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Mechanism of Human Lymphotoxin and Tumor Necrosis Factor Induced Destruction of Cells In Vitro: Phospholipase Activation and Deacylation of Specific-Membrane Phospholipids

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The role of phospholipase (PLase) activation and lipid metabolism in lymphotoxin (LT)- and tumor necrosis factor (TNF)-mediated destruction of murine L929 cells was examined. At the levels of LT and TNF employed, cell destruction began at 4–6 h and was 99% complete by 30 h. Cell membrane phospholipids (PL), labelled in situ at the C2 position with ^{14}C arachidonic acid, were analyzed by two-dimensional thin-layer chromatography and quantitated over a 30 h time course after LT or TNF treatment. The ratio of radiolabel incorporation relative to the actual amount of each PL present was determined by inorganic phosphate analysis. Radiolabelled arachidonic acid, eicosanoids, and neutral lipids were released into the medium prior to the onset of cell death (4–6 h) and continued to accumulate linearly throughout the destructive reaction. There was a quantitative relationship between the appearance of radiolabelled metabolites in the media and the loss of radiolabelled cellular PL. Cellular phosphatidylethanolamine was the primary PL deacylated by PLase action, showing a 75% reduction in radiolabel. The PLase inhibitors—quinacrine, hydrocortisone, dexamethasone, and indomethacin—were potent inhibitors of LT- and TNF-mediated cell destruction, suggesting that selective deacylation of specific membrane PL by PLase activation is an important step in the events that lead to LT- and TNF-mediated cellular destruction in vitro.

The molecular mechanism of how lymphotoxin (LT) and tumor necrosis factor (TNF) cause selective destruction of certain transformed cells in vitro is beginning to become apparent. It is now clear that both normal and transformed cells express a small number (2,000–5,000) of high-affinity LT and TNF cell surface receptors (Sugarman et al., 1981; Aggarwal et al., 1985; Hass et al., 1985). Aggarwal et al. (1985) reported that human LT and TNF (which share 28% amino acid homology) may bind to the same plasma membrane receptor(s). After binding to the membrane, the receptor-associated LT or TNF complex is rapidly internalized, and a large portion is degraded (Hass et al., 1985; Tsujimoto et al., 1985; Baglioni et al., 1985; Kull Jr. and Cuatrecasas, 1981). However, selective destruction of transformed cells does not appear to be due to differences in the number or affinity of membrane receptors nor the ability of cells to internalize and degrade membrane-associated forms (Sugarman et al., 1985; Kull Jr. et al., 1985). In addition to selective tumor cell destruction, both LT and TNF have been shown to cause a wide variety of effects on different types of cells in vitro (Sugarman et al., 1985; Camussi

et al., 1987; Nawroth and Stern, 1986; Philip and Epstein, 1986; Bertolini et al., 1985; Pujol-Borrell et al., 1987; Jelinek and Lipsky, 1987).

Although the actual steps are not known, it is apparent that interaction of LT or TNF with membrane receptors initiates a cascade of intracellular events that lead to cell destruction. Several investigators reported that a target cell esterase is required for destruction (Weitzen and Granger, 1980; Ruggiero et al., 1987). Kobayashi et al. (1979) and Yagisawa and Osawa (1982) showed that partially purified preparations of guinea pig LT induced phospholipase (PLase) A_2 activation when L-P3 cells were killed in vitro. These authors were the first to suggest that PLase activation was an important step in LT-induced cell destruction. Recently, PLase A_2 has been shown to be involved in the TNF-mediated changes in endothelial cell morphol

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ogy (Clark et al., 1988) and in destruction of L929 cells (Neale et al., 1988) in vitro. PLase A₂ deacylates phospholipids to release arachidonic acid, which then can be metabolized into eicosanoids via the cyclooxygenase and lipoxygenase pathways. Recent studies have given contradictory data on the question of whether agents that inhibit the cyclooxygenase and lipoxygenase pathways block human TNF-induced destruction of L929 cells in vitro (Neale et al., 1988; Suffys et al., 1987; Matthews et al., 1987).

In a preliminary report, we demonstrated that PLases were activated in both murine L929 and human HeLa target cells undergoing destruction induced by recombinant human LT in vitro (Fitzgerald et al., 1987). In contrast, PLase activation was not observed in these same cells deliberately killed by other methods. The present study is the first quantitative analysis of the changes in lipid metabolism due to PLase activation in murine L929 cells treated with lethal levels of LT or TNF. The deacylation of membrane phospholipids was specific, and the primary target phospholipid was phosphatidylethanolamine. Once activated, PLase remained active throughout the destructive phase, even after most of the cell culture was killed. This resulted in an increase in free arachidonic acid and arachidonic acid metabolites in the supernatant as destruction of the culture proceeded. Furthermore, agents that inhibited PLase activation blocked LT- and TNF-mediated destruction of L929 cells in vitro. Thus, PLase activation appears to be an important step in the process of LT- and TNF-mediated cell destruction.

MATERIALS AND METHODS

Cell culture

Monolayers of murine L929 fibrosarcoma and ME180 human carcinoma cells were passaged bi-weekly in RPMI 1640 media (Grand Island Biological Co., Grand Island, NY) supplemented with 10% heat-inactivated (56°C, 1 h) fetal calf serum (Grand Island Biological Co.) (RPMI + 10%) in an atmosphere of 95% air, 5% CO₂ at 37°C.

Cell viability assay

Parallel test and control L929 cell cultures were established in glass culture tubes as monolayers of 2×10^5 cells in 1 ml RPMI + 10%. Test cultures then were treated with 10 ng/ml of recombinant human α LT or 50 ng/ml TNF- α (Genentech Corp., South San Francisco, CA) and incubated for various times. Replicate cultures were assayed for cell viability as previously described by Granger et al. (1980). Briefly, after visual examination, medium was decanted and phosphate-buffered saline (PBS) was added to wash off dead cells. Adherent live cells were trypsinized and counted via hemacytometer or model F Coulter counter. Percent viability was calculated from the average cell count of triplicate LT or TNF-treated cultures and cells treated in the same way in the absence of LT or TNF (control), according to the following equation.

$$\text{Percent viability} = \left(\frac{\text{Average cell count with sample}}{\text{Average control cell count}} \right) \times 100$$

Radiolabelling of L929 monolayer cell cultures with ¹⁴C arachidonic acid (*AA)

Twenty-four hours prior to LT or TNF treatment, 1 ml target L929 cell cultures were established in glass culture tubes at a concentration of 2×10^5 cells/ml. One tenth μ Ci/ml of ¹⁴C arachidonic acid (*AA) (Amersham, Arlington Heights, Ill., 54.9 mCi/mmol) and 0.5 μ g/ml mitomycin C (Sigma Chemical Co., St. Louis, MO) were added directly to the media. After 8 h, excess unincorporated radiolabel was removed by washing each culture twice with 3 ml RPMI + 10% containing 1% fatty acid-free bovine serum albumin (Sigma Chemical Co.). Scintillation counting of the radioactivity in the wash solution indicated that 98% of the radiolabel had been taken up by the monolayer cell cultures. One milliliter of fresh RPMI + 10% media without radiolabel then was added to each culture, and the tubes were incubated for another 16 h prior to the start of LT or TNF treatment.

LT and TNF treatment

Media was decanted from each monolayer tube culture, and 1 ml of fresh media was added. Half of the tubes were treated with lethal levels of LT (1–10 ng/ml) or TNF (5–50 ng/ml) added directly to the media. The variation in amount of LT or TNF necessary to achieve a lethal dosage for all the cells in a culture was caused by variation in cell sensitivity to LT or TNF activity. Parallel control and treated cultures were incubated at 37°C for various intervals, and then the lipids were extracted and analysed as described below.

Lipid extraction and thin-layer chromatography

At each time point, parallel LT-treated and untreated control tube cultures were centrifuged at 300g for 10 minutes, and supernatants were decanted and spun for 5 minutes at 14,000g. Cell pellet and supernatant lipids were extracted separately using modifications (Hajra et al., 1968) of the procedures of Bligh and Dyer (1959). Analytical solvents purchased from commercial sources (Fisher Scientific, Aldrich Chemical Co., Mallinckrodt) were used without further purification. The extract of the supernatant or cells from a single tube was dried under nitrogen and resuspended in 50 μ l chloroform:methanol (95:5). The 50 μ l lipid suspensions from each tube were spotted separately on silica gel 60 plates (Merck, Darmstadt) and separated by thin-layer chromatography (TLC). Two-dimensional TLC was performed using the three solvent system of Yavin and Zutra (1977). Samples were spotted in the lower left corner of separate silica gel 60 plates cut to 10 cm $\times$ 10 cm. Plates were subjected to TLC in the first dimension using a solvent system consisting of 50 ml of chloroform:methanol:methylamine (34:13:3). Plates then were exposed to concentrated hydrochloric acid vapors for 3 minutes to remove residual methylamine, dried for 3 minutes, rotated 90° so that the origin was now located in the lower right corner, and run in the second ascending solvent system consisting of 50 ml of ethyl ether:acetic acid (95:5). Plates were dried 3 minutes, and the third ascending solvent system consisting of 50 ml chloroform:acetone:methanol:acetic acid:water (108:36:20:27:9) was run in the same direction as the second solvent system. This method

resolves individual phospholipids and lysophospholipids and separates them from the neutral lipids and fatty acids. Lipids were visualized by staining in iodine vapor, and the positions of radiolabelled components were detected by autoradiography. The amount of material in radioactive spots was quantitated by scraping the spot (visualized by backlighting through the autoradiograph) off the plate and counting in a Beckman LS-233 liquid scintillation counter. Calibration of this TLC system was accomplished by separation of purified known lipid standards visualized by iodine staining.

Nine different lipid components were visualized and quantitated on each TLC plate. These components were identified as NL = neutral lipid, AA = free arachidonic acid, EP = ethanolamine plasmalogen, PE = phosphatidylethanolamine, LPC = lysophosphatidylcholine, PI = phosphatidylinositol, PS = phosphatidylserine, LPE/SPH = comigrating lysophosphatidylethanolamine and sphingomyelin, and PC = phosphatidylcholine. Supernatant and cell pellet extracts of both LT-treated and control cultures were assayed individually. Total radiolabel was calculated as the sum of radioactivity in spots from the supernatant extract and spots from the cell pellet extract of each individual culture. The amount of each species is reported as a percentage of the total radiolabel scraped from these two plates.

Inorganic phosphate quantitation of specific phospholipids

The percentage of total phospholipid accounted for by each individual phospholipid, independent of the radiolabel content, was quantitated by phosphorus analysis using the procedures of Rouser et al. (1966). Briefly, nondividing L929 cultures (10^7 cells/150 cm² Corning tissue culture flask) were established for 24 h, the media decanted, and the membrane lipids extracted and run in a two-dimensional TLC system as described above. Individual phospholipid spots were identified by iodine staining and comparison to known lipid standards run in parallel. Spots were scraped directly off the silica gel plate into phosphate-free 16 × 125 mm screw cap test tubes and then assayed for phosphorus content by the method of Rouser et al. (1966). Absorbance was read at 660 nm and the amount of phosphate determined by comparison to a standard curve generated from known phosphate standards.

Inhibitor studies

The effects of various inhibitors of PLase A₂ activation on LT- and TNF-induced lysis of L929 cells were examined using the microtiter plate technique described by Yamamoto et al. (1985). Briefly, monolayers of nondividing L929 cells were established in 96-well microtiter plates (2×10^4 cells per 100 µl medium per well) and incubated for 24 h. Various dilutions of quinacrine hydrochloride, hydrocortisone, dexamethasone, and indomethacin (Sigma Chemical Co.), up to the maximum nonlethal level, were added to triplicate culture wells. Cultures then were treated with serial dilutions of LT or TNF. Controls consisted of triplicate culture wells treated with each concentration of LT or TNF alone and with each concentration of inhibitor alone. The microplates were then incubated for 16–24 h.

Cell destruction was quantitated by two independent methods: 1) crystal violet staining of the viable adherent cells or 2) measurement of the uptake and metabolism of neutral red dye by viable cells. Both methods were used for each of the inhibitors tested, and the results with both methods were found to correlate closely. Crystal violet staining was quantified by reading the absorbance at 580 nm on a Titertek Multiskan microplate reader (Flow Laboratories). Neutral red dye uptake was quantified in the same fashion at 540 nm. Percent viability was calculated as:

$$\frac{(\text{Mean absorbance of test triplicates})}{(\text{Mean absorbance of untreated control triplicates})}$$

The absorbance of triplicate wells located toward the center of the plate were averaged with the results of identically treated triplicate wells located near the perimeter in parallel plates resulting in less than 5% variation between duplicate experiments. Experiments were repeated at least three times for each concentration of LT, TNF, and inhibitor tested. Titration curves were plotted for each dilution of inhibitor, and the concentration of LT or TNF required to achieve 50% cytotoxicity was extrapolated from each curve.

RESULTS

Relationship between L929 cell viability and the release of metabolically incorporated ¹⁴C arachidonic acid

To evaluate the temporal relationship between the killing action of LT or TNF and the release of ¹⁴C arachidonic acid (*AA) that had been metabolically incorporated into cellular lipids, kinetic studies were performed. Parallel cultures were incubated with optimal doses of TNF or LT over a 30 h time course. For direct comparison, parallel control cultures were incubated identically in the absence of LT and TNF. At various time points after LT or TNF treatment, replicate samples from control and treated cultures were assayed for the amount of ¹⁴C released into the supernatant, and the cultures were scored for viability. Viability of LT-treated cultures is reported as a percent of control culture viability which remained at 100% throughout the 30 h assay (Fig. 1). Significant cell death began at 6–8 h after LT treatment and progressed rapidly over the next 7–8 h resulting in 90% killing by 16 h and 99% at the final time point (30 h). The release of radiolabel from LT-treated cultures preceded cell death and was significantly elevated over controls by 6 h. As of 8 h, more than one third of the total radiolabel released during the experiment was already present in the media, whereas cell viability had decreased only about 10%. Released radiolabelled material continued to increase throughout the 30 h time course. By 30 h, approximately 50% of the total radiolabel originally incorporated was present in the media. In contrast, control untreated cultures released less than 10% of their total radiolabel into the media throughout the entire 30 h time course. Parallel experiments using TNF instead of LT showed nearly identical results for both cell viability and radiolabel release (data not shown).

Fig. 1. Parallel test and control cultures of L929 cells were assayed for both cell viability and release of ^{14}C radiolabel into the media. Cultures (2×10^5 cells per ml) were prelabelled with ^{14}C arachidonic acid (for details see Materials and Methods). At the indicated time points following LT treatment, replicate cultures were assayed for cell

viability (Granger et al., 1980). Samples (100 μl) of the supernatant of test and control cultures were assayed for ^{14}C by liquid scintillation counting. Viability is reported as a percentage of control cultures that did not receive LT treatment. Throughout the 30 h assay, control untreated cell culture viability remained at $100\% \pm 5\%$.

Quantitation of cellular phospholipids (PL) and distribution of ^{14}C arachidonic acid (*AA) into PL of L929 cells in vitro

Mammalian and bacterial cells labelled in culture with free ^{14}C arachidonic acid (*AA) incorporate 90–98% of the free fatty acid into the C2 position of the phospholipids (PL): phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) (Yagisawa and Osawa, 1982; Chang et al., 1986). To identify individual lipids and establish the proportion of radiolabel incorporation into each, L929 cells were labelled in culture for 24 h with free *AA. The supernatant was removed after centrifugation, and cell membrane lipids in the cell pellet and supernatant were extracted separately, concentrated by evaporating under nitrogen and run in a TLC system that separates components in two dimensions as visualized by autoradiography or iodine vapor (Fig. 2). Known lipid standards were run and visualized with iodine vapor to identify the position of individual lipids. The quantity of lipid present in each PL spot was measured by scraping each PL spot and assaying total inorganic phosphate present in the scraping. The amount of *AA incorporated into each PL was quantitated by scraping the radioactive PL spot off the plate and counting in a liquid scintillation counter. The percent of total cellular PL calculated from phosphate analysis and the percent of total cellular PL radiolabel incorporated into each individual PL are tabulated in Table 1. These data represent the combined results of three separate experiments in which all six PL were quantitated each time.

By comparing the percent of total cellular PL to the percent of radiolabel (*AA) incorporated, it is apparent that phosphatidylserine (PS) and comigrating lysophosphatidylethanolamine and sphingomyelin (LPE/SPH) each show proportional amounts of *AA incorporation relative to the amount of phospholipid present. How-

Fig. 2. To identify individual lipids and establish the proportion of radiolabel incorporation into each, L929 cells were labelled in culture for 24 h with free *AA as described in Materials and Methods. The supernatant was removed after centrifugation, and cell membrane lipids in the cell pellet and supernatant were extracted separately, concentrated under nitrogen, and run in a TLC system that separates the major lipid components into two dimensions using the three-solvent system of Yavin and Zutra (1977). The origin is located at the lower left. Lipids were visualized by autoradiography. Known lipid standards were run and visualized with iodine vapor to identify the position of individual lipids. Nine different lipid components were quantitated on each TLC plate. These components were identified as NL, neutral lipid; AA, free arachidonic acid; EP, ethanolamine plasmalogen; PE, phosphatidylethanolamine; LPC, lysophosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; LPE/SPH, comigrating lysophosphatidyl-ethanolamine and sphingomyelin; and PC, phosphatidylcholine. Supernatant and cell pellet extracts of both LT-treated and control cultures were assayed individually.

TABLE 1. Quantitation of phospholipid vs. radiolabel incorporation¹

PC	PE	LPE/SPH	PI	PS	LPC
Percent of total phospholipid in cell pellet					
54.3 ± 5.9	18.1 ± 3.6	15.1 ± 1.5	3.8 ± 2.0	5.0 ± 0.5	3.7 ± 1.3
Percent of radiolabel in total phospholipid of cell pellet					
37.4 ± 6.0	33.6 ± 3.6	14.3 ± 3.6	8.2 ± 2.3	4.7 ± 0.5	1.8 ± 0.2

¹L929 cells were cultured at 10⁷ cells per 150 cm² Corning tissue culture flask for 24 h. Cellular lipids were extracted, separated by two-dimensional TLC, located, and identified by staining with iodine vapor and comparison to known standards as described in Materials and Methods. The quantity of lipid present in each of the six PL spots (PC, PE, PI, PS, LPC, and LPE/SPH) was measured by scraping each PL spot and assaying total inorganic phosphate present in the scraping by the method of Rouser et al., 1966. Absorbance was read at 660 nm on a Hitachi spectrophotometer and the amount of phosphate determined by comparison to a standard curve generated from known phosphate standards. The amount of each PL species is reported as a percentage of total PL scraped from the plate. Phosphorous content was compared with ³H-AA incorporation in 1 ml tube cultures labelled for 24 h as described in Materials and Methods. Labelled lipids were extracted, separated, and visualized by autoradiography as described in Figure 1. The amount of ³H-AA incorporated into each of the six PL was quantitated by scraping the radioactive PL spot off the plate and counting in a liquid scintillation counter. The percent of ³H-AA in the total phospholipid of the cell pellet was reported for each PL species as a percentage of the total PL radiolabel scraped from the plate. These experiments were repeated three times, and the average result and standard deviation is shown for each PL.

ever, PE constitutes 18% of the total cellular PL, but it incorporates 34% of the ³H-AA, whereas PI constitutes 4% of the total cellular PL, but incorporates 8% of the ³H-AA. In contrast, PC, and LPC, which constitute 54% and 4% of total cellular PL, respectively, incorporate a slightly lower proportion of ³H-AA (37% and 2%, respectively). Thus, both PE and PI appear to be significantly enriched in the amount of ³H-AA incorporation, whereas PC and LPC have lower levels of ³H-AA incorporation.

Treatment with LT or TNF induces PLase activation, deacylation of AA, and changes in lipid metabolism

Next, quantitative changes in the PL radiolabel distribution over the 30 h time course of LT or TNF killing were examined. Parallel L929 cultures radiolabelled for 24 h with ³H-AA were established. At time zero, half of the cultures were treated with LT or TNF and, at intervals up to 30 h, the lipids from parallel LT- or TNF-treated and untreated control cultures were extracted, separated by two-dimensional TLC, visualized, and quantitated as described in Materials and Methods. Although exact percentages varied between duplicate experiments, the overall trend of changes in the distribution of radiolabel was very consistent. The complete 30 hour experiment was repeated three times and the 0, 2, 8, and 16 h time points were repeated in eight separate experiments. Figures 3A and B summarize the results of the three complete 30 h experiments using LT. TNF experiments showed similar results (data not shown).

The change in radiolabel distribution between the supernatant and cell pellet then was further analyzed for changes in each individual lipid component. Radioactivity in individual lipids was quantitated as a percentage of total radiolabel for each culture assayed (supernatant + cell pellet extracts). Control and LT-treated values from parallel cultures for each of the nine identified lipid components were compared directly at 0, 2, 8, 16, and 30 h. Changes in the percentage of total culture radiolabel over time for the four lipids—PE, AA, NL, and PI—showing the greatest effect are diagrammed in Figure 3A. The percentage of total radiolabel in PE from LT-treated cell pellets decreased linearly from 28% at 0 h to 7% at 30 h. Thus, approximately 75% of the ³H-AA radiolabel originally

incorporated into PE was lost through deacylation over the 30 h period. In contrast, control (untreated) cell pellets showed no deacylation of PE throughout the 30 h experiment. In the cell pellet fraction, both AA and NL showed no change in radioactivity between controls and LT-treated cultures. However, the percent of total radiolabel present in the LT-treated supernatant fraction running as free AA increased from 0 to 24%, whereas control supernatant-free AA plateaued at 4%. Similarly, the percent of total radiolabel in the LT-treated supernatant fraction of NL increased from 0 to 11%, whereas control supernatant NL plateaued at 3%. Phosphatidylinositol did show a loss of radiolabel in LT-treated cell extracts; however, due to the low levels of radiolabel incorporated into PI (7% of total radiolabel), this change is difficult to document consistently using this method of analysis. Figure 2B diagrams the changes in percentage of total radiolabel for the other five radiolabelled lipids assayed. Although slight variations in the percentage of radiolabel did occur, these changes did not appear to be significantly different in control and LT-treated cultures.

Inhibitors of phospholipase A₂ block LT- and TNF-induced cytotoxicity

The PLase inhibitors—quinacrine, hydrocortisone, dexamethasone, and indomethacin—were tested for their effect on LT and TNF-induced cytotoxicity. Quinacrine is a specific inhibitor of PLase A₂ (Matthews et al., 1987; Abe et al., 1988); hydrocortisone and dexamethasone are glucocorticoids that inhibit PLase A₂ (Abe et al., 1988; Blackwell et al., 1980), and indomethacin is an inhibitor of PLase A₂ at high concentrations and of cyclo-oxygenase at low concentrations (Franson et al., 1980). The results of these studies are shown in Table 2.

Ninety-six well microplate cultures of L929 cells were tested using each of these inhibitors added 24 h prior to LT or TNF treatment. Control wells within each of these plates were treated identically to the treated wells, but without the inhibitor present. Triplicates were assayed at the indicated concentrations of each of the inhibitors using various concentrations of LT or TNF. A titration curve from 0–100% cytotoxicity was generated for each concentration of inhibitor from which the amount of LT or TNF necessary to achieve

Fig. 3. **A:** Parallel L929 tube cultures were radiolabelled for 24 h with 3 H-AA. At time zero, half of the cultures were treated with LT or TNF and, at intervals up to 30 h, the lipids were extracted, separated by two-dimensional TLC, visualized, and quantitated for radiolabel by scraping spots and liquid scintillation counting as described in Materials and Methods. Supernatant and cellular lipids were assayed separately, and then total radioactivity for each test and control culture was determined by adding supernatant and cellular radioactive counts. Radioactivity in individual lipid components was quantitated as a percentage of total radiolabel for each tube culture assayed (supernatant + cell pellet extracts). Control and LT-treated values from parallel cultures for each of the nine identified lipid components in

both the supernatant and the cell pellet were compared directly at 0, 2, 8, 16, and 30 h. The changes in percentage of total culture radiolabel over time for the four lipids—PE, AA, NL, and PI—showing the greatest effect are shown. **B:** Changes in the percentage of total culture radiolabel over time for the five lipids—PC, PS, EP, LPC, and LPE/SPH—showing little or no effect. The complete 30 h experiment was repeated three times, and the 0, 2, 8, and 16 h time points were repeated in eight separate experiments. **A** and **B** together represent the compiled data (average and standard deviation) for the three complete 30 h experiments using LT. TNF experiments showed similar results (data not shown).

Fig. 3B.

50% lysis of the cell culture could be calculated. Each titration curve was generated from the average results of at least three separate experiments. For each inhibitor, the amount of LT necessary to achieve 50% lysis of the culture alone was between 1 and 2 pg/ml, whereas

for TNF 50%, lysis was achieved with 8–9 pg/ml of the recombinant protein. Each of the inhibitors was found to be significantly cytotoxic alone, at concentrations above those used. The highest dose of each inhibitor shown in the table was just below the level causing

TABLE 2. Phospholipase inhibitor studies¹

Inhibitor	Concentration of inhibitor (μM)	LT required for 50% cell lysis (pg/ml)	TNF required for 50% cell lysis (pg/ml)
Quinacrine	5	625.0	357.1
	1	15.2	33.3
	0.5	3.8	11.6
	0	1.7	8.3
Hydrocortisone	1	58.8	
	0.5	15.2	
	0.1	2.2	
	0	1.5	
Dexamethasone	5	11.1	
	0.5	9.1	
	0	1.6	
Indomethacin	1	5.9	
	0.1	4.5	
	0	1.4	

¹The effects of four inhibitors of PLase A₂ activation on LT- and TNF-induced lysis of L929 cells were examined using the microtiter plate technique described by Yamamoto et al. (1985). Briefly, monolayers of nondividing L929 cells were established in 96-well microtiter plates (2 × 10⁴ cells per 100 μl medium per well) and incubated in 5% CO₂, 95% air at 37°C for 24 h. Various dilutions of quinacrine hydrochloride, hydrocortisone, dexamethasone, and indomethacin (Sigma Chemical Co.), up to the maximum nonlethal level, were added to triplicate culture wells. Cultures then were treated with serial dilutions of LT or TNF. Controls consisted of triplicate culture wells treated with each concentration of LT or TNF alone and with each concentration of inhibitor alone. The microplates then were incubated at 37°C in 5% CO₂ for 16–24 h. Cell destruction was quantitated by two independent methods (see Materials and Methods for details). Percent viability was calculated as: [(Mean absorbance of test triplicates)/(Mean absorbance of untreated control triplicates)] × 100. Experiments were repeated at least three times for each concentration of LT, TNF, and inhibitor tested. Titration curves were generated and used to calculate the reported concentrations of LT or TNF required for 50% lysis of the cell cultures.

cytotoxicity alone, with the exception of dexamethasone, which was used at its maximum solubility.

Each of the inhibitors caused a significant increase in the concentration of LT or TNF required to achieve 50% cell lysis, and they are listed in the order of their efficacy. The most significant effect was seen with quinacrine, which caused a 400-fold shift in dose for LT and a 40-fold shift in dose for TNF to achieve 50% lysis. Indomethacin and dexamethasone were least effective, but showed a fourfold to sixfold shift in dose response at their maximal inhibitory dosage. Hydrocortisone was less effective than quinacrine in increasing the dose of LT required for 50% lysis, but similar to the effect of quinacrine in inhibiting TNF. In preliminary studies using ME180 human carcinoma cells as the target for LT or TNF cytolysis, we have shown that quinacrine has an equal or greater effect (unpublished observation).

To verify that the inhibitor was actually blocking the action of the PLase(s), studies using prelabelled L929 and ME180 cell cultures were performed. Cells were allowed to incorporate the ¹⁴C arachidonic acid for 24 h prior to LT treatment. Four hours prior to LT treatment, quinacrine was added to test cultures. Both L929 and ME180 cells released approximately 50% of the radiolabel into the media by 16 h after LT treatment (Table 3). In contrast, control untreated cultures released 9% and 23% of the total radiolabel, respectively. However, in the presence of quinacrine, the release of radiolabel to the media was decreased significantly for both LT-treated and untreated cultures. Taken together, these data indicate that there is a strong correlation between the action of PLase-mediated arachidonic acid release and LT/TNF-induced cytotoxicity.

TABLE 3. Effect of quinacrine on radiolabel release as percent of total CPM released to the supernatant after 16 h¹

Cell Type	Without quinacrine		With quinacrine	
	No LT	+ LT	No LT	+ LT
L929	9.0 ± 2.7	42.3 ± 5.6	6.2 ± 2.3	24.7 ± 1.1
ME180	23.2 ± 6.3	57.6 ± 7.0	11.2 ± 1.9	33.7 ± 6.1

¹ME180 and L929 cellular phospholipids (2 × 10⁶ cells/culture) were prelabeled with *AA for 20 h as described in Materials and Methods. Where indicated, quinacrine (5 μM final concentration) was added during the last 4 h of labeling, so that the cells received a 4 h quinacrine pretreatment prior to LT addition. The shorter quinacrine pretreatment was necessary because the simultaneous addition of quinacrine at the beginning of the 24 h prelabeling drastically reduced the amount of label incorporation into cellular phospholipids; 16 h after the addition of LT, control and quinacrine-treated culture supernatants were collected and assayed for released CPM, as described earlier. The mean values and standard deviations reported in the table represent the averages of three independent data points for each of the experimental conditions.

DISCUSSION

Treatment of murine L929 cells with human LT or TNF induces deacylation of membrane phospholipids and, therefore, activation of intracellular phospholipases (PLase). It has been shown that mammalian cells labelled *in situ* with ¹⁴C arachidonic acid (*AA) rapidly esterify this fatty acid almost entirely to the second carbon of membrane PL and a small amount into sphingolipids. Analysis of lipids from L929 cells labelled with *AA in two-dimensional TLC revealed that the label was distributed 31% into PC, 28% into PE, 7% into PI, 12% into neutral lipids (monoacylglycerol, diacylglycerol, triacylglycerol, etc.), and 12% into sphingomyelin or the comigrating lysophosphatidylethanolamine, respectively. The remaining 10% of label is distributed among minor components (less than 5% for each), such as ethanolamine plasmalogen, lysophosphatidylcholine, phosphatidylserine, and free arachidonic acid (AA). Deacylation of AA from PL can occur by two enzymatic mechanisms: 1) PLase A₂, which hydrolyses the PL into AA and the corresponding lysophospholipid; and 2) PLase C, which hydrolyses the PL into diacylglycerol and the corresponding phosphorylated headgroup. AA is then released by diacylglycerol and monoacylglycerol lipases. Once AA is formed within a cell, it is usually secreted or rapidly metabolised via cyclo-oxygenase or lipo-oxygenase pathways into highly active compounds (i.e., prostaglandins, thromboxanes, leukotrienes) collectively termed *eicosanoids*. Our studies reveal that LT- and TNF-treated cells, whose PL are labelled with *AA, release radiolabelled products, which arise by activation of PLase(s) and deacylation of specific PL.

Phospholipase activation begins soon after LT or TNF interaction as evidenced by the elevated levels of radiolabelled products released into the supernatant by 4 h after LT or TNF treatment. Release of these products continues at a steady rate, increasing slightly once cell destruction begins at 6–8 h. During the time required for complete destruction of the culture, about 50% of the radiolabel is released to the media. More than one third of these counts are released as of 8 h, whereas only about 10% of the cells have been killed at that time. At 10 h, more than half of the final released counts are present in the media, yet only 30% of the culture has died. Initial characterization studies of released products indicates that they are predominantly

free arachidonic acid, eicosanoids (e.g., prostaglandin E_2 , (PGE_2)), and some neutral lipids (predominantly triacylglycerol). The presence of radiolabelled triacylglycerol indicates the re-esterification of some of the radiolabelled arachidonic acid released by PLase activation. Small amounts of other lipids, perhaps due to membrane debris from lysed cells, are also present in the supernatant extract. PLase activation, and thus released radiolabelled products, continue to accumulate in a linear fashion even to 30 h, when 99% of the cells have been killed.

LT- or TNF-induced PLases deacylate specific membrane PL in the murine L929 cell. The released radiolabelled products mentioned above are derived from the cellular PL, which start to disappear as the radiolabelled products in the supernatant begin to be detected. These products are derived predominantly from PE, as the decrease in intracellular radiolabelled PE correlates with the quantity and rate of product formation. This relationship was determined by quantitative studies of autoradiographs of the PL extracts from LT- or TNF-treated and untreated cells separated by two-dimensional TLC. The majority of lipids retain a constant amount of radiolabel when LT- or TNF-treated extracts are compared with untreated control extracts. PE is a notable exception, losing 75% of the radiolabel originally incorporated into this PL, which also represents 28% of the total cellular label. PI also showed some deacylation, although, due to the low amount of radiolabel incorporated originally, it is difficult to determine if this radiolabel loss is significant. It should be noted that, of the cellular PL, PE and PI are the richest sources of AA (greatest amount of AA per amount of PL) as determined by comparison of radiolabelled AA incorporation by each PL with inorganic phosphate quantitation of each PL. However, PI stores only a small amount of AA relative to PC and PE, because it comprises only 4% compared with 54% and 18% of the total cellular PL, respectively.

LT or TNF treatment in L929 cells mobilizes large amounts of AA from specific membrane stores of PE. It should be mentioned here that membrane PE and PI are selectively deacylated by PLase(s) in other cells and tissues in response to a variety of stimuli (Johnson and Hoffman, 1985). This deacylation response mobilizes intracellular stores of AA for eicosanoid production. The mobilization of AA for eicosanoid production may also be occurring in the L929 cell, as preliminary experiments indicate that approximately half of the released AA has been metabolized to PGE_2 . Although these studies clearly indicate that PLase(s) are activated in these cells, we cannot at this time determine whether PLase A_2 or PLase C has been activated. The inhibitor studies support the activation of PLase A_2 , but cannot exclude the involvement of PLase C. However, this question is currently under active investigation. PLase activation and the subsequent deacylation of membrane PL is an important step in the destructive reaction. Inhibitors of PLase(s) suppressed the destructive effects of both LT and TNF *in vitro*. Quinacrine was the most effective inhibitor of cell destruction, followed by hydrocortisone, dexamethasone, and indomethacin. Other investigators have also found that these inhibitors block TNF-induced cytotoxicity, with even more potent effects (close to 100% inhibition of

cytotoxicity) (Abe et al., 1988; Tsujimoto et al., 1988). These inhibitor studies rule out the possibility that PLase activation occurs after cell destruction as a result of death. Rather, PLase activation is required for cell destruction, as inhibition of PLase blocks cytotoxicity significantly.

Activation of PLases is an intracellular event common to the action of hormones, growth factors, and activating agents on cells and tissues (Sheir and Durkin, 1982; Sheir, 1980). It is likely that this event may also be a common step in the mechanisms by which LT and TNF induce their wide range of effects on cells (Clark et al., 1988; Neale et al., 1988; Godfrey et al., 1987; Suffys et al., 1988). Transformed cells may be killed because of some modification in the ability of these cells to control PLase action or the products it generates. PLase activity induced by LT or TNF continues unabated through at least 30 h even though the majority of cells (approximately 95%) are dead as of 16 h. The induced activity (indicated by the release of AA) continues in a linear fashion and is not shut off even after cell death occurs. This suggests an inability of these cells to control PLase activation, and the release of too much AA and/or other products may overload the normal control mechanisms and ultimately lead to cell death. The manner in which LT and TNF cause this PLase activation probably involves a cascade of intracellular signalling events. Thus, there may not be a one-to-one relationship between the dosage of LT or TNF and the degree of PLase activation. However, at some threshold level of PLase activity, the cell may be unable to control the lethal products or processes resulting from that activity.

PLase(s) and/or products generated by PLase-induced PL deacylation must have a role in the destructive reaction. Because the biochemistry of PL deacylation and AA metabolism are well known, we can predict products generated by these reactions that could have a role in LT-induced cell destruction. Arachidonic acid itself can function as a calcium ionophore (Beaumier et al., 1987; Wolf et al., 1986), causing the release of calcium ion from intracellular stores. Therefore, free AA may function as a second messenger that signals a subsequent lethal reaction. Lysophospholipids, generated by PLase A_2 action on PL, are detergent-like and membrane disruptive. Preliminary inorganic phosphate quantitation studies at 16 h showed a slight increase (approximately 3%) in the amount of LPE/SPH component. This may accumulate at key intracellular sites disrupting cellular function. AA can be further metabolized into one or more eicosanoids (i.e., prostaglandins, thromboxanes, leukotrienes) via the cyclo- or lipo-oxygenase pathways. As mentioned previously, about half of the released radiolabelled AA was found to be metabolized into PGE_2 in preliminary experiments. Malondialdehyde is a toxic byproduct of the cyclo-oxygenase pathway. Matthews et al. (1987) reported an increase in this product when cells were treated with TNF; however, we were unable to detect malondialdehyde in preliminary experiments using both LT and TNF. Active oxygen radicals are lethal byproducts that can be generated by either the cyclo- or lipo-oxygenase pathways. Additional studies are necessary to determine if any or all of these materials are involved in the destructive reaction; however, it has recently been reported that radical scavengers can in-

hibit in vitro destruction of L929 cells mediated by human TNF (Matthews et al., 1987). In addition, we have preliminary data to indicate that LT-induced destruction of L929 cells is also blocked by certain radical scavengers in vitro. Wong and Goeddel (1988) have found that the sensitivity or resistance of different transformed cell lines correlates with the ability of these cells to increase their production of manganous superoxide dismutase (MnSOD) mRNA upon treatment with TNF. MnSOD scavenges potentially toxic superoxide radicals produced in the mitochondria. These data suggest that free radicals produced perhaps by metabolism of AA could be the ultimate cause of cell destruction.

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