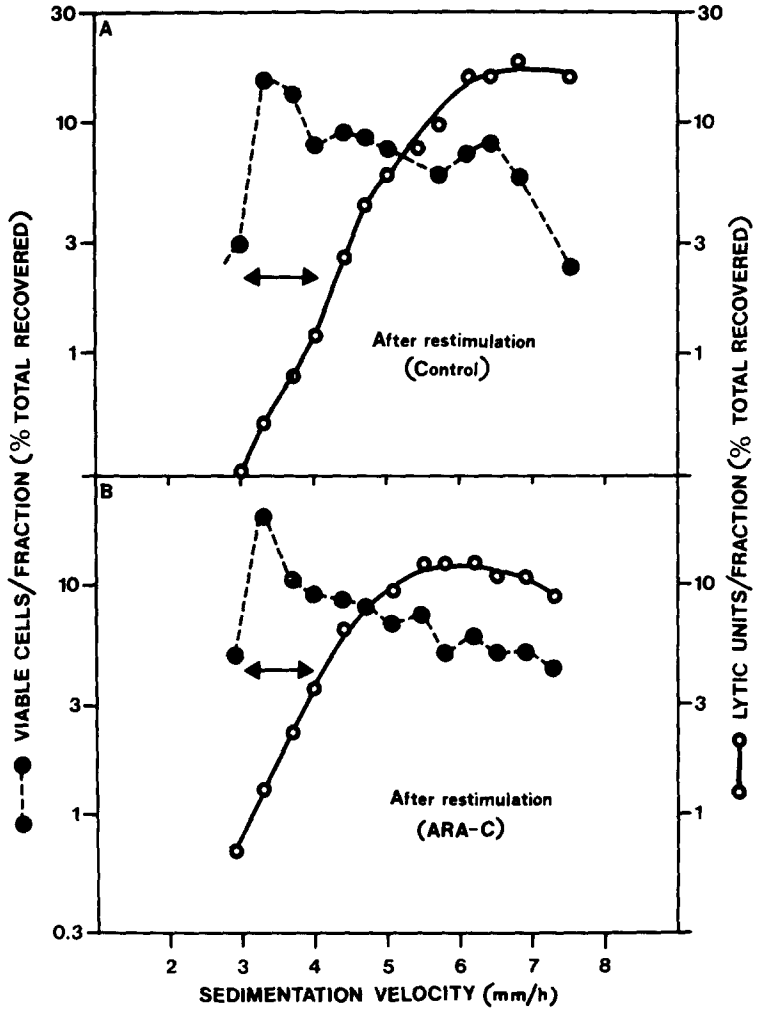

FIG. 3. Sedimentation properties of CTL formed within 24 h after restimulation of long-term MLC cells either in the presence or the absence of ARA-C. Equal numbers of C57BL/6 spleen cells and irradiated DBA/2 spleen cells were cultured for 14 days. The MLC cells were separated by velocity sedimentation at 1 *g*, and a pool of small lymphocytes encompassing the range of sedimentation velocity indicated by the *arrow* was incubated with equal numbers of irradiated DBA/2 spleen cells in the absence (Fig. 3 A) or presence (Fig. 3 B) of ARA-C (10 μ/ml). After incubation for 24 h, each restimulated MLC population was separated by velocity sedimentation at 1 *g*, and the cytotoxic activity of each fraction assayed on ^{51}Cr-labeled P-815 (DBA/2) target cells. The distribution of viable cells (●) and of LU (○) are expressed in terms of percent of total number recovered.

To further compare the large lymphoid cell populations formed in the presence or absence of ARA-C, the fractions with the highest lytic activity were pooled and processed for electron microscopy. As evident on semithin sections (Fig. 4), both cell populations contained >90% large lymphoid cells (most, if not all, contaminating small lymphocytes were pyknotic). The large lymphoid cells were relatively homogenous in size, with an oval- or kidney-shaped nucleus, and

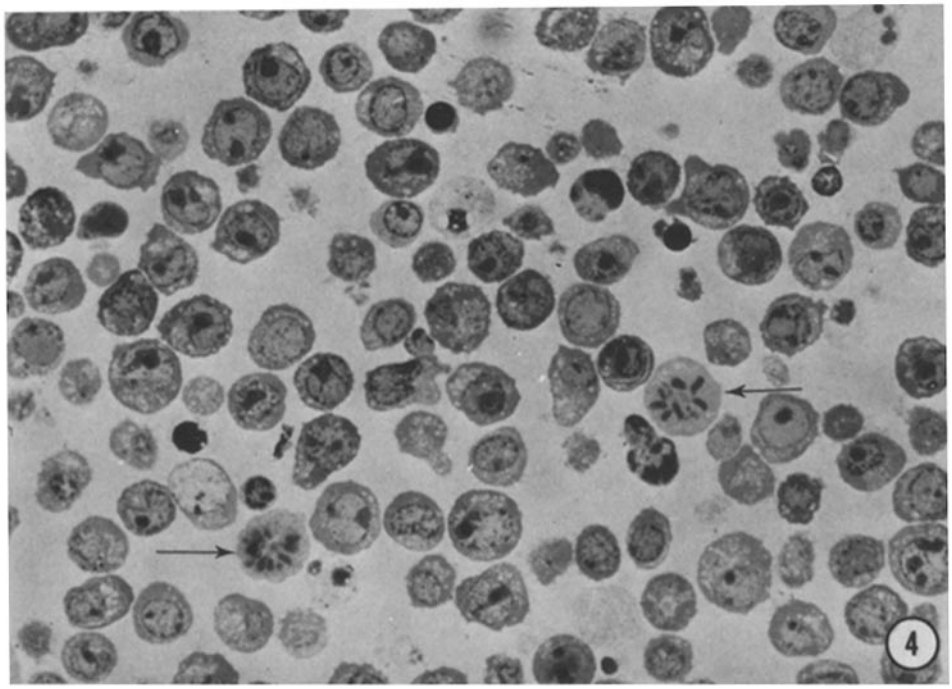

FIG. 4. Micrograph of a semithin section of the cell population obtained after two successive separations of long-term restimulated MLC cells. Note the relative homogeneity in size, most of the cells being large lymphoid cells with a few pyknotic small lymphocytes. *Arrows* indicate two mitotic figures. Giemsa staining. × 850.

a cytoplasm containing numerous ribosomes, scattered profiles of rough endoplasmic reticulum, mitochondria, and well developed Golgi complexes. Clear vacuoles located mostly in the Golgi region, as well as electron-dense osmiophilic inclusions were also observed. Based on relatively minor morphological differences, two types of large lymphoid cells could be recognized: (*a*) cells with an abundant dispersed chromatin, 2–3 large nucleoli, and ribosomes mostly in clusters (Fig. 5); and (*b*) cells, slightly smaller, with a more peripheral condensed chromatin, a larger amount of ribosomes evenly distributed in the cytoplasm, and a more developed filamentous network under the cell membrane (Fig. 6). With the exception of the absence of mitotic figures and division form of centrioles, the selected population obtained from restimulated cultures containing ARA-C showed no major morphological difference as compared to that of control cultures. In particular, the percentage of large lymphoid cells of one type or the other was identical in both groups (84 and 16%, respectively).

Discussion

The experiments described herein extend previous findings indicating that highly active CTL populations were generated within 24 h after re-exposure of day 14 MLC cells to the original stimulating alloantigen (4). Direct evidence has been obtained that the appearance of CTL activity could be dissociated from DNA synthesis since concentrations of ARA-C which were sufficient to com-

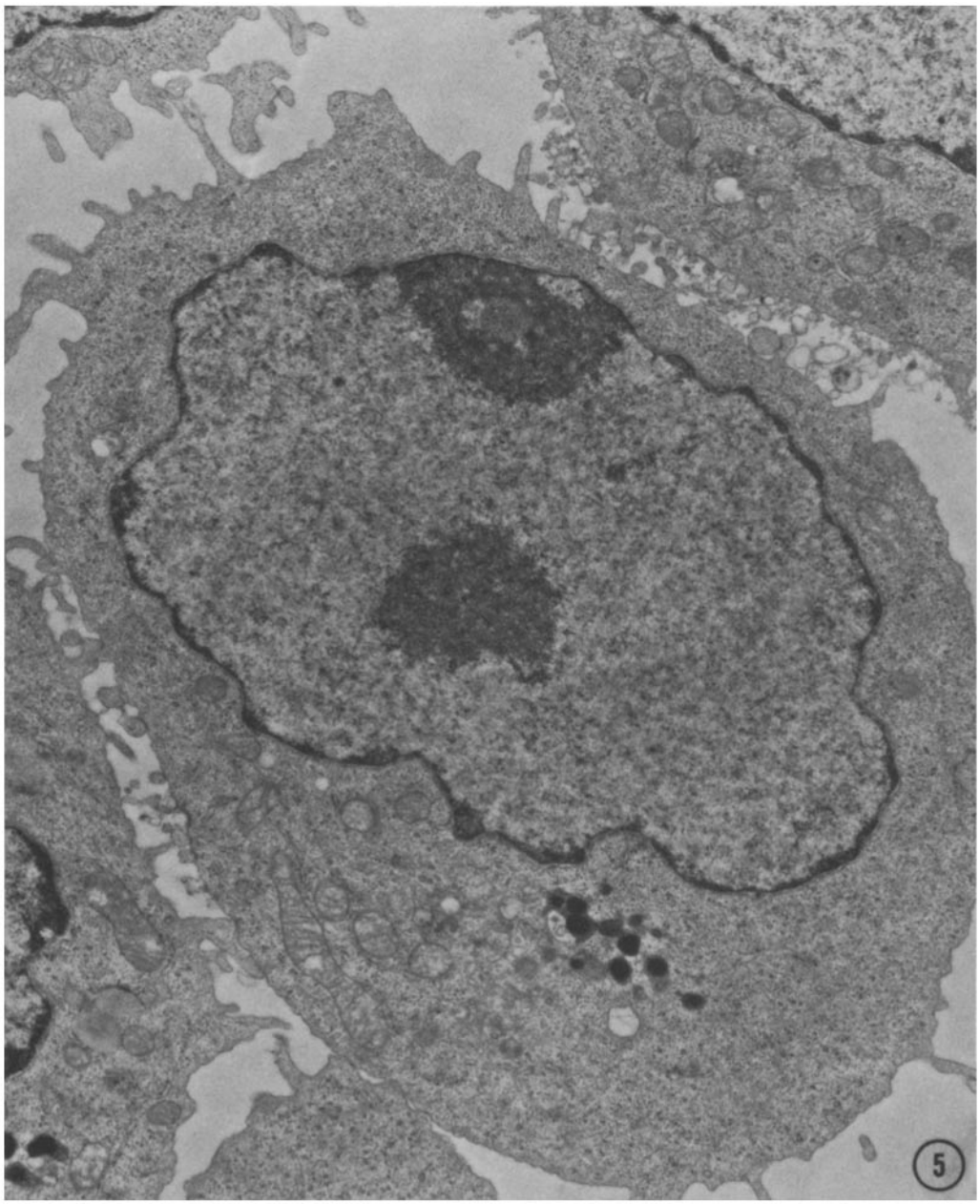

FIG. 5. Electron micrograph of a large lymphoid cell in the ARA-C-treated population. The nucleus is characterized by a dispersed chromatin and two nucleoli with a fibrillar center. The cytoplasm is abundant, with polyribosomes and ergastoplasmic profiles, several mitochondria, and inclusions in the vicinity of the Golgi region. 84% of the large lymphoid cells were of this type, irrespective of the presence or absence of ARA-C during restimulation. × 14,400.

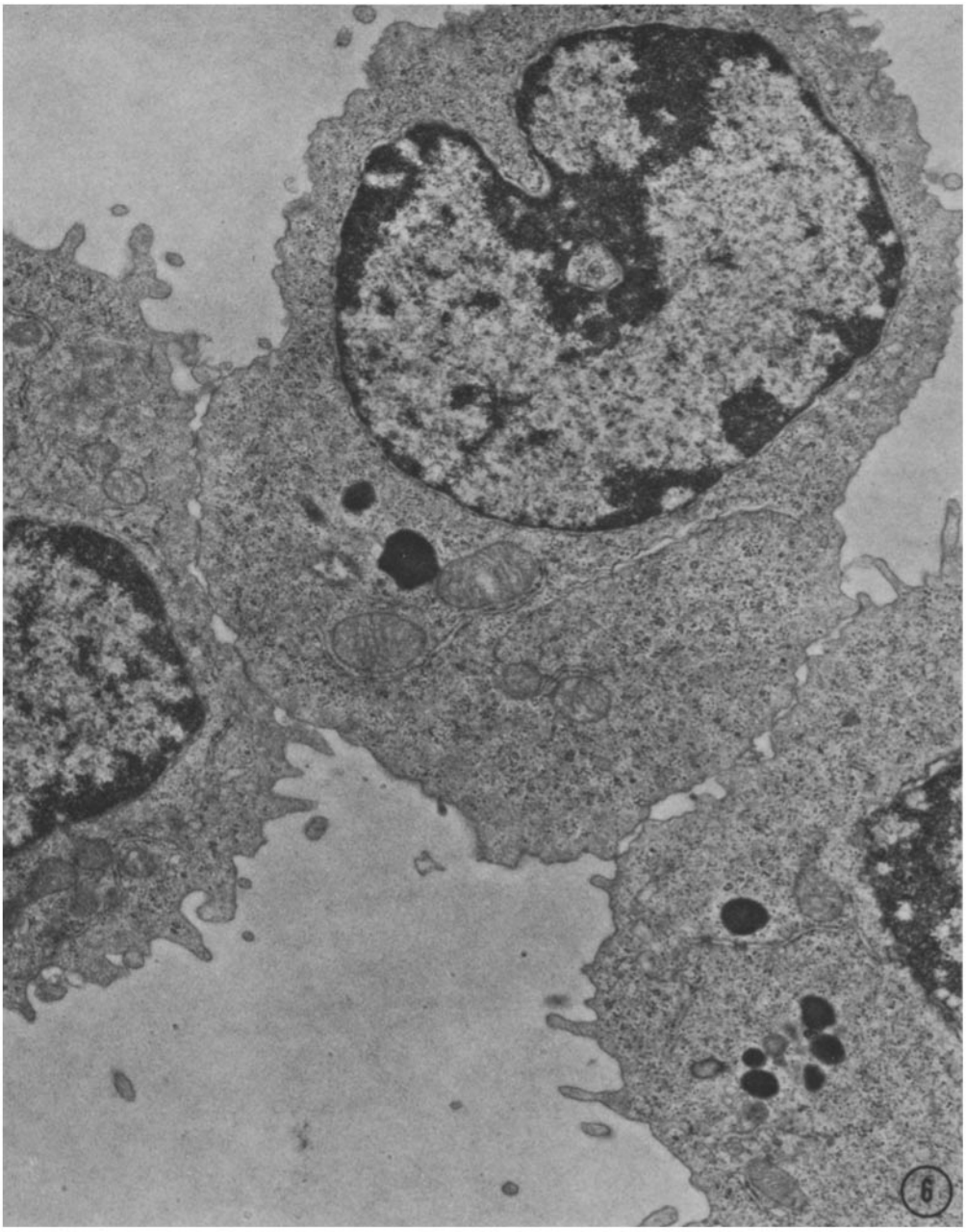

FIG. 6. Electron micrograph of a large lymphoid cell in the control population. The cell is representative of the second type of large lymphoid cells. It is slightly smaller, with a more condensed chromatin, dispersed ribosomes, and a more developed filamentous network. In both groups, 16% of the large lymphoid cells were of this type. × 18,000.

pletely inhibit cellular DNA synthesis (as assessed either by uptake of ^{125}IUdR or by autoradiographic analysis after incubation with low specific activity [^{3}H]TdR) had no effect on the increase in lytic activity observed during this time period.

The cellular events accompanying the appearance of CTL activity within 24 h after restimulation were characterized by cell separation and morphological analysis. Restimulation of a population containing primarily small lymphocytes (obtained after separation of day 14 MLC cells by velocity sedimentation at 1 *g*) in the presence or absence of ARA-C ended up 24 h later in a population containing 60, or 70% medium- to large-sized lymphoid cells, respectively. Identification of cells positive for MTLA, a T-cell marker, indicated that >99% of the restimulated large cells were T cells. Since no marker for CTL exists, it was necessary to directly establish the relationship between the formation of CTL and the appearance of large lymphoid cells. By using two successive cell separations, before and after restimulation, direct evidence was obtained that the CTL formed under these conditions had the sedimenting properties of medium- and large-sized lymphoid cells, irrespective of the presence or absence of ARA-C during restimulation. It is thus clear that the formation of active CTL, while independent of DNA synthesis, is associated with enlargment of small lymphocyte progenitors within the 24 h period. It should be stressed, however, that the actual frequency of CTL within the selected large lymphoid T-cell population remains to be determined.

A dissociation between morphological transformation and DNA synthesis has been observed previously after stimulation of T lymphocytes by mitogens (11). Häyry and Andersson (12) also found that the formation of large lymphoid cells after restimulation of long-term MLC cells with the relevant alloantigens was unaffected by the presence of hydroxyurea, an inhibitor of DNA synthesis, but no data on CTL function under such conditions were reported. Using a virus plaque assay, Jimenez et al. (13) observed that the antigen-dependent activation of presumably immune T cells was unaffected by the presence of a mitotic inhibitor. More recently, the differentiation of mitogen-stimulated B lymphocytes into functional antibody-producing cells in the absence of DNA synthesis has been demonstrated by Andersson and Melchers (14). In the latter study, the antibody-producing cells formed in the absence of DNA synthesis appeared to be relatively immature by morphological criteria. In the present study, since no CTL-specific marker exists, it was not possible to directly compare the morphology of CTL generated in the presence or absence of DNA synthesis. However, no major morphological difference was apparent when selected populations enriched in CTL generated either in the presence or the absence of ARA-C were compared.

A major problem in the interpretation of the present data is the lack of single cell assays for the detection of CTL. In this context, the increase in lytic activity of restimulated cultures observed within 24 h could reflect either the generation of CTL from noncytotoxic precursor cells or an increase in the per cell lytic capacity of residual CTL. In the former case, one could operationally define the increase in CTL activity differentiation (i.e., acquisition of differentiated function) whereas if the latter were true it would be more accurate to describe the

enhancement in activity in terms of maturation (i.e., quantitative increase in differentiated function). Methods for the quantitative estimation of CTL frequency are required to distinguish between these alternatives.

The presence of ARA-C for periods of longer than 24 h in restimulated cultures inhibited any further increase in CTL activity. Indeed, incubation of restimulated cells for 6 days in the presence of ARA-C resulted in reduction of LU per culture to below those values found in control unstimulated cultures. A nonspecific toxic effect of ARA-C after such extended incubation times seems unlikely in view of the fact that the lytic activity of long-term cultures exposed to equivalent concentrations of ARA-C for as long as 7 days in the absence of alloantigenic restimulation were completely unaffected by the drug (data not shown). On the contrary, the ultimate quantitative disappearance of lytic activity in the present of ARA-C in stimulated cultures strongly suggests that essentially all CTL underwent DNA synthesis at sometime during the secondary culture. The presence of 90% labeled cells in the medium and large lymphoid cell population 24 h after restimulation in the absence of ARA-C (Table III), and the even higher proportion of labeled cells found 1-2 days thereafter (data not shown) are consistent with this latter hypothesis.

As previous studies have suggested that the small lymphocytes in long-term MLC which differentiated into CTL after alloantigenic stimulation were themselves derived from large CTL present at the peak of the primary response (15, 16), the present findings support the hypothesis that CTL, or a fraction thereof, are not end cells but may become "memory" cells, which, although they are devoid of direct lytic activity, can be functionally reativated and morphologically transformed within 24 h after restimulation in the absence of DNA synthesis. Thereafter, under normal conditions, these large CTL undergo proliferation. As already stressed (17), since individual CTL cannot as yet be detected and isolated the relationship between CTL formed during the primary MLC response and those generated after restimulation of long-term MLC populations has not been directly established. Therefore, the possibility that CTL progenitors in long-term MLC are derived from an indepedent (but physically similar) responding population unrelated to primary CTL has to be excluded before the differentiation pathway mentioned above can be clearly established.

The experiments described in this report demonstrate a clear dissociation between DNA synthesis and the appearance of CTL activity at early times during a secondary in vitro allograft response. An interesting question raised by these findings is whether or not such a dissociation exists during the primary response to alloantigens. In this respect the dependence of CTL generation on cell proliferation has been documented in a number of experiments where cytostatic agents such as [^{3}H]TdR (18, 19), 5-bromo-2′-deoxyuridine (1, 20), and ARA-C (10) were found to strongly inhibit the development of optimal lytic activity in a primary in vitro response. However, it is significant that the sensitivity of the culture methods used in these studies was not sufficient to measure CTL activity at times as early at 24 h. Studies using extensively purified primary MLC cells are currently in progress to resolve this important question.

Summary

Re-exposure of day 14 mixed leukocyte culture (MLC) cells to the original stimulating alloantigens (secondary response) has previously been shown to result in significant proliferation and in rapid reappearance of high levels of cytolytic T-lymphocyte (CTL) activity within the next 4 days. Moreover, evidence has been presented that CTL precursor cells in day 14 MLC populations, while they derived from cells which were large at the peak of the primary response (day 4) were themselves small lymphocytes which developed into large CTL after restimulation.

In this study, inhibition of DNA synthesis by cytosine arabinoside (ARA-C) was used to investigate whether CTL formation could be dissociated from proliferation during the secondary response. It was found that within the first 24 h after restimulation (*a*) CTL activity increased 6-to-20-fold, (*b*) 60-70% of the small T lymphocytes became medium- to large-sized cells, and (*c*) both events were independent of DNA synthesis. By using two successive cell separations by velocity sedimentation at unit gravity, before and after restimulation of day 14 MLC cells for 24 h in the presence or absence of ARA-C, direct evidence was obtained that small CTL precursor cells developed into large CTL, irrespective of DNA synthesis.

The presence of ARA-C for periods longer than 24 h inhibited any further increase in CTL activity, in contrast to a parallel increase in lytic activity and cell number from day 1 to day 4 in control restimulated cultures. Taken together with the finding that 90% of the medium- and large-sized lymphoid cells in control restimulated cultures underwent DNA synthesis within 24 h, these results thus suggest that during a secondary MLC response there is initially a differentiation step leading to the formation of CTL which, although it can be clearly dissociated from DNA synthesis, is under normal conditions followed by proliferation of these effector cells.

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References

1. Häyry, P., L. C. Andersson, S. Nordling, and M. Virolainen. 1972. Allograft response in vitro. *Transplant. Rev.* **12**:91.
2. G. Möller, editor. 1973. Effector cells in cell-mediated immunity. *Transplant. Rev.* **17**:1.
3. Cerottini, J.-C., and K. T. Brunner. 1974. Cell-mediated cytotoxicity, allograft rejection, and tumor immunity. *Adv. Immunol.* **18**:67.
4. MacDonald, H. R., H. D. Engers, J.-C. Cerottini, and K. T. Brunner. 1974. Generation of cytotoxic T lymphocytes in vitro. II. Effect of repeated exposure to alloantigens on the cytotoxic activity of long-term mixed leukocyte cultures. *J. Exp. Med.* **140**:715.
5. Andersson, L. C., and P. Häyry. 1973. Specific priming of mouse thymus-dependent lymphocytes to allogeneic cells in vitro. *Eur. J. Immunol.* **3**:595.

6. Brunner, K. T., J. Mauel, J.-C. Cerottini, and B. Chapuis. 1968. Quantitative assay of the lytic action of immune lymphoid cells on ^{51}Cr labelled allogeneic target cells in vitro. Inhibition by isoantibody and by drugs. *Immunology*. **14**:181.
7. Cerottini, J.-C., H. D. Engers, H. R. MacDonald, and K. T. Brunner. 1974. Generation of cytotoxic T lymphocytes in vitro. I. Response of normal and immune mouse spleen cells in mixed leukocyte cultures. *J. Exp. Med.* **140**:703.
8. Miller, R. G., and R. A. Phillips. 1969. Separation of cells by velocity sedimentation. *J. Cell. Physiol.* **73**:191.
9. Cottier, H., J. Turk, and L. Sobin. 1972. A proposal for a standardized system of reporting human lymph node morphology in relation to immunological function. *Bull. W. H. O.* **47**:375.
10. Röllinghoff, M., J. Schrader, and H. Wagner. 1973. Effect of azathioprine and cytosine arabinoside on humoral and cellular immunity in vitro. *Clin. Exp. Immunol.* **15**:261.
11. Polgar, P. R., and S. Kibrick. 1970. Origin of small lymphocytes following blastogenesis induced by short-term PHA stimulation *Nature (Lond.)*. **225**:857.
12. Häyry, P., and L. C. Andersson. 1974. Generation of T memory cells in one-way mixed lymphocyte culture. II. Anamnestic responses of "secondary" lymphocytes. *Scand. J. Immunol.* **3**:823.
13. Jimenez, L., B. R. Bloom, M. R. Blume, and H. F. Oettgen. 1971. On the number and nature of antigen-sensitive lymphocytes in the blood of delayed-hypersensitive human donors. *J. Exp. Med.* **133**:740.
14. Andersson, J., and F. Melchers. 1974. Maturation of mitogen-activated bone marrow-derived lymphocytes in the absence of proliferation. *Eur. J. Immunol.* **4**:533.
15. MacDonald, H. R., J.-C. Cerottini, and K. T. Brunner. 1974. 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. *J. Exp. Med.* **140**:1511.
16. Andersson, L. C., and P. Häyry. 1974. Generation of T memory cells in one-way mixed lymphocyte culture. I. Selective recovery of activated cells and their reversion to "secondary" lymphocytes. *Scand. J. Immunol.* **3**:461.
17. Cerottini, J.-C., H. R. MacDonald, H. D. Engers and K. T. Brunner. 1974. Differentiation pathway of cytolytic T lymphocytes: in vivo and in vitro studies. *In* Progress in Immunology II. L. Brent and J. Holborow, editors. North-Holland Publishing Co. Amsterdam, The Netherlands. **3**:153.
18. MacDonald, H. R., R. A. Phillips, and R. G. Miller. 1973. Allograft immunity in the mouse. II. Physical studies of the development of cytotoxic effector cells from their immediate progenitors. *J. Immunol.* **111**:575.
19. Cantor, H., and J. Jandinski. 1974. The relationship of cell division to the generation of cytotoxic activity in mixed lymphocyte culture. *J. Exp. Med.* **140**:1712.
20. Clark, W., and J. Nedrud. 1974. Effect of BUdR on proliferation and development of cytotoxicity in mixed leukocyte culture. *Cell. Immunol.* **10**:159.