

---

**Brief Definitive Report**

---

**CYTOLYTIC T LYMPHOCYTE FUNCTION  
IS INDEPENDENT OF GROWTH PHASE AND POSITION  
IN THE MITOTIC CYCLE**

BY RAFICK P. SEKALY, H. ROBSON MACDONALD, PIERRE ZAECH,  
ANDREW L. GLASEBROOK, AND JEAN-CHARLES CEROTTINI

*From the Ludwig Institute for Cancer Research, Lausanne Branch, Epalinges, Switzerland; and the  
Department of Immunology, Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland*

The influence of cell cycle on antigen expression has previously been demonstrated for a variety of lymphocyte surface antigens including murine and human histocompatibility antigens (1-2) and tumor-associated antigens (3). Other studies have shown that immunological reactions such as lysis of target cells by cytolytic T lymphocytes (CTL) are also related to the cell cycle status and growth of the target cells. For instance, L cells (4) and P815 mastocytoma cells (5) were reported to have an increased susceptibility to CTL-mediated lysis during the G1 phase, although the latter results are controversial (6). In contrast, due to the difficulty of obtaining homogeneous populations of effector T cells, very few attempts have been made to correlate CTL function with the cell cycle phase. However, with the recent demonstration (7) that CTL clones can be derived and maintained in medium containing soluble growth factor(s) (usually referred to as T cell growth factor [TCGF] or interleukin 2), this question is now amenable to experimentation.

In the present study we have investigated a possible cell cycle and growth phase regulation of the lytic activity of two CTL clones. To circumvent the difficulties encountered in conventional synchronization techniques, we have used the recently described (8) Hoechst 33342 bisbenzimidazole dye, which stains cells quantitatively according to their DNA content without affecting viability, and a fluorescence-activated cell sorter (FACS) to obtain pure populations of G1, S, and G2 cells. It will be shown that the cytolytic activity of the clones tested remained unchanged throughout the cell cycle.

**Materials and Methods**

*Cells.* 88/20 and L3C5 are independently derived C57BL/6 anti-DBA/2 CTL clones obtained under limiting dilution conditions as described previously (9, 10). The clones were kept in continuous culture by weekly passage in flat-bottomed 16-mm-Diam wells (Costar, Data Packaging, Cambridge, Mass.) containing 2 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% (vol:vol) fetal bovine serum (FBS),  $5 \times 10^{-5}$  M 2-mercaptoethanol, and additional amino acids.  $5 \times 10^6$  irradiated allogeneic (DBA/2) spleen cells and secondary mixed lymphocyte culture supernate (2° MLC SN) (11) at a final concentration of 20% were also added in each well.

*Cell Staining Procedure.* Cells were harvested from the culture 2 and 5 d after their initial plating. Dead cells and aggregates remaining from the stimulating population were removed after centrifugation over a Ficoll-Hypaque gradient ( $d = 1.085$ ). The interface cells were collected, washed twice, and then resuspended in DMEM containing 2% FBS and 6  $\mu$ g/ml of

Hoechst 33342 (a gift from Dr H. Loewe, Hoechst AG, Frankfurt, Germany). After a 90-min incubation period at 37°C, the cells were washed once and resuspended in cold DMEM for analysis and sorting.

*Flow Cytometry and Cell Sorting.* DNA analysis and sorting of cells were performed using a Spectra-Physics argon ion laser (Spectra-Physics, Inc., Mountain View, Calif.) and conventional FACS II system (B-D FACS Systems, Mountain View, Calif.) modified to allow three-parameter sorting. The laser was adjusted to emit 50 mW at 351 and 363 nm. Fluorescence was detected without any intervening optical filters. For sorting, narrow angle forward light scatter was used to identify viable cells. G1, S, and G2 populations were selected on the basis of their fluorescence intensity. The fraction of random contaminants could be reduced by limiting the flow rate to ~2,000 cells/s and applying a three-drop deflection criterion. The deflected cells were collected over a period of 30 min in 5-ml tubes coated with 0.5 ml of FBS. Cell viability in all experiments was always >90% and the maximum contamination of sorted cells was <5%.

*Target Cells.* P815 mastocytoma cells were maintained in culture and labeled with Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> as described previously (12).

*Assay for Cytolytic Activity.* Cytolytic activity was measured in a short-term <sup>51</sup>Cr release assay as described in detail elsewhere (13). Briefly, varying numbers of effector cells were mixed in 0.2 ml of assay medium in conical-bottomed microplates (C. A. Greiner and Söhne GmbH and Co., K. G. Nürtingen, West Germany) with 3 × 10<sup>3</sup> <sup>51</sup>Cr-labeled P815 mastocytoma target cells. Plates were centrifuged at 200 g for 3 min. After a 30-min incubation at 37°C, 20 μl of EDTA was added to a final concentration of 10 mM in each microwell. This was followed by a further 90-min incubation at 37°C to allow <sup>51</sup>Cr release from damaged target cells. To terminate the assay, the plates were centrifuged at 500 g for 5 min and 150 μl of supernate was removed from each well and counted in a well-type scintillation counter. The percentage of specific <sup>51</sup>Cr release was calculated as described previously (12), where spontaneous release was determined in the absence of effector cells and maximal release was determined in the presence of 0.5 N HCl.

*Analysis of DNA Distribution.* A semiquantitative method was used to estimate the relative number of cloned CTL in the different phases of the cell cycle. The G1 and G2 plus M phase populations were assumed to be normally distributed, and the mean fluorescence intensities were determined directly from the DNA distribution. Standard deviations were calculated by dividing the full width at half maximum of each peak by 2.35. The G1 population was defined as comprising the region of the DNA distribution within 2 SD above and below the mean fluorescence intensity of the G1 peak. The G2 plus M population was similarly ascribed to the region of the distribution within 2 SD of the G2 plus M peak. S-phase cells were defined as having fluorescence intensities distributed between the G1 and G2 plus M regions.

## Results and Discussion

L3C5 and 88/20 are two independently derived CTL clones with doubling times of about 10 h as calculated from the exponential phase of their growth curves. The two CTL clones were stained under supravital conditions with the DNA-binding dye Hoechst 33342 2 d after their initial plating with irradiated allogeneic cells in medium supplemented with 2° MLC SN as a source of TCGF. Preliminary studies established that the clones were growing exponentially at this time. Cells remained fully viable after 90 min incubation with dye as determined by trypan blue exclusion. Flow cytometric analysis of the DNA content of 50 × 10<sup>3</sup> cells from CTL clone L3C5 (Fig. 1A) was consistent with what would be expected for a proliferating cell population. The peak of high fluorescence intensity (G2 plus M) was approximately double that of low fluorescence intensity (G1) and the proportion of cells in the G1, S, and G2 plus M phases of the mitotic cycle was calculated to be 45, 27, and 28%, respectively. CTL clone 88/20 showed similar growth and cell cycle kinetics (data not shown). When harvested 2 d after initial plating, 48% of 88/20 cells were in G1, 28% were in S, and 24% were in G2 plus M. The data illustrated in Fig. 2A (clone 88/20)

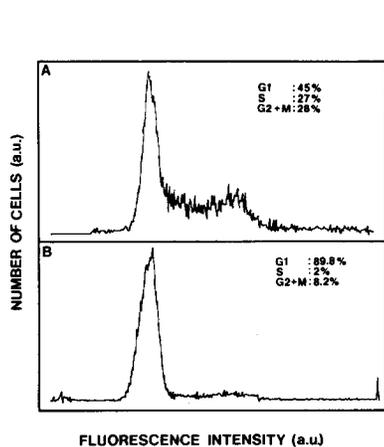


FIG. 1

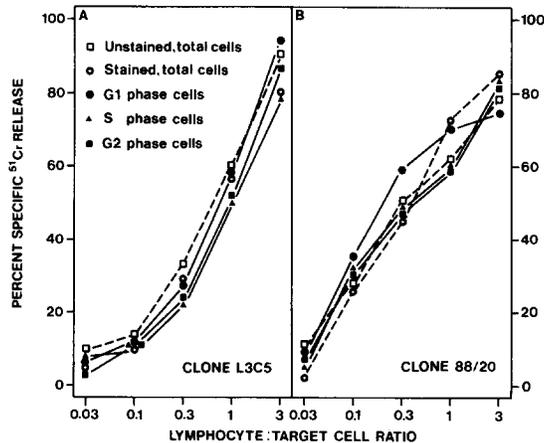


FIG. 2

FIG. 1. DNA distribution of a viably stained CTL clone in exponential or plateau phase of growth. Cells from CTL clone L3C5 were harvested 2 (A) and 5 (B) d after their initial plating. After Ficoll-Hypaque density sedimentation, the interface cells were stained with Hoechst 33342 (6  $\mu\text{g}/\text{ml}$  for 90 min at 37°C) and run on a FACS II gated to exclude nonviable cells. Percentages of cells in G1, S, and G2 plus M phases of the mitotic cycle were calculated as detailed in Materials and Methods.

FIG. 2. Cytolytic activity of CTL clones as a function of position in the mitotic cycle. Exponentially growing CTL clones L3C5 (A) and 88/20 (B) were stained with Hoechst 33342 and analyzed for DNA content as described in Fig. 1A. G1, S, and G2 cells were then sorted and tested for lytic activity against  $3 \times 10^3$   $^{51}\text{Cr}$ -labeled P815 target cells using a short-term (30 min) assay. Open symbols represent the cytolytic activity of control unstained ( $\square$ ) or stained ( $\circ$ ) CTL that had been passed through the FACS but not separated according to fluorescence intensity. Closed symbols illustrate cytolytic activity of sorted G1 ( $\bullet$ ), S ( $\blacktriangle$ ) and G2 ( $\blacksquare$ ) cells.

and 2B (clone L3C5) indicate that unstained sorted CTL clones had high cytolytic activity as measured in a short-term  $^{51}\text{Cr}$  release assay. In fact, cells from clone 88/20 and clone L3C5 lysed 50% of  $^{51}\text{Cr}$ -labeled P815 targets within 30 min at effector to target cell ratios of 0.3:1 and 1:1, respectively. It should also be noted that the lytic activity of the cloned cells stained with Hoechst 33342 was not reduced when compared with the respective unstained populations (Fig. 2A and 2B). To test the possible cell cycle dependence of cytolytic function,  $40 \times 10^3$  G1, S, or G2 cells were sorted from both clones and tested at several lymphocyte to target cell ratios. The data obtained for both 88/20 (Fig. 2A) and L3C5 (Fig. 2B) indicated that cytolytic activity remained unchanged throughout the different phases of the mitotic cycle. In interpreting these results, it is important to note that the addition of EDTA after 30 min prevents any de novo initiation of target cell lysis (13), thus restricting the cytotoxic assay to a short time relative to the duration of the cell cycle. Previous reports have provided indirect evidence that there was no correlation between cytolytic activity and cell cycle stage. Thus, MacDonald (14) has demonstrated that there was no difference in the lytic activity of different fractions of asynchronously cycling populations of restimulated MLC lymphocytes separated by velocity sedimentation at unit gravity. Using the same separation method, von Boehmer et al. (15) obtained identical results with a CTL clone specific for H-Y antigen. It should be noted, however, that velocity sedimentation methods, which separate cells according to the square of their radius (16), allow only a partial synchronization of exponentially growing cells because size dispersion of cells at a given stage of the cell cycle cannot

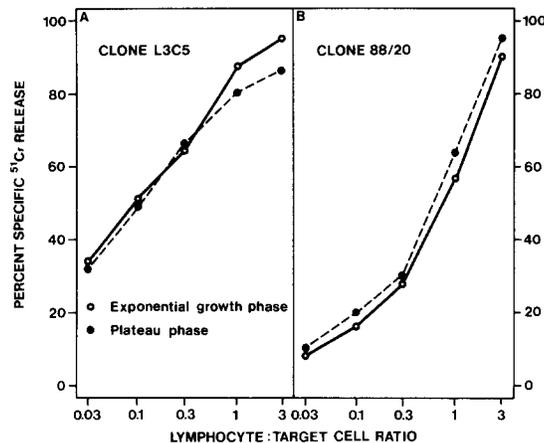


FIG. 3. Cytolytic activity of CTL clones in exponential or plateau growth phase. Cells from CTL clones L3C5 (A) and 88/20 (B) were collected either 2 (○) or 5 (●) d after initial plating and tested for lytic activity against  $3 \times 10^3$  <sup>51</sup>Cr-labeled P815 target cells using a short-term (30 min) assay.

be overcome by this technique (17). On the other hand, sorting asynchronous cells according to their DNA content, as performed in the present study, is a direct method of obtaining cells at a given position in the mitotic cycle.

Growth stimulation of quiescent cells has been shown to lead to profound changes in their metabolic state (17). Therefore, we have studied the cytolytic activity of CTL clones 88/20 and L3C5 arrested in quiescent cultures or restimulated to growth upon addition of fresh medium and TCGF. In particular, CTL clone L3C5 was grown for 5 d, thus reaching a quiescent state that (as illustrated in Fig. 1B) was characterized by a twofold increase in the relative number of cells in the G1 phase of the cell cycle as compared with exponentially growing cultures (Fig. 1A). Fig. 1B demonstrates the small number of cells (<5%) in the DNA synthetic phase, thus further confirming the quiescent state of these cultures. Flow cytofluorometric analysis of the DNA content of clone 88/20 after 5 d of culture was similar to that obtained for clone L3C5 (data not shown). Each clone, growing exponentially (i.e., after 2 d) or arrested in plateau phase (i.e., after 5 d), was assayed for lytic activity on <sup>51</sup>Cr-labeled P815 target cells. The results obtained from these experiments (Fig. 3A and B) demonstrate that the cytolytic activity of CTL from quiescent or exponentially growing cultures was identical. It is thus evident that lytic activity was not correlated to the growth phase of the two CTL clones tested.

Previous reports relating T cell-mediated cytolysis to the mitotic cycle have been difficult to interpret because drug treatment or physical separation methods were used for synchronization (4-6). Such drugs may by themselves alter the susceptibility of target cells to CTL activity, whereas physical separation methods (as mentioned above) allow only partial synchronization of either effector or target cells. These difficulties have been overcome in the present study by using viable DNA staining and sorting of effector cells according to their position in the mitotic cycle. Furthermore, the use of CTL clones that represent highly cytotoxic and homogeneous populations excludes possible artefacts that might be due to modulation of immune function by contaminating subpopulations of T cells or other leukocytes. T cell clones

with other distinct immunological functions have been recently developed in our laboratories. They will enable us to look at possible correlations between the mitotic cycle and immunological activities that can be measured in short-term assays.

### Summary

We have investigated mitotic cell cycle and growth phase regulation of homogeneous cytolytic T lymphocytes (CTL). Two independently derived CTL clones were stained with the DNA-binding dye Hoechst 33342, sorted in a fluorescence-activated cell sorter according to their position in the cell cycle, and then assayed for specific lytic activity using a short-term (30 min)  $^{51}\text{Cr}$  release assay. Results show that lytic activity remained unchanged throughout the cell cycle. Furthermore, there was no significant difference in the lytic activity of CTL clones growing exponentially or arrested in a plateau phase. These results demonstrate that T cell-mediated cytolysis is independent of growth phase and position in the cell cycle.

We thank C. Horvath and A.-L. Peitrequin for expert technical assistance.

*Received for publication 26 May 1981.*

### References

1. Pasternak, C. A., A. M. H. Warmsby, and B. Thomas. 1971. Structural alterations in the surface membrane during the cell cycle. *J. Cell. Biol.* **50**:562.
2. Everson, L. K., B. A. Plocinik, and G. N. Rogentine. 1974. HL-A expression in the G1, S, and G2 cell cycle stages of human lymphoid cells. *J. Natl. Cancer Inst.* **53**:913.
3. Cikes, M., and S. Friberg, Jr. 1971. Expression of H2 and Moloney leukemia virus determined cell surface antigens in synchronized cultures of a mouse cell line. *Proc. Natl. Acad. Sci. U. S. A.* **69**:566.
4. Leneva, N. V., and G. J. Svet-Moldavsky. 1974. Susceptibility of tumor cells in different phases of the mitotic cycle to the effect of immune lymphocytes. *J. Natl. Cancer Inst.* **52**:696.
5. Sanderson, C. J., and J. A. Thomas. 1976. The mechanism of T cell mediated cytotoxicity. III. Changes in target cell susceptibility during the cell cycle. *Proc. R. Soc. Lond. B Biol. Sci.* **194**:417.
6. Ching, L. M., J. B. Gavin, J. Marbrook, and M. Skinner. 1976. The surface morphology and the cell cycle of mastocytoma target cells: no apparent effect on cell-mediated killing. *Aust. J. Exp. Biol. Med. Sci.* **54**:137.
7. Gillis, S., and K. A. Smith. 1977. Long-term culture of tumor specific cytolytic T cells. *Nature (Lond.)* **268**:154.
8. Arndt-Jovin, D. J., and T. M. Jovin. 1977. Analysis and sorting of living cells according to deoxyribonucleic acid content. *J. Histochem. Cytochem.* **25**:585.
9. Glasebrook, A. L., M. Sarmiento, M. R. Loken, D. P. Dialynas, J. Quintans, L. Eisenberg, C. T. Lutz, D. Wilde, and F. W. Fitch. 1981. Murine T lymphocyte clones with distinct immunological functions. *Immunol. Rev.* **54**:225.
10. MacDonald, H. R., J.-C. Cerottini, J.-E. Ryser, J. L. Maryanski, C. Taswell, M. B. Widmer, and K. T. Brunner. 1980. Quantitation and cloning of cytolytic T lymphocytes and their precursors. *Immunol. Rev.* **51**:93.
11. Ryser, J.-E., J.-C. Cerottini, and K. T. Brunner. 1978. Generation of cytolytic T lymphocytes in vitro. IX. Induction of secondary CTL responses in primary long-term MLC by supernatants from secondary MLC. *J. Immunol.* **120**:370.
12. 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.
13. MacDonald, H. R. 1975. Early detection of potentially lethal events in T cell mediated cytotoxicity. *Eur. J. Immunol.* **5**:251.
  14. MacDonald, H. R. 1978. Restimulation of cytotoxic T lymphocytes in long-term mixed leukocyte cultures. I. Physical and proliferative characteristics of cytotoxic T lymphocytes restimulated at low cell density. *Cell. Immunol.* **35**:414.
  15. von Boehmer, H., H. Hengartner, M. Nabholz, W. Lernhardt, M. H. Schreier, and W. Haas. 1979. Fine specificity of a continuously growing killer cell clone specific for H-Y antigen. *Eur. J. Immunol.* **9**:592.
  16. Miller, R. G., and R. A. Phillips. 1969. Separation of cells by velocity sedimentation. *J. Cell. Physiol.* **73**:191.
  17. MacDonald, H. R., and R. G. Miller. 1970. Synchronization of mouse L cells by a velocity sedimentation technique. *Biophys. J.* **10**:834.
  18. Rudland, P. S., and L. J. de Asua. 1979. Action of growth factors in the cell cycle. *Biochim. Biophys. Acta.* **560**:91.