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RELATIONSHIP OF CIF, LT, AND PIF RELEASED IN VITRO BY ACTIVATED HUMAN LYMPHOCYTES

II. A Further Functional Comparison of LT and PIF Activities on HeLa and L-929 Target Cells[1]

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Lymphotoxin (LT), and proliferation inhibition factor (PIF) activities found in 5-day supernatants of mitogen-activated human lymphocytes (SAL) were further compared. In agreement with previous results, the activities could not be distinguished functionally. Quantitative differences in the amount of activity detected in the SAL could be accounted for on the basis of target cell differences, concentration of the lymphocyte effector molecules in the supernatant, and the parameter employed to assess cell function. Growth inhibitory activity detected at high supernatant dilutions was completely reversible, whereas the cytotoxic activity detected at low supernatant dilutions was irreversible. When the active medium was fractionated on DEAE, two peaks of inhibitory activity were detected. Depending upon the amount of activity and target cell, both peaks of activity were growth inhibitory or cytotoxic. Since both peaks of material affected HeLa and L-929 cells, the materials were not species specific. Thus, it appears that cloning inhibitor factor, LT, and PIF activities may actually be measures of the same stable materials found in 5-day activated lymphocyte supernatants.

The present report is the second in a series concerned with a group of lymphocyte effector molecules (LEM)[3] that have a cytotoxic or cytostatic effect on continuous cell lines *in vitro*. In a previous manuscript, it was shown that the LEM, lymphotoxin (LT) (1-3), proliferation inhibitory factor (PIF) (4-7), and cloning inhibitior factor (CIF) (8, 9) present in mitogen-activated human lymphocyte supernatants appear to be similar by various functional criteria (10). The present manuscript further defines the *in vitro* assay systems employed to detect these activities and shows that although there are multiple molecules in 5-day supernatants, additional functional measures could not separate them.

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[3] Abbreviations used in this paper: LEM, lymphocyte effector molecules; LT, lymphotoxin; PIF, proliferation inhibitory factor; CIF, cloning inhibitor factor; NCS, newborn calf serum; SAL, supernatant from activated lymphocytes, MC, mitomycin-C; HLT, human lymphotoxin.

MATERIALS AND METHODS

Culture medium and cell lines. The stock culture media in these experiments was Eagle's minimal essential medium (MEM) (Flow Labs, Los Angeles, Calif.), supplemented with 0.29 mg/m glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5% newborn calf serum (NCS) (Microbiological Associates, Bethesda, Md.).

The cell lines employed were maintaned with biweekly passages in the above stock media. The L-929 cell used was obtained from Grand Island Biological Company, Grand Island, N. Y. and the HeLa cell line was generously donated by Dr. Edward Wagner.

LT production. Fresh human adenoid lymphocytes were prepared as a single-cell suspension as described previously (10). The lymphocytes in these suspensions were adjusted to 5×10^6 cells/ml in MEM containing 10% NCS, nonessential amino acids (100 X, Flow Labs) and I mM pyruvate (Grand Island Biological Co.). Phytohemagglutinin-P (PHA) (Difco, Los Angeles, Calif.) was then added, bringing the total concentration to 20 μg/ml and the cultures were incubated in 32-oz bottles for 5 days at 37°C in a 5% CO_2 to 95% air atmosphere. The medium was cleared of cells by centrifugation and was stored frozen at −20°C until use.

Lymphotoxin assay. In this assay, target cells (10 to 50,000 HeLa, 100,000 α L-929)were established as 1-ml monolayer tube cultures during an overnight preincubation at 37°C. The LT dilutions were made just before testing at 2- to 5-fold serial dilutions in fresh MEM + NCS. These tube cultures were incubated at 37°C for an additional 24 to 48 hr, after which the total adherent cell number was determined. This was accomplished by discarding the serum-containing media from each tube, washing the monolayer with 5 ml of phosphate-buffered saline (0.14 M NaCl, 0.01 M PO_4, pH 6.9) (PBS), adding 0.1 ml of 0.05% trypsin, 10^{-3} M EDTA solution, incubating these cultures for 5 min at 37°C, and then vortexing the tube cultures. Five milliliters of PBS were added to each single-cell suspension and then counted using a Model F Coulter Counter as previously described (11). The reciprocal human lymphotoxin (HLT) dilution that results in a 50% reduction in cell number will be referred to in this text as the "titer", or the number of units of activity per milliliter in the active supernatant.

Assay of DNA synthesis. Tube cultures were pulsed by adding 0.1 ml media containing 2 μCi ^{3}H-TdR (6 Ci/mM) during the last 4 hr of incubation at 37°C. The labeling media were discarded and 10 ml of cold 10%-trichloroacetic acid (TCA) were added to each tube. The cells that were fixed to the glass after the first TCA rinse were washed with an additional

10 ml of TCA. After draining, the adherent cells were dissolved in 0.5 ml Nuclear Chicago Solubilizer. Fifteen milliliters of Omnifluor scintillation fluid were then added, and the solution was counted in a Beckman LS-100 Scintillation Counter.

Column Chromatography

Diethylamino ethyl cellulose (DEAE). Previously washed Whatman DE-11 resin was poured into 3.4- x 60-centimeter columns and allowed to pack to a bed height of 30 cm. The columns were equilibrated with 10 mM Tris-HCl (pH 8.0) containing 25 mM NaCl. Protein was eluted with a 2.0 liters continuous gradient of NaCl from 25 to 350 mM in 0.01 M Tris, pH 8.0, and 10^{-5} M EDTA. Ten-milliliter fractions were collected at a flow rate of 1 ml/min, the conductivity of every other fraction was assessed, and the absorbance at 280 nm was monitored.

Concentrated toxic and control medium, which had been subjected to DEAE-cellulose separation techniques, were tested for the presence of cytotoxic (LT) and growth inhibitory factors (PIF, CIF). Monolayers of indicator α L cells or HeLa cells were established in tubes 24 hr before use at densities of either 25,000 or 50,000 cells per tube. At the beginning of each assay, a random group of control tubes was counted for total cell number on a Model F Coulter Counter. The cells in each tube were then allowed to grow for either 48 hr (2-day assay with 50,000 cell starting number) or 96 hr (4-day assay with 25,000 cell starting number). At the end of each assay period, the total number of adherent cells in each tube was determined. Cytotoxicity was defined when the final cell number was less than the initial cell innoculum. Growth inhibition was defined as a final cell number greater than the beginning control values, but less than the final control values (10).

Sephadex. A 1-cm column was packed with Sephadex G-150 to a bed height of 25 cm, and washed with 200 ml of PBS. Molecular weight markers were added to the column in 1 ml of PBS, 2-ml fractions were collected from the column at a flow rate of 1 ml/3 min and the fractions were tested for the presence of the markers by absorbance at 280 nm. In the same manner, 1 ml of concentrated toxic or control media was applied to the column and eluted with PBS. Two-milliliter fractions were collected, and 0.1-ml aliquots of the fractions were assayed for *in vitro* activity, as previously described.

RESULTS

Inhibition of ^{3}H-TdR incorporation into DNA of HeLa Cells in vitro by HLT-containing supernatants. In a previous manuscript, it was observed that the amount of DNA synthesis per HeLa cell was lower in cultures treated with various dilutions of supernatant from activated lymphocytes (SAL) when compared to untreated controls (10). The exact nature of the reduction in ^{3}H-TdR incorporation into DNA in HeLa cells, and the relationship to cytotoxicity and growth inhibition was not clear. The present experiments were designed to examine this relationship further.

A series of parallel tube cultures containing 50,000 HeLa target cells were established as monolayers during an overnight incubation of 37°C. Dilutions of SAL were added to these tubes, then incubated at 37°C an additional 48 hr, and then either the total cell number or the ability of the cells to incorporate ^{3}H-TdR into DNA during the last 4 hr of culture was determined. The data presented in Figure 1 demonstrate that the incorporation of ^{3}H-TdR into DNA does not correlate with cell number. Toxicity induced by LT can account for the reduced incorporation of ^{3}H-TdR at 1:2 and 1:4 dilutions, since the cell number in these cultures was reduced below the initial plating density. At higher SAL dilutions, up to 1:64, growth inhibition greatly reduced cell numbers (Fig. 1*A*) and likewise reduced isotope incorporation (Fig. 1*B*) in HeLa cells, when compared to nontreated control cultures, but at dilutions above 1:64, incorporation of ^{3}H-TdR into DNA was still reduced to 50% of the control values, even though the total cell number was similar in both the SAL-treated and control cultures. Additional studies (not described here) have shown that the reduced incorporation of ^{3}H-TdR in DNA could also

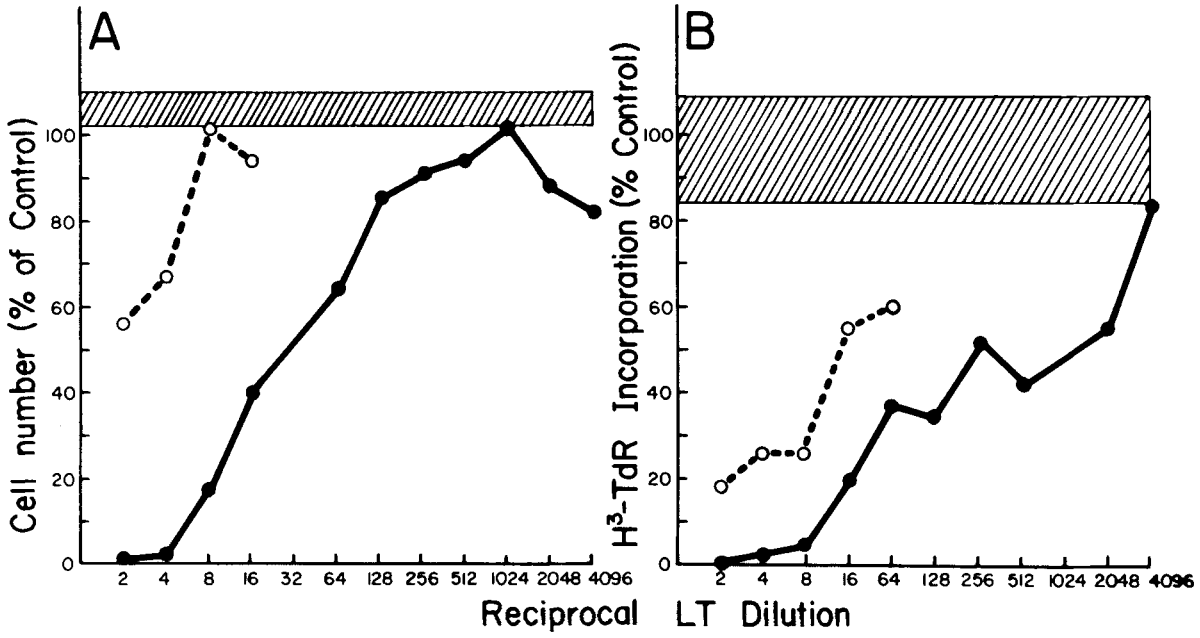

Figure 1. Relationship between cell number and incorporation of ^{3}H-TdR into DNA in HeLa cells treated with supernatants from either PHA-activated human lymphocytes (SAL) (—●—); or unstimulated human lymphocytes (- -○- -). The HeLa cells were exposed to the supernatants for 6 days. The initial cell number was 5000 HeLa cells per tube, the final control cell number was 241,200 HeLa cells, and there were 195,238 cpm per tube culture. *A*, total viable cell number per culture; *B*, incorporation of ^{3}H-TdR into DNA.

be measured in cultures incubated 1 to 4 days with SAL. It was not clear whether the reduced incorporation of ^{3}H-TdR into DNA was a reflection of slowed DNA synthesis or reduced ^{3}H-TdR isotope transport into the HeLa target cell. However, comparison of cell number and isotope incorporation into DNA suggests transport processes may be inhibited. Additional experiments suggest the latter situation may be the case. It appears that inhibition of ^{3}H-TdR incorporation into DNA may be a more sensitive measure of low levels of LEM activity employing HeLa target cells.

The above results were not obtained when the same SAL was tested on L-929 cells. These experiments, shown in Table I, demonstrated that DNA synthesis and cell number did correlate, regardless of the biologic activity measured (i.e., toxicity to growth inhibition) and SAL dilution. The data presented in Table I are typical of three experiments. Walker and Lucas have also reported that cell number and ^{3}H-TdR incorporation into DNA correlated in HLT-treated L-929 cultures (1). Target cell differences (e.g., perhaps cell type and species of origin) thus exist after exposure to SAL with respect to the correlation of inhibition of ^{3}H-TdR incorporation into DNA and cell number.

Reversibility of LT-induced effects. Conflicting reports exist in the literature about the reversibility of PIF, CIF, and LT activities found in mitogen-activated human lymphocyte supernatants. Several studies have shown that if Chang, HeLa, or L-929 cells were treated with low concentrations of SAL (i.e., about 1:5 dilution of crude supernatant), washed, and placed in fresh media, the remaining cells resume cell division (3, 5). If L-929 cells were treated with high concentrations of SAL, washed, and placed in fresh media, the target cells underwent cytolysis (3). Support for a concentration-dependent effect comes from our previous studies, in which it was demonstrated that various dilutions of SAL had a variety of activities that range from cytotoxicity to temporary growth inhibition when present on the targets continuously (10). The factors in this last study that influenced what activity was measured included: a) the concentration of the SAL; b) the target cell employed in the assay; and c) the length of the assay period. It was the purpose of these next studies to determine if reversibility of LT action on target cells depended upon the SAL concentration.

In the present experiments, tube cultures containing 10,000 HeLa cells were established as monolayers during an overnight preincubation. The media on the monolayers were discarded and 1 ml of the SAL dilutions was added. The tube cultures were next incubated 24 hr at 37°C, and then parallel sets of LT-treated cultures were either 1) untouched; or 2) washed, and fresh media were added. Total adherent cell number was determined at 24-hr intervals during the above experiment. As is shown in Figure 2, the HeLa target cells "recovered" from

TABLE 1

Correlation between cell number and incorporation of ^{3}H-TdR into DNA when growing L-929 target cells are exposed to SAL for 48 hr

SAL Dilution	Total Viable Cell No. (% of Control) ± SD	Incorporation of ^{3}H-TdR[a] into DNA (% of Control) ± SD
1:4	2 ± 1	12 ± 6
1:16	9 ± 1	20 ± 2
1:64	59 ± 6	52 ± 6
1:256	75 ± 5	61 ± 4
Control	100%	100%
	(51,736 ± 3,160 cells)	(10,521 ± 235 cpm)

[a] One microcurie of ^{3}H-TdR was added for the last 5 hr of culture.

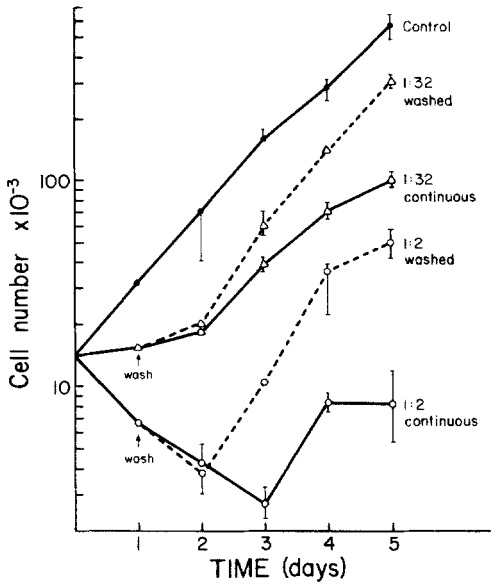

Figure 2. Reversibility of the inhibitory activities in dilutions of SAL on human HeLa target cells *in vitro*. The test cells were exposed to the dilutions of SAL for 24 hr, and then placed in fresh culture media.

the growth inhibitory or cytotoxic effects when washed out of the active supernatants, although there was a 24-hr lag before they resumed growth. Controls containing mitogen at levels found in 1:32 dilutions of LT had no effect on the growth rate of the target cells; however, an amount of mitogen equivalent to that present in the 1:2 LT dilution (i.e., 10 μg/ml PHA-P) was significantly growth inhibitory, although not cytotoxic. Further work has also shown the growth-inhibitory activity found in Con A-stimulated lymphocyte supernatants was reversible in exactly the same manner described for SAL generated with PHA.

Modulation of sensitivity of L-929 cells to LT during growth in stock cultures. The amount of LT activity that can be detected in any assay system depends upon several factors that include: a) target cell type; b) length of assay; and c) whether the target cells have been treated with metabolic or biosynthetic inhibitors (2, 11). Peter *et al.* (12) have shown that LT-mediated destruction of L-929 cells appears to be unrelated to how long the stock cultures were maintained before assaying their LT sensitivity. Spofford *et al.*, (11) have shown that L-929 pretreated with mitomycin-C (MC) were 13 times more sensitive to LT-mediated cytodestruction than untreated target cells. It also appeared that this increase in sensitivity by MC was a stable property of the α L-929 target cells. In contrast, we observed a wide variation in the sensitivity of MC pretreated L-929 cells, even when the same lymphocyte supernatant was repeatedly tested. We, therefore, investigated the possibility that target cells may change sensitivity to LT-mediated cytolysis during growth in stock cultures.

Monolayer cultures of α L-929 cells were initiated by adding 1 × 10^6 cells in 16-oz prescription bottles. Eight parallel cultures were established in this manner for each experiment. These stock cultures were grown for periods of 1 to 5 days and at intervals, their sensitivity to HLT was tested. Each day several bottles containing stock cell monolayers were washed with PBS, trypsinized, and media containing 3% serum were added to stabilize the cell suspension. These cells were then employed in the LT assay.

Two types of LT assay were employed: a) one that allowed the target cells to divide during the exposure to the toxin (as

described in *Materials and Methods*) and b) one that inhibited cell division with MC. Before exposure to the SAL, 1-ml tube cultures containing 100,000 target L-929 cells and 0.5 μg/ml MC (when used) were established as monolayers during a 24-hr preincubation at 37°C. After inspecting for viability and uniformity, the media were discarded and various dilutions of the SAL in new tissue culture media were added to the monolayers. Serial dilutions of SAL employed in each experiment were prepared on day 1, and stored at 4°C until use. These cultures were incubated at 37°C for an additional 24 hr before counting the total adherent cell number. Units of activity in the original SAL is defined as the reciprocal of the dilution which gave 50% destruction.

As shown in Figure 3, the L-929 target cells significantly change sensitivity to HLT-mediated cytolysis during growth in stock cultrues. Two peaks (days 2 and 4) of LT sensitivity were noted in the assays employing MC-pretreated target cells, whereas only one peak in sensitivity (day 4) was observed in assays not containing MC. An additional six experiments employing MC-treated targets consistently demonstrated a peak in sensitivity on day 2 and day 4 of growth over a 3-month period. Additional studies over 6 months have shown that the drop in sensitivity of the L-929 cells on day 3 was not as reproducible as the initial experiments seemed to indicate. The general overall trend in MC-treated cultures was an increase in sensitivity to SAL-mediated destruction with length of stock culture. It appeared that the stock L-929 cells changed their sensitivity to SAL-mediated destruction during routine subculture during the 9 months in which these experiments were done. When the L-929 targets were assayed in the absence of MC, significant change in sensitivity to LT-mediated cytolysis was only seen in two out of the seven experiments. Pretreatment of the L-929 target cells with MC increased the sensitivity of the LT assay in these experiments from 2.5 to 40 times that of non-MC treated target cells.

An experiment was performed in exactly the same manner as described above to determine if the HeLa target cells change sensitivity to LT during growth in culture. The results of this experiment demonstrated that the HeLa cells do not dramatically change sensitivity to HLT when assayed with untreated or MC-treated targets. This observation was consistent with data obtained from other assays in which the assay consistently yielded the same level of activity, regardless of how the HeLa cells were maintained *in vitro*.

Column chromatography. In recent reports, human LT activity has been dissociated into two separable families by column chromatography on DEAE (12) and on Sephadex.[4] In the present studies, we decided to examine the two families resolvable on DEAE with respect to their ability to cause cytotoxicity or growth inhibition on several types of target cells.

The SAL employed for these studies was obtained by stimulating human adenoid lymphocytes with 20 μg/ml PHA-P in MEM containing 5% NCS at 37°C for 6 days. The supernatants were cleared of cells and concentrated 20 times by Amicon filtration employing a PM 10 filter. Careful monitoring of the concentration process accounted for all of the activity, since no growth inhibitory or cytotoxic activity was demonstrable in the filtrate. Forty milliliters of this concentrate were eluted from DEAE-cellulose with a salt gradient and a 1:8 dilution of each fraction was assayed for activity as is shown in Figure 4. Two peaks of activity were demonstrable both on HeLa and L-929 targets.

The material found in fractions 7 and 8 on L-929 was cytotoxic (Fig. 4*B*), whereas on HeLa cells, it was only growth inhibitory (Fig. 4*A*) when tested at the same concentration. The material found in fractions 16–24 was only growth inhibitory on both target cell types in this particular test, but upon additional testing, we have shown that both peaks of activity

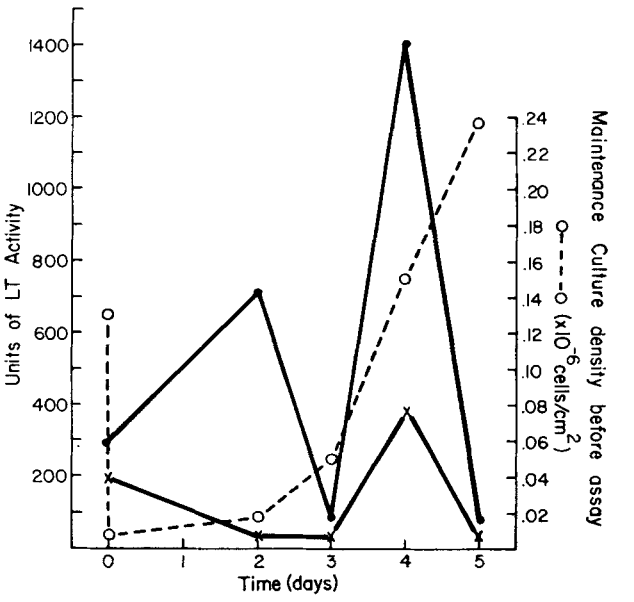

Figure 3. Change in sensitivity of alpha L-929 target cells to SAL activity during maintenance in stock cultures. - -O- -, Assay done in the presence of MC; —x—, assay done in the absence of MC.

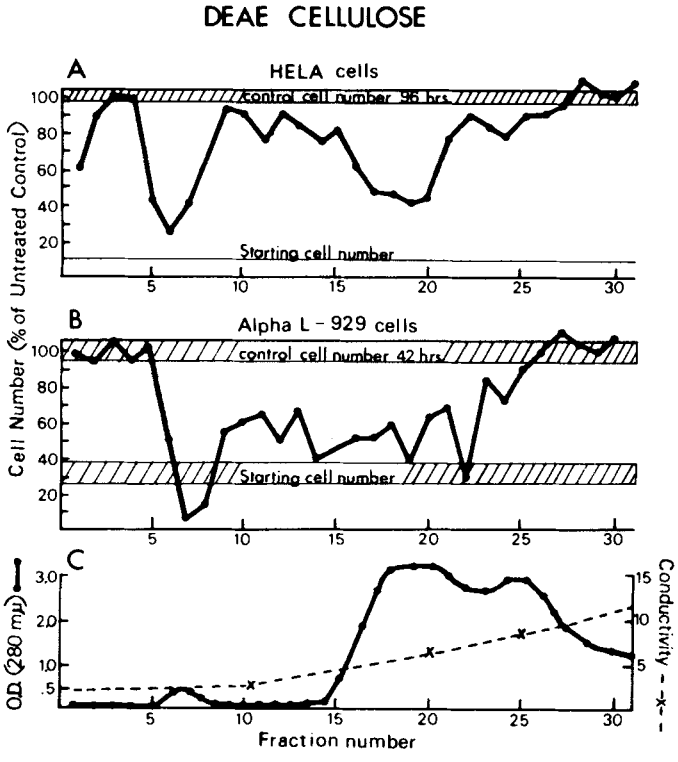

Figure 4. DEAE-cellulose chromatography of SAL containing LT, CIF, and PIF activities.

[4] Hiserodt, J. C., A.-M. Prieur, and G. A. Granger. 1976. *In vitro* lymphocyte cytotoxicity, I. Evidence of multiple cytotoxic molecules secreted by mitogen activated human lymphoid cells *in vitro*. Submitted for publication.

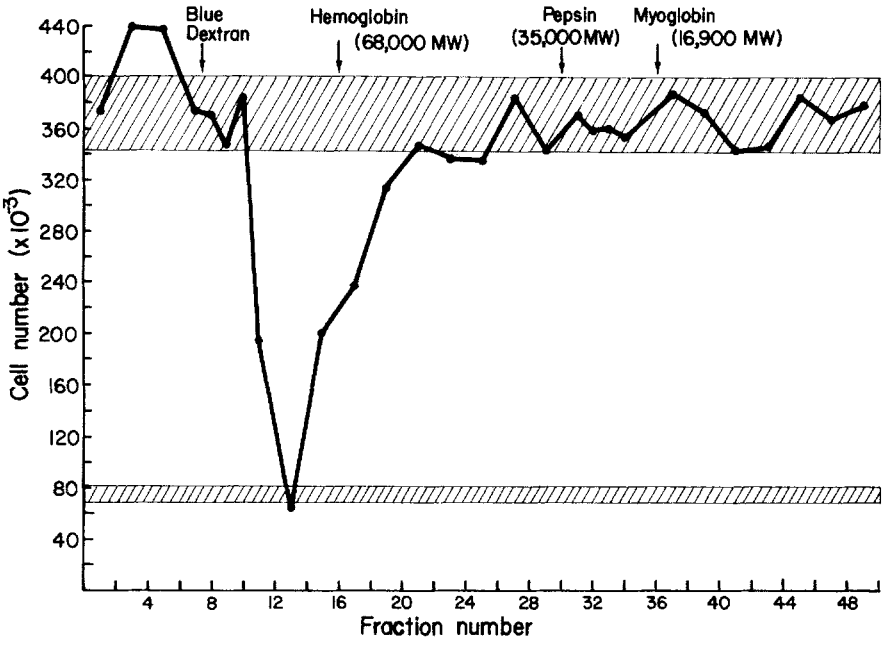

Figure 5. Sephadex G-150, A, B, C chromatography of the second peak obtained from DEAE-fractionated SAL (fractions 16 to 24). The hashed areas indicate range of the untreated control at the beginning and end of the 48-hr assay period.

when concentrated are cytotoxic. The first weakly charged DEAE peak represented 90% of the activity found in crude supernatants.

Previous purification schemes have shown that the DEAE-cellulose peak associated with the γ-globulins (fractions 7 and 8) has a m.w. of about 80 to 100,000 as measured on Sephadex, although the m.w. of the second peak was unknown. The m.w. of the material found in fractions 16–24 was next determined on Sephadex G-150. These fractions were pooled, concentrated, separated on Sephadex G-150, and the activity was then tested on L-929 cells (Fig. 5). The m.w. of the globulin material was 80 to 90,000, as was found in both the crude and the weakly charged material eluted off the DEAE column. It again appears that the HLT after 5 days of mitogenic stimulation of human lymphocytes consists of at least two stable components that have a m.w. of 80 to 100,000.

DISCUSSION

Many *in vitro* systems have been used to describe the activities found in the cell-free supernatant of activated lymphocytes. Each assay technique is given a name usually descriptive of the assay (e.g., migration inhibition factor, chemotactic factor) and is assumed to measure a unique molecule. Certain activities appear to be physically separable (e.g., guinea pig LT and migration inhibition factor) (13, 14). Many other activities, however, have only been functionally separable. It is not clear, therefore, whether several activities could be the result of a single material and the different activities observed by the many investigators reflecting differences in the *in vitro* assay systems.

A group of inhibitory activities has been described in the supernatant of the mitogen or antigen-activated human lymphocyte cultures. This group of inhibitory activities, LT, PIF, and CIF may be the result of the same or similar materials. The kinetics of appearance and method of production of these activities appear to be similar (6, 9, 15, 16), and a variety of species can produce all these effector molecules (16, 17). Although there are these similarities among LT, PIF, and CIF, several functional differences seemed to separate them into at least two subgroups. CIF and PIF were growth inhibitory and species specific. LT, on the other hand, was cytotoxic to cells and not species specific.

Further criteria distinguishing these activities were that LT-mediated cytolysis was irreversible (3), and PIF-mediated growth inhibition was reversible (5). The present studies demonstrated that depending upon the SAL concentration employed and target cell employed, a wide range of activities in a single supernatant was observed, ranging from growth inhibition to cytotoxicity. The possibility was examined that the irreversible cytodestructive and reversible growth-inhibitory effects could be explained on the basis that the particular effect one observed was again dependent on the concentration of the active media. The irreversible cytodestruction associated with LT was the result of multiple materials. When the molecules were present in high concentrations, they damaged a portion of the target cells to an extent that even when placed in fresh media, they could not recover. There was, however, a population of HeLa cells that recover 24 hr after removing the SAL. The growth inhibitory activity associated with PIF, on the other hand, was the result of these same molecules present in lower concentrations. In these more dilute SAL, we again noted a 24-hr period after removing the SAL and replacing it with fresh media before the targets resumed cell division. Polet (18) has also observed that Chang cells recovered from growth inhibition only 12 to 24 hr after removing the SAL. However, Walker and Lucas (1) have shown that L-929 cells recover immediately when low concentrations of SAL were removed and replaced with fresh media.

The amount of activity detected in SAL was found to depend not only on what target cell was employed, but what parameter was used to assess cell function. Growth inhibitory dilutions of SAL measured on HeLa cells were cytotoxic when assayed upon L-929 cells. Furthermore, the amount of activity in the same supernatant did not correlate when cell number and ^{3}H-TdR incorporation into DNA were employed as measures of

SAL activity on HeLa cells. In contrast, the amount of SAL activity detected employing growing L-929 targets was the same whether cell number or DNA biosynthesis was measured.

Additional target cell differences were observed when MC was employed to increase the sensitivity of the assay systems. The amount of the LT activity measured on MC-treated L-929 changed dramatically depending on how the stock cultures were maintained before establishing the tube cultures employed in the assay. Assays employing growing α L-929 targets showed no large changes in LT sensitivity during growth. HeLa cells, on the other hand, did not change sensitivity during growth in the culture when assayed in the presence or absence of MC. The situation, therefore, was quite complex and a variety of activities were demonstrable, depending upon the lymphokine concentration, parameter employed to assess cell function, and details of the particular assay (e.g., use of inhibitors, target cell type). Repair processes, receptor density, and cell cycle were but few of the complicated reasons that could be proposed to explain the differences in the *in vitro* assay employed (2).

The physical characteristics of the molecules, i.e., LT, PIF, CIF, in the lymphocyte supernatants were studied in an attempt to separate the growth inhibitory and cytotoxic activities. Recent studies have revealed the existence of two separate families of cell-toxins in SAL, the first one, termed LT_1, is heat stable and 90,000 m.w., and the second, termed LT_2, is highly heat labile and 50,000 m.w.[3] The present studies deal primarily with the high m.w. stable or LT_1 family of LEM. We found only one broad peak of stable activity reproducible on Sephadex G-150 approximately 80,000 m.w., and two peaks of growth inhibitory activity on DEAE chromatography. Both peaks of activity separated on DEAE appear to be approximately 80,000 m.w. The growth inhibitory activity measured on HeLa and L-929 cells after fractionation correlated and thus were not species specific. In addition, quantitation has shown that the weakly charged material accounts for approximately 10% of the activity in the SAL. Both materials, however, were capable of a spectrum of effects ranging from growth inhibition to cytotoxicity, depending upon the particular concentration and target cell employed. Others have obtained similar fractionation results employing a cytotoxicity assay to detect the activity of the lymphocyte supernatant (12, 19–21). Thus, it appears that there were at least two active molecules in the LT_1 family that could inhibit cell growth and/or be cytotoxic, regardless of the target cell employed.

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REFERENCES

1. Walker, S. M., and Z. J. Lucas. 1972. Cytotoxic activity of lymphocytes. II. Studies of lymphotoxin-mediated cytotoxicity. J. Immunol. 109:1233.
2. Williams, T. W., and G. A. Granger. 1973. Lymphocyte *in vitro* cytotoxicity: Mechanism of human lymphotoxin-induced target cell destruction. Cell. Immunol. 6:171.
3. Hessinger, D. A., R. A. Daynes, and G. A. Granger. 1973. Binding of human lymphotoxin to target-cell membranes and its relation to cell-mediated cytodestruction. Proc. Natl. Acad. Sci. 11:3082.
4. Green, J. A., S. R. Cooperband, J. A. Rutstein, and B. Kibrick. 1970. Inhibition of target cell proliferation by supernatants from cultures of human peripheral lymphocytes. J. Immunol. 105:48.
5. Badger, A. M., S. R. Cooperband, and J. A. Green. 1971. Direct observations on the effect of "proliferation inhibitory factor" on the clonal growth of target cells. J. Immunol. 107:1259.
6. Badger, A. M., S. R. Cooperband, and J. A. Green. 1973. Culture conditions affecting induction and release of lymphocyte produced proliferation inhibitory factor (PIF). Cell. Immunol. 8:12.
7. Badger, A. M., S. R. Cooperband, V. J. Merlazzi, and J. A. Green. 1973. Sensitivity of non-human cells to human lymphocyte "proliferation inhibitory factor" (PIF) *in vitro*. Clin. Exp. Immunol. 15:435.
8. Holzman, R. S., A. S. Lebowitz, F. T. Valentine, and H. S. Lawrence. 1973. Preparation and properties of cloning inhibitory factor. 1. Inhibition of HeLa cell cloning by stimulated lymphocytes and their culture supernatants. Cell Immunol. 8:249.
9. Holtzman, R. S., F. T. Valentine, and H. S. Lawrence. 1973. Preparation and properties of cloning inhibitory factor, II. Factors affecting its production and assay. Cell. Immunol. 8:259.
10. Jeffes, E. W. B., and G. A. Granger. 1975. Relationship of cloning inhibitory factor (CIF), lymphotoxin (LT) and proliferation inhibitory factor released *in vitro* by mitogen activated human lymphocytes. J. Immunol. 114:64.
11. Spofford, B. T., R. A. Daynes, and G. A. Granger. 1974. Cell-mediated immunity *in vitro:* A highly sensitive assay for human lymphotoxin. J. Immunol. 112:2111.
12. Peter, J. B., J. A. Stratton, K. E. Stempel, D. Yu, and C. Cardin. 1973. Characteristics of a cytotoxin ("lymphotoxin") produced by stimulation of human lymphoid tissue. J. Immunol. 111:770.
13. Ruddle, N. H. 1972. Approaches to the quantitative analysis of delayed hypersensitivity. Curr. Top. Microbio. Immunol. 57:75.
14. Granger, G. A. 1972. Lymphokines—The mediators of cellular immunity. Ser. Haematol. 5:8.
15. Kolb, W. P., and G. A. Granger. 1970. Lymphocyte *in vitro* cytotoxicity: characterization of mouse lymphotoxin. Cell. Immunol. 1:122.
16. Williams, T. W., and G. A. Granger. 1969. Lymphocyte *in vitro* cytotoxicity: Correlation of derepression with release of lymphotoxin from human lymphocytes. J. Immunol. 103:170.
17. Badger, A. M., S. R. Cooperband, and V. J. Merlazzi. 1974. The production of proliferation inhibitory factor (PIF) by mouse spleen cells *in vitro*. J. Immunol. 112:1435.
18. Polet, H. 1974. The effect of lymphokines on the metabolism and the functions of the cellular membrane of Chang liver cells *in vitro*. Cell Immunol. 11:247.
19. Russell, S. W., W. Rosenau, M. L. Goldberg, and G. Kunitomi. 1972. Purification of human lymphotoxin. J. Immunol. 109:784.
20. Granger, G. A., E. C. Laserna, W. P. Kolb, and F. Chapman. 1973. Human lymphotoxin: Purification and some properties. PNAS 70:27.
21. Eifel, P. J., S. M. Walker, and Z. J. Lucas. 1975. Standardization of a sensitive and rapid assay for lymphotoxin. Cell. Immunol. 15:208.