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# THE LT SYSTEM IN EXPERIMENTAL ANIMALS

## IV. Rapid Specific Lysis of $^{51}$CR-Labeled Allogeneic Target Cells by Highly Unstable High M.W. Lymphotoxin-Receptor Complex(es) Released *in Vitro* by Activated Alloimmune Murine T Lymphocytes[1]

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**Lymphocytes or purified T cells obtained from the spleens of alloimmune C57BL/6, DBA/2, or C3H/DiSn mice, when placed on monolayers of lectin-coated allogeneic (L-929 or 3T3) fibroblasts, release into the supernatant various forms of cell lytic material. One form appears to be a high m.w. complex containing an antigen-binding receptor(s) that is highly labile and capable of causing rapid and specific lysis of allogeneic target cells *in vitro*. Material(s) that could mediate these cell lytic effects were detected in culture supernatants as early as 3 hr after stimulation, peaked at 6 to 9 hr, and declined thereafter. The specific cell lytic activity appeared to be due to high m.w. LT-receptor complexes for the following reasons: a) antisera that could neutralize murine LT activity *in vitro* could inhibit this effect; b) absorption of supernatants on the specific target cells at 4°C removed both the specific lytic activity and nonspecific LT activity detectable on L-929 cells *in vitro*; c) this material(s) was highly unstable, as are LT complex forms; and d) fractionation by molecular sieving of these supernatants revealed that cell lysis was mediated by material(s) in the high (>200,000) m.w. complex form(s). The lytic effect did not appear to be due to Ab + C because supernatants that had lost their specific lytic activity could not be reconstituted with fresh sources of C. Since purified alloimmune T lymphocytes yielded more active supernatants than unseparated nonadherent spleen cells, and polyspecific goat anti-mouse Ig sera had virtually no effect on this lytic activity, we feel the receptor(s) in these complexes originates from the T cell(s). The data support the concept that the short-lived specific cell lytic material in these supernatants is a high m.w. complex containing $\alpha_H$ m.w. LT subunits in functional association with specific (T cell?) antigen-binding receptor(s) molecules.**

**These findings strongly corroborate analogous findings in the human and support the concept that the smaller m.w. LT molecules represent a system of weakly lytic but related subunits released by cells that can associate together and functionally associate with antigen-binding receptor(s) to form highly effective cell lytic complexes. Furthermore, these lytic LT-receptor complexes can be directed by the specificity of the receptor with which they are associated.**

The previous manuscripts of this series demonstrated that cell lytic activity, lymphotoxin (LT),[2] detectable on LT sensitive L-929 cells was released *in vitro* when nonadherent murine splenic lymphocytes or purified T cells were stimulated on lectin-coated target cell monolayers (1). Physical and immunologic studies on the material(s) in these supernatants having cell lytic activity revealed that these various molecules were physically heterogeneous but interrelated forms (2). Based on these findings and similar findings with LT molecules isolated from other animal species (3) and man (4, 5), we proposed that these materials represent a system of related subunits, the smaller m.w. forms of which can interact with one another to form high m.w. complexes (6). Furthermore, as clearly demonstrated in the human system, LT molecules in the high m.w. complex form have the unique capacity to associate with antigen-binding receptor molecule(s) (7, 8).

Studies conducted to begin to analyze the relative lytic effectiveness of the various m.w. human LT classes revealed that the Cx form(s) are *much* more effective at inducing nonspecific cell lysis *in vitro* than the smaller m.w. ($\alpha_L$, $\beta$, $\gamma$) forms (9). Furthermore, these data also indicated that when, in association with specific receptor molecules, the lytic effects of the high m.w. LT-receptor complexes could be more effective when directed or localized by the binding specificity of the receptor (9). The above data support the concept that the smaller m.w. LT forms ($\alpha_L$, $\beta$, $\gamma$) are only weakly lytic by themselves and therefore are only detectable on certain sensitive target cells *in vitro*; however, their cell lytic effects become greatly increased when they are assembled together in the complex forms. In addition, when the complex form(s) is associated together with receptor molecules, the nonspecific lytic effects of these materials become more effective and may be directed by the specificity of the antigen binding receptor(s).

The present studies examine the functional activities of labile cell lytic molecules identified in fresh supernatants obtained from nonspecifically activated alloimmune or normal murine splenic lymphocytes or purified T cells *in vitro*. The findings

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[2] Abbreviations used in this paper: LT, lymphotoxin; Cx, high m.w. LT forms; NA, nonadherent; NRS, normal rabbit serum.

reveal that alloimmune T cells, when activated on PHA-coated nontarget-related allogeneic monolayers, release highly unstable materials into the supernatant that can cause rapid specific $^{51}Cr$ release from the sensitizing target cells *in vitro*. Physical and immunologic studies suggest the lytic material(s) in these supernatants, which appears to be responsible for these effects, is a high m.w. material containing LT molecules functionally associated with specific antigen-binding T cell receptor(s).

## MATERIALS AND METHODS

*1. Culture media and cell lines.* Culture media employed in these studies consisted of RPMI 1640 supplemented with 3 or 10% heat-inactivated (56°C, 60 min) fetal calf serum (FCS) (GIBCO, Grand Island, N. Y.), 20 μg/ml streptomycin and 100 μg/ml penicillin (1640-S). Murine L-929 and 3T3 cells were grown in 32-oz prescription bottles in 1640-S at 37°C, in a 95% air, 5% $CO_2$ atmosphere, and passed biweekly. P815 and EL4 cells were a generous gift of Dr. Benjamin Bonavida, Department of Microbiology, University of California, Los Angeles, and were grown in RPMI 1640, supplemented with 10% FCS.

*2. Immunization protocol and harvesting murine spleen cells.* Normal and immune lymphoid cells were collected from C57BL/6, DBA/2, or C3H/DiSn 8- to 10-week-old mice (Jackson Labs, Bar Harbor, Maine). The animals were immunized by i.p. injection of $10^7$ P815 mastocytoma (H-$2^d$) or EL4 lymphoma (H-$2^b$), suspended in 500 μl PBS. After 11 or 12 days, the spleens were aseptically removed, and a single-cell suspension prepared as described previously (10). Lymphoid cells from nontreated normal animals were obtained by identical methods. Cell viability was determined by exclusion of 0.1% eosin Y in PBS, and was routinely 90 to 100%. Spleen cells from immune and nonimmune animals were routinely placed in 90 ml or 250 ml plastic T flasks at a density of approximately $5 \times 10^6$ cells/ml, for 1 to 2 hr at 37°C in media containing 10% FCS, to remove plastic adherent cells. The nonadherent cells were then collected, pooled, sedimented by centrifugation at 300 × G for 4 min, and resuspended at a density of $2.5 \times 10^6$ cells/ml in 1640-S. Differential and viable cell counts revealed these cells were 90 to 95% lymphocytes and 90 to 100% viable.

*3. Production of cell lytic culture supernatants.* The details of these procedures are explained in the first manuscript of this series (1). Briefly, $10^8$ nonadherent normal or immune murine splenic lymphoid cells (NA lymphocytes) or nylon wool-purified T cells in 50 ml 1640-3% FCS were placed into 250-ml culture flasks containing $4 \times 10^6$ L-929 or 3T3 cells that were previously coated with PHA-P (100 μg/ml/$10^5$ cells for 1 hr at 37°C) and washed with PBS. These cultures were then allowed to incubate for 3 to 10 hr at 37°C, and the culture supernatant was collected and either 1) immediately assayed for toxic activity on L-929 cells or $^{51}Cr$-labeled allogeneic target cells; or 2) immediately concentrated 5 to 15 × with an Amicon PM 30 membrane, and then tested for toxic activity.

*4. Lymphotoxin assay.* The details of these procedures are explained in the first manuscript of this series (1).

*5. Isolation of splenic T lymphoid cells.* Purified T cell populations were obtained from nylon wool columns according to the procedures described by Julius *et al.* (11) and detailed in the first manuscript in this series.

*6. Treatment with antiserum + C.* C3H anti-AKR serum was a generous gift of Dr. Raymond Daynes, Department of Pathology, University of Utah. NA lymphocytes were suspended in a 1/10 dilution of antiserum for 30 min at 4°C, washed, and resuspended in a 1/10 final dilution of guinea pig serum as a source of C for 1 hr at 37°C. This procedure generally killed 40 to 45% of the NA splenic lymphoid cells. These cells were then washed two times with 50 ml 1640-S, and were employed as a source of T cell-depleted effector cells.

*7. Gel filtration chromatography.* The methods employed in this study are identical to those described in the second manuscript of this series, except the columns used were 2.5 × 50 cm to shorten elution time.

*8. $^{51}Cr$-release assay.* P815 or EL4 tumor target cells were labeled with $^{51}Cr$ identical to methods already described. Typically, $10^7$ cells in 1 ml of 1640 were labeled with 100 μCi $Na_2{}^{51}CrO_4$ for 1 hr at 37°C. After washing four times, 25,000 cells in 50 μl were placed into microtest II plates containing various amounts of supernatants or fractions to be tested for lytic activity, during a 5- to 10-hr-incubation period at 37°C. After this time, supernatants were harvested using a microtest II harvester and counted on a Beckman Biogamma Counter. Total $^{51}Cr$ release was determined by harvesting cells suspended in 2% sodium dodecyl sulfate (SDS). In general, 25,000-labeled P815 or EL4 cells contained 10 to 15,000 counts and spontaneous release was never higher than 1 to 2%/hr for P815 or 2 to 3%/hr for EL4. All assays were done in triplicate with standard deviations consistently less than 5%. Percent $^{51}Cr$ release was calculated by the following formula:

$$\%\ ^{51}Cr\ \text{release} = \frac{\text{Counts of test supernatant} - \text{spontaneous counts}}{\text{Total counts} - \text{spontaneous counts}}$$

We consider less than 5% $^{51}Cr$ release in 10 hr as nonsignificant in these experiments. When anti-LT sera were employed, percent inhibition of $^{51}Cr$ release was calculated by the following formula:

$$\%\ \text{Inhibition} = \frac{\%\ ^{51}Cr\ \text{release} + \text{NRS} - \%\ ^{51}Cr\ \text{release} + \text{Ab}}{\%\ ^{51}Cr\ \text{release} + \text{NRS}}$$

## RESULTS

*1. Specific lysis of $^{51}Cr$-labeled allogeneic target cells by supernatants collected from nonspecifically activated alloimmune murine lymphoid cells in vitro.* Experiments were designed to determine if alloimmune murine lymphoid cells could be induced to release soluble products with the capacity to cause specific lysis of target cells. Immune murine spleen cells were activated on monolayers of PHA coated L-929 cells for the following reasons: a) this induces high levels of LT release; b) if LT molecules *could* associate with receptor molecules having specificity for a given alloantigen, then theoretically the stimulating cell(s) should *not* possess the same H-2 haplotype as the specific target, because this would presumably result in the absorption of receptor-LT complex activity, and it would not be detectable in the supernatant; and c) it has been reported that alloimmune T lymphocytes can be nonspecifically activated in a secondary reaction by nonrelated target cells or lectins to become fully specifically cytotoxic (12–14).

C57BL/6 mice were immunized with the P815 mastocytoma and their spleen cells harvested 11 days later. Nonadherent lymphocyte-rich cells from immune or nonimmune animals were activated on monolayers of PHA-P coated L-929 cells (PHA/L cells) at a 50:1 ratio for 8 hr at 37°C, as described in *Methods*. The supernatants were collected and immediately tested for LT activity on L-929 cells (18 hr assay) or $^{51}Cr$ release on P815 target cells during an 8 to 10 hr microplate assay. Shown in Figure 1 is the result of an experiment in which fresh supernatants collected from P815 immune C57BL/6 lymphocytes were tested on $^{51}Cr$ labeled P815 cells. As can be seen, significant $^{51}Cr$ release (9%) occurred from these target cells by 5 hr of incubation at 37°C, which increased to 21% by 10 hr.

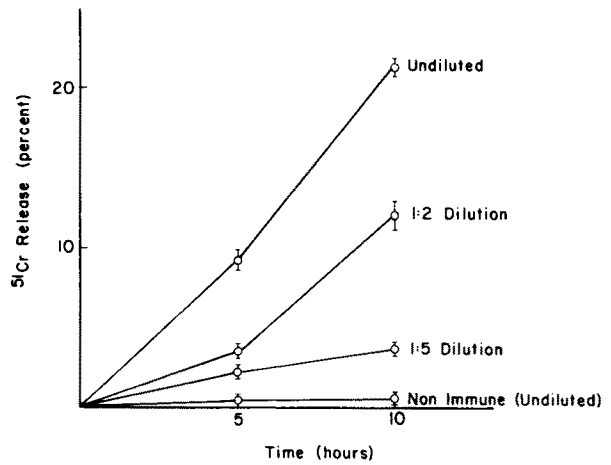


*Figure 1.* Lysis of $^{51}$Cr-labeled P815 target cells by supernatants collected from PHA/L cell activated alloimmune C57BL/6 lymphocytes *in vitro*. Normal or P815 alloimmune C57BL/6 lymphocytes were activated on PHA/L cell monolayers for 8 hr, and various amounts of the supernatant were tested for lytic activity on $^{51}$Cr-labeled P815 target cells, as described in *Materials and Methods*.

Furthermore, this effect was removed by dilution of the supernatant. In contrast, supernatants collected from activated nonimmune C57BL/6 lymphoid cells were totally ineffective at inducing $^{51}$Cr release. However, as shown in Table I, supernatants collected from immune or nonimmune lymphoid cells contain similar levels of nonspecific LT activity detected on L-929 cells *in vitro*. Also shown in Table I are the cumulative results of 22 separate experiments. In 15 of 22 experiments, significant specific target cell lysis (>5%) was observed with supernatants collected from immune lymphoid cells. Many of these supernatants were also simultaneously tested for specificity of cell lysis by incubation on nonrelated or syngeneic target cells. Only insignificant (<5%) to low levels (5 to 8%) of nonspecific lysis were detected when supernatants from immune mice were tested on nonrelated lymphoid targets (i.e., anti-P815 tested on EL4 and *vice versa*), or when nonimmune supernatants were tested on either lymphoid target. However, supernatants collected from immune lymphoid cells were capable of causing significant (5 to 39%) $^{51}$Cr release on the specific target. In several experiments, we found that release of specific supernatant lytic activity only occurred when the aggressor lymphocytes were cultured in the presence of lectin-coated allogeneic or xenogeneic monolayers and was *not* detected with lectin stimulation alone. However, on several occasions, we detected specific cell lytic materials from cultures of alloimmune cells in the presence of nontarget, nonlectin-coated allogeneic cells. We could not demonstrate the presence of these specific toxic material(s) when alloimmune lymphocytes were cultured together with their respective target cells.

2. *Kinetics of lysis of specific and nonrelated target cells by supernatants collected from activated alloimmune murine lymphocytes in vitro.* Initial experiments revealed that the specificity and degree of cell lysis induced by these supernatants was related to the length of the cytolytic assay. To investigate this finding further C3H/DiSn(H2$^k$) were immunized with P815 (H2$^d$) or EL4 (H2$^b$) tumor cells, the immune spleen cells collected after 12 days, and the lymphocytes were activated on monolayers of PHA-coated 3T3 cells, as described in *Methods*. We chose 3T3 monolayer cells for these experiments, because they gave a consistently better response than PHA/L cell monolayers when employed with lymphoid cells from C3H mice. Shown in Figure 2 is a representative result of three such experiments. Two important points should be made from these experiments. First, C3H alloimmune spleen cells were activated to release supernatant lytic activity that only lysed the specific sensitizing target cell. This suggests that these lytic effects were not due to the sensitivity of a particular target cell to "nonspecific" effects. Secondly, since these data also reveal that specificity of lysis was clearly time dependent, supernatant-induced cell lysis was specific only up to 8 to 10 hr of incubation on these target cells. After this time (i.e., 12 to 16 hr) cell lysis also became apparent in the cultures of nonrelated target cells. Furthermore, in data that are not presented here, we have observed low levels of nonspecific lysis (5 to 10%) of both P815 and EL4 target cells when nonimmune supernatants are assayed on these cells for longer (15 to 18 hr) intervals.

3. *Kinetics of release of specific lytic activity from alloimmune C57BL/6 lymphocytes activated on monolayers of PHA coated L-929 cells in vitro.* The kinetics of release of soluble material(s) causing specific lysis of P815 cells *in vitro* was investigated. Normal or P815 alloimmune C57BL/6 NA splenic lymphocytes were activated on monolayers of PHA-coated L-929 cells for various periods of time at 37°C, and the supernatants were collected and immediately tested for lytic activity on $^{51}$Cr-labeled P815 target cells during a 10-hr microplate assay. As can be seen in Figure 3, significant release of lytic activity occurs after 3 hr, peaks by 6 to 9 hr, and declines at times thereafter. After 24 hr of incubation, specific lytic activity is virtually undetectable (data not shown). In addition, similar kinetic curves have been observed with alloimmune C57BL/6 anti-P815 or DBA/2 anti-EL4 effector lymphocytes *in vitro*.

4. *Specific removal of nonspecific LT activity by absorption of immune supernatants on the specific allogeneic target cells in vitro.* The next experiments were designed to test the concept that LT molecules were associated with specific Ig or Ig-like receptor molecule(s) having specificities for a given alloantigen. If this were true, then absorption of immune supernatants on the specific sensitizing target cell should remove a certain degree of nonspecific LT activity detectable on L-929 cells. Normal or alloimmune C57BL/6 or DBA/2 NA lymphocytes or alloimmune C57BL/6 T cells were activated on PHA/L cell monolayers, and the cell-free supernatants were collected and treated as follows: 1 ml of supernatant was incubated with various numbers of specific or nonrelated target cells or left untreated for 30 min at 4°C. The cells were then removed by centrifugation and the supernatants were tested for "nonspecific" LT activity on mitomycin C-treated L-929 cells. Shown in Table II are data of five separate experiments. It is clear that absorption of alloimmune C57 anti-P815 or DBA/2 anti-EL4 supernatants on the specific sensitizing target cell removes LT activity detectable on L-929 cells *in vitro*. Furthermore, removal of LT activity was dose dependent, reaching a plateau at about 45%, when $20 \times 10^6$ P815 cells were employed. However, at very high levels of absorbing cells (i.e., $10^8$), we did begin to observe nonspecific removal of LT activity. In contrast, absorption of these same "immune" supernatants or supernatants from nonimmune lymphoid cells with $20 \times 10^6$ nonrelated target cells had little effect.

5. *Stability of specific lytic activity present in PHA/L cell-activated alloimmune supernatants at various temperatures.* Supernatants from PHA/L cell activated C57 anti-P815 NA splenic lymphocytes were collected and *immediately* tested for lytic activity on $^{51}$Cr-labeled P815 target cells during an 8-hr assay at 37°C, or allowed to incubate at 4 or 37°C for 30 min, 1, 2, or 4 hr. After those time periods, they were then again tested for lytic effectiveness on $^{51}$Cr-labeled P815 during an 8-

TABLE I

*Capacity of immune murine lymphoid cells and supernatants from nonspecifically activated immune and nonimmune murine lymphoid cells to induce lysis of specific and nonrelated target cells in vitro*[a]

| Expt. No. | Effector Cells | Method of Nonspecific Activation | Supernatant Concentration | Supernatant Killing (Microplate Assay) % $^{51}$Cr Release on: P815 | Supernatant Killing (Microplate Assay) % $^{51}$Cr Release on: EL4 | LT Act. (22 hr L cell assay) | Lymphocyte Killing (Direct Kill) |
|---|---|---|---|---|---|---|---|
| | | | | | | *units/ml* | *% $^{15}$Cr release* |
| 1 | C57BL/6 anti-P815 | PHA/L cell | Unconc. | 19 ± 1 | 4 ± 0.1 | 86 | 48 ± 2 |
| | C57BL/6 anti-P815 | PHA/3T3 | Unconc. | 24 ± 1 | 5 ± 0.8 | 94 | |
| | C57BL/6 anti-P815 | PHA (50 μg/ml) | Unconc. | 0 | 0 | 7 | |
| | C57BL/6 anti-P815 | Con A (10 μg/ml) | Unconc. | 0 | 1 | 5 | |
| 2 | C57BL/6 anti-P815 | PHA (50 μg/ml) | 5 × | 0 | 0 | 24 | 42 ± 3 |
| | C57BL/6 anti-P815 | PHA/L cell | 5 × | 21 ± 0.8 | 3 ± 0.6 | 600 | |
| | C57BL/6 nonimmune | PHA/L cell | 5 × | 0 | 0.8 | 510 | 2 ± 0.2 |
| 3 | C57BL/6 anti-P815 | PHA/L cell | 10 × | 16 ± 1 | NT[b] | 710 | NT |
| | C57BL/6 anti-P815 | PHA/3T3 | 10 × | 19 ± 0.8 | NT | 805 | |
| | C57BL/6 nonimmune | PHA/3T3 | 10 × | 1 | NT | 690 | |
| 4 | C57BL/6 anti-P815 | PHA/L cell | Unconc. | 12 ± 0.2 | 2 ± 0.3 | NT | 69 ± 4 |
| | (T cells) anti-P815 | PHA/L cell | 10 × | 28 ± 1 | 5 ± 0.7 | NT | |
| | (T cells) nonimmune | PHA/L cell | 10 × | 1.4 ± 0.1 | 2.6 ± 0.1 | NT | 2 ± 0.1 |
| 5 | C57BL/6 anti-P815 | PHA/L cell | Unconc. | 2 ± 1 | 1 ± 0.6 | 20 | 16 ± 4 |
| 6 | C57BL/6 anti-P815 | PHA/L cell | 8 × | 0 | 0 | 105 | 12 ± 1 |
| 7 | C57BL/6 anti-P815 | PHA/L cell | 15 × | 21 ± 1 | 6 ± 0.5 | 945 | 56 ± 2 |
| 8 | C57BL/6 anti-P815 | PHA/L cell | Unconc. | 0 | 0 | 10 | 14 ± 0.4 |
| 9 | C57BL/6 anti-P815 | PHA/L cell | Unconc. | 9 ± 0.8 | 2 ± 0.4 | 60 | 40 ± 2 |
| 10 | C57BL/6 anti-P815 | PHA/L cell | 10 × | 39 ± 2 | 8 ± 0.4 | 395 | 81 ± 0.9 |
| 11 | C57BL/6 anti-P815 | PHA/L cell | Unconc. | 0 | 0 | 14 | 18 ± 0.8 |
| | (T cells) nonimmune | PHA/L cell | Unconc. | 0 | 0 | 22 | NT |
| 12 | C57BL/6 anti-P815 (T cells) | PHA/L cell | 2.5 × | 14 ± 0.8 | 2 ± 0.1 | NT | NT |
| 13 | DBA/2 anti-EL4 | PHA/L cell | 10 × | 7 ± 0.6 | 36 ± 2 | 900 | 49 ± 2 |
| | DBA/2 nonimmune | PHA/L cell | 10 × | 0 | 0 | 820 | 3 ± 0.2 |
| 14 | DBA/2 anti-EL4 | PHA/L cell | Unconc. | 3 ± 0.4 | 10 ± 0.4 | 96 | NT |
| 15 | DBA/2 anti-EL4 | PHA/L cell | 5 × | NT | 22 ± 0.8 | NT | NT |
| 16 | DBA/2 anti-EL4 | PHA/L cell | Unconc. | 3 ± 0.6 | 5 ± 0.1 | 21 | 12 ± 0.8 |
| 17 | DBA/2 anti-EL4 | PHA (50 μg/ml) | Unconc. | 0 | 1 | 6 | NT |
| | (T cells) EL4 | PHA/L cell | Unconc. | 3 ± 0.6 | 23 ± 1 | 95 | NT |
| | (T cells) nonimmune | PHA/L cell | Unconc. | 0 | 1 ± 0.1 | 75 | NT |
| 18 | C3H/DiSn anti-P815 | PHA/L cell | 5 × | 3 ± 0.1 | 2 ± 0.2 | NT | NT |
| | C3H/DiSn anti-P815 | PHA/3T3 | 5 × | 18 ± 1 | 3 ± 0.1 | NT | NT |
| | C3H/DiSn nonimmune | PHA/3T3 | 5 × | 0 | 0 | NT | NT |
| 19 | C3H/DiSn anti-EL4 | PHA/L cell | Unconc. | 0 | 0 | NT | NT |
| | C3H/DiSn anti-EL4 | PHA/L cell | 10 × | 3 ± 0.1 | 10 ± 0.7 | NT | NT |
| 20 | C3H/DiSn anti-EL4 | PHA/L cell | 5 × | 2 ± 0.1 | 9 ± 0.4 | 290 | 40 ± 0.9 |
| | C3H/DiSn anti-EL4 | PHA/3T3 | 5 × | 2 ± 0.2 | 16 ± 1 | 386 | |
| 21 | C3H/DiSn anti-EL4 | PHA/HeLa | 10 × | 2 ± 0.1 | 18 ± 2 | NT | NT |
| | (T cells) nonimmune | PHA/HeLa | 10 × | 1 ± 0.1 | 1 ± 0.1 | NT | |
| 22 | C3H/DiSn anti-P815 | PHA/3T3 | 10 × | 15 ± 1 | 4 ± 1 | 780 | 54 ± 3 |
| | (T cells) nonimmune | PHA/3T3 | 10 × | 0 | 2 ± 0.1 | 650 | |

[a] NA alloimmune spleen cells or purified alloimmune T cells or normal controls from various strains of mice were activated by various techniques as indicated for 8 hr at 37°C, as described in *Materials and Methods*. Supernatants were collected and tested for a) LT activity on L929 cells during a 16-hr assay as described; or b) $^{51}$Cr release on P815 or EL4 target cells during a 9-hr microplate assay. Also indicated is the % $^{51}$Cr release by freshly isolated NA alloimmune spleen cells or T cells assayed only on the sensitizing target cell at a 10:1 ratio during a 4-hr microplate assay at 37°C.

[b] NT, not tested.

hr assay. As can be seen in Figure 4, anti-P815 lytic activity is extremely unstable at both 4 and 37°C. Seventy-five or 90% of the lytic activity is lost after incubation of these supernatants for 1 hr at 4 or 37°C, respectively. Virtually 100% of this activity is lost after 2 hr of incubation at either temperature. These results are the average values obtained from six separate experiments. In no case was the lytic activity stable for more than 10 to 20 hr, even at 4°C. When supernatants are immediately frozen at −70°C, upon thawing, the lytic activity is lost within a few hours (data not shown).

*6. Identification of the cell population responsible for the release of specific lytic activity in vitro.* Spleen cells from alloimmune C57BL/6 mice were collected and subjected to the following cell separation procedures. Spleen cells were allowed to adhere to glass to remove glass adherent cells, and either 1) the NA cells were treated with anti-$\theta$ serum, plus guinea pig C to remove $\theta^+$ cells (T cells), or 2) lymphocytes were passed over nylon wool columns to yield T cell-enriched populations. These T cells were assessed for contaminating surface Ig-positive B cells by using a fluorescently (FITC) tagged polyvalent rabbit anti-mouse Ig serum as described in *Methods*. The purity of these nylon wool-purified T cells in three experiments was 95

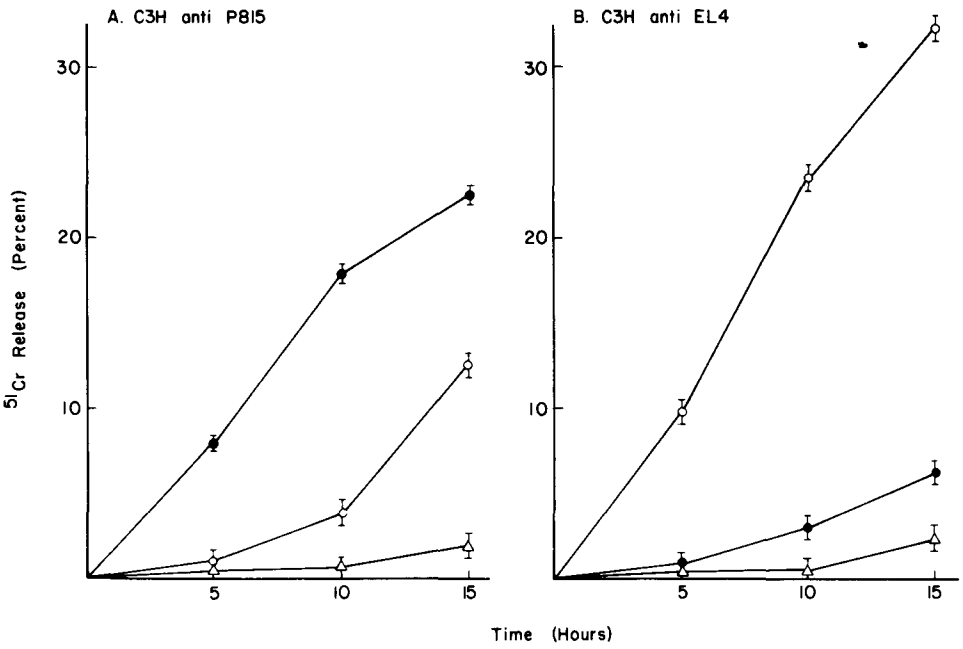


*Figure 2.* Kinetics of lysis of $^{51}$Cr-labeled P815 or EL4 target cells by supernatants collected from activated alloimmune C3H anti-P815 (Fig. 2*A*) or C3H anti-EL4 (Fig. 2*B*) lymphocytes *in vitro*. Nonadherent lymphocytes collected from normal or P815 or EL4 alloimmune C3H/DiSn spleen cells were activated on PHA/L cell monolayers for 8 hr. The supernatants were then collected, concentrated 5×, and 100 μl tested for lytic activity on $^{51}$Cr-labeled P815 or EL4 targets for the various periods of time indicated. ●——●, Lysis of $^{51}$Cr-labeled P815 target cells by "immune supernatants"; ○——○, lysis of $^{51}$Cr-labeled EL4 target cells by "immune supernatants"; △——△, lysis of $^{51}$Cr-labeled P815 or EL4 target cells by "nonimmune supernatants.

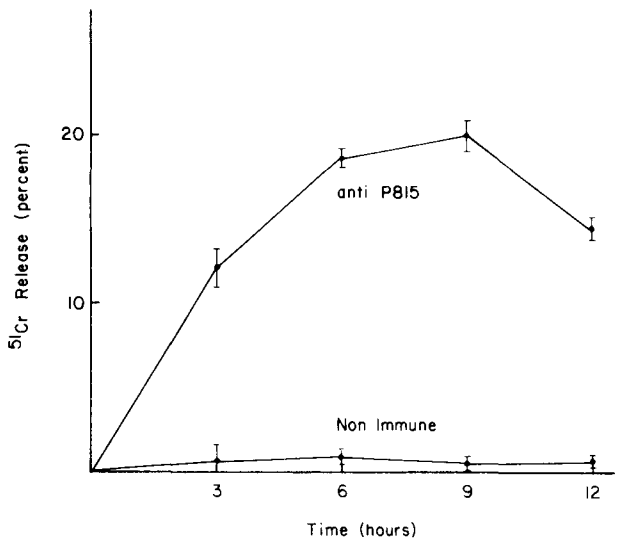


*Figure 3.* Kinetics of release of specific supernatant lytic activity from activated P815 alloimmune C57BL/6 lymphocytes *in vitro*. Nonadherent lymphocytes from normal or P815 alloimmune C57BL/6 mice were activated on PHA/L cell monolayers for various periods of time as indicated. The cellfree supernatants were then tested for lytic activity on $^{51}$Cr-labeled P815 target cells during a 10-hr microplate assay.

to 97%. As can be seen in Table III, in three separate experiments, the specific lytic activity was obtained with alloimmune splenic T lymphocytes. Although lytic activity was present in supernatants of unseparated spleen, NA splenic lymphocytes, and nylon wool-purified (96% pure) T cells, it was removed when the cells were treated with anti-$\theta$ serum + C.

*7. Evidence that the lytic activity present in alloimmune supernatants is due to LT or "LT-like" molecules.* Supernatants collected from activated P815 alloimmune C57BL/6 lymphocytes, as already described, were assayed for lytic activity on $^{51}$Cr-labeled P815 targets in the presence of various amounts of: 1) normal rabbit serum (NRS); 2) rabbit anti-C57BL/6 whole supernatant (anti-WS); or 3) anti-C57 $\alpha_H$ serum (anti-$\alpha_H$); or 4) no added serum as controls. As can be seen in the data presented in Table IV, incubation with anti-WS or anti-$\alpha_H$ sera will effectively inhibit lytic activity in this system. Anti-WS serum inhibited lysis up to 100% (1:8 final serum dilution), anti-$\alpha_H$ inhibited up to 87% (1:2 final serum dilution), and NRS

TABLE II

*Specific absorption of nonspecific LT activity detectable on L-929 cells by treatment of immune supernatants with specific target cells in vitro*[a]

| Expt. No. | Responder Lymphocyte | No. of Absorbing Cells Used/Ml | % of LT Activity Absorbed on[b] | | |
|---|---|---|---|---|---|
| | | | P815 | EL4 | L-929 |
| 1 | C57BL/6 anti-P815 | $2 \times 10^6$ | *19 ± 1* | 0 ± 0.5 | 1 ± 0.5 |
| | C57BL/6 anti-P815 | $10 \times 10^6$ | *39 ± 3* | 1 ± 1 | 3 ± 0.5 |
| | C57BL/6 anti-P815 | $20 \times 10^6$ | *45 ± 3* | 5 ± 0.5 | 5 ± 0.5 |
| | C57BL/6 nonimmune | $10 \times 10^6$ | 5 ± 3 | 11 ± 2 | 4 ± 0.2 |
| 2 | C57BL/6 anti-P815 T lymphocytes | $10 \times 10^6$ | *37 ± 3* | 5 ± 0.1 | 0 ± 0.1 |
| | C57BL/6 nonimmune T lymphocytes | $10 \times 10^6$ | 8 ± 0.2 | 9 ± 0.5 | 4 ± 0.1 |
| 3 | C57BL/6 anti-P815 | $10 \times 10^6$ | *50 ± 3* | 7 ± 0.5 | 4 ± 0.5 |
| 4 | DBA/2 anti-EL4 | $10 \times 10^6$ | 5 ± 1 | *40 ± 3* | 3 ± 0.1 |
| 5 | DBA/2 anti-EL4 | $10 \times 10^6$ | 5 ± 0.1 | *46 ± 2* | 3 ± 0.1 |

[a] Supernatants were collected from normal or alloimmune C57BL/6 anti-P815 or DBA/2 anti-EL4 nonadherent lymphocytes or alloimmune C57BL/6 anti-815 T cells after 9 hr of incubation on monolayers of PHA-coated L-929 cells as described in *Materials and Methods*. One milliliter of these monolayers of PHA-coated L-929 cells was then added to various numbers of packed P815, EL4, or L-929 cells or left untreated, and the mixtures were allowed to incubate at 4°C for 30 min. The cells were then removed by centrifugation and the number of units of LT activity in the cellfree supernatants was determined as described in *Materials and Methods*.

[b] The total number of LT units/ml in supernatants was Expt. 1—126, 2—163, 3—76, 4—142, and 5—97. Average per cent of LT bound in all experiments was: a) immune supernatant on specific targets—39.42%; b) immune supernatant on non-targets—3.39%, and c) nonimmune supernatant on all targets—6.83%.

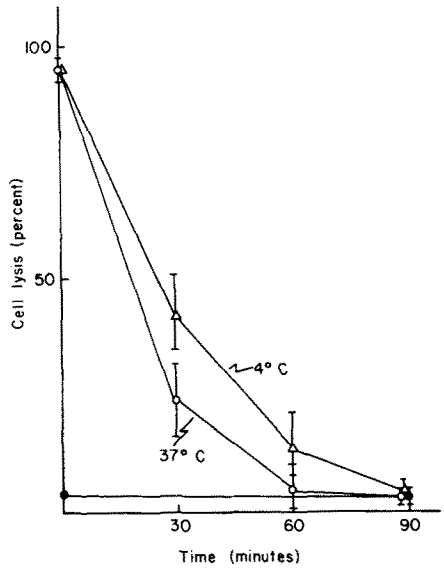


*Figure 4.* Stability of specific supernatant lytic activity at 4 or 37°C. Supernatants collected from PHA/L cell-activated P815 alloimmune C57BL/6 lymphocytes were immediately tested for lytic activity on $^{51}$Cr-labeled P815 cells or allowed to incubate at 4° or 37°C for various periods of time as indicated, and then tested for P815 lytic activity.

TABLE III

*Evidence that the soluble specific P815 cell lytic activity released in vitro by P815 alloimmune C57BL/6 spleen cells requires T lymphocytes*[a]

| Treatment of Lymphoid Cells | % $^{51}$C5 Release from P815 Targets | | | | | |
|---|---|---|---|---|---|---|
| | Expt 1 | | Expt. 2 | | Expt. 3 | |
| | I | NI | I | NI | I | NI |
| Unseparated spleen | 18 ± 1 | 0 | | | 23 ± 1 | |
| Non-glass adherent lymphocytes | 28 ± 3 | 1 | 15 ± 1 | 1 | 30 ± 1 | 1 |
| NGA Ly + anti-$\theta$ + C | 4 ± 0.8 | 0 | 2 ± 0.1 | | | |
| NGA nylon wood purified (T cells) | 21 ± 2 | 1 | 19 ± 2 | 0 | 29 ± 1 | 1 |

[a] P815-immune C57BL/6 spleen cells were collected 11 days postimmunization and treated as follows: a) the cells were placed on glass bottles in 10% FCS at 37°C and the nonadherent cells collected after 1½ hr (non-glass adherent (NGA) lymphocytes); b) the cells were treated with anti-$\theta$ serum (1:25) plus fresh guinea pig serum (1:10) as a source of C for 1 hr at 37°C; or c) NGA lymphocytes were passed over nylon wool columns as described in *Materials and Methods* to yield T cell enriched populations. Each of these populations was then stimulated on PHA-P-coated L-929 cells for 9 hr at 37°C, and the cellfree supernatants were immediately collected and tested on $^{51}$Cr-labeled P815 targets during an 8-hr $^{51}$Cr release assay.

inhibited up to 13% (1:2 final serum dilution). Furthermore, inhibition of lytic activity by anti-WS or anti-$\alpha_H$ serum was equally effective when these sera were previously absorbed on P815 target cells or on affinity beads containing normal mouse serum proteins. Thus, these blocking effects could not be localized to interaction of these sera with target cell antigens or serum proteins.

*8. Evidence that soluble anti-P815 lytic activity released by P815 immune spleen cells or isolated T cells is not due to classical Ab + C.* Three different types of experiments were performed to examine whether these effects were due to classical Ab + C: 1) populations of T-enriched alloimmune lymphoid cells were used as a source of responding cells; 2) since the lytic activity was highly unstable at 4 or 37°C, possibly due to loss

TABLE IV

*Effect of heterologous rabbit anti-mouse LT sera on the soluble specific cell lytic activity from cultures of activated alloimmune C57BL/6 spleen cells or purified T cells in vitro*[a]

| Supernatant Source | Treatment | % Inhibition of $^{51}$Cr Release (P815)[b] (Expts. 1 to 3) |
|---|---|---|
| NA lymphocytes | No added serum | 0 |
| NA lymphocytes | NRS (100 µl) | 10 ± 3 |
| NA lymphocytes | Anti-WS (25 µl) | 99 ± 1 |
| NA lymphocytes | Anti-$\alpha_H$ (100 µl) | 80 ± 5 |
| NA lymphocytes | NRS[c] (100 µl) | 5 ± 5 |
| NA lymphocytes | Anti-WS[c] (25 µl) | 95 ± 5 |
| NA lymphocytes | Anti-$\alpha_H$[c] (100 µl) | 79 ± 8 |
| T cells | NRS (100 µl) | 10 ± 5 |
| T cells | Anti-WS[c] (25 µl) | 93 ± 2 |
| T cells | Anti-$\alpha_H$[c] (100 µl) | 75 ± 5 |

[a] On day 11, spleen cells were collected from P815 immunized C57BL/6 mice, allowed to adhere to glass (NA lymphocytes) or passed over nylon wool columns to yield T cell enriched populations. Each of these populations was then placed on PHA-P coated L-929 monolayers as described in *Materials and Methods.* Cellfree supernatants were then collected and assayed for toxic activity on 25,000 $^{51}$Cr P815 target cells during a 10-hr assay. The sera employed had no effect on the spontaneous release of $^{51}$Cr from the target cells.

[b] Per cent $^{51}$Cr release calculated from the average values of three separate experiments. Total release induced by untreated immune supernatants in each experiment was 28%, 14%, and 26%, respectively.

[c] These sera were sequentially absorbed on NMS affinity beads and $10^8$ P815 target cells/ml serum before use. The treatments had no demonstrable effect on the LT-neutralizing capacity of the anti-WS or anti-$\alpha_H$ sera.

of unstable C components, we allowed cytolytically active supernatants to become inactive by incubation at 37°C for 2 hr, then attempted to reconstitute the lytic activity with fresh sources of C; and 3) a commercially available polyspecific rabbit anti-mouse globulin serum was employed to test if classical globulin mediated these effects. Shown in Table V are the averages of four experiments. As can be seen when supernatants collected from alloimmune C57 anti-P815 lymphocytes or nylon wool column purified T cells were tested on $^{51}$Cr-labeled P815 target cells during an 8-hr $^{51}$Cr release assay, 15 to 27% lysis occurred. Furthermore, when supernatants from these two cell sources were allowed to incubate for 2 hr at 4°C, a complete loss of lytic activity was observed. When these inactive supernatants were tested in the presence of fresh guinea pig serum or normal C57 mouse serum as sources of C, no reconstituted lytic activity was observed. As a positive C control, C57BL/6 anti-P815 serum, although not cytotoxic to P815 target cells by itself, caused 83% lysis of these cells in the presence of a 1/10 final dilution of guinea pig C during a 2-hr incubation at 37°C. Finally, anti-mouse immunoglobulin had little or no effect on inhibition of lysis when a 1/10 dilution was used.

*9. Evidence that soluble anti-P815 lytic activity is present in the high m.w. LT complex fraction after separation by gel filtration chromatography with Ultrogel AcA 44.* A 2.5 x 75 cm column was packed to a bed height of 55 cm with Ultrogel AcA 44 and equilibrated in RPMI 1640, pH 6.8. This experiment was coordinated in such a way that minimal time delay occurred between collection of supernatants, concentration, column chromatography, and assay on target cells. Thus, alloimmune supernatants were collected, immediately concentrated (employing Amicon PM30 membranes) (200 ml of supernatant could be concentrated to 4 ml in approximately 30 min), and 2.5 ml were

TABLE V

*Relationship of soluble specific P815 cell lytic activity released by P815 alloimmune spleen cells or isolated T cells to classical Ab + C*[a]

| Supernatant Source | Treatment | C Source | % $^{51}$Cr Release[b] (P815) |
|---|---|---|---|
| C57 anti-P815 (NA Ly) | | | 23 ± 4 |
| C57 anti-P815 (NA Ly) | R α mouse Ig (1:4) | | 19 ± 3 |
| C57 anti-P815 (NA Ly) | 4°C—2 hr | | 2 ± 1 |
| C57 anti-P815 (NA Ly) | 4°C—2 hr | Guinea pig serum | 1 ± 0.2 |
| C57 anti-P815 (NA Ly) | 4°C—2 hr | Normal C57 serum | 1 ± 0.3 |
| C57 anti-P815 (T cells) | | | 19 ± 4 |
| C57 anti-P815 (T cells) | 4°C—2 hr | | 1 ± 0.5 |
| C57 anti-P815 (T cells) | 4°C—2 hr | Guinea pig serum | 2 ± 0.5 |
| C57 anti-P815 (T cells) | 4°C—2 hr | Normal C57 serum | 1 ± 0.5 |
| C57 anti-P815 (T cells) | R α mouse Ig (1:4) | | 18 ± 2 |
| C57 anti-P815 hyperimmune serum (1:10) | 56°—1 hr | | 0 |
| C57 anti-P815 hyperimmune serum (1:10) | 56°—1 hr | Guinea pig serum | 83 ± 7 |

[a] Alloimmune C57BL/6 spleen cells and isolated T cells were stimulated for 9 hr on PHA-coated L-929 cells, and the supernatants immediately collected, and 100 μl tested on 25,000 $^{51}$Cr-labeled P815 target cells during an 8-hr assay at 37°C. An aliquot of this same supernatant was heated (37°C, 1 hr) and subsequently tested for lytic activity in the presence or absence of a 1:10 final dilution of guinea pig serum or normal C57 serum as sources of C. As a positive C control, hyperimmune C57 anti-P815 serum (1:10) was tested in the presence of a 1:10 dilution of guinea pig serum during the assay.

[b] Per cent $^{51}$Cr release is the average of four separate experiments.

applied to this column. Fractions were collected at a flow rate of 50 ml/hr (this rate allowed the entire column volume to elute in approximately 5 hr) and alternate fractions were immediately assayed for LT lytic activity on: 1) L-929 target cells; or 2) the fractions were collected into three pools corresponding to Cx (Pool 1), $\alpha_H$ (Pool 2) and $\alpha_L$ plus the rest of the column (Pool 3) as indicated by the horizontal bars in Figure 5. Each pool was concentrated to 2 ml and tested on $^{51}$Cr-labeled P815 or EL4 target cells *in vitro*. As can be seen in the data presented in Figure 5, lytic LT activity detected on L-929 cells eluted from this column in three individual peaks labeled Cx, $\alpha_H$, and $\alpha_L$, as previously noted. In addition, the elution peaks of the reference m.w. markers, blue dextran, IgG, hemoglobin (Hb) and α chymotrypsin (αCT) are indicated by vertical arrows. Individual pools, 1, 2, and 3, were tested on $^{51}$Cr-labeled P815 or EL4 targets during a 10-hr microplate assay. The only significant cell lytic activity was present in the Cx fraction (Pool 1). Finally, when the Cx and $\alpha_H$ pools were assayed on P815 targets in the presence of NRS, anti-WS or anti-$\alpha_H$ serum (1/8 final serum dilution) significant inhibition of lysis was observed (Table VI).

## DISCUSSION

*LT appear to form a complex but interrelated system of cell lytic molecules.* Biochemical and immunologic studies of various LT forms obtained from human, mouse, and other animal species suggest they are components of a common system of interrelated subunits that can form high m.w. complexes via noncovalent association (2-6). Moreover, it is apparent that certain forms of human LT complex have the capacity to functionally associate with an antigen-binding receptor(s) (7). Studies of individual m.w. classes of human LT molecules reveal the smaller forms (α, β, and γ) are only weakly lytic or growth inhibitory to most cells, whereas the higher m.w. forms are capable of inducing nonspecific lysis of different cell types *in vitro* (9). In addition, these studies revealed that complexes,

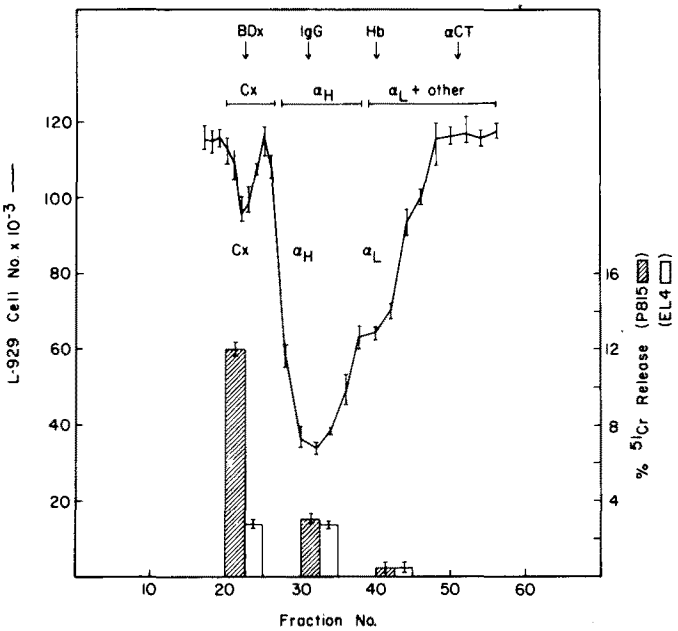


*Figure 5.* Gel filtration chromatography of material(s) having specific anti-P815 cell lytic activity or nonspecific LT activity of Ultrogel AcA 44. Supernatants were collected from P815 alloimmune C57BL/6 lymphocytes after 7½ hr of PHA/L cell activation, concentrated 40×, and 2 ml applied to a 2.5- × 55-cm Ultrogel AcA 44 column. Fractions were collected and assayed for LT activity on mitomycin C-treated L-929 cells and then gathered into three pools, corresponding to the Cx, $\alpha_H$, and $\alpha_L$ plus the rest of the column. Each pool was then concentrated to 1 ml, and 100 μl was tested for cell lytic activity on $^{51}$Cr-labeled P815 or EL4 target cells during a 10-hr microplate assay.

TABLE VI

*Effect of heterologous rabbit anti-LT serum on the specific in vitro lysis of $^{51}$Cr-labeled P815 target cells mediated by high MW supernatant components released by activated alloimmune murine lymphoid cells*[a]

| Fraction Tested | % $^{51}$Cr Release in the Presence of: NRS (100 μl) | Anti-WS (25 μl) | % *Inh.* | Anti-α (100 μl) | % *Inh.* |
|---|---|---|---|---|---|
| C57 anti-P815 whole supernatant (50 × conc.) (25 μl) | 40 ± 2 | *12 ± 1* | (65) | *19 ± 3* | (50) |
| Pool 1 (Cx) (100 μl) | 11 ± 1 | *2 ± 1* | (85) | *3 ± 1* | (75) |
| Pool 2 ($\alpha_H$) (100 μl) | 2 ± 1 | 0 | (100) | 0 | (100) |
| Pool 3 ($\alpha_L$ + Rest) (100 μl) | 0 | 0 | | 0 | |

[a] Pooled fractions containing Cx, $\alpha_H$, or $\alpha_L$ m.w. LT classes were obtained after gel filtration chromatography on Ultrogel AcA 44 pooled fractions as indicated (Fig. 5). Each m.w. LT class or the unfractionated whole supernatant was then tested on $^{51}$Cr-labeled P815 targets in the presence of NRS, anti-WS, or anti-$\alpha_H$ serum during a 10-hr microplate assay.

in association with target cell-specific receptor(s), released by lectin-activated alloimmune human lymphoid cells, were most lytically active on the specific sensitizing cells *in vitro*. These studies provided the first evidence to suggest that nonspecific LT molecules, when functionally associated with target cell specific receptors might be capable of inducing "specific" cell destruction. We decided to test the possibility that alloimmune murine lymphoid cells could be activated *in vitro* to release LT forms with capacity to cause specific cell destruction, for the following reasons: a) the physical similarity of human and murine LT forms; b) the well defined alloimmune murine lymphocyte-killing reactions *in vitro*; and c) development of a method for inducing high levels of LT activity from murine cells.

*The present results indicate there are different types of cell lytic activity detectable in fresh supernatants obtained from nonspecifically activated alloimmune or nonimmune lymphocytes.* As previously reported, all types of lytic activity were highest in cultures activated by co-culture of murine lymphocytes with lectin-treated L929 or 3T3 monolayer cells. Immune lymphoid cells were shown to release somewhat higher levels of nonspecific LT activity than nonimmune cells when 8-hr supernatants were tested on the highly sensitive L929 cell in a 16- to 24-hr assay. The amounts of activity in certain supernatants were quite high, up to 950 units/ml, and activity was detected in all experiments. In contrast, fresh immune supernatants were capable of causing significant specific target cell lysis (5 to 39% $^{51}Cr$ release) in 15 of 22 experiments (Fig. 1) when they were tested in a shorter (6 to 10 hr) assay on P815 or EL4 target cells. Moreover, control experiments (Fig. 2) indicated this appears to be specific lysis and not simply due to a unique sensitivity of a target cell to a nonspecific material(s). Kinetic studies revealed specific supernatant killing activity appears within 2 to 3 hr, peaks by 6 to 9 hr, and rapidly declines thereafter. Specific cell lytic activity was extremely labile with a half-life of 30 to 40 min, and efforts to stabilize activity to date have been unsuccessful. We have no direct explanation for experiments in which we failed to find activity, except results appeared to correlate to low levels of direct cell killing ability by immune lymphocyte populations and failure to process supernatants rapidly. A significant degree of nonspecific lysis of both P815 nd EL4 targets was noted if the assays were continued for 18 to 24 hr (Fig. 2). Thus, fresh immune supernatants possess labile, rapid, and specific cell lytic component(s) and both immune and nonimmune supernatants contain slow killing nonspecific components moderately effective on P815 and EL4, and highly cell lytic for murine L929 cells.

*Physicochemical and immunologic studies suggest the cell lytic activity present in supernatants from immune or nonimmune murine cells is due to various LT forms.* The previous study indicated nonspecific L cell lytic activity in similar supernatants from nonimmune lymphoid cells was due to various m.w. classes of LT molecules (2). The nonspecific cell lytic molecules in immune supernatants appear similar, since they fall into the same m.w. classes, and while not shown in this report, they are neutralized by anti-$\alpha_H$ sera. It appears that the short-lived specific cell lytic material(s) released by activated immune cells is a high m.w. LT form in functional association with a specific antigen-binding receptor(s). Physical evidence that murine LT molecules and specific receptor molecules were functionally linked in these supernatants was shown by absorption of alloimmune T cell supernatants with the specific sensitizing target cells that removed up to 56% of the total detectable nonspecific supernatant LT activity measured on L929 cells *in vitro*. Furthermore, this absorption was antigen specific since treatment of the same supernatants with similar numbers of nonrelated lymphoid target cells or on L929 cells caused little or no reduction in detectable LT activity. These absorptions were performed at 4°C to minimize loss of LT activity due to "nonspecific" processes, such as cell surface protease activity or pinocytosis. That the specific lysis was due to high m.w. forms was evident, since activity eluted from molecular sieving columns in the void volume or in the region of the high m.w. LT complex forms. Immunologic association of LT with specific killing was supported by the finding that anti-LT sera of several types, which are potent inhibitors of soluble LT activity *in vitro*, blocked specific lysis mediated by these alloimmune supernatants or by the high m.w. Ultrogel fractions. These same antisera could not be shown to react with normal mouse serum proteins. Therefore, we feel they are not inhibiting specific lysis by interacting with classical serum Ig molecules or serum C components. Because these sera might nonspecifically interfere with cell lysis by binding to the target cell surface, they were absorbed extensively on P815 and EL4 cells, such that no immunofluorescence on these cells was detectable. These procedures neither affected the anti-LT neutralizing capacity of these sera nor their ability to inhibit lysis mediated by these alloimmune supernatants or LT complexes. Finally, specific lysis mediated by these supernatants or high m.w. fractions was extremely unstable, as are murine LT complexes obtained in this system.

*It does not appear that the specific cell lytic activity present in supernatants from nonspecifically activated alloimmune murine lymphoid cells is related to classical Ig and C.* Because these supernatants were generated by lectin-activated immune lymphoid cells and conceivably could contain specific anti-target cell antibody and C, we conducted further experiments to determine if these components were responsible for specific lysis. We feel the data in the preceding paragraph supporting that this effect is mediated by high m.w. LT-receptor complexes do not lend support to the rule of Ig and C. Additional results, which also negate the role of Ig and C as effectors in these supernatants, were the findings that: a) polyvalent rabbit anti-Ig sera did not inhibit specific supernatant lysis; b) the inhibitory effect of the two classes of anti-murine LT sera available was not affected by absorption with normal mouse serum; c) alloimmune supernatant cell lytic activity was not affected by addition of active C components; and d) the appearance of the active material requires T cells. Collectively, we feel these data do not lend support to the concept that traditional Ig or C components are the lytic effectors in these supernatants.

*The release of soluble specific cell lytic material by alloimmune lymphoid cells appears to require unique cellular activating signal(s).* This conclusion is based on the observation that supernatants from alloimmune cells cultured without stimulation, supernatants collected from alloimmune lymphocytes cultured with lectin alone or with specific target cells alone were all ineffective. However, specific lytic complexes were released when alloimmune lymphocytes were placed on lectin treated, nontarget-related allogeneic or xenogeneic cell monolayers. It should be mentioned that nonlectin-treated allogeneic cells were capable of stimulating some release of specific lytic complexes, but they were generally less effective than lectin-treated monolayers. Thus, specific cell lytic complexes were released from alloimmune T cells upon activation by nonspecific means. It is germaine to relate these findings to the results of several other investigators who reported that lectins can stimulate specific alloreactive murine T cells to express both nonspecific and specific cell lytic activity *in vitro* (12, 13). It was also shown that contact with nontarget-related allogeneic cells

induced recall of specific cytotoxic activity of alloimmune murine lymphoid cells (14). Thus, seemingly nonspecific signals can induce alloimmune lymphoid cells to promote specific cell lysis and release specific killer complexes. Because of the unique triggering signals required to cause specific complex release, we feel the lytic complexes may not be normally found in the soluble phase, but rather are delivered to a specific target cell by membrane contact. Although not yet established, it is tempting to speculate that specific cell killing and release of specific killing complexes are related phenomena.

*Other investigators have attempted to demonstrate specific cytolytic materials in supernatants obtained from lymphocyte-killing reactions in vitro.* While there are a few positive reports, the majority of attempts have been negative (15, 16). We believe the present studies may provide some logical reasons why these materials, if released during specific T cell killing reactions, have not been detected previously. Unless supernatants were handled and tested quickly, these forms would have decayed and not been detected. In addition, it appears that such materials, upon release by the immune cell induced by the presence of the specific target cell, would be rapidly bound to the target cell and not be detectable free in the soluble phase. It should be noted that we also were unable to detect specific complexes in supernatants from direct killing reactions. Our ability to reproducibly detect these forms was dependent on a nonspecific signal that triggered release of specific killing complexes by immune cells into the milieu and a testing situation that has been optimized for their detection.

*The physical nature of the receptor(s) associated with the specific complexes in these supernatants is unclear, but appears to be derived from or require the presence of alloimmune T cells.* This conclusion was established on the following considerations: a) supernatants obtained from nylon wool-purified immune T cells, which were 96% surface Ig negative by immunofluorescent staining, were equally as effective as supernatants obtained from unseparated spleen cells; and b) treatment of alloimmune spleen cells with anti-$\theta$ serum plus C abrogated the ability of the remaining cells to release these cytolytic materials. In no cases did we observe specific cytolytic activity in supernatants when nonimmune cells were employed.

*Whether the small m.w. LT forms ($\alpha$, $\beta$, $\gamma$) are the actual lytic material(s) in the high m.w. specific cell-lytic molecules is not clear.* The more effective cell lytic capacity of the complex forms *in vitro* could be due to a number of different mechanisms. One possibility is that the smaller m.w. LT subunits may be focused or nucleated at a discrete site(s) and thus become more effective due to a high local concentration. Another possibility is that together these molecules induce cell lysis by a synergistic mechanism, perhaps different from that induced by the smaller m.w. subunits alone. It is clear that complex forms, when functionally associated with a target cell-specific receptor(s), can cause much more rapid and effective cell lysis than complexes lacking specific antigen receptors. However, forms of complex-containing receptors that do not recognize the target cell or forms that are apparently devoid of receptors appear to be equally effective at causing nonspecific protracted lysis. Thus, the presence of a receptor(s) *per se* does not make the complex forms more effective, unless it recognizes the specific target cell antigen, and by virtue of its antigen-binding site apparently can specifically direct the lytic potential of these molecules.

*The experiments presented in this series of manuscripts taken collectively indicate the following new concepts.* First, LT molecules released *in vitro* by activated lymphoid cells are physically heterogeneous and represent a related system of subunits that have been conserved through evolution because similar m.w. forms can be found in many animal species. The subunits have the capacity to form high m.w. complexes, which in turn can functionally associate with antigen-binding receptor(s). The smaller m.w. forms of these molecules appear to be weakly active. They are nonspecific and detectable only on certain indicator target cells *in vitro.* The most lytically effective LT forms appear to be those present in the high m.w. classes, which if in functional association with receptor molecule(s), can become highly effective and directed by the specificity of the receptor with which they are associated. These findings are new and describe a unique lymphoid cell-associated lytic system. Although not clearly proven, the extreme instability of the highly effective complex LT forms may be due to dissociation into subunits that are less effective. To define the role of these molecules in lymphocyte-killing reactions will require that we understand at the molecular level the nature of the components involved, the effector cells involved, and the events leading to the delivery of these molecules to a susceptible target cell.

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