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Lymphocyte *In Vitro* Cytotoxicity: Mechanism of Human Lymphotoxin-Induced Target Cell Destruction¹

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These *in vitro* studies were conducted in an attempt to elucidate the mechanism of how cell-free supernatant fluids obtained from PHA-stimulated human lymphocytes cause destruction of cells. The undiluted supernatant fluids with high activity exerted a nonspecific cytotoxic effect on many different continuous cell lines. However, upon dilution, a wide spectrum of cell sensitivities was observed. These studies suggest human lymphotoxin acts by first absorbing to receptors on the target cell plasma membrane. The next effect is shut-down of cellular DNA synthesis, followed later by a decrease in cell numbers and finally, cellular destruction. Once sufficient LT has bound to the target cell surface, the cytopathic effect is irreversible. A role for LT in lymphocyte-mediated tissue destruction is discussed.

INTRODUCTION

Immune lymphoid cells from experimental animals cause destruction of donor target cells *in vitro* (1-4), with the necessary first step in the destructive reaction being contact of the immune aggressor cell with the target cell (5, 6). This cell destruction is usually highly specific (1-3), but recent evidence suggests nonspecific destruction can also occur (7). Target cell destruction can also occur *in vitro*, when lymphoid cells obtained from animals immunized with unrelated soluble proteins are cultured with the immunogen and allogeneic or syngeneic target cells (8-10). Initiation of these reactions is highly specific for the antigen employed, but target cell destruction is nonspecific and apparently does not require contact between the antigen-activated lymphoid cell and the target cell (10-13). Nonimmune aggressor lymphoid cells can induce a cytolytic reaction when incubated *in vitro* with target cells in the presence of various agents which are known to cause cellular aggregation and/or lymphocyte transformation³ (14-16). Depending on the system, contact

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³ This refers to the conversion of a small lymphocyte from a resting cell, to a large biosynthetically active blast cell (14-16).

between the nonimmune aggressor lymphoid cell and target cell may or may not be required. However, target cell destruction occurs by a nonspecific mechanism (17, 18). The relationship of these systems to one another and the mechanism of how cell destruction occurs is still not known. However, in most instances it appears that lymphoid-target cell contact *per se* is not sufficient to cause the cellular destruction (12, 19–21), but an active lymphocyte process independent of DNA synthesis (22–24) involving energy metabolism and perhaps protein biosynthesis is required (21, 25–27). There is no satisfactory explanation as yet for the actual mechanism of how destruction occurs. However, there are at least three possibilities which have been suggested: (a) interaction of toxic membrane sites on the aggressor and target cell (allogeneic inhibition) (28); (b) release, by the activated aggressor cell, of complement or some component which, in the presence of antibody on the target cell surface, causes lysis (29); and (c) release of a nonspecific cell toxin by the activated lymphocyte (30). This communication deals with information relevant to the latter proposal.

It has been demonstrated that immune lymphocytes can be specifically stimulated with cellular or soluble antigens, or nonimmune lymphocytes can be nonspecifically activated with mitogens, to secrete a variety of cell-free soluble effector molecules (31). One of these substances is a nonspecific cell toxin termed "lymphotoxin (LT)." Secretion of LT by the PHA-activated human lymphocyte *in vitro* requires active cellular protein synthesis and energy metabolism (32). Lymphotoxin secreted *in vitro* by PHA-activated human (33) and mouse (34) lymphocytes are unrelated proteins, nonantibody in nature, with molecular weights between 85,000 and 150,000 daltons. Our previous studies revealed that LT secreted by PHA-activated mouse lymphocytes *in vitro* induces nonspecific cytolysis of a variety of mammalian and nonmammalian cells (18). The present report is an examination of the mechanism of how LT secreted by PHA-activated human lymphocytes caused *in vitro* cytolysis of various mammalian cells.

MATERIALS AND METHODS

The following cells were employed in these studies: L (mouse fibroblast), Chang (human liver), KB (human carcinoma of the nasopharynx), HeLa (human carcinoma of the cervix), WISH (human amnion), and MBK (Moden bovine kidney). They were maintained as monolayer cultures in 16- and 32-oz prescription bottles in an atmosphere of 95% air, 5% CO₂. The culture medium used was RPMI 1640 (kindly supplied by Dr. George Moore, Roswell Park Memorial Institute, Buffalo, NY or purchased from Associated Biomedic Systems, Buffalo, NY, Flow Laboratories, Los Angeles, CA and Grand Island Biological Supply, Grand Island, NY) supplemented with 10% fetal bovine serum (Flow Labs., Los Angeles, CA), penicillin (100 U/ml), streptomycin (100 µg/ml), and mycostatin (50 µg/ml).

Lymphotoxin Preparation

Suspensions of human small lymphocytes were obtained from surgically removed adenoids of normal children as previously described (32). The LT preparations used in these experiments were prepared from adenoid samples 220–235 and 376–385.

We have found a great variation in the capacity of the different commercial lots of 1640 to support maximal LT secretion. Certain batches of powdered media are as good as premade media from commercial suppliers. Lymphocyte suspensions were adjusted to 5×10^6 cells/ml in 1640 Medium and 100-ml vol were added to 32-oz prescription bottles. Activation was effected by the addition of PHA-P (Lots 529236, 55110, 549125, Difco Laboratories, Detroit, MI) to a final concentration of 20 or 30 $\mu\text{g}/\text{ml}$, at the time the suspensions were placed in culture. Each lot of PHA-P was prescreened and the dosage which stimulated the highest levels of LT secretion was selected for that particular lot. In addition, we routinely added 30 $\mu\text{g}/\text{ml}$ of Garamycin (Shering Corp., Bloomfield, NJ) to these cultures. The bottles were sealed and incubated in an atmosphere of 95% air, 5% CO_2 at 37°C . After 5 days, the medium was collected and cleared of cells and debris by centrifugation at 300g and passage through Millipore filters (0.45- μm pore size). The medium was stored at -20°C until use. Since levels of toxicity varied from batch to batch, a number of batches were prepared and screened for LT activity. Several with the highest activity as measured by dilution were selected and used for most of these experiments. The batches chosen caused 50% destruction of target L cell monolayers at dilutions greater than 1:20. Control medium was prepared by culturing lymphoid cells under similar conditions in the absence of PHA. After incubation at 37°C for 5 days, the cultures were cleared of cells and debris, then PHA was added to a final level of 20–30 $\mu\text{g}/\text{ml}$. A second type of control employed was heat-killed lymphocytes (56°C for 30 min) plus PHA cultured as described for active supernatant fractions. Medium prepared in this manner was routinely nontoxic. Occasionally control samples from certain patients have been toxic without stimulation with mitogens. These media were not employed in the present studies.

Cytotoxicity Assay and Dilution Experiments

Medium harvested from the various lymphocyte cultures was tested for LT activity in the following manner: monolayers of the aforementioned indicator cell lines were established in tube cultures ($16 \times 125\text{mm}$) 24 hr prior to use at a density of 50,000–75,000 cells/tube in 1.0 ml medium. Each tube was examined microscopically and selected for monolayer uniformity. The medium was then discarded, and 1.0 ml of test or control medium was added. The tubes were sealed, placed in racks at a five degree slant and examined every 12 hr under the $100\times$ objective of the light microscope. Viability in certain experiments is presented as direct counts per minute (cpm) incorporated into cellular protein, calculated by the following formula: $\text{cpm exp/cpm control} \times 100$. In all experiments, $^{14}\text{C-AA}$ labeling corresponded to microscopic evaluation of cell destruction. To determine the actual number of viable cells in these cultures, tubes were removed at various intervals and the culture media decanted and saved. Cells still attached to the glass were removed by treatment with 0.1 ml of a 0.05% trypsin–0.01 M EDTA solution for 5–10 min. The original medium and trypsin-suspended monolayer cells from each tube culture were then recombined and sedimented by centrifugation (200g for 10 min). The resulting cell pellet was resuspended in a saline solution containing 0.1% Eosin Y. Total and viable cell counts were then performed under the light microscope in a Neubauer chamber. There was little difference between viable and total cell counts.

Kinetics of Target Cell Destruction

Indicator L cell tube cultures were established 24 hr prior to use at 200,000 cells/tube in 1.0 ml 1640. At time zero, the medium was replaced with 1.0 ml LT or control medium. Duplicate tubes of control and test cultures were then assessed at various intervals over the next 72 hr for levels of protein synthesis, DNA synthesis, and total remaining viable cells. To assay DNA synthesis, the cultures were centrifuged at 100g for 2 min and the cell-free medium discarded. One milliliter of 1640 containing 2 μ Ci tritiated thymidine, specific activity 6.7 Ci/mmol (New England Nuclear) was added. The tubes were sealed and reincubated for 30 min. Incorporation was stopped by adding 1.0 ml of sodium dodecyl sulfate solution (SDS). The nucleic acid was extracted with SDS-phenol as previously described (32), solubilized in Nuclear Chicago Solubilizer, and counted in a Beckman LS-233 Liquid Scintillation Counter. Tube cultures were centrifuged as described above, and the cell-free medium discarded. One milliliter of protein labeling medium containing 1.0 μ Ci 14 C-amino acid hydrolyzate was added and the tubes incubated 20 min. Incorporation was stopped by adding 1.0 ml of 0.1 M KOH and the protein was extracted as described previously (33). The resulting protein precipitate was caught on Millipore filters and counted in a Beckman CPM-100 Liquid Scintillation Counter. Total viable cells/tube were determined as described in the previous section. All procedures in this series of experiments were performed in a 37°C warm room.

Inhibitor Experiments

One milliliter nondiluted LT and control medium were added to tube cultures containing 75,000 indicator cells with and without the addition of 0.05 ml of 0.05 M 2,4-dinitrophenol (DNP, an inhibitor of energy metabolism) in 0.15 M NaCl. The final concentration of DNP was 2.5×10^{-3} M. Cultures were removed at various time intervals, rinsed twice with 2.0 ml warm 1640, and replaced with 1.0 ml fresh medium. The tubes were reincubated for 24 hr, and then viability was assayed with 14 C amino acids as previously described. All manipulations were performed at 37°C.

Binding Experiments

Ten 8-oz prescription bottles containing L, HeLa, MDBK, and Chang cell monolayers at a uniform density of 10×10^6 cells/bottle were separated into two groups. Then two bottles of cells were washed four times with 50 ml warm 0.15 M NaCl and allowed to drain completely. Ten milliliters of LT medium was added to one bottle, and 10 ml of control medium added to the other. The bottles were sealed and gently rocked from side to side on a rocker platform. After 10 min, the media were removed and the LT medium was absorbed on four successive monolayers in an identical fashion. After the last incubation, the media were collected, cleared of cells and debris by centrifugation at 300g for 10 min, filtered, and serial dilutions tested for cytotoxicity on L cells. The level of cytotoxicity of test and control samples was compared with the original batch of HLT, which had been exposed to identical treatment with bottles containing no cell monolayer or not treated at all.

Preparation for light microscopy. Coverslip cultures of target L cells (1.5×10^6 cells) were established in milk dilution bottles and treated with LT and control

medium for 24–48 hr. At each time interval, a coverslip was removed, air dried, fixed, and stained by the method of May–Grünwald–Giemsa. Stained coverslips were examined with the light microscope and then photographed with a Ziess autophotomat mounted on an Olympus microscope.

RESULTS

The effect of test and control medium on target cell protein synthesis and total cell numbers. Target L cells exposed to undiluted LT-containing medium for 48 hr showed a marked decrease in cell numbers and incorporation of ^{14}C amino acids into protein (Table 1). The amount of cellular debris present in the culture medium increased progressively over the 24- to 48-hr period. Vital stains and amino acid incorporation studies revealed that this material was composed of only dead cells and debris. In contrast, cells treated with control preparations demonstrated normal levels of protein synthesis and cell numbers. Several types of controls were employed in these studies, i.e., (a) normal culture medium, (b) medium from unstimulated cells to which PHA was added after incubation, and (c) medium from cultures to which was added heat-killed (56°C –30 min) lymphoid cells and PHA.

Morphologic alterations of LT-treated L cells—Light microscopy. The time required to cause cell destruction varied from one lot of LT to another. Several studies suggested that this was apparently related to the concentration of LT in the particular medium and the number of target cells employed in the test cultures. For example, in a given concentration of LT, small numbers of target cells were destroyed rapidly (within a few hours), while larger numbers of cells required 48–76 hr. Rapid cell destruction was observed in high-activity samples obtained from various steps in purification procedures, which will be reported in another article. The major morphologic alterations induced by LT on large numbers of cells in monolayer culture are seen in Fig. 1A, B, and C. At the end of 14 hr, distinct cell borders could not be visualized (Fig. 1C), and membrane blebbing was apparent. After 24 hr, vacuoles were present in the cytoplasm, cells borders were irregular (Fig. 1B), and destruction of large numbers of cells was evident. The process of target cell disintegration continued until only a limited amount of cytoplasm sur-

TABLE 1
THE EFFECT OF TEST AND CONTROL MEDIA ON TARGET CELL
PROTEIN SYNTHESIS AND CELL VIABILITY^a

Medium tested	Counts per minute incorporated into target cell protein	Number of viable cells attached to glass and in suspension
PHA-activated lymphocytes	236 $\pm$ 100%	1,000 $\pm$ 100%
MEM control	7,100 $\pm$ 5%	270,000 $\pm$ 8%
Nonactivated lymphocytes + PHA	6,850 $\pm$ 10%	250,000 $\pm$ 8%
Heat-killed lymphocytes + PHA	6,899 $\pm$ 9%	255,000 $\pm$ 10%

^a Tube cultures of target cells were exposed for 48 hr to various cell-free media. Parallel cultures were then pulse labeled with ^{14}C amino acids and total viable cell counts were performed as described in text.

FIG. 1. The appearance of mouse L cells *in vitro* after treatment with cell-free supernatants from control and PHA-stimulated human lymphocytes. A. Twenty-four-hour control treated with medium from unstimulated lymphocytes with PHA added after incubation. B. Identical monolayer 24 hr after treatment with medium from a PHA-activated lymphocyte culture. C. Higher power of 14-hr LT-treated L cells.

rounding a pyknotic nucleus remained which finally detached and could be detected in the culture medium. However, cell destruction was not synchronized, and cells at many different stages of destruction could be visualized in the same monolayer. These changes were not evident in control L cultures treated with normal medium or medium from unstimulated cultures observed during the same time intervals (Fig. 1A). Cells in high activity LT medium did not exhibit distinct visible cytopathic changes, as described here, but underwent a rapid destruction resembling cytolysis.

Binding and Temperature Dependence of HLT on Target Cells

We wished to determine if LT released by mitogen-activated lymphocytes underwent binding to the target cells. Volumes of LT-containing and control media were sequentially adsorbed with confluent target cell monolayers. After this treatment, media were collected and various dilutions tested for toxicity on L cells. Positive control preparations consisted of the same batch of LT, which was unadsorbed or adsorbed with empty bottles. These experiments were repeated a total of three separate times with essentially identical results in each repetition. The results of Expt 2, in which 10-ml aliquots of HLT medium were sequentially adsorbed on five monolayers in 8-oz prescription bottles (20×10^6 cells/bottle) are shown in Fig. 2. It is clear that a significant amount of HLT activity was lost during the repeated contact with the various target cells. To insure that this was adsorption and not inactivation/degradation, the same experiments were performed on heat killed (56°C , 1 hr) cells with essentially identical results.

Previous studies revealed that mouse LT-induced target cytolysis is a temperature-dependent process (18). Similar experiments were performed on L cell tube

FIG. 2. Adsorption of LT *in vitro* to monolayers of target cells from several animal species. Lymphotoxin-containing media were exposed to monolayers of various cells. The levels of LT remaining in the media was ascertained by testing dilutions of adsorbed and unadsorbed media on fresh sensitive target L cells.

FIG. 3. Irreversibility of LT-induced L cell destruction. Target L cells were exposed to LT at 4°C for various time intervals, then washed and further incubated in fresh media. Cell destruction was assayed after 48 hr of incubation at 37°C.

cultures exposed to HLT. When L cell tube cultures were incubated in the presence of LT-containing medium at 4°C, cytolysis was not inhibited but was greatly delayed. In the next series of experiments, tube cultures of L cells, at 4°C, were exposed to HLT and control medium. Individual tubes were removed at various times, washed gently with 1640 medium, and reincubated at 37°C in fresh complete medium. Viability was assayed after 48 hr of additional incubation. The data shown in Fig. 3 reveals that enough LT bound to the L cells at 4°C to cause lysis when washed cells were incubated at 37°C.

The Effect of LT on Macromolecular Synthesis and Cell Numbers

It seemed possible that LT might induce destruction by inhibiting target cell macromolecular biosynthesis. To test this possibility, tube cultures of L cells were established (200,000 cells in 1-ml culture) and treated with LT and control medium. At intervals over a 48-hr period, the various test and control tubes were pulsed for protein synthesis, DNA synthesis and assessed for the total number of remaining viable cells. These experiments were performed in a 37°C warm room and were repeated a total of four times with several different batches of toxic medium. An example of the results of this experiment is shown in Fig. 4A-C. It can be seen in 4A, that cell numbers began declining at 12 hr and continued rapidly downward to 24 hr, with a small number of cells remaining until 48 hr. Protein synthesis began declining after 10-12 hr and then paralleled the disappearance of intact cells (Fig. 4B). However, the earliest detectable change was in cellular DNA synthesis, which was affected at 8 hr, 4 hr before any reduction in cell numbers (Fig. 4C).

FIGURE 4A.

FIGURE 4B, C.

FIG. 4. The effect of LT on macromolecular synthesis and total cell number in parallel cultures of L cells. A. Tube cultures of L cells were treated with LT and control media. Then the number of remaining cells was assayed at different time intervals as described in Materials and Methods. B. The effect of LT on the incorporation of ^{14}C amino acid into L cell protein. Tube cultures of L cells were exposed to LT and control media. Then at intervals, samples were removed and the level of protein synthesis determined. C. The effect of LT on the incorporation of tritiated thymidine into target L cell nucleic acid. Tube cultures of cells were exposed to LT and control media. Then at intervals, a tube was removed and the level of nucleic acid synthesis determined as described in Materials and Methods.

The reduction of cell numbers and macromolecular synthesis was highly reproducible for each individual batch of LT. In general, the more potent the batch, the more rapid and dramatic the drop in the numbers of viable cells and macromolecular synthesis. The points shown are representative of duplicates at each point. There was less than 10% variation between experiments performed with one batch of LT.

FIG. 5. Variation in the sensitivity of various continuous cell lines to different dilutions of LT-containing media. Each cell line was established as a monolayer at identical numbers of cells/tube and exposed to LT and control media for 48 hr. The number of viable cells/tube was determined as described in text.

Concentration Dependence of LT-induced Cell Destruction

Experiments were designed to investigate the effect of different concentrations of LT on various continuous cell lines. Parallel tube cultures of target cells were established and treated with various dilutions of HLT and control medium. After 48-hr incubation, they were assayed for viability by ^{14}C amino acid incorporation. The data in Fig. 5 show the results of such an experiment, utilizing L, HeLa, Chang, MBK, KB, and WISH cell lines. It is clear that almost all continuous cell lines tested were susceptible to the undiluted LT medium. However, a spectrum of sensitivities is evident in parallel cultures exposed to lower dilutions of LT.

Mechanism of Cellular Resistance to LT-Induced Destruction

It seemed possible that the variable resistance to levels of LT could be based on processes linked to cellular metabolism and biosynthesis. Experiments were designed in an attempt to test this premise. Various monolayers were treated with LT medium and $2.5 \times 10^{-3}\text{M}$ 2,4-DNP for 2, 4, and 6 hr, after which they were washed thoroughly and reincubated with fresh control medium. After 24-hr further incubation at 37°C , viability was assayed by pulse labeling for 10 min with $0.5 \mu\text{Ci/ml}$ ^{14}C amino acids. Figure 6A shows the results of this treatment on target L cells. The L cell was rapidly destroyed by LT in the presence of DNP, evidenced by complete monolayer cytolysis after only a 2-hr exposure. The data in Fig. 6B shows that HeLa cell destruction is also dramatically accelerated. However, they were not destroyed as rapidly as L cells. Similar treatment of WISH cells (Fig. 6C) revealed that while cytolysis was accelerated, it leveled off after 4-hr exposure to 50% reduction in viability. The most resistant cell, MBK, became fully sensitive to LT in the presence of 2,4-DNP (Fig. 6D). In contrast, the Chang cell was apparently

TABLE 2
THE EFFECT OF METABOLIC INHIBITORS ON L CELL PROTEIN SYNTHESIS^a

Tubes		Counts per minute incorporated into L cell protein
1	No treatment	4520
2	No treatment	4950
3	DNP, 2.5×10^{-3} M	620
4	DNP, 5.0×10^{-3} M	505
5	Puromycin, 50 μ g/ml	3270
6	Cycloheximide, 50 μ g/ml	895

^a Tube cultures of L cells were established at 75,000 cells/tube 24 hr prior to use. At time zero, 0.5 μ Ci/ml of a ¹⁴C-amino acid mixture, containing the various inhibitors, was added to each tube. After 10 min of incubation, the amount of amino acids incorporated into cellular protein was determined as described in Materials and Methods.

not rendered more sensitive to LT-induced cytolysis by this treatment (Fig. 6E). Similar, but less dramatic results were obtained when puromycin or cycloheximide was substituted for DNP in these studies.

In order to verify that the inhibitors employed did, in fact, inhibit cellular biosynthesis, the following controls were performed: L cell tube cultures at 75,000/tube were pulse labeled for 10 min with 0.5 μ Ci/ml ¹⁴C amino acids with and without DNP, puromycin, and cycloheximide. The inhibitors were added along with the labeling medium. The data in Table 2 represent the CPM incorporated into extractable L cell protein. These results show that in as little as 10 min, 2.5×10^{-3} M DNP will inhibit protein synthesis by approximately 80%. It is of interest to note that puromycin is not nearly as quick in this as DNP or cycloheximide. The acceleration of cytolysis of certain cell lines by an inhibitor probably depends on how rapidly and to what extent the inhibitor stops metabolic processes.

DISCUSSION

Macromolecular synthesis and cell viability rapidly decrease in LT-treated target monolayer cultures. The time required for complete cell destruction appears to depend on the relative concentration of LT present in the particular batch of medium tested. We observed with high activity medium that target L cells were destroyed in a few hours, whereas, destruction was delayed with lower activity medium or upon dilution of high-activity medium. While there is variation in the timing and degree of cell destruction induced by one lot of LT when compared to a different lot, repeated testing of dilutions of individual lots revealed that the degree of destruction is relatively constant within a lot. Reduction in the cytotoxicity of a particular LT lot did appear after storage of the material for more than a week at 4°C, and after 20 weeks at -20°C.

The present data suggest that LT released by PHA-stimulated human lymphocytes probably causes cell destruction by effects on the cell plasma membrane. The first step in this destructive reaction is the attachment of LT to the cell membrane. This is supported by the observations that: (a) target cells could be exposed to LT in the cold, washed thoroughly, and were still destroyed upon subsequent incubation

at 37°C in fresh medium, and (b) absorbing LT medium with various live or non-viable target cells reduced the level of toxic activity. This latter situation substantiates that it is an absorption process and not inactivation of the LT molecule. Once attached to the cell, LT does not apparently act by inhibiting macromolecular synthesis. If the opposite situation had been observed, pretreatment of target cells with inhibitors of macromolecular synthesis would have protected the cell, and destruction, although slowed, would not have occurred at 4°C. In addition, the appearance of LT-treated cells in both the light and electron microscope reveal changes, such as membrane blebbing, suggestive of an action on the plasma membrane. With these results in mind, we feel the early shutdown of DNA synthesis is probably not the primary site of LT action but secondary and most likely due to the effects on the cell membrane. However, it is clear that LT-induced cell destruction is a complex, phenomenon, and there are other possible alternatives.

While LT is nonspecific in high concentration and causes destruction of a wide variety of continuous cells *in vitro*, upon dilution a whole spectrum of sensitivities becomes apparent. All cells tested in the present experiments were affected to some degree in undiluted LT-containing medium. The most resistant cell in these studies was MBK, and the most sensitive, our strain of L cells. In additional experiments, we have noticed differences in sensitivity even among various "strains" of L cells obtained from different laboratories. The present experiments revealed that timing and degree of target cell destruction can be greatly affected by pretreating the cells with the metabolic inhibitor 2,4-DNP. However, the effects on individual cell lines varied, i.e., treatment of MBK, WISH, HeLa, and L cells rendered them more sensitive, while the same treatment of Chang cells had little or no effect. These experiments suggest at least two possible mechanisms of target cell resistance to LT-induced *in vitro* cytolysis: (a) an energy-requiring cell process, perhaps a

FIGURE 6A, B.

FIGURE 6C, D, E.

FIG. 6. The effect of exposing various target cells to LT when they have been metabolically inhibited. Tube cultures of various target cells were exposed to LT and the metabolic inhibitor 2,4-DNP. Samples were removed at intervals, and the cells washed in 1640, fed with fresh medium, and incubated at 37°C. The number of viable cells in each tube culture was assessed after 24 hr. A. The effect on L cells. B. The effect on HeLa cells. C. The effect on WISH cells. D. The effect on MBK cells. E. The effect on Chang cells.

mechanism of repair, and (b) a nonenergy-requiring resistance mechanism, explained perhaps by the absence or masking of LT-sensitive receptor or substrate sites on the cell surface. This latter situation appears to be the case for the nonactivated small lymphocyte, which was not rendered more sensitive to LT-induced lysis by treatment with metabolic inhibitors.

The preceding experiments reveal that the interaction of LT with the target cell is a complex phenomenon. The outcome of this reaction is controlled by a number of factors: First, the concentration of LT present in the immediate area of the target cell; second, the nutritional and metabolic state of the target cell; and third, the kind of target cell employed. These results may explain the negative reports of various authors who have attempted to find evidence of toxic factors active in *in vitro* cellular immune reactions (35, 36). In the case of lymphoid cells obtained from immune animals activated *in vitro* with antigens, it has been calculated that a very small number, perhaps 2–5% of the cells, actually respond to the antigen (37), and from tests in our laboratory, we know that the amounts of LT released into the cell-free medium under these conditions is low and detectable only with sensitive test systems such as the microplate method (38). In situations in which cells are activated with strong activating agents, such as phytohemagglutinin, the activity in the undiluted medium can be detected with a wide variety of target cells, but even then could be missed with a relatively resistant indicator cell, such as the bovine kidney cell.

The observation that LT binds to the target cell may provide new insight into the mechanism of its possible role in *in vitro* lymphocyte-induced target cell destruction. The cell toxin, once released, would presumably be rapidly bound and under normal circumstances never occur free. This would obviously serve as one means of localizing LT once released and insure that it is consumed in the toxic reaction. The specificity in the reaction would presumably then reside in the activation step(s) and physical disposition of the effector molecule. It is envisioned that the magnitude of the tissue destruction induced by the lymphoid cell would be related to the degree of initiation and the amount of LT released.

The present results would predict that not all cells in a complex tissue would be uniformly sensitive to lymphocyte-induced destruction and, therefore, certain elements would be destroyed, while others could persist. It has long been recognized that there is a differential rate of rejection of various tissues and organs (39). This was first attributed to a differential expression of histocompatibility antigens on the cells which comprise that tissue. Thus, cells with high levels of transplantation antigens on their surface would be more easily recognized and thus destroyed before other cells in that tissue expressing less antigen. There may, however, be other possible mechanisms to account for this observation, for small lymphocytes express the highest levels of transplantation antigens on their surface, yet they are highly resistant to lymphocyte-mediated destruction (20). These differences could be explained by the presence of blocking antibodies (40). However, the present studies suggest yet another alternative, namely, the differential resistance to LT.

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