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THE MECHANISM OF CELL-MEDIATED CYTOTOXICITY
I. Killing by Murine Cytotoxic T Lymphocytes Requires Cell Surface Thiols and Activated Proteases[1]

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Murine allogeneic cell-mediated lympholysis (CML) involves steps requiring cell surface sulfhydryl groups and separate steps that appear to require one or more cell surface proteases that become activated during the lytic process. Chloromethyl ketone derivatives of amino acids, which are specific irreversible inhibitors of *isolated* serine-dependent esterases, inhibit a variety of cellular functions including cell-mediated cytotoxicity. These reagents, such as the lysine derivative (TLCK), inhibited CML when the effectors were briefly treated and washed before the assay. However, the inhibition did *not* appear to result from inactivation of a serine-dependent esterase, because we found that TLCK inhibition was not reversed by including 10- to 30-fold molar excesses of protease substrates. Chloromethyl ketone derivatives are also alkylating agents that nonspecifically react with thiol groups. Iodoacetamide ($IAcNH_2$), an alkylating agent with little reactivity for serine-dependent proteases, also inhibited CML. Furthermore, we showed that several other thiol-reactive compounds, including diamide, which penetrates cells, and oxidized glutathione and thiolyte monoquat, which do not penetrate cells, also inhibited CML. Therefore, a cell surface thiol group appeared involved in an obligatory step in CML. We obtained evidence in other experiments that proteases also appeared to participate in CML. CML was inhibited when low m.w. trypsin or chymotrypsin ester substrates or protein anti-proteases were included in the assay. Pretreatment of effector cells with serum α_1AT, which irreversibly inactivates serine-dependent esterases, did not inhibit killing. Inhibition of CML by α_1AT included in the assay increased as the assay time was lengthened. We interpreted these results to mean that CML involves one or more cell surface proteases that become activated as a consequence of effector-target interactions.

The mechanism of cell-mediated cytotoxicity remains unclear despite extensive study (reviews, 1–6). We have addressed this question by using murine T lymphocytes (CTL)[4] stimulated by allogeneic differences. Murine CTL are specific and bear receptors that recognize structures coded by the K and D regions of H2. The identity of the CTL receptor is not known, but it appears to share idiotype(s) with immunoglobulin (7, 8). The receptor probably provides more than a simple Mg^{++} dependent (4) adherence mechanism between CTL and target cells (9). Perturbation of the receptor initiates the remainder of the lytic sequence which requires energy (10), is Ca^{++} dependent (3, 4, 11), unidirectional (12), and ultimately results in the osmotic lysis of the target (4, 11).

One or more serine esterases may be involved in the lytic process. Ferluga *et al.* (13) reported that CTL-mediated lysis (CML) could be blocked by phosphonates and organophosphorus fluoridates that are serine esterase inhibitors. The fluoridates, such as diisopropyl phosphorofluoridate (DFP), were inhibitory both when present in the assay and when the CTL were pretreated and washed. Strom *et al.* (10) confirmed these data by using rat CTL, although they found that much lower concentrations of DFP were inhibitory. However, it is puzzling that phenyl methyl sulfonyl fluoride (PMSF), which has properties very similar to DFP, has not been found to inhibit CML (4, 14). Chloromethyl ketone derivatives, which specifically alkylate a histidyl residue in the active site of isolated serine proteases (15, 16), also inhibit CML. Matter (14) reported that the lysine derivative, tosyl lysine chloromethyl ketone (TLCK),[5] was considerably more inhibitory than the phenylalanine derivative, tosyl phenylalanine chloromethyl ketone (TPCK), even though others have reported that both compounds were inhibitory (17). The implications of these data are unclear, since the chloromethyl ketones can also inhibit thiol-dependent enzymes by alkylating sulfhydryl groups (16, 18–20). Macromolecular protease inhibitors, such as soybean trypsin

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[4] Abbreviations used in this paper: ADCC, antibody-dependent cell-mediated cytotoxicity; $B6_m$, C56BL/6 (mitomycin C treated); CTL, cytotoxic T lymphocyte(s); CML, CTL-mediated lysis; DFP, diisopropylfluorophosphate; $IAcNH_2$, iodoacetamide; NK, natural killer; PMSF, phenyl methyl sulfonyl fluoride; TIC, trypsin inhibitor capacity; SBTI, soybean trypsin inhibitor; 2-Me, 2-mercaptoethanol; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid; ATEE, acetyl tyrosine ethyl ester; PABAM, *p*-aminobenzamidine; diamide, diazenedicarboxylic acid *bis*(*N*,*N*-dimethylamide); α_1AT, α_1-anti-trypsin; GSSG, glutathione in the oxidized (disulfide) form; GT, glycyltyrosine; LBTI, lima bean trypsin inhibitor; TM, thiolyte monoquat; TLCK, tosyl lysine chloromethyl ketone; TLME, tosyl lysine methyl ester; TPCK, tosyl phenylalanine chloromethyl ketone; TAME, tosyl arginine methyl ester; BSS, balanced salt solution; E:T, effector:target; IAcOH, iodoacetate; ATP, adenosine triphosphate.

[5] Although TLCK was identified as the leucine derivative (14), Dr. Matter has verified that the lysine derivative was actually used.

inhibitor (SBTI), have been reported to be inhibitory in some (21) but not other studies (14).

The current experiments were undertaken to reexamine the participation of proteases in CML. We found that both TPCK and TLCK inhibited CML. However, the inhibition was not due to inactivation of serine proteases, but was probably caused by thiol alkylation. Other experiments confirmed that thiols were essential for CTL activity and that these sulfhydryl groups appeared to be on the cell surface. Although chloromethyl ketones did not inhibit killing via inactivation of proteases, we found that low m.w. substrates of trypsin and chymotrypsin-like enzymes did inhibit CML if present during the assay. Furthermore, macromolecular protease inhibitors also blocked CML, but only if they were included in the CML assay. Our results imply that CML activity requires both a cell surface thiol-dependent step and one or more serine-dependent proteases that become activated by effector-target cell interaction.

MATERIALS AND METHODS

Media and reagents. Secondary mixed lymphocyte cultures and CML assays were performed by using RPMI 1640 prepared by the University of California, San Diego (U.C.S.D.) tissue culture core facility and supplemented with 1% penicillin-streptomycin-glutamine (22). Culture medium was also supplemented with 5×10^{-5} M 2-mercaptoethanol (2-Me) and 5% fetal bovine serum (FBS; Flow Laboratories, Rockville, Md.; Lot 4055865, which was a supportive lot of FBS, but nonsupportive lots appeared to work as well in CTL generation). Assay medium was supplemented with 5% heat-inactivated FBS (56°C for 30 min) and 1.5% 1.0 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; Sigma Chemical Co., St. Louis, Mo.).

The following compounds were obtained and used without further purification: acetyl-L-tyrosine ethyl ester (ATEE; Calbiochem, La Jolla, Calif., Lot 920162); *p*-aminobenzamidine (PABAM; Sigma, Lots 47C-0323 and 127C-0345); human serum α_1-anti-trypsin (α_1AT[6]; Worthington, Freehold, N. J., Lots 58K591 and 58K592); chymotrypsin (Worthington, Lot 36J835); diazenedicarboxylic acid *bis*(*N,N*-dimethylamide) (diamide; Calbiochem, Lot 110060); glutathione in the oxidized (disulfide) form (GSSG; Calbiochem, Lot 400414); glycyltyrosine (GT; Sigma, Lot 58C-0091); lima bean trypsin inhibitor (LBTI; Sigma, type II-L, Lot 127C-8010); PMSF (Sigma, Lot 28C-0039); SBTI (Sigma, type I-S, Lot 77C-8000); thiolyte monoquat (TM; Calbiochem, Lot 940002); TLCK (Calbiochem, Lot 740122, and Sigma, Lot 67C-0047); tosyl lysine methyl ester (TLME; Sigma, Lot 127C-0436); TPCK (Calbiochem, Lot 800051); tosyl arginine methyl ester (TAME; Calbiochem, Lot 410149, and Sigma, Lot 93C-2230); and trypsin (Worthington, Lot 38H682). Iodoacetamide ($IAcNH_2$), Lot 081767, was from the Aldrich Chemical Co., Milwaukee, Wis. All reagents were stored at 4°C or −20°C and freshly prepared in balanced salt solution (BSS) (22) from the U.C.S.D. tissue culture core facility at a concentration 2- to 4-fold greater than the final stated concentrations. ATEE was less soluble in aqueous solutions, so it was initially solubilized in ethanol and then diluted with BSS. The highest reported concentration of ATEE (3.3 mM) also contained 0.8% (v/v) ethanol. Ethanol solutions at this concentration were tested in the same experiments and had no effect on CTL. All inhibitor solutions were kept at ice temperature until they were added to CTL assays.

Trypsin inhibitory capacities (TIC) of anti-proteinases were determined as described (23).

Animals, tumors, and culture conditions. BALB/c and CBA mice were obtained from L. C. Strong Laboratories (San Diego, Calif.). DBA/2J mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) and C56BL/6 (B6) animals were obtained from both sources. All mice were housed in the U.C.S.D. animal quarters and furnished with food and water *ad libitum*. EL4 tumors cells (B6 thymoma) were generously provided by J. Coha (Biology Dept., U.C.S.D.) and maintained in ascites form in B6 mice. BALB/c or CBA mice that were to be used as responder spleen cell donors were immunized one or more times with 1 to 3×10^7 viable EL4 i.p. and sacrificed 4 to 8 weeks after the final immunization.

CTL were generated in secondary mixed lymphocyte cultures. Mice were sacrificed by cervical dislocation, and single-cell suspensions were prepared from the spleens. Stimulator cells were incubated with 25 μg/ml mitomycin C (Sigma) for 60 min at 37°C and washed four to six times. Cell suspensions containing $\sim 1 \times 10^7$ responder and 5×10^6 mitomycin C-treated B6 ($B6_m$) in 5 ml were incubated in 60 x 15 mm tissue culture dishes (Falcon No. 3002). Cultures were incubated in air-tight boxes with continuous rocking (4 to 8 cycles/min) under an atmosphere of 10% CO_2, 7% O_2, 83% N_2 for 4 to 7 days.

^{51}Cr-labeled target cells and CML assays. Freshly obtained EL4 ascites cells (2 to 4×10^7) were suspended in assay medium and incubated with 100 to 200 μCi $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, Mass.) for 1 to 3 hr followed by extensive washing. Just before use, ^{51}Cr-EL4 target cells were adjusted to contain 5×10^4 cells/100 μl with 8,000 to 30,000 cpm. The targets were then added to effector cells alone or with various concentrations of inhibitors to give a final volume of 0.40 to 0.50 ml. Effector cell titrations with 3-fold serial dilutions were performed with each inhibitor concentration. All assays were performed in plastic tubes, 12 x 75 mm (Falcon or Sarstedt) or 10 x 75 mm (Falcon) in size. After the times indicated in the results, 0.50 or 0.60 ml ice cold assay medium was added to terminate the reaction, the tubes were centrifuged at 4°C, and 0.5 to 0.7 ml of supernatant was removed for gamma counting. Spontaneous release was determined by incubating target cells in the absence of effectors, and total release was that caused by 4 cycles of freeze-thaw.

$$\text{Specific release} = \frac{\text{Experimental} - \text{spontaneous}}{\text{Total} - \text{spontaneous}} \times 100\%$$

All assay points were set up in duplicate or triplicate tubes, and the replicates were within 2 to 4 % of each other.

CML inhibition assays were performed in two ways. Cultured effector cells were either pretreated with inhibitor and washed before assay, or the inhibitor was included during the 37°C incubation with target cells. After pretreatment, the effector cells were counted with trypan blue before being used in CML assays. In the other type of inhibition protocol, effectors were preincubated with inhibitor for 0 to 60 min at 37°C or room temperature before adding targets. These variations in the preincubation conditions produced no detectable effect on the degree of inhibition and are not described in detail. Controls included inhibitors incubated alone with effector cells that were counted with trypan blue at the end of the assay, and target cells incubated alone with inhibitor. The inhibitors had no detectable effect on the number of viable effector cells and elevated the spontaneous release ≤15%, as noted in *Results*. In

[6] The commercial α_1AT used in these studies was examined by polyacrylamide gel electrophoresis and was found to contain a minimum of four to six components with α_1AT comprising ~30% of the total protein.

no case could the observed inhibition be explained by toxicity as determined by these criteria.

RESULTS

Inhibition of CTL-mediated killing by TLCK and TPCK included in the assay. Both TLCK (trypsin inhibitor) and TPCK (chymotrypsin inhibitor) inhibited CML when included in the assay at concentrations of 0.1 to 0.3 mM. These effects were observed without reductions in the viability of effector cells and in the absence of any significant elevation of spontaneous ^{51}Cr release from target cells. The two compounds appeared to have similar activity (Fig. 1), although TPCK appeared to be slightly more inhibitory than TLCK. Comparisons of TLCK and TPCK were complicated by their differential solubilities and susceptibilities to hydrolysis. TLCK was readily soluble at a concentration of 4 mM in BSS, whereas TPCK was only partially soluble at ~1 mM. In an experiment not shown, both compounds were incubated ~2 hr at 37°C in an attempt to solubilize TPCK and then were titrated. TPCK retained its inhibitory activity, but TLCK required ~1 mM final concentration to inhibit as well as 0.1 mM in the results presented in Figure 1, suggesting TLCK had undergone hydrolysis. Therefore, in subsequent experiments TLCK was used quickly after preparation and/or TLCK solutions were kept at ice temperature until used.

Pretreatment with TPCK and TLCK. CTL that were exposed to TPCK or TLCK for 5 to 30 min and then extensively washed were inhibited. In an experiment not shown, CTL were incubated with 0.33 or 0.11 mM TPCK for 5, 10, 15, or 30 min at 37°C or at ice temperature for 30 min, centrifuged, and washed 2 × 3 ml at 0–4°C, resuspended, and assayed. The CTL treated with 0.33 mM TPCK at 37°C all had ~9-fold reductions in activity, i.e., treated cells had CML activity equivalent to a 1:9 dilution of untreated effectors. However, the cells that were treated in the cold were also significantly inhibited by TPCK (~3- to 6-fold). This suggested that TPCK, which was poorly soluble, was either crystallizing out of solution upon chilling and/or binding to cells such that it could not be effectively removed by centrifugation. Therefore, TPCK yielded ambiguous results in pretreatment protocols. Conversely, cells exposed to TLCK at ice temperature and washed in the cold were not inhibited, as shown in Figure 2*A*. The experiment summarized in Figure 2*A* demonstrated that inhibition by TLCK was temperature and concentration dependent, and that cells briefly exposed to TLCK at 37°C remained inhibited after removing the compound by centrifugation.

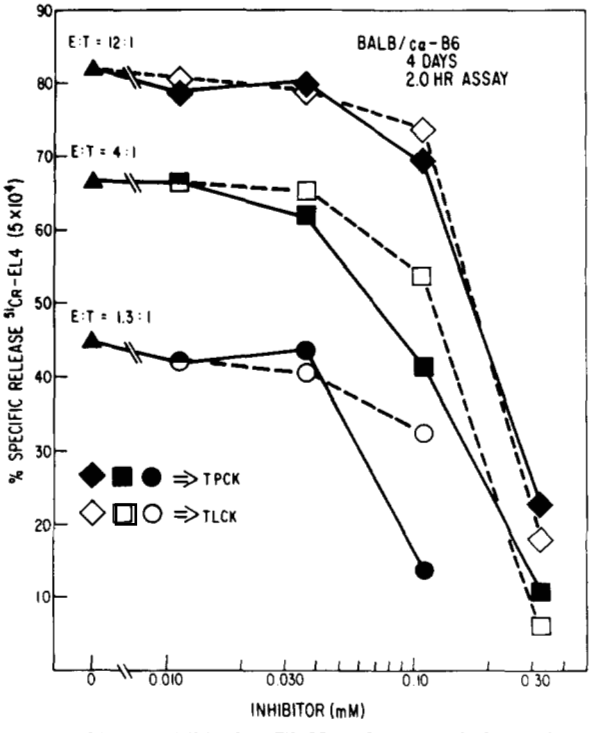

Figure 1. CML is inhibited by TLCK or TPCK included in the assay. BALB/c spleen cells were cultured with $B6_m$ for 3 days and then tested in a 2-hr assay with varying concentrations of TLCK or TPCK. Spontaneous release was 1055 cpm (5.08%/hr) and total release was 8945 cpm (88.48%). TLCK caused 2 to 3% elevations in spontaneous release and TPCK caused as much as 12% additional spontaneous release at 0.33 mM. The indicated values were corrected for these changes in background release.

The target of chloromethyl ketone derivatives. TLCK and TPCK are specific inhibitors of trypsin (16) and chymotrypsin-like (15) enzymes, respectively. Chloromethyl ketones are also nonspecific alkylating agents that react with sulfhydryl groups and can inhibit thiol-dependent enzymes such as ficin, papain, or cathepsin B1 (16, 18–20). Therefore, the observation that TPCK and TLCK were nearly equally inhibitory (Fig. 1) suggested that both trypsin and chymotrypsin-like enzymes were obligatory and/or that CTL required thiol groups to be able to kill targets. The results of the following experiments indicated that the chloromethyl ketone derivatives were probably inhibiting CTL activity by reacting with essential thiol groups.

$IAcNH_2$, an alkylating agent with little trypsin-inhibitory capacity (24) and TLCK were equally effective as CML inhibitors (Fig. 2). Furthermore, CTL could not be protected from inhibition by treating in the presence of a 10- to 30-fold molar excess of a protease substrate. Figure 2 summarizes one experiment of this type comparing TLCK (Panel A) and $IAcNH_2$ (Panel B) pretreatments and showing that a 10-fold excess of the trypsin substrate TAME did not protect the CTL. The inability of TAME to prevent inhibition by TLCK or $IAcNH_2$ did not represent carryover of these materials, because CTL pretreated in the cold were not inhibited. TLCK inhibition was also not prevented by a 30-fold molar excess of TAME or a 10- to 30-fold molar excess of ATEE or PABAM (not shown). Similar experiments were attempted with TPCK instead of TLCK. However, since CTL exposed to TPCK at ice temperature and then washed in the cold were inactivated, one could not determine whether the ester substrates were actually not protective. Use of TLME instead of TAME yielded ambiguous results, since TLME alone exerted some irreversible inhibitory effects (see below).

Cells treated with TLCK could not be reactivated by exposure to trypsin. It has been reported that CTL activity could be enhanced by treating the effectors with trypsin or papain (25). Therefore, we attempted to reverse TLCK-induced inhibition by incubating cells first with TLCK and then with trypsin at concentrations ranging from 3.3 μg/ml to 1.0 mg/ml. The TLCK-induced inhibition was not reversed by trypsin treatment, and cells treated with trypsin did not have enhanced activity (not shown).

Inhibition of CTL by thiol-reactive compounds. The data summarized above suggested that thiol groups were obligatory in CTL-mediated killing. Therefore, it was of interest to determine whether other thiol reactive compounds were inhibitory. We found that diamide, GSSG, and TM (26) were all capable of inhibiting CML. The latter two compounds do not readily penetrate cell membranes (R. Fahey, personal communication,

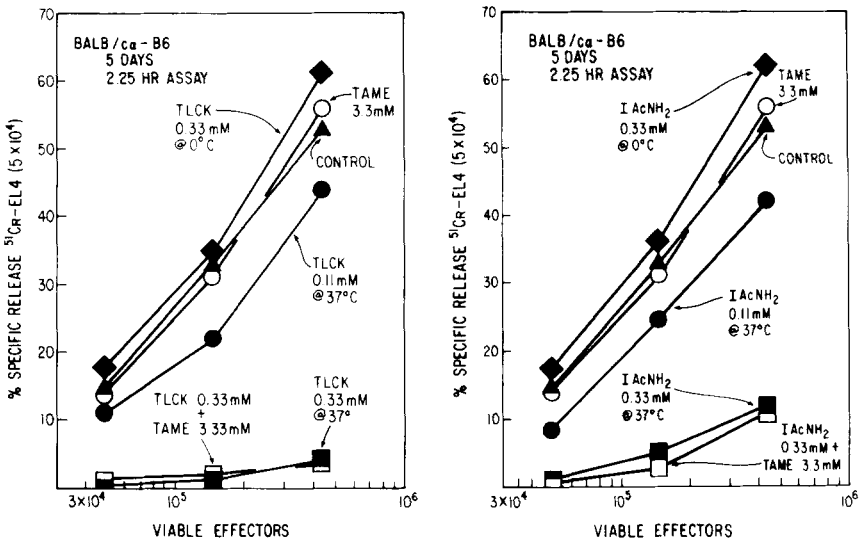

Figure 2. Excess protease substrate, TAME, does not protect CTL against inactivation by TLCK or by $IAcNH_2$. BALB/c spleen cells were cultured for 5 days with $B6_m$ and aliquots containing 0.80 ml with ~2.3×10^6 cells were mixed with TAME or BSS followed by BSS, TLCK (*A, left panel*), or $IAcNH_2$ (*B, right panel*) as indicated. After treatment, the cells were washed, counted with trypan blue, and tested in a normal 2-hr CML assay. Spontaneous release was 1196 cpm (5.0%/hr) and total release was 8706 cpm (83.56%).

and 27), suggesting that the obligatory thiol groups were on the CTL surface.

The diazene compound diamide (28) inhibited CML when included in the assay at 0.1 to 0.4 mM (Fig. 3). The concentrations of diamide required to inhibit CML were a function of the number of cells present. For example, 0.10 mM inhibited the lower effector:target (E:T) ratios, but had virtually no effect on the highest E:T ratio (Fig. 3). Likewise, 0.20 mM diamide caused progressively greater inhibition as the total number of effector and target cells was decreased. This was expected, since diamide is known to cause a quantitative oxidation of cellular sulfhydryl groups. The concentrations of diamide reported in Figure 3 did not cause detectable changes in the number of viable cells but did cause some elevation in the rate of ^{51}Cr release, as noted in the Figure 3 legend. Higher concentrations of diamide, e.g., 1 mM, were clearly toxic and caused 40 to 60% cell death. Inhibition was reversible when cells were treated for 15 to 30 min with 0.1 to 0.3 mM diamide. After washing, these cells regained CML activity (not shown). The presence of 0.2 mM diamide during pretreatment partially protected cells from inactivation by 0.33 mM TLCK in an experiment analogous to that summarized in Figure 2 (not shown). This was consistent with TLCK inhibition of CML being caused by alkylation of sulfhydryls instead of inactivation of serine esterase(s).

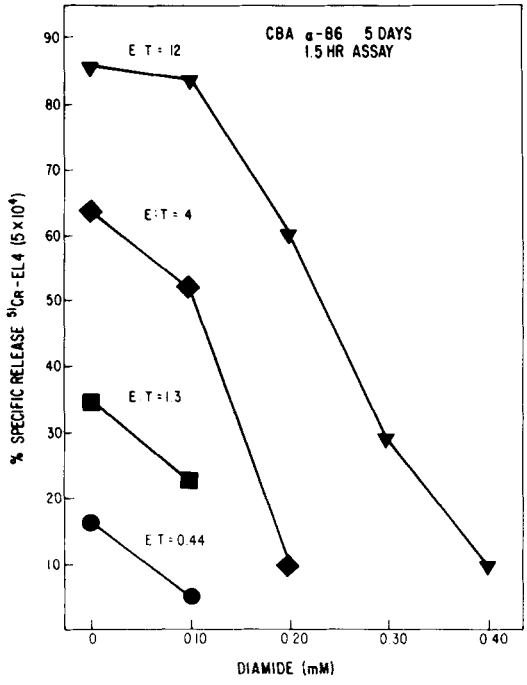

Figure 3. Diamide included in the CML assay inhibits cytolysis. CBA spleen cells were cultured with $B6_m$ stimulators for 5 days and then tested in a 1.5-hr CML assay with diamide added to yield the stated concentrations. Spontaneous release was 847 cpm (4.3%/hr) and total release was 9606 cpm (80.77%). Diamide did not affect the number of viable effector cells but it did elevate the spontaneous release as much as 15% at 0.40 mM. The indicated values were compensated for the increased background release.

Diamide-induced CML inhibition confirmed that thiols were necessary for CTL function but did not indicate whether the essential thiol groups were on the cell surface or intracellular, since diamide could penetrate cell membranes (28). Therefore, we tested GSSG and TM, to which cells are impermeable. GSSG pretreatment did not cause CML inhibition, but inclusion of 5 to 20 mM GSSG in the CML assay did inhibit cytolysis (Fig. 4). GSSG at ~15 mM was sufficient to cause a 3-fold reduction in activity. None of the tested GSSG concentrations caused any detectable loss of viable effector cells or any increase in spontaneous release from ^{51}Cr-EL4. TM caused a 3-fold reduction in CML activity when included in the assay at 0.5 mM (not shown). TM also inhibited CML when the CTL were pretreated (Fig. 5). TLCK and TM had similar inhibitory activity, and none of the tested concentrations reduced the number of viable effector cells.

Inhibition of CTL-mediated killing by low m.w. protease substrates or inhibitors. ATEE, a substrate of chymotrypsin-like enzymes, TAME, and TLME, substrates of trypsin-like enzymes, and PABAM, a reversible trypsin inhibitor, were each capable of inhibiting CML reactions when included in the assays at concentrations of 1 to 10 mM. ATEE at a concentration of ~3 mM caused an inhibition in ^{51}Cr release, equivalent to a 1:3 dilution of the effector population (Fig. 6). The ester linkage on the tyrosine was important because GT at 10 mM caused no CML inhibition (not shown). TAME was approximately one-half as effective as ATEE and required a 5 to 6 mM concentration to achieve a 3-fold inhibition (Fig. 6). TLME was of intermediate activity, with a concentration of ~4 mM causing a 1:3 reduction in CML activity (not shown). PABAM also inhibited CML when included in the assays in the same range of concentrations at which the ester substrates were effective

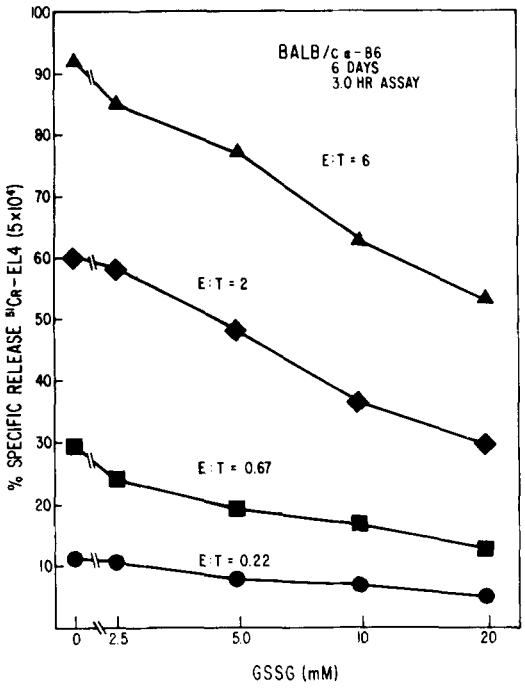

Figure 4. GSSG inhibits CML when included in the assay. BALB/c spleen cells were cultured with $B6_m$ stimulator cells for 6 days and then tested in a 3-hr CML assay. GSSG was solubilized in BSS, neutralized with NaOH, and added to produce the stated concentrations. Spontaneous release was 739 cpm (1.54%/hr) and total release was 11,314 cpm (77.79%). GSSG caused no detectable increase in spontaneous release and did not reduce effector cell viability.

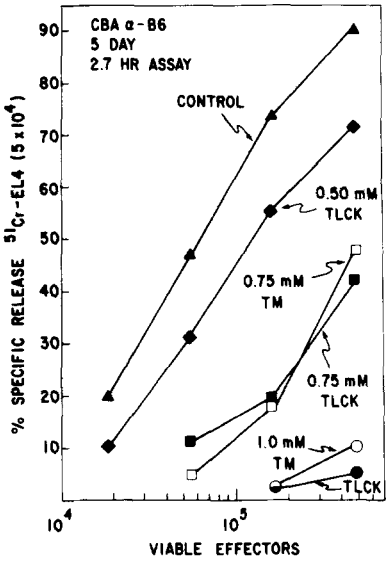

Figure 5. CML is inhibited by pretreatment of effectors with equivalent concentrations of TLCK or TM. CBA spleen cells were cultured with $B6_m$ stimulators for 5 days. Aliquots containing ~2.5 × 10^6 effector cells were incubated with BSS or the stated concentrations of TM or TLCK for 15 min at 37°C, washed 2 x 3 ml with cold medium and tested in a standard CML assay. None of the treatments reduced the number of viable cells. Spontaneous release was 4033 cpm (5.54%/hr) and total release was 21,597 cpm (86.10%).

(Fig. 7). Effector cells were incubated with the compounds and then counted with trypan blue at the conclusion of the assay to determine toxicity. None of the compounds as tested in the experiments summarized in Figures 6 and 7 caused a detectable decrease in viable effector cells. The inhibitors were also added to ^{51}Cr-EL4 and incubated in the absence of effectors. None of the compounds caused more than 5 to 10% increase in spontaneous release, as noted in the figure legends. Therefore, it appeared unlikely that the inhibitory effects were caused by toxicity. It was also possible that the ethanol or methanol hydrolysis products of the esters could be inhibitory. However, this appeared to be unlikely, because alcohol concentrations as great as 100 mM (ethanol) or 300 mM (methanol) had no detectable effects on CML (not shown).

Two anomalous results were observed with these compounds. First, TAME lost 25 to 50% of its inhibitory activity after 2 to 3 months of repeated tests from the same bottle of dry reagent. Similar deterioration of TAME as a trypsin substrate has been reported by others (29). Second, TLME was observed to have some irreversible effects on CTL after pretreatment, whereas the other compounds had no effects unless they were present during the assay. Only one lot of TLME was tested, so it remains to be determined whether the inhibitory effect of pretreatment was caused by a contaminant in this particular preparation.

Inhibition of CTL-mediated killing by macromolecular trypsin inhibitors. The experiments reported above demonstrated that CTL-mediated killing could be inhibited by substrates of both trypsin and chymotrypsin-like enzymes (Figs. 6 and 7). If such enzymes were involved in cytolysis, the preceding experiments did not permit one to determine whether they were on the surface and/or intracellular, or whether they were preactivated or activated as a consequence of target cell contact. Therefore, we tested macromolecular trypsin inhibitors both in pretreatment protocols and by including them in the assay.

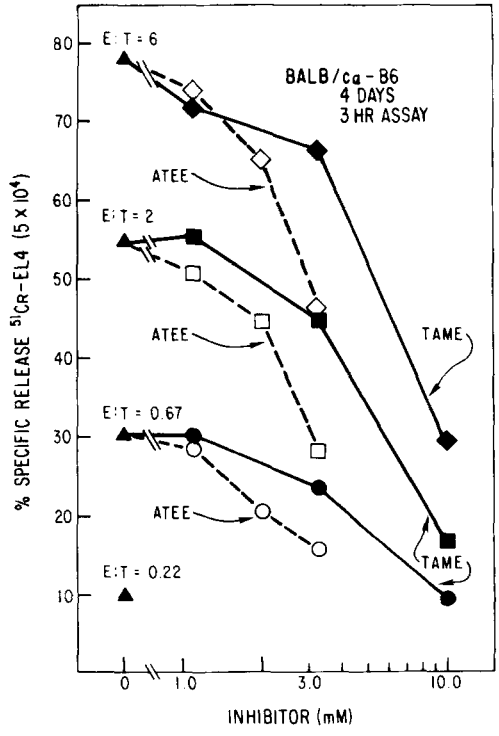

Figure 6. TAME or ATEE included in the assay inhibits CML. BALB/c effectors were generated by 4 days of culture with $B6_m$ stimulator cells. Serial 3-fold dilutions of effectors were mixed with TAME or ATEE to yield the stated concentrations and incubated 3 hr with ^{51}Cr-EL4. Spontaneous release was 1418 cpm (3.64%/hr), and total release was 10,526 cpm (87.28%). The inhibitors caused a 1 to 6% total increase in spontaneous release, and the indicated values were corrected.

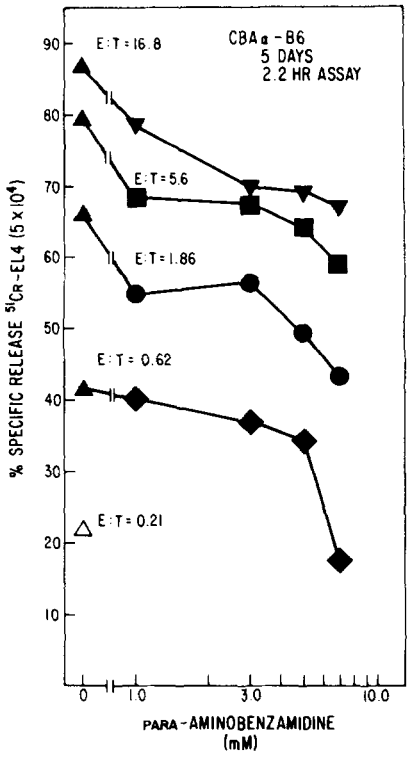

Figure 7. PABAM included in the CML assay inhibits killing. CBA effectors were generated by culturing with $B6_m$ stimulators for 5 days and tested in a 2.2-hr assay. Spontaneous release was 1800 cpm (6.0%/hr) and total release was 11,748 cpm (90.59%). PABAM had no effect on CTL viability or spontaneous release.

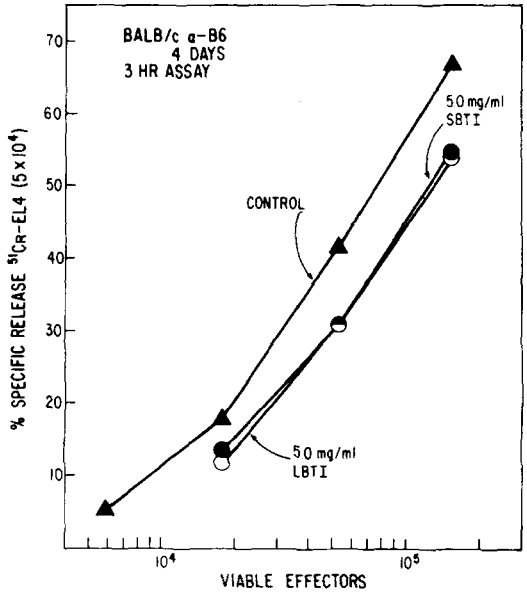

Figure 8. SBTI and LBTI block CML when included in the assay. BALB/c effector cells were generated and tested as described in Figure 6. The inhibitors were dialyzed for 2 days and retested with similar results. Neither inhibitor reduced the number of viable effector cells nor increased the spontaneous release from ^{51}Cr-EL4.

Protein trypsin inhibitors derived from soybeans (SBTI), lima beans (LBTI), and human serum α_1 antitrypsin (α_1AT) inhibited CML when included in the assay (Figs. 8 and 9). None of these materials appeared to be toxic for the effectors or for the targets when tested as described above for the low m.w. substrates. SBTI and LBTI both retained inhibitory activity after dialysis by using dialysis tubing that retained materials larger than 3000 daltons (not shown). Pretreatment of CTL with α_1AT did *not* inhibit CML activity, even though α_1AT is

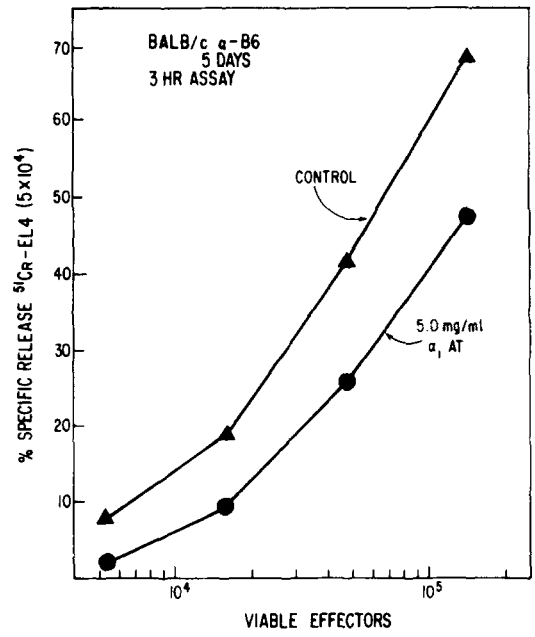

Figure 9. Human α_1AT inhibits CML when included in the assay. BALB/c spleen cells were cultured with $B6_m$ stimulator cells and tested in a 3-hr assay. Spontaneous release was 762 cpm (2.42%/hr) and total release was 7618 cpm (83.38%). Although, α_1AT did not change the number of viable effector cells, it did increase the spontaneous release by a total of 8.6% which was compensated in the indicated values.

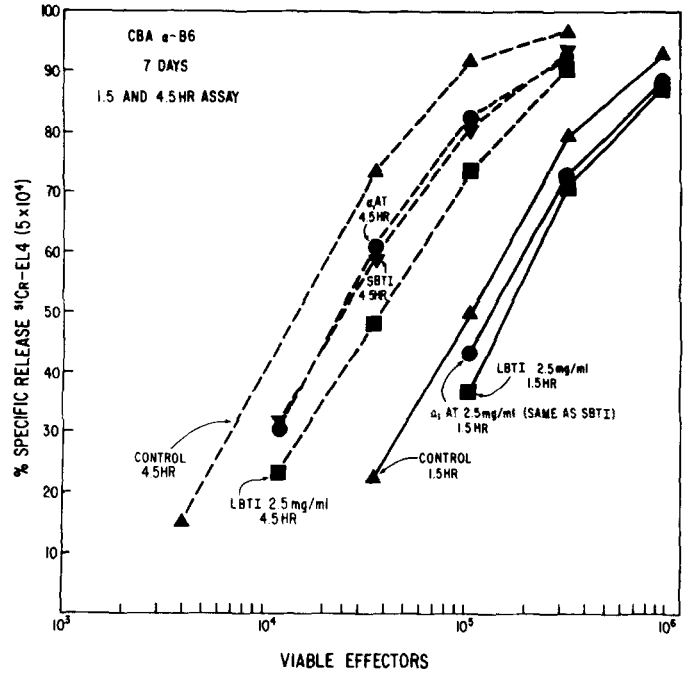

Figure 10. Inhibition by SBTI, LBTI, or α_1AT increases as the assay time is lengthened. CBA spleen cells were cultured with $B6_m$ stimulators for 7 days and then tested in 1.5- (*solid lines*) or 4.5-hr (*dashed lines*) CML assays. Spontaneous release was 507 cpm (5.53%) for 1.5 hr and 1088 cpm (11.94%) for 4.5 hr and total release was 7550 cpm (89.29%). None of the inhibitors affected the number of viable effector cells or the spontaneous release.

known to form stable complexes with serine proteases (30) and all three inhibitors were able to block trypsin activity after 5 to 15 min in the TIC assays. Furthermore, all three inhibitors blocked killing to a greater extent as the assay incubation time was increased (Fig. 10). This was in contrast to inhibition by TAME, which did not become greater with time (not shown). In the experiment summarized in Figure 10, inhibitors at 2.5 mg/ml had little effect at 1.5 hr, but caused marked inhibition after 4.5 hr. At this later time, the observed ^{51}Cr release reflected multiple lytic events by individual effector cells because of the low E:T ratios. The three inhibitors were tested at equal protein concentrations, but they possessed different trypsin inhibitory capacities, which were LBTI = 4.0, SBTI = 3.1, and α_1AT = 0.36. The actual solutions used in this experiment were tested for TIC, comfirming that LBTI and SBTI were similar, and that α_1AT had a TIC at least one order of magnitude lower (not shown). Furthermore, crude α_1AT preparations consisting of 50 to 70% saturated ammonium sulfate fractions from human serum or plasma, which had relatively low TIC, were even more inhibitory than the commercial (Worthington) α_1AT used in Figures 9 and 10. This suggested that one or more of the other serum anti-proteases contained in these fractions, such as α_1-antichymotrypsin, were also active as CTL inhibitors.

DISCUSSION

The current results suggest that killing by murine CTL includes steps requiring cell surface thiol groups and one or more cell surface proteases that become activated during the lytic process. Brief treatment of CTL with alkylating agents, including chloromethyl ketone derivatives of amino acids, $IAcNH_2$, or TM at concentrations from 0.1 to 1.0 mM, inhibited their ability to lyse allogeneic target cells. In addition, the thiol oxidant diamide and the disulfide compound GSSG inhibited CML when included in the assay at 0.1 to 0.3 mM or 5 to 20 mM, respectively. GSSG (27) and TM (26) (probably also TLCK because of its charge) do not readily permeate cells, suggesting that the essential thiols are on the cell surface. CTL-mediated killing also appears to involve one or more proteases. Low m.w. substrates of trypsin or chymotrypsin-like proteases such as TAME or ATEE inhibited CML when included at 3 to 10 mM. PABAM, a nonhydrolysable arginine analog and a trypsin inhibitor, also blocked CML when included at a similar concentration. Macromolecular irreversible protease inhibitors such as LBTI and human serum α_1AT also blocked CML, but only when included in the assay at concentrations of 2.5 to 5.0 mg/ml. These findings implied that the protease(s) blocked by these inhibitors occurred on the cell surface and became activated only during the killing process. However, the current data do not allow us to determine whether the protease(s) is associated with the effector and/or the target cell.

Chloromethyl ketone derivatives of amino acids have been shown to inhibit a variety of cellular functions, such as antibody-dependent cellular cytotoxicity (ADCC) by human lymphocytes (31) and eosinophils (32), murine and human CML (14, 21), and cell division by cultured cell lines (33). These findings have been interpreted to mean that proteases were obligatory in these processes, since these compounds are specific inhibitors of isolated serine-dependent proteases, such as trypsin or chymotrypsin. However, these compounds are also nonspecific alkylating agents that react with thiol groups and can therefore inactivate a variety of thiol-dependent enzymes. To our knowledge, no other reports of cellular functions being inhibited by TLCK have included the appropriate control showing protection by the presence of a trypsin substrate. For example, TLCK inactivation of trypsin is significantly reduced by including an equimolar concentration of a reversible inhibitor such as benzamidine (16). However, we found that TLCK inactivation of CTL was not prevented by as much as a 30-fold molar excess of TAME (Fig. 2). Any experiments purporting to demonstrate the involvement of a serine-dependent esterase based on the susceptibility of cell function to inhibition by chloromethyl ketone derivatives must be interpreted with extreme caution.

The thiol requirement in CML could also explain some of the inconsistencies reported with other serine esterase inhibitors. DFP, an irreversible serine esterase inhibitor (34), was reported to inhibit CML by two groups (10, 13), but significantly different concentrations were required in these studies. Commercially available DFP is frequently contaminated with a thiol reactive compound (35, 36), which could have been responsible for the observed inhibition. This could also explain why PMSF, which has reactivity similar to that of DFP, has been shown not to inhibit CML (4).

Thiols are involved in a variety of metabolic processes including glycolysis, protein synthesis, and nucleic acid synthesis (37). Cellular adenosine triphosphate (ATP) levels can also be reduced by alkylating agents. However, Trinchieri and DeMarchi (31) demonstrated that inhibition of ADCC by TLCK or $IAcNH_2$ did not correlate with the reduction of ATP concentration. Likewise, Golstein and Smith (11) found that CML inhibition by iodoacetate (IAcOH), which inhibits glycolysis by blocking glyceraldehyde-3-phosphate dehydrogenase (38), could not be overcome by including pyruvate, which should be able to bypass the glycolytic blockade (39). Our data indicated that the essential thiols were on the cell surface and could be regenerated, since inhibition by GSSG or diamide was reversible. Extracellular sulfhydryl groups are unusual because of the redox potential of an oxygen-based atmosphere (37). The cell surface thiol could be in a protected site, e.g., as part of a thiol-dependent protease. If CTL do possess cell surface thiol-dependent protease(s), such enzyme(s) would have to possess substrate specificities different from arginine or tyrosine, because we could not protect the thiols with TAME or ATEE. Whatever the thiol-dependent step is, thiol alkylation appears to cause a complete inhibition of a cell's ability to mediate cytolysis. One can see in Figures 2 and 5 that effectors that were partially inhibited by alkylation killed with an efficiency similar to the untreated cells. That is, the slopes of the percent release *vs* the number of effector cells remained relatively constant, as if one had reduced the *number* of CTL by the treatment. This suggests that the thiol-dependent step is an absolute requisite for the lytic mechanism to be initiated.

The effects of the macromolecular anti-proteases suggested that the serine-dependent proteinase(s) were also on the cell surface. Pretreatment of CTL with α_1AT had no effect on their CML activity, even though α_1AT forms a stable complex with serine-dependent proteases, causing irreversible enzyme inactivation (30). This implied that the enzyme was not present in an active form on the CTL in the absence of targets. We also noticed that the macromolecular protease inhibitors became increasingly inhibitory as the assay incubation time was lengthened (Fig. 10). This was consistent with the susceptible step being a protease that became activated during the CML process. If one or more cell surface proteases become activated only in the region of effector:target cell contact, then it could be difficult to deliver a macromolecular protease inhibitor because of the intimate cell-to-cell contact (40). One also might

not observe any inhibition if the E:T ratio were high. These points could explain previous reports stating that CML was not inhibited by macromolecular inhibitors (14). Activatable cell surface proteases have been proposed in other systems, such as the chemotactic response of neutrophils (41) and degranulation of mast cells (42), although more recent studies indicate these concepts may need to be reconsidered (43). "Activation" could occur in several ways. The relevant enzyme(s) could exist on the cell surface in zymogen form and become activated by hydrolysis. Nonenzymatic activation is also possible, as has been recently reported with sperm acrosin (44). "Activation" could be a conformational change rendering the active site more available to its substrate.

The current data can be fit into a minimal model for the mechanism of cell-mediated lysis. We propose that the thiol-dependent step is a sulfhydryl-dependent protease which is brought to its substrate when the specific receptor is perturbed. Green *et al.* (9) have recently shown that effector-target cell contact alone is not sufficient to initiate CML. The substrate of this thiol-dependent protease could be the zymogen form of a serine esterase. There could be one such enzyme with extremely broad substrate specificity that could be inhibited by ATEE or TAME or two enzymes, perhaps with the first activating the second. Obviously, each step in which a zymogen is activated offers the possibility of amplification. The ultimate lytic event could be mediated directly by a protease, as Hatcher *et al.* (45) have proposed. However, we think this is less likely, because protease inhibition does not appear to reduce CTL efficiency. Figures 8–10 show that reductions in lytic activity resemble a reduction in the number of active cells, as noted above with thiol-reactive compounds. Partial inactivation of the ultimate lytic mechanism should alter the slope of the percent release *vs* effector cell graph rather than the parallel shift we have observed. Therefore, we would propose that the ultimate mechanism is analogous to complement- (C) mediated lysis. The terminal protease would then hydrolyze a substrate leading to the nonenzymatic assembly of a membrane attack complex. Mayer *et al.* (46) have pointed out the similarities between C-mediated and cell-mediated lysis, even though direct attempts to demonstrate the involvement C proteins in CML have been negative (47). Similar mechanisms that utilize completely separate components could exist for the two types of cell lysis.

The same mechanism could be shared among many types of cell-mediated lysis, including CML, ADCC, and natural killer (NK) cells. The results of Trinchieri and DeMarchi (31) showing that chloromethyl ketones and protease substrates could block human ADCC would be consistent with such a model. NK cells have been less studied, but Kiessling and Wigzell (48) have shown that DFP blocks murine NK cells. Haverty *et al.* (49) demonstrated inhibitions of human NK activity with macromolecular protease inhibitors, and one of the authors (Hudig, unpublished observations) has shown that human NK lysis can be blocked by TAME or by chloromethyl ketones. The feature distinguishing the various types of effector cells would then be the receptor and the structure it recognizes on the target cell. Furthermore, all types of cell-mediated cytolysis could be sensitive to inhibition by the plasma anti-proteinases, which are acute phase proteins (30) and could constitute an additional regulatory mechanism.

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