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### Authors

Kramer, James J  
Granger, GA

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## Relationship of Lymphotoxin Secretion and DNA Synthesis in the Human Mixed Lymphocyte Reaction *in Vitro*<sup>1</sup>

JAMES J. KRAMER AND G. A. GRANGER

*Department of Molecular Biology and Biochemistry,  
University of California, Irvine, California 92664*

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One-way mixed lymphocyte cultures employing human adenoid or peripheral blood lymphocytes activate lymphotoxin (LT)-secreting cells. Kinetic analysis of lymphocytes stimulated in mixed culture demonstrates that LT is secreted before the onset of DNA synthesis, but that maximum levels of LT secretion are reached simultaneously with maximum levels of DNA synthesis. Although the response of peripheral leukocytes is qualitatively similar to the response of adenoid-derived lymphocytes, unexplained high nonspecific background levels of toxic material(s) obscure early events in the former response. While cytochalasin B reversibly inhibits LT secretion, mitomycin C treated cultures are still capable of LT secretion. The results suggest that a population of cells exists, which does not require DNA synthesis to develop into effector cells. The requirement for DNA synthesis for the maximal development of effector cells may reside in a separate helper cell population as postulated by the two cell model of the mixed lymphocyte reaction.

### INTRODUCTION

The mixed lymphocyte culture reaction (MLC), observed when genetically incompatible lymphocytes are cultured together, is thought to be an *in vitro* correlate of a primary cell-mediated immune response (CMI) with proliferation and the development of cytotoxic effector cells (1-3). The proliferative phase, as measured by the ability of cells to incorporate increased levels of [<sup>3</sup>H]thymidine into DNA, has been postulated to relate to the afferent or initiation phase of the immune response (4, 5); while cytotoxic activity is thought to relate to the efferent or effector phase of the response (6-8). While the relationship between the lymphocytes which synthesize DNA and the cytotoxic cells is not clear, it is generally accepted that DNA synthesis is required for the development of the cytotoxic effector cells (9, 10). It has also been demonstrated in many species (11), including man (12), that the lymphocytes involved in MLC are thymus-derived.

Many investigators have demonstrated the presence of soluble factors (lymphokines) in the cell-free supernatants from MLC (13-16). These lymphokines have a wide range of effects on cells *in vitro* and include blastogenic factor, potentiating factor, migration inhibitory factor, and lymphotoxin (LT). There has also been

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described a medium-conditioning factor, presumably released by macrophages, which is necessary to support the MLC response. These factors have been postulated to be mediators of CMI reactions *in vitro* (17, 18).

Lymphotoxin has been described in a number of *in vitro* models of cellular immune systems and may be a primary effector in the cytotoxic action of activated lymphoid cells on target cells. The relationship between the appearance of LT in the medium and the proliferation of stimulated lymphocytes is still unclear. Lymphotoxin can be detected in the cell-free supernatants of phytohemagglutinin-stimulated human lymphocyte cultures 18–20 hr before DNA synthesis begins (19). Moreover, the daughter cells in these cultures do not appear to be involved in LT secretion (19, 20). It is also the case that lymphoid cells from immunized animals do not require DNA synthesis to secrete LT or to lyse target cells when cultured with antigen *in vitro* (17, 21). It has been suggested, however, that DNA synthesis is a prerequisite for the generation of cytotoxic cells following primary sensitization *in vitro* (9, 22).

The results presented here suggest that in mixed cultures, DNA synthesis and CMI effector function as measured by LT secretion may be separable but related phenomena, which may be associated with different cell populations.

## MATERIALS AND METHODS

### *Cells and Media*

The maintenance of mouse alpha L-929 cells used in these experiments has been described elsewhere (23, 25). The medium used in the mixed lymphocyte cultures consisted of Eagle's minimum essential medium supplemented with 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, 3.2  $\mu\text{g}/\text{ml}$  garamycin, 20  $\mu\text{g}/\text{ml}$  amphotericin B, 4 mM glutamine, nonessential amino acids, 1 mM sodium pyruvate,  $5 \times 10^{-5}\text{M}$  mercaptoethanol (EtSH), and 10% pooled A+ heat-inactivated human serum (HS), (MEM + 10% HS). Cell washes were performed with medium containing 3% HS (MEM + 3% HS). When peripheral blood cells (PBC) were used, the medium was supplemented with 10% fresh pooled plasma from responder and stimulator blood.

### *Preparation of Suspensions of Peripheral Blood and Adenoid Cells*

Forty milliliters of blood obtained from health donors was suspended in 10 U/ml heparin and centrifuged at 900g for 10 min. Ten milliliters of the plasma was removed, filter-sterilized, and added to the MLC medium. The blood cells were then resuspended in the remaining plasma, one-third volume of Plasma Gel (HTI Corp., Buffalo, NY) added, and the mixture incubated at 37°C for 30 min to sediment the red blood cells (RBC). After incubation, the supernatant was removed, leukocytes pelleted at 325g for 10 min, and washed twice with MEM + 3% HS. Typical differential cell counts revealed 35–50% lymphocytes, 45–65% polymorphonuclear leukocytes, 2–10% monocytes, and RBC:leukocyte ratios of 2–3:1.

Fresh adenoid tissues obtained from unrelated healthy children were minced and suspended in MEM + 3% HS. The mixture was vortexed thoroughly and the tissue fragments were removed by centrifugation at 900g for 2 min. The cells were pelleted at 325g for 5 min, washed once with MEM + 3% HS, and suspended finally in MEM + 10% HS. These cells were 85–95% viable and consisted of 90–98% lymphocytes (24).

Either PBC or adenoid cells were suspended in MEM + 3% HS, containing 25  $\mu\text{g/ml}$  mitocycin C (MC) Sigma, St. Louis, Mo., lot 122C-0320) at a concentration of  $2-4 \times 10^6$  mononuclear cells/ml, and incubated for 30 min at  $37^\circ\text{C}$  in the dark. The cells were then pelleted at  $325g$  for 5 min, washed twice with MEM + 3% HS, and finally suspended in MEM + 10% HS, at a concentration of  $2 \times 10^6$  mononuclear cells/ml. Then 0.5 ml of responder cells (R) and 0.5 ml of stimulator cells (S) were added to appropriate plastic tubes (Falcon Plastics, Oxnard, Ca.) and incubated at  $37^\circ\text{C}$  in an atmosphere of 95% air + 5%  $\text{CO}_2$  for the indicated time.

#### *Assay for [ $^3\text{H}$ ]Thymidine Incorporation into DNA*

Each 1.0 ml culture to be assayed for DNA synthesis received 0.1 ml MEM containing 2  $\mu\text{Ci}$  [ $^3\text{H}$ ]thymidine (6 c/mM, Schwarz/Mann, Orangeburg, NY). The cultures were then incubated 4 hr at  $37^\circ\text{C}$ . The cultures were then chilled to  $4^\circ\text{C}$ , and the cells sedimented by centrifugation at  $900g$  for 4 min. The supernatants from duplicate culture tubes were pooled and later assayed for LT activity. The cell pellets were washed once with ice cold 0.15 M NaCl, resuspended in 2.0 ml of 0.15 M NaCl, vortexed, and the suspensions transferred to glass tubes. The plastic culture tubes were washed once with 2.0 ml 0.15 M NaCl, and the wash added to the glass tube. The cells were again pelleted and the supernatant discarded. Two drops of phenol-extracted glycogen carrier and 1.0 ml of 5% trichloroacetic acid were added to each cell pellet. The resulting suspensions were centrifuged at  $1400g$ , and the pellets were thoroughly drained. Following this, 0.4 ml Nuclear Chicago Solubilizer (Nuclear Chicago, Chicago, Ill.) was added to each pellet and the tubes incubated at  $56^\circ\text{C}$  for 2 hr. Finally, 10 ml of Onmifluor scintillation cocktail (New England Nuclear, Boston, Mass.) was added to each tube, the contents thoroughly mixed and transferred to glass scintillation vials and counted on a Beckman LS-100 spectrophotometer, using a wide-open channel.

#### *Lymphotoxin Assays*

The highly sensitive assay for the detection of LT, involving the use of MC-treated alpha L cells, has been described elsewhere (25, 26). A brief description of this assay follows. Mouse alpha L cells were established in  $16 \times 125$  mm glass screw-capped culture tubes in the presence of 0.4  $\mu\text{g/ml}$  MC for 18-24 hr. Uniform monolayers were selected and test and control mediums were diluted as indicated. The cultures were then incubated for 48 hr and visually examined for evidence of target cell destruction. The medium was then discarded, and the monolayers washed once with 4.5 ml 0.15 M NaCl buffered with  $1.5 \times 10^{-3}$  M phosphate, pH 7.2 (PBS). After washing, 0.5 ml PBS containing 0.01% trypsin and  $10^{-4}$  M EDTA was added to each tube. After incubation at  $37^\circ\text{C}$  for 3-5 min, each culture was agitated vigorously to mechanically dislodge all cells from the glass surface. Then 4.5 ml PBS containing 0.1% formaldehyde solution was added to each tube, the suspensions were again thoroughly mixed, and the total number of cells enumerated on a Coulter Counter. The results are expressed as units of LT present per milliliter of culture supernatant where one unit of LT represents the amount of LT necessary to destroy 50,000 L cells in 48 hr, and is expressed as the inverse of the  $\text{LD}_{50}$  LT dilution.

## RESULTS

*Kinetics of DNA Synthesis and LT Secretion in PBC and Ad Mixed Leukocyte Cultures*

Cells from peripheral blood (PBC) or adenoids (Ad) were established in tube culture as described in Materials and Methods. Each set consisted of duplicate cultures of R:S<sub>m</sub> cell combinations. Each day for 8 or 9 days, one set of cultures was assayed for DNA synthesis and LT secretion. The experiment was repeated a total of four times, utilizing cells from different donors. The data which represents a typical experiment can be seen in Figs. 1 and 2. The proliferative response of Ad in mixed culture (Fig. 1) as measured by DNA synthesis was first detected on day 3. The number of cells involved in DNA synthesis in allogeneic mixes increased until days 6 or 7, and then decreased rapidly to near control levels by day 9. On the first day of culture, Ad cells showed a slight degree of spontaneous DNA synthesis in both syngeneic and allogeneic mixes. Cultures containing double MC-treated cells (R<sub>m</sub>:S<sub>m</sub>) always incorporated equal or less amounts of [<sup>3</sup>H]TdR into DNA than R:R<sub>m</sub> cultures. The kinetics of LT release by Ad cells in these cultures are shown in Fig. 1. In contrast to DNA synthesis, low levels of specific LT (that amount of LT found in allogeneic mixes above background syngeneic levels) could be detected in R:S<sub>m</sub> cultures on day 2. Peak LT concentrations, however, were reached by days 6 or 7, in concert with peak DNA synthesis in R:S<sub>m</sub> cultures and remained constant for the remainder of the incubation period, although DNA synthesis had decreased to control levels. The cultures containing R<sub>m</sub>:S<sub>m</sub> cells were able to secrete some LT, although significant levels were not detected until days 4 or 5. The cultures containing R:R<sub>m</sub> cells released little or no LT into the supernatant.

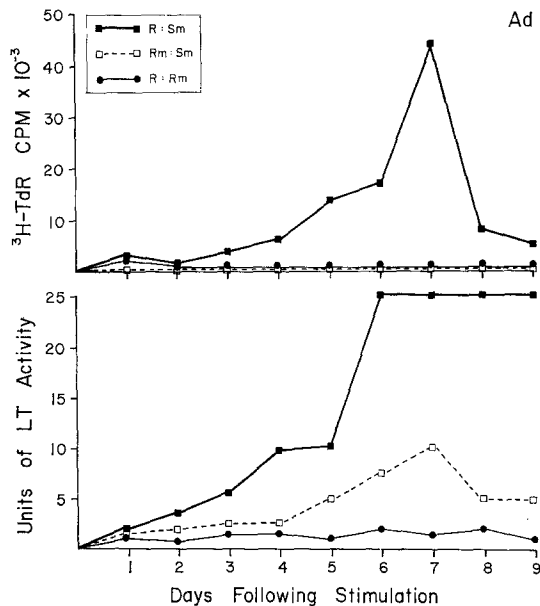


FIG. 1. Kinetics of the mixed lymphocyte response of human adenoid-derived lymphocytes in terms of DNA synthesis (top) and LT secretion (bottom).

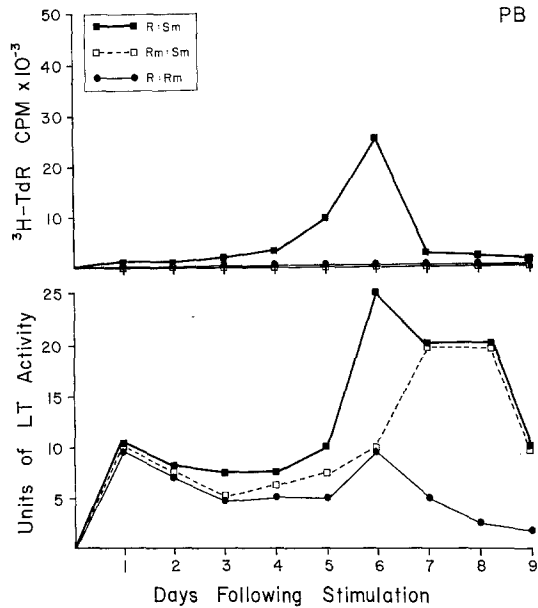


FIG. 2. Kinetics of the mixed lymphocyte response of human peripheral blood leukocytes, in terms of DNA synthesis (top) and LT secretion (bottom).

The results obtained from similar studies on mixed cultures containing PBC can be seen in Fig. 2. The proliferative response for PBC was essentially the same as that observed with Ad cells. Synthesis of DNA commenced on day 3, and reached a peak on days 6 or 7. Spontaneous DNA synthesis on day 1 was low in PBC-MLC, and [<sup>3</sup>H]TdR incorporation in R<sub>m</sub>:S<sub>m</sub> cultures was lower than or equal to incorporation by R:R<sub>m</sub> cultures. Spontaneous secretion of "LT-like" substances by PBC was high (5–10 units) for all cultures on day 1, following mixing. This level of nonspecific cytotoxic activity remained constant or dropped slightly by day 2. Specific LT secretion by R:S<sub>m</sub> cultures became detectable by days 3 or 4; however, secretion could have begun earlier, but may have been obscured by the high level of spontaneous activity. The highest levels of LT were observed in these cultures on days 6 or 7, at times which also corresponded to peak DNA synthesis. After reaching a peak, the LT levels slowly decayed over the next 3 days. Levels of LT in supernatants from R<sub>m</sub>:S<sub>m</sub> or 1 day later than R:S<sub>m</sub> cultures. The levels of LT in R<sub>m</sub>:S<sub>m</sub> cultures decayed at a rate similar to that observed in R:S<sub>m</sub> cultures. The background "nonspecific" LT levels from R:R<sub>m</sub> cultures remained high until days 5 or 6, when they, too, decayed.

Experiments were designed to determine if glass-adherent cells are involved in the secretion of LT in MLC. A standard MLC was established, and on day 4 following mixing, one set of PBC and one set of Ad cultures were assayed for DNA synthesis and LT activity. The remaining culture tubes were centrifuged at 325g for 4 min, the nonadherent cells suspended in 1.0 ml MEM + 3% HS, transferred to fresh culture tubes, pelleted again, and resuspended in 1.0 ml fresh MEM + 10% HS. After incubation for the indicated times, the cultures were assayed for DNA synthesis and the supernatants for the presence of LT. Microscopic observations revealed that the mechanical manipulations did not dislodge the glass-adherent cells. The results shown in Fig. 3 indicate that the removal of adherent

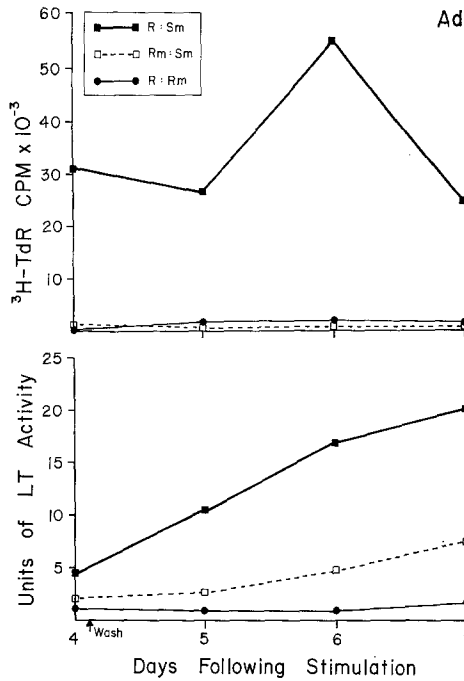


FIG. 3. The effect of removing glass-adherent cells on DNA synthesis (top) and LT secretion (bottom) in the human adenoid-derived mixed lymphocyte response. Glass adherent cells were removed from the cultures at day 4, then the nonadherent cells were washed and transferred to new culture tubes. The levels of LT and DNA synthesis were determined each day up to day 7.

cells from these cultures had little or no effect on the levels of DNA synthesis or LT secretion of the nonadherent cell population. While not evident in this figure, in a few experiments, the background cytotoxic activity in supernatants from R:R<sub>m</sub> cultures were reduced. These results would indicate that adherent cells do not participate directly in LT secretion in Ad cultures once the responder cells have been activated. In contrast, results obtained with PBC-MLC revealed that while DNA synthesis was not affected, the level of LT in allogeneic R:S<sub>m</sub> mixtures was somewhat reduced and may have been responsible for some of the nonspecific toxicity. In addition, studies were performed which revealed that in contrast to PB cells, MLC reactions initiated with adenoid cells did not require the presence of glass adherent cells during the initiation phases of this reaction.

#### *Inhibition of DNA Synthesis and LT Secretion by Cytochalasin B*

A set of standard MLC, using Ad cells, was established and incubated for 4 days. Following this incubation, the cultures were centrifuged at 325g for 4 min, and the cells resuspended in MEM + 10% HS containing 10  $\mu\text{g/ml}$  cytochalasin B (CB) (Imperial Chemical Industries, Ltd., Macclesfield, Cheshire, England) and 0.33% dimethyl sulfoxide (DMS) (Spectro grade, Eastman Kodak Co., Rochester, NY). This concentration of CB was chosen after extensive experiments checking various levels of the agent on lymphocyte responsiveness. This level was employed because it reversibly inhibited DNA synthesis and LT secretion in Con A and PHA stimulated cells. Control cultures contained 0.33% DMSO. Previous experiments had shown that this concentration of DMSO had no effect on the MLC in terms of

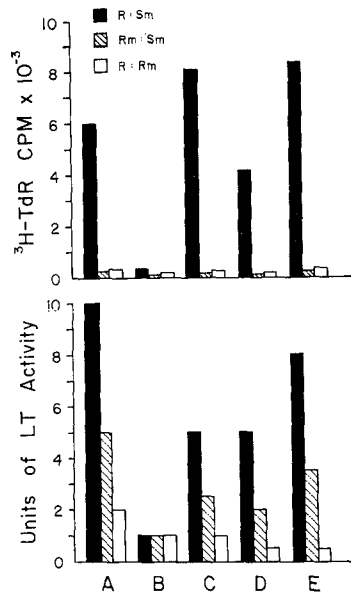


FIG. 4. The effect of cytochalasin B on the uptake of [ $^3\text{H}$ ]TdR (top) and secretion of LT (bottom) by adenoid-derived human lymphocytes stimulated in mixed culture. A—untreated control; B—CB-treated for 24 hr; C—DMSO-treated for 24 hr; D—CB-treated for 24 hr, washed, incubated an additional 24 hr in fresh medium; E—DMSO-treated for 24 hr, washed, incubated an additional 24 hr in fresh medium. See text for details.

DNA synthesis and LT supernatant activity. These cultures were preincubated 6 hr in DMSO or CB at 37°C, to ensure complete inhibition by the CB, and then each culture was centrifuged at 325*g* for 4 min, and resuspended in MEM + 10% HS, containing fresh CB or DMSO. After 20 hr incubation, 2  $\mu\text{Ci}$  [ $^3\text{H}$ ]TdR in 0.1 ml was added to test and control cultures. The tubes were incubated an additional 4 hr, the supernatants removed, and the cells assayed for DNA synthesis. The supernatants were dialyzed against four changes of 1 liter each of MEM over a 12-hr period, to remove the CB and DMSO. They were then filter-sterilized and assayed for the presence of LT. Parallel sets containing CB and DMSO were washed three times with MEM + 3% HS, resuspended in fresh MEM + 10% HS, and incubated for an additional 20 hr. Then 2  $\mu\text{Ci}$  of [ $^3\text{H}$ ]TdR in 0.1 ml was added to each culture, the tubes incubated for an additional 4 hr, the supernatants removed for LT assays, and the cells assayed for DNA synthesis. From Part A of Fig. 4, it can be seen that the normal [ $^3\text{H}$ ]TdR and LT levels were observed prior to treatment. After incubation in the presence of CB for 24 hr, shown in Part B, DNA synthesis and LT secretion in R:S<sub>m</sub> cultures was reduced to control levels. Cytochalasin treatment also inhibited the release of LT from R<sub>m</sub>:S<sub>m</sub> cultures. Parallel mixed cultures incubated in the presence of DMSO were unaffected in their ability to incorporate [ $^3\text{H}$ ]TdR into DNA or secrete LT (Part C). Cultures which were incubated in the presence of CB for 24 hr, washed and reincubated in fresh medium for 24 hr, regained most of their DNA-synthesizing and LT-secreting abilities (Part D), when compared to parallel cultures which had been incubated in and washed from DMSO (Part E). These data strongly suggest that LT release is an active membrane process, since it is reversibly inhibited with CB.



## DISCUSSION

It is becoming increasingly apparent that the MLC reaction may be an *in vitro* model which typifies how "nonimmune" lymphoid cells become sensitized to become effector cells and participate in cell-mediated immune reactions. The response may be divided into at least three stages: (1) the first is recognition and stimulation or activation of the responder lymphoid cells upon contact with foreign antigens on the stimulator lymphoid cells, which (2) stimulates cell proliferation (DNA synthesis), followed by (3) the appearance of specific cytotoxic effector cells. The relationship of these stages, one to another, is not yet clearly understood. The response of the generated effector cells can be measured by the effects of the direct interaction of lymphocytes on target cells (direct lymphocyte cytotoxicity) or the effects of cytotoxic supernatants obtained from activated effector cell cultures on target cells (indirect lymphocyte cytotoxicity). It has been previously shown that LT is released in human and murine MLC reactions (19, 30).

Our studies with cytochalasin B verify that the appearance of LT in the medium is due to an active cell secretory process, and not a product(s) released by dead or dying cells. Similar inhibition of the release of LT has been observed in populations of immune rat lymphocytes stimulated by soluble Ag (27). This is a lymphocyte product for removal of glass-adherent cells and had little or no effect on LT release in cultures of activated adenoid cells.

Glass-adherent cells have been reported to be important in the initiation of an MLC reaction in PB lymphoid cells of experimental animals and humans by many investigators (31-33). It appears that the role of the monocytic cell is nonspecific, for it can be replaced by supernatants from cultures of these cells (34). We found that glass-adherent cells are not needed to initiate or maintain an MLC response when human adenoid lymphocytes are employed. When these cells are removed from MLC adenoid cultures after stimulation and proliferation are initiated, DNA synthesis and LT secretion are unaffected.

The relationship of DNA synthesis to the development of effector cells as measured by LT secretion or direct lymphocyte-mediated cytotoxicity is at present a matter of controversy. The kinetics of the MLC response can be divided into three distinct phases: (1) the events which precede DNA synthesis, (2) the events which commence with the beginning of, and end with, the peak of DNA synthesis, and (3) the events which occur subsequent to the peak proliferative response.

The first phase extends to the third day following stimulation in mixed cultures of both Ad and PB cells. We observed levels of LT in allogeneic stimulated Ad cultures which are above the LT levels observed in control syngeneic cultures (specific LT levels) consistently 1 or 2 days before the initiation of DNA synthesis. Early levels of LT are low, however, and cell proliferation is required for maximal levels to be reached. The early levels of LT secretion may be the first detectable event of a lymphocyte-mediated cytotoxic reaction. The observation that an indirect lymphocyte cytotoxic response occurs prior to proliferation in cultures of mitogen-stimulated lymphocytes supports this view (19). In contrast, direct cytotoxic effector cells have not been detected in murine MLC until after peak DNA synthesis has occurred (36). This has been interpreted to mean that maturation of the killer cell must occur before it can function to cause destruction of a target cell (37). It is possible, however, that the assay systems employed to measure killer cells are not sensitive enough to detect the functional capabilities of these cells at the early

stages of activation, as indicated by the large lymphocyte:target cell ratios which are required to detect target cells lysis. The large numbers of lymphocytes present in the cultures could also result in high nonspecific background levels of cytotoxicity which would obscure low levels of cytotoxic activity, which could be manifested by effector cells early in the reaction. In contrast, our LT assay system for measuring indirect lymphocyte cytotoxicity is manifold more sensitive and is able to detect low levels of LT secreted early in the response (25, 26). In MLC cultures employing PBC, the early specific release of LT is not observed, but most likely is obscured by the high background levels of "LT-like activities" observed in these cultures. These high nonspecific levels of cytotoxic materials may be due to the disintegration of and release of toxic lysozymes of polymorphonuclear leukocytes (PMN). Such toxic materials have been reported by other investigators (35).

The second phase of the response begins on day 3 and terminates on days 6 or 7, following stimulation when maximal levels of DNA synthesis have been reached. During this phase, LT levels in allogeneic PB and Ad cultures increase rapidly and reach maximal levels simultaneously with DNA synthesis. This suggests that no new LT-secreting cells are generated after DNA synthesis ceases, and that DNA synthesis may influence the development of LT secreting cells or vice versa. The possible control of LT secretion by DNA synthesis is further substantiated by allogeneic cultures where both R cells and S cells are MC-treated. This treatment greatly reduces the levels of LT (40-70% of controls) in Ad mixed cultures, and, although LT levels in PBC mixed culture are comparable to control allogeneic cultures, the rate of secretion is retarded. The need for DNA synthesis may represent the proliferation of a helper cell population, proliferation of effector cells or gene amplification in effector cells. It has been recently shown that cytotoxic cells can be separated from proliferating cells in both human and murine MLC reactions. This separation was accomplished by cell adsorption on immunospecific monolayers (5), separation of cells differing in the densities of the thymus-specific Ag,  $\theta$  (22), and separation by tissue of origin (38). These results indicate that although effector cells may undergo limited DNA synthesis, the majority of cells involved in DNA synthesis do not develop into effector cells.

The third phase of the response is characterized by the inhibition of DNA synthesis and cessation of the increase in LT in the culture medium. Mixed cultures containing Ad cells maintain high levels but do not increase the levels of LT several days following the shutdown of DNA synthesis, while in the cultures containing PBC, the LT levels decay slowly. The amount of LT released is directly related to the degree of stimulation in terms of DNA synthesis, and we observe final levels of LT in supernatants derived from separate MLC of 10-100 units, where the greatest levels of LT were observed in cultures where DNA synthesis is the greatest. The halting of LT secretion could be either the result of the destruction of the responder lymphocytes involved in the response or control exerted by some component of the culture. The latter supposition is supported by the observation that when the medium from allogeneic cultures containing either adenoid cells or PBC is replaced with fresh medium (data not shown), the levels of LT return to the original level within 24 hr. There is the possibility that the LT secretion is under control of a type of feedback regulation system. Alternatively, another regulatory lymphokine may exist which stops these reactions, and either affects the LT-secreting cells directly, or the helper cell. Finally, a repressor cell popula-

tion might be involved in regulating the degree of activation and final levels of direct and indirect lymphocyte cytotoxicity observed in the MLC response.

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