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Inducible Macrophage Cytotoxins. I. Biokinetics of Activation and Release In Vitro^{1,2}

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ABSTRACT—Peritoneal exudate macrophage monolayers (PEMM) from C57BL/6 and DBA/2 mice inoculated ip with tumor allografts were induced to release in vitro labile cell toxin(s), herein called “macrophage cytotoxin(s)” (MCT). Macrophages released MCT spontaneously for a short interval when initially established as monolayers, and they were reinduced to secrete MCT by exposure to allogeneic and syngeneic tumor cells (but not to normal cells) and by exposure to polyinosinic-polycytidylic acid (poly I·poly C) and lipopolysaccharide (LPS). PEMM from normal mice treated ip 3 days previously with thioglycollate were also induced to release toxins in vitro. These cells did not release MCT spontaneously before or after treatment with neoplastic cells but were induced to release MCT by exposure to poly I·poly C or LPS. Resident peritoneal macrophages did not release MCT either spontaneously or after treatment with tumor cells, poly I·poly C, or LPS. MCT released from alloimmune mice stimulated with syngeneic or allogeneic tumor cells were resolved by molecular sieving into a major peak at 140,000–160,000 daltons, called “ α -MCT,” and into a minor peak at 60,000 daltons, called “ β -MCT.” However, supernatants from thioglycollate-induced PEMM, stimulated with poly I·poly C or LPS, appeared to be composed entirely of the α -class. α -MCT from poly I·poly C-stimulated PEMM caused 31–56% lysis of syngeneic EL-4 and allogeneic L-929, NS-1, and YAC-1 tumor cells in vitro but was not cytotoxic for normal cells. Secretion of the MCT by PEMM derived from thioglycollate-treated animals stimulated with poly I·poly C was inhibited by colchicine, emetine, iodoacetic acid, trypan blue, and cytochalasin B.—JNCI 1982; 69:879–887.

The activated state of a murine PEM is characterized by increases in size, in motility, in phagocytic capacity, and in production of lysosome enzymes; by greater adherence to glass; and by an increased ability to nonspecifically destroy allogeneic and syngeneic neoplastic cells over normal cells in vitro (1–8). The mechanism by which the activated murine PEM destroy tumors is not known. However, in vitro evidence suggests that this mechanism requires contact between effector and target cells, occurs in 24–72 hours, and does not involve phagocytosis (9–12).

In a recent review, Hibbs Jr. et al. (13) described various treatments that induce the activation of murine PEM to tumoricidal activity in vitro. The first treatment induces the macrophage to a “stimulated” but nontumoricidal stage. This inducement can be brought about by ip injection of thioglycollate before harvest of the PEM or by maintenance of the macrophage in vitro. Secondary stimulation in vitro is initiated by addition of LPS, macrophage activating factor, or various “serum factors,” which raise the PEM to the final tumoricidal stage. Most investigators believe that activated macrophages selectively destroy neoplastic over normal cells (1, 5–7, 11–13); however, evidence indicates that such macrophages may also destroy normal cells (14).

The destruction of neoplastic cells by activated macrophages normally requires contact between effector and target cells. Several investigators (15) have suggested that

tumor cell lysis subsequent to contact with the effector is mediated by materials derived from the activated macrophage lysosome system. It has been demonstrated that PEM from BCG-immune mice, obtained with or without purified protein derivative challenge, or that PEM from normal mice stimulated with LPS and poly I·poly C release cell toxins in vitro (16–19). These toxins have not been well characterized; however, their involvement in the lytic event has been suggested.

The present study describes an inducible system of cell lytic molecule(s) called “MCT” that are released by activated murine PEM in response to different in vitro stimuli.

MATERIALS AND METHODS

Culture media and cell lines.—Culture media used in these studies consisted of RPMI-1640 supplemented with 3 or 10% heat-inactivated (56°C, 60 min) FCS (GIBCO, Grand Island, N.Y.) or NBCS (GIBCO), 100 μ g streptomycin/ml, and 100 U penicillin/ml. Murine α -L-929 (C3H-H-2^k) transformed fibroblasts, which had previously been selected in our laboratory from a number of L-cell strains for extreme sensitivity to lysis by human lymphotoxins, were grown in 32-oz prescription bottles in RPMI-1640 with 3% NBCS at 37°C in a 95% air–5% CO₂ atmosphere and passed twice a week, indefinitely. Murine C57BL/6 EL 4 (H-2^b) lymphoma, Balb/c NS-1 (H-2^d) myeloma, P815 DBA/2 (H-2^d) mastocytoma, A/Sn YAC-1 (H-2^a) lymphoma, and human K562 erythroid leukemia cell lines were grown in RPMI-

ABBREVIATIONS USED: Con A=concanavalin A; EMEM=Eagle's minimum essential medium; FCS=fetal calf serum; HBSS=Hanks' balanced salt solution; LAH=lactalbumin hydrolysate; LPS=lipopolysaccharide; MCT=macrophage cytotoxin(s); α -MCT=MCT detectable at 140,000–160,000 daltons; β -MCT=MCT detectable at 60,000 daltons; NBCS=newborn calf serum; OD₆₀₀=optical density at 600 nm; PBS=phosphate-buffered saline; PEC=peritoneal exudate cell(s); PEM=peritoneal exudate macrophage(s); PEMM=PEM monolayers; PHA=phytohemagglutinin; PHA-P=aglycan protein form of PHA; poly I·poly C=polyinosinic-polycytidylic acid; SBTI=soybean trypsin inhibitor; SDS=sodium dodecyl sulfate.

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1640 supplemented with 10% FCS. Naive BALB/c (*H-2^d*) spleens were aseptically removed and prepared on the day of the experiment. Spleens were minced, and a single cell suspension was formed and then transferred to a T-25 culture flask (Corning Glass Works, Corning, N.Y.) at 4×10^6 cells/ml in RPMI-1640 supplemented with 10% FCS. The cells were allowed to incubate for 1 hour at 37°C for removal of adherent cells. Viability was 93–98% as determined by eosin Y dye exclusion. Blasts were induced by Con A (1 µg/ml) treatment of normal nonadherent splenocytes for 4 hours at 37°C.

Induction of PEC.—PEC were obtained by three methods: a) P815 mastocytoma cells (10^7) suspended in RPMI-1640 or PBS were injected ip into C57BL/6 mice; 10–14 days later the PEC were harvested as previously described (20). PEC induction was similarly performed by ip injection of EL 4 lymphoma cells into DBA/2 mice. Thioglycollate (3 ml) (BBL, Cockeysville, Md.) was prepared and injected ip into C57BL/6 mice. Approximately $15\text{--}20 \times 10^6$ PEC/mouse was harvested 3 days later. c) Resident PEC ($2\text{--}4 \times 10^6$ /mouse) from nonmanipulated C57BL/6 or DBA/2 mice were harvested by peritoneal lavage 24 hours before in vitro stimulation.

Preparation of PEMM.—PEMM were obtained from C56BL/6 (Kingston Colony, Charles River Breeding Laboratories, Wilmington, Mass.) and DBA/2 (ARS, San Diego, Calif.) mice 6–8 weeks old. Mice were killed by cervical dislocation, and each peritoneal cavity was irrigated aseptically with 7–8 ml cold PBS (pH 6.9). The exudate was pooled and centrifuged for 5 minutes at $1,500 \times g$ at 4°C. The cell pellet was resuspended in 5–10 ml of either cold-modified EMEM in HBSS or RPMI-1640 (GIBCO) supplemented with 10% FCS or NBCS, 100 U penicillin/ml, and 100 µg streptomycin/ml. The PEC were then suspended in either EMEM in HBSS or RPMI-1640 and placed in T-culture flasks at $1 \times 10^6/5 \text{ cm}^2/\text{ml}$. The flasks were incubated at 37°C in 95% air–5% CO₂ for 2 hours and then washed four times with serum-free RPMI-1640 or sterile PBS to remove nonadherent cells. Monolayers were maintained in RPMI-1640 at 37°C. The composition of the adherent cells was determined by examination of tetrachrome-, eosin Y-, and neutral red-stained cover slip PEMM. The monolayers were 99% or more macrophages and 100% viable.

Assay for direct macrophage-mediated cytotoxicity.—PEC obtained as described above were pelleted by centrifugation, resuspended in RPMI-1640 supplemented with 10% heat-inactivated NBCS, and plated in 16-mm diameter Costar plates (Costar, Cambridge, Mass.) at a density of 0.5×10^6 cells/well. After 2 hours, the monolayers were washed and allowed to incubate for an additional 24 hours in RPMI-1640 supplemented with 10% FCS before use.

The PEMM were then treated with 40 µg poly I·poly C/ml (P-L Biochemicals, Inc., Milwaukee, Wis.) in RPMI-1640 containing 10% NBCS, incubated for 1 hour, and washed four times with serum-free media. Various tumor target cells were added at a 10:1 effector-to-target cell ratio (total vol, 0.5 ml). After 12 and 36 hours of incubation at 37°C, we removed the remaining target cells from the monolayers by aspirating five to seven times, followed by a short trypsinization. Upon visual inspection, less than 5% of

the tumor cells remained associated with the monolayers after this treatment. The washes and trypsin supernatant were then pooled and centrifuged. The pelleted tumor cells were resuspended in 0.1% eosin Y in PBS solution, and viable cells were enumerated in a hemacytometer. All targets were nonadherent cells.

Production of MCT.—Alloinduced PEMM were incubated in RPMI-1640 containing either 10% FCS, 10% NBCS, or 0.01% LAH (GIBCO) for various lengths of time. Supernatants were then collected, rendered cell-free by centrifugation, and assayed for MCT activity. We attempted restimulation of alloinduced macrophages by adding 2.5×10^6 EL 4 or P815 tumor cells, poly I·poly C (P-L Biochemicals, Inc.), LPS (Sigma Chemical Co., St. Louis, Mo.), Con A (Sigma), or PHA-P (Difco Laboratories, Detroit, Mich.) to PEMM in serum-free RPMI-1640. After incubation for 1–2 hours, the PEMM were washed thoroughly with serum-free medium and then the medium was replaced; the supernatant was assayed for MCT activity as described later.

Thioglycollate-elicited or resident macrophages were treated in a similar fashion with either EL 4 or NS-1 tumor cells, poly I·poly C, LPS, Con A, or PHA-P for 1–2 hours; cultured; and assayed for MCT activity. Media were either assayed for MCT immediately or frozen at –20°C and analyzed within 48 hours.

Assay for MCT and determination of the number of units of activity in a supernatant.—Methods are detailed in (20). Serial dilutions of MCT-containing or control supernatants in RPMI-1640 were added to duplicate 1-ml slant-tube cultures, which were seeded with 10^5 mitomycin C-treated α-L-929 cell targets. These nondividing targets were used for evaluation of cytotoxicity only and not cytostasis. After 14–24 hours at 37°C, the remaining viable adherent cell number was then enumerated on a model F Coulter counter. Nonattached cells were determined to be greater than 95% nonviable by eosin Y staining. One unit of MCT activity is defined as that amount of material that destroys 50% of the target L-cells (50,000). The reciprocal of the highest dilution that destroys 50% of the target L-cells denotes the number of MCT unit per milliliter in a given supernatant.

Generation of activated macrophage supernatants, molecular sieving fractions containing α-MCT, and assay for MCT-induced tumor cell cytotoxicity.—The 10×10^6 thioglycollate-elicited PEC were established as monolayers in T-75 culture flasks as described above. The PEMM were washed and stimulated with 40 µg poly I·poly C/ml. Following the last wash, 10 ml RPMI-1640 supplemented with 0.01% LAH was added, and the monolayers were incubated for 2 hours at 37°C. The supernatant was collected and either: a) immediately assayed for units of lytic activity on L-929 cells or b) concentrated 20 times by ultrafiltration on an Amicon PM10 membrane and passed through an Ultrogel AcA 44 column. We applied 2 ml of twentyfold to thirtyfold concentrates of MCT supernatants to an Ultrogel AcA 44 column (2.5×60 cm) equilibrated in 10 mM potassium phosphate (pH 7.2) and 10^{-4} M EDTA. Fractions of 4 ml were collected at a flow rate of 40 ml/hour. The peak of cytotoxic activity detectable on murine L-929 cells at 140,000–160,000 daltons, called “α-MCT,” was pooled and concentrated as described above. The α-MCT was filtered through a cellulose nitrate membrane

with a 0.2- μ m pore (Sartorius membrane filter; Science Essentials, Fullerton, Calif.) and then added to the various tumor targets. Following 12 and 36 hours of incubation at 37°C, the remaining cells were removed and viable cells enumerated as described previously.

Determination of macrophage viability.—We added 25 μ Ci $\text{Na}_2^{51}\text{CrO}_4$ /ml RPMI-1640 to 0.5×10^6 macrophages seeded in 16-mm Costar plates (Costar) and incubated them for the release period (4–6 hr) at 37°C. PEMM were then washed thoroughly for removal of the nonincorporated counts, solubilized with 0.5 ml 10% SDS in PBS, and enumerated in a Beckman biogamma counter.

Inhibitors.—The inhibitors colchicine, emetine, iodoacetic acid, SBTI, and trypan blue were dissolved directly in RPMI-1640. Trypan blue was dialyzed against 500 vol PBS before use. Inhibitor cytochalasin B was first dissolved in dimethyl sulfoxide and then diluted with RPMI-1640. All inhibitors here came from Sigma.

Use of trypan blue.—Thioglycollate-elicited macrophage monolayers were established as described previously. Trypan blue was added to monolayers at various concentrations as described by Hibbs Jr. (15). After 24 hours, the monolayers were washed four times; then they were incubated for 2 hours in 0.01% LAH, after 1 hour of poly I-poly C stimulation. The supernatant was removed and assayed for lytic activity. To evaluate the amount of trypan blue incorporated by the macrophages, we solubilized the monolayer with 10% SDS in PBS and determined the light absorption at 600 nm with a Beckman model 25 spectrophotometer.

RESULTS

Spontaneous MCT release in vitro from PEMM obtained from C57BL/6 allografted mice.—PEMM, from C57BL/6 mice that had received an ip injection of P815 tumor, were established as described in "Materials and Methods." Samples of medium were removed at various intervals and tested for MCT activity on L-929 cells. As seen in text-figure 1, low levels of MCT activity were detected in the initial 1- to 4-hour culture; then between 4 and 6 hours, activity climbed to a peak, followed by a decline to less than 6 U MCT by 24 hours. While the data are not shown, additional experiments revealed that release of MCT rapidly ceased after 6–8 hours in these cultures. Resident macrophages released less than 6 U MCT/ml during the same culture period.

Restimulation of MCT release in vitro from C57BL/6 and DBA/2 alloimmune PEMM after a short exposure to P815 or EL 4 tumors.—PEMM from alloimmune C57BL/6 or DBA/2 mice were cultured in vitro for 6–8 hours, as described in "Materials and Methods." The monolayers were then washed thoroughly and reexposed to allogeneic or syngeneic tumor cells for 1 hour. The data shown in table 1 reveal that alloimmune macrophages released from 75–110 U MCT/ml in 4–6 hours when reexposed to EL 4 or P815 tumors for 1 hour. Resident or naive macrophages, however, did not release detectable levels of activity under the same conditions. Moreover, when exposed to allogeneic or syngeneic normal lymphocytes, these alloimmune macrophages did not release detectable levels of activity (data not shown). Exposure of alloimmune macrophages to Con A lympho-

TEXT-FIGURE 1.—Spontaneous release of MCT from alloimmune and resident PEMM. Alloimmune (●—●) and resident (○—○) C57BL/6 PEM were collected, cultured as monolayers, and then allowed to release MCT up to 24 hr. Supernatants were collected at various intervals of time and assayed for lytic activity as described in "Materials and Methods."

blasts caused the release of low levels (10–15 U/ml; data not shown) of a toxin into the supernatant. However, the nature of this toxin is currently unclear (see "Discussion").

Next, experiments were designed to determine if MCT could be induced from alloimmune and thioglycollate-elicited PEMM maintained in culture for various periods of time without stimulation. In numerous experiments, we were able to restimulate alloimmune cells up to 12 days in culture by reexposure to tumor cells for 1 hour. Moreover,

TABLE 1.—Release of MCT by PEMM^a obtained from alloimmune and normal C57BL/6 and DBA/2 mice following a 1-hour exposure to allogeneic and syngeneic tumor cells

PEMM	Treat-ment	MCT U/ml
Alloimmune C57BL/6 immunized with P815	EL 4	110±60 ^a
	P815	90±30 ^b
	None	12±3 ^b
	EL 4	80±40 ^b
DBA/2 immunized with EL 4	P815	75±25 ^b
	None	8±4 ^b
Resident ^c C57BL/6	EL 4	<6
	P815	<6
	None	<6
DBA/2	EL 4	<6
	P815	<6
	None	<6

^aPEMM were obtained from C57BL/6 and DBA/2 mice 10–15 days after intraperitoneal allografts of P815 and EL 4 tumors, respectively. PEMM were treated as described in "Materials and Methods" and supernatants were collected 6 hr after exposure to tumor cells and tested for MCT activity.

^bValues are means ± range.

^cResident PEMM were obtained from nonimmunized mice and treated as stated in footnote a.

the release of MCT approached original maximal levels. One restimulation experiment with PEMM maintained for 5 days in culture is shown in text-figure 2. Control resident macrophages did not acquire the capacity to release MCT during this period in vitro.

Thioglycollate-elicited PEMM could also be restimulated with poly I·poly C. After 3–4 days in culture, these macrophages were reexposed to 40 µg poly I·poly C or LPS/ml for 1 hour and then allowed to release MCT as described previously (data not shown). We observed MCT release to 90–95% of the original level.

Induction of MCT secretion by PEMM from alloimmune or thioglycollate-treated C57BL/6 mice treated with various stimulating agents.—Thioglycollate- or tumor-elicited macrophages from C57BL/6 mice were cultured as described in "Materials and Methods." After 8 hours, the PEMM were treated with either allogeneic or syngeneic tumors or poly I·poly C, LPS, Con A, or PHA that were tested at levels from 0.1 to 75 µg/ml. After 1 hour the monolayers were washed thoroughly as described previously, and then supernatants were collected after 4 hours and assayed for lytic activity. As seen in table 2, syngeneic or allogeneic tumor cells stimulated release from PEMM from allografted animals but not thioglycollate-elicited PEMM. However, poly I·poly C and LPS were capable of inducing the release of cell-lytic material from thioglycollate-elicited and alloinduced macrophages. In contrast, the lectins Con A and PHA did not induce MCT release in any of these cultures. While not shown, release by poly I·poly C and LPS was dose dependent; maximal activity was observed with 40 µg of either agent/ml; however, as little as 0.1 µg LPS/ml was sufficient to achieve a comparable response. Finally, resident macrophages could not be stimulated to release cell lytic molecules with any stimulating agent or treatment used.

TEXT-FIGURE 2.—Restimulation of C57BL/6 alloimmune PEMM by short exposure to allogeneic tumor cells. Alloimmune (●—●) and resident (○—○) C57BL/6 macrophages were cultured for 5 days, exposed to P815 allogeneic tumors for 1 hr, washed free of tumors, and then allowed to release material for 24 hr. Supernatants were collected at various times and assayed for MCT activity as described in "Materials and Methods."

TABLE 2.—Release of MCT by C57BL/6 PEMM treated in vitro with different stimulating agents^a

PEMM	Treatment ^b	MCT released in U/ml
Alloinduced, immunized with P815	EL 4	70±35 ^c
	P815	85±40 ^c
	NS-1	65±30 ^c
	Poly I·poly C	143±50 ^c
	LPS	125±25 ^c
	PHA	<15
	Con A	<15
Thioglycollate elicited	None	<15
	EL 4	<6
	NS-1	<6
	Poly I·poly C	47±25 ^c
	LPS	33±5 ^c
	PHA	<6
	Con A	<6
Resident	None	<6
	EL 4	<6
	NS-1	<6
	Poly I·poly C	9±3 ^c
	LPS	<6
	PHA	<6
	Con A	<6
	None	<6

^aPEMM were established and 8 hr later were treated with various stimulating agents as described in "Materials and Methods." Each culture was then thoroughly rinsed and fresh RPMI-1640 containing 10% FCS added. After 2–4 hr, supernatants were removed and assayed for MCT activity.

^bPoly I·poly C, LPS, PHA, and Con A were used at 40 µg/ml.

^cValues are means ± range.

Kinetics of MCT release from thioglycollate-elicited C57BL/6 PEMM in vitro under various stimulation and culture conditions.—Thioglycollate-elicited PEMM were treated with poly I·poly C or LPS for 1 hour and placed in fresh medium, and MCT release was measured at various time intervals as described in "Materials and Methods." As seen in text-figure 3, there was a lag period of 30–60 minutes following poly I·

TEXT-FIGURE 3.—Kinetics of MCT release from C57BL/6 poly I·poly C-stimulated PEMM in vitro. Thioglycollate-elicited C57BL/6 PEMM were stimulated with 40 µg poly I·poly C/ml for various lengths of time (■—■, 30 min; ×—×, 60 min; ●—●, 90 min; ○—○, 120 min; ▲—▲, 180 min; △—△, 240 min) and then allowed to release MCT over 24 hr. Resident PEMM (□—□) was treated similarly. Supernatants were collected at various time intervals and assayed for lytic activity as described in "Materials and Methods."

poly C stimulation, and maximal levels of MCT were detected after 2 hours of culture. When macrophages were exposed to poly I·poly C for either 90 or 120 minutes, the lag period disappeared; if exposed for greater than 180 or 240 minutes, no release was observed.

Production of MCT from thioglycollate-elicited C57BL/6 PEMM under various culture conditions.—To generate quantities of MCT for biochemical characterization, we explored the capacity of thioglycollate-elicited PEMM monolayers to release MCT under various in vitro conditions. When poly I·poly C-stimulated macrophages were allowed to release MCT in medium containing 10% FCS, 10% NBCS, 0.01% LAH, 200 μ g serum albumin/ml, or 200 μ g ovalbumin/ml, levels of lytic activity were comparable, averaging between 21 and 48 U MCT/ml (data not shown). Only under protein-free conditions were both MCT activity levels and macrophage viability lowered.

Definition of the molecular classes of murine MCT.—Supernatants from PEMM obtained from C57BL/6 mice immunized with EL 4 lymphoma were concentrated and chromatographed on Ultrogel AcA 44. A typical elution profile is shown in text-figure 4A. Also shown are elution profiles of supernatants obtained from LPS or poly I·poly C-stimulated thioglycollate-induced macrophages as described in "Materials and Methods" (text-figs. 4B, 4C). The elution profiles of the various molecular weight markers blue dextran, IgG, α -hemoglobin, and phenol red are also indicated.

Clearly, supernatant MCT stimulated by all induction methods can be resolved into one major class of approximately 140,000–160,000 daltons known as α -MCT and a smaller class of activity of approximately 60,000 daltons called " β -MCT." The β -MCT class was only observed when supernatants from PEMM from alloimmune mice were stimulated by tumor cells.

TEXT-FIGURE 4.—Ultrogel AcA 44 chromatography of MCT-containing supernatants obtained from C57BL/6 PEMM stimulated in various ways. Molecular mass markers: Blue dextran (BD), $>2 \times 10^6$ daltons; IgG, 140,000–160,000 daltons; hemoglobin (Hb), 64,000 daltons; and phenol red (PR), 354 daltons. Columns were equilibrated and eluted with 10 mM phosphate (pH 7.2) containing 10^{-4} EDTA. A) Alloinduced: A 1-ml sample of a twentyfold-concentrated supernatant generated by incubation of PEMM from P815 alloimmune C57BL/6 mice stimulated with P815 cells was chromatographed over a 60-cm gel bed on Ultrogel AcA 44 in a 2.5×75 -cm column. Four-ml fractions were collected at 40 ml/hr, and 200- μ l samples were tested from every other fraction for MCT activity. For details, see "Materials and Methods." Toxic activity of each sample is expressed as percent viability. B) LPS induced: A 1-ml sample of a twentyfold-concentrated supernatant generated by LPS stimulation of PEMM from thioglycollate-induced C57BL/6 mice was chromatographed as above. Samples were collected and tested, and results were expressed in a manner identical to those in text-fig. 4A. C) Poly I·poly C induced: A 1-ml sample of a thirtyfold-concentrated supernatant generated by poly I·poly C stimulation of PEMM from thioglycollate-induced C57BL/6 mice was chromatographed and tested for MCT activity as described in text-fig. 4A.

TABLE 3.—Effect of whole supernatants and ultragel AcA-44 fractions containing MCT activity from poly I·poly C-stimulated C57BL/6 PEMM on different tumor targets *in vitro*^a

MCT preparation	Percent of control cells remaining; incubation time:							
	12 hr				36 hr			
	EL 4	NS-1	YAC-1	K562	EL 4	NS-1	YAC-1	K562
Whole supernatant, U/ml ^b								
175	90 ^c (82±3)	91±2 (90±4)	92±2 (96±2)	104±3 (96±2)	55±3 (73±4)	44±5 (63±3)	53±2 (84±2)	96±0.7 99±2
17.5	97±6 (84±1.9)	98±0.7 (93±12)	94±4 (99±6)	98±4 (102±3)	74±3 (93±2)	80±7 (91±2)	77±5 (89±2)	103±8.0 (99±2)
α-MCT, U/ml ^d								
150	90±6 (90±2)	88±4 (93±2)	94±2 (97±1)	99±3 (101±0.4)	53±2 (72±0.5)	48±3 (67±2)	69±3 (83±2)	100±5 (103±2)
15	94±2 (95±6)	93±1 (97±7)	98±3 (94±1)	105±6 (98±3)	79±3 (97±3)	76±2 (83±1)	79±2 (92±0.1)	100±6 (100±2)

^aTarget cells, 0.5×10^5 , were added to supernatants containing various MCT lytic activities (total vol, 0.5 ml). After 12 and 36 hr, the remaining cells were removed and enumerated on a hemacytometer, as described in "Materials and Methods." These data are representative of two expts. Values are means ± range. Values in parentheses represent viabilities of the remaining tumor targets ± range.

^bSupernatant was generated from poly I·poly C-stimulated peritoneal macrophages, rendered cell-free by centrifugation, and added directly to various target cells. Units of MCT in these supernatants were determined by an L-cell assay as described in "Materials and Methods."

^cPercent of control number, untreated targets.

^dPrepared by molecular sieving of supernatants obtained as stated in footnote b.

Cytolysis-cytostasis of various target cells by whole supernatants and by α-MCT elaborated by poly I·poly C-stimulated macrophages in vitro.—We next tested whether whole supernatants or column fractions from poly I·poly C-stimulated macrophages containing MCT were capable of affecting these various tumor cells. Materials with MCT activity were added directly to 0.5×10^6 target cells and incubated up to 36 hours at 37°C. The remaining tumor cells were vitally stained and enumerated. Table 3 indicates that both whole supernatants and the α-MCT fractions from molecular sieving columns containing 175 and 150 U of L-cell-lytic material, respectively, reduced cell number from controls by 20–52% for the EL 4, NS-1, and YAC-1 murine targets. These targets were also affected to a lesser extent by a 1:10 dilution of MCT activity. Interestingly, MCT at these levels did not affect human K562 targets. Furthermore, the results shown in table 4 indicate that murine spleen lymphocytes, normal counterparts of these lymphoid tumor cells, were resistant to lysis.

TABLE 4.—Effect of supernatants from poly I·poly C-stimulated C57BL/6 PEMM on normal and transformed NS-1 cells *in vitro*^a

MCT activity, U/ml ^b	Percent of control cells remaining; ^c incubation time:			
	12 hr		36 hr	
	Normal spleen cells	NS-1	Normal spleen cells	NS-1
50	102±7.3 (101±4.9)	98.2±3.2 (92.5±2.6)	95.5±2 (98.9±9.4)	48.9±6.4 (78.9±4.8)
5	97±3.1 (100±3.0)	99.6±2.0 (95.6±3.4)	102.2±6.8 (104±9.8)	84.5±5.1 (87.7±1.6)

^aSee footnote a in table 3.

^bSee footnote b in table 3.

^cValues are means ± range of viable treated cells as compared to values for untreated controls. Values in parentheses represent mean viabilities ± range of treated cells as compared to viabilities of untreated controls.

Direct cytolysis of various target cells by poly I·poly C-stimulated macrophages.—When syngeneic, allogeneic, or xenogeneic target cells were exposed to intact poly I·poly C-stimulated macrophages, reduction in all tumor cell numbers was observed. As shown in table 5, from 23 to 70% of the cells were removed by the stimulated PEMM at 36 hours. In contrast to the above experiments with MCT, K562 targets were destroyed by the macrophages. The reduction in tumor cell numbers in these and the previous studies could be due to either cytostasis or cytotoxicity or to both effects.

Effect of various inhibitors on MCT secretion in vitro by poly I·poly C-stimulated macrophages.—PEMM from thioglycollate-induced C57BL/6 mice were treated with various inhibitors during the 1-hour poly I·poly C stimulation, washed thoroughly, and then allowed to release materials for 2 hours in the absence of inhibitors. Supernatants were collected and tested for cytotoxicity, as described in "Materials and Methods." Colchicine and iodoacetic acid inhibited release, in a dose-dependent manner, at levels of 5×10^{-5} and 1.2×10^{-5} M, respectively (text-figs. 5A, 5B, 5C). The effect of these inhibitors on cell viability is shown in text-figure 5. At the above levels, these inhibitors were not toxic to the PEMM. Cytochalasin B, however, was not as effective in inhibiting release (text-fig. 5C). Text-figure 5D demonstrates that emetine inhibited 75% of the release at 10^{-5} M. However, it also reduced macrophage viability to 83%. At this level, protein synthesis (⁷⁵selenium-methionine incorporation) was inhibited approximately 30%. Further, the inhibition by emetine was not dose dependent; at a concentration two orders of magnitude higher, 10^{-3} M, MCT release was inhibited only 80%, whereas viability was reduced to 57%. An inhibitor of trypsin-like proteases, SBTI, was also tested. Neither viability nor release was significantly affected at a concentration of 10^{-4} M (data not shown).

Finally, trypan blue, a vital stain reported to be an inhibitor of lysosomal hydrolysis (15), was tested. Macrophages were pretreated with various concentrations of try-

TABLE 5.—Direct cytotoxicity and/or cytostasis of target cells by poly I·poly C-stimulated C57BL/6 PEMM^a

Expt No.	Percent of control cells remaining; incubation time:							
	12 hr				36 hr			
	EL 4	NS-1	YAC-1	K562	EL 4	NS-1	YAC-1	K562
1	78.9±15.4	97.3±5.6	81.4±6.2	91.6±8.4	55.2±8.4	77.2±8.7	30.7±5.9	39.1±13.7
2	85.6±14.4	102.5±3.9	96.1±8.3	105.6±8.8	64.7±1.4	65.1±2.7	60.2±6.2	76.5±1.9

^aTarget cells, 0.5×10^5 , were added to 5×10^5 poly I·poly C-stimulated C57BL/6 PEMM (10:1 effector-to-target cell ratio). After 12 and 36 hr, the remaining tumor cells were removed, and total viable cells were enumerated on a hemacytometer, as described in "Materials and Methods." Values are means ± range.

pan blue. After 24 hours, the residual vital stain was washed from the macrophages, poly I·poly C was added, and release was allowed to proceed, as described in "Materials and Methods." The incorporation of trypan blue by the macrophages, as determined by OD₆₀₀, is shown in table 6. Clearly, incorporation was enhanced with increasing concentrations of the dye. Trypan blue inhibited approximately 50% of the release of MCT at 2.2×10^{-3} M, and cell viability was approximately 70% (text-fig. 6). Upon restimulation with poly I·poly C, a similar pattern of inhibition and effector cell viability was observed.

TABLE 6.—Incorporation of trypan blue by thioglycollate-elicited C57BL/6 PEMM after primary and secondary stimulation by poly I·poly C

Trypan blue concentration, M	OD ₆₀₀	
	Primary	Secondary
5.5×10^{-4}	0.075	0.069
1.1×10^{-3}	0.082	0.080
2.2×10^{-3}	0.093	0.093
4.4×10^{-3}	0.120	0.118

TEXT-FIGURE 5.—Effect of inhibitors on the release of MCT from thioglycollate-elicited C57BL/6 PEMM in vitro. Thioglycollate-elicited C57BL/6 PEMM monolayers were pretreated for 1 hr with A) colchicine, B) iodoacetic acid, C) cytochalasin B, and D) emetine at various molar (M) concentrations. The monolayers were then washed thoroughly, exposed to $40 \mu\text{g}$ poly I·poly C/ml for 1 hr, and then allowed to release MCT for 2 hr. Supernatants were collected and then assayed for MCT activity. Numbers in parentheses represent cell viability of the PEMM. Viability is determined by measurement of the amount of ^{51}Cr incorporated by the cells during the release interval. For details, see "Materials and Methods."

TEXT-FIGURE 6.—Effect of incorporated trypan blue on the primary and secondary stimulation and release of MCT from thioglycollate-elicited C57BL/6 PEMM in vitro. Following solubilization of the monolayer as described in "Materials and Methods," the PEMM were examined for cell viability by ^{51}Cr incorporation and trypan blue incorporation by absorbance at 600 nm.

DISCUSSION

PEMM obtained from alloimmune mice can be stimulated to release cell-lytic molecule(s) in vitro. The cell toxin(s) are herein termed "macrophage cytotoxin(s)" MCT. Furthermore, PEMM from animals that had rejected the tumor allograft spontaneously released MCT when initially established as monolayers, whereas PEMM from an occasional animal that did not reject the tumor did not release detectable levels of MCT. Release in vitro from PEMM begins 4 hours after a 1-hour contact with tumor cells. Release ceases at 4–6 hours; in cultures left at 37°C , activity declines by 12–24 hours. After 6–8 hours, or even 4–5 days in culture, release can be reinitiated by a 1-hour exposure to fresh allogeneic or syngeneic tumor cells but *not* by exposure to normal allogeneic or syngeneic lymphocytes. Moreover,

the second round of release follows similar patterns of kinetics. Treatment of the alloimmune macrophages with Con A lymphoblasts caused release of low levels of a cytotoxin. However, the treated PEMM were associated with bound lymphoblasts (presumably binding via free valences of lectin) during the release period, and in this case the toxin may in fact be lymphocyte derived. This is clearly an inducible system of cell-lytic molecules. No MCT is detectable in these cultures if the tumor cells are not removed, which will be discussed later. In contrast to the response of alloimmune macrophages, resident macrophages release negligible MCT under all conditions.

Although we were able to generate significant levels of lytic activity from alloimmune macrophages, other methods were examined for induction of these molecules for further study. We found that thioglycollate-elicited PEMM treated with 40 μ g poly I·poly C/ml responded quite uniformly and released high levels of MCT. Following the removal of poly I·poly C, there was a 30- to 60-minute lag period, after which the levels climbed to a peak by 2 hours and then declined to very low levels by 24 hours. The loss of MCT activity in the supernatant is clearly due to two effects: *a*) cessation of biosynthesis and secretion and *b*) degradation of already secreted forms.

Upon chromatography of supernatants from stimulated C57BL/6 macrophages on Ultrogel AcA 44, MCT can be resolved into two molecular species: α - and β -classes—140,000–160,000 and approximately 60,000 daltons, respectively (text-fig. 4A–C). The α -class is observed in supernatants from alloimmune PEMM and in poly I·poly C-stimulated and LPS-stimulated thioglycollate-induced PEMM. However, the β -class is observed rather infrequently and *only* in supernatants from alloimmune macrophages (text-fig. 4A). Although not examined, this smaller β -form may be a subunit or degradation product of the larger α -class.

When supernatants from poly I·poly C-stimulated PEMM and α -MCT obtained from these supernatants are added to murine EL 4, NS-1, and YAC-1 tumor targets, a 30–55% reduction in cell number is observed. However, there was little or no effect either on the human K562 tumor or on normal BALB/c spleen cells. When the above transformed cells are added to poly I·poly C-stimulated thioglycollate-elicited PEMM at a 10:1 ratio, 24–70% of the targets are removed. Whether cytolysis or cytostasis is occurring is difficult to prove unequivocally. As observed in table 3, the reduction in cell number does not directly correlate with the reduction of cell viability, which would suggest cytostasis occurring. An appropriate suggestion is that both cytolysis and cytostasis are occurring simultaneously.

A common feature of MCT and previously described macrophage-derived cell-lytic materials is their instability in supernatants (7, 21, 22). Once released, MCT is unstable at 37°C, which might contribute to the fall in supernatant activity levels. At –20 or 4°C, activity is partially preserved for an additional 2–4 days (data not shown). However, the activity is stable enough for various parameters of production and release from the PEMM to be examined.

The release of MCT after stimulation appears to require cellular protein synthesis and an active secretory system, because treatment of stimulated PEMM with emetine (to

block protein synthesis), colchicine (to disrupt microtubules), and iodoacetic acid (an alkylating agent to block phagocytosis) at levels that were shown not to significantly affect cell viability in a 3-hour period dramatically affected the secretion of MCT. Treatment with cytochalasin B affected release to a lesser extent, and SBTI had no measurable effect. However, trypan blue at a concentration of 2.2×10^{-3} M inhibited release by approximately 55% while decreasing macrophage viability to 70%. With a twofold-higher concentration, release of MCT was still approximately 50% while cell viability dropped to 55%; these results obtained with trypan blue might indicate existence of both lysosomal and nonlysosomal release mechanism(s). In other words, the inhibition by trypan blue would indicate that an alternative secretory route might exist—possibly one that does not require a vacuolar system.

There are several parameters that are operative and control the *in vitro* release of MCT by murine PEMM. The first raises macrophages to a particular state of activation, which permits them to respond to further stimulation. In our experiments this is represented as an *ip* injection of tumor cells or thioglycollate. As an alternative explanation, these treatments may induce responsive cells to migrate into the peritoneum from other tissue sites. The second is a stimulating signal *in vitro* represented in our studies by tumor or poly I·poly C treatment. This treatment induces rapid release of cell-lytic material(s). Removal of the stimulating signal results in cessation of secretion. Resident or naive macrophages did not release MCT under any *in vitro* conditions we used. While the data are not shown, starch- or glycogen-induced PEMM also could not be induced to release MCT, *in vitro*, with the agents we employed. In our studies, poly I·poly C or LPS treatment stimulated both alloimmune and thioglycollate-elicited macrophages to release MCT. In contrast, we were unable to induce MCT release by thioglycollate-induced macrophages via tumor stimulation. Thus poly I·poly C and LPS appear to be stronger, less-selective inducers in stimulating the release of MCT. However, mitogens Con-A or PHA-P cannot apparently serve as a second signal to stimulate MCT release from any of the PEMM we tested.

The three classes of peritoneal macrophages we examined appear to be at different stages of activation and capable of eliciting differential responses to a second stimulus. Macrophages from alloimmune mice are at an advanced stage, inasmuch as contact with tumor cells or poly I·poly C can potentiate the release of MCT. However, thioglycollate-elicited macrophages may be at an intermediate stage and thus require a stronger signal (i.e., poly I·poly C or LPS) to release. Finally, the resident macrophage appears to represent an early stage and does not respond even to strong signals.

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