PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **183**, 231–236 (1986)

Potentiation of Cytotoxic Effect of Lymphotoxin by Anti-Cancer Drugs and Elevated Temperatures (42410)

SATHIT PICHYANGKUL AND AMANULLAH KHAN

Department of Immunotherapy, The Cancer Center at Wadley Institutes, Dallas, Texas 75235

Abstract. The cytotoxic lymphokine, lymphotoxin (LT), has been shown to possess antitumor effect *in vitro* and *in vivo.* We examined the effect of the combination of partially purified LT with anti-cancer drugs and elevated temperatures on mouse transformed fibroblast cell line, L-929, and two human carcinoma of the cervix cell lines, HeLa and ME180. The cells were treated for 7 hr with Adriamycin, cisplatin, or bleomycin. These cells were then incubated for 24 hr in the presence of LT. At the end of the incubation period, cytotoxicity was measured by the neutral red dye uptake assay. There was 10- to 47-fold potentiation of cytotoxicity of LT on L-929 cells. The potentiation of cytotoxicity on human carcinoma of cervix cell lines ranged from 3- to 23-fold. L-929 cells and ME180 cells were incubated for 7 hr at 40 or 42°C followed by 24 hr of incubation in the presence of LT. The elevated temperature treatment also enhanced (5- to 9-fold) the cytotoxic effect of LT. DNA, RNA, and protein syntheses of the ME180 cells was measured following incubation at 42°C. It was observed that all three parameters were suppressed by incubation at this temperature. It was, therefore, possible that the repair of LT damaged cells was hampered by the elevated temperature treatment. It is suggested that LT may have a potential as an anti-tumor agent in combination with selected therapeutic drugs and hyperthermia. © 1986 Society for Experimental Biology and Medicine.

Lymphotoxin (LT) is a cytotoxic lymphokine produced by lymphocytes in response to mitogenic, antigenic, or allogenic stimulation (1, 2). Many lymphoblastoid cell lines can also release LT (3–6). We have shown that OKT4+ cells were the major source of this lymphokine (7). The cytotoxic effect of LT, on tumor cells, has been observed *in vitro* (8–10). It has also been shown to possess anti-tumor activity in mice and a dog (11, 12). Phase 1 clinical trials of LT are now underway.

The exact mechanism of cytotoxic action of LT is still undetermined. Specific binding of human LT to the target cells (L-929) has recently been shown (13). Evidence suggested that LT acted directly on the target cells and caused cell membrane damage (14–16). The potentiation of this effect by cytotoxic drugs could offer therapeutic advantage. Therefore, we investigated the effect of some of the widely used anti-cancer drugs and elevated temperatures on the cytotoxic activity of LT against tumor cells.

Materials and Methods. *Cell line.* HeLa Cells (human carcinoma of cervix), ME-180 cells (human carcinoma of cervix), and L-929 cells (mouse transformed fibroblast) were obtained from American Type Culture Collections (Rockville, Md.). All cell lines employed in this study were maintained in RPMI 1640 (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (GIBCO) and 50 μg/ml gentamycin (Schering Corp., Kennilsworth, N.J.). The stock cultures were maintained in 75-cm^2 flasks (Costar, Cambridge, Mass.) and subcultured twice a week.

Assay system. A semi-automated computer-assisted microassay for Lt (17) was used in this study. Briefly, a suspension of a sensitive clone of L-929 cells, at a concentration of 1×10^5 cells/ml containing 0.5 μg/ml of mitomycin C (Sigma, St. Louis, Mo.), was plated into 96-well plates (150 μl/well). The plates were incubated overnight. The following day, LT samples were added and serial dilutions were made. after 24 hr of incubation, the viability of target cells was assessed by the uptake of neutral red dye (17). A unit of LT activity was defined as the reciprocal of the highest dilution that caused 50% cytotoxicity as compared to the control (17).

A similar procedure was used to measure the cytotoxicity of LT, in combination with anti-cancer drugs or elevated temperature, against different cell lines. The L-929 cells (1.5×10^4 cells/well) and the human tumor cells

0037-9727/86 $1.50
Copyright © 1986 by the Society for Experimental Biology and Medicine.
All rights reserved.

(3×10^4 cells/well) were seeded into 96-well plates and incubated overnight at 37°C. The next day, target cells were treated with different concentrations of anti-cancer drugs: Adriamycin (doxorubicin), cisplatin (Platinol), and bleomycin (bleonoxane). After 7 hr of incubation in the presence of drugs, the cells were incubated for another period of 24 hr with different amounts of LT. In experiments designed to investigate the effect of elevated temperatures, L-929 and ME180 cells were incubated at 40 and 42°C, respectively. These were the highest temperatures which did not kill the cells after 48 hr of incubation. After 7 hr of incubation at the elevated temperatures, the cells were incubated for another period of 24 hr with different amounts of LT. The viability of the target cells was determined and cytotoxicity was calculated as described above. The potentiation index was calculated as follows:

Potentiation index

$$= \frac{\text{LT (units/ml) causing 50\% cytotoxicity in the untreated cells}}{\text{LT (units/ml) causing 50\% cytotoxicity in the treated cells}}.$$

LT and drugs. LT was produced as described previously (7). Briefly, the buffy coats were obtained from the Wadley Central Blood Bank and red blood cells were hemolyzed with 0.83% NH_4Cl in Tris–HCl buffer (0.017 *M*, pH 7.2). The white blood cells were applied to a nylon wool column (19×19 cm) and incubated for 1 hr at 37°C in 5% CO_2. The nonadherent cells were then eluted using prewarmed (37°C) culture medium. These cells (1×10^6 cells/ml) were then cultured in RPMI 1640 containing 80 μg/ml of PHA-P (Difco, Detroit, Mich.), 1 μg/ml of indomethacin (Sigma), and 50 μg/ml of gentamycin at 37°C for 5 days. The supernatant was obtained after removing cells by centrifugation at 1000*g* for 20 min. The pool of supernatants was concentrated and dialzyed by Pellicon System (Millipore, Bedford, Mass.) using 10,000 mol wt cut-off membrane. The crude concentrated material was partially purified by Blue Agarose and Con-A Sepharose chromatography (7). This preparation of LT, with specific activity of 5×10^4 units, was used in the study.

Adriamycin, cisplatin, and bleomycin were obtained through Morton Cancer and Research Hospital, Dallas, Texas. The drugs were diluted with 0.9% NaCl solution to obtain the desired concentration, immediately before use.

Effect of elevated temperature on DNA, RNA, and protein syntheses. ME180 cells at a concentration of 1×10^5 cells/ml were seeded into 24-well plates (1 ml/well) and incubated overnight at 37°C. The next day, plates were incubated at the elevated temperature of 42°C for 24 hr. After the incubation, 1 μCi of [^{3}H]thymidine (2.0 Ci/mmole), 2 μCi of [^{3}H]uridine (25.2 Ci/mmole), or 4 μCi of [^{3}H]leucine (142.3 Ci/mmole) was added and incubated for another 4 hr. The materials were precipitated with 15% tricholoroacetic acid (TCA) and the precipitate was collected on Millipore filters. The amount of radioactivity in the TCA-insoluble material was determined by liquid scintillation counting.

Results. *Potentiating effect of drugs on the cytotoxic activity of LT.* The concentration of the drugs for these experiments was predetermined. The highest concentration which did not cause cytotoxicity on target cells after 48 hr of exposure (in the absence of LT) was used. The data in Table I show that all three drugs, Adriamycin (0.8 μg/ml), cisplatin (0.8 μg/ml), and bleomycin (0.24 units/ml), potentiated the cytotoxic activity of LT against L-929 cells (47-, 10-, and 16-fold, respectively).

ME180 cells were quite sensitive to cytotoxic activity of LT. Lymphotoxin at a concentration of 7 units/ml caused 50% cytotoxicity to the target cells (Table II). Following drug treatment, the cytotoxic activity of LT against ME180 cells was significantly potentiated. Treating the cells with Adriamycin (0.1 μg/ml), cisplatin (0.6 μg/ml), and bleomycin (0.0005 units/ml) potentiated the cytotoxic activity of LT, 5, 5, and 3 times, respectively (Table II).

The second carcinoma of cervix cell line (HeLa cells) was sensitive to the cytotoxic activity of LT at high concentration. These cells required 623 units/ml of LT to cause 50% cytotoxicity (Table 2). Treatment of HeLa cells with Adriamycin (0.6 μg/ml), cisplatin (2 μg/ml), and bleomycin (0.24 units/ml) also potentiated the cytotoxic activity of LT by 17, 23, and 6 times, respectively (Table II).

Experiments were carried out to study whether potentiating effect is dose (drug) dependent. HeLa cells were treated with different

TABLE I. POTENTIATION EFFECT OF DRUGS ON CYTOTOXIC ACTIVITY OF LT AGAINST MOUSE TRANSFORMED FIBROBLAST CELLS (L929)

Treatment	LT (units/ml) required to kill 50% of cell populations	Potentiation index
Control	14 ± 4[a]	—
Adriamycin (0.8 μg/ml)	0.3 ± 0.2	47
Cisplatin (0.8 μg/ml)	1.4 ± 0.2	10
Bleomycin (0.24 units/ml)	0.9 ± 0.4	16

[a] Mean ± SD.

concentrations of Adriamycin (0.1–0.6 μg/ml). A fixed amount of LT (125 units/ml) was then added. The data in Fig. 1 indicate the drug dose-dependent effect. The percentage cytotoxicity on HeLa cells was found to increase with the increased concentration of Adriamycin.

Potentiating effect of elevated temperature on the cytotoxic activity of LT. The cytotoxic effect of LT at elevated temperature was studied on L-929 and ME180 cells. The data in Table III show that the cytotoxic activity of LT on both cells was potentiated by the elevated temperatures. The cytotoxic activity of LT on L-929 cells was potentiated by nine times at 40°C and five times on ME180 cells at 42°C.

The effect of elevated temperature on the DNA, RNA, and protein synthesis of ME180 cells was also studied. It was found that DNA, RNA, and protein synthesis were greatly suppressed at 42°C as compared to the control temperature of 37°C (Table IV).

Discussion. Lymphotoxin has been shown to have cytotoxic effect on certain cell lines (1, 2, 8–10, 23, 24). The mechanism of cytotoxic action of LT is not clear at present. It has been suggested that LT causes cell membrane damage (10, 15, 16). Loss of prelabeled plasma membrane proteins was observed in LT-treated target cells. This loss was general and did not involve any particular protein (18). Recently, Leopardi *et al.* (19) have shown that LT can induce changes in the arrangement of f-actin and cell configuration, but not in the total amount of f-actin.

Studies on LT-treated cells showed increase in the RNA synthesis, plasma membrane protein synthesis and RNA polymerase activity (18, 20, 21). The increase in these parameters appears to be part of the repair process of the target cells in response to LT-induced membrane damage (18, 22). Inhibition of target cell RNA synthesis or protein synthesis by actinomycin D or cycloheximide enhances the cytotoxicity of LT (15, 23). In this study, we

TABLE II. POTENTIATION EFFECT OF DRUGS ON CYTOTOXIC ACTIVITY OF LT AGAINST HUMAN TUMOR CELLS (HeLa, ME180)

Treatment	LT (units/ml) required to kill 50% of cell populations	Potentiation index
ME180 cells		
Control	7 ± 1[a]	—
Adriamycin (0.1 μg/ml)	1.4 ± 0.1	5
Cisplatin (0.6 μg/ml)	1.3 ± 0.3	5
Bleomycin (0.005 units/ml)	2.4 ± 0.2	3
Hela cells		
Control	623 ± 157	
Adriamycin (0.6 μg/ml)	37 ± 6	17
Cisplatin (2 μg/ml)	27 ± 6	23
Bleomycin (0.24 units/ml)	109 ± 25	6

[a] Mean ± SD.

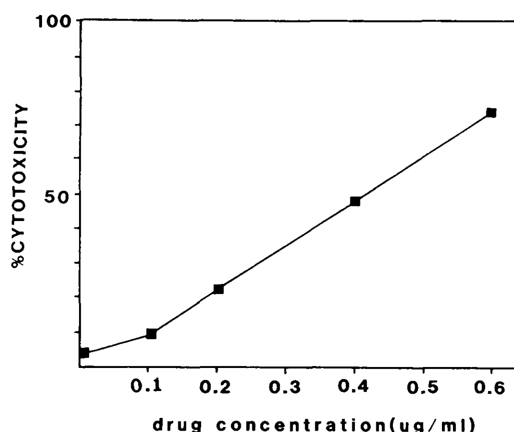

FIG. 1. Potentiation effect of adriamycin on cytotoxic activity of LT against Hela cells. The target cells were treated with different concentrations of adriamycin (0.1–0.6 μg/ml). Fixed amounts of LT at a concentration of 125 units/ml were then applied. Percentage cytotoxicity was determined by using neutral red dye uptake assay.

investigated the potentiating effect of widely used anti-cancer drugs (Adriamycin, cisplatin, and bleomycin) which are known to inhibit the synthesis of macromolecules, including DNA, RNA, and proteins. These drugs greatly potentiated the cytotoxic activity of LT against mouse transformed fibroblasts (L-929 cells) and two human carcinoma of the cervix (HeLa and ME180) cells. The potentiating effect of Adriamycin was found to be dose dependent. The potentiation of cytotoxic activity of LT, by elevated temperatures (40 and 42°C) was also observed on L-929 and ME180 cells. Similar potentiation was also seen when cells were exposed to LT, at the same time that drug or elevated temperature treatment was initiated (data not shown).

The potentiating effect of cytotoxic activity of LT by drugs or elevated temperatures employed in this study may be explained on the basis of inhibition of RNA and protein synthesis which in turn inhibits the repair mechanism of target cells, resulting in increased cell kill by LT. The LT preparation used in these experiments was partially purified. However, it is unlikely that other substances such as interferon and tumor necrosis factor (TNF) contributed substantially to this effect. Our preparation contained less than 10 units/ml of interferon γ, which is far below the amount of interferon γ (16,384 units/ml) shown to potentiate the effect of LT on HeLa cells (26). TNF is another cytotoxic protein, produced by monocytes and known to be present in PHA-stimulated mononuclear cell cultures (26). Both TNF and LT are indistinguishable in the *in vitro* assay. However, unlike LT, TNF did not bind to Con-A–Sepharose (27). Since Con-A–Sepharose was used for LT purification in this study, the presence of TNF in the LT preparations can be ruled out.

Chemoimmunotherapy combining lymphokines and chemotherapeutic drugs offers

TABLE III. POTENTIATION EFFECT OF ELEVATED TEMPERATURE ON CYTOTOXIC ACTIVITY OF LT AGAINST L-919 CELLS AND ME180 CELLS

Treatment	LT (units) required to kill 50% of cell population	Potentiation index
L-929 cells		
Control (37°C)	24.3 ± 4[a]	—
Elevated temperature (40°C)	2.6 ± 0.2	9
ME180 cells		
Control (37°C)	6 ± 1	—
Elevated temperature (42°C)	1.2 ± 0.1	5

Note. L-929 cells (1.5 × 10^4 cells/well) and ME180 cells (3 × 10^4 cells/well) were seeded on 96-well plates and incubated overnight at 37°C. The next day, L-929 cells and ME180 cells were incubated at elevated temperature, 40 and 42°C, respectively (temperature at these elevated temperatures did not kill the cells after 4 hr). After 6 hr of incubation, the appropriate amount of LT was then added and incubated at elevated temperature for a further 24–28 hr. The viability of the target cells and potentiation index were calculated as described under Materials and Methods.

[a] Mean ± SD.

TABLE IV

	Treatment	[^{3}H]Thymidine incorporation (cpm)	[^{3}H]Uridine incorporation (cpm)	[^{3}H]Leucine incorporation (cpm)
Control	37°C	20598 ± 1632[a] (100)[b]	58326 ± 5754 (100)	144029 ± 23930 (100)
	42°C	4098 ± 756 (20)	17932 ± 2429 (31)	84266 ± 4034 (59)

[a] Mean ± SD.
[b] % of control.

an attractive approach to cancer management. This study suggests that such an approach may be feasible.

1. Ruddle NH, Saksman BH. Cytotoxicity mediated by soluble antigen and lymphocytes in delayed hypersensitivity. III. Analysis of mechanism. J Exp Med **129**:1267–1280, 1968.
2. Granger GA, William TW. Lymphocyte cytotoxicity *in vitro:* Activation and release of a cytotoxic factor. Nature (London) **218**:1253–1254, 1968.
3. Amino N, Lim ES, Pysher JJ, Mier R, Moor GE, Degroot LJ. Human lymphotoxin obtained from established lymphoid cell lines; Purification, characterization and inhibition by anti-immunoglobulin. J Immunol **13**:1334–1345, 1974.
4. Rosenau W, Stiter D, Jentrud S. Elaboration of lymphotoxin by freshly isolated human T lymphocytes and continuous lymphoid cell line. Cell Immunol **43**: 235–244, 1979.
5. Granger GA, Moor GE, White JG, Matzinger P. Sundsmo JS, Shipe S, Kolb WP, Kramer J, Glade PR. Production of lymphotoxin and migration inhibitory factor by established human lymphocyte cell line. J Immunol **104**:1476–1485, 1970.
6. Fair DS, Jeffer EWB, Granger GA. Release of LT molecule with restricted physical heterogeneity by a continuous human lymphoid cell line *in vitro.* Mol Immunol **16**:185–192, 1979.
7. Pichyangkul S, Miller, JE, Waldrop S, Khan A. Cellular origin of human lymphotoxin and its purification. Clin Immunol Immunopathol **35**:22–34, 1985.
8. Gately MK, Mayer MM, Henney CS. Effect of antilymphotoxin on cell-mediated cytotoxicity: Evidence for two pathways, one involving lymphotoxin and the other requiring intimate contact between the plasma membrane of killer and target cells. Cell Immunol **27**: 82–93, 1976.
9. Evans CM, Rabin ES, DiPaolo JA. The susceptibility of guinea pig cells to the colony-inhibitory activity of lymphotoxin during carcinogenesis. Cancer Res **37**: 898–903, 1977.
10. Weitzen ML, Yamamoto RS, Granger GA. Identification of human lymphocyte-derived lymphotoxins with binding and cell-lytic on NK-sensitive cell lines *in vitro.* Cell Immunol **77**:30–41, 1983.
11. Papermaster BW, McEntire JE, Gilliland CD. Lymphokine adjuvant therapy: Bioassay of human lymphokine fractions in a mouse tumor model. In: Khan A, Hill NO, eds. Human Lymphokines. New York, Academic Press, pp459–475, 1982.
12. Khan A, Martin ES, Webb K, Weldon D, Hill NO, Duvall J, Hill JM. Regression of malignant melanoma in a dog by local injection of a partially purified preparation containing human lymphotoxin (41345). Proc Natl Exp Biol Med **169**:291–294, 1982.
13. Hass PE, Hotchkiss A, Mohler M, Aggarwal BB. Characterization of specific high affinity receptors for human tumor necrosis factor on mouse fibroblasts. J Biol Chem **260**:12214–12218, 1985.
14. Friend DS, Rosenau W. Target-cell membrane alterations induced by lymphotoxin. Amer J Pathol **86**: 149–162, 1977.
15. Rosenau W, Goldberg ML, Burk GC. Early biochemical alterations induced by lymphotoxin in target cells. J Immunol **111**:1128–1135, 1973.
16. Kobayashi Y, Sawada J, Osawa T. Activation of membrane phospholipase by guinea pig lymphotoxin (GLT). J Immunol **122**:791–794, 1979.
17. Khan A, Weldon D, Duvall J, Pichyangkul S, Hill NO, Muntz D, Lanius R, Ground M. A standardized automated computer assisted microassay for lymphotoxin. In: Khan A, Hill NO, eds. Human Lymphokines. New York, Academic Press, pp23–30, 1982.
18. Rosenau W. Effect of human α-lymphotoxin on target cell plasma membranes. In: Khan A, Hill NO, eds. Human Lymphokines. New York, Academic Press, pp487–491, 1982.
19. Loepardi E, Friend DS, Rosenau W. Target cell lysis: Ultra-structural and cytoskeletal alterations. J Immunol **133**:3429–3436, 1984.
20. Rosenau W, Burk GC, Moy J. Lymphotoxin-induced loss of plasma membrane protein. Amer J Pathol **94**: 473–482, 1979.
21. Kunitomi G, Rosenau W, Burk GC, Goldberg ML. Alteration in RNA synthesis in lymphotoxin-treated target cells. Amer J Pathol **80**:249–260, 1976.
22. Rocklin RE, Bendtzen K, Greineder D. Mediators of

immunity: Lymphokines and monokines. Adv Immunol **29**:56–127, 1980.

23. Wallach D. Preparation of lymphotoxin induced resistance to their own cytotoxic effect. J Immunol **132**: 2464–2469, 1984.
24. Jeffer EWB, Granger GA. 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. J Immunol **117**:174–179, 1976.
25. Gray PW, Aggarwal BB, Benton CV, Bringman TS, Henzel WJ, Jarrett JA, Leung DW, Moffat B, Ng P, Svedersky LP, Palladino MA, Nedwin GE. Cloning and expression of cDNA for human lymphotoxin, a lymphokine with tumor necrosis activity. Nature (London) **312**:721–724, 1984.
26. Stone-Woff DS, Yip YK, Kelker HC, Le J, Henriksen-DeStefano D, Rubin BY, Rinderknecht E, Aggarwal BB, Vilcek J. Interrelationship of human interferon-gamma with lymphotoxin and monocyte cytotoxin. J Exp Med **159**:828–843, 1984.
27. Kelker HC, Oppenheim JD, Stone-Wolff D, Henriksen-DeStefano D, Aggarwal BB, Stevenson HC, Vilcek J. Characterization of human tumor necrosis factor produced by peripheral blood monocytes and its separation from lymphotoxin. Int J Cancer **36**:69–73, 1985.

Received March 13, 1986. P.S.E.B.M. 1986, Vol. 183.
Accepted July 14, 1986.