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LYMPHOCYTE EFFECTOR MOLECULES: AN IN VITRO PRODUCTION METHOD FOR OBTAINING LITER VOLUMES OF SUPERNATANTS FROM MITOGEN-STIMULATED HUMAN LYMPHOCYTES *

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An in vitro method has been developed utilizing phytohemagglutinin (PHA) activated lymphocytes obtained from human tonsils and adenoids which permit the accumulation of multi-liter quantities of cell-free supernatants containing lymphotoxin and other lymphocyte effector molecules (LEM). An enriched media is employed which contains a large molecular weight, heat stable bovine serum fraction which supports lymphoid cell activation and levels of LEM secretion equal to that of cultures maintained in medium supplemented with whole serum. Elimination of whole serum from the media greatly reduces overall protein concentrations and facilitates concentration and purification studies. Various technical aspects of these cultures have been examined, i.e.: 1) cell concentration, 2) kinetics of LT production over a ten-day period, 3) mitogen dosage, and 4) types of media. Supernatants can be harvested repeatedly from a single culture over the ten day period, thus doubling the yield of LEM collected from a single culture.

INTRODUCTION

There are a broad range of in vitro activities associated with soluble molecules, collectively termed lymphokines (LK) (Dumonde et al., 1969) or lymphocyte effector molecules (LEM), detectable in the cell-free supernatant media obtained from antigen or mitogen activated lymphocytes, mixed lymphocyte culture or certain continuous human lymphoid cell lines (Granger, 1972). It is generally regarded that LEM may be mediators in cell-mediated immune reactions (CMI). Because LEM are present in trace amounts, and are difficult to obtain in large quantities of active culture supernatants containing the various LEM, studies on their identity, physical characteristics and mode of action have been slow. We have studied one of the human LEM activities, the lymphotoxins (LT), and have shown that they are a family of non-specific cell-toxins which may be involved in lympho-

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cyte-mediated cytostatic and cytotoxic reactions (Granger et al., 1975). In attempts to study human LT, continuous human lymphoid cell lines have been explored as potential sources of secreting cells (Glade et al., 1969; Granger et al., 1970; Amino et al., 1974). Most of the cell lines tested secrete very low levels of LT. In contrast, mitogen-activated human lymphoid cells release high levels of LT as compared to human lymphoid cell lines (Granger et al., 1970). Mitogen-activated human lymphoid cells, however, release higher levels of LEM in medium supplemented with serum than in protein-free medium (Kolb et al., 1971; Shacks and Granger, 1971). Various investigators have attempted to bypass this problem by first establishing cultures in serum-containing medium and then, once activated, transferring the cells to a protein-free medium (Walker and Lucas, 1974), or a serum substitute (Kolb et al., 1971; Shacks and Granger, 1971). The present studies employed lymphotoxin as an indicator human LEM and reveal that an enriched media, supplemented with a large molecular weight fraction from heat stable bovine serum, supports phytohemagglutinin (PHA) induced human lymphocyte activation and LEM secretion, equal to that when cells are cultured in medium with whole serum. A semi-quantitative bioassay for the measurement of human LT (Spofford et al., 1974) has permitted study of some kinetic parameters associated with the production and secretion of this effector molecule in these cultures.

MATERIALS AND METHODS

Tonsil and adenoid collection

Tonsils and adenoids (T and A) were collected aseptically after surgery and placed in a media containing high concentrations of antibiotics. The tissue samples were maintained at 4°C during collection and transportation to the laboratory. We found single cell suspensions and/or sliced whole lymphoid tissues in media could be held at 37°C for 24 h or up to two days at 4°C. Viability was increased if storage media was supplemented with 10% bovine serum. Medium used for suspending the tissues during their collection was powdered Hanks minimal essential media (MEM - Gibco, Grand Island, NY), supplemented with non-essential amino acids, 2 mM glutamine, 1 mM sodium pyruvate, 200 U/ml penicillin, 200 μg/ml streptomycin, 5 μg/ml amphotericin B, and 62 μg/ml of amikacin. The antibiotic concentration in this media was twice the level normally employed in order to minimize bacterial and fungal contamination (2X-AM).

Preparation of single lymphoid cell suspensions

Manipulation of the samples was performed under aseptic conditions in a laminar air flow hood. The whole tissues were washed twice with protein-free 2X-AM before removal from the collecting containers. Each tonsil was placed in a disposable Petri dish, trimmed of non-lymphoid tissue and tho-

roughly rinsed with 2X-AM. Lymphocytes were released from solid tissues by scalpel mincing in MEM + 2X-AM. After mincing the tissue into fragments of 1 mm or smaller, portions of the Petri dish contents were poured into a sterile fine-mesh tissue strainer and gently pressed through the screen mesh using a ceramic pestil with frequent rinses of 2X-AM. The filtrate, collected in a sterile Petri dish, was then pipetted into a 50 ml tube and centrifuged at 50 *g* for 30—60 sec to sediment large tissue debris. The supernatant was aseptically aspirated into another 50 ml tube and the cells sedimented by centrifugation at 300 *g* for 5 min. The supernatant was aspirated and discarded. The cell pellet was resuspended in 10 ml of MEM + 2X-AM. The number of viable cells/ml was determined by visual count in a Neubauer Chamber of an aliquot of the suspension suspended in 0.1% Eosin Y in 0.15 M NaCl buffered with 1.5×10^{-3} phosphate, pH 7.2 (PBS). Lymphocyte cell suspensions having less than 60—70% viability were not usually responsive in vitro. Most preparations were from 75 to 85% viable and consisted of 95—98% lymphocytes, 1 to 5% monocytes and 0—1% cells of connective or epithelial tissue origin.

Lymphocyte culture

Lymphoid cell suspensions were cultured in powdered MEM, supplemented with non-essential amino acids, 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B and 5×10^{-5} mercaptoethanol. Lymphocytes were cultured at a density of 4 to 5×10^{6} cells/ml in 32 oz screw-top glass prescription bottles containing 200 ml of culture media/btl. Boiled serum (BS) fraction was added to the medium at 10 μg/ml as a substitute for whole serum. However, the amount employed varied (5 to 10 μg/ml) from lot to lot of BS employed. Cells were stimulated by the addition of 20 μg/ml of phytohemagglutinin-P (PHA-P, Difco Laboratories, Detroit, Mich.) and incubated in a 95% air 5% CO_2 environment at 37°C.

Preparation of boiled serum component

The material was prepared in a manner similar to that described by Kemp et al. Five hundred ml of newborn or calf serum (Microbiological Associates, Inc., Bethesda, MD; Gibco, Grand Island, NY) was diluted 1 : 2 with PBS. The solution was poured into 150 ml glass tubes and held in a boiling water bath for 15 min. The heated serum was centrifuged at 9,000 rpm for 5 h in a Beckman I-21 centrifuge. The supernatant from this step was concentrated four-fold by pressure filtration through a PM-30 Amicon membrane. Twenty ml in PBS was chromatographed on a Sephadex G-150 column (5 × 50 cm). Ten ml fractions were collected from the column at a flow rate of 1 ml/min with PBS. Protein determinations (Folin—Lowry) were performed on each fraction. There were essentially three fractions: a) greater than 100,000; b)

50,000 to 100,000; and c) smaller than 50,000. The fractions in each protein peak were then pooled and filter sterilized. Only the materials present in peak 1 were active.

Lymphotoxin assay

These techniques have been described in detail previously (Spofford et al., 1974). An LT sensitive strain of mouse L-929 fibroblast cells was employed as the target cell (9, 10). Briefly, LT cytolytic activity was determined in the following manner. Monolayers of mouse alpha L-cells were established in 16 × 125 mm glass screw-capped culture tubes at a density of 100,000 cells/ml in MEM + 3% FCS containing 0.4 μg/ml of mitomycin C (Sigma Chemical Co., St. Louis, Mo.), and incubated in 95% air, 5% CO_2 for 24 h. Uniform monolayers were selected and test and control media were diluted by five-fold dilutions in MEM + 3% FCS. Media covering the cell monolayers was discarded, and the varying dilutions of test and control media added to the cultures and incubated for an additional 24 h. Controls consisted of media from cultures of unstimulated cells or MEM containing cell additives. The number of viable cells remaining was determined by passing trypsinized samples through a Coulter Counter. The reciprocal of the LT dilution killing 50% of the target cells in 24 h yields the number of units of LT/ml in the original undiluted supernatant.

Supernatant collection

Lymphocyte supernatants were cleared of cells by either: a) centrifugation at 300 *g* for 5 min, or passage through one layer of glass fiber filter paper (Gelman, Ann Arbor, MI) in a Buchler sunction funnel. The glass filter was first washed with 20 ml of a 1% bovine serum albumin solution in PBS. The cell-free supernatants were pooled and immediately frozen. When reculturing the cells for multiple harvest, the supernatants were centrifuged in sterile centrifuge bottles at 300 *g* for10 min and the pellet resuspended in fresh medium plus 5 μg PHA-P and cultured for various intervals at 37° C. Intracellular LT was collected by adding 100 ml of distilled-deionized water to the cell pellet on the glass filter. After 10 to 15 min, these filtrates were collected by suction and pooled separately from the active supernatants.

Lymphocyte washes and subcellular fractions

Lymphocytes were stimulated with PHA-P and cultured at 37°C for 5 days. The 200 ml culture media was aliquoted into samples. All samples were centrifuged at 300 *g* for 5 min to pellet the lymphocytes. Each supernatant was gently aspirated and saved. The pellet from sample 1 was resuspended in 50 ml of PBS containing 2% NBCS and washed by alternate centrifugation and resuspension. Each supernatant was saved for LT titering. At the com-

pletion of the fourth wash, the lymphocyte pellet was sonicated for 10 min, examined microscopically to verify cell lysis and then centrifuged at 100 *g* for 30 min.

RESULTS

Human tonsils and adenoids are lymphoid tissues, readily obtainable on a daily basis and composed primarily of lymphocytes. We found the mean yield of lymphocytes obtained from a single tonsil and adenoid was 2.5×10^9 and 1.4×10^9, respectively, based on approximately 2000 samples. Thorough washing and preparation of the solid tissues in MEM + 2X-AM markedly diminished contamination problems. For example, a single month's yield of 20 lots of 42 liters of active supernatants had only a 3% rate of contamination.

Initial studies of various culture parameters have been reported previously (Kolb et al., 1971; Shacks and Granger, 1971). These experiments revealed that data collected from 1—2 ml lymphocyte tube cultures was not applicable when larger volume culture methods were employed. In these experiments, we employed 32 oz screw-capped prescription bottles as culture vessels, because individual culture units minimized contamination of an entire lot and was easily expanded to accomodate variable sized lots derived from different amounts of lymphoid tissue. We were not able to elicit a reproducible cellular response in the 200 ml cultures when lymphoid cell numbers were below 3×10^6 cells/ml. While cell numbers could be increased, the best yield of LT/cell over a five-day period was at a density of $4—5 \times 10^6$ cells/ml. Powdered MEM (Gibco, Grand Island, NY) was employed in all cultures, and individual samples of several large lots were tested before selecting one, for there was variation from lot to lot. Phytohemagglutinin most reproducibly stimulates higher levels of LT secretion in vitro from human lymphocytes than other methods of activation (Kolb et al., 1971). Dose response curves of PHA-P induced human lymphocyte stimulation, measured by increased levels of DNA synthesis and LT secretion, revealed a broad peak which plateaued between 20—75 μg PHA-P/ml (Kolb et al., 1971; Shacks and Granger, 1971). A PHA concentration of 20 μg/ml was employed for all subsequent cultures.

A number of substitutes for the medium serum component have been employed, i.e., polyethylene glycol, lactalbumin hydrolysate, bovine serum albumin (Kolb et al., 1971; Shacks and Granger, 1971; Granger, 1972). Kemp et al. (1971) reported a heat resistant, large molecular weight fraction of bovine serum supported target cell destruction in vitro mediated by immune murine lymphocytes, as well as whole serum. Various fractions of a boiled bovine sera were isolated and tested and an active fraction was identified similar to that reported by those investigators. Dose curves employing the high MW fraction of BS in MEM with PHA-P (20 μg/ml) stimulated human lymphocytes revealed that levels from 5 to 20 μg/ml were as effective as 5%

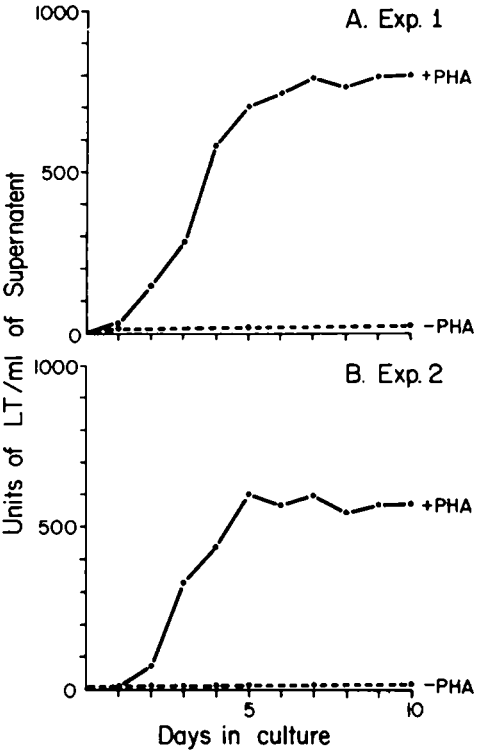

Fig. 1. Kinetics of lymphotoxin (LT) production in vitro by two cultures of PHA-activated human lymphoid cells over a 10-day period. Cells were activated with PHA-P, and the amount of LT in a supernatant was assayed each day, as described in Methods. (+ PHA refers to the activated cultures and PHA to non-treated controls.)

bovine serum for lymphocyte stimulation, depending on the particular lot of BS employed.

Kinetics of LT production and leukocyte viability

Cultures were established as described in Methods with lymphoid tissues from single donors. Each day, 5.0 ml samples were aseptically removed and frozen. The accumulated samples were all tested for the level of LT activity on the same day after collection of the last sample. The kinetics of LT production in two of these cultures over a 10 day period is shown in fig. 1. This data is representative of data collected from 10 different batches of lymphoid cell cultures from both single and mixed donors. While the cells in each culture respond in the same general way, the actual levels of LT secreted are quite variable from culture to culture and range from 400 to 6000 units/ml of LT activity on day 4 or 5. Fluctuation in levels of LT secreted was observed whether the cultures were established with the cells from single

or multiple donors. Hereafter, we cultured cells from single or multiple donors together. While cytotoxic molecules can be detected in the supernatant collected from PHA-P activated human lymphoid cells within an hour after stimulation (Williams and Granger, 1969), the final levels in cell cultures reach a plateau on day 4 or 5. Usually the major accumulation of LT in the supernatant occurs between days 2 and 4. Cultures of unstimulated human lymphocytes from single donors routinely give a background LT titer of 20—30 after five days in culture. However, certain tissue samples have an unexplained high spontaneous level of DNA synthesis and also secrete relatively high levels of LT in vitro, i.e., 1 : 100, without activation.

Studies were performed to determine the state of lymphoid cell viability during a 15 day culture period. Lymphoid cells were established in culture and samples were removed at various intervals over a 15 day period. Viability was determined by visual examination of the cells suspended in 0.1% Eosin Y in PBS. Total cell counts were not performed because of extensive cell clumping in these cultures. A total of 7 different cultures were examined, and the results of these experiments are shown in fig. 2. It appears that even though the levels of LT in the culture supernatant reach a plateau on day 5, the lymphoid cells remain viable for 8 to 10 days in culture. Previous experiments performed on one and two way human MLC cultures (Kramer and Granger, 1975) and Con A-stimulated human lymphocyte cultures (Daynes and Granger, 1974) suggested the existence of a possible feedback mechanism(s) which might regulate the final levels of LT reached in a culture supernatant. These results and the previous data suggested that this type of effect may also be operative in these culture systems.

Collection of multiple supernatants containing LEM from single mitogen-stimulated lymphocyte cultures

Experiments were designed to explore the possibility of harvesting increased amounts of human LEM by collecting multiple supernatants from single cultures of lymphoid cells. Previous experiments in tube culture had revealed that Con A-activated and alpha methyl mannoside-deactivated human lymphoid cells required much less mitogen for reactivation (Daynes and Granger, 1974). With these results in mind, one set of lymphoid cell cultures was established under standard culture conditions and supernatants collected at 5 days. The supernatants from individual bottles were collected by centrifugation as described in Methods, and the cells resuspended in 200 ml fresh medium containing variable amounts of PHA-P, i.e., from 5 to 20 μg/ml. After 3 days at 37°C, all the supernatants were collected, and the amount of LT present in each was determined. These results revealed: a) PHA-activated human lymphoid cells, after 5 days in primary culture, can secrete more LT, but they must be restimulated with fresh PHA, and b) there is an optimum level of PHA-P required for restimulation which is 1/3 to 1/2 that of the initial primary level. These experiments were repeated with cells from two

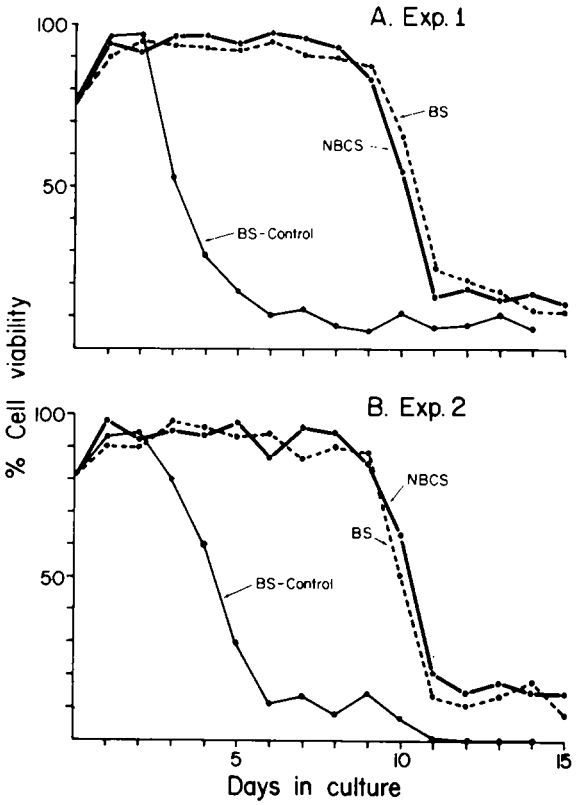

Fig. 2. Viability of two samples of human lymphoid cells cultured in media supplemented with boiled serum (BS) and newborn calf serum (NBCS) over a 15 day period. The cells were activated by addition of 20 μg/ml of PHA-P to the cultures on day 0. Controls were incubated in medium + BS without mitogen.

separate donors with similar results in each experiment. Lymphocyte cultures were initiated under standard conditions, and supernatants collected from primary and restimulated cells at different time intervals. The levels of LT secreted by the stimulated cells into the supernatant was determined by the standard method. Three of seven separate experiments involving 10 liters of supernatant are shown in table 1. These data illustrate that up to twice the amount of LT-containing supernatant can be collected with two consecutive 5 day harvests by primary and secondary stimulation of single cell cultures, as compared with the amount found in 0 to 5 or 0 to 10 day intervals. Additional experiments also verified that supernatant collected at two consecutive 5 day intervals consistently yielded higher LT levels than supernatants collected at other intervals. It appears that the major amount of LT secretion occurs when cellular viability is above 50—80% during the first 8 to 10 days in culture.

TABLE 1

LT secretion during sequential stimulation and collection of supernatants from PHA-stimulated human lymphoid cells in vitro. Two hundred ml cultures of human lymphoid cells were activated with PHA and established in culture as described in Methods. At various intervals, supernatants were collected from the primary cultures and the cells resuspended in fresh MEM-BS. Five μg/ml PHA-P was added, and the cultures incubated at 37° C. After various intervals, this procedure was repeated.

	Stimulation	Days in culture	Units of LT (ml)	Total units ($\times 10^4$)	% of total collected
Experiment 1	Primary	0– 6 (6)	1265 ± 30	6.33	72.6
	Secondary	6– 9 (3)	412 ± 12	2.06	23.7
	Tertiary	9–12 (3)	53 ± 5	0.27	0.03
	Quaternary	12–15 (3)	10 ± 2	0.05	0.005
				(871)	
Experiment 2	Primary	0– 5 (5)	682 ± 17	3.41	34.7
	Secondary	5–10 (5)	573 ± 8	2.87	29.3
	Tertiary	0–10 (10)	705 ± 18	3.53	35.9
				(981)	
Experiment 3	Primary	0– 5 (5)	972 ± 20	4.86	56.1
	Secondary	5– 8 (3)	632 ± 17	3.16	36.4
	Tertiary	8–11 (3)	112 ± 10	0.56	0.06
	Quaternary	11–15 (3)	20 ± 10	0.10	0.01
				(866)	

Presence of LT in multiple washings and cell sonicates from PHA-P stimulated cultures of human lymphoid cells

Experiments were conducted to determine if additional amounts of LT could be collected from additional washing of cell pellets after collection of the primary supernatant. Human lymphoid cells were PHA-activated and established in culture. After 5 days, the cells were sedimented, washed and sonicated, and the levels of LT in each supernatant determined, as described in Methods. The results of 3 separate experiments are shown in table 2. It is apparent that 90% of the LT is collected in the original supernatant. A small amount of intracellular LT can be collected from cells one day past stimulation, however, within the limits of these experiments, little or no intracellular LT is present in cells 5 days after stimulation.

The effect of medium supplements on LT secretion by PHA-stimulated human lymphocytes in vitro

Experiments were designed to further increase the yield of LT/culture by adding various medium supplements. A number of substances were tested, i.e., glucose, essential and non-essential amino acids, co-factors. Each addi-

TABLE 2

The effect of multiple washings on collection of LT from PHA-stimulated cultures of human lymphoid cells in vitro. Human lymphoid cells were activated with PHA-P and established in 50 ml cultures in 32 oz. prescription bottles at 37°C. After 1 or 5 days of incubation, the cells were sedimented and supernatant (SAL) collected as described in Methods. The cell pellet was resuspended in 50 ml of PBS + 2% NBCS and resedimented. The supernatant was collected and the cell pellet resuspended in a similar fashion. The suspension was sonicated and the cellular debris removed by centrifugation. All supernatants were tested for LT activity by the standard method.

Days in culture	Treatment	LT units		
		ml	total collected	% of total
1 (A)	SAL	25	1250	92.6
	1st wash	2	100	7.4
	Cell-sonicate	5	250	18.0
(B)	SAL	18	900	94.7
	1st wash	1	50	5.3
	Cell-sonicate	3	150	15.0
5	SAL	625	31250	98.4
	1st wash	10	500	1.6
	Cell-sonicate	0	0	0

tive had a slight affect on the final LT levels reached in a 5 day supernatant. However, more pronounced effects were obtained when a number of additives were mixed together and an aliquot added directly to the culture medium. One of these mixtures which was quite effective contained tissue culture

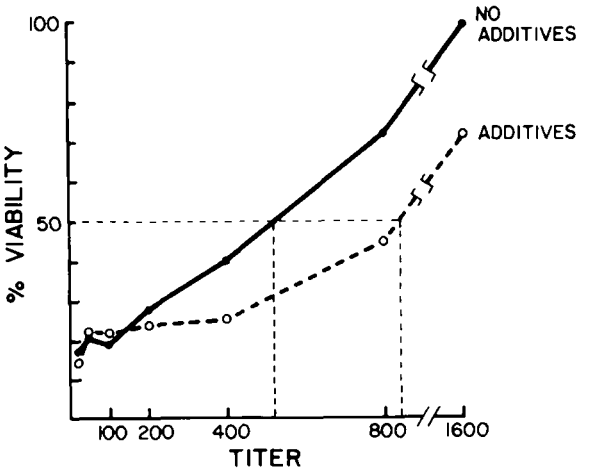

Fig. 3. The effect of mercaptoethanol (EtSH), non-essential amino acids (NEAA) and Na pyruvate on LT elaboration in vitro by PHA-activated human lymphoid cells. The agents were added directly to cultures of human lymphoid cells as described in Methods. Supernatants were collected after 5 days and assayed for LT activity in culture.

levels of nonessential amino acids, sodium pyruvate and 0.05 M mercaptoethanol. We tested the results of adding the cocktail to the cultures at various intervals and found that the final LT levels in 5 day supernatants were essentially the same when the cocktail was added on day 0 or at various intervals over a 5 day period. The effect of this cocktail on LT secretion by PHA-activated human lymphocytes is shown in fig. 3. It is clear that cultures which received the cocktail had significantly higher levels of LT after 5 days.

DISCUSSION

This manuscript describes in vitro methods for generating relatively large volumes of culture supernatant containing lymphocyte effector molecules from PHA-activated normal human cells. The supernatant has a low protein concentration which permits high multiplicities of concentration and facilitates the use of small volumes with high levels of biologic activity. While not reported here, we found that PHA-induced LEM secretion is much more reproducible than Con A in the present culture system. Studies on many individual batches and pools of supernatant show that LT is not the only human LEM present. These supernatants contain migration inhibitory factor, leukocyte inhibitory factor, interferon and stimulatory and inhibitory factors for human B-cells and T-cells (Lundak et al., unpublished results). However, we have evidence that there is sequential secretion of different LEM which indicates that supernatants should be collected at different times during the culture. Additional studies are under way to better define these in vitro situations. Methods have been employed by other investigators in attempts to collect human LEM in medium containing low or no extraneous protein (Kolb et al., 1971; Walker and Lucas, 1974). In addition, certain continuous human lymphoid cell lines have been identified as secreting LEM (Glade and Broder, 1969). Obtaining LEM from continuous cell lines obviously has advantages, i.e., they are uniform cell sources, can be experimentally manipulated and presumably could be releasing a uniform material. However, it has been our experience that they release from 20—100 times less LT than PHA-activated normal human lymphocytes (Granger et al., 1970). In the present studies, we obviated the requirement for serum by employing small quantities (10 μg/ml) of a heat stable, large molecular weight serum fraction (BS) which was as effective as serum in sustaining lymphocyte activation and secretion of LT. The active component in the BS fraction is currently under investigation.

Different procedures were employed in the current studies to increase the yield of LEM from primary PHA-activated human lymphocyte cultures. An important new observation is that supernatants with high LT activity may be harvested more than once from a single culture. Kinetic studies of LT secretion revealed that there were plateaus reached after 4—5 days in culture, yet studies of the activated cells indicated that they were still viable and appear-

ed healthy for another 3—4 days. Resuspending these cells in fresh culture media and mitogen revealed they were fully capable of releasing additional high levels of LT during a subsequent culture interval. Moreover, the second harvest required 75% less mitogen required to continue cell stimulation necessary for optimum levels of LT secretion. Additional studies, to be reported elsewhere, have indicated that the cessation of LT secretion represents a form of control mechanism(s) which stops intracellular LT synthesis as well as secretion. However, this inhibition is quickly reversed by placing the cells in fresh medium and PHA. The use of the various additives in a nutritional cocktail generally increased the amount of LT collected anywhere from 10 to 30%. The production of large quantities of SAL has generated the necessity for additional studies dealing with the handling, storage and stability of human LEM. These studies are currently under way.

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