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THE ROLE OF LYMPHOTOXIN IN TARGET CELL DESTRUCTION INDUCED BY MITOGEN-ACTIVATED HUMAN LYMPHOCYTES IN VITRO

II. The Correlation of Temperature and Trypsin-Sensitive Phases of Lymphotoxin-Induced and Lymphocyte-Mediated Cytotoxicity[1]

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The in vitro destruction of phytohemagglutinin (PHA) coated β L cells by non-immune human lymphocytes was resolved into two distinct phases—lymphocyte dependent and lymphocyte independent. The initial or lymphocyte-dependent phase occurred within the first 2 hr and proceeded equally well at 34 and 37°C. The amount of lymphotoxin (LT) secreted by PHA-activated human lymphocytes in vitro to PHA stimulation was the same at 34 and 37°C. Antiserum and complement inactivation of the aggressor lymphocytes at various intervals revealed that target cell lysis was lymphocyte independent. However, the latter phase was temperature dependent, i.e., proceeding at the permissive temperature of 37°C, but inhibited at the restrictive temperature of 34°C. Further experiments revealed that LT-induced destruction had the same temperature sensitivity as target cell cytolysis occurring during the lymphocyte-independent step. Trypsin treatment of target cells during an early period of the lymphocyte-independent phase protected the target cell from subsequent death, indicating the aggressor lymphocyte has deposited a cytotoxic effector material on its surface. These results suggest the lymphocyte-dependent stage involves the processes required for the induction of LT synthesis and secretion. The actual cytolysis occurring during the lymphocyte-independent stage may be caused by LT or LT-like material(s) deposited on the target cell surface by the mitogen-activated human lymphocyte.

Our understanding of the cell and molecular mechanisms involved in the target cell destruction mediated by immune and mitogen-activated effector lymphocytes *in vitro* is of central importance to our understanding of the mechanisms of the cell-mediated tissue destructive immune reactions *in vivo*. The latter are typified by such reactions as tumor and transplantation immunity, delayed hypersensitivity, and the tissue destruction seen in certain autoimmune disease states (1-4).

Studies by numerous investigators reveal that there are several steps involved in the *in vitro* cytolytic reaction: a) the first step in all of these reactions involves some form of recognition event, typified by the interaction of target cell antigens with immune lymphocyte surface receptors or interaction of a mitogen with the lymphocyte surface receptors (5, 6); b) this interaction triggers "activation," which results in a transformation of the lymphocyte into the effector cell; c) it is the activated effector cell which then induces cytolysis of the target cell *in vitro* (5, 6). The actual mechanism involved in the cytolytic reactions is still not clear.

Direct lymphocyte-mediated cytotoxicity (DLC),[3] which requires physical contact of the aggressor and target cells (TGC) was described as early as 1967 as having temperature-dependent step(s) (7, 8). Wilson (7) described the clustering of immune rat lymphocytes about the appropriate TGC as relatively temperature independent, whereas the subsequent destruction of the TGC occurred only at 37°C. Berke and associates (9, 10) more recently have reported that immune murine lymphoid cells induced cell cytolysis *in vitro* which exhibited an early temperature-independent phase and a subsequent temperature-dependent phase. Martz and Benacerraf (11) have resolved the action of immune murine lymphocytes on tumor TGC into an early lymphocyte-dependent phase and a subsequent lymphocyte-independent phase which began 1 to 2 hr after the initial contact between lymphocyte and target cell. Although the actual lytic mechanism involved in these systems is still not clear, it was proposed that it was due to the damage inflicted during the contact of lymphocyte and TGC. In previous publications, we have implicated a lymphocyte released cell-toxin as an important effector molecule involved in lymphocyte-mediated lysis of cells *in vitro* (12-14). In the present study, we report that there are lymphocyte-dependent and independent phases in the destruction of cells *in vitro* induced by phytohemagglutinin- (PHA) activated human lymphoid cells from a lymphocyte product deposited on the TGC surface, which causes destruction of the TGC during the lymphocyte-independent phase.

MATERIALS AND METHODS

Many of the methods and materials employed in this study were described in detail in the first manuscript of this series (15).

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[2] G. A. Granger is supported by a career award from N.I.A.I.D. of the NIH.

[3] Abbreviations used in this paper: DLC, direct lymphocyte-mediated cytotoxicity; TGC, target cells; PHA, phytohemagglutinin; MEM, minimum essential medium; LT, lymphotoxin; MEM-S, serum-free MEM; AS, antisera.

Target cell and culture medium. The β subline of mouse L 929 cells, maintained in monolayer culture in our laboratory, were used as TGC throughout these studies. The *in vitro* characteristics of this particular L cell line routinely maintained in our laboratory have been described in detail elsewhere (15). The culture medium employed throughout these studies was Eagle's minimum essential medium (MEM) supplemented with nonessential amino acids, 1 mM sodium pyruvate (Grand Island Biological Company, Berkeley, Calif.), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. All assays for lymphotoxin (LT) or direct lymphocyte-mediated cytotoxicity contained 5×10^{-5}M mercaptoethanol. Both serum-free MEM and medium containing 3% heat-inactivated (56°C for 30 min) newborn calf serum (Microbiological Associates, Inc., Bethesda, Md.) (MEM-S) were used.

Lymphoid cells. Human lymphocytes were isolated in single-cell suspension from adenoid tissue after surgical removal from healthy children, as described previously (15). Briefly, the adenoid tissues were minced, then tissue debris were removed by centrifugation, and finally, the adherent cells were removed from the suspension by overnight incubation in plastic or glass flasks at 37°C. This procedure routinely yielded a nonadherent cell suspension consisting of 95 to 100% small to medium lymphocytes which were 90 to 95% viable. These lymphocytes were routinely maintained and cultured in MEM-S.

Mitogen. Purified PHA (PHA-W, Lots K4944 and K5764) was obtained from Wellcome Reagents, Ltd., Bechenham, England. The optimal dose for stimulation of lymphocyte DNA synthesis assayed at 72 hr was determined to be 2.0 μg/ml. The optimal dose for inducing direct destruction by lymphocytes of mitogen precoated TGC monolayers was determined to be 10 μg/ml.

Production and assay of human LT. Lymphocytes were cultured in 2.0 ml tube cultures (2×10^6 cells/ml) in MEM-S in the presence or absence of 2.0 μg/ml PHA-W. After 72 hr at 34 or 37°C, the medium was cleared of cells and debris by centrifugation at 2,000 rpm in an International PR-2 refrigerated centrifuge. Four-fold dilutions of the supernatants were then assayed for LT activity at 34 and 37°C, on 1.0-ml tube cultures containing 10^5 β L cells. Monolayer target L cell viability was first assessed microscopically after 24 hr, and then adherent cells were removed from the glass with trypsin and enumerated by using a Coulter Counter (Model F) as previously described (15). Percentage of TGC cell destruction was calculated from the following formula:

$$\frac{\text{Control} - \text{Experimental}}{\text{Control}} \times 100.$$

The LD_{50} endpoint or "titer" was employed when it was necessary to compare the actual amount of LT in a supernatant and is defined as the reciprocal of the supernatant dilution causing 50% cytolysis of the TGC monolayer after 24 hr.

Direct lymphocyte cytotoxicity assay. Target β L cells were established 48 hr before assay in 1.0 ml tube cultures at 1.5×10^5 cells per tube, at 37°C on a 5° slant in an atmosphere of 5% CO_2, 95% air. These conditions resulted in confluent monolayer cultures. All the following procedures were conducted in a 37°C warm room. The medium was dicarded and each tube was gently washed once with MEM and then 1.0 ml MEM containing 10 μg/ml PHA-W or control MEM was added to test and control tubes. After 1 hr at 37°C, the monolayers were rinsed with 1.0 ml MEM to remove the unabsorbed PHA-W, and 1.0 ml of the cell suspension containing a predetermined number of cells was added. Ratios of 1, 2, 4, and 10:1 lymphocytes to 1 TGC were used as indicated in the text. Parallel non-mitogen-coated control cultures were treated in an identical fashion or received 1.0 ml cell-free MEM-S. The tube cultures were then incubated for 24 hr at two different temperatures, either 34 or 37°C. Target cell viability was determined on suspensions of trypsinized cells by counting the total number of viable cells/ml in a Neubauer chamber in 0.1% eosin Y. Percentage of destruction was derived by comparing viable cell counts from experimental cultures to counts from parallel control cultures. Non-mitogen-induced or background cytotoxicity was determined in cultures in which the target cells were treated with lymphocytes but not precoated with PHA-W.

Preparation of anti-lymphocyte serum and inactivation of effector human lymphocytes. Two young female New Zealand white rabbits were injected i.v., each with a pooled suspension of 4×10^8 viable human lymphocytes from several donors. Two weeks later, each animal received a second i.v. injection of 3×10^8 human lymphocytes. One week later, the rabbits were bled from the marginal ear vein. The serum from each rabbit was collected and heat inactivated at 56°C, 30 min. The antisera were diluted and the dilutions were tested for cytotoxic activity on human lymphocytes in the presence of 1:2, 1:5, 1:10, and 1:20 dilutions of normal rabbit serum as a complement source. The LD_{50} dilution of each of the two samples were determined, and the sera were pooled, dispensed in small aliquots, and stored at −20°C. Additional *in vitro* tests verified these two antisera (AS) were nontoxic to the target L cell. Repeated testing revealed that a 1:20 dilution of the normal rabbit serum complement preparation was fully effective in causing destruction of antibody-coated human lymphocytes in the numbers routinely employed in the present studies.

The kinetics of antibody and C-induced cytolysis of human lymphocytes *in vitro* was determined. Lymphocytes (1.25×10^6 cells/ml) were incubated at 37°C for 1 hr on a 5° slant just as they would be if employed in the cytotoxicity assay. Various dilutions of AS and C were then added to each culture and the tubes incubated at 37°C. After 40 min, 0.2 ml of the suspension was removed and mixed with 0.2 ml of 0.2% eosin Y. Viability of the treated and control lymphocytes was then determined by counting the cells in a Neubauer chamber. These studies revealed that at 34 and 37°C, an antiserum dilution of 1:100 and a complement dilution of 1:20 killed 50% of the lymphocytes within 5 min, 80% in 15 min, and after 30 min, total inactivation was complete.

RESULTS

The effect of altering the temperature on the capacity of human lymphocytes to induce in vitro TGC destruction. Tube cultures of target cells were pretreated with PHA-W or control MEM as described in *Materials and Methods.* The unbound mitogen was then removed by washing the cells, then lymphocyte suspensions containing various numbers of cells were added to each monolayer culture. These cultures contained lymphocyte to TGC ratios of 1, 2, 4, and 10:1. Parallel cultures were incubated at various temperatures. After 24 hr, TGC viability was determined as described in *Methods.* These preliminary studies were surprising since only a few degrees drop in temperature dramatically affected the cytolytic reaction. The results from one experiment conducted at two

temperatures, 34 and 37°C, are illustrated in Figure 1*A* and *B*. These particular experiments were repeated with the lymphoid cells from eight separate donors, and although the magnitude of the responses varied slightly, the results were essentially identical in all tests. It is obvious there is a greatly reduced level of TGC destruction at 34°C, when compared to that which occurred at 37°C.

The effect of altering the temperature on LT production by PHA-activated lymphocytes. Experiments were designed to determine the effect of the shift from 37° to 34°C temperature variation upon the secretion of LT in cultures of PHA-stimulated human lymphocytes. Lymphocyte tube cultures were activated by adding predetermined optimal doses of PHA-W. The cultures were incubated at 34 and 37°C, then supernatants were collected after 72 hr, and dilutions were tested for LT activity on two cells at 37°C. The result of one of these experiments is presented in Figure 2, and indicate that essentially the same levels of LT are secreted by PHA-activated human lymphocytes incubated at 34 or 37°C. In four repetitions of this experiment, we found lymphocyte preparations from different donors yielded mediums with slightly different LD_{50} endpoints, however, there was no difference in the level of LT secreted at 34 and 37°C by lymphocytes from a single donor.

The effect of altering the temperature on LT activity. At this point we reasoned that if LT-like material(s) are involved in the cytolytic reaction, and lymphocyte activation and LT secretion occur equally well at the permissive and non-permissive temperature, then temperature inhibition may affect the action of LT on the TGC. Experiments were designed to examine the effect of incubation at 34 and 37°C, on LT-induced cytotoxicity. Dilutions of various supernatants generated by PHA-stimulated lymphocyte cultures incubated at

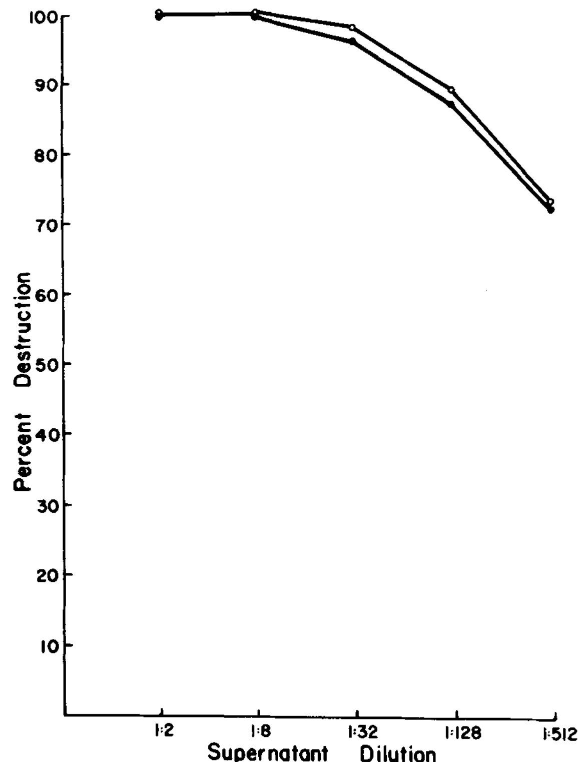

Figure 2. Temperature independence of LT production. Lymphocytes were stimulated by PHA-W at 34°C (●——●) or 37°C (O——O) for 72 hr, at which time supernatants were collected and assayed for LT activity at 37°C.

37°C for 72 hr were assayed after 24 hr for cytolytic activity at 34 and 37°C. The results of one of five assays are illustrated in Figure 3. The cytolytic activity of LT at the non-permissive temperature is greatly reduced, *i.e.*, the LD_{50} endpoints differ from 1/400 at 37°C to 1/9.5 at 34°C (a 42-fold reduction). In additional experiments not shown, the LT secreted by PHA-stimulated lymphocytes incubated at 34°C yielded identical results when assayed for activity at 34 and 37°C, ruling out the possibility that a different cytotoxin is secreted by lymphocytes at 34°C.

Delineation of early lymphocyte-dependent and late independent step(s) and the effect of varying the temperature on these step(s). Effector lymphocytes were specifically inactivated at various times during their interaction with target cells at 34 and 37°C by the addition of rabbit AS and rabbit C, as described in *Methods*. After destruction of the aggressor cell, the reaction was allowed to continue, and viability of the TGC was determined after 24 hr, at both 34 and 37°C. Exhaustive experiments verified that the rabbit AS and C were found to be nontoxic to the β L cell, and inactivation of the aggressor lymphocytes was complete after 30 min. The results of one of these experiments is visualized in Figure 4. The experiment was repeated a total of five times with lymphocytes from separate donors for each experiment. The results demonstrate clearly that the lymphocyte-dependent phase of the reaction is completed at 34 and 37°C, after 2 to 3 hr of contact, at which time target cell lysis does not require the presence of the viable activated lymphocyte. To eliminate the possibility that the destruction of the lymphocyte by antiserum was either incomplete or that these product(s) were released by the killed lymphocytes after inactivation, the following experiment was designed. Lymphocytes (2×10^6 cells/ml) were stimulated with 2.0 μg/ml PHA-W or control MEM for 6 hr at 37°C. Cells were

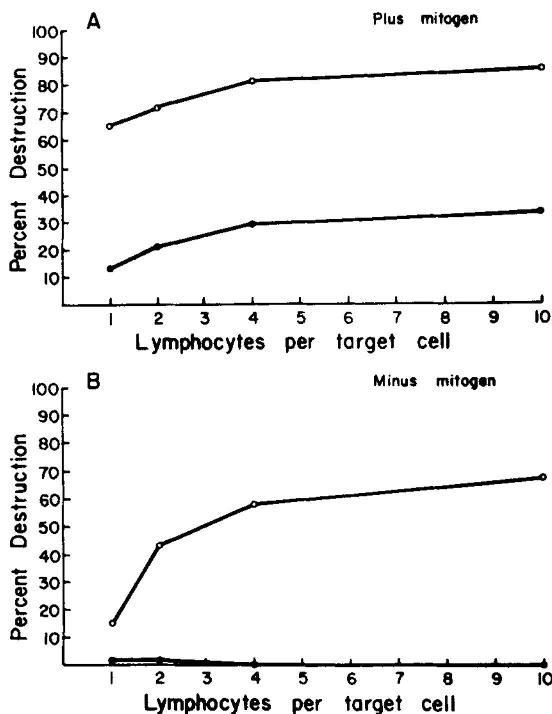

Figure 1. Temperature dependence of PHA-induced DLC. Lymphocyte suspensions of various lymphocyte to target cell ratios were incubated with *A*) PHA-W or *B*) control MEM-coated TGC monolayers at 34°C (●——●) or 37°C (O——O). Target cell viability was determined after 24 hr.

then sedimented by centrifugation and washed once with MEM. Test and control lymphocytes were resuspended in MEM-S and then treated with AS (1:100), C (1:20), or control MEM. One milliliter of the appropriate lymphoid cell suspension (5:1 ratio) was then added to PHA-W or control MEM-coated TGC monolayers. The cultures were incubated at 37°C for 24 hr and then assayed for TGC viability. Percentage of destruction was determined by the viability of parallel cultures

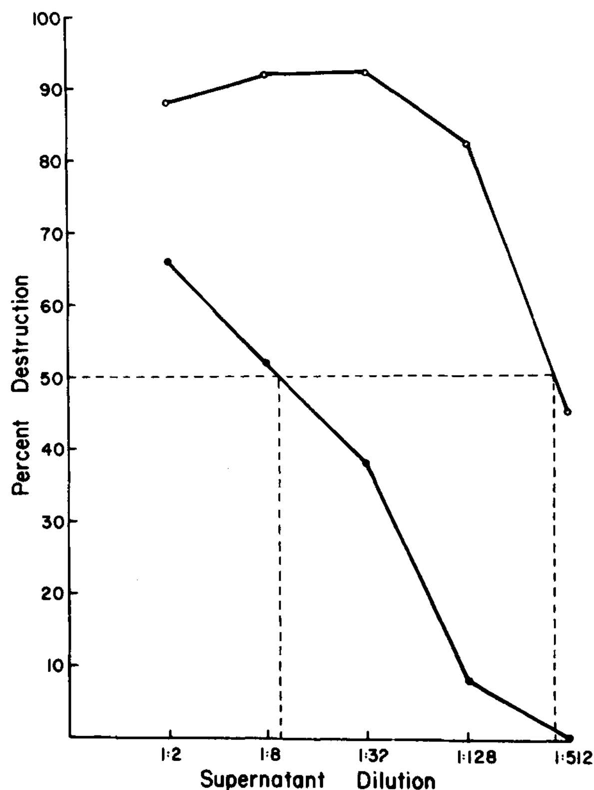

Figure 3. Temperature dependence of LT activity. Supernatant medium generated by PHA-W-stimulated lymphocytes at 37°C in 72 hr was assayed for LT activity at 34°C (●——●) or 37°C (○——○).

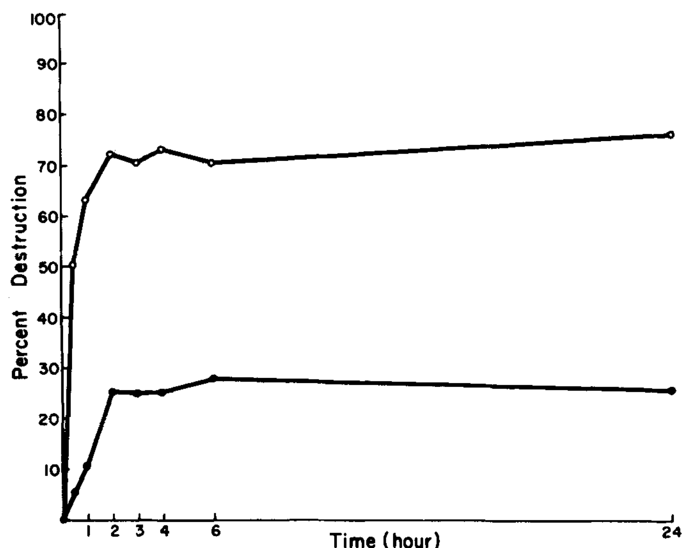

Figure 4. Kinetics of lymphocyte-dependent function. Lymphocytes (5:1 ratio) were incubated with PHA-W coated TGC monolayers at 34°C (●——●) or 37°C (○——○). At various times, AS (1:100) and C (1:20) in a total volume of 0.06 ml were added to the appropriate cultures. Control cultures received 0.06 ml MEM. After 24 hr, all cultures were assayed for TGC viability.

that received no lymphocytes. The results are depicted in Figure 5, and demonstrate that lysing activated lymphocytes does not affect the viability of TGC. In addition, as previously reported, heat-killed cells are not effective as killer cells (8, 16, 17).

The effect of dropping the temperature from 37 to 34°C on the lymphocyte-independent step of TGC lysis. After having determined that TGC destruction mediated by PHA-activated human lymphocytes is at least a two phase phenomenon, the first phase completed during the first 1 to 2 hr of interaction between the lymphocyte and TGC, termed lymphocyte-dependent, and the second phase termed lymphocyte-independent, it was of critical importance to determine the temperature dependence of the second phase. We reasoned that if LT is an important mediator of TGC destruction in this *in vitro* system, the lymphocyte-independent phase might represent the situation in which LT has bound to the TGC membrane and cellular lysis would be predicted to be the stage which is temperature-dependent. The following experiment was designed to test this hypothesis. Lymphocytes were incubated on PHA-W-pretreated target L cell monolayers at a 5:1 ratio for 2 hr at 34°C. The aggressor lymphocytes in certain cultures were then destroyed by the addition of AS (1:100) and C (1:20) to the cultures and reincubated for an additional 1 hr at 34°C, at which time one set of parallel tubes were shifted to 37°C, and one set kept at 34°C. Target cell viability was assayed after 24 hr and percentage of destruction was determined by comparison with the viability of parallel cultures which received no lymphocytes. Results from one representative, of three separate experiments, are described in Figure 6. Destruction was inhibited in cultures maintained at 34°C after lymphocyte inactivation, whereas untreated control cultures incubated at 34°C exhibited 16% destruction. In contrast, TGC cytolysis occurred in the cultures which were shifted to 37°C.

The effect of trypsin treatment on cytolysis of TGC, the lymphocyte-independent phase. There are at least two possible explanations that might account for the death of TGC after 2 hr of contact with viable aggressor lymphocytes: 1) the lymphocyte irreversibly damages the TGC, or 2) the lymphocyte releases a substance(s) that binds to the membrane surface of the TGC, and this material(s) causes cytolysis in the absence of the lymphocyte. Hessinger, *et al.* reported that LT binds to the target cell and the initial phases of human LT-induced cytolysis can be reversed by trypsin treatment (10). If the former explanation is valid, then treatment of TGC with trypsin after the first phase of DLC might be expected to enhance the subsequent destruction of the TGC. If the second explanation is valid, trypsin treatment of the TGC after incubation with activated lymphocytes might be expected to strip off the bound LT and protect the cell from destruction. The following experiment was designed to distinguish between these two possibilities.

Lymphocytes were incubated on PHA-W or control MEM-treated TGC monolayers at a 5:1 ratio for 1 hr at 34°C, at which time the lymphocytes were destroyed by the addition of AS and C, and the tubes were returned to 34°C for an additional 45 min. Appropriate monolayers were washed once with PBS and 1 ml of trypsin solution (0.1% trypsin, 10^{-4}M EDTA) was added to each tube. After an 8-min incubation at 34°C, 1 ml of MEM-S was added and the detached cells were sedimented by centrifugation and washed once with MEM-S. The cells were then resuspended in MEM-S and test and control tubes were incubated for an additional 24 hr at 37°C.

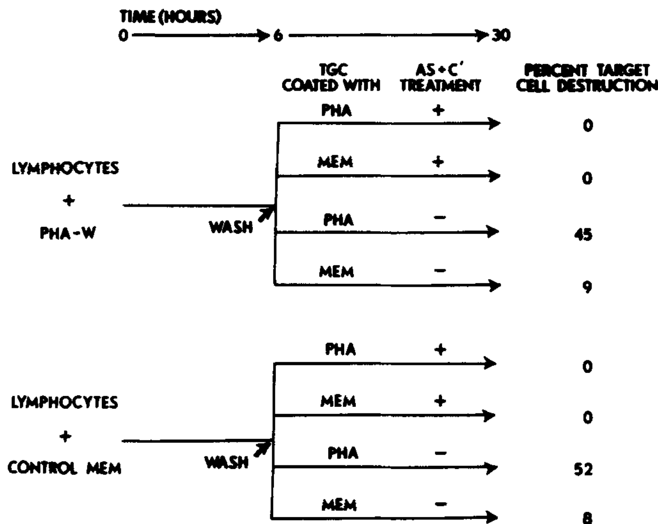

Figure 5. Lysis of activated lymphocytes on TGC monolayers. Lymphocytes were PHA stimulated for 6 hr, then washed and added to appropriate monolayers at a ratio of five lymphocytes per TGC. Inactivation of the lymphocytes was affected by antiserum-mediated complement-dependent lysis at the time of addition. After 24 hr incubation at 37°C, TGC viability was determined.

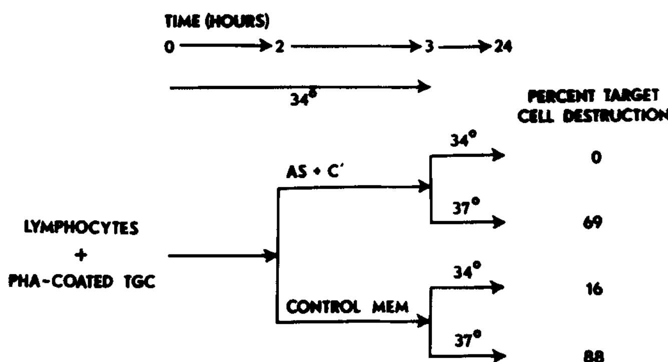

Figure 6. Temperature dependence of the lymphocyte-independent phase of DLC. Lymphocytes (5:1 ratio) were incubated on PHA-W-coated target L cell monolayers for 2 hr at 34°C. Effector lymphocytes were then inactivated by the addition of AS and C or control MEM for one additional hour at 34°C. Appropriate cultures were then shifted to 37°C, and TGC viability was assayed after 24 hr.

Target cell viability was assayed and percentage of destruction was determined. The results of one of three experiments are shown in Figure 7. Clearly, trypsin treatment of parallel cultures reduced TGC destruction by 48%. In addition, trypsin treatment protected control TGC treated with non-mitogen-activated lymphocytes. These results dramatically support the second of the two hypothetical explanations, that is, the lymphocyte has deposited a toxin(s) on the membrane surface of the TGC, which then goes on to cause actual cytolysis.

DISCUSSION

There appears to be a lymphocyte-independent and dependent phase in the destruction of TGC by PHA-activated human lymphocytes. Effector cell function is completed rapidly within the first hour of contact with the TGC at both the restrictive (34°C) and unrestrictive temperature (37°C). However, lymphocytes pretreated with AS and C or inactivated by heat treatment were completely ineffective at mediating TGC destruction in agreement with results of other investigators (9, 11, 16, 17). It is also evident that equivalent amounts of LT are secreted at both 34°C and 37°C by PHA-activated human lymphocytes.

Target cell cytolysis observed during the lymphocyte-independent phase of cytolysis appears similar in many respects to cellular lysis mediated by LT. Binding of LT to trypsin-sensitive TGC membranes is temperature-independent (14), however, we found actual cytolysis is essentially blocked by shifting the temperature from 37 to 34°C. The lymphocyte-independent phase of destruction was shown to have the same temperature dependence as that of LT. The situation is, however, more complex when we consider other target cells, for the same temperature that blocked direct and indirect cytotoxicity on L cells is different for other TGC.

There are two hypothetical explanations for the destruction of TGC during the lymphocyte-independent step. The first is that the lymphocyte irreversibly damages the TGC during cell contact. Klein and Perlmann (18) report that trypsin treatment accelerates the lysis of cells damaged by heat or by specific antibody in the presence of complement, whereas undamaged cells are unaffected by a similar trypsin protocol. If the TGC is irreversibly damaged during the dependent phase, then trypsin treatment of the TGC during this time would be expected to enhance the subsequent TGC destruction. On the other hand, if the lymphocyte has deposited LT or LT-like materials on the membrane surface of the TGC, trypsin treatment of the L cells after a 2-hr incubation with viable lymphocytes at the restrictive temperature could be expected to inhibit destruction. The present results favor the latter possibility. However, the protective effect of trypsin was not as dramatic for the lymphocyte-independent step as for LT-treated α L cells. As a partial explanation of these results, one can envision that TGC incubated with LT supernatants probably binds the LT molecules evenly about its surface. In contrast, the interaction of TGC with activated lymphocytes may result in a high local concentration of LT molecules placed on the TGC surface in the close proximity of the lymphocyte. Thus, these LT molecules might not be as labile to trypsin removal. Also, in the latter situation, the LT molecules deposited by the lymphocyte in close proximity to one another would be expected to be more highly efficient at causing local

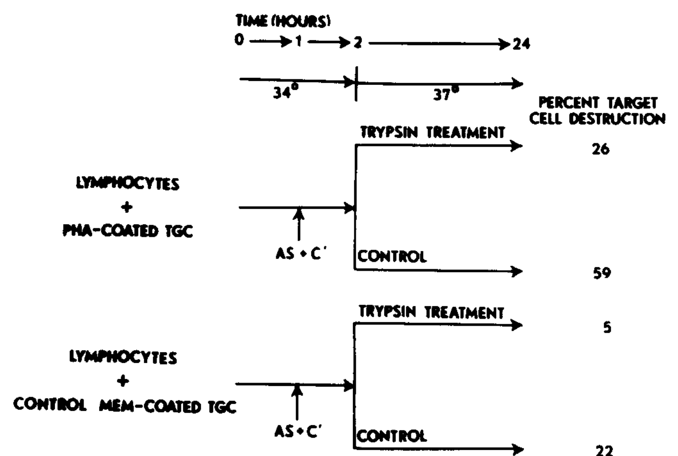

Figure 7. Trypsin treatment of TGC during the lymphocyte-independent phase of DLC. Lymphocytes (5:1 ratio) were incubated on PHA-W or control MEM-coated TGC monolayers for 1 hr at 34°C, at which time lymphocytes were inactivated by the addition of AS and C for 45 additional min at 34°C. Target cells of appropriate monolayers then received a brief trypsin treatment and were resuspended in fresh medium. All cultures were then permitted to incubate for an additional 24 hr at 37°C at which time TGC viability was determined.

TGC lesions and even at the lower temperature of the pre-incubation, the residual LT activity might be sufficient to damage the cell in such a way as to render it sensitive to trypsin enhancement of lysis. It is unclear at this time whether the trypsin protective effect causes removal of the LT membrane receptor complex from the TGC surface or by inactivation of LT itself.

The cytolysis induced *in vitro* by mitogen activated cells may consist of the following step(s): a) the initial phase of lymphocyte-mediated target cell destruction, which is lymphocyte-dependent, encompasses the phases of recognition, activation, and placement of LT or LT-like material(s) on the TGC surface by the activated lymphocyte; b) the subsequent phase which culminates in TGC death is lymphocyte-independent and, in these studies, temperature-dependent, and although there are alternatives, may represent the action of LT or LT-like materials bound on the TGC surface.

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