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Inducible Macrophage Cytotoxins. II. Tumor Lysis Mechanism Involving Target Cell-Binding Proteases [1, 2]

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ABSTRACT—Thioglycollate-elicited C57BL/6 peritoneal exudate macrophage monolayers (PEMM) stimulated with poly I·poly C or LPS released a macrophage cytotoxin (MCT) that rapidly bound to syngeneic (EL 4) or allogeneic (NS-1, YAC-1) tumor cells but did not bind to normal splenocytes. No binding to human (K562) tumor cells was observed. PEMM stimulated with poly I·poly C destroyed allogeneic tumor cells (NS-1) when separated by cell-impermeable Millipore filters in vitro; in contrast, PEMM *not* stimulated with poly I·poly C were incapable of lysing targets when separated by membranes. The reversible inhibitors *Nα-p*-tosyl-L-arginine methyl ester and soybean trypsin inhibitor and the irreversible inhibitors *Nα-p*-tosyl-L-lysine chloromethyl ketone and phenylmethylsulfonyl fluoride, of trypsin-like proteases, significantly or totally inhibited MCT cell-lytic activity for L-929 cells in vitro. Furthermore, modification of MCT-associated arginine residues by 1,2-cyclohexanedione completely blocked lytic activity. MCT was concluded to be an inducible nonspecific cell-lytic effector molecule elaborated by activated macrophages, which could bind to potential target cells, and was itself or was associated with a protease.—JNCI 1982; 69:889–894.

Activated peritoneal macrophages from mice are capable of inducing nonspecific cytotoxicity to a variety of syngeneic, allogeneic, and xenogeneic tumor targets in vitro (*1–3*). The cytotoxic reaction occurs within a microenvironment existing at the contact region(s) between the effector and the target. One mechanism of lysis has been proposed to involve a vectorial release of a monokine (e.g., lysosomal enzymes) from the macrophage to the target cell (*4–5*). Yet very little is known about the putative cytotoxic molecule(s) involved in this reaction and the mechanism by which they act on tumor cells.

In the previous manuscript of this series (*6*) we demonstrated that PEMM from alloimmune C57BL/6 mice and thioglycollate-elicited macrophages from nonimmune C57BL/6 mice can be induced in vitro to release a cell-lytic material known as MCT. This is an inducible toxin, and activated macrophages can be stimulated to release MCT when briefly exposed to tumor cells or poly I·poly C in vitro. Release begins within a few hours after stimulation and then rapidly ceases. Physical studies have indicated that MCT from macrophages obtained from alloimmune or thioglycollate-induced mice appears on molecular sieving columns principally as one species of 140,000–160,000 daltons. Biochemical studies indicated that MCT is homogeneous with respect to charge and is heat- and pH-labile (Klostergaard J, Reidarson TH, Granger GA: Manuscript in preparation).

We report here that MCT binds rapidly to murine tumor targets but not to normal cells. Additional studies reveal that stimulated PEMM can destroy NS-1 allogeneic targets in the absence of effector-to-target cell contact. Both reversible and irreversible inhibitors of trypsin-like proteases block MCT activity. Modification of MCT arginine but not lysine residues also blocks lytic activity. These findings suggest that a proteolytic reaction is necessary for the cell-lytic event. Several model(s) are proposed to explain how MCT may act as an effector of in vitro tumor cell lysis mediated by macrophages.

MATERIALS AND METHODS

Culture media and cell lines.—Culture media consisted of RPMI-1640 medium supplemented with 3 or 10% heat-inactivated (56°C, 60 min) FCS (GIBCO, Grand Island, N.Y.) or newborn calf serum (GIBCO), 100 μg streptomycin/ml, and 100 U penicillin/ml. Murine C3H L-929 ($H\text{-}2^k$) transformed fibroblasts were grown in 32-oz prescription bottles in RPMI-1640 with 3% serum at 37°C in 95% air and 5% CO_2 atmosphere and passed twice a week. C57BL/6 EL 4 ($H\text{-}2^b$) lymphoma, BALB/c NS-1 ($H\text{-}2^d$) myeloma, A-Sn YAC-1 ($H\text{-}2^a$) lymphoma, and human K562 erythroid leukemia cell lines were grown in RPMI-1640 supplemented with 10% FCS. Naive BALB/c ($H\text{-}2^d$) spleens were asceptically removed and prepared on the day of the experiment as previously described (*6*).

Binding of MCT to target cells.—Tumor and normal cells, 15×10^6 and 3×10^6, respectively, which were 93–98% viable, were pelleted by centrifugation at $300\times g$ for 5 minutes and washed four additional times with serum-free RPMI-1640. The cells were then resuspended in 300 μl MCT obtained from supernatants of poly I·poly C-stimulated PEMM containing 39–62 U lytic activity and incubated at 4°C. After 30 minutes the cells were pelleted by centrifugation, and

Abbreviations used: ALME=*Nα*-acetyl-L-lysine methyl ester; CF=cytolytic factor; CHD=1,2-cyclohexanedione; FCS=fetal calf serum; IAM=iodoacetamide; MA=maleic anhydride; MAI=methylacetimidate hydrochloride; MCT=macrophage cytotoxin; α-MCT=MCT detectable at 140,000–160,000 daltons; PBS=phosphate-buffered saline; PEMM=peritoneal exudate macrophage monolayers; PMSF=phenylmethylsulfonyl fluoride; poly I·poly C=polyinosinic-polycytidylic acid; SBTI=soybean trypsin inhibitor; TAME=*Nα-p*-tosyl-L-arginine methyl ester; TLCK=*Nα-p*-tosyl-L-lysine chloromethyl ketone.

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[2] Guidelines for the care and use of laboratory animals were followed as set forth by the National Research Council.

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250 μl supernatant was removed and then assayed for lytic activity on L-929 cells. Percent adsorption or removal of units of activity was determined by comparison of the control level of activity with the activity remaining after the supernatants were removed from the target cells.

Lysis of NS-1 targets by poly I·poly C-activated macrophages through membrane filters.—Cellulose nitrate membranes with 0.2 μm-pores (Sartorius membrane filter; Science Essentials, Fullerton, Calif.) were cut into circles and washed with sterile PBS, and then one or two were fitted tightly over 0.5×10^6 adherent macrophages in wells of Costar plates 15 minutes before 50×10^3 target cells were added. After 12- and 36-hour incubation periods at 37°C in a CO_2 incubator, the remaining tumor cells were enumerated and viability was determined by eosin Y exclusion in a hemacytometer.

Protein modification reactions.—MCT partially purified by molecular sieving, as previously described (*6*), was used for protein modification studies. The active fractions were pooled, concentrated on an Amicon PM10 membrane to 5 ml, and then dialyzed overnight against more than 100 volumes of approximately 0.2 *M* (pH 9) carbonate buffer. Aliquots of this pool (250–1,500 μl) were used in the chemical modification reactions described below.

MA (0.2–4.0 mg; Aldrich Chemical Co., Milwaukee, Wis.) was dissolved directly in the MCT preparation. The solution was agitated until dissolution was complete (≈60 sec). After 30 minutes at room temperature, the preparation was dialyzed against approximately 1,000 volumes of PBS for 4–16 hours. The dialyzed preparation was then assayed for toxicity on L-cells.

MAI (Pierce Chemical Co., Rockford, Ill.) was either added directly to the buffered MCT or first diluted in PBS and then an aliquot was added to the MCT. The final level was 0.1–11 mg/ml. After 30 minutes at room temperature, the preparation was dialyzed and assayed as above.

We reacted MCT with CHD (Pierce Chemical Co.) by making the MCT preparation 0.06 *M* in CHD. The reaction was quenched at various times (30 min–5 hr) by dialysis; after dialysis against more than 1,000 volumes of PBS for 4–16 hours, cytotoxic activity of the modified MCT was determined by assay on L-cells, as above.

Protease inhibitors.—MCT was purified as described before. Similarly, the active fractions were pooled, concentrated, and dialyzed overnight against 100 m*M* (pH 7.2) phosphate buffer. Aliquots (250–1,500 μl) were employed in the reaction described below.

SBTI at 0.1 and 1.0 m*M* (Sigma Chemical Co., St. Louis, Mo.) was dissolved in the MCT preparation of 6–15 U/ml. After 30 minutes at room temperature, the sample was assayed directly on L-cells. As a toxicity control, SBTI was diluted in PBS to the concentration used for the protease-inhibition experiments and added directly to L-cells.

Modification of MCT by TLCK and PMSF (Sigma Chemical Co.) was conducted according to the following protocol. Aliquots (400–1,500 μl) of MCT corresponding to approximately 10–40 U/ml *were reacted directly* with TLCK at final levels of 0.4–10 m*M*. The modification was performed at 37°C for 1 hour. The samples were then dialyzed against approximately 1,000 volumes of PBS for 4–6 hours at room temperature. After dialysis, the lytic activity of each sample was determined by assay on L-cells, as previously described.

TAME and ALME (Sigma Chemical Co.) were used in inhibition studies by *concurrent incubation* of MCT prepared as above, with various levels (10^{-4} to 10^{-2} *M*) of the inhibitor on L-cells. After 20 hours, the level of MCT lysis in the presence of TAME was compared to the level of control MCT lysis. TAME was not toxic to L-cells at levels up to 10^{-2} *M*; ALME was occasionally toxic at this level.

MCT was prepared as above and treated with various levels (1–10 m*M*) of IAM (Sigma Chemical Co.) for 2 hours at room temperature. After dialysis against 500 vol PBS for 24 hours, the samples were assayed on L-cells and their titers determined.

The percent inhibition of MCT by MA, MAI, CHD, PMSF, and TLCK was calculated according to the following equation: Percent inhibition=[(control MCT, U/ml − experimental MCT, U/ml)/control MCT, U/ml]×100%. The percent inhibition of MCT by SBTI, TAME, or IAM was expressed as follows: Percent inhibition=1−[(control L-cell No. − experimental MCT L-cell No.)/(control L-cell No. − MCT L-cell No.)]×100%.

RESULTS

Reduction of MCT activity by adsorption of whole macrophage supernatant on various target cells.—Cell-free whole supernatants containing approximately 39–102 U L-cell lytic activity ml were collected from poly I·poly C-stimulated macrophages as described in "Materials and Methods" and treated as follows: Supernatant, 300 μl, was incubated with various targets or left untreated for 30 minutes at 4°C. The cells were then removed by centrifugation, and supernatants were retested for lytic activity. Shown in tables 1 and 2 are four separate experiments. Both EL 4 and NS-1 removed approximately the same amount of MCT (40–50%), and YAC-1 adsorbed an even greater amount (≈60%). In contrast, the human K562 targets and normal BALB/c spleen

TABLE 1.—*Removal of supernatant MCT activity from poly I·poly C-stimulated C57BL/6 PEMM on normal BALB/c spleen cells in vitro*[a]

Expt No.[b]	Target cell	Percentage of MCT activity adsorbed on targets:	
		3×10^6	15×10^6
1	Normal BALB/c spleen	15.7	12.1
	NS-1	18.7	54.2
2	Normal BALB/c spleen	−2.4	7.9
	NS-1	27.4	38.6

[a] A 300-μl sample of whole supernatant from poly I·poly C-stimulated PEMM containing various levels of MCT activity was added to 3×10^6 and 15×10^6 target cells, allowed to incubate at 4°C for 30 min, and then rendered cell-free. A 250-μl sample of this cell-free supernatant was collected and assayed for MCT as described in "Materials and Methods."

[b] Total numbers of MCT (U/ml) in supernatant were 52 for expt 1 and 102 for expt 2.

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TABLE 2.—*Removal of supernatant MCT activity from poly I·poly C-stimulated C57BL/6 PEMM on various target cells in vitro*[a]

Expt No.[b]	Target cell	Percentage of MCT activity adsorbed on targets:	
		3×10^6	15×10^6
1	EL 4	27.8	43.9
	NS-1	20.8	42.8
	YAC-1	22.2	62.5
	K562	13.0	10.8
2	EL 4	24.3	52.8
	NS-1	25.1	38.8
	YAC-1	19.7	54.3
	K562	6.7	7.2

[a] *Refer to* table 1 for details.
[b] Total numbers of MCT (U/ml) in supernatants were 62 for expt 1 and 39 for expt 2.

cells had little effect; neither target cell type removed greater than 16% of the MCT activity.

Cytolysis and/or cytostasis of allogeneic NS-1 target cells in vitro through Millipore membranes by poly I·poly C-stimulated macrophages.—The preceding results raised the question of whether stimulated macrophages could inhibit growth or kill tumors in the absence of direct cellular contact. We separated effector macrophages and target tumor cells by a 0.2-μm Millipore membrane. After the macrophages were stimulated with poly I·poly C, as described in "Materials and Methods," the filter was placed over the macrophage monolayer and tumor targets were added at a 10:1 effector-to-target cell ratio. In both experiments shown in table 3, little effect was seen after 12 hours, whether or not effectors and targets were separated by filters. However, after 36 hours, reduction in the number of viable tumor cells was evident in control cultures without filters (47–75%) as well as in tests separated by filters (40–55%).

Effect of protease inhibitors on MCT activity.—Several investigators have suggested that serine proteases are involved in the expression of lytic activity by activated macrophages (*5, 7–9*). We tested the effects of several inhibitors of trypsin-like proteases: SBTI, TLCK, PMSF, TAME, and ALME on α-MCT-containing fractions, as described in "Materials and Methods." The results are presented in table 4. α-MCT showed a very striking susceptibility to direct treatment with TLCK. Although TLCK blocked MCT activity, it was important for us to establish whether this blockage occurred by inactivation of a trypsin-like protease or by modification of a vital sulfhydryl group (*10–13*). IAM at levels as high as 10 m*M* was totally without effect on the lytic activity (table 4), which suggested that the former mechanism (trypsin-like protease) and not the latter (vital sulfhydryl group) was operative. This probability was further supported by the blocking observed with PMSF. Higher doses of PMSF (>2.5 m*M*) could not be examined due to insolubility of the drug.

When MCT was incubated with TAME during target cell lysis, as described in "Materials and Methods," a dose-dependent inhibition of lysis was observed (table 4). When MCT was co-incubated with SBTI during target cell lysis, as described in "Materials and Methods," there was a decrease in lytic activity (table 4). If inhibition were occurring at the same active site as that affected by TLCK and TAME, inhibition beyond that seen at 0.1 m*M* (40%) should be attainable. However, at levels higher than this (table 4), toxicity of SBTI for the cells was apparent, thereby overriding the effect of MCT-mediated cytolysis being inhibited. The specificity of the protease was further probed with ALME. No blocking could be observed up to 10^{-2} *M*, the highest dose usually nontoxic for the L-cell (table 4).

Protein modification of MCT.—The effects of modification of MCT partially purified by being sieved on Ultrogel AcA 44 with amino acid side chain-specific reagents MA (which modifies lysine and tyrosine), MAI (which modifies lysine), and CHD (which modifies arginine) are presented in table 5. In none of these modification experiments were we able to quantitate the extent of reaction with a particular type of amino acid side chain, because the MCT preparation was impure and the lytic moiety present was in extremely low amounts; these factors precluded the necessary total amino

TABLE 3.—*Cytolysis and/or cytostasis of NS-1 tumor cells by poly I·poly C-stimulated C57BL/6 PEMM through Millipore membranes*[a]

Expt No.	Treatment	Percent targets[b] remaining after incubation time[c]:	
		12 hr	36 hr
1	Macrophages	93.6±3.2	53.4±4.8
	Macrophages + filters	96.7±1.2	59.8±3.9
2	Macrophages	92.1±1.8	25.6±5.8
	Macrophages + filters	91.3±10.0	44.8±5.6

[a] NS-1 target cells, 5×10^4, were added to 5.0×10^5 PEMM (poly I·poly C treatment described in "Materials and Methods"). An intact Millipore filter with 0.2-μm pores was placed over the macrophages 15 min before the targets were added.
[b] Expressed as percent of tumor targets incubated in the presence of untreated macrophages.
[c] After 12 and 36 hr, the remaining cells were removed and enumerated on a hemacytometer, as described in "Materials and Methods."

TABLE 4.—*Effect of protease inhibitors on lytic activity of α-MCT*[a]

Inhibitor	Concentration, m*M*	Reaction factors	Percent of inhibition[b]
TLCK	0.4	1 hr, 37°C	2
	2		96
	10		100
IAM	1	3 hr, ambient temperature	0
	10		0
PMSF	1	1 hr, 37°C	53
	2.5		61
TAME	0.1	Simultaneous incubation on L-cells	10
	1		52
	10		100
SBTI	0.1	Simultaneous incubation on L-cells	41
	1		23[c]
ALME	0.4	Simultaneous incubation on L-cells	0
	2		0
	10		0

[a] α-MCT was purified by molecular sieving, as described in "Materials and Methods." Concentrated, pooled fractions were dialyzed against 100 m*M* (pH 7.2) phosphate buffer and then treated with the reagents.
[b] Calculated as described in "Materials and Methods."
[c] Toxicity of SBTI on L-cells reduced apparent inhibition of MCT.

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TABLE 5.—*Effect of chemical modification on lytic activity of α-MCT*[a]

Reagent	Amino acid modified	Reagent concentration	Reaction time	Percent inhibition[b]
MA	Lysine and tyrosine	0.2 mg/ml	30 min	88.8
		4 mg/ml		98.7
MAI	Lysine	0.1 mg/ml	30 min	15.1
		0.4 mg/ml		39.2
		1.0 mg/ml		43.1
		11 mg/ml		59.1
CHD	Arginine	60 m*M*	30 min	65.6
			3 hr	75.7
			5 hr	89.3

[a] α-MCT purified by molecular sieving was used. The active fractions were pooled, concentrated, and dialyzed overnight against 0.2 *M* (pH 9) carbonate buffer. Various protein modifiers were dissolved directly in the MCT preparation and then assayed for lytic activity, as described in "Materials and Methods."

[b] Various percentages are representative of several experiments in which 10–70 U control MCT activity/ml was observed.

acid analysis for determination of the extent of modification. However, in an attempt to obtain reasonable levels of substitution, we used wide ranges of exposure to the reagents.

Modification of MCT with MA was achieved with the use of a twentyfold range of MA (0.2–4.0 mg). Although multiple experiments were performed, only the results from the extremes of reagent concentration are shown. In every case, total, or nearly total, abrogation of lytic activity was observed (table 5).

When MCT was allowed to react with the lysine-specific reagent MAI, there was an initially progressive loss of lytic activity, which tended to plateau. However, even at extremely high reagent concentrations, blocking of lysis was incomplete.

The guanido-specific reagent CHD caused progressive loss of MCT lytic activity. Loss of activity corresponded to increased time of exposure to and reaction with the CHD, until after 5 hours when lytic activity was abolished. This was a remarkable rate of modification, when one considers that the pH of the reaction (9) was at least 3 U lower than the pK of arginine side chains (≈12).

DISCUSSION

In the first manuscript of this series (*6*), we demonstrated that alloimmune macrophages stimulated with tumor cells, poly I·poly C, or lipopolysaccharide release MCT in vitro. The principal form of MCT from this source appeared to have biochemical and functional characteristics indistinguishable from those of MCT obtained by poly I·poly C or LPS stimulation of thioglycollate-elicited macrophages. MCT had selective cytotoxic effects for murine tumor cells when compared to normal cells.

In this report, we demonstrate that when supernatants from C57BL/6 ($H\text{-}2^b$) thioglycollate-elicited PEMM stimulated with poly I·poly C are added to normal BALB/c ($H\text{-}2^d$) spleen cells, syngeneic EL 4 ($H\text{-}2^b$) lymphoma, allogeneic NS-1 ($H\text{-}2^d$) myeloma, YAC-1 ($H\text{-}2^a$), and human K562 erythroid leukemia targets, approximately 10, 48, 44, 57, and 9%, respectively, of the MCT activity is removed (tables 1, 2). This removal occurs rapidly at 30 minutes and 4°C, suggesting that adsorption, rather than degradation, has occurred. Adsorption of MCT to the target cell appears to be a prerequisite for cell cytotoxicity. That the human K562 targets were not affected by the levels of MCT used in these studies might be explained by either target insensitivity or target selectivity on the part of MCT. Target insensitivity is most likely, in view of the work by Kramer and Granger (*14*), who have demonstrated that another human target cell (HeLa) was destroyed by activated murine macrophage supernatants. It is also possible that a mechanism not involving MCT is used by the macrophage in cytolysis of K562.

Although direct cell-to-cell contact is considered necessary for cytolysis of targets by activated macrophages (*1–5, 7–9, 15–17*), this contact appears not to be necessary in certain situations, provided the effectors are appropriately activated. We separated poly I·poly C-stimulated macrophages from the NS-1 targets by a membrane filter with a 0.2-μm pore and found that normal levels of cytolysis and/or cytostasis are observed in 36 hours (table 3). To control for the possibility that effector macrophages could project a cytoplasmic process(es) through the filter and affect target cells through direct contact, we separated the cells by two filters and observed identical results (data not shown). Clearly, the signal presented to the macrophage by poly I·poly C overrides the necessity for contact with the tumor in this system. In contrast, when non-poly I·poly C-stimulated macrophages were employed, they were unable to induce cell lysis through the filter. If MCT is involved in tumor cell lysis, these studies suggest that contact between a tumor and effector macrophage is a necessary first step to provide the inducing signal for MCT release, followed by rapid binding of MCT to the target. However, poly I·poly C alone is capable of triggering; once induced, effector macrophages may not require contact with the target cell to induce lysis. The rapidity with which the MCT binds to the tumor target might explain why MCT was not observed to be spontaneously released into the supernatant from alloimmune macrophages if residual tumor cells were present in the exudate (*6*). This rapid binding is compatible with a role for MCT only in the immediate microenvironment between a macrophage and its conjugated tumor target.

Our evidence further suggests that the manner in which MCT expresses cell-lytic activity involves protease-dependent steps. Previous in vitro studies by Hibbs and co-workers (*5*) and by other groups (*7–9*) have suggested a role for serine proteases in the expression of cytolysis by activated macrophages. In particular, the reagent TLCK, which alkylates proteases with trypsin-like specificity, has been shown to effectively block cytolysis in these cell-lytic reactions. This blockage suggested to us that, if MCT were in fact a toxic mediator employed by the activated macrophage in cytolysis, MCT's activity might also be protease dependent. Experiments with partially purified MCT and TLCK do show that this alkylating reagent destroys lytic activity of the soluble molecule (table 4). The blocking of MCT activity also observed with TAME, PMSF, and SBTI and the lack of effect of the sulfhydryl alkylating agent IAM (table 4) further suggest the vital role of a trypsin-like

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protease in the expression of lytic activity.

On the basis of these results, we attempted to determine the nature of the specific substrate of this protease. We believe that MCT could either be a protease or be associated with a protease. Although a reasonable model for the mechanism of MCT-induced lysis could invoke direct protease action on the target cell, we favor a second model. In this model, the substrate for the protease is within the MCT itself, and it must itself be cleaved for lytic activity to be expressed. Since inactivation of MCT by TLCK suggested the protease has trypsin-like specificity, we approached the chemical characterization of the substrate by using reagents capable of modification of positively charged amino acid side chains. If the protease were juxtaposed in the MCT in such a way as to cleave an arginine- or lysine-containing loop of the polypeptide chain, then chemical modification of the arginine or lysine residue should prevent "activation" by the protease and therefore block cell lysis. We used the reagents MA (which acylates lysine and tyrosine residues), MAI (which modifies lysine), and CHD (which reacts with arginine).

Our results suggest that if MCT were associated with a protease, the protease would cleave an MCT-associated polypeptide chain containing arginine regions. The most compelling evidence for this supposition is the time-dependent reaction of MCT with CHD, which results in a progressive and virtually complete loss of lytic activity (table 5). The kinetics of this reaction appear to be similar to that reported by Toi et al. (*18*) for CHD and hemoglobin. Their studies were conducted in strongly alkaline conditions (0.05–0.10 *N* NaOH), whereas extensive modification of the MCT was successful for us in 0.2 *M* (pH 9) carbonate buffer. This hyperreactivity is fortuitous because we could not subject the MCT to such extremes of pH to promote chemical modification since the biologic activity would be lost at this pH (Klostergaard J, Reidarson TH, Granger GA: Manuscript in preparation).

In contrast to our results from modifying arginine, attempts to ascertain a role for a lysine-containing region of the protein were equivocal. MAI, which modifies lysine side chains while preserving the positive charge, showed some blocking effect on the lytic activity of MCT when a wide range of reagent levels was used (table 5). This effect appears to plateau at high reagent levels. Had, in fact, a lysine residue been the target of the protease, lytic activity should have been *totally* blocked, when the extremely high levels of MAI were used. The partial effect observed might be explained by a general denaturation effect upon modification of MCT lysine residues with MAI. In view of the report by Hunter and Ludwig (*19*), side reactions with other amino acid side chains and MAI are not likely.

In this light, the blocking of MCT by MA (table 5) does not suggest a role for lysine. On the contrary, two alternate explanations are more tenable. First, MA also modifies tyrosine and possibly serine. Thus these residues might be of importance, not as substrates, but as contact or catalytic residues in the protease. Serine is an important catalytic residue in some extensively characterized enzymes [for review, *see* (*20*)]. The other distinct and reasonable explanation for the MA inactivation of MCT is the electrostatic repulsion introduced by the negatively charged maleyl-lysine adducts where previously positively charged lysine side chains had been. This repulsion, which is widely reported for proteins reacting with dicarboxylic anhydrides (*27–33*), could easily lead to perturbation of critical tertiary or quarternary structure.

Although it is also possible that CHD and MAI react directly with the protease active site rather than the protease's substrate to inactivate MCT, we believe this model is less likely, since basic amino acids are poor candidates to occupy binding or catalytic regions of a trypsin-like protease [for review, *see* (*20*)].

The distinct effects of the two reversible inhibitors TAME and ALME further suggest that the protease cleaves at an arginine rather than lysine residue. TAME, an arginine derivative, was a very effective inhibitor (table 4), whereas ALME, a lysine derivative, had no measurable effect. We cannot discount, however, that TAME may be superior at least in part due to its hydrophobic binding in the ρ_1 region of the protease active site (*21, 22*). This hydrophobicity could account for the blocking observed by TLCK, a lysine derivative. However, TLCK and the protease may initially form complexes with only a low association constant, comparable to the poor association with ALME; the subsequent covalent bond-forming step with TLCK would then serve to effectively block the active site.

Contrast of the characteristics of MCT with those of CF described by Adams and co-workers (*8*) is noteworthy. They described CF found in the supernatants of peritoneal macrophages from BCG- or thioglycollate-treated mice, following endotoxin stimulation, to be principally approximately 40,000 daltons (*7, 8*), whereas MCT is approximately 150,000 daltons. Furthermore, the lytic activity CF is strongly blocked by serum (*17, 23*), whereas MCT can lyse all susceptible tumor targets in the presence of 3–10% bovine serum. This difference may reside in the inability of α_2-macroglobulin to effectively "bury" MCT in its cleft(s) (*24–26*). Both CF (*8*) and MCT are dependent on protease activity for expression of their tumoricidal function. Further studies for characterization and comparison of CF and MCT are warranted and their possible subunit relationship should be considered.

Finally, these reports provide initial documentation of a molecular mechanism that may be operative in the target-injury phase of macrophage-mediated cytolysis. Further aspects of this possible mechanism can be only elucidated pending the purification and characterization of the MCT itself.

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