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Inhibition of the Lytic Phase of Murine T-Cell-Mediated Alloimmune Cytotoxicity by a Rat Antiactivated T-Cell Antiserum¹

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Antisera produced in rats by immunization with alloimmune murine C57Bl/6 anti-P815 splenic lymphocytes or purified T cells activated *in vitro* by coculture with phytohemagglutinin-coated L-929 cells were found to inhibit the *in vitro* cytolytic action of *in vivo* and *in vitro* alloimmune C57Bl/6 anti-P815 cytotoxic T cells in a 4-hr chromium-51 release assay. The rat anti-murine-activated lymphocyte (anti-MAL) or antiactivated T-cell (anti-ATC) serum inhibited lysis in the absence of exogenously added complement activity and were not directly cytotoxic to CTL. Absorption of anti-MAL with target cells P815, L-929, EL-4, and normal C57Bl/6 lymphocytes removed a limited amount of the CTL-inhibitory activity. In contrast, lectin-activated alloimmune lymphocytes fully absorbed the inhibitory activity indicating these antisera preferentially recognize unique antigenic determinants associated with the activated CTL cell surface. The anti-ATC was found to block alloimmune lysis by CTL from several inbred mouse strains suggesting these antisera recognized antigenic determinants of a common lytic mechanism. A kinetic analysis of the inhibitory activity of the anti-MAL on the CTL reaction scheme revealed this antiserum inhibited lysis at a post-Ca²⁺-dependent step, presumably during the target cell lytic phase. This result suggests the rat antiserum can neutralize the CTL lytic mechanism.

INTRODUCTION

The destruction of alloantigen-bearing target cells by murine cytotoxic T lymphocytes (CTL)⁴ *in vitro* can be resolved into three discreet phases at the cellular level (1-4). The first event in the killing reaction is *recognition* of target-associated H-2K or D antigens by specific receptors present on the CTL surface (5, 6). The CTL antigen-specific receptors appear to share idiotypic determinants with humoral

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⁴ Abbreviations used; Anti-MAL, anti-murine activated lymphocyte serum; anti-ATC, anti-activated T-cell serum; ⁵¹Cr, Na₂⁵¹CrO₄; CTL, cytotoxic thymus-derived lymphocyte; EGTA, ethylene glycol-bis-(β -amino-ethylether) *N,N'*-tetraacetate; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; PHA, phytohemagglutinin.

alloantibodies (7, 8), but not classical Ig heavy-chain determinants. Additional cellular processes requiring Mg^{2+} ions (9), energy generating metabolic pathways (10), and a functional cytoskeletal system (11) lead to the formation of firmly attached *contact* regions between the CTL and target cell membranes. Electron micrograph studies have revealed numerous membrane interdigitations between CTL:target conjugates and have established the close juxtaposition between opposing cell surfaces (12, 13). The second stage of the reaction is an *activation* event requiring Ca^{2+} ions (1, 14, 15) and has been defined as the phase of the CTL reaction during which the target cell becomes irreversibly "programmed" to lyse (1). Recent evidence has suggested that a thiolreactive esterolytic enzyme(s) may function during the activation phase (16, 17). The third phase is defined by target cell lysis during which the cell undergoes progressive loss of membrane integrity leading to cell death (18, 19). The recognition and activation events occur quite rapidly (1) and are absolutely *dependent* on the presence of the CTL (20). In contrast, the target cell lytic phase is *independent* of the presence of a functional CTL and may be protracted in time (20).

Numerous substances, including metabolic inhibitors, metal ion chelators, and antibodies with reactivity to target cell H-2 antigens, have been shown to block CTL lytic activity (1). These reagents appear to inhibit the reaction by blocking target cell recognition and/or activation phases of the reaction. Our goals have been to develop reagents capable of blocking the *lytic phase* of the cytotoxic reaction. Such reagents would be important for understanding the basic mechanism of lysis as well as their potential use in clinical immunotherapy regimens.

We wish to report here the production and initial characterization of a xenogeneic antiserum produced against lectin-activated alloimmune CTL that inhibits the cytotoxicity of allogeneic target cells in the absence of exogenously added complement and appears to block the CTL reaction during the *lytic* phase.

MATERIALS AND METHODS

Mice. All mice were bred and maintained in the UTHSC animal facility. Breeding pairs of inbred and congenic mice strains were originally obtained from the following colonies: the Jackson Laboratory, Bar Harbor, Maine—C57Bl/6 (H-2^b) and CBA/HT6J (H-2^k); E. A. Boyse, Sloan-Kettering Memorial Cancer Institute, New York, New York—C57/H2K (H-2^k) and C3H/AN H-2^k; P. Chauvenet—H(z1) mutant of C57Bl/6 (H-2^{ba}) (21).

Target cell lines. P815 DBA/2 mastocytoma (H-2^d) and EL-4 C57Bl/6 T lymphoma (H-2^b) cell lines were maintained *in vitro* with periodic *in vivo* passage every 4 to 6 weeks in their respective syngeneic host. The C3H fibroblast L-929 cell line (H-2^k) was maintained *in vitro*.

Tissue culture medium. Tissue culture medium for routine passage and culture of target cells and lymphocytes was RPMI 1640 (Grand Island Biological Co., Grand Island, N.Y.), supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) (Gibco), and antibiotics penicillin (100 units/ml), streptomycin (100 μ g/ml), and gentamycin (50 μ g/ml). Cytotoxicity assays were performed in the above medium except that Hepes buffer (25 mM) (Sigma Chemical Co., St. Louis, Mo.) was substituted for bicarbonate.

Chemical reagents. Ethylene glycol-bis-(β -amino-ethylether) *N, N'*-tetraacetic

acid (EGTA) (Sigma) was dissolved in phosphate-buffered saline (PBS) (150 mM NaCl, 10 mM Na H₂PO₄, pH 7.0) to a final concentration of 200 mM.

Generation of alloimmune cytotoxic T cells. Cytotoxic splenocytes were generated *in vivo* by immunization of C57Bl/6 (H-2^b) mice with 1×10^7 P815 tumor cells as described by Brunner and Cerrotini (22) or *in vitro* as described previously (23). Cytotoxic assays were performed in round-bottom microtiter plates (Flow Laboratories, Inglewood, Calif.) containing a total volume of 0.2 ml. Target cells (1×10^4) prelabeled with Na ⁵¹Cr (sp act ~ 305 mCi/mg, New England Nuclear, Boston, Mass.) were mixed with various numbers of alloimmune CTL. The reaction was carried out at 37°C for 2–4 hr. The release of ⁵¹Cr label was determined by the uptake of cell-free supernatant with the Titer-Tek Supernatant Collection System (Flow Laboratories) and quantitated in an automated gamma counter (Beckman, Fullerton, Calif.). The percentage of lysis was determined by the formula:

$$\% \text{ lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100. \quad (1)$$

Experimental release is defined as the counts per minute released by targets in the presence of CTL. Spontaneous release represents the counts per minute released by targets incubated alone. Spontaneous release by P815 cells was between 5 and 12% over the reaction period. Total releasable counts per minute was determined by lysis with sodium dodecyl sulfate (3%). Data represent the mean \pm SD of triplicate samples.

In studies employing the antisera, CTL were added to various dilutions of the antisera in medium followed immediately by the addition of labeled target cells. The various antisera showed no capacity to inhibit the spontaneous or total release of ⁵¹Cr label from target cells incubated in the absence of CTL. In certain experiments, the data are represented as the percentage inhibition of lysis and were calculated according to the formula:

$$\% \text{ inhibition of lysis} = 1 - \frac{\% \text{ lysis in experimental serum}}{\% \text{ lysis in normal serum}} \times 100. \quad (2)$$

The percentage of inhibition was calculated in each case with results obtained with identical concentrations of normal serum.

Production and preparation of rat anti-C57Bl/6-activated lymphocyte serum. Lectin activation of alloimmune C57Bl/6 anti-P815 lymphocytes: C57Bl/6 anti-P815 splenocytes taken from day-11 through day-13 *in vivo*-immunized mice were depleted of adherent cells by absorption on plastic tissue culture flasks for 60 min at 37°C in medium. Nonadherent spleen cells were harvested and washed once with serum-free medium. Nonadherent lymphocytes were further purified by nylon wool chromatography as described (24) and were 88–97% T cells as determined by treatment with anti-theta serum and guinea pig complement. Lymphocytes or T-cell-enriched populations of alloimmune lymphocytes were activated by incubation with murine L-929 (H-2^k) cell monolayers in serum-free medium. The L-929 cell monolayer had been previously coated with the lectin phytohemagglutinin-P (PHA-P) (Difco, Los Angeles, Calif.) as described (25). After 6–10 hr in culture, activated lymphocytes or T-cell cultures were harvested and washed once with cold serum-free medium. Lewis male rats were immunized by intraperitoneal (ip) injection according to the following schedule: Animals received an initial immuni-

zation of 1.5 to 2×10^8 activated lymphocytes or T cells/animal, followed by immunization with 5×10^7 cells/animal every 10 to 20 days for 3 months. Rat antiserum directed against alloimmune murine-activated lymphocytes (unseparated) will be designated anti-MAL and antiserum produced against activated T cells will be designated anti-ATC. A pool of normal (preimmune) sera was obtained by pooling sera from 5 rats prior to immunization.

Preparation of antisera: Antisera preparations were obtained after the eighth week of immunization. Normal and immune sera were heat inactivated at 56°C for 60 min and dialyzed against 2000 vol of cold Hanks' balanced salt solution. Sera were cleared of debris by centrifugation at $13,000\text{ g}$ for 10 min and frozen at -20°C until use. Absorption of the antisera with various cell populations was performed by incubating various numbers of cells with serum for 1–2 hr on ice with mixing. Antisera were routinely absorbed with $2\text{--}4 \times 10^8$ cells/ml serum. Activated alloimmune lymphocytes used for absorption were obtained as described above. Normal lymphocytes were obtained from nonimmune C57Bl/6 spleens. Both *in vivo* and *in vitro* passaged L-929, P815, or EL-4 target cells were used for absorption. Cells were removed from the absorbed sera by centrifugation at $13,000\text{ g}$ for 5 min. Fresh guinea pig complement was used where indicated at a 1:5 final dilution. Guinea pig serum was preabsorbed with agarose before use.

Immunofluorescence. Indirect immunofluorescence studies were performed by incubating 1×10^6 lymphocytes with a 1:5 dilution of the absorbed or unabsorbed antisera for 30 min on ice. Cells were washed twice with cold PBS containing 1% FCS and incubated for an additional 30 min on ice with goat IgG anti-rat IgG conjugated to fluorescein isothiocyanate (FITC). FITC-goat anti-rat IgG was a gift of Dr. Toni Neri, University of California, Irvine, California. A total cell count of 100–200 cells was used to determine the percentage of cells with positive staining. The FITC-goat anti-rat IgG did not nonspecifically stain lymphoid or target cells.

RESULTS

Capacity of Anti-MAL and Anti-ATC to Inhibit the Lysis of P815 by Alloimmune C57Bl/6 Effector Lymphocytes

Xenogeneic antisera produced against lectin activated alloimmune C57Bl/6 lymphocytes or purified T cells were tested for their capacity to block the cytolytic activity of C57Bl/6 anti-P815 CTL *in vitro*. Prior to these experiments all antisera were exhaustively absorbed with P815 and L-929 target cells (5×10^8 cells/ml of serum). Absorbed sera were tested for completeness of absorption and showed no reactivity with P815 or L-929 cells as judged by agglutination, complement lysis, and indirect immunofluorescence. Antisera were heat inactivated, dialyzed, and clarified before use. Immune CTL were added to various dilutions of the anti-MAL, anti-ATC, or control sera followed immediately by the addition of ^{51}Cr -labeled P815 target cells and incubated at 37°C in the continuous presence of the antisera. The reaction was terminated after 4 hr and the percentage lysis was calculated as described under Materials and Methods. The results of five representative experiments are presented in Table 1. The data clearly show these antisera to be potent inhibitors of murine CTL activity in the absence of exogenously added complement. Complete inhibition of lysis was observed at dilutions of 1:4 to 1:20, and the degree of inhibition was dependent upon the dilution of the antisera em-

TABLE 1

Inhibition of *in Vitro* T-Cell-Mediated Lysis of P815 Mastocytoma by Rat Antiserum to Activated C57Bl/6 Alloimmune Lymphocytes (Anti-MAL) and Purified T Lymphocytes (Anti-ATC)

Experiment	Effector to target ratio	Dilution of antiserum	Preimmune serum ^a (% lysis)	Anti-MAL	
				Lysis ^b (%)	Inhibition of lysis (%)
1 ^c	16:1	1:4	28 ± 1	-1 ± 2	100
		1:8	34 ± 2	-2 ± 3	100
		1:16	30 ± 0.5	0 ± 2	100
		1:32	30 ± 2	12 ± 1	60
2 ^c	20:1	1:20	21 ± 3	0.2 ± 1	100
		1:40	20 ± 1	4 ± 2	80
		1:80	22 ± 1	13.5 ± 1	39
3 ^c	20:1	1:8	44 ± 2	3 ± 1	93
		1:24	42 ± 1	11.5 ± 2	72
		1:72	41 ± 4	36 ± 3	12
		1:216	44 ± 1	40 ± 0.5	9
Anti-ATC					
4 ^d	20:1	1:12	61 ± 4	8 ± 1.5	87
		1:24	—	22 ± 2	64
		1:96	—	51 ± 4	16
5 ^d	20:1	1:16	20 ± 1	2 ± 1	90
		1:32	—	6.5 ± 2	68
		1:64	—	13 ± 0.5	35
		1:128	—	23 ± 4	0

^a Preimmune serum was a pool from two Lewis rats prior to immunization expts 1-3. Normal rat serum was a pool of six Lewis rats (expts 4 and 5) tested at the lowest dilution.

^b Percentage lysis in medium alone was: expt 1, 26 ± 3%; expt 2, 21.7 ± 3%; expt 3, 40.5 ± 1%; expt 4, 62 ± 3%; expt 5, 19.5 ± 1%. Spontaneous release was less than 9% in all experiments.

^c *In vivo*-immunized C57Bl/6 anti-P815 splenic lymphocytes were incubated with 1×10^4 ⁵¹Cr-labeled P815 mastocytoma targets cell in the continuous presence of rat anti-MAL. Dilutions of heat-inactivated antiserum or preimmune serum were made in RPMI 1640 containing 10% FCS and 10 mM HEPES buffer. Cytotoxic reaction was carried out for 3 to 4 hr at 37°C. Data represent mean ± SD of triplicate samples.

^d Same as in footnote c except *in vitro*-generated (Day 6) CTL were employed with the rat anti-ATC.

ployed during the cytotoxic reaction. Both *in vivo*- and *in vitro*-generated CTL were inhibited to a similar degree. Preimmune rat serum had no inhibitory effect on lysis and levels of cytolysis obtained in the presence of control sera were comparable to those obtained with medium alone. Furthermore, these antisera were not directly cytotoxic to the immune lymphocytes as judged by eosin-Y dye exclusion test. C57Bl/6 CTL reactive with L-929 (H-2^k) cells were inhibited by rat anti-MAL or anti-ATC to a similar degree (data not shown). This result implied that blocking activity of these antisera was not directed at target cell structures and that blocking was effective with C57Bl/6 CTL irrespective of the strain used for immunization.

Capacity of Rat anti-ATC Serum to Inhibit Alloimmune CTL Activity from Different Inbred Mouse Strains

The next experiments performed tested the capacity of this rat anti-ATC to inhibit alloimmune effector cell activity obtained from several mouse strains differing predominately at the H-2 locus. The following strains were used: CBA/HT6J (H-2^k); C57/H2K, (H-2^k); C3H/AN (H-2^k); H(z1) (mutant H-2^{ba}); and DBA/2 (H-2^d). *In vitro*-generated killer cells from these strains reactive to P815 mastocytoma or DBA anti EL-4 were tested for cytolytic activity in the continuous presence of the anti-ATC as described in the previous section. The rat anti-ATC inhibited the lysis of P815 or EL-4 by all strains tested and a representative experiment is shown in Fig. 1. The only significant difference observed in the capacity of the anti-ATC to inhibit CTL activity in the various strains tested was a quantitative effect between C57Bl/6 and the mutant C57Bl/6 strain H(z1) (Fig. 1A). Although similar levels of killer cell activity were observed for both CTL populations, the dilution of the anti-ATC-inhibiting lysis by 50% occurred at 1:27 for H(z1) and 1:75 for C57Bl/6. In contrast, equal inhibitory activity of the anti-ATC was observed between H-2 disparate CBA/HT6J and C57Bl/6 (Fig. 1B).

Effect of Absorption with EL-4, Normal murine Lymphocytes, and Activated Alloimmune Effector Cells on the CTL Inhibitory Activity of Rat Anti-MAL

The rat antiactivated lymphocyte serum was further absorbed on the C57Bl/6 T lymphoma EL-4 or C57Bl/6 splenocytes to remove antibodies directed against nonimmune lymphoid cell surface components. These results are presented in Figs. 2 and 3. Extensive absorption of the antiserum (previously absorbed with P815 and L-929 cells) with increasing numbers of T-cell line, EL-4, removed approximately 30% of the inhibitory activity when the antiserum was tested at 1:10 or 1:20 dilution. Following absorption with EL-4 (2×10^8 cells/ml serum) the anti-MAL showed no reactivity for EL-4 by indirect immunofluorescence. Absorption of the anti-MAL with increasing numbers of normal nonimmune C57Bl/6 lymphocytes showed

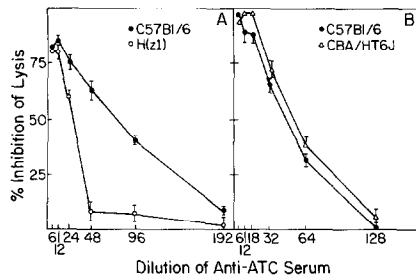


FIG. 1. Inhibition of alloimmune CTL activity obtained from different strains of mice by anti-ATC. *In vitro*-generated anti-P815 CTL from (A) C57Bl/6 and H(z1) or (B) CBA/HT6J and C57Bl/6 were tested against P815 target cells in a 4-hr ⁵¹Cr release assay in the continuous presence of various dilutions of the rat antiactivated T-cell serum (preabsorbed with P815, L-929 and EL-4 target cells) at a 20:1 lymphocyte:target cell ratio. The percentage inhibition of lysis was calculated as described under Materials and Methods. Data represent mean of triplicate samples \pm SD. The percentage lysis in medium alone was: (A) $55.5 \pm 2\%$ for C57Bl/6 and $60.5 \pm 3\%$ for H(z1); (B) $21.2 \pm 2\%$ for C57Bl/6 and $30 \pm 1\%$ for CBA/HT6J.

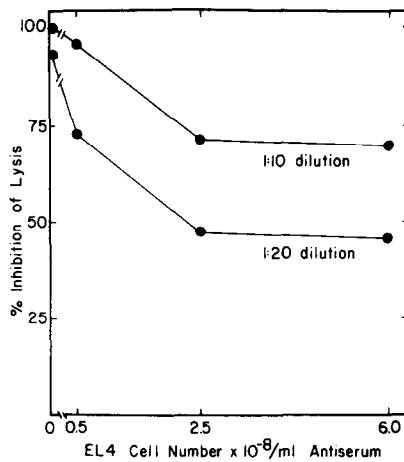


FIG. 2. The effect of absorption with C57Bl/6 EL-4 T-cell line on the inhibitory activity of rat anti-MAL serum. Rat anti-MAL (preabsorbed with P815 and L-929 cells) was absorbed with varying numbers of EL-4 lymphoma cells and then tested for its cytolytic inhibitory activity on C57Bl/6 anti-P815 CTL in a 3-hr assay (20:1 lymphocyte: target ratio). Percentage lysis in medium alone was 83 ± 2 . Data represent the mean of triplicate samples. The standard deviation did not exceed $\pm 4\%$ lysis.

a limited capacity of these cells to remove the CTL-inhibitory activity of this antiserum. However, when the absorbed antiserum was tested at dilutions of 1:20 or greater, the anti-MAL showed a linear loss of the CTL-inhibitory activity over the same range of absorbing cells. Analogous results were obtained with the anti-ATC. Absorption of the antisera with PHA-P or FCS covalently linked to Sepharose 4B beads did not remove any of the CTL-inhibitory activity of the anti-MAL or anti-ATC (data not shown).

Following extensive absorption of the rat anti-ATC with target cells followed by normal C57Bl/6 splenic lymphocytes, the anti-ATC reacted *in vitro* with 2 to

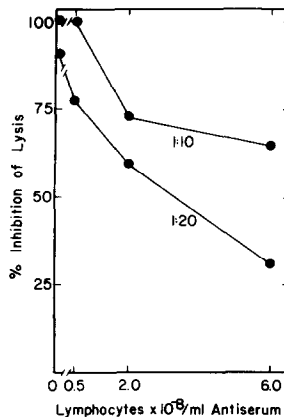


FIG. 3. The effect of absorption with normal C57Bl/6 splenocytes on the cytolytic inhibitory activity of rat anti-MAL serum. Rat anti-MAL (preabsorbed with P815 and L-929 cells) were absorbed with varying numbers of C57Bl/6 normal splenocytes and the antiserum was tested for its cytolytic inhibitory activity as described in Fig. 2. Percentage lysis in medium alone was $34 \pm 1\%$.

5% of the lymphocytes harvested from an alloimmune C57Bl/6 spleen as detected by indirect immunofluorescence. The rat anti-MAL, after this two-stage absorption, did not have any agglutinating activity for lymphocytes.

The previous results indicated that the antigens presented on normal lymphocytes or EL-4 did not contain a full set of the antigenic determinants relevant to the CTL-inhibitory activity of these antisera. Since these antisera were developed against lectin-activated alloimmune C57Bl/6 lymphocytes, an experiment was designed to compare the relative efficiency of activated vs nonactivated lymphocytes in absorbing the CTL-inhibitory activity from anti-MAL. The results of such an experiment are presented in Fig. 4. The data indicate that lectin-activated immune cells were 8 to 10 times more efficient than normal lymphocytes in removing the CTL-inhibitory activity of the antisera. These results demonstrate that the anti-MAL and anti-ATC have preferential reactivity with antigenic determinants associated with activated lymphocytes and, moreover, the CTL-inhibitory activity cannot be attributed to antilymphocyte serum-like effects.

Evidence that the Rat Anti-MAL Inhibits CTL Reaction during a Postrecognition-Post- Ca^{2+} -Dependent Phase

Experiments were designed to determine at what step the antiactivated lymphocyte serum inhibited this lytic reaction. To accomplish this, alloimmune C57Bl/6 effector cells were mixed with ^{51}Cr -labeled P815 target cells (20:1 ratio) and centrifuged together at 400 g for 2 min at 25°C to initiate contact. The reaction was allowed 3 min to equilibrate to 37°C. This time was defined at t_0 . At various times after contact was established, either EGTA (a Ca^{2+} -specific chelating agent) (5 mM) (26), rat anti-MAL (previously absorbed with P815, L-929, and normal C57Bl/6 lymphocytes) (1:10 dilution), or normal Lewis rat serum (1:10) was added

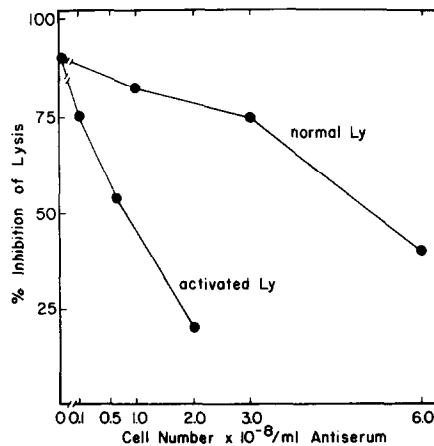


FIG. 4. The effectiveness of normal or activated C57Bl/6 lymphocytes to absorb the cytolytic inhibitory activity of rat anti-MAL serum. Rat anti-MAL (preabsorbed with P815, L-929, EL-4) serum was absorbed with varying numbers of either normal nonimmune C57Bl/6 lymphocytes or lectin-activated immune lymphocytes for 2 hr at 0°C. The absorbed or unabsorbed antisera were then tested at a 1:10 dilution to inhibit the lysis of P815 target cells by alloimmune C57Bl/6 lymphocytes (20:1 ratio) in a 3 hr ^{51}Cr release assay. Percent lysis in medium alone was 22 ± 2 .

to the cytotoxic reaction and incubation was continued at 37°C for 2 hr. At this time the reaction was halted and percentage lysis was calculated. All reagents were prewarmed to 37°C to insure that temperature fluctuations would not inhibit lysis. The results of such an experiment are presented in Fig. 5. The data have been normalized relative to percentage of inhibition obtained when these reagents were added 10 min prior to initiation of contact, $t_{(-10)}$. In this particular experiment, the maximum inhibition at $t_{(-10)}$ was 98% for EGTA, 83% for anti-MAL, and 0% for normal rat serum. This experiment has been repeated five times with similar results. Clearly, the data indicate that the anti-MAL inhibited lysis at a time(s) when EGTA was ineffective in blocking lysis. The addition of excess Mg^{2+} ions (2.5 mM) to the EGTA reagent did not influence the kinetics of inhibition indicating EGTA was inhibiting lysis by chelation of Ca^{2+} ions. Additional experiments not shown were performed with alloantisera directed against the P815 target cell (C57Bl/6 anti-P815 serum, 50% complement lysis titer for P815 of 1:254). This serum was ineffective at blocking lysis when added at t_0 and, in fact, required a 10-min *preincubation* with target cells to block lysis by CTL by 20–30%.

DISCUSSION

The results presented here demonstrate that xenogeneic antisera produced against lectin-activated alloimmune C57Bl/6 splenic lymphocytes (anti-MAL) or purified T cells (anti-ATC) can inhibit cytotoxic T-cell activity in the absence of exogenously added complement.

The lectin activation system used to pretreat the alloimmune lymphocytes used for immunization appears to be a key requirement in obtaining an antiserum with specificities capable of inhibiting CTL activity. This activation system was developed from observations demonstrating the capacity of lectins to nonspecifically activate the CTL lytic mechanism (27). This view is supported by the unsuccessful

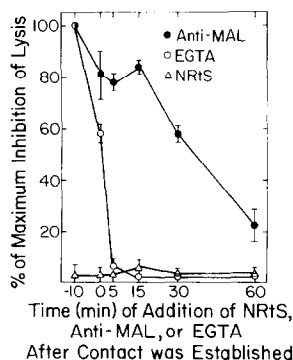


FIG. 5. Kinetics of inhibition of cytolysis by EGTA or rat anti-MAL serum after establishment of CTL-target cell contact. Alloimmune C57Bl/6 lymphocytes were mixed with 1×10^4 ^{51}Cr labeled target cells (20:1 ratio) and were centrifuged to initiate contact (t_0). At various times before or after contact was established, either EGTA (5 mM), rat anti-MAL or normal rat serum (NRtS) was added to the ongoing cytotoxic reaction. The reaction was allowed to proceed for 2 hr. Percentage lysis in medium alone was $58 \pm 1.5\%$. Data represent the mean \pm SD of triplicate samples. The percentage maximum inhibition of lysis was defined for each reagent as the percentage inhibition of lysis relative to the percentage inhibition obtained at $t_{(-10)}$ and was 98% for EGTA; 83% for rat anti-MAL; and 0% for normal serum.

attempts of several other investigators to produce xenogeneic antisera that inhibit CTL activity in the absence of added complement (28–30). In those studies both rat and guinea pig antisera made against enriched CTL populations did not block CTL activity in the absence of complement (K. Sullivan, personal communication) (28, 30). Although the cells used for immunization in those studies contained high levels of CTL, the antigenic determinant(s) associated with the lytic mechanism was apparently not expressed. Therefore, lectin “triggering” of CTL populations may have induced expression of the relevant antigens sufficient to elicit a measurable antibody response. We feel this system represents a new approach in developing reagents specific for CTL-associated antigens.

A comparison of results presented in this study with the observations of several other laboratories employing a variety of anti-T-cell antisera of varying specificities reveal the uniqueness of the anti-MAL and anti-ATC in their capacity to inhibit murine CTL activity. First, exhaustive absorption of the anti-MAL or anti-ATC with P815, or L-929 target cells did not remove the CTL-inhibiting activity of these antisera. This result rules out the possibility that the antisera were blocking target cell antigens recognized by CTL and indicates the inhibitory affect of these antisera was at the effector cell level. This finding was important since it is well documented that anti H-2 antibodies directed at target cell antigens will inhibit CTL activity (31). Second, quantitative absorption studies of the anti-MAL with the T-cell lymphoma, EL-4, and normal C57Bl/6 lymphocytes revealed a limited capacity of these cells to absorb the CTL-inhibitory activity. These results, coupled with the observation that activated alloimmune lymphocytes were 8 to 10 times more effective in absorbing the inhibitory activity of the anti-MAL than were normal lymphocytes, indicate that a significant proportion of the antibodies in these sera are preferentially reactive with antigenic determinant(s) unique to activated CTL. These findings, in conjunction with the *inability* of other xenogeneic or allogeneic anti-CTL antisera (28, 29) as well as anti H-2 (32) and anti-theta antiserum (33) to block CTL activity (in the absence of complement) support the contention that the anti-MAL or anti-ATC are not simply antilymphocyte sera.

Both anti-ATC and anti-MAL appear to recognize a set of antigenic determinants common to CTL effector cells. This contention appears to be valid since the anti-ATC was shown to be effective in blocking cytotoxicity against P815 targets by CTL derived from several different mouse strains. Neither the H-2 type of the effector CTL nor the target cell was a factor in determining the effectiveness of the blocking activity of the anti-ATC since both H-2^b, H-2^d, and H-2^k CTL were equally inhibited. Although CTL from the mutant C57Bl/6 strain H(z1) (H-2^{ba}) were completely inhibited by the anti-ATC, a quantitative difference in the effective inhibitory titer of the antiserum was observed. The basis of this quantitative difference is currently being studied.

The capacity of the anti-ATC to inhibit CTL from several mouse strains further distinguishes this antiserum from other xenogeneic and allogeneic antilymphocyte sera which were recently reported to block CTL activity (in the absence of complement) in a strain-specific fashion (34–37). The antisera investigated in those studies were proposed, by virtue of their reactivity with Lyt-2, Lyt-3, or idiotypic determinants, to block lytic activity by binding to CTL antigen recognition structures. As yet, we cannot rule out the possibility that the anti-ATC or anti-MAL

also inhibit lysis by blocking CTL antigen receptors. However, additional studies discussed below indicate the anti-MAL inhibition of CTL activity occurs at a postrecognition-activation step, and not by blocking antigen recognition structures.

Martz and Benacerraf (1, 20) and other workers (2-4) have elegantly shown that the cytolysis of P815 mastocytoma by alloimmune murine T lymphocytes proceeds through several discrete phases. In this report we have investigated the inhibition of the CTL reaction by antitarget cell serum, EGTA, and anti-MAL serum and the results indicate these reagents can also define three discrete phases to the CTL reaction. The first phase involves *recognition* and contact between the killer lymphocyte and the target cell and is inhibited by antitarget cell alloantiserum (31). The second phase involves a lymphocyte dependent process during which the lytic mechanism is *activated* and the lethal event is delivered to the target cell. The second phase is dependent upon the presence of Ca^{2+} ions and is defined in this system by the inhibition of lysis with EGTA. Experiments in progress are determining the possibility that the activation step defined by EGTA here is synonymous with the "programming for lysis" step as defined by Martz (1, 39). The first two steps occur quickly in this CTL system (~ 10 min) as shown in Fig. 5 and by other techniques (1). The third phase is independent of the lymphocyte and is the step in which the target cell undergoes *lysis* (1). Thus, reagents that inhibit lysis after a Ca^{2+} -dependent step may be thought of as inhibiting the lytic phase. In the experimental system employed in this study the rat anti-MAL inhibited the lysis of P815 targets at times *after* a Ca^{2+} -specific chelating agent, EGTA, and antitarget antisera could mediate their inhibitory activities. This result implies that the anti-MAL serum contains antibody specificities for determinants involved in the lytic phase of the cytotoxic reaction. However, the experimental system employed in these studies cannot rule out the possibility that recognition and activation steps were also inhibited by these sera. We propose that the anti-MAL inhibits CTL activity by virtue of its capacity to neutralize the lytic effector mechanism employed by CTL.

The hypothesis derives support from the studies of Hiserodt and Bonivida (38) who, in the same manner, developed an analogous anti-ATC antiserum (termed anti-T* in their studies) by our methods described here. Employing the dextran dispersion method of Martz (39) to characterize the site of inhibition by their antiserum, these investigators have shown the anti-T* to be capable of inhibiting target cell lysis after delivery of the lethal hit during the lymphocyte independent phase. This result indicates that a transfer of "material" from the CTL to the target cell takes place during cytolysis. Moreover, this result implies that the CTL effector mechanism employs effector molecules in the lytic mechanism and thus argues against the hypothesis that lysis occurs via mechanical disruption or tearing of the target cell membrane (13).

Further studies of the anti-MAL will focus on defining the antigenic specificities recognized by this antiserum. The use of immunoabsorbent columns and traditional biochemical techniques will be employed to remove and isolate the cytolytic-inhibitory antibodies from this antiserum, thus yielding information on the relevant antigenic determinants involved in lytic mechanism. Work in progress is defining the inhibitory antigenic specificities of this antiserum by application of the monoclonal antibody-producing hybridoma technique (40).

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