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Binding of Human Lymphotoxin to Target-Cell Membranes and Its Relation to Cell-Mediated Cytodestruction

(cytotoxin/immune reactions)

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ABSTRACT **One of the lymphocyte effector molecules produced *in vitro* by antigen- and mitogen-stimulated lymphocytes is lymphotoxin, which has been proposed to be the cytodestructive mediator in cell-mediated immune reactions. Our *in vitro* findings suggest that lymphotoxin binds to sites on the plasma membrane of sensitive target cells, and this binding represents the earliest detectable effect in cell destruction induced by lymphotoxin. The kinetics of binding are rapid, and the affinity of lymphotoxin for binding sites is strong. Furthermore, cytodestruction can be localized, for lymphotoxin-coated cells cannot release lymphotoxin to affect adjacent ^{51}Cr-labeled, noncoated cells in mixed cultures. This evidence suggests how a nonspecific soluble cytotoxin could cause highly specific localized tissue destruction.**

Studies of the basic mechanism(s) involved in cell-mediated immune reactions have been greatly facilitated by the advent of various *in vitro* models, which may typify *in vivo* phenomena (1–4). Lymphocytes and/or macrophages appear to be the main effectors in these reactions. While the various *in vitro* models cover a broad range of reactions and their relationship to each other is unknown, they do share several common steps. The initial event in all cell-mediated immune reactions is a recognition phenomenon facilitated, in immune lymphocytes, by specific surface receptor(s) (1, 5). These events, in some unknown manner, trigger the second phenomenon, termed activation. Nonimmune lymphocytes can also be activated by interaction with various phytomitogens, e.g., phytohemagglutinin (PHA), with saccharide moieties on the cell membrane. This activation results in conversion of the normally quiescent small lymphocyte to a biosynthetically active and morphologically enlarged blast cell (5, 6). The activated cell functions in cell-mediated immune reactions as the cytodestructive or killer cell. The actual mechanism(s) involved in the cytodestructive reaction is unknown and a subject of controversy.

The same treatment(s) that induce the appearance of the activated lymphocyte also cause *in vitro* synthesis and release of a series of soluble factors termed "lymphocyte effector molecules." These substances, which are relatively undefined chemically, have a wide range of effects on other cells *in vitro* (4, 7). One of these is a cell toxin "lymphotoxin" (8). Human lymphotoxin is a proteinaceous molecule of about 90,000 molecular weight (9, 10). While it is nonspecific in action, we have found that cell strains differ widely in their *in vitro* sensitivities to lymphotoxin (11). This report describes studies that demonstrate the binding of lymphotoxin to target L-cell membrane sites and suggests that this binding may be one of the reasons for the differential cell sensitivity. In addition, a model is proposed to explain how an apparently nonspecific cytotoxin can cause highly localized and specific cell destruction.

Abbreviation: PHA, phytohemagglutinin.

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MATERIALS AND METHODS

Culture of Continuous Cell Lines. Maintenance and culture of the target cells (mouse fibroblast L-929, or L cells) used in these studies have been described (12). Briefly, all cells were cultured in RPMI 1640 medium (Gibco, Grand Island, N.Y.) containing 10% fetal-bovine or calf serum. All cell lines were maintained in monolayer cultures in 8- or 16-oz prescription bottles and passed biweekly. In several studies, we used cell suspensions rather than cell monolayers. These suspensions were prepared from washed monolayer cells treated for 10 min with 0.05% trypsin (1:250; Difco, Detroit, Mich.) in 1 mM EDTA and 150 mM NaCl. Cells were used directly or else incubated for 16 hr at 37° in RPMI 1640 on a Bellco (Vineland, N.J.) rocking platform, containing 20% fetal-bovine serum at a density of 2×10^6 cells per ml.

Lymphocyte Culture and Lymphotoxin Production. We obtained lymphotoxin-containing media by stimulating cultures of human adenoid lymphocytes with PHA, as described (13). Suspensions of human lymphocytes were culture with 20–30 μg/ml of PHA-P (Difco, Detroit, Mich., lot 561827) at a density of 3 to 5×10^6 cells per ml. After incubation at 37° for 5 days, cells and debris were removed by centrifugation. The supernatants were then stored at −20° until used. In some cases, supernatants from several cultures were pooled to form 2-liter batches, e.g., lot numbers B-20 and B-21. The titer or level of lymphotoxin activity in each lot was the dilution that would reduce cell numbers by 50%, as related to control cultures after 48 hr of incubation. Controls consisted of testing medium from unstimulated lymphocyte cultures, fresh medium, or fresh medium plus PHA.

Preparation and Use of Medium Containing Labeled Lymphocyte Products. Lymphocytes prepared and cultured in the usual manner were incubated for 5 days in the presence of 0.5, 0.6, and 1.0 μCi/ml, respectively, of [^{14}C]leucine, [^{14}C]-lysine, and [^{14}C]phenylalanine (Schwartz, Orangeburg, N.Y.). Although lymphotoxin production is dependent upon protein synthesis (14), it is not the only protein produced and secreted by stimulated lymphcytes (7). After incubation, the culture supernatants were cleared of cells and debris, then dialyzed extensively to remove free amino acids, and stored at −20°.

Monolayers of L cells were exposed to ^{14}C-labeled superna-

tant medium for different times. Labeled medium was then aspirated off the tubes, and the remaining cells were washed with saline. Then either fresh culture medium was added, when cell viability was to be ascertained 48 hr later, or the washed cells were dissolved in 100 mM KOH–150 mM saline–10% trichloroacetic acid 1:2:3 (v/v). After samples were heated in a 90° water bath for 10 min, the flocculated precipitate was collected on glass fiber filters (Whatman, Clifton, N.J.), rinsed with cold 10% trichloroacetic acid, and put into scintillation vials with 0.5 ml of NCS (Nuclear Chicago, Chicago, Ill.). 2 hr later 15 ml of scintillation fluid (0.4% Omnifluor in toluene) was added and the samples were counted.

Cytotoxic Assays. Cytotoxic activity of test and control culture medium was determined by several methods: (*i*) viable cell counts in a hemocytometer; (*ii*) total cell counts determined in a Coulter Counter, (*iii*) total cells remaining attached to monolayers in microtiter plates, and (*iv*) ^{51}Cr release. Monolayer tube cultures were washed once with saline and trypsinized until the cells detached. Then Eosin Y, to a final concentration of 0.1%, was added and the dye-excluding cells were enumerated in a Neubauer chamber. When microtiter plate cultures were used, target cell viability was determined by direct counts of cells remaining on the monolayer at the end of 48 hr, as described (15). The number of monolayer cells remaining in tube cultures was also determined by passing trypsinized preparations through the Coulter Counter. In the bystander experiments, L cells were prelabeled with $Na_2{}^{51}CrO_4$ (ICN, Irvine, Calif.), by the procedure described by Perlmann and Perlmann (16). 1 ml of labeled or unlabeled cells was then added to sterile, siliconized screw-capped culture tubes and incubated at 37° in an upright position. After 30 hr, the tubes were removed, and the cells were sedimented by centrifugation at 300 × *g*. The entire supernatant was decanted into test tubes, and the cell pellet and supernatant were counted for 1 min in a manual gamma counter (Baird-Atomic, Los Angeles, Calif).

RESULTS

It appeared to us that even the simplest scheme of lymphotoxin-induced cell lysis must include at least two steps: (*i*) interaction of lymphotoxin with the target-cell membrane, and (*ii*) subsequent biochemical effects that result either directly or indirectly in membrane permeability changes leading to cell lysis. Studies of the complement and animal-venom lytic systems have demonstrated that binding of cytolytic factor(s) to target-cell membranes is preliminary and obligatory to all subsequent events leading finally to cytolysis, and moreover, that binding can be isolated and studied independently of the terminal lytic reactions (17–19). This reasoning, together with previous findings from this laboratory (11), led us to speculate that binding of lymphotoxin to the target-cell membrane may be the first step in lymphotoxin-induced cytodestruction.

Removal of Lymphotoxin Activity from Supernatants Absorbed with L Cells. In initial experiments, we could not remove cytotoxic activity by incubating lymphotoxin-containing medium at 37° with suspensions of L cells freshly treated with trypsin. Consequently, we devised a means of obtaining suspension cells 16 hr after trypsin treatment which did possess the ability to absorb lymphotoxin. Studies on four separate lymphotoxin preparations revealed that the

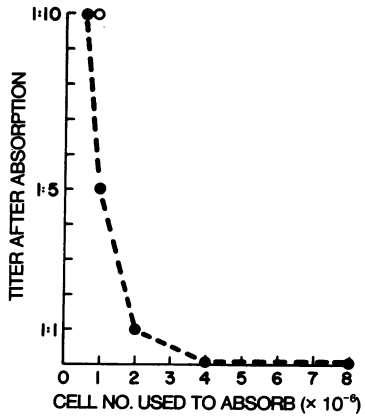

FIG. 1. Removal of lymphotoxin activity by absorption of lymphotoxin-containing medium with various numbers of L cells. Various numbers of L cells were added to 1.0 ml of lymphotoxin-containing B-20 medium in screw-capped tubes. The tubes were incubated for 4 hr at 37° on a rocking platform, cells were removed by centrifugation, and supernatant medium was tested for lymphotoxin activity by titration on target cells in microplates. These experiments were repeated three times with duplicates at each point. This is experiment no. 2, which is typical of all three.

amount of lymphotoxin removed from the supernatant medium was dependent upon three variables: (*i*) the initial activity of the medium; (*ii*) the number of cells per ml used to absorb; and (*iii*) the type of cell used to absorb. The data in Fig. 1 illustrate that the amount of lymphotoxin removed is directly proportional to the number of L cells per ml used.

Since the reduction of lymphotoxin activity in supernatant medium could also be explained by pinocytotic internalization or destruction of lymphotoxin activity by an active cellular process, these experiments were performed at reduced temperature. Results of three separate experiments indicate that the amount of lymphotoxin absorbed was the same at 4°, 15°, 25° and 37° (data not shown). Hence, the loss of activity is probably not due to pinocytosis or active degradation of lymphotoxin, since these processes should be reduced or inhibited at lower temperatures.

Adsorption of Lymphotoxin Activity to Target Cells. The previous experiments demonstrated removal of lymphotoxin activity from supernatants. The next set of experiments was designed to study the adsorption of lymphotoxin to cells in tube-culture monolayers. We exposed glass-adherent monolayers to lymphotoxin medium for various intervals, washed them with minimal disturbance or loss of cells, and incubated them at 37° for 48 hr. The kinetics of absorption under these conditions are shown in Fig. 2. The data indicate that a very short exposure to lymphotoxin medium is effective in reducing the total number of viable cells. Apparently, lymphotoxin adsorption to target cells is very rapid, then reaches a maximum and levels off within a brief period. Furthermore, the extent of cell destruction appears to be directly related to the initial lymphotoxin concentration. It is apparent that periods of exposure to lymphotoxin in excess of 8 hr result in increased cytotoxicity, which is dependent upon time rather than the initial lymphotoxin concentration. These results, when considered in combination with the previous experiments, suggest that lymphotoxin is binding to the target cell, and as a result of this binding, it is being removed from the superna-

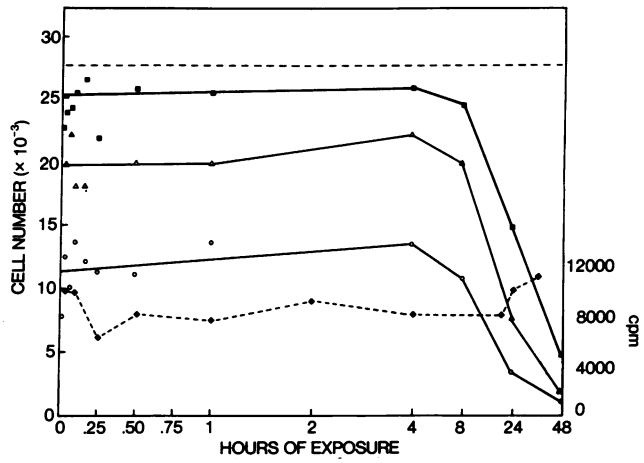

FIG. 2. Kinetics of adsorption of cytotoxic activity from lymphotoxin-containing medium. Tube cultures of L cells were set up at 50,000 cells in 1 ml of culture medium. The culture medium was discarded 20 hr later, and 1-ml aliquots of various dilutions of lymphotoxin (B-21) were put onto the monolayer cultures. The tubes were gassed and sealed; at various times the lymphotoxin-containing medium was decanted, the monolayers were washed twice with isotonic saline, and fresh culture medium was put on. After regassing and sealing, the cells were allowed to incubate the remainder of the 48-hr period, commencing with addition of lymphotoxin-containing medium. After 48 hr, the remaining adherent cells were enumerated on the Coulter Counter. ^{14}C-Labeled lymphocyte medium was used in the same way, except that when it was removed at various times, the monolayers were washed and then dissolved in 1 ml of 100 mM KOH. ^{14}C counts (●- - -●) associated with the monolayer were then determined in a scintillation counter. Dilutions of lymphotoxin: ○, 1:2; △, 1:8; ■, 1:32. *Dashed line*, control.

tant medium. In addition, they support the concept that there may be phases in the cell cycle when there may be a greater sensitivity or resistance to lymphotoxin.

Direct Evidence of Lymphocyte Products Binding to Target Cells. A direct demonstration of lymphocyte products binding to target L cells was provided by incubating monolayers for various times in ^{14}C-labeled lymphotoxin-containing medium. After exposure to labeled medium, the cells were thoroughly washed, and the number of ^{14}C counts associated with target cells was determined (Fig. 2). The binding kinetics of cell-associated counts parallel the adsorption of cytotoxic activity from the previous experiment. Binding of labeled counts appears to be complete by 1 min, with no significant increase after prolonged exposure to the labeled medium. To determine whether the observed counts reflect specific binding of lymphocyte products, we first briefly exposed cell monolayers to unlabeled lymphotoxin medium or nonstimulated lymphocyte medium. Cells were rinsed and then briefly exposed to ^{14}C-labeled lymphotoxin-containing medium. The results reveal that prior exposure of cells to unlabeled lymphotoxin medium reduces the counts that bind to cells upon subsequent exposure to labeled medium, whereas nonstimulated control medium has no effect. These experiments are being repeated, with highly purified, unlabeled lymphotoxin (10) for pretreatment of target cells.

Cellular Site of Lymphotoxin Binding. The rapidity with which lymphotoxin associates with target cells and the temperature insensitivity of lymphotoxin adsorption suggest that lymphotoxin is bound at the external surface of cell membranes. This view is substantiated by treatment of target cells that had been used to absorb lymphotoxin activity from supernatants with trypsin, washing them, and comparing their viability at 48 hr to lymphotoxin-coated cells that had not been treated with trypsin (Table 1*A*). The viability of the trypsinized cells was greater than that of cells that had been exposed to lymphotoxin, but not trypsinized. Trypsinization of cells before exposure to lymphotoxin also confers protection from subsequent cytotoxic effects (Table 1*B*). This effect is reversible, because restoration of sensitivity to lymphotoxin after trypsinization is complete within several hours (Fig. 3). The ability of trypsin to protect target cells from the cytolytic effects of either prior or subsequent exposure to lymphotoxin implies that: (*i*) lymphotoxin binds to the external surface of target cells; (*ii*) the binding site is probably a cell-membrane glycoprotein, since trypsin cleaves the sialomucopeptide por-

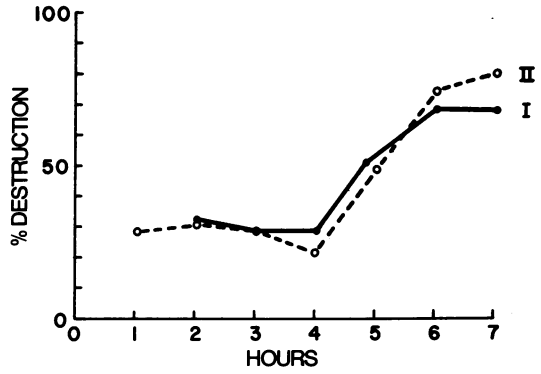

FIG. 3. Replacement of lymphotoxin receptors on L cells after trypsin treatment. Monolayer L cells were trypsinized, then placed in suspension culture. After various intervals, 3 × 10^6 cells were removed and added to 1.0 ml of lymphotoxin (B-20) and placed on the rocking platform for 2 hr. The cells were then washed free of all lymphotoxin medium and recultured in fresh medium at a density of 2 × 10^5 cells per ml in tube culture. They were assayed for viability after 44 hr. These experiments were repeated five times; results of two experiments are shown.

TABLE 1. *Effect of trypsinization on viability after and before exposure to lymphotoxin*

Trypsinization	Trypsin	Lympho-toxin	Trypsin and lymphotoxin
A. After lymphotoxin			
% Viability	100	19.5	108.5
B. Before lymphotoxin			
% Viability	100	13.4	96.8

Results were determined as remaining, adherent cells (Coulter Counter) 48 hr after treatment and presented as percent viability. In Exp. A, tube cultures were exposed to 1 ml of 1:1 lymphotoxin (B-20) on a rocker for 3 hr at 37°. The monolayers were then washed with saline and treated with trypsin for 10 min at 37°. The cells were rewashed and suspended in fresh medium for 48 hr. In Exp. B, tube cultures were first trypsinized for 10 min at 37°, washed, and then exposed to 1:1 lymphotoxin (B-20) for 1 hr on a rocker at 37°. The cells were washed and reestablished in tube cultures in fresh medium for 48 hr.

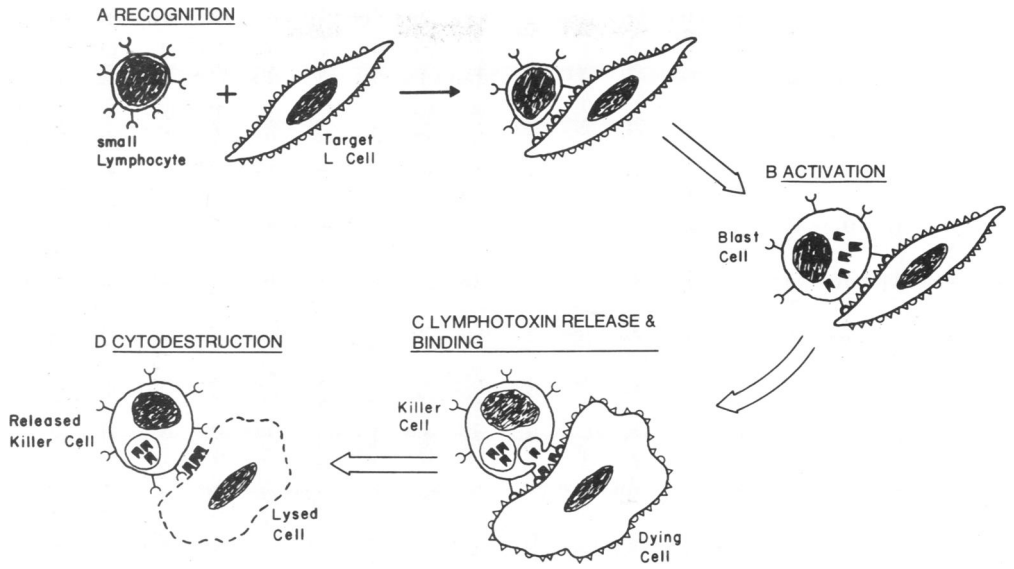

FIG. 4. Proposed model for role of lymphotoxin in cell-mediated immune cytodestructive reactions.

tion of the membrane glycoproteins (20); and (*iii*) protection of lymphotoxin-coated cells by trypsinization is not due to the direct action of trypsin on lymphotoxin, since lymphotoxin activity in protein-containing medium is insensitive to trypsin (9).

Restriction of Cytodestruction to Lymphotoxin-Coated Cells. To determine if cell-bound lymphotoxin is available to destroy adjacent cells not coated with lymphotoxin, we used techniques that had previously been used to demonstrate specific destruction of cells in mixed cell culture, commonly termed "innocent bystander" experiments (21). Target L cells were coated with lethal amounts of lymphotoxin, washed, and then suspended in fresh medium in various ratios with ^{51}Cr-labeled target L cells (innocent bystander) that had not been treated with lymphotoxin. The cells were cultured at 37° in tubes in an upright position for 30 hr. Both the supernatant and pelletable, cell-bound ^{51}Cr counts were determined. As a positive control, chromium-labeled cells were coated with lymphotoxin and their release was measured, in order to ensure that surface-bound lymphotoxin could cause release of the isotope from target L cells. The slight chromium release accompanying mixed lymphotoxin-coated cells and uncoated ^{51}Cr-labeled cells appears to be caused by the presence of dead or dying cells, because heat-killed L cells will stimulate the same amount of chromium release. These experiments were repeated three times. Clearly, the results indicate (Table 2) that lymphotoxin-coated cells undergo complete destruction, whereas innocent bystander cells of equal sensitivity are unaffected.

DISCUSSION

These data support the concept that human lymphotoxin attaches to binding sites expressed on the surface of target L cells *in vitro*. We have demonstrated that lymphotoxin activity is removed from supernatant medium after exposure to intact L cells and, conversely, that lymphotoxin is transferred and adsorbed to these cells. The adsorption of lymphotoxin to L cells is rapid, as indicated by both attachment of labeled lymphocyte products and cytotoxic activity, and is independent of temperature. The latter results are important because they suggest that attachment is due to binding and not simply internalization by pinocytosis. Binding to sites on the target-cell membrane is further supported by the observation that trypsin treatment renders L cells incapable of binding lymphotoxin as well as insensitive to lymphotoxin-induced destruction. However, the cells become fully sensitive again if allowed to replace their receptors.

There appear to be several variables regulating lymphotoxin binding: (*i*) the number of cells used; (*ii*) the initial level of

TABLE 2. *Effect of lymphotoxin-coated cells on the viability of innocent bystander cells*

Exp.	Unlabeled L cells	^{51}Cr-Labeled L cells	Specific ^{51}Cr release
A	2×10^5 noncoated	5×10^4 noncoated	0
B	1×10^5 LT-coated	5×10^4 noncoated	6.8
C	2×10^5 LT-coated	5×10^4 noncoated	5.4
D	2×10^5 heat-killed (56°, 30 min)	5×10^4 noncoated	4.1
E	2×10^5 noncoated	5×10^4 LT-coated	35.0
F	Total releasable ^{51}Cr counts (distilled water lysis)		40.8

Results are averages of six or seven separate tubes. Suspensions of L cells (2×10^6 cells) were coated with lymphotoxin (LT) by incubation in 10 ml of B-20 for 1–4 hr at 37°. Unbound lymphotoxin was removed by washing in fresh medium. Various numbers of these cells were mixed with ^{51}Cr-labeled untreated (Exps. B and C) or heat-killed L cells (Exp. D). Positive controls were both labeled with ^{51}Cr and treated with lymphotoxin (Exp. E). Cell mixtures were placed in silicon-coated tubes and cultured upright for 35 hr at 37°. Viability was determined by the percent of ^{51}Cr released into the supernatant. Specific ^{51}Cr release (percent destruction) was determined by subtracting the 30-hr background [Exp. A; (44.2 ± 4%)].

lymphotoxin in the medium; and (*iii*) the capacity of a cell to bind lymphotoxin, which seems to correlate with the cell's susceptibility to lymphotoxin-induced destruction (11). Preliminary experiments indicate that absorption of lymphotoxin from supernatants is greatest by such cells as selected strains of L-929 and DBA/2 mastocytoma, while lymphotoxin-resistant cells, i.e., human erythrocytes and small lymphocytes absorb little, if any, lymphotoxin. There is one other important variable, namely, the cells used must be free of microbial contamination and pleuropneumonia-like organisms. These variables, along with the tendency of workers to use trypsinized cells, might explain why other investigators (22), including our own initial report (23), were unable to detect lymphotoxin binding to target cells *in vitro*.

Apparently, the rate of cell destruction is related to the amount of lymphotoxin bound to the surface. This would explain the effect that different concentrations of lymphotoxin have on both the extent of binding and on the rate of lysis (22). These data support the findings of Walker and Lucas (22), who reported multiple-hit stoichiometry for human lymphotoxin-induced L-cell destruction *in vitro*. Thus, if a cell sustains relatively few lymphotoxin hits, it will undergo a protracted death or perhaps survive by repairing the lesion, whereas additional hits can commit it to a more rapid destruction. It is not known how many hits a cell must sustain before it is committed to destruction, but the sensitivity of a cell appears to be regulated by the total number of binding sites per cell and/or their topographical distribution. While binding is the first step in the reaction, data indicate that it is not sufficient itself to cause destruction.

Since the initial step in the lymphotoxin-induced cytodestructive reaction is the binding of lymphotoxin to the target cell, the possibility exists that the selective action of lymphocyte effector molecules on various cells may reside in the capacity of these molecules to selectively attach to sites differentially expressed on different cell types. This proposal is further supported by the recent report that guinea-pig macrophage-inhibition-factor binds to peritoneal, but not alveolar, macrophages (25).

Our approach to explaining the mechanism(s) of antigen- or mitogen-activated, lymphocyte-induced destructive reactions *in vitro* is based upon the following observations: (*i*) Lymphotoxin is synthesized and released from either antigen- or mitogen-activated lymphocytes (24). (*ii*) Binding of lymphotoxin is a necessary first step, although not sufficient itself for target cell destruction. The relative sensitivity and insensitivity of certain target cells to lymphotoxin-induced destruction *in vitro* may be explained by their corresponding ability or inability to bind lymphotoxin. (*iii*) The tightness or avidity of lymphotoxin binding is such as to functionally restrict it to act only upon the target cell to which it is bound.

The present results lead us to propose the following model (Fig. 4). The immune lymphocyte, by specific receptors, recognizes, attaches to, and is activated by membrane interaction with the specific target cell. During activation, lymphotoxin is synthesized and secreted by the killer cell into the localized, interstitial microenvironment. Maximal binding of lymphotoxin occurs on those target cells in closest proximity to the secreting lymphocyte, where lymphotoxin concentrations are highest. Once bound to a cell, lymphotoxin is functionally restricted to contribute to the destruction of only that cell. The degree and specificity of cell destruction observed in certain cell-mediated immune cytodestructive reactions *in vitro* (5) could be the cumulative effect of several processes: the degree of lymphocyte activation regulating the amount of lymphotoxin secretion; its selective release into a localized microenvironment; and the number and topographical configuration of target-cell lymphotoxin-binding sites. Cytolysis of the target cell might cause release of the immune lymphocyte, due to disruption of membrane recognition sites, freeing it to attach to and kill other target cells.

The lack of specificity associated with lymphotoxin-induced cytolysis has conceptually hampered its putative role as a mediator in cell-mediated immune reactions. The findings reported herein offer an explanation of how the lymphotoxin molecule could be used by the lymphocyte to cause effective and highly localized cell destruction.

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