0022-1767/81/1265-1934$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 126, No. 5, May 1981
Printed in U.S.A.

MECHANISMS OF LYMPHOCYTE-MEDIATED CYTOTOXICITY

III. Characterization of the Mechanism of Inhibition of the Human Alloimmune Lymphocyte-Mediated Cytotoxic Reaction by Polyspecific Anti-lymphotoxin Sera *in Vitro*[1]

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The mechanism of inhibition of one human cytotoxic T lymphocyte-mediated (CTL) reaction *in vitro* by a polyspecific antiserum directed against soluble products of activated lymphocytes was investigated. This antiserum was made against serum-free culture supernatants from lectin-activated human lymphocytes (anti-WS). The anti-WS shows strong neutralizing activities for both soluble and membrane forms of human lymphotoxins. The anti-WS was investigated for its site(s) of inhibition of the alloimmune CTL reaction relative to the calcium-dependent phase. The anti-WS was found to neutralize the CTL reaction subsequent to the calcium-dependent phase, suggesting the anti-WS neutralized a lytic effector mechanism. A fluid-phase immunoadsorption assay was developed to analyze the biochemical characteristics of the antigenic determinant(s) recognized by the anti-WS. Unfractionated supernatants or defined m.w. regions corresponding to the m.w. classes of the human LT system were used to adsorb the anti-WS. The data indicated that unfractionated supernatants and LT-Cx fractions (>200,000 m.w.) significantly reduced the capacity of the anti-WS to inhibit the alloimmune CTL reaction. α-LT fractions (70 to 90,000 m.w.) partially adsorbed the cytolytic inhibitory antibodies of the anti-WS, whereas β and γ LT fractions (40 to 50,000 and <20,000 m.w., respectively) were ineffective. These results support the concept that the mechanism of inhibition mediated by the anti-WS was by its capacity to interact with multiple antigenic species of LT molecules, and thus implies that lymphotoxins are involved as a multicomponent system of cytotoxins in the lytic effector mechanism of human alloimmune cytotoxic T lymphocytes. The LT-Cx appears to represent the most likely molecular candidate for the lytic effector mechanism. A model is presented that attempts to integrate the biochemical properties of the LT system with the cellular mechanism(s) of the alloimmune cytotoxic reaction scheme.

In vitro cytotoxic reactions mediated by alloimmune thymus-derived (T) lymphocytes occur as a multi-staged event (see References 1–3, for review). The first phase involves *recognition* and *contact* between the membranes of the effector lymphocyte and target cell. Recognition occurs via antigen-specific receptors present on the T cell surface and antigenic determinants associated with the target cell membrane. The recognition process is metabolically complex, requiring energy metabolism (4), a functional cytoskeletal system (5), and Mg^{++} ions (6). The second phase of the cytotoxic reaction is termed *activation*, and is characterized by its dependence on Ca^{++} ions (6, 7), and appears to involve the delivery of the lethal hit. The third phase represents *target cell lysis*. This is the stage at which the target cell undergoes a progressive loss of membrane integrity resulting in the release of electrolytes, followed by the loss of macromolecules (8, 9). Recognition and activation stages are dependent upon the presence of the lymphocyte and occur quite rapidly, e.g., 10 min (10). In contrast, the lytic phase may be protracted over several hours and can occur in the absence of the lymphocyte (11).

In the previous manuscripts of this series, we demonstrated the potent inhibitory activity of polyspecific antiserum directed at soluble products of activated lymphocytes (anti-WS) for a human alloimmune cytotoxic T lymphocyte (CTL)[3] reaction *in vitro* (12). This anti-WS was made against unfractionated serum-free culture supernatants from lectin-stimulated human lymphocytes, and these antisera have strong neutralizing activities for all the presently identified components of the human lymphotoxin (LT) system, including the precursor-LT form (13). Although the anti-WS represents a complicated immunochemical reagent, the usefulness of this reagent in analysis of the lytic effector mechanism employed by human CTL will be described. The first area of investigation with this antiserum was to define the stage(s) of the CTL reaction relative to the Ca^{++}-dependent activation step that is inhibited by the anti-WS. The second area of study was to characterize the molecules associated with the antigenic determinant(s) recognized by the polyspecific anti-WS serum that are relevant to the inhibitory activity of this antiserum in the CTL reaction. It would appear that the α and β LT-associated antigens are unlikely candidates responsible for the inhibitory activity, since anti-α and β antiserum showed no inhibitory activity in this cytotoxic reaction (12). However, a more likely candidate may be the antigenic determinant(s) associated with the precursor-LT form (13). An equally possible explanation would be that several antibody specificities, acting in a collective fashion against several distinct antigenic components, may be required to inhibit the alloimmune cytotoxic reaction. This latter hypothesis is consistent with the multi-component nature of the LT system (14, 15). In this regard, a functional assay has been developed that allows for the biochemical characterization of the antigenic determinant(s) recognized by the anti-WS serum. This assay system is based on the capacity of an antigenic determinant(s) to functionally adsorb the cytolytic-inhibitory antibodies from this antiserum. The use of this assay system will be examined to study the m.w. characteristics of the antigenic determinant(s) involved in the lytic mechanism of this human T cell-mediated cytotoxic reaction *in vitro*.

Received for publication July 11, 1980.
Accepted for publication January 20, 1981.
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[1] This work was supported by Grant AI-09460 from the Institute of Allergy and Infectious Diseases, National Institutes of Health, Rheumatic Diseases Research Foundation Grant 1882, and the National Institutes of Health Training Grant GM-07311.
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[3] Abbreviations used in this paper: Anti-WS, anti-whole supernatant; ASR, antigen-specific receptor; CTL, cytotoxic T lymphocyte; EGTA, ethyleneglycol-bis-(β-amino-ethylether) *N,N'*-tetraacetic acid; LT, lymphotoxin; PHA-P, phytohemagglutinin; PBL, peripheral blood lymphocytes; pre-LT, precursor LT; t_0, time zero.

MATERIALS AND METHODS

Culture medium and chemicals. The medium used was RPMI 1640 (GIBCO, Grand Island, NY), supplemented with 10% (v/v) fetal bovine serum (FCS) (GIBCO) and antibiotics (12). Ethyleneglycol-bis-(β-aminoethylether) *N,N'*-tetraacetic acid (EGTA) (Sigma, St. Louis, MO), was dissolved in phosphate-buffered saline (0.15 M NaCl, 0.01 M phosphate, pH 7.0).

Generation of effector lymphocytes and cytotoxic assay. Ficoll-Isopaque isolated human peripheral blood or adenoid lymphocytes were employed to generate killer cells to human lymphoblastoid cell line, WI-L2, in a one-way mixed lymphocyte culture (MLC), as previously described in detail (13).

LT assay and anti-LT antisera. LT was assayed for its cytolytic action on 1×10^5 mitomycin-C treated murine L-929 cells as previously described (16). Goat and rabbit anti-whole supernatants (anti-WS) were produced and characterized as described in Reference 12. The goat and rabbit anti-WS used in the studies presented here had been previously heat inactivated (56°C, 50 min) and absorbed with 2×10^8 human WI-L2 target cells/ml antiserum.

Fluid-phase immunoadsorption assay detecting antigens recognized by the cytolytic-inhibitory antibodies in anti-WS. Human LT activity used for immunoadsorption of the anti-WS was obtained from 5-day phytohemagglutinin-P (PHA-P) (Difco, Los Angeles, CA) activated human adenoid lymphocytes (allogeneic pool of 3 to 5 adenoids) cultured in medium containing a serum substitute (17). Cell-free supernatants were concentrated 50-fold by membrane ultrafiltration (PM-10, Amicon). Concentrated supernatants were separated by gel filtration chromatography into various m.w. regions on a 2.5 × 100 cm column containing Ultrogel AcA 44 (LKB, Uppsala, Sweden) (18). Elution was carried out in 10 mM phosphate, pH 7.0, 0.1 mM EDTA buffer at a flow rate of 20 ml/hr. LT activity peaks corresponding to: LT-Cx (>200,000 m.w.), α LT (70 to 90,000 m.w.), β LT (40 to 50,000 m.w.) were pooled, concentrated, and rechromatographed over a similar Ultrogel column. This method functionally purifies an LT m.w. class from other contaminating LT classes (18). The γ LT fraction and the α LT obtained from PGLC-33h lymphoblastoid cell line (19, 20) were isolated by a single chromatographic step as described above. All preparations were concentrated by ultrafiltration and then reconstituted to 150 mM NaCl. Goat anti-WS (300 μl) was mixed with 300 μl of the various LT preparations or as control, PBS, and then allowed to incubate for 1 hr at 4°C. These proportions were employed to give the maximum level of LT activity without over diluting the antiserum. These mixtures were centrifuged at 13,000 × G for 10 min (Beckman Microfuge, Fullerton, CA). Various dilutions of these mixtures were then tested for their capacity to inhibit the lysis of WI-L2 targets by alloimmune lymphocytes in a 2- to 3-hr ^{51}Cr-release assay. As controls, the WI-L2 target cells were also incubated in these mixtures, but in the absence of effector cells.

RESULTS

The kinetics of the inhibitory action of the anti-WS in the human alloimmune cytotoxic reaction. In the murine alloimmune cytotoxic reaction, Ca^{++} chelating agents have been employed to distinguish between the lymphocyte-dependent and independent phases of the cell-killing reaction (6, 7). These findings were applied to this human alloimmune cytotoxic system to test the capacity of anti-WS or a Ca^{++} specific chelator, EGTA, to distinguish among the various phases of cytolytic reaction. Effector lymphocytes were added to microtiter wells with their specific ^{51}Cr-labeled target cells (20:1 ratio) in cold medium and centrifuged at 4°C for 2 min at 400 × G to initiate contact. The reaction mixtures were then allowed to equilibrate temperature to 37°C for 3 min. The end of equilibration period was arbitrarily defined as time zero (t_0). At various times after t_0, either EGTA (5 mM final concentration) or goat anti-WS (1:4 final dilution) (prewarmed to 37°C) were added to the cytotoxic reaction. The cytotoxic reaction was then terminated at the end of 180 min. The results from a representative experiment are presented in Figure 1. The data have been normalized to the level of inhibition obtained when the antisera or EGTA were added to lymphocytes and target cells before centrifugation. The maximum inhibition of lysis obtained with the anti-WS in this experiment was 68%, provided the anti-WS was added before t_0. EGTA inhibited this reaction 98%. Therefore, these values were defined as 100% of the maximum inhibitory level. The data indicate that anti-WS inhibited the lysis of target cells by immune lymphocytes at times after the Ca^{++} chelator, EGTA was effective. In 3 such experiments, the time (at 50% maximum inhibition) that anti-WS inhibited lysis after an EGTA-sensitive step ranged from 2 to 18 min (mean, 12 min).

Inhibitory action of anti-WS after lymphocyte:target cell conjugation. Immune lymphocytes were mixed with ^{51}Cr-labeled target cells (20:1 ratio) in the presence of EGTA (5 mM), centrifuged (800 × G, 4 min) to initiate contact, and incubated for 15 min at 37°C. The conjugates were washed in cold Ca^{++}-containing medium and then added to various dilutions of anti-WS in microtiter plates. The degree of inhibition of lysis in this situation was then compared with the inhibitory action of the anti-WS when lymphocytes and targets were added to the antiserum before conjugation. Such a comparison of the inhibitory activities of the anti-WS, tested before and after the conjugation step, should provide a relative measure of the proportion of antibodies capable of blocking the recognition (conjugation) phase of the reaction. The results testing both rabbit and goat anti-WS are presented in Table I. The anti-WS antisera significantly inhibited the lysis of WI-L2 target cells before conjugation, and the anti-WS retained a majority of its inhibitory activity after conjugate formation indicating the conjugation step was not the primary site of inhibition by the anti-WS. Visual observation of the reaction wells revealed lymphocyte-target cell clusters were present after incubation in both control or anti-WS sera.

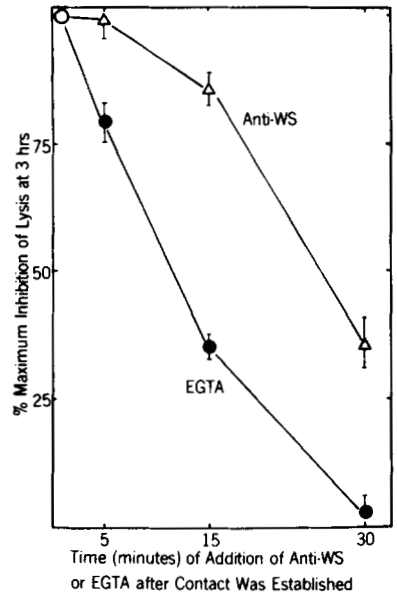

Figure 1. Kinetics of the inhibitory activity of goat anti-WS or EGTA in the human alloimmune cytotoxic reaction. Effector PBL were mixed with 1×10^4 ^{51}Cr-labeled WI-L2 (20:1 ratio), centrifuged for 2 min at 400 × G to initiate contact, and the reaction mixture was equilibrated to 37°C for 3 min. Anti-WS (1:4 dilution) or EGTA (5 mM) were added to the wells at various times (in minutes) after contact was established. The reaction was terminated after 3 hr and the percent lysis and percent inhibition of lysis was calculated as described in reference 12. Percent maximum inhibition was calculated as ratio of the values from the percent inhibition of lysis when antiserum or EGTA was added to the reaction wells before centrifugation to the percent inhibition values at the indicated times of addition. Anti-WS inhibited lysis 68%; EGTA inhibited lysis 98% when added before t_0. Lysis in the absence of inhibitors was 72 ± 4%.

A fluid phase immunoadsorption assay detecting the antigen(s) recognized by the cytolytic-inhibitory antibodies in the anti-WS. The m.w. characteristics of the antigenic determinant(s) recognized by the goat anti-WS that are responsible for the inhibitory action of this antiserum in the alloimmune cytotoxic reaction was investigated by immunoadsorption of these antibodies in the fluid phase. To accomplish this, supernatant fluids from 5-day PHA-activated lymphocytes were fractionated by gel filtration chromatography as described in *Materials and Methods.* The m.w. fractions or unfractionated whole supernatants were then mixed with goat anti-WS and incubated for 1 hr at 4°C to allow primary antigen-antibody complexes to form. No visible precipitates were observed to occur. However, these preparations were centrifuged at 13,000 × G for 10 min to remove any insoluble complexes that may have formed. These mixtures were then tested for their capacity to inhibit the lysis of WI-L2 target cells by MLC-generated killer cells. The results of 2 such experiments are presented in Figures 2 and 3. Unfractionated whole supernatants from activated lymphocytes showed the greatest capacity to abrogate the inhibitory activity of goat anti-WS in this cytotoxic system. Fractions containing LT-Cx activity (>200,000 m.w.) also showed a dramatic capacity to adsorb the

TABLE I

The effect of anti-WS on the cytolytic activity of alloimmune PBL before or after conjugation

Antiserum Dilution	Before Conjugation:[a] % Lysis of WI-L2 in medium containing: Goat anti-WS	Rabbit anti-WS	EGTA (5 mM)	Medium alone
1:2	12.4 ± 0.2 (77)	25.5 ± 0.6 (53)	10.6 ± 1.0 (80)	53.8 ± 2.0
1:6	20.5 ± 0.3 (62)	50.2 ± 3.0 (7)		
1:18	46.3 ± 1.8 (14)	58.2 ± 0.9 (0)		
Antiserum Dilution	After Conjugation:[b] Goat anti-WS	Rabbit anti-WS	Medium alone	
1:2	12.9 ± 1.3 (68)	29.5 ± 3.0 (29)	41.2 ± 0.5	
1:6	16.4 ± 0.2 (40)	40.3 ± 1.0 (2)		
1:18	28.9 ± 2.0 (30)	47.6 ± 0.1 (0)		

[a] Before conjugation: Immune PBL were mixed in microtiter wells with 1 × 10^4 ^{51}Cr-labeled WI-L2 target cells (20:1 ratio) in the presence of various dilutions of goat or rabbit anti-WS, or 5 mM EGTA or medium alone and incubated for 3 hr at 37°C. Percent lysis was determined as described in *Materials and Methods*. Data represent the mean ± SD of triplicate samples. Numbers in parentheses are the % inhibition of lysis.

[b] After conjugation: Immune PBL from the same population as described in (a) were mixed with ^{51}Cr-labeled WI-L2 target cells (20:1 ratio) in medium containing 5 mM EGTA. This mixture was centrifuged for 5 min at 800 × G and incubated at 37°C for 15 min to form conjugates. The reaction was then cooled on ice for 5 min and then diluted with 3 volumes of cold (4°C) medium containing Ca^{++}. Centrifugation step was repeated, the supernatant was discarded, and the pellet was tapped lightly to resuspend the pellet. Conjugates were washed again in cold medium and resuspended in medium (Hepes buffered) such that 100 μl contained 2 × 10^5 lymphocytes and 1 × 10^4 WI-L2 target cells. Aliquots of conjugates (100 μl) were then dispensed into microtiter wells containing various dilutions of anti-WS or into medium alone. The reaction was then allowed to incubate for 3 hr at 37°C. Spontaneous release was calculated from values obtained in (a) for 1 × 10^4 targets.

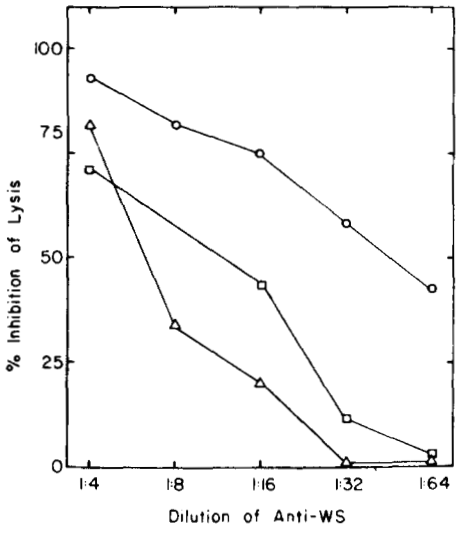

Figure 2. Abrogation of cytolytic-inhibitory activity of anti-WS by LT-Cx containing fractions or whole supernatant. Goat anti-WS (300 μl) was preincubated with 300 μl of either LT-Cx (1000 units/ml), unfractionated whole supernatants (6000 units/ml), or PBS for 1 hr at 4°C and centrifuged. Various dilutions of these antisera preparations were then tested for their capacity to inhibit the lysis of ^{51}Cr-labeled WI-L2 target cells by immune human PBL in 3 hr at a 20:1 lymphocyte:target cell ratio. Goat anti-WS incubated with: LT-Cx (□——□); whole supernatant (△——△); PBS (○——○). Percent lysis in presence of normal goat serum was 55.5 ± 2 (1:4 dilution). Data represent mean of triplicate determinations. Standard deviation did not exceed ±5% in all cases.

cytolytic-inhibitory activity of goat anti-WS. Both types of preparations of α LT (70 to 90,000 m.w.) demonstrated an intermediate capacity to reduce the inhibitory activity of this antiserum. It should be noted that α LT obtained from PGLC-33h cell line appears to share antigenic and biochemical identity with α LT obtained from lectin-activated human lymphocytes (19, 20). Fractions containing β and γ LT forms showed no capacity to abrogate the inhibitory activity goat anti-WS. Within an individual m.w. class, the level of LT activity, as measured on L-929 cells, correlated with its ability to adsorb in a dose-dependent fashion the inhibitory activity of anti-WS. However, the levels of LT activity between the α LT and LT-Cx containing classes did not correlate with their capacity to adsorb the inhibitory activity of the anti-WS. For example, in Figure 3 the α LT preparation from lectin-stimulated lymphocytes, although containing more LT activity than the LT-Cx fraction, was not as effective as the LT-Cx fractions in adsorption of the cytolytic-inhibitory antibodies present in the anti-WS. This result indicated

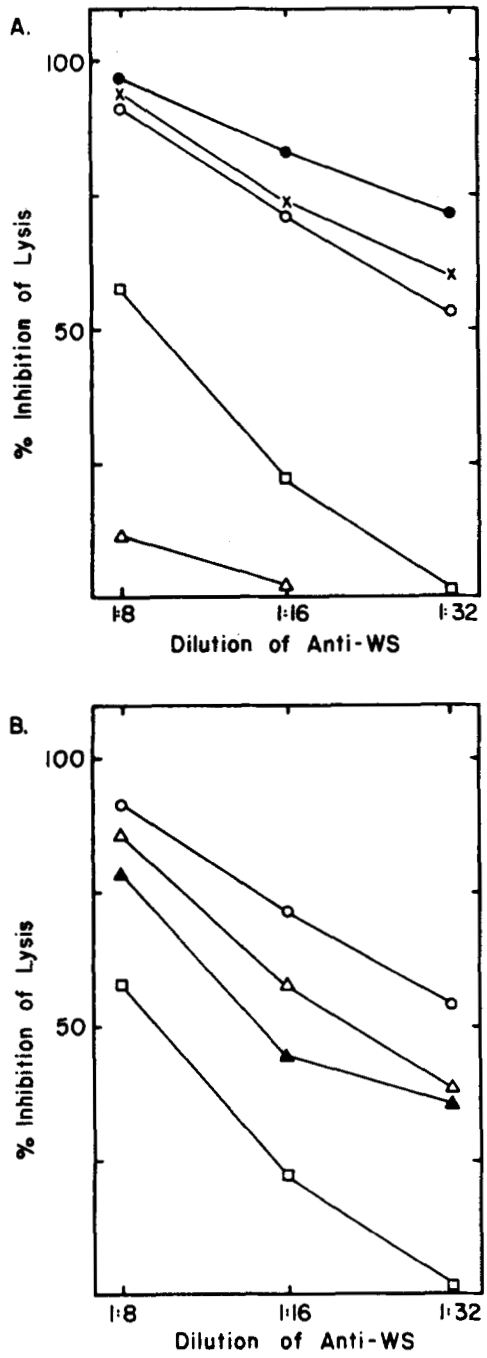

Figure 3. Abrogation of the cytolytic-inhibitory activity of goat anti-WS by LT-Cx, α-LT, β-LT, and γ-LT containing fractions. *A*, goat anti-WS was preincubated with m.w. fractions containing either LT-Cx (2,600 units/ml), β-LT (400 units/ml), γ-LT (11 units/ml), unfractionated whole supernatant (10,000 units/ml) or PBS. These preparations were then tested for their capacity to inhibit lysis of WI-L2 target cells by immune adenoid lymphocytes in 3 hr at a 20:1 ratio. Goat anti-WS preincubated with: LT-Cx (□——□); β-LT (×——×); γ-LT (●——●); unfractionated whole supernatant (△——△); PBS (○——○). *B*, same experiment as in (*A*), except goat anti-WS was preincubated with α-LT obtained from either human adenoid lymphocytes (3,100 units/ml) (▲——▲), or PGLC-33h human lymphoblastoid cell line (200 units/ml) (△——△). Percent lysis in presence of normal goat sera was 43 ± 2 at a 1:4 dilution.

TABLE II

The effect of LT: anti-WS mixtures on the spontaneous release of ^{51}Cr from labeled target cells[a]

Expt. No.	Spontaneous Release of ^{51}Cr (cpm) from WI-L2 after Incubation in Mixtures of Goat Anti-WS and:						
	Medium Only	WS	LT-Cx	α-LT[b]	α-LT[c]	β-LT	γ-LT
1	1128 ± 137	1022 ± 69	1208 ± 107				
2	1825 ± 126	1879 ± 126	2000 ± 20	2042 ± 237	1972 ± 172	1828 ± 75	1877 ± 234

[a] 10^4 ^{51}Cr-labeled WI-L2 target cells were incubated with mixtures of goat anti-WS and various LT fractions (prepared as described in *Figs. 1* and 2) or medium only for 3 hr at 37°C. Target cells were centrifuged and the release of ^{51}Cr label into the supernatant was determined as described in *Materials and Methods*.

[b] α-LT obtained from lectin-stimulated adenoid lymphocytes.

[c] α-LT obtained from PGLC-33h lymphoblastoid cell line.

a qualitative difference existed between the antigenic composition of these preparations.

The possibility existed that these LT:anti-WS mixtures may have nonspecifically increased the spontaneous release of the ^{51}Cr label from the target cell in the absence of effector cells. This would result in the artifactual appearance of these preparations of being able to reduce the inhibitory activity of goat anti-WS. To control for this type of result, the LT:anti-WS preparations were tested for their capacity to increase the spontaneous release of ^{51}Cr label from WI-L2 target cells. This was done by incubating the LT:anti-WS mixture with the target cells for 3 hr at 37°C. These experiments were performed simultaneously with those presented in Figures 2 and 3, and are presented in Table II. The results indicated that the LT:anti-WS mixtures did not increase the spontaneous release of the ^{51}Cr label during this time period.

DISCUSSION

The experiments presented in this manuscript have attempted to answer 2 questions concerning the inhibitory activity of the polyspecific anti-WS serum on the human alloimmune cell-mediated cytotoxic reaction. First, at what phase(s) of the cytolytic reaction does the anti-WS mediate its inhibitory action? Second, what are the biochemical characteristics of the antigen(s) recognized by the anti-WS and are these antigens related to components of the LT system of cytotoxins?

The present study indicates the anti-WS inhibits the *in vitro* cell-killing reaction subsequent to a calcium-dependent step (Fig. 1). The post-Ca^{++}-dependent step(s) is thought to represent the phase(s) of the cytotoxic reaction after the effector cell delivers the *lethal hit*, when the target cell undergoes cytolysis (3, 7). Therefore, these results imply that a major portion of the inhibitory antibodies in the anti-WS are directed at the lytic effector mechanism employed by alloimmune killer cells. Additional evidence shown in experiments presented in the preceding article (12, Table VII) and in this article (Table I) indicated that *recognition* and or *activation* phases of the cytolytic reaction were *not* the primary site of inhibition mediated by the anti-WS. Those experiments showed that: 1) the lymphocyte-target cell conjugation step (recognition phase) was not the major site of inhibition of the anti-WS; 2) effector lymphocytes preincubated in anti-WS and washed free of the antiserum were still capable of mediating direct-contact cytotoxicity and releasing LT in response to lectin stimulation (recognition and activation phases). The basis of the conclusion that anti-WS neutralized the lytic phase, however, rests on the supposition that this human alloimmune cytotoxic reaction proceeds in an analogous fashion to the murine alloimmune cytotoxic reaction in which the calcium-dependent phase occurs after recognition and contact have been established (6, 7). This assumption appears to be valid for several reasons: 1) both murine and human alloimmune cytotoxic reaction systems involve T cells (21–23); 2) the effector cells require active immunization and react specifically with target cell structures controlled by the major histocompatibility complex (24, 25); 3) both systems are inhibited by a variety of reagents that block energy metabolism and membrane function (5, 6, 26); 4) target cells are lysed with similar kinetics (27, 28); and 5) both cytotoxic reactions are multi-phasic and require calcium ions as defined for the human system in these studies.

A fluid phase immunoadsorption assay was developed to characterize the molecules associated with the antigenic determinants relevant to the *lethal hit* that are being recognized by the anti-WS. Unfractionated supernatants obtained from lectin-activated alloimmune lymphocytes contain the relevant molecules, since these supernatants were able to completely block the inhibitory antibodies in the anti-WS (Figs. 2 and 3). Several lines of evidence indicated the loss of the inhibitory activity of the anti-WS was due to the blocking of antibody active sites with the relevant antigens, and not due to nonspecific mechanisms. First, the various LT preparations adsorbed with anti-WS did not cause nonspecific lysis of the target cells (Table II). Second, an increase in the cytolysis of WI-L2 by induction of lectin-dependent cytotoxicity by residual PHA appears highly unlikely, since supernatants obtained from concanavalin A-stimulated lymphocytes completely abrogated the inhibitory activity of the anti-WS (data not shown). In addition, the active component in PHA-P is 140,000 m.w. (29), and therefore, was not present in any m.w. fraction tested in this study. Third, it is also unlikely that soluble antigen-antibody complexes formed in these mixtures were inhibiting lysis, since specific lysis of allogeneic target cells has been reported to be insensitive to the effect of such complexes (30). In fact, if antigen-antibody complexes were active in this assay system, they should enhance the blocking activity of anti-WS.

Supernatants from lectin-activated alloimmune lymphocytes were fractionated by gel filtration chromatography into various m.w. regions corresponding to the classes of the LT system, and these fractions were tested for their capacities to adsorb the inhibitory activity of the anti-WS. We found that the LT-Cx containing fractions (>200,000 m.w.) were capable of adsorbing the cytolytic-inhibitory activity of the anti-WS (Figs. 2 and 3). α LT-associated antigenic determinants in the 70 to 90,000 m.w. region were slightly active in removing the inhibitory antibodies, whereas the smaller m.w. β and γ LT-containing fractions (40 to 50,000 m.w. and <20,000 m.w., respectively) were totally ineffective. The inability of the β and γ LT-containing fractions to absorb the cytolytic-inhibitory activity of the anti-WS points out a limitation of this fluid-phase immunoadsorption assay. The limitation being dependent on preserving the antigenic structure of the molecules during separation procedures. The instability of β and γ LT cytotoxic activities may offer an alternative explanation as to why these fractions were unable to adsorb the activity of the anti-WS (31). This point is also germaine to our findings on the antigenic variability exhibited by the precursor-LT form after various manipulations (13).

A good correlation was observed between the capacity of both lymphocytes and various m.w. fractions to express LT-associated antigens and their capacity to adsorb the cytolytic inhibitory activity of the anti-WS. Clearly, both MLC-sensitized and lectin-activated alloimmune T lymphocytes express cell-surface antigens associated with the pre-LT and α LT antigenic determinants (13). Recent serologic evidence has revealed that the larger m.w. LT-Cx and α_H classes contain alloantigenic determinants associated with normal human lymphocyte cell surfaces (15). This finding may explain why normal lymphocytes showed a partial capacity to adsorb the inhibitory activity of the anti-WS (12). In contrast, cells and molecules not expressing LT antigens, such as human B lymphocytes and nonlymphoid target cells, and serum proteins did not adsorb the cytolytic-inhibitory antibodies in the anti-WS (12). It has been previously shown that the LT-Cx class expresses all of the presently identified antigenic determinants associated with the lytically active α, β, and γ, and the precursor-LT forms (13, 32). In addition, the LT-Cx contains antigenic determinants associated with nonclassic antigen-specific receptor(s) with $F(ab')_2$-like determinant(s) and lymphocyte cell-surface components recognized by certain human alloantisera (15, 33, 34). The capacity of the high m.w. fractions from gel filtration columns and unfractionated supernatants to adsorb the inhibitory antibodies in the anti-WS may be due to the

presence of high m.w. LT-Cx and precursor-LT forms. The ability of the 70 to 90,000 m.w. fractions containing α LT to partially adsorb the inhibitory activity of the anti-WS, whereas anti-α LT antiserum itself failed to block the direct lymphocyte-mediated cytotoxic reaction (12) indicates the mechanism of inhibition of the alloimmune cytotoxic reaction by the anti-WS involves multiple antibody specificities. This contention is further supported by our findings that antibodies against the pre-LT form present as a membrane component were not sufficient to block lysis (Table VII, Reference 12). These findings are consistent with the hypothesis that the lytic mechanism of alloimmune cytotoxic T cells involves "LT" as a multi-component system of effector molecules.

When taken together, we feel these data support the concept that a form similar to the LT-Cx represents the most likely candidate for the molecular mediator employed by alloimmune cytotoxic effector cells. Our findings here support similar conclusions drawn from observations on the functional properties of the LT-Cx class (34, 35). In those studies, the LT-Cx class showed an enhanced capacity to mediate rapid (4 to 12 hr) lysis of a variety of target cells when compared to the smaller m.w., α, β, or γ LT classes, and when obtained from immune lymphocytes, the LT-Cx class contained functional antigen-binding receptor(s). Similar results have recently been described for murine lymphotoxin (36). It should be noted that the lytic activity of gel filtration purified LT-Cx is neutralized by anti-α_2-LT and thus distinguishes it antigenically from the pre-LT form (35). In view of our findings that the anti-α_2 LT did not directly block T cell-mediated lysis (12), we would suggest the form delivered by the effector lymphocyte may be antigenically similar to the pre-LT form in that the α-LT antigen(s) are masked.

With our current understanding of the biochemical and functional properties of the LT system and the cellular mechanisms known for the T cell-killing reaction, a preliminary working model of the molecular mechanism of this cytotoxic reaction can be developed and subjected to testing. A highly schematic representation of this model is presented in Figure 4. Initially, the alloantigen induces the differentiation of a clone(s) of T lymphocytes into cytotoxic T effector cells (CTL). During the induction phase, the CTL precursor synthesizes components of the cytotoxic mechanism (LT) and transports those components to the cell surface, where they would be expressed in the precursor-LT (pre-LT) antigenic form (13, 37, 38). We currently envision the pre-LT form to represent a membrane macromolecular complex that consists of 3 essential subunits: 1) an antigen-specific receptor that functions as the recognition unit; 2) *cytolytic* unit formed by the α, β, and γ LT moieties; presumably the cytotoxic unit would reside in a nonactive form; 3) an *activation* unit that would transmit and interpret the antigen-recognition signal to the cytotoxic unit, and thus initiate cytolytic function (33–36). The LT-receptor complex described here is conceptually similar to that envisioned by Marchalonis (39) for a generalized T cell receptor-complex as it resides on the cell membrane. Our hypothesis is based largely on the biochemical properties of the LT-Cx obtained in the fluid phase (33–36). It is entirely possible that the receptor, activation unit, or the lytic moiety may reside on the membrane as separate entities and may only form a complex when released from the membrane surface.

The *recognition* phase of the cell-lytic sequence is initiated by the collision between the CTL and target-cell membranes. A stable interaction between these cells would be brought about by the interaction of the CTL antigen-specific receptor (ASR) and the target cell antigen. We would speculate that a functional-structural change in the ASR would result from the ASR-antigen binding interaction and provide 2 signals to the CTL that may be qualitatively or quantitatively distinct. The first signal would instruct the CTL to redistribute additional ASR-LT complexes to the portion of the CTL membrane in contact with target antigens. This cellular process(es) would require Mg^{++} ions, energy metabolism, and a cytoskeletal system to move additional ASR-complexes into the contact region and thus may explain the sensitivity of this recognition-contact phase to a variety of drugs that inhibit energy and membrane processes (5, 6, 26). These cellular process(es) would thereby establish a multipoint high avidity contact region with an increased density of ASR-LT complexes between opposing membranes.

The ASR-antigen binding interaction would provide a second signal that results in the *delivery* and *activation* of the *lethal hit*. The molecular events or components involved in the delivery and activation steps in the CTL reaction are unknown. We feel the activation phase represents the release of the ASR-LT complexes from the CTL membrane, and thus the effector cell delivers the cytotoxic complex directly onto the target cell membrane in a unidirectional mode. Complexes free in the fluid phase, which don't make contact with the target cell, are disarmed by rapidly dissociating into the smaller α, β, and γ LT classes. The LT subunits would then be liberated in a cytolytically active form directly onto the target-cell membrane and thus be localized to the region of

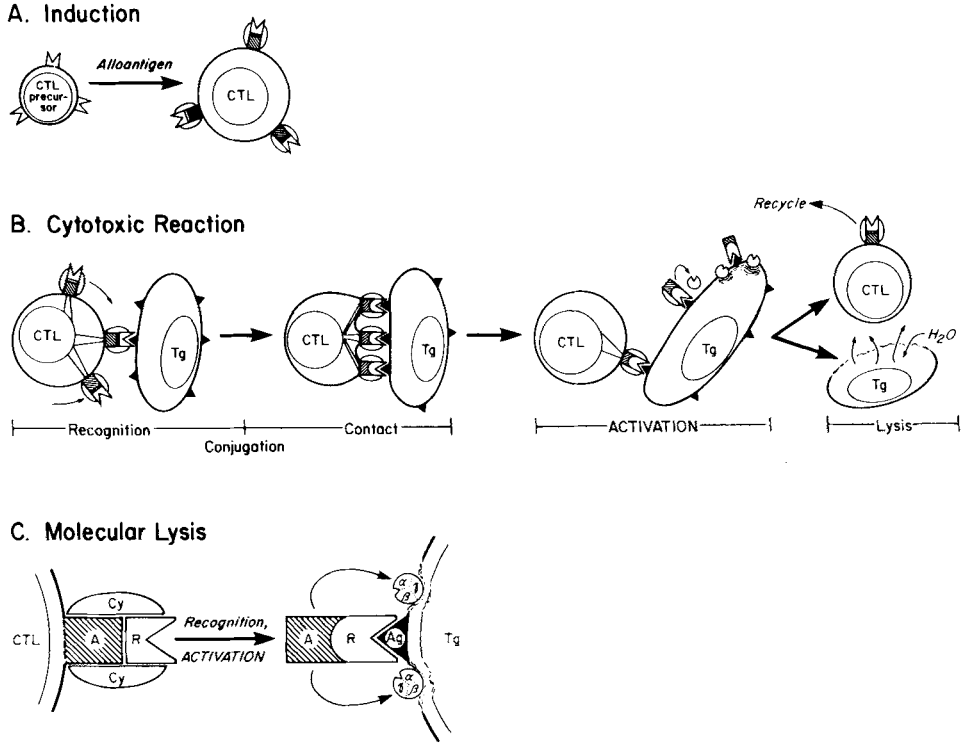

Figure 4. Schematic representation of the cellular and molecular events in alloimmune T cell-mediated cytotoxicity. *A* and *B*, CTL, cytotoxic T lymphocyte; Tg, alloantigen-bearing target cell. C, R, antigen-specific receptor; A, molecular activating unit; Cy, nonactive cytotoxic (LT) subunits; Ag, alloantigen; α, β, γ lytically active LT components. See the text for further explanation.

contact not to diffuse into the extracellular milieu. Our preliminary evidence studying the release of pre-LT from lectin-activated alloimmune T lymphocytes indicates the release of pre-LT into the fluid phase is strictly dependent upon the presence of Ca^{++} ions and 1 or more esterase-like enzymes (C. Ware and M. Weitzen, manuscript in preparation). Those studies indicate the presence of an "activation" component(s) associated with the ASR-LT complexes as proposed in this model. The sensitivity of the CTL reaction to inhibition by EGTA (Ca^{++} chelator) and a variety of protease inhibitors (7, 40, 41) would represent in this model inhibition of the LT delivery mechanism and activation of toxic moieties. However, it is not yet clear whether Ca^{++} ions and/or esterase(s)-like enzymes function as essential co-factors for LT activity or function in a more general role in a cellular process associated with a stimulus-secretion response. The molecular form of the ASR-LT complex delivered to the target cell is not known, but may exist as the 140 to 160,000 m.w. α_H form, where it then assembles into the high m.w. LT-Cx on the target-cell surface, or perhaps the large m.w. complexes are formed first on the CTL surface and then transferred to the target membrane as a large complex.

The next phase of the reaction sequence is *target cell lysis.* It is currently unknown how LT exerts its cytolytic action, although the presently available information indicates that the target-cell membrane may be the primary site (14, 42). When delivered to the target cell membrane, the α, β, and γ subunits may interact in a synergistic fashion resulting in the disruption of the target-cell membrane, leading to the loss of osmotic integrity and eventual cell death. Our results indicate that the anti-WS blocks the lytic reaction at this stage by reacting with several antigenic determinants, including the pre-LT form, LT-Cx, α- and perhaps β- and γ-associated antigens.

Several general features of this model can be defined and are germane to the proposed role of LT in the CTL reaction: 1) only antigen accommodated ASR-complexes would be released from the CTL surface where they would be restricted to the target cell via ASR-antigen complex, and thus LT release would be under strict control and require continuous presence of the antigen for release. This is in keeping with the earlier findings of Daynes and Granger (43), showing that LT release from mitogen-activated cells required the continuous presence of the lectin. This concept is consistent with the kinetics of target-cell lysis by CTL (27, 28), and explains why soluble toxins are not detected after CTL-target cell interaction (2), and the lack of "innocent bystander" lysis (44). 2) The release and delivery of ASR-LT complexes onto the target cell implies a direct transfer of CTL products and indicates that the later stages of lytic reaction should become independent of the lymphocyte. This concept is consistent with the observations that defined the lymphocyte-*dependent* and *independent* phases of the CTL reaction (11). 3) The release of the ASR-toxin complex from the CTL surface would provide a mechanism by which the CTL could disengage the target cell, thus freeing the effector cell for recycling to other targets (45). 4) The model indicates that the lytic mechanism is consumable and should require protein synthesis at some stage of the reaction to replenish the CTL lytic activity. This is in apparent conflict with the results of Thorn and Henney (46), who showed protein synthesis was not required for CTL-mediated lysis. Recently, Hiserodt *et al.* (37) have eliminated this argument by providing evidence that the effector cells, during the *induction phase*, synthesize intracellular pools of the LT and thus do not require protein synthesis to mediate lysis once these pools have accumulated.

Acknowledgments. The authors would like to acknowledge the excellent technical assistance of Mr. Bob Yamamoto. The expert secretarial assistance of Mrs. Gloria Heidenfelder is greatly appreciated.

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