QUANTITATIVE STUDIES ON THE PRECURSORS OF CYTOTOXIC LYMPHOCYTES

III. The Lineage of Memory Cells*

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The establishment of long-term mixed lymphocyte cultures (MLC)[1] (1–3) has made possible the characterization of cells that participate in a secondary in vitro cytotoxic response. The objective of this study was to determine if the lymphocytes which respond to a second antigen stimulation are derived from the same precursors that generate CL during a primary response to the same antigen. Previous studies involving the physical separation of various MLC populations on the basis of their sedimentation velocity at unit gravity have shown that cytotoxic lymphocytes (CL) in a primary MLC are derived from small- to intermediate-sized lymphocytes and have become large cells on day 4. On day 4 cells of similar size (perhaps CL) are present which, by day 14, have reverted to small noncytotoxic memory lymphocytes. These cells can be stimulated into large secondary effector cells upon re-exposure to alloantigen (2, 4, 5). Although these studies show that memory lymphocytes are derived from blasts generated during a primary response, the precise relationship between these blasts and the cytotoxic blasts or their precursors is unknown.

We have investigated this problem using a culture system (6) capable of detecting clones of CL derived from single precursors (CLP). The experiments described below indicate that this culture system is also suitable for a study of secondary in vitro responses. The results show that memory CLP, defined as those cells able to give a secondary in vitro response, and primary CL are derived from the same precursors.

Materials and Methods

Mice. Homozygous, athymic RNC-*nu*/*nu* ($H\text{-}2^k$) mice and their heterozygous, normal littermates were bred in the animal facility at the Ontario Cancer Institute as previously described (7). F_1 hybrids of C57BL/6J × DBA/2 (BDF_1) ($H\text{-}2^{b/d}$) were purchased from The Jackson Laboratory, Bar Harbor, Maine. Only male mice were used.

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* Supported by the Medical Research Council of Canada (grant MA-3017) and by the National Cancer Institute of Canada.

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[1] *Abbreviations used in this paper*: A cell, accessory cell; BDF_1, F_1 hybrid of C57BL/6J × DBA/2J; CL, cytotoxic T lymphocytes; CLP, precursor of cytotoxic T lymphocytes; LN, lymph node; 2-Me, 2-mercaptoethanol; MLC, mixed lymphocyte cultures; MLR, mixed lymphocyte reaction.

Target Cells. P815, a DBA/2 (H-2^d) mastocytoma and EL4, a C57BL/6 (H-2^b) leukemia, were maintained by weekly intraperitoneal passage through DBA/2J and C57BL/6J mice, respectively. The target cells were labeled with $Na_2{}^{51}CrO_4$ as previously described (6).

Mixed Lymphocyte Cultures. Microcultures (200 μl total volume) containing 2 × 10^5 RNC-*nu/nu* spleen cells, 2 × 10^5 irradiated (2,000 rads from a ^{137}Cs source) BDF_1 spleen cells, and limiting numbers of RNC-*nu/+* lymph node (LN) cells were set up in V-bottom trays (Linbro IS-MVC-96) as previously described (6). The medium used was α-minimal essential medium (Flow Laboratories, Inc., Rockville, Md.) supplemented with 10% (vol/vol) fetal bovine serum (Flow Laboratories, lot no. 455664), 5 × 10^{-5} M 2-mercaptoethanol (2-Me), and 10 mM HEPES buffer. After 7 days, 100 μl of each culture was removed and assayed for a primary cytotoxic response against both H-2^d and H-2^b targets. The other half of each culture was returned to the incubator and cultured for a further 7 days. At this time, i.e. on day 14, each culture was restimulated by addition of 2 × 10^5 RNC-*nu/nu* spleen cells and 2 × 10^5 irradiated BDF_1 spleen cells. 4 days later each culture was again divided into two equal parts and assayed for cytotoxicity against H-2^d or H-2^b targets.

Cytotoxic Assay. Two samples of 50 μl were taken from each culture. One was assayed for cytotoxicity against 1,000 ^{51}Cr-P815, and the other against 1,000 ^{51}Cr-EL4 target cells in V-bottom microtiter trays in a vol of 200 μl. The trays were centrifuged at 170 g for 5 min before being incubated for 4 h at 37°C. 100 μl of supernate were then counted in an LKB 80,000 gamma counter (LKB Instruments, Inc., Rockville, Md.). Secondary MLC were assayed in a similar manner except that each culture was divided into two equal parts before the addition of ^{51}Cr-labeled target cells.

Calculations. Percent specific lysis, p, is calculated as

$$\frac{\text{experimental cpm released} - \text{spontaneous release}}{\text{maximum cpm released} - \text{spontaneous release}} \times 100.$$

Spontaneous release was determined by taking the mean of ^{51}Cr counts from 24 cultures that received RNC-*nu/nu* spleen cells and BDF_1 stimulator cells but no LN cells. Maximum releasable counts were determined by addition of 1.90 ml of 1% acetic acid to 0.10 ml of target cells. A culture was scored as positive if its count was greater than the mean of the spontaneous counts by more than two standard deviations. This usually represented greater than 5% specific lysis.

Results and Discussion

Fig. 1 demonstrates the feasibility of using our clonal assay for CLP in a study of the lineage of memory cytotoxic cells: memory lymphocytes are produced and they can be activated. Cultures containing 2 × 10^3 RNC-*nu/+* LN cells (responder), 2 × 10^5 irradiated BDF_1 spleen cells (stimulator), and 2 × 10^5 RNC-*nu/nu* spleen cells were set up in microtiter trays. All CL are generated from precursors in the *nu/+* LN cell population; the *nu/nu* spleen cells provide a required accessory cell lost by dilution in the *nu/+* LN cells (8, 9). Sets of 12 cultures were examined for cytotoxicity against P815 (H-2^d) or EL4 (H-2^b) on days 7 and 14. Additional sets of 12 cultures were restimulated on day 14 with freshly irradiated BDF_1 spleen cells with or without RNC-*nu/nu* spleen cells. These cultures were assayed again on day 17, 18, 19, or 20. As indicated in Fig. 1, the cytotoxic activities of the cultures were substantially reduced by day 14. On restimulation, the activities of these cultures against both P815 and EL4 rose rapidly and remained high 3–5 days later. Unlike the primary stimulation, activation of these secondary cultures was not dependent on the addition of *nu/nu* spleen cells. Thus, it is possible that the activation of memory cells does not require the accessory cell present in *nu/nu* spleen. Alternatively, there may be enough of these cells left from the primary stimulation to activate the memory cells. Other workers have evidence to

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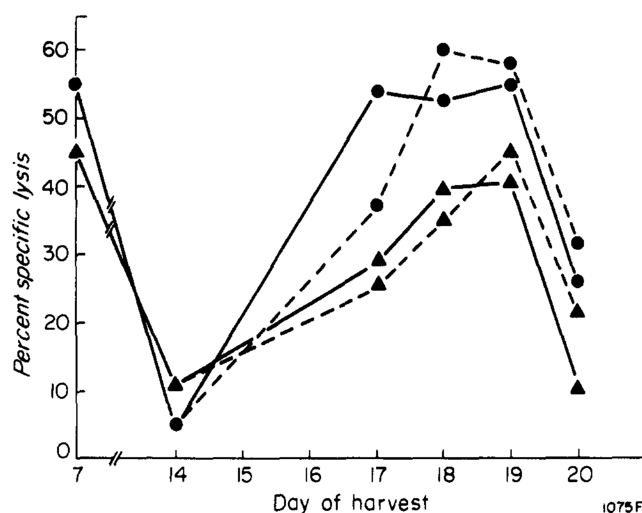

FIG. 1. Kinetics of the secondary cytotoxic response. Each culture initially contained 2 × 10^5 RNC-*nu/nu* spleen cells, 2 × 10^5 irradiated BDF_1 spleen cells ± 2 × 10^3 RNC-*nu/+* LN cells. The cultures were assayed for cytotoxicity against P815 or EL4 on days 7 and 14. Additional cultures were restimulated on day 14 with 2 × 10^5 irradiated BDF_1 spleen cells ± 2 × 10^5 RNC-*nu/nu* spleen cells. These cultures were assayed for anti-P815 and anti-EL4 activities 3-6 days after restimulation. The values given are the means of 12 cultures. (●), P815; ▲, (EL4); (——) *nu/nu* spleen cells added in the secondary stimulation; (- - -) *nu/nu* spleen cells omitted in the secondary stimulation.

TABLE I
Lineage of Memory Cytotoxic T Lymphocytes

No. LN cells per culture	Cultures positive for P815*			Cultures positive for EL4*			Double positives	
	Day 7	Day 18	New clones	Day 7	Day 18	New clones	Day 7	Day 18
200	9	5	0	4	2	0	1 (1.5)‡	0 (0.4)
400	13	11	0	5	4	0	4 (2.7)	3 (1.8)
800	22	13	1	12	7	1	10 (11.0)	3 (3.8)
CLP/10^6 cells§	2,426 (1,765-3,336)	1,174 (808-1,706)	–	783 (511-1,199)	439 (257-751)	–	–	–
χ^2	1.35	1.17	–	0.65	0.01	–	–	–
400	11	10	2	11	6	0	4 (5.0)	2 (2.5)
800	22	16	0	16	10	0	15 (14.7)	7 (6.7)
1,600	23	19	0	21	13	0	20 (20.1)	11 (10.3)
CLP/10^6 cells	2,127 (1,552-2,916)	1,188 (863-1,634)	–	1,384 (1,010-1,895)	584 (402-850)	–	–	–
χ^2	2.74	1.08	–	0.17	0.88	–	–	–

* Out of a total of 24 cultures.
‡ The numbers in parentheses are the expected number of double positives if the anti-P815 or EL4 clones were randomly associated.
§ The CLP frequencies were calculated according to the method of Porter and Berry (16). The 95% confidence limits are given in parentheses.

suggest that memory lymphocytes differ in their activation requirements from unprimed lymphocytes (10, 11).

Table I summarizes two experiments by studying the relationship between CL, memory lymphocytes, and CLP. Cultures were set up with limiting numbers of LN cells so that all groups had measurable numbers of nonresponding cultures, i.e. cultures lacking CLP for the alloantigen being tested. The LN

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TABLE II
*Percent Specific Lysis of Individual Cultures for P815 or EL4 on Days 7 and 18**

Culture number	Specific lysis for P815		Specific lysis for EL4	
	Day 7	Day 18	Day 7	Day 18
	%	%	%	%
1	1	−2	−3	4
2	−1	−1	−1	0
3	55‡	3	−4	−3
4	11	4	0	0
5	1	2	−2	2
6	−2	1	5	2
7	−1	−2	1	3
8	−1	3	−3	0
9	−2	5	2	2
10	1	0	3	0
11	54	48	48	−4
12	3	0	27	3
13	2	4	2	1
14	33	4	0	0
15	3	0	−3	2
16	1	−1	2	1
17	1	4	14	41
18	23	27	−2	−2
19	51	47	−2	3
20	35	16	−1	−3
21	3	1	−2	1
22	3	−1	35	11
23	9	−2	0	0
24	26	10	2	2

* Percent specific lysis values for individual cultures for P815 or EL4 on days 7 and 18. Each culture received initially 2 × 10^5 RNC-*nu/nu* spleen cells, 2 × 10^5 irradiated BDF_1 spleen cells, and 200 RNC-*nu*/+ LN cells, and was restimulated with 2 × 10^5 RNC/*nu/nu* spleen cells and 2 × 10^5 irradiated BDF_1 spleen cells on day 14. Spontaneous lysis in 4 h ranged from 8% to 15% of the maximum releasable counts.

‡ The positive cultures are underlined.

cells were RNC (*H-2*^{k}) and the stimulator cells were BDF_1 spleen (*H-2*$^{b/d}$). On day 7, a quarter of each culture was assayed with P815 or EL4 targets to identify the cultures reactive to H-2^d or H-2^b alloantigens. The remaining cells in these same cultures were restimulated on day 14 with irradiated BDF_1 spleen cells and, on day 18, each culture was assayed for anti-P815 and anti-EL4 activities. By comparing the pattern of the cytotoxic response on day 18 with that on day 7, one can determine directly if cultures positive for P815 or EL4 on day 18 were derived from cultures positive for these two target cells on day 7. With few exceptions (4 out of 120), cultures positive for P815 or EL4 on day 18 were derived from cultures that were previously positive for these two targets (see Table I). The significance of the four new clones detected on day 18 is not clear. Since only a quarter of the culture was assayed on day 7, one explanation is that the cytotoxic activity of these clones was below the detection limit of our assays. Table II gives a set of experimental data showing ^{51}Cr

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release in individual cultures for P815 and EL4 on days 7 and 18. Although the distinction between positive and negative cultures seems quite clear here, ambiguities do sometimes arise. Another explanation is that these clones might also be primary clones with an unusually long latent period. Finally, we cannot rule out the possibility that these CLP acquired specificity after prolonged exposure to alloantigen. Neglecting these four adherent clones, we conclude that memory lymphocytes are produced only in clones containing CL specific for the same alloantigen.

The design of the experiments of Table I allows a more direct test of whether CLP and memory cells are specific. The number of clones observed to be positive for both P815 and EL4 on days 7 and 18 is in good agreement with that predicted on the basis of random association of independent precursors (compare last two columns of Table I). This implies that both primary CLP and memory cells are specificity restricted and is in agreement with our earlier observation (12, 13) that CL clones are specific. The frequency estimates for CLP specific for H-2^d and H-2^b alloantigens reported in Table I are also in agreement with other measurements (12–15).

The data presented here indicate clearly that both primary and secondary CL are progeny of a common CLP. The only alternative model consistent with the data and also allowing for independent CLP is as follows: secondary CL are derived from an independent CLP present in much higher frequency than the primary CLP, and the activation of a secondary CLP requires the prior activation of a primary CLP in the same culture. This rather convoluted model can also be discarded. It predicts that the frequency of primary cultures capable of being reactivated in a secondary response should increase with the number of responder cells cultured. In contrast, we observed that about half of the primary cultures could be reactivated independently of the number of cells plated. The reasons that only half the primary clones could be restimulated have not yet been investigated. While it is clear from these experiments and from the work of others that memory CLP are derived from blasts produced from a primary CLP, the relationship between the blasts that are cytotoxic and the blasts that differentiate into memory CLP is not clear. There are two possibilities. First, some CL may further differentiate into memory CLP. The observation that only 50% of the primary clones produce memory CLP would then indicate that not all CL become memory CLP. Second, the blasts produced by a single CLP may be heterogeneous with some blasts becoming CL and other differentiating into memory CLP. Our data do not allow one to distinguish between these two possibilities.

Summary

In the course of a mixed lymphocyte culture, memory cells are produced which can give rise to a large secondary cytotoxic lymphocyte response on re-exposure to the sensitizing alloantigen. We have studied the lineage of these memory cells using a clonal assay for precursors of cytotoxic T lymphocytes (CLP). Our data provide conclusive evidence that individual CLP, upon stimulation with alloantigens, gives rise to clones which contain memory cells of the same specificity as the CLP. Only half of the clones that responded in the

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primary stimulation could be reactivated upon exposure to the original priming alloantigen.

We thank R. Course, R. Kuba, and H. Derry for technical assistance.

Received for publication 2 June 1977

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