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# Isolation and Initial Characterization of Tumoricidal Monokine(s) From the Human Monocytic Leukemia Cell Line THP-1<sup>1,2</sup>

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**ABSTRACT**—A cloned subline of the human monocytic leukemia cell line, THP-1, was induced to produce high levels of cytotoxic activity following an 18-hour phorbol myristate acetate (CAS: 16561-29-8) stimulation in vitro. This activity, termed monocyte cell line cytotoxin(s) (MCCT), was tested in vitro on different human continuous cell lines (Chang, ESH-7, GM3104A, HeLa, HT-1080, K562, Mel, T-24) and on primary human fibroblasts (GM3468, Manz). The continuous cell lines exhibited a spectrum of sensitivity to MCCT-containing supernatants whereas the primary fibroblasts were resistant to lysis. Enzymatic degradation and heat denaturation studies indicate that MCCT is a protein. Its bioactivity can be resolved into three lytic peaks after molecular sieving on Ultrogel AcA 44. The major peak, designated  $\alpha$ MCCT, eluted with a molecular weight of 100,000–140,000 daltons. A minor peak,  $\beta$ MCCT, was seen at 60,000–80,000 daltons, and a third, unstable minor peak,  $\gamma$ MCCT, eluted at less than 10,000 daltons. The  $\alpha$ -lytic peak was examined further and was found to migrate as a single peak in 7% native polyacrylamide gel electrophoresis tube gels with an  $r_f$  of 0.25–0.30. None of the MCCT forms were immunologically cross-reactive with human  $\alpha$ -lymphotoxin. Various protease inhibitors known to inhibit monokine- and macrophage-mediated direct cell lysis in vitro were tested for their inhibitory effects on  $\alpha$ MCCT activity. The irreversible binding inhibitor *N* $\alpha$ -*p*-tosyl-L-lysyl chloromethyl ketone inhibited the biologic activity of  $\alpha$ MCCT. The reversible binding inhibitors *N* $\alpha$ -*p*-tosyl-L-arginine methyl ester and soybean trypsin inhibitor were able to block in vitro lytic activity when added to  $\alpha$ MCCT in the presence of the target cell. In contrast, the inhibitors phenylmethylsulfonyl fluoride, L-1-tosylamide, 2-phenylethyl chloromethyl ketone, and *N* $\alpha$ -acetyl-L-lysine methyl ester were not effective in blocking cytotoxicity. Finally, the hydrogen peroxide scavenger catalase inhibited  $\alpha$ MCCT lytic activity in vitro; however, the hypochlorous acid scavengers alanine, serine, and valine were without effect.—*JNCI* 1985; 74:1–9.

Human peripheral blood monocytes and alveolar macrophages can be raised to a cell-lytic state by treatment in vitro with lymphokines and/or bacterial components (1–5). These activated human mononuclear cells exhibit a greater capacity to lyse neoplastic cells rather than primary cells in vitro. In addition, recent reports have demonstrated the release of CLM from peripheral blood monocyte and alveolar macrophage cultures [(6–8); Klostergaard J: In preparation; Sone S, Lopez-Dertein G, Fidler IJ: In preparation). Supernatants containing CLM from these cultures were also found in general to preferentially lyse continuous cells over primary cells in vitro.

For the in-depth investigation of CLM, the production of large amounts of human supernatant would be necessary from a homogeneous monocyte-macrophage source, preferably from a single donor. Primary cultures

of monocytes from many individual donors do not fulfill these criteria. To circumvent these problems, attention has been focused on recently derived human cell lines of monocytic and histiocytic origin as possible sources of these cytolytic monokines. The U937 line has proven to be a valuable model for several aspects of human monocytes-macrophages after lymphokine activation (9–11). Other cell lines appear to be arrested at a very early stage in monocyte-macrophage differentiation and attempts to activate them with lymphokines or LPS have not resulted in release of CLM (12–14). However, two well-characterized human monocytic cell lines in the presence of PMA will differentiate in vitro into cells that express many of the functional characteristics of mature macrophages. Specifically, the HL-60 (12) and THP-1-0 (14) cell lines have been shown to stop cell division, become glass adherent, increase phagocytosis, and express increased cell surface esterase staining. In addition, the HL-60 cell line becomes lytically active after incubation with PMA (15, 16).

In the present study, we describe the isolation of a clone of the THP-1 cell line termed AY-23, which, upon PMA stimulation in vitro, releases high levels of CLM activity. This activity, termed MCCT, exhibits the functional ability to lyse continuous cells and not primary

**ABBREVIATIONS USED:** ALME=*N* $\alpha$ -acetyl-L-lysine methyl ester; BD=Blue Dextran; CLM=cell-lytic monokine(s); Ct= $\alpha$ -chymotrypsin; Hb=hemoglobin; LAH=lactalbumin hydrolysate; L-cells=L-929 murine transformed fibroblasts; LPS=lipopolysaccharide; LT=human lymphotoxin(s); MCCT=monocyte cell line cytotoxin(s); NRS=normal rabbit serum; PAGE=polyacrylamide gel electrophoresis; PBS=phosphate-buffered saline; PMA=phorbol myristate acetate; PMSF=phenylmethylsulfonyl fluoride; PR=phenol red;  $R_f$ =retardation factor; RPMI-0%=serum-free RPMI-1640 medium; RPMI-3%=RPMI-1640 medium supplemented with 3% heat-inactivated (56°C, 60 min) fetal calf serum; RPMI-10%=RPMI-1640 medium supplemented with 10% heat-inactivated (56°C, 60 min) fetal calf serum; RPMI-LAH=RPMI-1640 medium supplemented with 0.01% lactalbumin hydrolysate; SBTI=soybean trypsin inhibitor; TAME=*N* $\alpha$ -*p*-tosyl-L-arginine methyl ester; TLCK=*N* $\alpha$ -*p*-tosyl-L-lysyl chloromethyl ketone; TPCK=L-1-tosylamide-2-phenylethyl chloromethyl ketone.

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fibroblasts in vitro. The biologic activity of MCCT can be attributed to distinct proteins that can be obtained in sufficient quantity for purification and for future functional and biochemical studies.

## MATERIALS AND METHODS

**Culture media and cell lines.**—Culture media used in these studies consisted of RPMI-1640 medium (GIBCO, Grand Island, NY) supplemented with RPMI-3% or RPMI-10% (GIBCO), 100  $\mu$ g streptomycin/ml (Sigma Chemical Co., St. Louis, MO), and 100 U penicillin/ml (Sigma Chemical Co.).

Target cells were grown in 16-ounce prescription bottles in either RPMI-3% for murine cells or in RPMI-10% for human cells at 37°C in 5% CO<sub>2</sub> and passed biweekly. The adherent continuous lines used were L-929 cells, referred to as L-cells (17); HeLa, human cervical carcinoma; HT-1080, human skin fibrosarcoma; Chang, human liver carcinoma; T-24, human bladder carcinoma; ESH-7, a cell line derived from the fusion between HeLa cells and WIL-2, a human B-cell lymphoma; and Mel, human melanoma. The adherent human primary cell strains used were GM3468, primary fetal foreskin fibroblasts, and Manz, primary skin fibroblasts. The primary strains were passed at confluence and were replenished from frozen (−80°C) stock after 30 passages. Nonadherent human target cells were also employed; they were, GM3104A, a human B-cell lymphoma, and K562, a human erythroblastoma. These lines were maintained in RPMI-10% at 37°C in 5% CO<sub>2</sub> atmosphere and passed biweekly.

**Human monocytic cell line.**—The human monocyte leukemia cell line THP-1 (13), a gift from Dr. Jun Minowada, New York State Department of Health, Buffalo, NY, was used as the source of cell-lytic materials in these studies. This cell line was cultured in RPMI-10% in Corning T-75 cm<sup>2</sup> flask (Corning, NY) in an atmosphere of 5% CO<sub>2</sub> at 37°C and subcultured every third day at an initial density of 3×10<sup>4</sup> cells/ml. A clone of this cell line was isolated by the limiting dilution method in the presence of a feeder layer of 2×10<sup>4</sup> BALB/c peritoneal macrophages per microtiter well in 96 flat-bottomed well microtiter plates in RPMI-10% (Flow Laboratories, Inglewood, CA). After 3 weeks in culture, several wells from the limiting dilution step contained single colonies of cells. These colonies were selected and the cells recloned in 96-well microtiter plates by the limiting dilution technique in preconditioned media from BALB/c peritoneal macrophage cultures. After 3 weeks in culture, colonies of cells appeared. These clones were then transferred into 1-ml cultures in Costar 24-well plates containing 50% preconditioned media and 50% fresh RPMI-10%. These clones were subsequently expanded in RPMI-10% and tested for production of cytolytic material.

**Production of cytolytic material from THP-1 and clones.**—THP-1 cells and clones were grown to a cell density of 5×10<sup>5</sup> cells/ml in RPMI-10%. These cultures were then pelleted by centrifugation at 450×g for 8 minutes and 5×10<sup>5</sup> cells/ml were resuspended in 40 ml of

fresh RPMI-10% in 32-ounce prescription bottles in a 5% CO<sub>2</sub> atmosphere. Varying amounts of PMA (Sigma Chemical Co.) at an initial concentration of 50  $\mu$ g/ml dissolved in 95% ethanol were then added to the cultures. After incubation at 37°C for various time intervals, the PMA-containing media were discarded and the now adherent monolayer was washed three times with RPMI-0% to remove any residual PMA. At this point, 40–50 ml of RPMI-LAH (GIBCO), which served as a serum substitute, was added to the culture. After incubation for an additional 2–72 hours at 37°C, supernatants were collected, and cell debris was removed by centrifugation at 500×g for 10 minutes, filter sterilized, then used immediately or stored at −70°C. Concentration of supernatants or partially purified fractions was achieved by ultrafiltration in an Amicon 8200 Stirred Cell (Amicon Corp., Danvers, MA) with the use of a 62-mm PM-10 Diaflo ultrafiltration membrane (Amicon Corp.).

**Cytolytic cell assays.**—Two types of assays were employed: One determined quantitatively the amount of cytolytic activity present in a given supernatant, and the other indicated only the presence or absence of activity in the supernatant as percent lysis. *a*) Tube assay: The details of this method have been reported previously (17). Briefly, 1×10<sup>5</sup> mitomycin target cells in 1.0 ml were established as nondividing monolayers in 16×125-mm culture tubes. Either serial dilutions of the test or control medium (quantitative assay) or a single aliquot of a sample (qualitative assay) was added to duplicate tubes. After an additional 24-hour incubation at 37°C, the remaining adherent cells were trypsinized and enumerated in a Model F Coulter Counter (Coulter Electronics, Hialeah, FL). Units of cell-lytic activity per milliliter of a given supernatant were obtained by determining the reciprocal of the dilution killing 50% of the target cells.

*b*) Microplate assay: Mitomycin-treated cells at a density of 1×10<sup>4</sup> cells/100  $\mu$ l were dispensed into flat-bottomed 96-well microtiter plates (Corning) and incubated at 37°C in a humidified air and 5% CO<sub>2</sub>-containing incubator. After 24 hours, serial dilutions of MCCT-containing supernatants or fractions and control media were added to triplicate wells. After an additional 24 hours of incubation, the media were aspirated and the remaining viable adherent targets fixed to the substrate and stained with a 1:1,000 crystal violet solution (18). Percent lysis was calculated according to the formula: Percent lysis = [(cell No. control well) − (cell No. well + MCCT)]/(cell No. control well) × 100, where cell number represents an average of the number of cells enumerated in three microscope fields counted per well. A microscope field contained approximately 75–100 cells. We found, by eosin Y-dye exclusion from a nontreated or control well, that 90% of the detached targets were nonviable, as reported previously (19). Suspension targets were removed from the wells by aspiration and stained with 0.1% eosin Y-dye as described previously (19), and the viable and nonviable cells enumerated in a hemacytometer. The percent cytolysis in these cultures was determined as follows: Percent lysis = [% viable cells in control well] − (% viable cells in well + MCCT)/(%

viable cells in control well)]  $\times 100$ , where the average percent was determined by the mean of triplicate wells. L-cells were used only in tube assays; HeLa cells were used in both tube assays and in microplate assays, as indicated. All other target cells were used only in microplate assays.

**Antiserum neutralization assays.**—Production, characterization, and testing of rabbit antisera that neutralize LT forms *in vitro* have been previously reported (20). All antisera were heat inactivated for 1 hour and centrifuged at 20,000 $\times g$  for 30 minutes before use. Neutralization of MCCT activity was performed by adding 25–50  $\mu$ l of antiserum or NRS to 10 U of MCCT activity from a supernatant or fraction, preincubation for 30 minutes at 25°C, then testing the remaining activity on L-cells. The percent neutralization of MCCT activity was determined by the following formula: Percent neutralization = [(cell No. + MCCT + antibody) – (cell No. + MCCT + NRS)] / [(cell No. + NRS) – (cell No. + MCCT + NRS)]  $\times 100$ .

**Molecular sieving.**—Two milliliters of twentyfold concentrated supernatant was chromatographed on an Ultrogel AcA 44 molecular sieving column (LKB, Rockville, MD) poured to a bed height of 95–100 cm in a 2.5 $\times$ 120-cm siliclad column equilibrated in 10 mM potassium phosphate, pH 7.2, and 10<sup>-4</sup> M EDTA buffer. The column was calibrated with the molecular weight markers BD, 2 $\times$ 10<sup>6</sup> daltons; IgG, 150,000 daltons; Hb, 64,000 daltons;  $\alpha$ -Ct, 23,000 daltons; and PR, 364 daltons. Fractions of 6.5 ml were collected at a flow rate of 26 ml/hour at 4°C.

**Discontinuous PAGE.**—A 150- $\mu$ l sample of MCCT in 20% sucrose and 0.001% bromphenol blue was applied to a 0.5 $\times$ 8-cm gel column consisting of a 1-cm 3% acrylamide stacking gel in 50 mM Tris-glycine, pH 9.0, and a 7-cm 7% acrylamide separation gel according to the method of Davis (21). The gels were then cut in 1-mm slices, and cytolytic material was eluted by incubation for 10–12 hours in 250  $\mu$ l RPMI-3% at 4°C. A 100- to 200- $\mu$ l sample was then tested for cytolytic activity.

**Protease inhibitors.**—Pooled molecular sieving fractions containing active  $\alpha$ MCCT buffered with a potassium phosphate (100 mM PO<sub>4</sub>, pH 7.2) buffer were employed in these studies. The methods employed for protease inhibitor treatment have been described previously (29). The irreversible binding inhibitors used were TLCK, TPCK, and PMSF, all from Sigma Chemical Co. A 1.5-ml sample containing approximately 10–20 U of  $\alpha$ MCCT/ml was reacted for 1 hour at 37°C with varying concentrations (0.4–10 mM) of the different inhibitors. The sample was then dialyzed against approximately 1,000 volumes of PBS for 10–12 hours at 4°C. After dialysis, the lytic activity of each sample was determined. Samples with  $\alpha$ MCCT activity were also incubated for 1 hour at 37°C without inhibitor, dialyzed against 500 volumes of PBS for 10–12 hours, and assayed for toxicity. As a control, 10 mM TLCK, 1 mM PMSF, or 1 mM TPCK was added to RPMI-LAH, incubated for 1 hour, dialyzed against PBS overnight, and assayed for toxicity.

The reversible inhibitors TAME (Sigma Chemical

Co.) and ALME (Sigma Chemical Co.) were added with 10–20 U of  $\alpha$ MCCT directly to the target cells. After 20 hours, the level of lysis in these cultures was compared to levels of  $\alpha$ MCCT-induced lysis in the absence of inhibitor. Extensive testing revealed that neither drug was toxic to the target cells at levels up to 10 mM. SBTI (grade 4; Sigma Chemical Co.) at 0.1 and 1.0 mM was incubated directly with 10–20 U of MCCT for 30 minutes at room temperature. The samples were then assayed directly on target cells. As a control, the same amount of SBTI in 100 mM phosphate buffer was added directly to target cells. The percent inhibition of lysis in all studies was determined by the following formula: Percent lysis = [(cell No. + MCCT + inhibitor) – (cell No. + MCCT)] / [(cell No. + inhibitor) – (cell No. + MCCT)]  $\times 100$ .

**Inhibitors of active oxygen radicals.**—Catalase (33,900 U/ml, beef liver extract; Sigma Chemical Co.) at a known level of enzymatic units per milliliter was added to L-cell assays simultaneously with  $\alpha$ MCCT or with control supernatants containing no MCCT. The percent inhibition of  $\alpha$ MCCT activity by catalase was determined with the same formula used with the protease inhibitors described above.

The amino acids alanine, serine, and valine (Sigma Chemical Co.) were dissolved in PBS, pH 7.2. These solutions were added simultaneously with  $\alpha$ MCCT from molecular sieving to L-cell assays, and the percent inhibition was calculated. The final concentration of added amino acid was 30 mM.

## RESULTS

**Release of CLM *in vitro*.**—THP-1 cells grown to a density of 5 $\times$ 10<sup>5</sup> cells/ml were exposed to a lymphokine-rich supernatant from 3-day cultures of phytohemagglutinin-stimulated human tonsil and adenoid lymphocytes (23) with a procedure similar to that used by Fisher et al. (4) to produce cytotoxin(s) from human peripheral blood monocytes *in vitro*. This supernatant was added to THP-1-0 cell cultures at ratios of 1:5 or 1:10 (vol/vol). After 24 hours, 500 ng LPS/ml was added directly to the cultures for either 1 or 2 hours, the LPS was removed by washing the cells in serum-free media, and the cells were resuspended in fresh RPMI-10% for an additional 24 hours. Samples from both the lymphokine-enriched supernatant without LPS and the supernatant obtained 24 hours after LPS removal were collected and assayed on L-cells for cytotoxicity. No cytolytic activity was detected in either of these supernatants.

THP-1 cells were reported to differentiate into cells expressing the characteristics of mature macrophages after exposure to PMA *in vitro* (14). When cultures of 2 $\times$ 10<sup>5</sup> THP-1 cells/ml were exposed to 10 ng PMA/ml, the cells were observed to become glass adherent after 3 hours while maintaining high (>90%) viability as determined by eosin Y-dye exclusion. However, 24-hour supernatants from these cultures were not cytotoxic. We subsequently tested various levels of PMA and found that supernatants from THP-1 cultures after exposure to 100 ng PMA/ml for 24 hours exhibited cytotoxic effects when

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tested on L-cells. In contrast, no toxicity was detected when 100 ng PMA/ml was added to either RPMI-10% or RPMI-0% and these media were assayed on L-cells. The results shown in table 1 summarize these initial data. It is obvious that cytotoxic activity was found in the supernatants only after PMA was added to the THP-1 cultures.

**Kinetics of cytotoxin release.**—Cell density, dosage of PMA, and incubation times necessary to achieve maximum MCCT release were examined. We first tested various cell numbers and found that a density of  $5 \times 10^5$  cells/ml gave the optimal levels of MCCT production; this cell density was used throughout further experiments. Subsequently, we incubated the cells in RPMI-10% with various concentrations of PMA for the indicated time periods. The levels of MCCT were quantified on L-cells; data from these studies are presented in table 2. The lowest concentration of PMA that induced MCCT release was 50 ng/ml. At this and higher levels of PMA, lytic activity appeared between 18 and 24 hours and increased to greater than 1,000 U/ml after 48 hours. To facilitate biochemical studies, it was important to establish if MCCT would be released into a serum-free medium. Cells were incubated with PMA for 18 hours in RPMI-10% as described above; then the cultures were transferred into RPMI-LAH without PMA. Under these conditions LAH serves as a serum substitute. Supernatants from these cultures were assayed for levels of MCCT activity at various intervals. The results are presented in table 3. The data show that MCCT activity plateaus at 48 hours after removal of PMA. Also, 100 ng PMA/ml induced higher levels of MCCT release than 50 ng/ml. In all following experiments, 100 ng PMA/ml was used to stimulate the cells for 18 hours followed by a 48-hour release period.

TABLE 1.—Attempts to induce toxin release from THP-1 cells in vitro

Cells	Treatment	Total incubation time, hr	Cell-lytic activity, U/ml <sup>a</sup>
None	RPMI-10% <sup>b</sup>	0	0 <sup>c</sup>
None	LPS 500 ng/ml <sup>b</sup>	0	0
None	Lymphokine-rich <sup>d</sup>	0	0
THP-1 <sup>e</sup>	Lymphokine-rich <sup>d</sup>	24	0
THP-1 <sup>d</sup>	Lymphokine-rich + LPS 500 ng/ml (24 hr), RPMI-10% (24 hr)	48	0
None	PMA 100 ng/ml <sup>b</sup>	0	0
THP-1 <sup>f</sup>	PMA 100 ng/ml	24	50-75 <sup>g</sup>
THP-1 <sup>f</sup>	PMA 100 ng/ml + LPS 500 ng/ml	24	50-75 <sup>g</sup>

<sup>a</sup>One unit of cell-lytic activity is the reciprocal of the dilution of MCCT that produces 50% lysis of  $1 \times 10^5$  L-cells in a 1-ml culture.

<sup>b</sup>Control media assayed directly on L-929 cells.

<sup>c</sup>Zero units of activity indicates no detectable cytolytic activity.

<sup>d</sup>Lymphokine-rich medium was prepared from PHA-stimulated human tonsil and adenoid cultures as explained in "Results." THP-1 cultures were treated with lymphokine-rich medium according to the method of Fischer et al. (4).

<sup>e</sup> $5 \times 10^5$  THP-1 cells/ml in RPMI-10%.

<sup>f</sup> $2 \times 10^5$  THP-1 cells/ml in RPMI-10%.

<sup>g</sup>Range of values from 3 separate expts.

TABLE 2.—Induction of MCCT activity from THP-1 cells by incubation with different levels of PMA in vitro<sup>a</sup>

PMA dose, ng/ml	MCCT activity, U/ml <sup>b</sup> after incubation with PMA for the interval, hr: <sup>c</sup>					
	3	6	12	18	24	48
0	0	NT	NT	0	0	NT
10	<5	<5	<5	<5	23	<5
50	<5	<5	<5	6	858	>1,000
75	<5	<5	<5	6	614	>1,000
100	<5	<5	<5	10	778	>1,000
200	<5	<5	<5	10	668	>1,000

<sup>a</sup> $5 \times 10^5$  THP-1 cells/ml stimulated with PMA for various intervals and supernatants collected. The number of cytolytic U/ml were then determined as described in "Materials and Methods." NT=not tested.

<sup>b</sup>One unit is the reciprocal of the dilution of MCCT that produces 50% lysis of  $1 \times 10^5$  L-929 cells in a 1-ml culture. See "Materials and Methods."

<sup>c</sup>Zero units of activity indicates no detectable cytolytic activity.

**PMA stimulation of nonlymphoid cell lines.**—Studies were performed to determine if PMA would induce cytotoxin release from continuous and primary cell lines of nonlymphoid origin. Different cell lines were stimulated under the conditions that were found optimum for CLM production from the THP-1 cells. The continuous cell lines tested were Chang, Mel, HT-1080, HeLa, and T-24. The primary line tested was the GM3468. There was no detectable lysis of L-cells with any of the supernatants except those from the T-24 cells which gave approximately 10-15 U/ml on L-cells.

**Cloning of a high-MCCT-producing cell subline.**—Clones of the THP-1 cell were isolated by limiting dilution with the intent of isolating a subline that produces high levels of MCCT activity. With the use of the methods that induced optimal release with the parent THP-1 cells, we found four clones, termed AY-16, AY-23, AY-56, and AY-84 that released high levels of MCCT. The data presented in table 4 are the means  $\pm$  standard errors of three separate experiments. The AY-23 clone was the highest MCCT-producing subline and was used in all further experiments.

**Lytic effects of MCCT on various human continuous and primary cell lines in vitro.**—Supernatants containing

TABLE 3.—Kinetics of the appearance of MCCT lytic activity in serum-free, PMA-free medium following PMA stimulation<sup>a</sup>

PMA dose, ng/ml	MCCT activity, U/ml <sup>b</sup> at release times <sup>c</sup> at, hr:						
	2	4	8	12	24	48	72
50	17.1	40.1	53.5	50.7	607.3	2,497.4	2,893.3
100	38.5	73.7	181.7	129.1	1,562.4	3,648.4	3,856.2

<sup>a</sup> $5 \times 10^5$  THP-1 cells/ml were stimulated with PMA for 18 hr. Monolayers were washed three times and allowed to release MCCT in RPMI-LAH for various intervals in the absence of PMA.

<sup>b</sup>Units of MCCT activity were determined as described in "Materials and Methods." One unit of lytic activity is defined in footnote b, table 2.

<sup>c</sup>Length of time after PMA removal.

TABLE 4.—Levels of MCCT activity released *in vitro* from PMA-stimulated cloned sublines of the THP-1 cell

Cell line <sup>a</sup>	MCCT activity, U/ml <sup>b,c</sup>
THP-1	3,285.3±47.2
Clone AY-16	4,716.9±85.1
Clone AY-23	5,135.1±78.4
Clone AY-56	4,441.8±110.4
Clone AY-84	4,907.5±112.3

<sup>a</sup>THP-1 cells or clones at  $5 \times 10^5$  cells/ml were stimulated with PMA for 18 hr, washed three times, and allowed to release MCCT in RPMI-LAH for 48 hr.

<sup>b</sup>Units of activity were determined as described in footnote b, table 2.

<sup>c</sup>Data are mean values from 3 experiments  $\pm$  standard error.

100–300 U/ml of MCCT activity were generated, collected, concentrated twentyfold to thirtyfold, filter sterilized, and supplemented with 10% fetal calf serum. The concentrates were then simultaneously tested on L-cells to determine the number of units of bioactivity per milliliter and assayed in triplicates on different target cell types previously treated with mitomycin. The percent lysis of the continuous and primary adherent targets was determined in the microtiter assay employing 10,000 cells/well. Additional studies revealed that more than 90% of detached cells were nonviable judged by staining with 0.1% eosin Y-dye. Lysis of the suspension target cells K562 and GM3104A was determined as explained in "Materials and Methods." Table 5 lists the various targets tested, the tissue from which they were isolated, and the amount of MCCT (expressed in L-cell units) necessary to produce 50% lysis. These data are the mean of four separate experiments and values in parentheses give the range of variation between experiments.

The data suggest that different cells exhibit a spectrum of sensitivity to MCCT. Clearly, the HeLa cell is the most sensitive. Cells expressing HeLa cell surface markers (Chang, ESH-7) were also sensitive but to a lesser degree. Of the continuous lines tested, the GM3104A and K562 cells showed no lysis. In none of the tests did the primary fibroblast strains (GM3468, Manz) show any lysis as measured by detachment, changes in shape, or viability staining with eosin Y-dye.

**Antibody neutralization.**—MCCT activity was also tested to determine if it could be neutralized by rabbit antisera made against LT. The anti-LT used were of two types: a) anti-WS, a polyspecific antiserum known to block the effects of most LT forms and b) anti- $\alpha_2$  made against highly purified  $\alpha_2$ -LT from phytohemagglutinin-stimulated human tonsils and adenoids (20, 23). Neither of these antisera significantly neutralized (>20%) MCCT activity.

**Determination of molecular weight of MCCT.**—The supernatant from AY-23 cells was collected, concentrated twentyfold, and chromatographed on Ultrogel AcA 44 molecular sieving columns. Samples from each fraction were assayed on L-cells for cytolytic activity. Molecular weight markers employed were: BD > 200,000, IgG 150,000, Hb 64,000,  $\alpha$ -Ct 23,000, and PR 364 daltons. As the data in text-figure 1 indicate, the major peak of lytic

activity eluted within a molecular weight range of 100,000–140,000 daltons and was designated as the  $\alpha$ -form. A second lytic peak, termed  $\beta$ -form, chromatographed with an approximate molecular weight of 60,000–80,000 daltons. A third peak of activity, termed  $\gamma$ -form, was occasionally detected just in front of PR. We found that the  $\gamma$ -peak was unstable and lost all activity within 48 hours after sieving.

Fractions were also assayed on HeLa cells in tube cultures. Text-figure 2 shows a representative elution profile with this target cell. As with the elution profile on the L-cell, both the  $\alpha$ - and  $\beta$ -sizes of MCCT activity were detected; however, no  $\gamma$ MCCT activity was observed.

$\alpha$ MCCT from molecular sieving in a low-salt phosphate buffer (20 mM  $\text{PO}_4^{2-}$ , pH 7.2) was stable for up to 2 weeks at 4°C. The addition of 0.05% LAH to the fractions did not prevent a loss of activity. Unfractionated supernatant activity was stable for up to 3 weeks at 4°C.

**Electrophoretic mobility on native PAGE.**—Fractions from molecular sieving containing  $\alpha$ MCCT activity, as indicated by the bars in text-figure 1, were pooled, concentrated 20 times and run on 7% native polyacrylamide tube gels. Gel slices were eluted with RPMI-3%, and eluates were then assayed on L-929 and HeLa cells in tube cultures. The data for these target cells are shown in text-figure 3. One peak of lytic activity was found on both the L-929 and HeLa targets. The  $R_f$  values ranged from 0.15 to 0.30 with the major peak at an  $R_f$  of 0.24–0.28 on both the L-929 and HeLa cells.

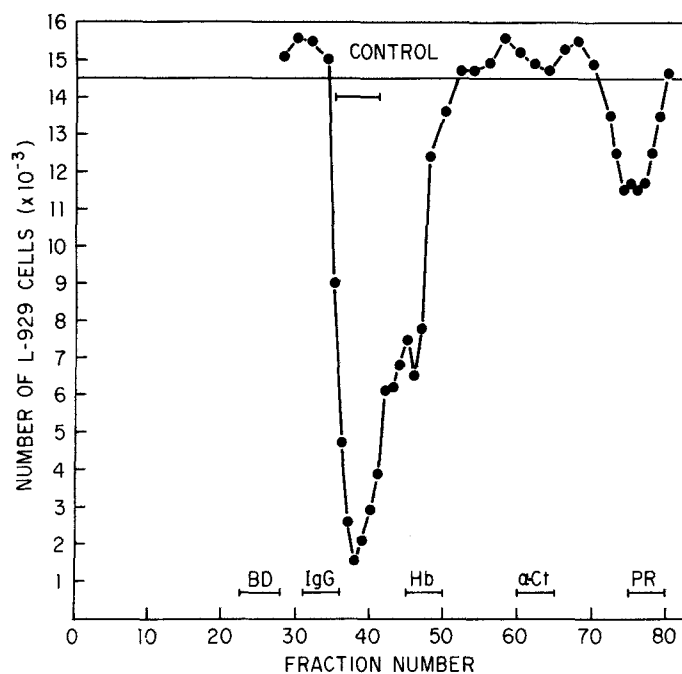
**Effects of protease inhibitors on MCCT activity.**—The reversible inhibitors tested were SBTI, TAME, and ALME; the irreversible inhibitors used were TLCK,

TABLE 5.—*In vitro* lytic effects of MCCT on continuous and primary target cells of human origin<sup>a</sup>

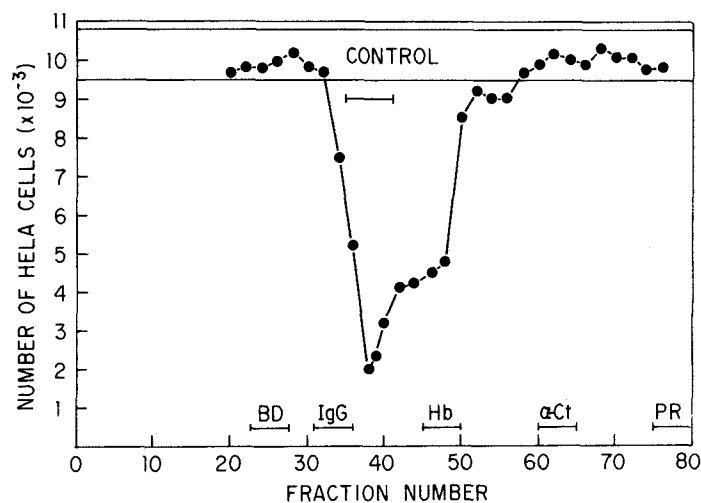
Cell line	Origin	Units needed to produce 50% cell lysis <sup>b</sup>
Continuous cells		
HeLa	Cervical carcinoma	18 (15–25)
HT-1080	Skin fibrosarcoma	100 (70–125)
CHANG	Liver carcinoma	73 (63–94)
T-24	Bladder carcinoma	115 (85–140)
MEL	Melanoma	75 (66–85)
ESH-7	Hela-Wil-2 fusion	100 (75–120)
K562	Erythroblastoma	No lysis with 150 U
GM3104A	B-cell lymphoma	No lysis with 150 U
Primary cells		
GM3648	Fetal foreskin fibroblast	No lysis with 150 U
MANZ	Skin fibroblast	No lysis with 150 U

<sup>a</sup>Target cells were established in microplates and described in "Materials and Methods" and then treated with various amounts of a concentrate of supernatant from AY-23 cells. After 24 hr the number of viable cells in each well was determined as described in "Materials and Methods."

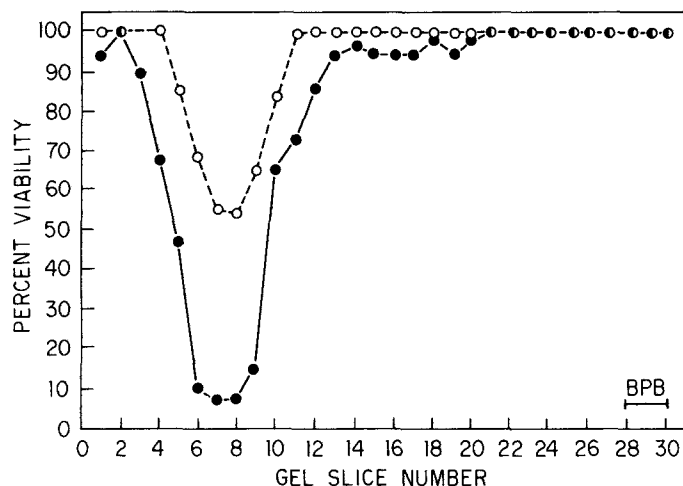
<sup>b</sup>The number of units of bioactivity determined on L-929 target cells necessary to induce 50% cell lysis of the indicated target cell(s). Data are expressed as the mean of 4 experiments. Values in parentheses indicate the range of values obtained in the 4 expts.



TEXT-FIGURE 1.—Ultragel Aca 44 chromatography of MCCT-containing supernatants obtained from *in vitro* PMA-stimulated AY-23 cells: L-929 target cells. A 2-ml sample of a twentyfold concentrated supernatant from PMA-stimulated AY-23 cells was chromatographed over a 95-cm gel bed on Ultragel Aca 44 in a 2.5×120-cm column; 6.5-ml fractions were collected at 26 ml/hr and 20- $\mu$ l samples were tested for MCCT activity in tube assays, as described in "Materials and Methods." The toxic activity of each sample is expressed as percent viability. Molecular weight markers: BD, >200,000 daltons; IgG, 150,000 daltons; Hb, 64,000 daltons;  $\alpha$ -Ct, 23,000 daltons; and PR, 364 daltons. Columns were equilibrated and eluted with 10 mM phosphate, pH 7.2, containing  $10^{-4}$  EDTA.



TEXT-FIGURE 2.—Ultragel Aca 44 chromatography of MCCT-containing supernatants obtained from *in vitro* PMA-stimulated AY-23 cells: HeLa target cells. Supernatant from PMA-stimulated AY-23 cells was chromatographed on Aca 44 column according to the procedure outlined in text-fig. 1. Samples of each fraction were assayed on HeLa cells as described in "Materials and Methods." Toxic activity is expressed as percent viability. Molecular weight markers employed were the same as in text-fig. 1. Columns were equilibrated and eluted with 10 mM phosphate, pH 7.2, containing  $10^{-4}$  EDTA.



TEXT-FIGURE 3.—Native PAGE analysis of an  $\alpha$ MCCT preparation after molecular sieving employing L-929 and HeLa target cells. Fractions from molecular sieving showing  $\alpha$ MCCT activity on L-cells (see bar on text-fig. 1) were pooled, concentrated fifteenfold, and adjusted to 20% sucrose plus 0.001% Bromophenol Blue (BPB). Samples of 150  $\mu$ l were applied to 0.5×8-cm tube gels consisting of a 1-cm 3% acrylamide stacking gel in 50 mM Tris-glycine, pH 9.0, and a 7% acrylamide separation gel and electrophoresed as explained in "Materials and Methods." One-millimeter gel slices were incubated in RPMI-3% at 4°C for 12 hr to recover lytic activity. Samples of 200  $\mu$ l were tested for lytic activity on both L-929 (●—●) and HeLa cells (○—○) in tube assays. Toxic activity of each sample is expressed as percent viability.

TPCK, and PMSF. The results of a single experiment are presented in table 6. Tests with the reversible inhibitors showed that 10mM TAME quite effectively blocked lytic activity. In contrast, ALME, which has a lysyl group in place of the arginyl group in TAME, did not block MCCT activity. BTI, which inhibits trypsin and trypsin-like proteases, showed an intermediate level of inhibition. Since TAME was incubated with  $\alpha$ MCCT and target cells, the possibility existed that TAME could block an L-cell membrane-associated protease that was required for MCCT action. Therefore,  $\alpha$ MCCT was incubated with the irreversible inhibitors TLCK, TPCK, and PMSF for 1 hour before addition to the L-cells. Samples were then dialyzed for 10–12 hours against PBS to remove unbound inhibitor. As the data in table 6 show, treatment with TLCK readily blocked  $\alpha$ MCCT activity. This finding is especially interesting in light of the results with ALME, since both TLCK and ALME contain lysyl groups. Identical experiments conducted with TPCK as the inhibitor showed only minimal inhibition of  $\alpha$ MCCT activity. Finally, when PMSF was employed as the inhibitor, we did not detect any decrease in the lytic effectiveness of  $\alpha$ MCCT. In additional experiments we found that these levels of inhibitors had no effect on target cell viability.

*Effects of active oxygen scavengers on  $\alpha$ MCCT activity.*—Different levels of catalase activity were coincubated with  $\alpha$ MCCT and target L-cells. The results of a single experiment are shown in table 7. In multiple experiments, however, we found that catalase at a

TABLE 6.—Effects of reversible and irreversible protease inhibitors on *in vitro* cell-lytic activity of  $\alpha$ MCCT<sup>a</sup>

Inhibitor	Inhibitor concentration, mM	Reaction conditions	Inhibition, % <sup>b</sup>
Irreversible inhibitors			
TLCK <sup>c</sup>	1	1 hr 37°C, dialysis	2
	5	12 hr against	65
	10	PBS	100
TPCK <sup>c</sup>	0.4	1 hr 37°C, dialysis	0
	1	12 hr against	16
PMSF <sup>c</sup>		PBS	
	1	1 hr 37°C, dialysis	0
	2	12 hr against	0
		PBS	
Reversible inhibitors			
TAME <sup>d</sup>	0.4	Simultaneous	2
	2	incubation on	10
	10	L-cells	76
SBTI <sup>d</sup>	0.1	Simultaneous	23
	1	incubation on	54
ALME <sup>d</sup>		L-cells	
	0.4	Simultaneous	0
	2	incubation on	0
	10	L-cells	0

<sup>a</sup> $\alpha$ MCCT was partially purified by molecular sieving as described in "Materials and Methods." The L-929 cell was used as a target in these studies.

<sup>b</sup>Data presented are from a representative experiment.

<sup>c</sup>15.5 U/ml of  $\alpha$ MCCT was added to target L-cells in these experiments.

<sup>d</sup>10.5 U/ml of  $\alpha$ MCCT was added to target L-cells in these experiments.

concentration of 4,000 enzyme U/ml inhibited 75% of the  $\alpha$ MCCT activity. The enzyme at this level had no detectable effect on L-cells. As a control, the enzyme preparation was dialyzed against 500 volumes of PBS for 24 hours; this preparation was equally effective in inhibiting  $\alpha$ MCCT lysis (data not shown). As a further control, catalase was heated to 90°C for 10 minutes before addition to  $\alpha$ MCCT. This treatment destroyed all enzymatic activity and removed the inhibitory effect on  $\alpha$ MCCT activity.

Hypochlorous acid (HOCl) is formed from hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and chloride ion (Cl<sup>-</sup>) by the enzyme myeloperoxidase and is scavenged by the amino acids alanine, serine, and valine (25). As table 7 illustrates, these amino acids at a concentration of 30 mM did not block lytic activity successfully when incubated simultaneously with  $\alpha$ MCCT on L-cells.

## DISCUSSION

Monocytes-macrophages can be promoted to a tumoricidal state by an appropriate combination of *in vivo* and/or *in vitro* priming and triggering events (1-5, 24, 26). Most studies have revealed that this state is characterized by a selective cytotoxic effect preferentially manifested on neoplastic-transformed targets compared to their normal counterparts (1-5, 24-27). The molecular basis for the target injury phase of the lytic mechanism is

currently unresolved; however, numerous mechanisms have been proposed, some of which invoke the extracellular release of a cell-lytic mediator by the macrophage into the fluid phase (6-8, 17, 22, 28-32).

The lack of a large-scale source of these monokines has been a major impediment to the complete functional and biochemical characterization of some of the candidate mediators and to a definition of their possible role in the lytic mechanism. In the murine system, the identification of the P388D<sub>1</sub> macrophage line as a source of lymphocyte activating factor (LAF-interleukin 1) has facilitated biochemical and functional studies of this important monokine (33). Recently, promising models of human monocyte-mediated tumor-cell killing *in vitro* have been forthcoming on the basis of phorbol ester-induced differentiation of monocytoid cell lines (12-16). Concomitant with differentiation, typical macrophage characteristics are acquired, such as a reduced replicative potential, increased adherence to substratum and phagocytic capacity, expression of Fc receptors for IgG, detectable staining for cell-surface esterase, and tumor cytolytic capacity. In this study, we have isolated and initially characterized a cell-lytic mediator or mediators from PMA-treated THP-1 monocytic leukemia cells.

As a biologic assay for cell-lytic molecules, we have employed mitomycin-treated target cells. Drug treatment increases the sensitivity of most targets to lytic effects of the mediators. This increased sensitivity has been reported in numerous cell-lytic systems and may reflect perturbed repair mechanisms and/or cell cycle-linked sensitivity of the targets to the mediators (34-37). A similar assay has recently been employed as a rapid, sensitive assay for human monocyte cytotoxicity (38). Furthermore, the use of nondividing targets in our bioassay allows the discrimination between cytolytic and cytostatic effects of the mediators. In this regard, Gaffney and colleagues (39) have described growth inhibitory substances released by merzerein-treated THP-1 cells. The major component described by them was in the 20,000- to 30,000-dalton range, and it is apparently distinct from the

TABLE 7.—Effects of active oxygen scavengers on *in vitro* cell-lytic activity of  $\alpha$ MCCT<sup>a</sup>

Scavenger	Concentration	Inhibition, % <sup>b</sup>
Catalase <sup>c</sup>	200 U/ml	0
	2,000 U/ml	30
	4,000 U/ml	75
	0 U/ml <sup>d</sup>	0
Alanine <sup>e</sup>	30 mM	0
Serine <sup>e</sup>	30 mM	5
Valine <sup>e</sup>	30 mM	0

<sup>a</sup> $\alpha$ MCCT was partially purified by molecular sieving as described in "Materials and Methods." The toxin was coincubated with the scavenger and L-929 target cells.

<sup>b</sup>Data presented are from a representative experiment.

<sup>c</sup>These studies were conducted with 18.8 U/ml  $\alpha$ MCCT.

<sup>d</sup>Catalase at 4,000 U/ml subjected to 90°C for 10 min before assay with  $\alpha$ MCCT. All enzymatic activity was destroyed by this process.

<sup>e</sup>These studies were conducted with 3.5 U/ml  $\alpha$ MCCT.



major lytic component in our study ( $\alpha$ MCCT $\approx$ 120,000 daltons).

A number of laboratories have reported the detection of cytotoxic molecules released by human monocytes obtained from peripheral blood or from alveolar lavage [(6-8); Klostergaard J: In preparation]. Nissen-Meyer and Hammerstrom (40) have initially characterized cytostatic factors termed CstF I and CstF II, of molecular weights 55,000 and 40,000, respectively, obtained from cultured peripheral blood monocytes. On the basis of molecular weight, these factors appear to be distinct from those released by the THP-1 cell line; however, given the high level of protease activity associated with the culture conditions used for the elicitation of CstF I and CstF II, one could not exclude their derivation from a higher molecular weight precursor. Furthermore, although Nissen-Meyer and Hammerstrom (40) did not detect cytolytic activity, only cytostatic activity, they employed the K562 erythroleukemia target in their assays. In our study, this target was highly resistant to lysis by activated THP-1 supernatant compared to the L-929 target cell (table 5). This raises the possibility that their CstF I and CstF II would be lytically active in a bioassay that uses a more sensitive target.

Matthews (6) has used a bioassay for cytolysis similar to that in our study in the isolation and characterization of an antitumor cytotoxin from cultured human peripheral blood monocytes. The toxin has a molecular weight of 34,000 by molecular sieving and 140,000 by gradient PAGE. In standard PAGE, this toxin appears to have mobility similar to that of  $\alpha$ MCCT. It is noteworthy that the K562 target is substantially less susceptible to lysis by the toxin than the actinomycin-D-treated L-929 cell; this finding is similar to our results.

Recently, Sone et al. (8) have reported the release of a cytolytic factor(s) from human alveolar macrophages. Although only initially characterized, their factor(s) could not be inhibited in lysis of the A375 melanoma by TAME or SBTI, which were effective in blocking the  $\alpha$ MCCT-mediated lysis of L-929 targets (table 6).

We have determined in our laboratory that human peripheral blood monocytes, isolated from platelet-pheresis residues by Ficoll-Hypaque sedimentation and counterflow elutriation, can be induced to release cell-lytic molecules active in the L-929 bioassay (Klostergaard J: In preparation). We have initially characterized these molecules and found two distinct molecular weight forms with biologic activity: The larger is approximately 100,000-140,000 daltons and represents approximately 20% of the total activity; the smaller is about 65,000 daltons and is the predominant form. The sizes agree very closely with those reported here for  $\alpha$ MCCT and  $\beta$ MCCT, although the proportions are nearly inverted. Further characterization of these toxins from peripheral blood monocytes and comparison to the THP-1-derived toxins are under way.

Williams et al. (41) have reported that human B-cell lines release a factor that is active in the in vivo assay for tumor necrosis factor. Although these investigators have tentatively defined this as the human counterpart to

tumor necrosis factor, we believe there is a strong likelihood that this factor is human  $\alpha$ -class lymphotoxin,  $\alpha$ -LT, on the basis of a number of characteristics including the molecular weight, the B-cell origin, and the augmentation in production with PMA (23, 41-43). It is noteworthy that in our studies a highly specific anti- $\alpha$ -LT antiserum did not block  $\alpha$ MCCT activity (data not shown). We propose that  $\alpha$ -LT may well be active in the tumor necrosis factor in assay, but is distinct from monocyte-macrophage-derived cell toxins, some of which may be the more appropriate human counterparts to tumor necrosis factor. This hypothesis should be testable in the near future.

Finally, it is an intriguing possibility that  $\alpha$ MCCT can mediate target cell lysis by both protease-dependent and oxidative mechanisms. These mechanisms have been proposed by a number of investigators as operative in monocyte-macrophage-mediated cell lysis (22, 24, 28, 29, 44-47). Indeed, in the murine system, Adams et al. (47) have shown that a macrophage-derived cytolytic protease and hydrogen peroxide may act synergistically in mediating target cell destruction. In the current studies, although any proposed model would have significant untested aspects to it, we can certainly eliminate the trivial possibility that H<sub>2</sub>O<sub>2</sub> is present in the 120,000-dalton molecular sieving fractions. Furthermore, since PMA is only added during the initial 18-hour phase during which the THP-1 cells become adherent and activated, and thereafter PMA-free media are replaced for the 48-hour release period, any residual PMA is most likely present at very low levels. This PMA could only arise by release from pretreated THP-1 cells and must not be degraded by esterases during the subsequent culture. Thus after the serum-free supernatants are concentrated and subjected to molecular sieving, we expect any PMA to be below the threshold for most PMA-inducible events. Therefore, in the absence of direct quantitation of residual PMA levels, we tentatively exclude the role of PMA in eliciting peroxide from the target cells on which  $\alpha$ MCCT activity is expressed. Further resolution of this mechanism, for example, to determine if  $\alpha$ MCCT induces H<sub>2</sub>O<sub>2</sub> production by the target cell subsequent to binding, will be possible when this mediator can be sufficiently purified.

In this context, this report is an initial phase of our studies of models for human monocyte-macrophage cytotoxicity. To some extent the THP-1 cell line satisfies the necessary criteria for such models and will provide sufficient sources for isolation, purification, characterization, and, if justified, gene cloning of molecular mediators of tumoricidal mechanisms.

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