

## *Brief Definitive Reports*

# THE RELATIONSHIP OF CELL DIVISION TO THE GENERATION OF CYTOTOXIC ACTIVITY IN MIXED LYMPHOCYTE CULTURE

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There is good evidence that the proliferative response of lymphocytes in the presence of allogeneic cells ("mixed lymphocyte response") is a reflection of specific recognition of alloantigens *in vitro* (1, 2) although the magnitude of the response is clearly influenced by nonspecific serum factors and the presence of non-lymphoid cells in the cultures (3). One consequence of *in vitro* exposure of lymphocytes to irradiated or mitomycin-treated allogeneic cells is the production of cytotoxic cells which are specific for the sensitizing alloantigens (4). However, the relationship between the proliferative response in mixed lymphocyte cultures (MLC) and the development of cytotoxic activity is not fully understood. It is now evident, for example, that the production of cytotoxic cells is not an invariable consequence of strong proliferative responses in allogeneic mixed culture (5). Moreover, although the proliferative response to alloantigens is virtually unimpaired after adsorption of lymphocytes on allogeneic monolayers before MLC stimulation, this procedure results in a specific reduction in the generation of cytotoxic cells after the MLC (6). This latter observation is consistent with the hypothesis that cytotoxic precursors may be distinct from cells that account for the bulk of proliferative activity in MLC. This hypothesis receives some support from the finding that high levels of cytotoxic activity may develop in the absence of substantial proliferative responses across certain histocompatibility barriers (7).

The present study was therefore undertaken to examine the importance of cell division to the *in vitro* development of specific cytotoxic activity. To this end, we examined the effects of eliminating dividing cells at different intervals during a mixed lymphocyte reaction upon the development of cytotoxic activity.

## Materials and Methods

Cell suspensions were prepared from peripheral and mesenteric lymph nodes of 10- to 14-wk old BALB/c mice, and the spleens of (C57BL/6 × BALB/c)<sub>F</sub><sub>1</sub> 10- to 13-wk old mice. The lymph node cells were passed through nylon columns so that the passed cells contained 85-95% Thy 1.2 bearing cells.

MLC were performed using RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) enriched with 10% fetal calf serum (Microbiological Associates, Inc., Bethesda, Md.). Each culture contained  $0.5 \times 10^6$  BALB/c lymph node (LN) cells and  $0.5 \times 10^6$  (C57BL/6 × BALB/c)<sub>F</sub><sub>1</sub> spleen cells that had been irradiated (2,500 R) *in vitro* just before culture. The cells were incubated in 0.2-ml vol in Falcon 3040 plates (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.).

Hot pulsing was accomplished by adding 0.7  $\mu$ Ci of [<sup>3</sup>H]thymidine (sp act, 56) (Schwarz/Mann Div., Becton, Dickinson & Co. Orangeburg, N. Y.) in 0.05 ml to each well at different times over a 5 day MLC period, according to the protocol shown in Table I. Since we wished to assess the

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TABLE I  
Protocol for Hot Pulse Experiment

	Day				
	1	2	3	4	5
A	$^3\text{H} - \text{T}$	T	→	→	→
B		$^3\text{H} - \text{T}$	T	→	→
C			$^3\text{H} - \text{T}$	T	→
D				$^3\text{H} - \text{T}$	T
E					$^3\text{H} - \text{T}$
A'	$^3\text{H} - \text{T} + \text{T}$	→	→	→	→
B'		$^3\text{H} - \text{T} + \text{T}$	→	→	→
C'			$^3\text{H} - \text{T} + \text{T}$	→	→
D'				$^3\text{H} - \text{T} + \text{T}$	→
E'					$^3\text{H} - \text{T} + \text{T}$
F	RPMI alone	→	→	→	→

importance of cell division during successive stages of the 5-day MLC, the radioactive thymidine was "chased" with 2  $\mu\text{g}$  cold thymidine after the cells had been exposed for a 24-h interval (Table I). The effectiveness of the chase was assessed by simultaneously adding hot and cold thymidine to cultures.

The development of cytotoxic activity was quantitated by a chromium release assay (7) described previously. Briefly, different numbers of sensitized cells were incubated with  $10^5$   $^{51}\text{Cr}$ -labeled EL-4 cells for 4 h, spun down at 2,500 rpm, and an aliquot of supernate was removed to assess  $^{51}\text{Cr}$  release. Cytotoxic activity was calculated according to the following formula:

$$\% \text{ Lysis} = \left( \frac{{}^{51}\text{Cr-labeled MLC cells} - {}^{51}\text{Cr-labeled unsensitized cells}}{{}^{51}\text{Cr-labeled freeze-thaw}} \right) \times 100.$$

In these experiments, background release ( $^{51}\text{Cr}$ -labeled unsensitized) was never greater than 10% of freeze-thaw values.

Total lytic activity generated per culture was determined by multiplying the specific lytic activity of  $0.5 \times 10^6$  sensitized cells by the number of aliquots of  $0.5 \times 10^6$  sensitized cells obtained from each culture.

$$\text{Total lytic activity} = \frac{\text{specific lytic activity} \times \text{cell yield/culture}}{0.5 \times 10^6}.$$

## Results and Discussion

*Tempo of Development of Cytotoxic Activity during MLC.* The development of cytotoxic activity with time is shown in Fig. 1. Activity was detectable by 3 days, and increased substantially over the subsequent 2 days of culture. The peak of cytotoxic activity was reached after 5 days of culture, with a falloff over the next 24 h.

*The Effect of Hot Thymidine during MLC upon Generation of Cytotoxic Cells.* The effect of eliminating dividing cells during successive intervals over the first 5 days of culture upon the development of peak cytotoxic activity (at 120 h) was examined (Fig. 2). Elimination of dividing cells 24 h before the addition of

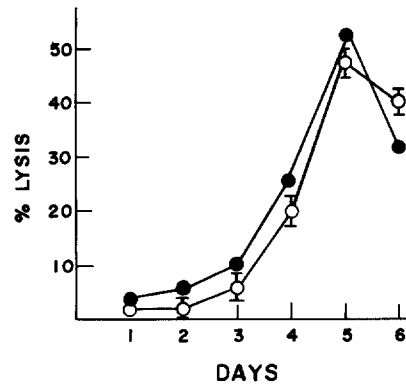


FIG. 1. Lytic activity produced with time during a one-way MLC, BALB/c LN-T plus (BALB/c  $\times$  C57BL/6) $F_1$  (2,500 R). Specific lytic activity (O) against  $H-2^b$  (EL-4) target cells produced after successive 24-h intervals during a 6-day culture is shown. Total lytic activity (see Materials and Methods) produced during this period is also shown ( $\bullet$ ). Vertical bars denote the limit of one standard error.

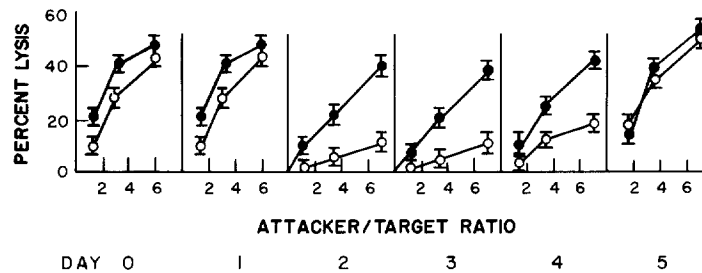


FIG. 2. The effects of eliminating dividing cells on successive days of MLC upon the development of cytotoxic activity. The effects of adding "hot" thymidine at various intervals during a one-way MLC upon the production of cytotoxic activity at the end of 5 days of culture is shown (O). 24 h after the addition of each pulse of hot thymidine, the cultures were "chased" with excess cold thymidine. That the chase was effective is shown by the virtually unimpaired reactivity of cultures simultaneously pulsed with hot and cold thymidine ( $\bullet$ ). The addition of hot thymidine alone 24 h before MLC stimulation or during the first 24 h of MLC (days 0 and 1) did not impair the development of cytotoxic activity. The addition of hot thymidine during the 2nd, 3rd, and 4th day of MLC culture markedly reduced subsequent cytotoxic activity. The vertical bars denote the limit of one standard error.

antigen (day 0) or over the 1st day of culture did not affect the cytotoxic response on day 5, indicating that (a) cell division during this period was not critical to the development of the cytotoxic response, and (b) the hot thymidine did not exert nonspecific toxic effects. Elimination of dividing cells during the 2nd and 3rd day of mixed cell culture, however, virtually abolished subsequent cytotoxic activity. Removal of dividing cells on day 4 substantially reduced the subsequent lytic activity, while a hot pulse during the 5th day of culture had no effect upon the subsequent cytotoxic response (Fig. 1). That the chase by cold thymidine after each 24-h interval was fully effective is shown by the unimpaired activity (compared with untreated cultures) of cells that had received the hot and cold thymidine simultaneously. A summary of the time-course of the division-

dependent and division-independent phases of generation of killer activity is shown (Fig. 3). It should be noted that the substantial rise in cytotoxic activity between the 4th and 5th day of culture shown in Fig. 1 was not susceptible to the effects of the hot thymidine pulse during that period.

The finding that the development of killer activity could be abolished by hot thymidine during the 2nd-4th day of culture is formally consistent with one of two interpretations. The first is that all cells necessary for the generation of effector cell activity are activated to divide, take up thymidine, and are "suicided." The second explanation is that although dividing cells are necessary

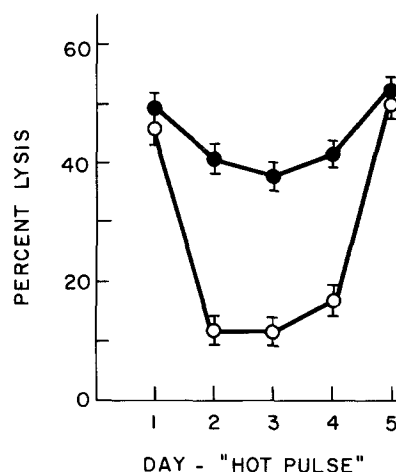


FIG. 3. A summary of the effects of thymidine hot pulse on different days of MLC upon the development of cytotoxic activity. Lytic activity (at a 7:1 attacker:target ratio) produced by cells that were hot pulsed at different intervals during MLC (O) is compared to the lytic activity produced by cells that had simultaneously received hot and cold thymidine during the same intervals (●) (for full protocol, see Table 1). Vertical bars denote the limit of one standard error.

for the production of cytotoxic activity, they are not cytotoxic precursors but serve only to amplify the differentiation of the latter. This latter possibility was directly tested: Cells depleted of cytotoxic precursor activity, but not MLC activity, after monolayer adsorption were combined with cells that had been suicided by hot thymidine during MLC on day 2 and sensitized *in vitro*. These mixtures did not produce significant cytotoxic responses after sensitization (Table II).

In sum, these experiments make three points: (a) The inability of hot thymidine pulsing during the 24 h before MLC to affect subsequent generation of cytotoxic activity indicates that precursors of cells that develop cytotoxic activity to mouse alloantigens are primarily long-lived, nondividing T lymphocytes. (b) We have demonstrated that the *in vitro* generation of cytotoxic activity is dependent upon cell division during a defined, critical period of sensitization occurring 24-72 h after the addition of antigen. Moreover, a subsequent division-independent period of differentiation is also required for full expression of cytotoxic activity. (c) These studies also indicate that cytotoxic precursor activity as well as MLC activity is susceptible to the thymidine hot pulse

TABLE II  
*Cytotoxic Activity Produced After Sensitization of Mixtures of "Hot Pulsed"  
 BALB/c Cells and Monolayer-Adsorbed BALB/c Cells*

	Hot pulse day 2	Adsorbed C57BL/6 monolayer*	Adsorbed CBA monolayer*	Lysis
				%
A.	$2.5 \times 10^5$	—	—	1.3
B.	—	$2.5 \times 10^5$	—	6.2
C.	—	—	$2.5 \times 10^5$	29.1
D.	$2.5 \times 10^5$	$2.5 \times 10^5$	—	2.2

\*  $5 \times 10^6$  BALB/c LN-T cells were adsorbed on monolayers of C57BL/6 or CBA peritoneal macrophage cells for 4 h at 37°C. Nonadherent cells (approximately 80% of starting cell numbers) were then sensitized with C57BL/6-2,500R cells alone or combined with BALB/c cells that had been "hot pulsed" on day 2 of MLC, and washed twice before mixing with nonadherent cells. Lytic activity of these MLC cultures was assessed 5 days later.

technique. In this experiment, the addition of MLC active cells (depleted of cytotoxic precursor activity by monolayer adsorption) to cells that had been depleted of dividing cells during MLC did not restore ability to generate a cytotoxic precursor activity by monolayer adsorption) to cells that had been alloantigens as well as MLC active cells divide to some extent during sensitization, although perhaps at different rates.

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## References

1. Wilson, D. B., and P. C. Nowell. 1971. Quantitative studies on the mixed lymphocyte interaction in rats. Tempo and specificity of the proliferative response and the number of reactive cells from immune donors. *J. Exp. Med.* **133**:442.
2. Zoschke, D. C., and F. H. Bach. 1971. Specificity of allogeneic cell recognition by human lymphocytes in vitro. *Science (Wash. D. C.)* **127**:1350.
3. Peck, A. B., E. Katz-Heber, and R. E. Click. 1973. Immune responses in vitro. IV. A comparison of the protein-free and mouse serum-supplemented mouse mixed lymphocyte interaction assays. *Eur. J. Immunol.* **3**:516.
4. Hayri, P., L. C. Andersson, S. Nordling, and M. Virolainen. 1972. Allograft responses in vitro. *Transplant. Rev.* **12**:91.
5. Abbassi, K., P. Demant, H. Festenstein, J. Holmes, B. Huber, and M. Rychilková. 1973. Mouse mixed lymphocyte reactions and cell mediated lympholysis. *Transplant. Proc.* **5**:1329.
6. Bach, F. H., M. Segall, K. S. Zier, P. M. Sondel, B. J. Alter, and M. L. Bach. Cell mediated immunity: separation of cells involved in recognitive and destructive phases. *Science Wash. (D. C.)*. **180**:403.
7. Nabholz, I., J. Vives, H. M. Young, T. Meo, V. Miggiano, A. Rijnbeek, and D. C. Shreffler. 1974. Cell mediated cell lysis in vitro: genetic control of killer cell production and target specificities in the mouse. *Eur. J. Immunol.* **4**:378.