QUANTITATIVE STUDIES ON THE PRECURSORS
OF CYTOTOXIC LYMPHOCYTES

VI. Second Signal Requirements of Specifically Activated Precursors
Isolated 12 h after Stimulation*

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In the mixed lymphocyte reaction (MLR),[1] cytotoxic T lymphocytes (CL) are generated after the activation and subsequent proliferation and differentiation of a specific subclass of T cells, cytotoxic T lymphocyte precursor cells (CLP) (1–3). The exact sequence of events in the activation of CLP remains controversial. The CLP appear to require two separate signals. In a murine MLR in which the strains differ at H-2, one of these is provided when specific CLP in the responder population recognize H-2 K and/or D molecules on cells of the stimulator (2). It is the nature of the second signal, particularly its source, which is not clear. A second subset of cells within the responder cell population, usually identified as T-helper cells (2, 4), has been widely implicated as the source of the second signal (2, 5, 6). Alternatively, it may be provided by all cells or some subset of cells within the stimulator cell population (3).

One limitation to the analysis of CLP activation and, therefore, the distinction between the different two-signal models, is that no method exists for isolating CLP at early times after allogeneic stimulation. We have previously reported (7) that activated lymphocytes can be detected in a flow cytometer-cell sorter using the nontoxic (8) and DNA-specific (9) fluorescent probe, Hoechst 33342. We show here that CLP specific for the stimulator can be detected and separated from both other CLP and stimulator cells as early as 12 h after the initiation of an MLR. The isolation of specifically activated CLP after this short time in culture, before the onset of DNA synthesis, permits the dissection of the early sequence of events in the CLP-induction process.

Materials and Methods

Mice. Athymic RNC nu/nu (H-2^k), their heterozygous (RNC $nu/+$) normal littermates, C57BL/6J $nu/+$ (B6) (H-2^b), BALB/cBom $nu/+$ (BALB) (H-2^d), and the F_1 hybrid C57BL/6J $nu/+$ × BALB/cBom $nu/+$ (B6 × BALB)F_1 (H-$2^{b/d}$) were bred in the animal facility at the Ontario Cancer Institute, Toronto, Ontario, Canada. CBA/J (H-2^k) and DBA/2J (H-2^d) were obtained from The Jackson Laboratory, Bar Harbor, Maine.

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[1] *Abbreviations used in this paper:* CL, cytotoxic T lymphocyte; CLP, cytotoxic T lymphocyte precursor; Con A, concanavalin A; CSCS, concanavalin A-induced spleen cell supernate; FBS, fetal bovine serum; HI, high intensity; LI, low intensity; LN, lymph node; α-MEM, α-minimum essential medium; MLR, mixed lymphocyte reaction; PBS, phosphate-buffered saline.

Mixed Lymphocyte Cultures. Cell suspensions from lymph nodes (LN) (inguinal, axillary, and brachial) or spleens of 8- to 16-wk-old female mice were prepared aseptically by pressing the organs through a wire mesh (60 gauge) into our standard culture medium (α-minimum essential medium [α-MEM]) (Flow Laboratories, Inc., Rockville, Md.), 10% (vol/vol) heat-inactivated (56°C, 45 min) fetal bovine serum (FBS) (International Biological Laboratories, Rockville, Md., lot No. 3059), 0.01 M Hepes buffer, and 5×10^{-5} M 2-mercaptoethanol.

MLR were initiated by culturing 3×10^5 RNC $nu/+$ LN cells with 3×10^4 γ-irradiated (1,500 rad) P815 (H-2^d) mastocytoma cells maintained by weekly i.p. passage through DBA/2J (H-2^d) mice, in microtiter trays (Linbro Chemical Co., Hamden, Conn.; IS-MVC-96) containing 0.2 ml of standard culture medium.

Cell-Staining Procedures. The cells from about 140 mixed lymphocyte microcultures (RNC $nu/+$ LN anti-P815) were collected and pooled either at the time the cultures were set up (0 h) or after a 12-h incubation at 37°C. The cells were washed once in phosphate-buffered saline (PBS) and treated with trypsin and DNAase I to remove dead cells and cell aggregates as previously described (7). The cells were then collected by centrifugation and resuspended in phenol red-free α-MEM containing 10% FBS and 2.5 μM Hoechst 33342 (a kind gift from Dr. H. Loewe, Hoechst AG, Frankfurt, West Germany). After a 90-min incubation period at 37°C, the cells were washed once in PBS and resuspended in cold (4°C) PBS for analysis and sorting. Before restimulation, the sorted cell fractions were incubated in dye-free medium for 30 min to remove excess dye.

Flow Cytometry and Cell Sorting. The Ontario Cancer Institute flow cytometer-cell sorter and the instrumental configuration used for analyzing Hoechst-stained cells are described elsewhere (10, 7). The instrument is presently equipped with bidirectional sorting electronics which permit the simultaneous separation of two subpopulations and rejection of a third (M. J. McCutcheon and R. G. Miller. Manuscript in preparation.). The subpopulations are selected by the operator on the basis of their fluorescence intensity and/or their forward light scatter ($\pm 12°$ half-angle). Random contamination of the sorted cell pools by unwanted cells increases with flow rate as a result of a 20-μsec dead time in the analog processing electronics (11). The fraction of random contaminants can be reduced to <3% in the sorted fractions by limiting the flow rate to ~2,000 cells/sec and applying a 3-drop purity window (M. J. McCutcheon and R. G. Miller. Manuscript in preparation.). Our instrumentation and procedures are basically similar to those in widespread use elsewhere (12).

Restimulation of Sorted Populations. After cell sorting, varying numbers (10^4, 3×10^3, 10^3, and 3×10^2) of cells from each sorted fraction were restimulated in vitro with 3×10^5 gamma-irradiated (1,500 rads) (B6 $\times$ BALB)F$_1$ spleen stimulator cells and 1×10^5 RNC nu/nu spleen cells. The nu/nu spleen cells are required when small numbers of responder LN cells are cultured and appear to provide a required accessory cell lost through dilution (1). Eight replicate microcultures of each restimulated group were set up in microtiter trays containing 0.2 ml of our standard culture medium supplemented with 50 μg/ml gentamycin (Roussel Laboratories Ltd., Montreal, Quebec, Canada).

Preparation of concanavalin A-induced Spleen Cell Supernate (CSCS). CSCS was prepared by the method of Talmage et al. (13). Briefly, 3×10^8 CBA/J (H-2^k) spleen cells were incubated in plastic culture flasks (No. 3024, Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) containing 15 ml of α-MEM supplemented with 5×10^{-5} M 2-mercaptoethanol and 5 μg/ml concanavalin A (Con A) (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.). After 2 h at 37°C, the adherent cells were washed three times with 10 ml of PBS. 30 ml of α-MEM containing 5×10^{-5} M 2-mercaptoethanol were then added to each flask. After an 18-h incubation at 37°C, the supernate was collected and concentrated 10-fold as previously described (13).

Cytotoxicity Assay. After a 5-d incubation at 37°C, each restimulated microculture was assayed for production of CL against both BALB (H-2^d) and B6 (H-2^b) target cells as previously described (14). Briefly, each culture is first divided into two halves of 0.10 ml each. 3×10^3 target cells, which are spleen Con A blasts grown as described previously (15), are labeled with Na[^{51}Cr]O$_4$ (New England Nuclear, Boston, Mass.) and added to each one-half culture. The trays are then centrifuged at 170 g for 5 min before being incubated for a further 4 h at 37°C. 100 μl of supernate are then counted in a well-type gamma counter.

The fractional specific lysis, p, is defined as: (observed counts − spontaneous counts)/(total
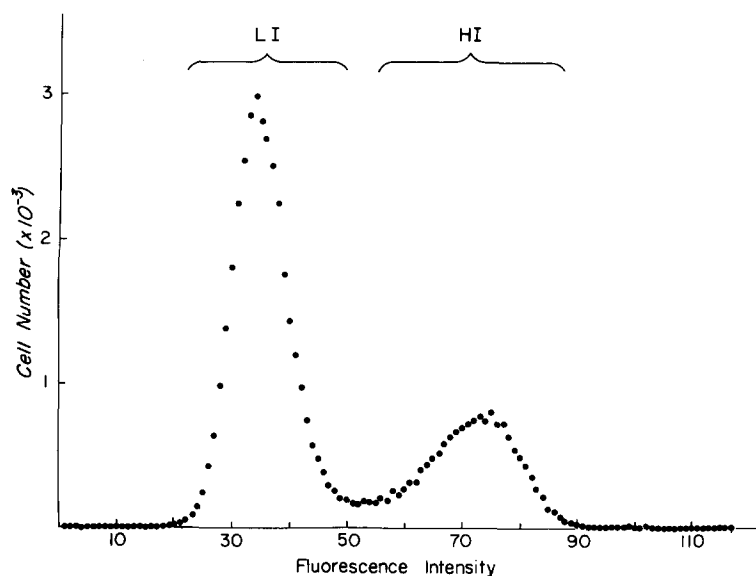
FIG. 1. Fluorescence analysis of Hoechst-stained lymphocytes. Cells obtained from 12-h priming (RNC nu/+ anti-P815) cultures were stained with 2.5 µM Hoechst 33342 dye and analyzed on the flow cytometer. The abscissa is a linear (128 channel) scale of fluorescence intensity and the ordinate is the cell number in thousands. The LI and HI fluorescence regions are defined by the brackets.

releasable counts − spontaneous counts). Spontaneous counts and total releasable counts were determined as described elsewhere (16). Under these assay conditions, the results can be reexpressed in terms of the cytotoxic activity: Nαt = −ln (1 − p), which is proportional to the number of CL generated and where N is the total number of sensitized cells, α is a constant proportional to the frequency of CL, and t is the assay time in hours (17).

Results

Flow Cytometry and Sorting of Hoechst-stained Cells. Fig. 1 shows the fluorescence distribution of cells obtained from RNC anti-P815 cultures 12 h after initiation and stained with Hoechst 33342 dye. As previously shown (7), the cells of the high-intensity (HI) fluorescence population contain responding T cells whereas the non-responding T cells are contained in the low-intensity (LI) fluorescence peak. Although Hoechst 33342 is a DNA-specific dye, the difference in fluorescence intensity between the LI and HI peaks is not a result of a difference in DNA content between the two populations. Instead (7), it appears to be a result of a physiological change occuring in the T cells as a result of the activation process. The two intensity peaks can be detected only when low concentrations of Hoechst dye (<5 µM) and short dye-incubation periods (<2 h) are used. The difference can be eliminated by increasing the dye concentration (7).

The cells obtained after either 0 or 12 h in culture were stained with Hoechst dye and the LI and HI fluorescence populations (shown in Fig. 1) were simultaneously sorted left and right using the Ontario Cancer Institute flow cytometer-cell sorter. For both time points, a second separation was performed in which LI and HI cells were sorted together as a control. In both separations, the stimulator P815 mastocytoma cells were excluded from the sorted fractions. This was possible because the P815
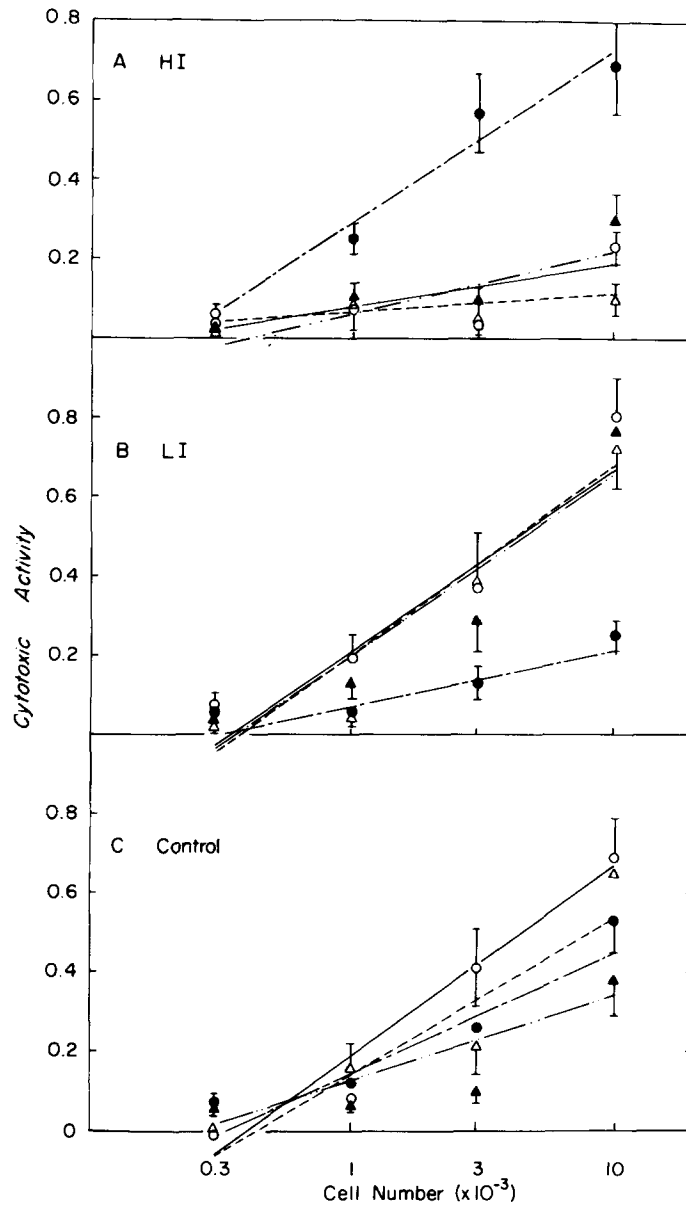
Fig. 2. Restimulation of sorted cell fractions. Cells obtained from RNC nu/+ anti-P815 cultures primed for 0 or 12 h were stained with Hoechst dye and the (A) HI, (B) LI, and (C) control (see text) fluorescence populations were sorted. Each fraction was then restimulated and assayed for production of CL as desribed in Materials and Methods. The ordinate is the cytotoxic activity, Nα (Materials and Methods), and the abscissa is a logarithmic scale of the number of sorted cells which were restimulated. The mean Nα values for eight replicate cultures as well as the curves giving the results of a logarithmic-linear fit to the data points are shown as: (▲—▲) anti H-2^d (specific) and (△– – –△) anti H-2^b (nonspecific) activity from 0-h priming cultures; (●- - -●) anti H-2^d and (○- · · -○) anti H-2^b activity from 12-h cultures.

TABLE I

Conditions for Obtaining CL from Activated CLP

Group	Additional cells or factor per culture*	Cytotoxic activity‡			
		Specific (anti-BALB)		Nonspecific (anti-B6)	
		HI	LI	HI	LI
1	None	-2 ± 1	1 ± 1	1 ± 1	3 ± 1
2	1×10^5 RNC nu/nu spleen	0 ± 1	1 ± 1	1 ± 1	2 ± 1
3	3×10^5 (B6 × BALB)F$_1$ stimulator	3 ± 2	2 ± 1	1 ± 1	3 ± 1
4	1×10^5 RNC nu/nu spleen + 3×10^5 (B6 × BALB)F$_1$ stimulator	57 ± 7	34 ± 7	9 ± 2	97 ± 13
5	CSCS	52 ± 6	5 ± 2	8 ± 2	10 ± 2

* 12 h after the initiation of RNC nu/+ anti-P815 priming cultures, the HI and LI peaks cells were sorted (see Fig. 1). For each group, 16 replicate cultures were set up, 8 containing 10^4 HI cells and 8 containing 10^4 LI cells. Additional cells or factor were added as indicated.

‡ After 5 d of incubation, each culture was split in two and assayed for cytotoxic activity against BALB and B6 target cells. The entries are Nα × 100 ± SE (Materials and Methods). An entry of 10 corresponds to 10% specific ^{51}Cr release, and an entry of 100, to 63% specific ^{51}Cr release.

mastocytoma cells have a larger cell volume than the lymphocytes and, therefore, can be distinguished separately on the basis of their increased forward light scatter. Microscopic examination of the sorted cell pools revealed that the fraction of P815 present was routinely <1%.

Restimulation of Sorted Cell Fractions. After cell sorting, the HI, LI, and control populations were restimulated in vitro as described in Materials and Methods. (B6 × BALB)F$_1$ (H-2^b × H-2^d) stimulator cells were used in the restimulation so that the cytotoxic activity against both the priming (H-2^d) alloantigen and a third-party (H-2^b) alloantigen, to which the H-2^k responder cells had not been primed, could be assessed. The results of the ^{51}Cr-release assay for the generation of CL against both alloantigens are presented in Fig. 2, which shows one of three experiments giving equivalent results.

In Fig. 2 A, it can be seen that the HI peak obtained from cells after 12 h of culture is enriched for CLP responsive to the priming antigen (H-2^d) as compared to the third-party response (H-2^b) or either the H-2^d or H-2^b response from 0-h cultures. Conversely (Fig. 2 B), the LI peak after 12 h is specifically depleted of anti H-2^d CLP compared to the other three groups. No significant changes in cytotoxic activity are seen in the control (Fig. 2 C). Using limiting dilution analysis (1), all CLP found in the control can be accounted for as being in either the HI or LI peak. It therefore appears that anti H-2^d CLP, and only anti H-2^d CLP, have specifically moved from the LI to the HI peak during the 12-h priming culture.

We next investigated the effect of varying the conditions for restimulating HI and LI cells obtained from 12-h priming cultures. In Table I, it can be seen that CL are not produced when the cells from either peak are cultured in medium only (group 1) or when either nu/nu spleen cells (group 2) or stimulator spleen cells (group 3) are added alone. When both nu/nu spleen cells and stimulator cells are added (group 4),

the HI and LI peaks are respectively enriched in and depleted of anti H-2^d cytotoxic activity as was observed in Fig. 2. The levels of nonspecific, third-party activity in both peaks are also similar to those observed in Fig. 2. In particular, third-party activity is present but is found almost exclusively in the LI peak.

A very different result was reproducibly obtained when CSCS, prepared as described in Materials and Methods, is used (group 5). When the sorted fractions were restimulated with CSCS, but without added nu/nu spleen cells or stimulator cells, the specific (anti H-2^d) CLP in the HI peak produced CL. Specific CLP remaining in the LI peak do not produce CL. There is little nonspecific, third-party activity produced from either the LI or HI peak. Thus, the specifically activated CLP present in the HI peak differ from other CLP with respect to their response to CSCS.

Discussion

The results shown in Fig. 2 indicate that the specific activation of CLP is associated with a transition of these cells from the LI to the HI fluorescence population. This transition occurs within 12 h of the CLP-P815 interaction. The enrichment for specifically activated CLP in the HI peak can be observed as early as 6 h after stimulation in an MLR, although it is less pronounced than at 12 h; after 18 h of culture the enrichment is only slightly better than at 12 h (data not shown). Whether the transition of the CLP from the LI to the HI peak reflects a cell cycle G$_0$ to G$_1$ transition is not known but it is known that detectable DNA synthesis in the MLR does not occur until after 24 h of culture (18). The physiological change which results in the increase of Hoechst fluorescence intensity of the activated CLP can however be used as a marker for an early event in the process of induction of CLP.

The relative number of cells in the LI (~70%) and HI (~30%) fluorescence population does not change significantly between 0 and 12 h after stimulation. The fraction of cells in the HI peak does not, therefore, reflect the total number of cells which have been activated during the 12-h MLR, although the activation of specific CLP can be demonstrated (Fig. 2). In freshly excised and unstimulated LN cells, the fraction of HI cells is reduced from 30 to 5% by passing the cells through a nylon-wool column before staining with Hoechst dye (unpublished data). As nylon-wool passed cells are enriched for T cells and depleted for B cells (19), the majority of the cells in the HI peak before stimulation appear to be non-T cells.

The specifically activated CLP (anti H-2^d) of the HI peak from 12-h cultures generate CL when both nu/nu spleen and stimulator cells are added to the restimulation cultures but not if either one is omitted (Table I). They will also produce CL if CSCS is added (Table I). The activated CLP seem, therefore, to require a second signal for production of mature CL. CSCS has been implicated to be a source of second signal (13, 20, 21). Because, in the presence of CSCS, specifically activated (anti H-2^d) CLP produce CL without a requirement for added stimulator cells (Table I), the activated CLP can apparently produce CL without any further requirement for alloantigen. The cells were treated with trypsin before staining and sorting and, therefore, any P815 alloantigens which could be attached to the surface of CLP after their interaction with P815 and which could account for the antigen-independent response of the activated CLP to CSCS have been removed.

We conclude that the CLP which undergo a transition to the HI peak as a result of alloantigenic stimulation are in a primed state of differentiation where the presence

of an apparently nonspecific second signal in the absence of either stimulator cells or helper cells is sufficient for proliferation and differentiation into mature CL. This primed state is analogous to the poised state of CLP differentiation described by Bach et al. (2). A factor similar to CSCS appears to be produced after ~15 h in a normal MLR (5). When CSCS is not added, both nu/nu spleen and stimulator cells must be added for production of CL by the CLP in the primed state. Earlier experiments (21) indicate that nu/nu spleen cells are providing a helper cell. Thus we tentatively conclude that the second signal for CL production is CSCS factor and that it is produced by helper cells after their stimulation by stimulator cells.

Summary

It is shown that, in a mixed lymphocyte reaction, the production of cytotoxic T lymphocytes (CL) from cytotoxic T lymphocyte precursors (CLP) requires two signals which are separated in time. Using a flow cytometer-cell sorter and a vital, fluorescent DNA stain, Hoechst 33342, CLP specific for the stimulator cells can be separated from other CLP and from stimulator cells 12 h after initiation of mixed lymphocyte cultures. These CLP are in a state of partial activation and can produce CL in the absence of stimulator cells if a second signal in the form of a concanavalin A-induced spleen cell supernate factor is added. Specific CL are also generated when the partially activated CLP are cultured with both nude spleen cells and stimulator cells. In this case it appears that an interaction between the stimulator cells and the nude spleen cells leads to production of the second signal.

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