Brief Definitive Report

PRIMARY GENERATION OF CYTOLYTIC T LYMPHOCYTES IN THE ABSENCE OF DNA SYNTHESIS

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Recent developments in tissue-culture methodology have greatly facilitated the detailed analysis of the differentiation pathway of cytolytic thymus-derived lymphocytes (CTL), particularly in mixed leukocyte cultures (MLC) in the mouse (1, 2). In such studies, particular attention has been paid to the functional activation in long-term MLC of the progeny of CTL which had been generated during a primary response. These cells, which are hereafter referred to as memory CTL, have little, if any, residual lytic activity, but respond by proliferation and differentiation after specific or nonspecific stimulation (1-3). Because of the enhanced reactivity of such cell populations in both quantitative and kinetic terms, it has been possible to analyze the early events in memory-cell activation in some detail. From such studies, it was established that memory CTL were small lymphocytes that could enlarge and acquire greatly increased cytolytic activity within 24 h in the absence of DNA synthesis (4). After this initial stage, subsequent increases in CTL activity were primarily related to cellular proliferation (5).

In contrast to the situation with memory CTL, little is known about the early events in the activation of putatively virgin CTL precursor cells (CTL-P). Although it has been shown that the appearance of detectable levels of CTL activity in MLC is dependent on cellular proliferation (6-8), it is difficult to exclude the possibility that this dependence may be quantitative (i.e., related to the presence of insufficient numbers of CTL-P to give rise to detectable CTL activity without proliferation). In addition, it is possible that the requirement for proliferation may not be related to the CTL-P themselves, but rather to another (helper) cell type that is required for efficient CTL generation.

In the present report, we have attempted to circumvent these difficulties in interpretation by investigating the polyclonal induction of CTL in the presence of concanavalin A (Con A). As shown initially by Bevan and Cohn (9), many, if not all, CTL-P in a heterogeneous population can be stimulated by Con A to give rise to CTL, and these CTL can be detected in a nonspecific cytolytic assay carried out in the presence of Bacto Phytohemagglutinin M (PHA). Using this sensitive system, we will demonstrate that primary CTL induction can occur in the absence of detectable DNA synthesis.

Materials and Methods

Mice. Adult C57BL/6 mice were obtained from the animal colony maintained at the Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland.

Generation of CTL in the Presence of Con A. C57BL/6 spleen cells (5-7 X 10⁶) were cultured in
flat-bottomed 16-mm wells (Costar, Data Packaging, Cambridge, Mass.) in 2 ml of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2-5% (vol/vol) fetal bovine serum (FBS), 5 × 10⁻⁴ M 2-mercaptoethanol, and additional amino acids (10). Con A (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.; 2 μg/ml) was added at the initiation of culture. In some experiments, cytosine arabinoside (ARA-C, Cytostar, The Upjohn Co., Puurs, Belgium) was also added. Cultures were maintained in a humidified atmosphere of 5% CO₂ in air. After 48 h, cells were washed and resuspended in 1 ml of DMEM containing 5% (vol/vol) FBS and 50 mM α-methyl-mannoside (assay medium).

**Target Cells.** P-815 mastocytoma cells were maintained in culture and labeled with Na₂⁵¹CrO₄ as described previously (10).

**Assay for Cytolytic Activity.** Cytolytic activity of Con-A-stimulated cells was assayed by a minor modification of the method of Bevan and Cohn (9). Briefly, varying numbers of effector cells were mixed in 0.2-ml assay medium in round-bottomed microplates (C. A. Greiner und Söhne GmbH and Co., K. G., Nurtingen, West Germany) with 10⁴ ⁵¹Cr-labeled P-815 mastocytoma target cells and an optimal final concentration (1:180) of PHA (Difco Laboratories, Detroit, Mich.). Plates were briefly centrifuged (200 g for 3 min) and incubated for 3-4 h at 37°C. The plates were then centrifuged again (500 g for 5 min) and 0.1 ml of supernate was removed and counted in a well-type sculllon counter. The percentage of specific Cr release was calculated as described previously (10), where spontaneous release was determined in the absence of effector cells and maximal release in the presence of 0.5 N HCl.

In some experiments, lytic units (LU) were calculated from dose-response curves as described previously (10). 1 LU is defined in the present study as the number of lymphoid cells required to lyse 30% of 10⁴ target cells under the assay conditions.

**Assay for DNA Synthesis.** DNA synthesis was assessed in Con-A-stimulated cultures by adding 0.5 μCi/ml [³H]thymidine (2.0 Ci/mmol; Radiochemical Centre, Amersham, England) for the duration of the culture. Aliquots of 0.2 ml (i.e., 1/10 culture) were harvested onto paper filters using a multiple-sample harvester (Skatron, Lierbyen, Norway). The filters were then dried, placed in scintillation fluid, and counted on a liquid scintillation counter programmed for quench correction (LKB Produkter, Bromma, Sweden). Results are expressed as the mean dpm ± 1 SD of quadruplicate aliquots.

**Removal of T Cells.** In some experiments, T cells were removed from populations of Con A-stimulated spleen cells by treatment with a heterologous anti-T-cell serum and complement as described previously (10).

**Results**

To assess the dependence of polyclonal CTL activation on DNA synthesis, we first determined the earliest time at which significant cytolytic activity could be detected after Con A stimulation. Fig. 1A summarizes an experiment in which normal C57BL/6 spleen cells were cultured in the presence of 2 μg/ml Con A for 24 or 48 h and then tested for cytolytic activity on ⁵¹Cr-labeled P-815 mastocytoma cells in the presence of PHA. High levels of cytolytic activity were detectable 48 h after Con A stimulation, whereas little or no activity was seen after 24 h. Spleen cells cultured in the absence of Con A for 24-48 h and fresh spleen cells were not significantly cytolytic under these conditions. In additional experiments, it was established that the lytic activity of 48-h Con-A-activated spleen cells was reduced from 23 to <1 LU/culture by treatment with rabbit anti-mouse T-lymphocyte antigen serum plus complement, indicating that only T cells were being detected in this system.

The role of DNA synthesis in the induction of CTL by Con A was then investigated. Fig. 1B shows the result of a representative experiment in which C57BL/6 spleen cells were cultured with Con A for 48 h in the presence or absence of 20 μg/ml of the DNA-synthesis inhibitor ARA-C and subsequently assayed for cytotoxicity in the PHA-dependent test. It is clear that Con-A-induced CTL generated in the presence
Kinetics of CTL induction by Con A. C57BL/6 spleen cells (7 x 10^6) were cultured with Con A (2 μg/ml) for 24 (■) or 48 h (●), and nonspecific CTL activity was measured at several lymphocyte:target-cell ratios against 10^4 51Cr-labeled P-815 tumor cells in the presence of PHA. Open symbols represent the cytolytic activity of fresh spleen cells (▲), or of spleen cells cultured without Con A for 24 (□) or 48 (○) h. (B) Effect of ARA-C on induction of CTL by Con A. C57BL/6 spleen cells were cultured with Con A for 48 h in the presence (A—A—A) or absence (●—●—●) of ARA-C (20 μg/ml), and assayed for CTL activity as in (A).

**Table I**

Effect of ARA-C on DNA Synthesis and Cytolytic Activity in Con A-Stimulated Spleen Cells *

| Dose of ARA-C (μg/ml) | Cell recovery (percentage of input) | [%] | [¹H]Thymidine incorporation (dpm ± SD) | Percentage of inhibition (%) | Cytolytic activity (LU/10⁶ cells) | LU/culture |
|-----------------------|-------------------------------------|-----|--------------------------------------|-----------------------------|-----------------------------------|------------|
| None                  | 47                                  | 57,368 ± 3,846 | —                                  | 11.1                        | 36                               |
| 5                     | 22                                  | 2,817 ± 146    | 95                                 | 7.1                         | 11                               |
| 10                    | 22                                  | 1,806 ± 158    | 97                                 | 9.5                         | 14                               |
| 20                    | 21                                  | 1,033 ± 29     | 98                                 | 16.7                        | 24                               |

* Aliquots of C57BL/6 spleen cells were stimulated with Con A for 48 h in the presence or absence of the indicated concentration of ARA-C. [¹H]Thymidine incorporation was assessed on triplicate samples for the entire culture period and the recovered cells were assayed for cytolytic activity against 51Cr-labeled P-815 target cells in the presence of PHA. 

of ARA-C were as cytolytic (on a cell-to-cell basis) as control CTL. Recovery of viable cells was, however, reduced in the ARA-C-treated cultures (15 vs. 33% in control cultures), thereby resulting in a decrease of total CTL activity by 32% (expressed as LU/culture). In a series of six experiments, the mean LU/10⁶ cells recovered in ARA-C-treated cultures was 108% of control and the mean LU/culture was 40%. That DNA synthesis was effectively inhibited by ARA-C under these conditions is shown in Table I. In this experiment, varying doses (5–20 μg/ml) of ARA-C were added to duplicate cultures containing C57BL/6 spleen cells stimulated by Con A in the presence or absence of [¹H]thymidine (1 μCi per culture). After 48 h, cultures were tested for either thymidine incorporation or cytolytic activity. It can be seen that
thymidine uptake was inhibited by >95% at all three doses of ARA-C tested; however, as observed previously (Fig. 1B), CTL activity developed in the presence of ARA-C. Interestingly, more LU were recovered from cultures in which higher doses of ARA-C were present. This unusual dose-response effect was observed in three independent experiments (data not shown).

Discussion

The acquisition of differentiated function by eukaryotic cells is a fundamental issue in cellular and molecular biology. In this context, it has been proposed by Holzer and his colleagues (11) that the derepression of genetic information required for cellular differentiation may occur preferentially during a particular round of DNA synthesis leading to a so-called quantal mitosis. Although evidence exists to support such a concept in various developmental systems (11, 12), only limited information is available for the differentiation of lymphocyte precursor cells into their functional progeny. Several studies of the antigen- or mitogen-induced differentiation of B lymphocytes have suggested a relationship with DNA synthesis in the sense that the development of plasma cells was inhibited by the thymidine analogue 5-bromo-2-deoxyuridine (BrdU) (13, 14), that has been shown to interfere with eukaryotic-cell differentiation in a number of systems (12, 15). Similarly, Nedrud et al. (8) reported that primary induction of specific CTL activity in MLC was inhibited by BrdU and also by hydroxyurea (an inhibitor of DNA synthesis), thereby suggesting that a quantal mitosis may be required for the initial expression of cytolytic function.

In contrast to the aforementioned study, the results presented in this report directly demonstrate that primary polyclonal induction of CTL activity by Con A in normal spleen populations does not require a round of DNA synthesis. Continuous exposure of Con-A-stimulated cells to doses of ARA-C which were sufficient to reduce [3H]-thymidine incorporation by 95-98% did not decrease (on a cell-to-cell basis) the ensuing CTL activity measured 48 h after initiation of culture. Furthermore, CTL generated in the presence of ARA-C were characterized as large cells by velocity-sedimentation cell separation (H. R. MacDonald, and R. K. Lees, unpublished data). Because the functional activation of memory CTL in a secondary response is known to be independent of DNA synthesis (4, 8, 16), it could be argued that the cells in normal spleen which are stimulated by Con A are memory CTL resulting from immunization with cross-reacting environmental antigens. This would seem unlikely, however, because no PHA-dependent cytolytic activity was observed either in fresh spleen cells or after 24-h culture in the presence of Con A. Memory CTL are known to differentiate into highly cytolytic cells within 24 h of restimulation (4, 8, 16).

Several explanations for the apparent discrepancy between the conclusions reached in the present study and in an earlier report (8) should be considered. Firstly, Nedrud et al. (8) studied CTL generation in MLC, whereas we have examined the appearance of CTL activity after polyclonal stimulation of CTL-P by the mitogen Con A. It is conceivable that induction of CTL by specific alloantigens involves a different molecular mechanism (or a greater dependence on proliferating accessory cells) than induction by nonspecific means. An alternative possibility, however, would be that the alloantigen-induced differentiation of specific CTL-P into CTL can also occur in the absence of DNA synthesis, but that the number of CTL so produced is too small to be detected without subsequent amplification by proliferation. By taking into
account recent estimates of the relative frequency of CTL-P in normal spleen populations (17, 18), it follows that up to 40-fold fewer CTL could be activated by specific alloantigens than by Con A under conditions where cell division is blocked. If this were the case, an apparent dependence of specific CTL induction on DNA synthesis would be observed.

Finally, it should also be noted that different metabolic inhibitors of DNA synthesis were used in the study of Nedrud et al. (8) and in the present report (i.e., hydroxyurea and ARA-C, respectively). In this context, we have recently found that hydroxyurea strongly inhibits CTL induction by Con A under conditions where ARA-C, at a comparable dose with respect to inhibition of DNA synthesis, has no inhibitory effect (H. R. MacDonald, and R. K. Lees. Manuscript in preparation). Although the mechanism of inhibition of CTL induction by hydroxyurea in this system remains unclear, this example serves to clearly illustrate the principle that a parallel reduction in two parameters (such as CTL induction and DNA synthesis) by a complex metabolic inhibitor is a necessary, but insufficient, criterion upon which to base a causal relationship. On the other hand, a dissociation between two parameters (such as the induction of CTL in the presence of DNA-synthesis-inhibiting doses of ARA-C) can be interpreted with greater certainty.

Summary

The requirement for DNA synthesis during the primary differentiation of cytolytic T lymphocytes (CTL) had been investigated. CTL were induced polyclonally in vitro by stimulation of normal C57BL/6 spleen cells with concanavalin A (Con A) and their cytolytic activity was tested against 51Cr-labeled target cells in the presence of Bacto Phytohemagglutinin M. With this system, CTL activity could first be detected 48 h after exposure of spleen cells to Con A. Addition of cytosine arabinoside at concentrations sufficient to reduce DNA synthesis by 95-98% in Con A-stimulated cultures did not significantly inhibit the generation of cytolytic activity on a cell-to-cell basis. These results demonstrate that derepression of the genetic information required for the expression of CTL function can occur in the absence of detectable DNA synthesis.

We wish to thank Eric Martz for helpful suggestions and Jean-Charles Cerottini for his critical reading of this manuscript.

Received for publication 15 March 1979.

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