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SHORT COMMUNICATION

***In Vitro* Mechanism of Human Lymphotoxin Action: Destruction of Nondividing Target Cells**

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Soluble molecules secreted by activated lymphoid cells have been implicated as important to our understanding of host lymphocyte function in cellular immune reactions. These mediators include migration inhibition factor (MIF) (1, 2), chemotactic factors (CF) (3), lymphotoxin (LT) (4), and others which have been collectively termed lymphokines by Dumonde *et al.* (5). Normal lymphocytes activated *in vitro* by mitogens or immune lymphocytes activated specifically with antigens are known to secrete lymphokines into the supernatant medium.

Lymphotoxin, a soluble nonspecific cell toxin, has been implicated in the destruction of target cells by both mitogen-stimulated and antigen-stimulated lymphocytes *in vitro*. There has been, however, concern expressed as to the action or effect of LT on target cells (4, 6, 7). It has been asserted by certain laboratories that LT may act only to inhibit the proliferation of target cells without causing actual cell death (8, 9). Advocates of this assertion further maintain that the ^{14}C -labeled amino acid incorporation technique may not measure cellular destruction but reflect only inhibition of target cell proliferation (8, 9). This study reports the *in vitro* effects of human LT on target L cells inhibited from cell division by treatment with mitomycin C (MC). These effects were measured by the release of chromium 51 (^{51}Cr) from prelabeled L cells, the incorporation of ^{14}C -labeled amino acids into TCA-precipitable L cell protein, and total viable cell counts.

Maintenance of the continuous cell lines and culture media employed have been described previously (10). Monolayer cultures of target L cells were established in tubes (20×123 mm) containing 1×10^5 cells in 1.0 ml of RPMI 1640 + 5% fetal bovine serum (1640). Cells to be used in the ^{51}Cr assay were trypsinized as described previously (10). Then, 10^7 cells in 2.0 ml of 1640 were incubated with $100 \mu\text{Ci}$ of Na^{51}Cr (International Chemical Nuclear, Irvine, CA) at 37° for 1 hr. The labeled cells were then centrifuged for 4 min at $400 g$ and washed four to five times with a total of 40-50 ml of 1640. One set of ^{51}Cr -labeled and one set of un-

labeled cells were established as tube cultures in 1640 containing 0.3 μg MC (Calbiochem, Los Angeles, CA). A second set of ^{51}Cr -labeled and unlabeled cells were established in 1640 lacking MC. After 18–24 hr, the medium was removed, the monolayers washed twice with warm 0.15 M NaCl, and various dilutions of LT-containing medium were added. The LT medium was prepared from cultures of human lymphocytes activated with 20 $\mu\text{g}/\text{ml}$ PHA-P in RPMI 1640 + 10% fetal bovine serum (FBS) as previously described (11). The target cell cultures were then incubated at 37° in an atmosphere of 5% CO_2 and 95% air for 44–48 hr.

Cell viability was assayed by three separate methods. *^{51}Cr release*: Total ^{51}Cr counts per minute (cpm) were established for each untreated tube culture in a Baird Atomic Gamma Counter. Then the cells were sedimented by centrifugation at 400 *g* for 5 min and 0.75 ml of the supernatant was removed and counted. The cpm were corrected for total counts released and % ^{51}Cr released was calculated from the following formula: % release = (cpm released)/(total cpm) \times 100. *^{14}C -labeled amino acid incorporation*: Cell cultures were assayed for their ability to incorporate ^{14}C -labeled amino acids into TCA-precipitable counts as previously described (10). Briefly, monolayer cells were washed twice with warm 0.15 M NaCl, and incubated for 30 min with 1.0 ml of medium containing 0.5 $\mu\text{Ci}/\text{ml}$ ^{14}C -labeled amino acid labeling mixture (Schwartz BioResearch, Van Nuys, CA). The monolayer cells on the glass were then digested with 0.1 M KOH, precipitated with 5% TCA and the precipitate filtered on a 0.45 μm Millipore filter. The filters were dried, placed in vials and radioactivity determined in a Beckman LS-233 Liquid Scintillation Counter. Percent destruction was calculated in the following manner: % destruction = (cpm control – cpm experimental)/(cpm control) \times 100. *Total viable cell count*: Each culture was washed twice with 1.0 ml of 0.15 M NaCl, and incubated for 1–2 min with 0.1 ml of a 0.1% trypsin and 10^{-8} M EDTA solution. The cultures were agitated vigorously to remove the cells from the glass and to ensure a single cell suspension. To each tube was then added 0.1 ml 1640 and 0.05 ml of a 0.2% Eosin Y solution. The total number of viable cells was then determined in a Neubauer hemacytometer. The percentage of destruction was deter-

TABLE 1
CYTOTOXIC EFFECT OF DILUTIONS OF HUMAN LT ON MITOMYCIN-TREATED
L CELLS AS MEASURED BY ^{51}Cr RELEASE

LT dilution	Mitomycin-treated		Untreated	
	% Release	Specific release	% Release	Specific release
Undiluted	45.4 \pm 2.86 ^a	12.8	49.4 \pm 2.76	21.9
1:2	57.1 \pm 4.80	24.3	63.2 \pm 0.24	35.7
1:5	60.3 \pm 0.60	27.5	61.4 \pm 0.73	33.9
1:10	53.6 \pm 0.40	20.8	57.6 \pm 1.52	30.1
Control	32.8 \pm 1.75	0.0	27.5 \pm 0.30	0.0

^a Results are expressed as mean \pm standard error for two experiments.

TABLE 2
CYTOTOXIC EFFECT OF DILUTIONS OF HUMAN LT ON MITOMYCIN-TREATED L CELLS
AS MEASURED BY ¹⁴C-LABELED AMINO ACID INCORPORATION
INTO TCA-PRECIPIITABLE CPM

LT dilution	Mitomycin-treated		Untreated	
	% Destruction	cpm	% Destruction	cpm
Undiluted	96.4 ± 2.17 ^a	342 ^b	98.4 ± 0.27	732
1:2	94.9 ± 2.33	780	97.2 ± 1.64	556
1:5	95.0 ± 2.08	924	96.9 ± 1.57	611
1:10	94.1 ± 2.14	1,278	95.8 ± 2.06	846
Control	0.0	19,721	0.0	35,992

^a Results given as mean ± standard error for three experiments. See text for expression of % destruction.

^b Results of a typical experiment in terms of ¹⁴C-labeled protein TCA-precipitable CPM.

mined by the following equation: % destruction = (total viable cells control—total viable cells experimental)/(total viable cells control) × 100.

The results obtained from MC-treated target cells versus untreated target cells when incubated in the presence of LT can be seen in Tables 1–3. The LT batch used in these experiments was a pool of three separate batches prepared from lymphocytes from human adenoid tissue and gave an LD₅₀ titer on monolayer tube cultures of L cells to a dilution of 1:50. The level of MC employed in the experiments ensured total inhibition of DNA synthesis and cell division, yet 80–90% of the treated cells remained viable for the duration of the experiment as judged by Eosin Y uptake (12). The percentage of destruction and percentage of ⁵¹Cr release are given as the mean of two to three experiments. Duplicate cultures were employed in each experiment at each LT dilution indicated in Tables 1–3. Cell counts

TABLE 3
CYTOTOXIC EFFECT OF DILUTIONS OF HUMAN LT ON MITOMYCIN-TREATED
L CELLS AS MEASURED BY TOTAL VIABLE CELL COUNTS

LT dilution	Mitomycin-treated		Untreated	
	% Destruction	Viable cell count	% Destruction	Viable cell count
Undiluted	84.7 ± 10.05 ^a	0.125 ^b	98.4 ± 1.15	0.0
1:2	98.6 ± 1.43	0.188	99.9 ± 0.09	0.0
1:5	96.7 ± 1.28	0.188	99.8 ± 0.07	0.031
1:10	96.3 ± 1.74	0.250	99.8 ± 0.07	0.031
Control	0.0	4.50	0.0	27.3

^a Results expressed as mean ± standard error for three experiments. See text for expression of % destruction.

^b Results of a typical experiment in terms of total viable cell counts (× 10⁻⁴) as measured by Eosin Y exclusion.

or ^{14}C cpm for a typical experiment are also shown for the viable cell count and ^{14}C -labeled amino acid incorporation assays. It can readily be seen that the effect of LT on cells treated with MC parallels that of untreated control cultures as measured by all three assay methods. In one experiment (data not shown) where dilutions of LT to 1:50 were employed the same end point was obtained for treated as for untreated target cells. While not reported here, results with MC-treated HeLa cells have also shown them to be as sensitive to LT as untreated controls. Microscopic examination of the cultures revealed in many cases no cells visible attached to the glass; while a search for viable cells in the supernatants revealed only debris and an occasional intact nonviable cell as judged by Eosin Y uptake. These results indicate that the cytotoxic effects of lymphotoxin on target cells is due to a mechanism other than inhibition of DNA synthesis and/or cell proliferation.

From our results it is clear that the ^{14}C -labeled amino acid incorporation assay is directly related to the number of viable cells remaining in the test and control cultures. The ^{51}Cr release assay, however, is less sensitive in detecting maximum cell destruction. This can be attributed to the high background release of label from control cultures. [Heat-killed cells are shown to release 65–70% of their label which may indicate maximum release for total cellular destruction (data not shown).] After 48 hr incubation control cultures release approx 30% of their label which leaves 35–40% specific release available for measuring cytotoxicity since 100% destruction = 65–70% release. Since background release is a function of time, our data suggest that the ^{51}Cr release technique may be best applicable to short-term experiments where background release is low.

It has been suggested that the ^{14}C -labeled amino acid incorporation assay does not measure cell destruction. This is in fact true; however, it clearly does accurately reflect the number of viable cells remaining in the culture. This assay might then measure either a true reduction in cell numbers *via* a cytotoxic reaction or inhibition of cell growth below that of control cultures. Results from these experiments and previous manuscripts from our laboratory implicate a cytotoxic function for LT for the following reasons: (1) The percentage of destruction of LT-treated cells as measured by viable cell counts and ^{14}C -labeled amino acid incorporation is essentially parallel in cultures inhibited from cell division and normal proliferating controls. (2) Most previously reported data reveal a greater than 90% reduction of ^{14}C cpm in LT-treated cultures over control cultures, which is clearly indicative of massive levels of cellular destruction. (3) The release of ^{51}Cr which is independent of cell proliferation clearly reveals measurable cellular destruction in both MC-treated and untreated cultures. (4) Microscopic observation of LT-treated cells, destruction of which the above assay systems are designed to quantitate, consistently reveals a cytolytic reaction resulting in the destruction of the target cells.

The mechanism(s) by which target cells are destroyed by aggressor lymphocytes has yet to be conclusively elucidated both *in vitro* and *in vivo*, but may include several processes including soluble cytolytic substances such as lymphotoxin. Lymphotoxin has been shown to be present in many experimental systems which involve lymphocytes from several mammalian species (13). It has also been shown to cause ^{51}Cr release from target cells *in vitro* (14). The results of these experi-

ments clearly show that the primary action of LT is not to inhibit cell proliferation but to destroy target cells probably by some lytic membrane action.

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