THE REQUIREMENT FOR DNA SYNTHESIS AND GENE EXPRESSION IN THE GENERATION OF CYTOTOXICITY IN VITRO*

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The terminal step in the differentiation of those T lymphocytes that are capable of becoming cytotoxic effector cells ordinarily occurs when the effector cell precursors encounter foreign cell antigens in vivo for which they are presumably already committed. The in vitro analog for this final step, the mixed leukocyte culture (MLC)[1] reaction, makes possible a detailed analysis of cellular and molecular events accompanying the generation of cytotoxic function in T cells.

It has been clearly established that the generation of cytotoxic effector cells in vitro is accompanied by extensive proliferation in the reacting cell population. Prevention of or interference with proliferation has a profound suppressive effect on development of a full cytotoxic response in vitro, as does selective elimination of dividing cells during sensitization (1–3). On the other hand, it has been shown by a number of investigators that proliferation of cells in response to alloantigenic stimulation is not in itself sufficient to develop a cytotoxic response (4, 5).

These studies, however, leave unanswered very important and fundamental biological questions. Is one or more rounds of cell division absolutely required for the differentiation of a resting T cell to the cytotoxic effector cell state? Or, is it the case that proliferation triggered by contact with cell-bound alloantigen serves simply to amplify a specific, already differentiated (cytotoxic) cell type within the general cell population? A second, related question is whether expression of new genetic information is required for initial development of cytotoxic function, or whether the transition from resting T cell to cytotoxic effector cell is regulated at some other level, for example requiring only a rearrangement of membrane components.

In the present paper, we examine both of these questions, using two systems for the generation of cytotoxicity in T cells: the primary MLC, in which reacting T cells encounter alloantigen for the first time, and the secondary MLC, in which previously sensitized but inactive T cells are restimulated by specific alloantigen and undergo renewed proliferation and development of cytotoxicity (6, 7). The primary MLC as utilized in our laboratory allows detection of target-specific cytotoxicity as early as 44 h of culture; we can detect regeneration of cytotoxic function in secondary MLC within 12–15 h after restimulation.

The necessity for a single round of cell division cannot be examined in the

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[1] *Abbreviations used in this paper:* BUdR, 5-bromo-2'-deoxyuridine; HU, hyroxyurea; MLC, mixed leukocyte culture; PBS, phosphate-buffered saline; TCA, trichloroacetic acid.

most direct way, using inhibitors of mitosis, since such inhibitors interfere with cytotoxic effector function which is itself the endpoint of the assay (8). Instead, we use the inhibitor of DNA synthesis, hydroxyurea (HU), to prevent synthesis of DNA and thus cell division. To examine the question of new gene expression, we have used the drug 5-bromo-2′-deoxyuridine (BUdR). In those eukaryote systems in which a critical round of cell division results in the expression of new genes associated with cell-specific function, BUdR has been shown to selectively inhibit expression of those genes without interfering with cell viability or propagation (see review in ref. 9).

Our results will show that in the primary MLC, at least one round of cell division is required for expression of cytotoxicity. Furthermore, expression of cytotoxicity in primary MLC can be totally suppressed by levels of BUdR 20- to 80-fold below the levels required to affect DNA synthesis and cell viability. On the other hand, neither HU nor BUdR have any effect on the generation of cytotoxicity in secondary MLC.

Materials and Methods

Animals. Mice used for these experiments were obtained from the Jackson Laboratories, Bar Harbor, Maine, or from L. Strong, San Diego, Calif. Only inbred females, age 6–10 wk, were used.

Mixed Lymphocyte Cultures. For the experiments involving the primary MLC only, cultures were initiated as follows. Regional lymph nodes were collected from exsanguinated mice. Cell suspensions were prepared by teasing the nodes with forceps and pressing them through a 100 mesh stainless-steel screen into sterile phosphate-buffered saline (PBS). The cells were washed, counted with Trypan Blue and resuspended in Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, N. Y. no. H-21/high glucose), supplemented as suggested by Cerottini (6) with arginine (108 mg/l), asparagine (36 mg/l), glutamine (216 mg/l), 5×10^{-5} M 2-mercaptoethanol, and 10% fetal calf serum. Stimulating cells were irradiated with 1,000 R from a 60 kV X-ray source. Responding and stimulating cells were mixed at a ratio of 1:1 and cultured in Falcon 17 x 125 mM tubes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) at a final concentration of $2.5–4.0 \times 10^6$/ml. The cultures were maintained at 37°C in 7.5% CO_2-in-air until assay.

For the secondary MLC experiments, cell suspensions were prepared from regional lymph nodes and spleens exactly as described above, and cultured in Falcon tissue culture flasks (standing). Medium was changed on day 6 of culture. Cytotoxicity was measured on about the 10th day of culture, and the cells were utilized for secondary MLC unless the cytotoxicity was still vigorous in which case the cultures were returned to the incubator for an additional 2–3 days. To establish the secondary MLC, cells were harvested, washed, counted with Trypan Blue, and resuspended to $2–3 \times 10^6$/ml in culture medium. Either irradiated responding strain cells (control) or irradiated stimulating strain cells at the same concentration ($2–3 \times 10^6$/m) were mixed in equal volume with the cells from the primary MLC, and cultured in 17 x 125 mm Falcon tubes for 24–48 h.

Measurement of Cytotoxicity. Cytotoxicity was determined using the ^{51}Cr release assay as previously described (10). Putative effector cells were incubated together at various ratios with ^{51}Cr-labeled target cells (P815 mastocytoma cells maintained by serial passage in DBA/2 mice). For assay of 48-h-generated primary MLC cytotoxicity, the assay period was 10–16 h. For other effector cells, the assays were between 3–5 h. Assays were carried out in 12 x 75 mm Falcon culture tubes, using 2.5×10^4 target cells and various numbers of effector cells. Assay tubes were centrifuged briefly at the start of the assay.

Concanavalin A, HU, and BUdR. Con A was added to a final concentration of 5 μg/ml. In experiments where transformed cells were to be counted in a hemacytometer, the cells were washed free of Con A and incubated for several hours with 0.1 M α-methyl mannoside (11). This allowed subsequent disruption of cell clumps by gentle pipetting.

HU was found to inhibit DNA synthesis in lymphocytes within minutes after addition, and the inhibition was relieved within minutes of removal of HU. 0.5 mM was the maximum DNA

synthesis-inhibiting dose that did not significantly affect cell viability.

BUdR was added to cultures just before incubation. The test tube racks containing BUdR-treated cultures were kept protected from light at all stages of the experiment, including the assay period.

Incorporation of [^{3}H]thymidine. Cell cultures in which proliferation was to be measured were centrifuged, resuspended in 1.0 ml of culture medium, and triplicate 250-μl aliquots were removed to 12 x 75 mm plastic culture tubes. 2 μCi of [^{3}H]TdR in 50 μl was added to each tube for 3 h. The cultures were flushed and washed with cold PBS, and precipitated with cold 10% trichloroacetic acid (TCA) in the presence of a small amount of protein carrier. The TCA precipitates were dissolved in NCS (Amersham/Searle Corp., Arlington Heights, Ill.) and counted in Omnifluor-toluene (New England Nuclear, Boston, Mass.). In the case of experiments involving BUdR, the procedure was identical except that labeling was with [^{3}H]dCTP (Schwartz/Mann no. 2433-33 Div. Becton, Dickinson & Co., Orangeburg, N. Y.).

Results

The Effects of HU and BUdR on DNA Synthesis in Stimulated Lymphocytes. At the time point we have chosen to look at initial turn-on of cytotoxicity in the primary MLC (44 h), there is very little measurable specific DNA synthesis. In order to test the effects of various metabolic inhibitors on DNA synthesis in lymphocytes shortly after they had been activated, therefore, we decided to trigger the cells with Con A, a polyclonal activator of T cells that has been shown not only to stimulate DNA synthesis, but to lead to the activation of receptor-bearing cytotoxic effector cells as well (12–14).

The effects of HU on DNA synthesis in Con A-stimulated lymph node cells is shown in Fig. 1. It has been reported that HU added to lymphocytes after DNA synthesis was initiated leads to the death of cells as they pass through S phase, as detected by the ultimate production of plaque-forming cells (15). Since in the primary MLC we do not know precisely where DNA synthesis begins, we tested the effect of HU on Con A-stimulated lymphocytes when added before or after the initiation of DNA synthesis in these cultures (22 h). We found that in either case, DNA synthesis was inhibited by at least 90% at a HU concentrations of 0.5 mM. To our surprise, we found the long-term inhibitory effect of HU reversible to the same extent whether present from 16–40 h or from 28–48 h. In either case, at 0.5 mM HU, DNA synthesis was restored to 75-80% of control values (no HU added) within minutes after washing out the HU. Furthermore, recovery of viable cells in treated cultures across the range of 0.05–1.0 mM HU was always 90% or more of control values. At doses up to 0.5 mM HU, the proportion of blast cells in treated cultures did not fall below 80% of control cultures. Similar results have been reported for the effect of HU on mitogen-stimulated B cells, although the period of exposure to HU was shorter (16).

The effect of BUdR on DNA synthesis was tested in two proliferating cell systems: Con A-stimulated lymph node cells, as just described, and in MLC "memory cells" 24 h after secondary restimulation. (Details of the kinetics of stimulation in secondary MLC are presented in a subsequent section.) The results are shown in Fig. 2. The precise location of the curves varies slightly from experiment to experiment, but in all cases tested, the 50% inhibition dose of BUdR in both systems lies between 150 and 250 μg/ml. The fall-off of DNA synthesis was almost exactly paralleled by a loss of cell viability, and thus probably does not represent a specific effect on DNA synthetic activity. No loss

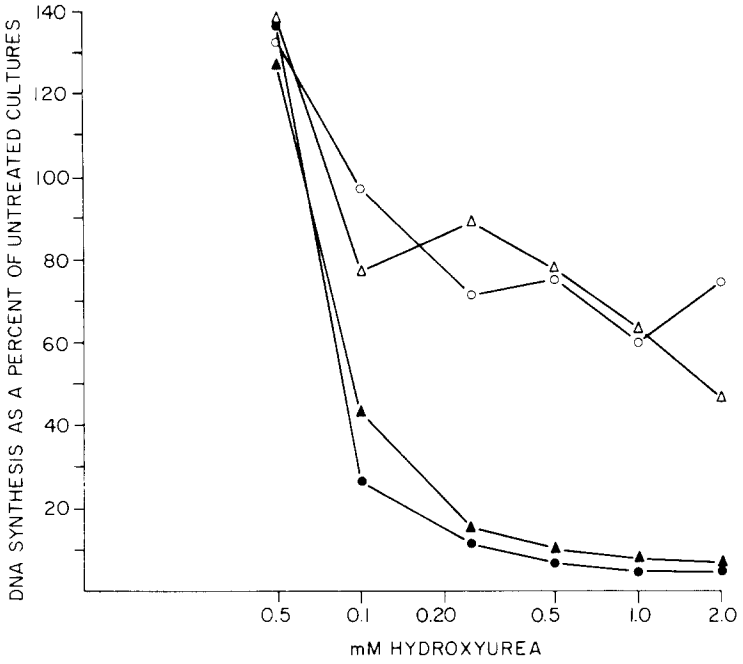

FIG. 1. The effect of hydroxyurea on DNA synthesis in Con A-stimulated lymph node cells. Lymph node cells were prepared and incubated with Con A as described in the Methods section. At 16 h (triangles) or 28 h (circles), HU was added to the indicated concentrations. 24 h later in either case, half of the cells at each dosage were washed and incubated in medium without HU for an additional 1–2 h (open symbols). At the end of this time period, [^{3}H]TdR was added to each of the cultures for 3 h as described in the Methods section. Cells that had not been washed free of HU (solid symbols) were also labeled in the presence of HU.

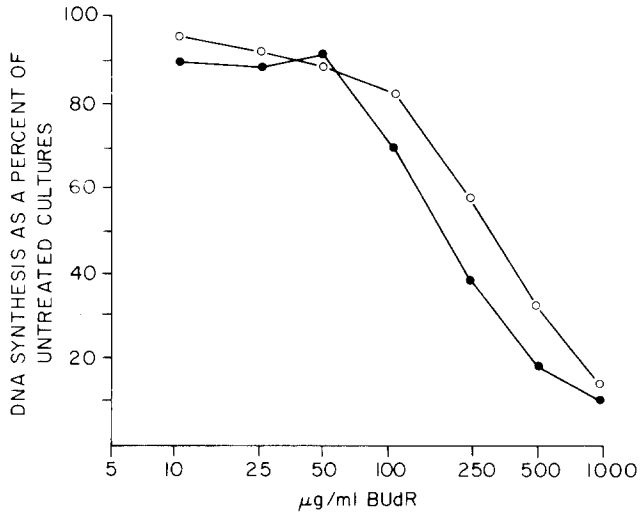

FIG. 2. The effect of BUdR on DNA synthesis in stimulated lymph node cells. Lymph node cells actively synthesizing DNA were obtained by Con A stimulation (see Fig. 1) or by restimulating MLC-generated "memory cells" (see text). The cells were incubated with varying doses of BUdR for at least 24 h after stimulation, and then with [^{3}H]CTP for 3 h.

in cell viability was noted at doses below 100 μg/ml.

The Effect of HU on Expression of Cytotoxicity in Primary MLC. The earliest time at which we can reproducibly detect significant, target-specific cytotoxicity in primary MLC is 44–48 h of culture (Table I). Ideally, one would like to be able to carry out the entire initial sensitization in the presence of an inhibitor of DNA synthesis in order to assess the need for DNA synthesis and cell division in the generation of cytotoxicity. However, HU and other inhibitors of DNA synthesis invariably exert nonspecific lethal effects on cells if left in culture more than 24 h. We therefore had to assess the effect of HU on generation of cytotoxicity in MLC over two different time periods: 0–24 h and 20–44 h. The latter time period was based on our observation that DNA synthesis in T-cell-specific lectin-stimulated lymphocyte cultures was undetectable before 22 h of culture. Thus, if the analogy is valid, HU present in MLC cultures from 20–44 h should cover the period from the beginning of DNA synthesis until first expression of cytotoxicity. Additional support for the notion that little if any DNA synthesis occurs in the first 24 h of MLC comes from studies showing that treatments that selectively kill cells synthesizing DNA have no effect on generation of cytotoxicity when applied during the first 24 h (3, 17). As shown by the data in Table II, HU present in MLC reactions from 0–24 h caused an average reduction of 33% in cytotoxicity measured at 48 h. On the other hand, when HU was present during the period 20–44 h, the reduction in cytotoxicity at 48 h approached 80%. These results would be consistent with the notion that DNA synthesis in primary MLC occurs mainly during the second day of culture. Possible interpretations of these effects will be presented in the Discussion section.

It is very important to determine whether the reduction in cytotoxicity caused

TABLE I

Development of Specific Cytotoxicity in Early Mixed Leukocyte Culture

Exp.	Sensitization period	Length of assay	Net ^{51}Cr release against P815	Net ^{51}Cr release against EL-4
	h	*h*	%	%
1	24	16	2.9	2.6
2	24	17	3.3	3.5
3	36	16	4.3	3.5
4	36	16	4.3	4.0
5	36	17	6.8	2.3
6	42	16	5.5	3.2
7	44	12	6.0	1.2
8	44	17	9.7	1.8
9	48	8	15.5	0.6
10	48	10	14.2	2.0
11	48	16	23.5	2.3

C57BL/6 lymph node cells were cultured together with irradiated DBA/2 lymphoid cells for various time periods from 24–48 h. At the end of the sensitization period, 10^6 cells from the various cultures were incubated for varying time periods with either ^{51}Cr-labeled P815 (DBA/2 origin) or ^{51}Cr-labeled EL-4 (C57BL/6 origin). The values reported are corrected for spontaneous release and are the average of triplicate assay samples.

by HU was due to prevention of a required metabolic step (DNA synthesis) on the part of the developing effector cells, or to a selective lethal effect on reacting cells. If the former is the case, then the inhibition of development of cytotoxicity ought to be reversible, and removal of the inhibitor should allow the generation of cytotoxicity to continue, with a displacement in time, toward maximal expression. As can be seen from the two experiments in Fig. 3, this is indeed the

TABLE II

The Effect of Hydroxyurea on Expression of Cytotoxicity at 44 H in Primary MLC

Exp.	Control 0–24 h*	HU (0–24 h)*	Reduction	Control (20–44 h)	HU (20–44 h)*	Reduction
			%			%
1	8.1	4.7	42.0	8.9	0.2	97.8
2	55.5	52.2	6.0	58.0	25.6	55.9
3	43.7	19.1	56.3	24.3	16.4	32.6
4	39.1	17.3	55.8	23.4	3.4	85.5
5	48.2	24.0	50.2	38.9	0	100.0
6	52.6	58.1	−10.4	49.5	6.6	86.7
7	—	—	—	58.9	2.8	95.3

Primary MLCs were initiated using C57BL/6 lymph node cells and irradiated DBA/2 lymph node cells. 0–24 h cultures received an additional ml of medium (control) or HU (final concentration 0.5 mM) at time zero. At 24 h both control and HU cultures were spun, washed and resuspended in fresh medium for an additional 20 h. 20–44 h cultures received medium or HU at 20 h (cells in both cultures were resuspended by pipetting to facilitate mixing). At 44 h, all four groups were harvested and tested by cytotoxicity against ^{51}Cr-labeled P815 target cells as described in the methods.

* Net percent ^{51}Cr release in assays ranging from 10–16 h, at an effector:target ratio of 40:1.

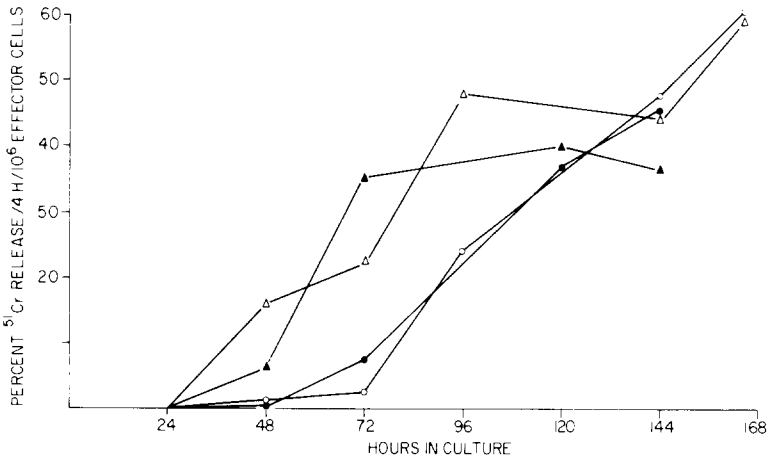

FIG. 3. Primary MLC's were initiated and treated with hydroxyurea from 20–44 h of culture as described in Table II. At 44 h, treated and untreated cultures were harvested, washed, and samples removed for cytotoxicity assay. The remaining cells were returned to the incubator, and samples removed for assay at 24-h intervals. Open and closed symbols represent two separate experiments. Triangles: untreated controls. Circles: exposed to HU from 20–44 h, reincubated without HU for the remainder of the experiment.

case. Cultures in which HU was present from 20–44 h (circles) had a cytotoxicity function that was 80–100% inhibited at 48 h compared with untreated controls (triangles). After release of the HU block, the treated cultures continued toward development of full cytotoxicity. The lag time between the two sets of curves is approximately 30 h, slightly longer than the period of exposure to HU. Once expression of cytotoxicity begins after release of the HU block, the rate of generation and the maximal value of cytotoxicity reached are essentially the same as in untreated cultures, suggesting that the number and proportion of reacting lymphocytes in the treated cultures is the same as in untreated controls.

The Effect of BUdR on the Generation of Cytotoxicity in Primary MLC. As noted in a previous section, BUdR has a significant effect on DNA synthesis and cell survival in Con A and secondary MLC-stimulated lymphocytes in the dosage range of about 100–300 μg/ml. The dosage range across which BUdR interferes with expression of cytotoxic function in cells undergoing sensitization in primary MLC is shown in Table III. In all experiments carried out, BUdR caused a 50% decrease in cytotoxicity at a dosage of <4 μg/ml, i.e. at levels 25- to 75-fold less than the average dose of BUdR required for a 50% decrease in DNA synthesis or cell survival. No loss of cell viability was detectable at BUdR concentrations of less than 100 μg/ml.

That the effect of BUdR is exerted through incorporation into DNA is shown

TABLE III
Effect of BUdR on Expression of Cytotoxicity in Primary MLC

Exp.	BUdR	Period of sensitization	Lysis	Reduction	50% dosage*
	μg/ml	*h*	*%*	*%*	
1	0	96	54.8	0	3.1
	1		44.7	18.4	
	7		15.9	71.0	
	50		8.0	85.4	
2	0	96	67.6	0	3.0
	1		69.3	0	
	7		12.3	87.7	
	50		0.3	99.7	
3	0	72	39.0	0	2.1
	1		26.5	32.1	
	7		7.9	79.8	
	50		4.7	88.0	
4	0	72	24.2	0	1.9
	1		16.2	33.0	
	7		4.4	81.8	
	50		2.2	90.9	

Primary MLCs were initiated using C57BL/6 lymph node cells and irradiated DBA/2 lymph node cells. BUdR at the indicated concentrations was included from the time of initiation of the cultures up to the time of assay.

* Interpolated dosage (μg/ml) required to reduce cytotoxicity by 50%.

TABLE IV

Thymidine Reversal of BUdR Inhibition of Generation of Cytotoxicity in Primary MLC

Exp.	BUdR concn.	Thymidine concn.	Net ^{51}Cr release	% control
	μg/ml	*M*		
1	0	0	30.4	100
	50	0	2.4	7.9
	50	7×10^{-4}*	26.6	87.5
2	0	0	37.5	100
	50	0	0.8	2.1
	50	7×10^{-4}*	34.5	92.0
	0	7×10^{-4}*	23.0	61.3

Primary MLCs were initiated using C57BL/6 lymphocytes and DBA/2 (irradiated) lymphocytes. BUdR and/or thymidine was added at time zero and remained in the cultures throughout the sensitization period (6 days for experiment 1, 7 days for Exp. 2).

* 4 molar excess over BUdR.

by the data of Table IV. The inhibition of cytotoxicity caused by 50 μg/ml BUdR is completely reversed by 7×10^{-4} M thymidine (a 4 molar excess).

The Effect of Hydroxyurea on Expression of Cytotoxicity in Secondary MLC. The detailed kinetics of the early secondary response in MLC (0–24 h after restimulation) are shown in Fig. 4. Significant differences between cultures exposed to lymphocytes syngeneic to the reacting cells and cultures exposed to lymphocytes syngeneic to the original stimulating cells, both in terms of increased proliferation and increased cytotoxicity, are readily apparent after 12–15 h of culture.

Cerottini had previously found that the reappearance of cytotoxicity in secondary MLC, obtained upon reexposure of primed MLC "memory cells" to the original stimulating antigen, could be detected fully in the presence of Ara C, an inhibitor of DNA synthesis (personal communication). A similar effect can be seen using HU under the conditions described earlier for the primary MLC. As shown in Table V, 0.5 mM HU, which inhibits DNA synthesis in Con A-stimulated lymphocytes by 90% or more, and which drastically inhibits development of cytotoxicity in primary MLC, has essentially no effect on initial regeneration of cytotoxicity in secondary MLC.

The Effect of BUdR on Expression of Cytotoxicity in Secondary MLC. The effect of BUdR on proliferation in the secondary MLC was shown in Fig. 2; a 50% decrease in DNA synthesis was obtained at a BUdR concentration of about 250 μg/ml. The effect of BUdR on the regeneration of cytotoxicity in the secondary MLC, as determined 48–72 h after restimulation, is shown in Table VI. While there was always some slight depression of cytotoxicity in the presence of BUdR, the effect was very small and not obviously dose-dependent. Estimated dosages required for a 50% depression of cytotoxicity range approximately between 10^3 and 10^4 μg/ml. This should be compared with the effect of BUdR on generation of cytotoxicity in the primary MLC (Table III), where a 50% depression was obtained between 1 and 3 μg/ml.

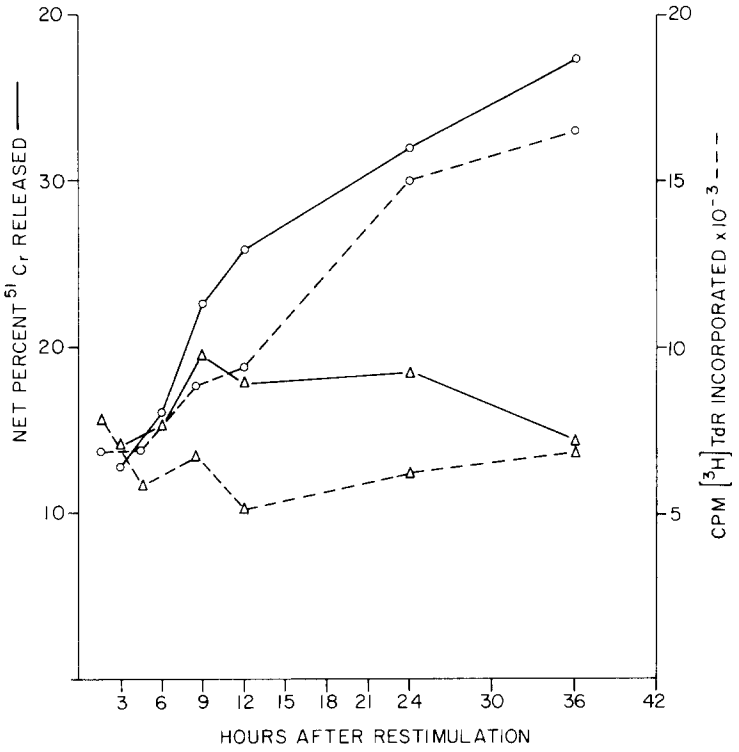

FIG. 4. Detailed kinetics of restimulation in secondary MLC. A primary MLC was initiated using C57BL/6 lymphocytes and irradiated DBA/2 lymphocytes. After 11 days, the cultures were harvested, counted and restimulated with equal numbers of either irradiated C57BL/6 (△) or irradiated DBA/2 (○) lymphocytes. At the indicated time intervals, proliferation (dashed line) and cytotoxicity (solid line) were measured in parallel cultures. Cytotoxicity assays were carried out at a 40:1 effector: target ratio for 4 h. Proliferation was measured as described in the Methods section. (Labeling time was 3 h; symbols represent the approximate mid-point of the labeling period.)

Discussion

With the apparently lone exception of hormonal activation of cell-specific genes, eukaryote cells require one or more rounds of cell division in order to express new (previously unexpressed) genetic information. Such cell divisions have been termed by Holtzer "quantal mitoses" (18), which are considered to be conceptually distinct from proliferative cell divisions. Whether or not this notion will prove to have universal application remains perhaps to be seen, but it is certainly true that the vast majority of nonhormonally-induced differentiative events in eukaryotes are preceded by at least one round of cell division. It is thus of considerable interest to establish not only that proliferation is a normal concommitant of the generation of cytotoxicity, or even that interference with proliferation leads to inhibition of cytotoxicity, but also to attempt to determine whether one or more rounds of cell division are absolutely required for the first expression of cytotoxicity.

In the strictest sense we have not answered that question here. HU blocks progress of cells through the normal cell cycle at the G1-S interface, not in

TABLE V

Effect of Hydroxyurea on Expression of Cytotoxicity after Restimulation in MLC

Exp.	Cytotoxicity − hydroxyurea*	Cytotoxicity + hydroxyurea*	Reduction
			%
1	17.6	17.1	2.3
2	46.6	47.7	−2.4
3	9.4	8.8	6.4

Primary MLCs of C57BL/6 lymph node cells versus DBA/2 irradiated lymph node cells were maintained until cytotoxicity per 10^6 effector cells in a 4-h assay fell below 10% (10–12 days). Cultures were harvested, counted and restimulated with an equal number of irradiated DBA/2 lymphocytes, in the presence or absence of 0.5 mM HU. The cells were assayed for cytotoxicity 24 h later.

* Percent ^{51}Cr released after 4 h at an effector: target ratio of 40:1. Residual cytotoxicity from primary MLC cultures was less than 15% of the restimulated values shown here.

TABLE VI

Effect of BUdR on Expression of Cytotoxicity after Restimulation in Secondary MLC

Exp.	BUdR	^{51}Cr release	Reduction	50% dosage*
	μg/ml	%	%	
1	0	64.7	—	
	7	63.7	1.6	$>3 \times 10^4$
	50	59.2	8.5	
2	0	21.1	—	
	7	16.2	23.2	$>3 \times 10^3$
	50	14.6	30.2	
3	0	48.5	—	
	7	42.8	11.8	$>3 \times 10^3$
	50	42.0	13.4	

10- to 12-day old primary MLC cultures of C57BL/6 vs. DBA/2 lymphocytes were restimulated as described in Table V. Cytotoxicity was measured at 48–72 h. BUdR was added at the time of restimulation to the indicated concentration.

* Estimated dosage (μg/ml) that would be required to reduce cytotoxicity by 50%.

mitosis. Thus it is possible that the effect we observe of HU on the primary MLC is related to inhibition of DNA synthesis per se and not to inhibition of cell division. At the present time we know of no satisfactory way around this problem, since as stated earlier inhibitors of mitosis may themselves interfere with the expression of cytotoxicity, and thus their effect on the generation of cytotoxicity cannot be determined. But taken at face value, the data strongly suggest that a round of cell division is needed for the initial expression of

cytotoxicity in primary MLC. The inhibitory effect of HU between 20–44 h in primary MLC is unlikely to be related to a nonspecific general cytotoxic action on reacting lymphocytes, for several reasons. As shown in Fig. 1, the effect of 0.5 mM HU on DNA synthesis per se in Con A-stimulated cells is reversible, whether it is added before or after DNA synthesis begins. The effect of 0.5 mM HU on the generation of cytotoxicity itself is also reversible (Fig. 3). Cells exposed to 0.5 mM HU between 20–44 h of MLC can be more than 90% inhibited in terms of expression of cytotoxicity at 44 h of culture, yet reach the level of cytotoxicity of control cultures within 72 h of release of the HU block. The displacement and kinetics of the rise in cytotoxic function in HU-treated cultures suggest that the treated cells had proceeded normally through whatever steps of the reaction take place in the first 20–24 h of culture, but were blocked from proceeding through the next phase by the HU. The alternative explanation, that the displaced rise in cytotoxicity in the treated cultures is due to first entry into reaction of cells that had not yet been triggered in the first 48 h of culture is unlikely, because in that case one would expect a lag time in recovery after removal of HU closer to 48 h. Finally, the lack of effect of HU on generation of initial cytotoxicity in secondary MLC (Table V) argues against a nonspecific lethal effect of the drug, either at the level of generation or expression of cytotoxicity.

In the experiments shown in Fig. 1, 0.5 mM HU present from 20–44 h in cultures treated with Con A caused almost no reduction in the number of blast cells per culture, indicating that HU does not block blastogenesis at levels which block DNA synthesis by 90% or more. It would seem fair to conclude, then, that blastogenesis per se is not sufficient for initial expression of cytotoxicity in MLC, since 0.5 mM HU, which does not block blastogenesis, does block initial expression of cytotoxicity. Thus, models which propose that cytotoxicity may be generated by exposure of cryptic receptor units or rearrangement of membrane components, simply as a result of blastogenesis, cannot hold.

The second question to which we have addressed ourselves is whether or not the generation of cytotoxicity requires the expression of new genes. In all cases of eukaryote cell differentiation that have been studied where new genetic information is expressed after one or more rounds of cell division, expression of the new, cell-specific genes can be selectively inhibited by the drug BUdR at levels which do not alter other cell functions, including proliferation (9). The exact mechanism by which BUdR exerts its selective effect in eukaryotes is not understood, although recent work with the Lac system of *E. coli* has shown that the Lac repressor protein binds about 10 times more tightly to the Lac operator in BUdR substituted *E. coli* DNA than in native DNA (19).

In the present system, we asked whether or not a level of BUdR could be found which would prevent the expression of cytotoxicity in lymphocytes reacting to alloantigens without affecting cell viability or normal function. In the primary MLC, the average concentrations of BUdR required for a 50% inhibition of cytotoxicity expressed at 44 h is about 2.5 μg/ml. On the other hand, the concentration of BUdR required for a similar effect on DNA synthesis and cell viability is approximately 200 μg/ml, nearly two orders of magnitude higher.

These results differ with a brief report issued by two of us (J. N. and W. C.) a year ago (20). In that communication we measured the effect of BUdR on cytotoxicity as measured at 6–7 days of culture. In the present experiments, a number of parameters have been changed, the most important being a more highly enriched culture medium, earlier determination of cytotoxicity, and use of [^{3}H]dCTP rather than [^{3}H]TdR to measure DNA synthesis. We thus feel that our earlier inability to obtain a selective effect with BUdR represented a technical failure rather than a true absence of such an effect.

In contrast to the selective effect of BUdR observed in the primary MLC reaction, essentially no effect of BUdR was observed in the secondary MLC. Some slight effect usually was observed, but extrapolation to estimated dosages required for a 50% reduction in cytotoxicity place this value well above the dosage leading to general cytotoxic effects of the drug.

In previous studies on cellular events accompanying generation of cytotoxicity in T cells in vitro, it was shown that a major functional change in reacting T cells was the acquisition of an efficient, specific binding capacity for target cell (10). This led to speculation that regulation of the development of cytotoxicity might lie at the membrane level, perhaps involving uncovering or rearrangement of antigen receptors. Such a model has been proposed for the initial expression of θ-antigen on mouse T cells, which does not require cell division and apparently is not genetically regulated (21). In the maturation of B cells in vitro to antibody-producing cells triggered by LPS, the B cells are able to mature through at least the first stages of differentiation, including antibody production, in the absence of cell division (16).

The present studies would seem to obviate such mechanisms for generation of cytotoxicity. We propose that resting, immunocompetent T cells, upon initial encounter with the alloantigens for which they are precommited, undergo at least one requisite round of cell division, and in so doing activate a new genetic program leading to the expression of cytotoxic function. Whether a single round of cell division brings the cell to its maximal cytotoxic potential, or whether subsequent rounds of mitosis refine the killing function on a per cell basis, remains to be explored. On the other hand, recent studies in our laboratory and others suggest that activation of this genetic program is not dependent on contact with specific alloantigen, but can be triggered polyclonally by T-cell mitogens such as Con A (12–14). This would suggest that the genetic program was certainly predetermined, if not expressed, and is unlikely to depend on the interaction of any external molecular signals directly, in an instructive way, with the T-cell genome.

The present study also makes clear that once the genetic program leading to cytotoxicity is expressed, it is apparently stable. The effector memory cells, which are presumably direct lineal descendants of the effector cells generated in primary MLC, can be brought from a quiescent to a fully active effector state without cell division and without expression of new genetic information, as indicated by refractoriness to HU and BUdR, respectively. The transition from resting memory cell to fully active effector cell is functionally the same as in the primary MLC, but would appear to be regulated in a different manner, perhaps

akin to the $\theta-$ to $\theta+$ transition of prothymocytes (21). The basis for this event in primed T cells is not presently resolved, but will hopefully be the subject of a future report.

Summary

The requirement for cell division and expression of new genes was examined in the primary and secondary mouse mixed leukocyte culture (MLC). Hydroxyurea (HU) was used to block DNA synthesis and cell division, and 5-bromo-2′-deoxyuridine (BUdR) was used to probe for the expression of new cell-specific genes.

In the primary MLC, inhibition of DNA synthesis and cell division by HU almost totally suppressed the generation of initial, target-specific cytotoxicity. When HU was washed out of the cultures, cytotoxicity was generated after a lag time approximately equal to the period of treatment with HU. The rate of development and maximal value of cytotoxicity in HU-reversed cultures was identical to untreated controls, suggesting that the inhibition was not due to a nonspecific lethal effect of the drug. Development of initial cytotoxicity in primary MLC was similarly suppressed by levels of BUdR 25 to 75-fold below the levels of this drug having nonspecific mutagenic effects in lymphocytes, indicating that development of cytotoxicity was also dependent on the expression of a new genetic program.

In the secondary MLC, regeneration of both DNA synthesis and cytotoxicity was apparent 12–15 h after re-exposure to initial stimulating antigen. In this reaction, however, generation of cytotoxicity was insensitive to both HU and BUdR. Thus, the cytotoxic program developed in the primary MLC appears to be genetically stable through the production of effector memory cells, and into regeneration of fully cytotoxic memory cells in secondary MLC.

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