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CHARACTERIZATION OF MACROPHAGE- AND GRANULOCYTE-INDUCING PROTEINS FOR NORMAL AND LEUKEMIC MYELOID CELLS PRODUCED BY THE KREBS ASCITES TUMOR

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Summary

Medium from serum-free cultures of Krebs ascites tumor cells contains two macrophage and granulocyte inducing (MGI) activities that can act on the myeloid precursors of these hematopoietic cells. One activity, MGI-1, induced the formation of macrophage and granulocyte colonies from normal myeloid precursors. The second activity, MGI-2, induced macrophage and granulocyte differentiation in myeloid leukemic cells that no longer required MGI-1 for colony formation. The medium contained one species of MGI-1 and two species of MGI-2. One species of MGI-2, MGI-2A, copurified through five stages of purification with MGI-1, but separated from the other MGI-2 species, MGI-2B, at an early stage in purification. MGI-1, MGI-2A and MGI-2B were purified 1490, 1140 and 678-fold, respectively. When bands with biological activity cut from non-denaturing polyacrylamide gels were run on SDS-polyacrylamide gel electrophoresis, MGI-1 and MGI-2A activities were associated with similar M_r and each activity showed two bands, one of 23 000 and the other 25 000. MGI-2B activity showed one band with a M_r of 45 000. Secretion did not appear to involve glycosylation, none of the species bound to concanavalin A, soybean agglutinin, or wheat germ agglutin agarose columns and they did not appear to contain carbohydrates. The assays for MGI-1 and MGI-2 activities were not affected by adding protease inhibitors. But MGI-2 was more readily destroyed by treatment with proteases and was more labile at high temperature and low pH than MGI-1. It is suggested that the level of cellular proteases may play a role in regulating the relative amounts of MGI-1 and MGI-2 that are present in vivo.

Abbreviations: Temed, *N,N,N',N'*-tetramethylethylenediamine; SDS, sodium dodecyl sulfate.

Introduction

Studies on the growth and differentiation of normal hematopoietic cells in culture have shown [1,2] that normal myeloid precursor cells incubated in culture with other cell types as a feeder layer can form colonies of macrophages and granulocytes and this was then confirmed by others [3]. It was also shown [2,4,5], that the formation of these colonies is due to secretion by the feeder layer cells of a specific inducer for the colonies containing either of these two types of hematopoietic cells. After this inducing activity was first detected in conditioned medium [4,5], this protein inducer has been referred to by a number of names [6] including mashran gm [7], macrophage and granulocyte inducer (MGI) [8], colony stimulating factor [9], and colony stimulating activity [10]. It is produced by various normal and malignant cells in culture and has also been detected in serum and in various tissues in vivo [6,11,12]. In addition to this MGI activity for normal myeloid colony forming precursors, there is also an MGI activity that can induce the normal differentiation of myeloid leukemic cells to macrophages and granulocytes both in culture [6,11,13–19] and in vivo [20].

Normal myeloid precursors require MGI for cell viability, multiplication and differentiation [6]. This activity on normal cells, which has been assayed by the induction of macrophage and granulocyte colonies, will be referred to as MGI-1 [21]. However, the myeloid leukemic cells used (MGI^+D^+) no longer require MGI for cell viability and multiplication, but can still be induced to differentiate by MGI [6]. These leukemic cells can thus form colonies but do not differentiate without adding MGI. This activity on the leukemic cells, which has been assayed by induction of the differentiation-associated marker lysozyme in the leukemic cells [22], will be referred to as MGI-2 [21]. There have been several reports on the characterisation of MGI-1 from a variety of sources, including conditioned medium from fibroblasts [8,23–25], lung [26,27], pancreatic carcinoma cells [28] and from urine [29]. The characterisation of MGI-1 and MGI-2 activities from fibroblasts has also been reported [8,15,23]. However, further progress requires a source of large amounts of serum-free material that contains both activities. We have now characterised both activities from medium conditioned by mouse Krebs ascites tumor cells. These ascites cells are conveniently passaged in mice and after 24 h in culture can yield large quantities of serum-free conditioned medium that is rich in both MGI-1 and MGI-2 activity [20].

Materials and Methods

Preparation of serum-free conditioned medium

Krebs ascites cells were passaged in ICR mice by intraperitoneal injections of about 10^6 cells in 0.2 ml phosphate-buffered saline. Routinely, cells were removed after 9 days in vivo by flushing the peritoneal cavity with 5 ml of phosphate-buffered saline containing 6 U · ml^{-1} heparin. Fluids containing high percentages of erythrocytes were rejected to avoid additional contaminating proteins. The harvested cells were washed three times with phosphate-buffered saline and seeded at $5 \cdot 10^6$ cells per ml Eagle's medium with a 4-fold concen-

tration of amino acids and vitamines (H-21, Grand Island Biological Co., Grand Island, NY) in spinner cultures at 37°C. The cells did not proliferate in the absence of serum. After 20 h, the cells were removed by centrifugation and the conditioned medium stored at −20°C until used. Repeated freeze-thawing resulted in loss of activity and was avoided.

For the preparation of ^{3}H-labelled conditioned medium, Krebs ascites cells were removed and washed as described above. They were then seeded at $5 \cdot 10^6$ cells $\cdot$ ml^{-1} in Eagle's medium without leucine. L-(4,5-^{3}H)leucine (57 Ci $\cdot$ mmol^{-1}), (Radiochemical Centre, Amersham) was added to the culture at a final concentration of 10 μCi $\cdot$ ml^{-1} and the cells cultured as described. Conditioned medium labelled in this way possessed MGI activity equivalent to unlabelled conditioned medium. The incorporation of label was 10—15% as measured after precipitation by trichloroacetic acid. This labelling was used to trace protein elution from the columns and to estimate protein concentration during the final step used for purification. Cell extracts ($2.5 \cdot 10^7$ cells $\cdot$ ml$^-$ in Eagle's medium) were prepared by 2 cycles of rapid freezing in liquid nitrogen and thawing at 37°C and removal of cellular debris by centrifugation at 10 000 $\times g$ for 20 min [30]. Freshly prepared samples were then assayed for MGI activities.

Assay for the activity of MGI on normal bone marrow cells (MGI-1 activity)

Colony formation in agar was measured using the technique described [2,31]. Briefly, $5 \cdot 10^4$ nucleated bone marrow cells from the femurs of 2—3 month old ICR mice were seeded in 0.8 ml soft agar (0.33%) on a 2.5 ml hard agar base (0.5%) in 35-mm tissue culture Petri dishes (Falcon Co., Rutherford, NJ). Both layers contained Eagle's medium (H-21) and 20% heat-inactivated fetal calf serum. The lower layer contained $2 \cdot 10^{-4}$ dithiothreitol and conditioned medium or fractions thereof. The column fractions were assayed at a concentration approx. equivalent to 5% starting material, assuming 100% recovery from the previous step. For determinations of purification factors and yields, pooled fractions were assayed at several concentrations and the values taken from the linear part of the assay (20—100 colonies per Petri dish). Colonies containing more than 50 cells were scored after 7 days. The reproducibility of the number of colonies in duplicate cultures was ±15%. Bone marrow from SL mice, the strain from which the myeloid leukemia used was isolated, gave similar results to those obtained with bone marrow from ICR mice.

Assay for the activity of MGI on myeloid leukemic cells (MGI-2 activity)

The myeloid leukemic cells used were from MGI$^+$D$^+$ clone 11 derived [16] from a cell line of myeloid leukemia originating in an SL mouse [32]. The cells were cultured in vitro in Eagle's medium (H-21) and 10% heat-inactivated horse serum. Induction of differentiation by MGI-2 was tested by incubation of 10^5 cells in 2 ml Eagle's medium with 10% horse serum and conditioned medium or fractions thereof in 35-mm tissue culture Petri dishes. The usual concentrations of conditioned medium were 1—20% v/v. After 4 days in culture, samples were screened for the onset of differentiation by checking for the attachment and spreading of cells on the petri dish and the degree of differentiation quantitated by assaying the secreted lysozyme by the decrease in turbidity at 540 nm of a

suspension in *Micrococcus lysodeikticus* as previously described [22]. For the screening of fractions from columns, MGI-2 was assayed at a final concentration in the linear range of the assay (equivalent to 10% original conditioned medium). For determination of purification factors and yields, pooled fractions were assayed at 3 or 4 concentrations and the value used was taken from a linear part of the assay. Duplicate cultures were reproducible to ±20%.

Protein analysis

Samples from pooled fractions were dialyzed against 20 mM Tris-HCl pH 7.0 (3 × 3 l) and protein determined [33], using bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as standard. Protein distribution in column fractions was estimated from the elution profile of [^{3}H]leucine. The protein concentration of the final purification step, polyacrylamide gel electrophoresis, was estimated by comparing the ration of radioactivity in this fraction to that of the previous step and assuming an equivalent yield of protein and radioactivity.

Purification of MGI from Krebs ascites conditioned medium

Conditioned medium (4—5 l batches) was made up to 40% saturated $(NH_4)_2SO_4$ (Schwarz/Mann, Orangeburg, NY), and stirred for 2 h at 4° C following dissolution of the $(NH_4)_2SO_4$. The material was centrifuged at 10 000 × *g* (Sorval GSA rotor) for 20 min to pellet the precipitated protein. The resulting supernatant was made up to 100% saturated $(NH_4)_2SO_4$ as above and the resulting precipitate collected by centrifugation. The pellet was solubilized in 200 ml of 10 mM Tris-HCl, pH 4.0 (pH at room temperature) and dialyzed against 3 × 5 l 1 mM Tris-HCl at 4°C for 36 h. Samples from each stage of the purification were assayed on normal bone marrow and leukemic cells and for protein determination. All further manipulations were carried out at 4° C.

The 40—100% saturated $(NH_4)_2SO_4$ fraction of the conditioned medium was chromatographed on a DEAE-cellulose (Whatman DE 52) column (35 × 4.5 cm) equilibrated with 10 mM Tris-HCl, pH 7.0. Activity was eluted with a 700 ml linear salt gradient (0—0.25 M NaCl in 10 mM Tris-HCl, pH 7.0). The two peaks of activity were pooled separately as indicated. Flow rate was 50 ml per h.

Each peak of MGI activity was loaded separately onto a hydroxy apatite (Bio-Gel HTP) column (29 × 1.5 cm) equilibrated with 10 mM Tris-HCl, pH 7.0. Following a wash with 100 ml of buffer, activity was eluted with a 400 ml linear salt gradient (10 mM Tris-HCl, pH 7.0 to 0.2 M sodium phosphate, pH 7.4). Flow rate was 30 ml/h. Active fractions were pooled and protein precipitated by bringing the fraction to 100% saturated $(NH_4)_2SO_4$. The protein pellet which remained after centrifugation at 10 000 × *g* for 20 min was dissolved in 10 ml 10 mM Tris-HCl.

The undialyzed concentrated activities from the hydroxyl apatite column were subjected to gel filtration on a column (92 × 2.6 cm) of Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala), equilibrated with 10 mM Tris-HCl. The column had previously been standardized with various markers of known molecular weight (Sigma Chemical Co., St. Louis, MO). Elution was reverse flow to maximize retention of calibration. Flow rate was 25 ml per h. The pooled

active fractions were brought to 100% saturated $(NH_4)_2SO_4$, the precipitate dissolved in 10 mM Tris-HCl and dialyzed against 2 × 3 l 10 mM Tris.

Non-denaturing polyacrylamide gel electrophoresis [34], was carried out in a slab gel apparatus (Tamar Equipment, Jerusalem). All chemicals used were electrophoresis grade (Bio-Rad Labs., Richmond, CA). Polymerization was initiated using Temed and ammonium persulfate. The gels of 10% acrylamide and 0.27% bisacrylamide were 12 cm wide, 25 cm long and 3 mm thick. The stacking gel was 3% acrylamide and 0.08% bisacrylamide. Activity fractions from the hydroxyapatite column were precipitated with 100% saturated $(NH_4)_2SO_4$. The precipitate was dissolved in 1 ml 10 mM Tris-HCl and dialyzed against 2 × 3 l of 10 mM Tris-HCl. Sample buffer (3 times concentrated) was added so that the final sample contained 10% glycerol, 62.5 mM Tris-HCl, pH 6.8, 1 mM phenylmethylsulfonyl fluoride and 0.001% bromphenol blue. The sample was warmed to 37°C for 15 min before applying it to the gel. When the marker bromphenol blue had reached the bottom, the gel was removed and side strips removed for staining with 0.25% Coomassi brilliant blue (Sigma Chemical Co., St. Louis, MO) in 50% methanol/7% acetic acid (v/v/v). The remaining gel was either sliced at 0.5-cm intervals or stored for 2 h at 4°C until staining and destaining with 50% methanol/7% acetic acid revealed the location of protein bands. Strips (0.5 cm) matching the positions of the bands were then removed. The gel slices were minced by forcing through a 5 ml syringe band and then shaken with 5 ml Eagle's medium at room temperature overnight. Gel fragments were removed by centrifugation.

Various fractions retained from the steps of the purification procedure were analyzed using SDS-polyacrylamide gel electrophoresis. Samples either directly, or after concentration by lyophylization following dialysis against 10 mM Tris-HCl, pH 7.0, were mixed with three times concentrated sample buffer to a final concentration of 10% glycerol (Fisher Scientific Co., Pittsburg)/5% 2-mercaptoethanol (Merck, Darmstadt)/3% SDS (Bio-Rad Labs., Richmond, CA)/62.5 mM Tris-HCl, pH 6.8/1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO) and 0.001% bromphenol blue (Bio-Rad Labs., Richmond, CA) [35], and applied to a slab gel with 3% acrylamide stacking gel [36] and a 10–20% acrylamide gradient in the resolving gel [37]. Staining and destaining was carried out as above.

For chromatography on Con A-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala) or Con A-agarose (Miles-Yeda, Rehovot), samples of condition medium were dialyzed against 3 × 200 vols. 0.1 M sodium acetate, pH 6.0/1 mM $CaCl_2$/1 mM $MgCl_2$/1 mM $MnCl_2$. 5-ml aliquots of dialyzed unconcentrated conditioned medium were loaded on a column (0.9 × 5 cm) and the column washed with the buffer. Elution was carried out with 0.1 M α-methylglucoside (Pfanstiehl Labs., Waukegan, IL) in buffer [38]. In some cases, dialyzed conditioned medium was incubated with 0.002 U · ml^{-1} α-L-fucosidase from bovine epididymis (Sigma Chemical Co., St. Louis, MO) and/or 2 U · ml^{-1} neuraminidase from *Vibrio cholerae* (Behringwerke, Marburg) for 60 min at 37°C before chromatography. Eluted fractions were then dialyzed against 3 × 100 vols. 0.1 M Tris-HCl, pH 7.0 (at room temperature) overnight at 4°C and assayed as described. Chromatography at low pH with Mg, Ca and Mn was essential as the concanavalin A leached out at neutral pH induces differentiation of MGI^+D^+

leukemic cells [6] (and Lotem and Sachs, unpublished data).

For chromatography on soybean agglutin-agarose (Miles-Yeda, Rehovot) or wheat germ agglutinin-agarose (Miles-Yeda, Rehovot) [39] conditioned medium was dialyzed against 3 × 200 vols. 0.1 M Tris-HCl, pH 7.0. 5-ml samples of the dialyzed unconcentrated conditioned medium were then chromatographed on columns (0.9 × 3 cm) of the above lectins. After washing with buffer, elution was carried out with the appropriate hexoseamine, 100 $\mu g \cdot ml^{-1}$ *N*-acetylgalactosamine in buffer in the case of soybean agglutinin and 100 $\mu g \cdot ml^{-1}$ *N*-acetylglucosamine (Pfanstiehl Labs., Wankegan, IL) in buffer in the case of the wheat germ agglutinin.

Protease sensitivity and protease inhibitors

Protease sensitivity was tested by incubating the conditioned medium or post-Sephadex G-200 MGI with the appropriate protease for 2 h at 37°C. The reaction was stopped by the addition of soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) in the case of trypsin or horse serum, or Trasylol (aprotinin) (Calbiochem, Lucerne) for other proteases. Trypsin 3 times crystallized was obtained from Worthington, pronase B grade from Calbiochem, chymotrypsin (Type II), papain (Type IV), thrombin (bovine grade I), subtilisin BPN', leucine aminopeptidase (Type III-CP), carboxypeptidase A (Type II) and carboxypeptidase B (chromatographically purified) were obtained from Sigma Chemical Co., St. Louis, MO.

Diisopropylfluorophosphate and benzamidine hydrochloride were obtained from Fluka, ϵ-aminocaproic acid, phenylmethylsulfonyl fluoride, L-1-tosylamide-2-phenylethyl-chloromethyl ketone, *N*-α-*p*-tosyl-L-lysinechloromethylketone hydrochloride, *p*-tosyl-L-arginine methyl ester hydrochloride, *N*-benzoyl-L-tyrosine ethyl ester, 2 nitro-4-carboxyphenyl-*N*,*N*-diphenyl carbamate and hirudin were obtained from Sigma, and *p*-nitrophenyl-*p'*-guanidobenzoate from Merck. Leupeptin and antipain were gifts from Dr. W. Troll and the U.S.-Japan Cancer Program. Pepstatin was a gift from Dr. R. Miskin.

Carbohydrate content

The effect of oxidation of carbohydrate was tested using periodic acid. Potassium meta periodate (Fluka) was added to MGI samples that had been dialysed against 3 × 100 vols. 0.2 M acetate-HCl, pH 5.0. Following incubation for 2.5 h at 4°C [40], the samples were dialyzed against 3 × 100 vols. 10 mM Tris-HCl, pH 7.2, and then assayed.

Neuraminidase from *Vibrio cholera* (Behringwerke), α-L-fucosidase (bovine epididymis), β-galactosidase (bovine liver), β-glucosidase (almond), α-glucosidase (yeast type I), and β-*N*-acetylglucosaminidase (jack bean) all from Sigma and mixed glycosidases (*T. cornutus*) from Miles Labs., Elkhart, IN, were tested on MGI samples in 0.2 M sodium acetate, pH 4.5, for 1—5 h at 37°C [41] and in 0.1 M sodium phosphate, pH 6.5, for 1—5 h at 37°C (based on the supplier's assessment of pH optimum for the glycosidases). All glycosidases were checked for activity at both pH values using the appropriate *p*-nitrophenol substrate (Sigma). MGI samples were dialyzed against 10 mM Tris-HCl before assaying.

Stability to temperature and pH

For determination of stability to temperature, 2 ml conditioned medium of

the post G200 MGI fraction was incubated at different temperatures for 30 min, cooled to 4°C and centrifuged at 10 000 × *g* for 20 min to remove precipitated protein. The sample was then assayed as usual. For determination of the stability to pH, 2 ml of conditioned medium or the post-G200 MGI fraction was mixed with the appropriate buffer and incubated at 37°C for 5 h. The samples were then dialyzed extensively against 0.1 M Tris-HCl, pH 7.0 and assayed as usual. For pH 2.5—6, the appropriate pH was achieved by mixing of 0.2 M Na_2HPO_4 with varying amount of 0.1 M citric acid for pH 7—8, 0.2 M NaH_2PO_4 and 0.2 M Na_2HPO_4, and for pH 9—10.5, 0.2 M glycine was adjusted to the appropriate pH with 0.2 M NaOH.

Effects of metabolic inhibitors on the production of MGI-1 and MGI-2

Actinomycin D (Calbiochem), cycloheximide (Sigma) or emetine (Sigma) were added to cultures at zero time. In some experiments, medium was discarded at 3 h and the cells washed twice with emetine and re-seeded at $5 \cdot 10^6$ cells · ml^{-1} in Petri dishes. In others, the inhibitors were left in the cultures for the entire culture period. Samples taken at all time points were then dialyzed against 5 × 500 vol. 0.1 M Tris-HCl, pH 7.4, at 4°C before assay. It was found that unless inhibitors were removed, killing of MGI^+D^+ clone 11 leukemic cells was observed and in certain cases induction of clone 11 by residual actinomycin D could occur [42].

Results

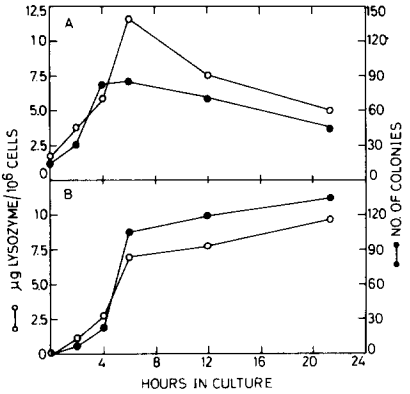
Production of MGI-1 and MGI-2 by Krebs ascites cells

Both MGI-1 and MGI-2 activity was found in the cell extracts when the Krebs ascites cells were removed from the mice, and after culture in serum-free medium the amounts of both activities increased in the cell extracts up to about 6 h (Fig. 1A). Both activities were detected in the culture supernatants

TABLE I

EFFECT OF ACTINOMYCIN D, CYCLOHEXIMIDE AND EMETINE ON THE APPEARANCE OF MGI-1 AND MGI-2 IN THE CULTURE SUPERNATANT

Inhibitor	Concentration	Time in culture (h)	Activity after 20 h (percent of control)	
			MGI-1	MGI-2
Actinomycin D	0.005 μg ml^{-1}	3	41	22
	0.05 μg ml^{-1}	3	24	16
	0.1 μg ml^{-1}	3	8	4
Cycloheximide	10^{-5} M	20	37	25
	$2 \cdot 10^{-5}$ M	3	33	18
	$2 \cdot 10^{-5}$ M	20	13	1
	10^{-4} M	20	10	0
	$2 \cdot 10^{-4}$ M	3	8	1
	$2 \cdot 10^{-4}$ M	20	0	0
Emetine	1 μg ml^{-1}	20	46	20
	10 μg ml^{-1}	20	14	3
	100 μg ml^{-1}	20	0	0



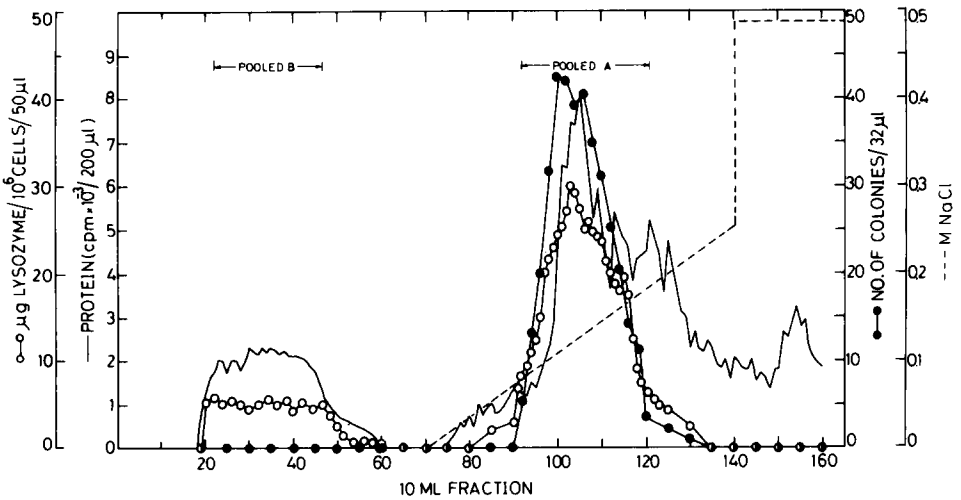


Fig. 1. Kinetics of production of MGI-1 and MGI-2 by Krebs ascites cells in culture. A, cellular MGI; B, secreted MGI on conditioned medium. Activity per ml of cell extract or conditioned medium.

Fig. 2. DEAE-Cellulose ion exchange chromatography of $(NH_4)_2SO_4$ precipitated MGI from Krebs ascites conditioned medium. Fractions designated A and B were pooled and used in the next step.

(conditioned medium) starting at 2 h and also almost reached a plateau at 6 h (Fig. 1B). The addition of actinomycin D, cycloheximide, or emetine at zero time (Table I), and the extensive dialysis of the samples before they were analyzed, showed that these compounds inhibited the appearance of MGI-1 and MGI-2 in the conditioned medium. This indicates that both activities were synthesized de novo by the cells in culture. Only the conditioned medium from the cultured cells was used for the purification of MGI-1 and MGI-2.

Stability of MGI during purification

During the purification procedure, care was taken to avoid procedures which would result in large losses of MGI activity. Freeze-thawing, particularly of partially purified fractions, or samples in low ionic strength buffers, resulted in losses of activity. This ranged from 10% in unprocessed conditioned medium to

80% in step 4 (Table II) of the purification. In general, low ionic strength buffers contributed to a slow loss of activity which was accelerated with reduced protein concentration. Dialysis against buffers lower in concentration than 10 mM Tris-HCl, pH 7.0, resulted in irreversible precipitation of much of the protein and activity. For example, dialysis against water resulted in a loss of 80% of the protein and 60—70% of the activity from unprocessed conditioned medium. Various compounds such as poly(ethylene glycol) or Triton X-100 used to prevent absorption of MGI-1 to glass and plastic surfaces [24,26] were not effective in preventing such losses. Losses of activity of 30—75% were observed when samples were dialyzed against buffer less than 10 mM Tris and hence were avoided. Only when samples were dialyzed for DEAE-cellulose or nondenaturing polyacrylamide gels, when low ionic strength was required, were samples dialyzed at 10 mM Tris. Usually, dialysis was against 0.1 M Tris where losses were less than 5%.

An even greater source of loss of activity was due to nonspecific binding to Millipore filters or Diaflo membranes under most conditions, particularly low protein or ionic strength. Although this could be reduced by adding bovine serum albumin or serum, this was not used during the purification procedure. In general, the entire procedure could be carried out over about a month-long period without contamination or the need to sterilize fractions before assay. Rates of contamination of less than 1% of the assays were observed under these conditions. The general procedure was to store fractions in plastic tubes at 4°C. Sodium azide (0.02%) and phenylmethylsulfonyl fluoride (1 mM) were added if the samples were to stand for more than a week. Samples not used in further purification, or for protein analysis, were made up to 1 mg · ml^{-1} bovine serum albumin or 10% horse serum and stored at 4°C.

Purification of MGI

$(NH_4)_2SO_4$ precipitation (40—100% saturation) provided a good reproducible method of concentrating the conditioned medium as well as an initial purification step. Recovery of MGI-2 activity was usually 100% and that of MGI-1 generally 80%. The remaining 20% of the MGI-1 activity remained soluble even at 100% saturation of $(NH_4)_2SO_4$. The activity precipitated over this broad range of $(NH_4)_2SO_4$ concentration and finer cuts gave additional loss of activity.

Unlike the results reported for MGI-1 from fibroblasts or lung [24—26], the MGI-1 or MGI-2 from the Krebs ascites cells did not bind to DEAE-cellulose at buffer concentrations of more than 50 mM Tris. Binding occurred at 10 mM Tris, pH 7.0, (Fig. 2) and elution of activity started at about 50 mM NaCl in Tris. An identical elution profile for the radioactive label and both biological activities was observed over a pH range of 5—8.5. Activity was seen to fractionate into two peaks. The first one, which did not bind to DEAE-cellulose and showed activity on leukemic cells only, was designated MGI-2B. Rechromatography of this peak on a second DEAE-cellulose column also showed no absorption. The peak of activity which absorbed to the column was found to contain both MGI-1 activity and 80% of the MGI-2 activity, which was designated MGI-2A. The MGI-2B activity was observed in about 70% of the preparation of conditioned medium. In the remaining 30% of the preparations, only one peak corresponding to MGI-1 and MGI-2 activity was found, and the MGI-2 activity

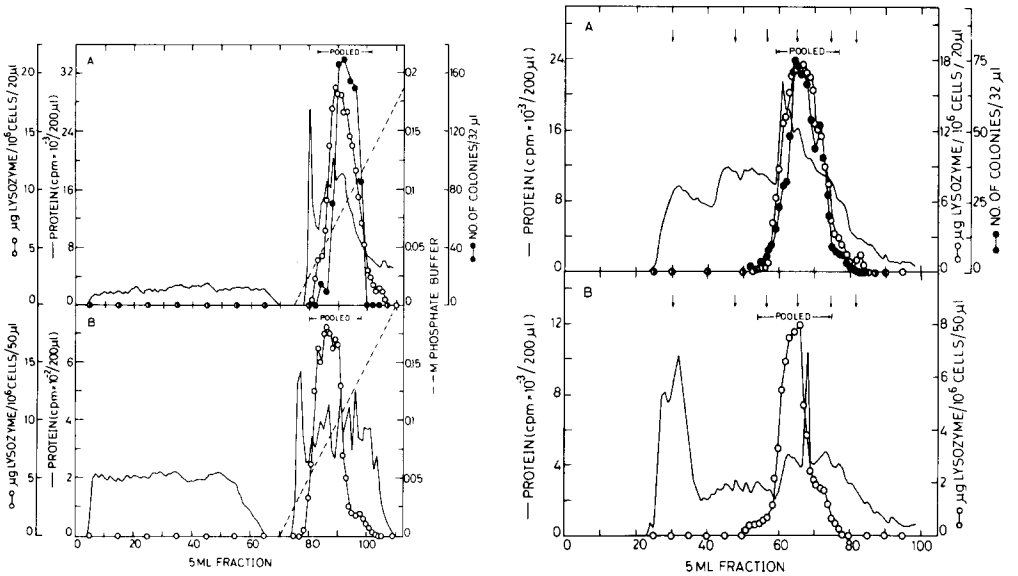


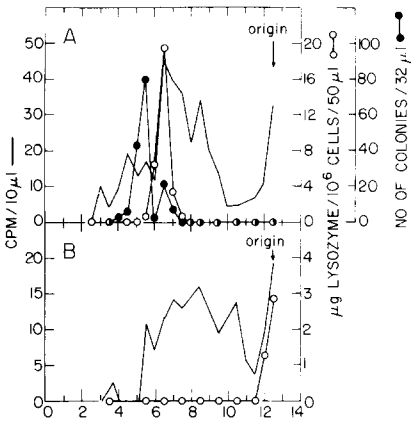
Fig. 3. Hydroxyapatite chromatography of the pooled fractions from the DEAE-cellulose column in Fig. 2. Pooled fractions used in the next step are indicated. A, MGI-1 and MGI-2A; B, MGI-2B.

Fig. 4. Sephadex G-200 gel filtration of concentrated pooled fractions from the hydroxyapatite column in Fig. 3. Pooled fractions are indicated. Arrows indicate the peak of elution of the blue dextran and proteins used in standardizing the column. A, MGI-1 and MGI-2A; B, MGI-2B. The six arrows in order of elution, represent blue dextran, aldolase, bovine serum albumin, ovalbumin, chymotrypsinogen and cytochrome *c*.

bound to the column under the same conditions as the MGI-2A fraction. Parallel purifications of both peaks were continued in those preparations where both MGI-2 activities were present.

Both the 2A and 2B peaks applied to hydroxyapatite columns showed similar elutions starting at approx. 50 mM sodium phosphate (Fig. 3). Following concentration of the pooled fractions by $(NH_4)_2SO_4$, 10-ml samples were loaded onto a Sephadex G200 column that had been precallibrated for molecular weight with a number of known standards (blue dextran, aldolase, bovine serum albumin, ovalbumin, chymotrypsinogen and cytochrome *c*). Elution profiles can be seen in Fig. 4, and the apparent molecular weights of MGI-1 and MGI-2A, and MGI-2B after this gel filtration were about 43 000.

Following concentration of the active fractions by $(NH_4)_2SO_4$ precipitation and dialysis against 10 mM Tris-HCl, pH 7, samples were electrophoresed on non-denaturing polyacrylamide gels, the gels were sliced and fractions were eluted and assayed for MGI-1 and MGI-2 activity. The results are shown in Fig. 5. In Fig. 5A, MGI-2A was partially separated from the bulk of the activity of MGI-1, but there was still some residual activity of MGI-1 associated with the MGI-2A peak. In Fig. 5B, MGI-2B activity remained associated with the activity that barely entered the stacking gel. The MGI-1 and MGI-2A activities corresponded to distinct bands after Coomassie blue staining. Mixing of the active fractions with MGI-1 and MGI-2 activity in equal volumes resulted in no change in either activity.



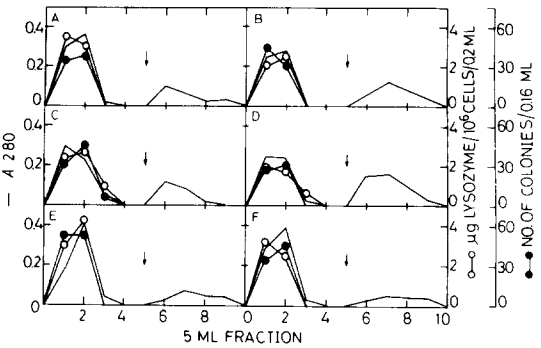


Fig. 5. Gel electrophoresis of dialyzed concentrated pooled fractions from the Sephadex G-200 column in Fig. 4. The anode is to the left. The gel front was determined from the position of the bromphenol blue tracking dye.

Fig. 6. Lectin-affinity chromatography of MGI-1 and MGI-2 from conditioned medium. Arrows indicate start of elution with appropriate sugar. A-D, Con A-Sepharose on untreated, fucosidase-treated, neuraminidase-treated, fucosidase plus neuraminidase-treated condition medium; E, wheat germ agglutinin agarose; F, soybean agglutinin agarose.

A typical purification profile for the various MGI activities is shown in Table II. At step 3 in the purification, the MGI-2 activity, which was assayed on leukemic cells, split into two peaks which were further purified. The peak designated MGI-2A was found to copurify with MGI-1, the activity measured on normal cells, until the final step in the purification in which the activities were partially separated. The final products were purified 1490-fold, 1140-fold and 678-fold for MGI-1, MGI-2A and MGI-2B, respectively. The lower purification of MGI-2B was probably because the activity barely entered the stacking gel in step 6.

The bands cut from non-denaturing polyacrylamide gels were run on SDS-polyacrylamide gels. Protein bands were visualised by staining and molecular weights determined by comparison with standards (bovine serum albumin, ovalbumin, chymotrypsinogen and lysozyme). The fraction with MGI-1 activity from the non-denaturing gels had two bands after staining of the SDS-polyacrylamide gels, with M_r 23 000 and 25 000 and with sometimes a contaminant at 50 000. MGI-2A activity was associated with two bands of the same M_r of 23 000 and 25 000, with sometimes a contaminating band of 12 000. MGI-2B activity was associated with a single band of 45 000.

The MGI-1 activity that was purified from fibroblast conditioned medium [8,23] was shown to have a cofactor requirement that could be replaced by adenine nucleotides [43]. Attempts to modify the activities of MGI-1, or of MGI-2, with medium containing the co-factor, 0.16 mM NAD, 5'AMP, or cyclic AMP [43] were not successful. Thus Krebs ascites conditioned medium seems to differ from this fibroblast conditioned medium in the involvement of a cofactor in the activity of MGI-1.

TABLE II

PURIFICATION OF MGI FROM KREBS ASCITES CONDITIONED MEDIUM

Fraction	Volume (ml)	Total units	Total proteins (mg)	Specific activity ($U \cdot mg^{-1}$)	Fold purification	Yield (%)
MGI-1						
1. Conditioned medium	4800	2 480 000 *	2570	970	1	100
2. 40—100% $(NH_4)_2SO_4$ precipitate	210	2 010 000	798	2 520	2.6	81
3. DEAE-cellulose	400	1 470 000	146	5 800	5.4	73
4. Hydroxyapatite chromatography	90	1 280 000	36.7	34 800	35.9	52
5. G-200 chromatography	85	925 000	5.48	169 000	174	37
6. Polyacrylamide gel electrophoresis	18	179 000	0.12	1 440 000	1490	7.2
MGI-2						
1. Conditioned medium	4800	185 000 **	2570	72	1	100
2. 40—100% $(NH_4)_2SO_4$ precipitate	210	192 000	798	295	4.1	104
3. DEAE-cellulose — peak A	300	117 000	146	798	11.8	63
— peak B	250	29 600	50.2	590	8.2	16
4. Hydroxyapatite — peak A	90	100 000	36.7	2 722	37.8	54
chromatography — peak B	85	22 200	6.65	3 340	46.4	12
5. G-200 chromatography — peak A	85	77 800	5.48	14 200	196.6	42
— peak B	110	15 000	0.90	16 700	231.2	8.1
6. Polyacrylamide gel — peak A	19	17 000	0.21 ***	82 300	1140	9.3
electrophoresis — peak B	17	2 220	0.045 ***	49 300	678	1.2

* MGI-1 units expressed as number of colonies.

** MGI-2 units expressed as μg lysozome produced per 10^6 cells.

*** Estimated on the basis of recovery of cpm in step 6.

Lack of carbohydrate content

The use of Con-A agarose affinity chromatography has proved to be a useful step in purifying MGI from some sources [24—26]. When Con A-Sepharose chromatography was examined as a possible purification step, no activity of MGI-1 or MGI-2 was found to bind to the column (Fig. 6A). Since concanavalin A binds to glucoside and mannoside residues which tend to be core residues in glycoproteins, attempts were made to remove fucose and sialic acid which are outer residues and may have masked the concanavalin A binding sites if present. Fig. 6B, C and D shows that neither activity binds to the lectin column after pre-treatment with fucosidase, neuraminidase or both enzymes, respectively. Similar results were obtained with wheat germ agglutin-agarose (Fig. 6E) and soybean agglutin-agarose (Fig. 6F) chromatography, lectins which bind *N*-acetylglucosamine and *N*-acetylgalactosamine, respectively, indicating an apparent absence from these carbohydrates. In addition, treatment of conditioned medium with neuraminidase had no effect on the apparent molecular weight of the two activities. In order to confirm the absence of carbohydrate involved in the activity of the two forms of MGI, the effects of a number of glycosidic enzymes and periodic acid on conditioned medium were examined (Table III). No alteration in activity was observed. Staining of SDS-polyacrylamide gels with periodic acid-Schiff (PAS) reagent indicated no carbohydrate containing proteins (PAS-positive bands) remaining after step 4 of the purification. The production of both MGI activities in these cells was also not affected by incubation with 1—10 $\mu g \cdot ml^{-1}$ deoxyglucose, 1—10 $\mu g \cdot ml$ tunicamycin or 5—10 $\mu g \cdot ml$ amphomycin, antibiotics [44,45] which are known to block glycosylation of glycoproteins and in some cases their secretion. Tunicamycin was a gift from Dr. Robert Hamill, Eli Lilly Laboratories, and amphomycin a gift from Dr. W. Minor, Bristol Laboratories.

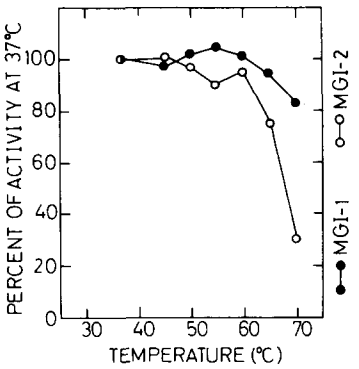
Sensitivity to temperature and pH

The effect of temperature on MGI activity is shown in Fig. 7. In unpurified conditioned medium, MGI-1 was basically unaffected by temperatures under

TABLE III

LACK OF EFFECT OF GLYCOSIDASES AND PERIODIC ACID ON MGI-1 AND MGI-2 ACTIVITY

Treatment	Percent of control activity	
	MGI-1	MGI-2 (A and B)
No treatment	100	100
Neuraminidase (2 $U \cdot ml^{-1}$) 1 h, 37°C	103	94
α-L-Fucosidase (0.002 $U \cdot ml^{-1}$) 1 h, 37°C	96	110
β-Glucosidase (100 $\mu g \cdot ml^{-1}$) 1 h, 37°C	113	105
α-Glucosidase (10 $\mu g \cdot ml^{-1}$) 1 h, 37°C	101	102
β-Galactosidase (100 $\mu g \cdot ml^{-1}$) 1 h, 37°C	99	99
β-*N*-acetylglucosaminidase (1 $U \cdot ml^{-1}$) 1 h, 37°C	94	93
Mixed glycosidases (1 $mg \cdot ml^{-1}$) 1 h, 37°C	101	104
Mixed glycosidases (1 $mg \cdot ml^{-1}$) 5 h, 37°C	92	95
1 mM IO_4^- (2.5 h, 4°C)	113	100
5 mM IO_4^- (2.5 h, 4°C)	96	110
10 mM IO_4^- (2.5 h, 4°C)	98	105



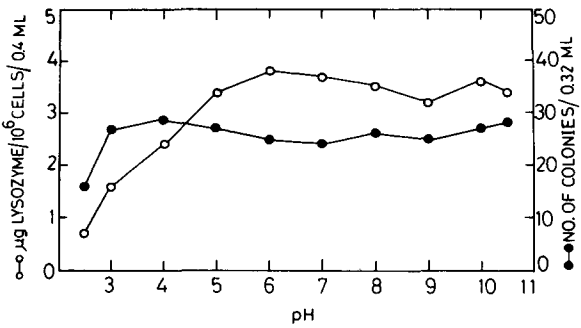


Fig. 7. Temperature sensitivity of MGI-1 and MGI-2 in conditioned medium. 100% MGI-1 activity was 61 colonies when assayed at 5%. 100% MGI-2 activity was 2.8 μg lysozyme per 10^6 cells when assayed at 10%.

Fig. 8. pH stability of MGI-1 and MGI-2 from conditioned medium.

65°C and at 70°C the activity was reduced by only 20%. MGI-2, however, showed a 25% loss in activity at 65°C and by 70°C, 70% of the activity was lost. Similarly, if the two MGI activities were treated following stage 5 of purification, MGI-1 showed a 32% loss in activity after 30 min at 70°C, while no activity remained in either MGI-2A or 2B. Lower protein concentration probably accounted for the enhanced sensitivity of cell activities after partial purification. In the case of MGI-1 activity on normal cells, an increase in the percentage of macrophage colonies was observed with both increased temperature or time of exposure to elevated temperature. No apparent alteration in the ratio of macrophage to granulocyte colonies was observed during purification of MGI-1.

The effect of pH on the stability of MGI activities is shown in Fig. 8. MGI-1 was stable at pH 3—10.5 and only below 3 showed some loss in activity. On the other hand, MGI-2 was unstable below pH 5. Following step 5 of purification, all the activities remained stable at pH 5—10.5. However, MGI-2A and 2B activity were reduced to zero at pH 3, whereas MGI-1 activity was reduced at pH 3 by only 23%. Below pH 4, insoluble precipitates were formed in the partially purified fractions. Although precipitation occurred in conditioned medium below pH 6 and above pH 9, most of the protein redissolved when the pH was returned to 7.4.

Sensitivity to proteases

Table IV shows the effect of incubation with various proteases on MGI-1 and MGI-2 activities. MGI-2 activity was extremely sensitive to all endoproteases except the rather specific thrombin. Neither MGI-1 nor MGI-2 were sensitive to any of the exoproteases (carboxypeptidase A and B and leucine aminopeptidase) tested. At concentrations of proteases which destroyed MGI-2 activity, MGI-1 was not affected or had slightly increased activity. MGI-2 from other sources has also been shown to be sensitive to trypsin [46,47]. When the molecular weight of MGI-1 from trypsin-treated (100 μg/ml) conditioned medium

TABLE IV

EFFECT OF PROTEASES ON MGI-1 AND MGI-2 ACTIVITY

Protease (37°C)	Percent of control activity	
	MGI-1	MGI-2 (A and B)
Untreated	100	100
Trypsin		
(100 $\mu g \cdot ml^{-1}$) 2 h	144	7
(1 $mg \cdot ml^{-1}$) 3 h	70	0
(1 $mg \cdot ml^{-1}$) 6 h	10	—
(10 $mg \cdot ml^{-1}$) 3 h	0	—
Chymotrypsin		
(100 $\mu g \cdot ml^{-1}$) 2 h	121	0
(0.5 $mg \cdot ml^{-1}$) 6 h	0	—
(3 $mg \cdot ml^{-1}$) 3 h	0	—
Pronase		
(20 $\mu g \cdot ml^{-1}$) 2 h	118	0
(0.5 $mg \cdot ml^{-1}$) 6 h	0	—
Subtilisin		
(20 $\mu g \cdot ml^{-1}$) 2 h	142	0
(2 $mg \cdot ml^{-1}$) 3 h	0	—
Papain (25 $\mu g \cdot ml^{-1}$) 2 h	113	5
Thrombin (1 $U \cdot ml^{-1}$) 2 h	99	97
Carboxypeptidase A (20 $\mu g \cdot ml^{-1}$) 2 h	108	98
Carboxypeptidase B (10 $\mu g \cdot ml^{-1}$) 2 h	106	108
Leucine aminopeptidase (100 $\mu g \cdot ml^{-1}$) 2 h	91	104

was determined by gel filtration, an increase of about 7000 in apparent molecular weight was observed. In addition, the fractionated MGI-1 was now labile and 50% of the activity was lost. Thus trypsin at this concentration may cleave MGI-1 somewhat opening up the structure. However, the possibly cleaved components remain associated unless fractionated and no loss of activity was observed. In addition, trypsinization increased the frequency of macrophage colonies induced by MGI-1 to almost 100%, indicating a lability in the granulocyte inducing ability of the molecule as was observed with temperature sensitivity. We have also noticed the greater lability of MGI-2 over MGI-1 on long term storage of conditioned media. This may result from the greater sensitivity of MGI-2 to proteases in the conditioned medium.

Effect of protease inhibitors

The addition of different protease inhibitors to the Krebs ascites cells during the production of conditioned medium has shown (Table V) that some of these inhibitors, including soybean-trypsin inhibitor, increased the yield of MGI-2 and did not affect the yield of MGI-1, presumably by preventing protease inactivation of MGI-2 at the time or after secretion. Experiments on the possible effect of the addition of different protease inhibitors to the assays for MGI-1 and MGI-2 activity showed no effect on either of these assays. Thus trasylol (5—500 $U \cdot ml^{-1}$), phenylmethylsulfonyl fluoride (10^{-5}—10^{-3} M),

TABLE V

EFFECT OF ADDING PROTEASE INHIBITORS ON THE LEVELS OF MGI-1 AND MGI-2 SECRETED BY KREBS ASCITES CELLS

Inhibitor	Concentration	Percent of control activity	
		MGI-1	MGI-2
Soybean trypsin inhibitor	0.01 mg ml^{-1}	106	165
	0.1 mg ml^{-1}	112	294
	1 mg ml^{-1}	102	350
Phenylmethylsulfonyl fluoride	0.1 mM	124	210
	1 mM	106	245
	5 mM	98	205
ϵ-Aminocaproic acid	1 mM	100	162
	10 mM	114	98
Benzamidine	100 μM	88	107
	1 mM	95	93
4-Nitrophenyl-4-guanido-benzoate	1 μM	104	110
	10 μM	110	105
Leupeptin	10 μM	99	104
	100 μM	104	98
Antipain	100 μM	89	106
	1 mM	103	112

diisopropylfluorophosphate (10^{-3}—10^{-2} M), soybean trypsin inhibitor (0.01—1 mg ml^{-1}), *N*-α-tosyl-L-lysine chloromethylketone ($5 \cdot 10^{-6}$—$5 \cdot 10^{-4}$ M), *p*-tosyl-L-arginine methyl ester ($5 \cdot 10^{-4}$—$5 \cdot 10^{-2}$ M), ϵ-aminocaproic acid (10^{-4}—10^{-2} M), 2-nitrocarboxyphenyl-*N*,*N*-diphenyl carbamate (0.1—10 $\mu g \cdot ml^{-1}$), *N*-1-tosylamide-2-phenylethylchloromethyl ketone (10^{-7}—10^{-5} M), *N*-benzoyl-L-tyrosine ethyl ester ($2 \cdot 10^{-6}$—$2 \cdot 10^{-4}$ M), benzamidine (10^{-5}—10^{-2} M), leupeptin (10^{-6}—10^{-4} M), *p*-nitrophenyl-*p'*-guanidobenzate (10^{-7}—10^{-3} M), antipain (10^{-4}—10^{-3} M), hirudin (0.5—2 $U \cdot ml^{-1}$), or pepstatin (10^{-5}—$5 \cdot 10^{-4}$ M), showed no effect on the assay for MGI-1 and MGI-2. Post Sephadex G-200 MGI-1 and MGI-2 also showed no plasminogen activator activity. Proteases such as trypsin, thrombin, fibrinolysin, urokinase and streptokinase, also did not induce differentiation in the MGI^+D^+ myeloid leukemic cells.

Discussion

The medium conditioned by Krebs ascites tumor cells is a good source of large amounts of the two MGI activities. 20 mice yielded sufficient cells (1—$1.2 \cdot 10^{10}$) to give 2—2.5 l of conditioned medium after 20 h in culture in medium without serum. Purification of MGI from this conditioned medium resulted in the separation of two factors with MGI-2 activity and one with MGI-1 activity. Both MGI-2 factors showed similar sensitivity to temperature, pH and proteolysis, but they differed in their charge properties as shown by DEAE-cellulose chromatography and polyacrylamide gel electrophoresis, MGI-1 copurified with MGI-2A for five steps of purification, partially separated on polyacrylamide gel electrophoresis and showed enhanced stability to temperature, pH

and proteolysis. Gel filtration indicated an apparent M_r of 43 000 for both MGI-1 and MGI-2. When bands with biological activity cut from non-denaturing polyacrylamide gels were run on denaturing SDS-polyacrylamide gels, MGI-1 ran as a dimer of 23 000 and 25 000 with sometimes a single contaminating band at 50 000 and MGI-2A had the same two bands, with sometimes a single low M_r contaminant of 12 000. An M_r of 23 000 has also been reported for MGI-1 from some other sources [26,48]. The differences between MGI-1 and MGI-2A from the Krebs ascites cells with different biological properties may involve cleavage resulting in alteration in a small peptide. On the other hand, MGI-2B ran as a single band of 45 000 and MGI-2B may be convertible to MGI-2A. Studies on the induction of MGI in myeloid leukemic cells have shown [30] that MGI-1 is produced before MGI-2 and it has been suggested that MGI-1 may be derived from a precursor that contains MGI-2 [21].

Proteins from some sources possessing MGI-1 activity contain carbohydrate [24—26,48], while both activities of MGI from fibroblasts [8,15,23] and the MGI-1 and MGI-2 from Krebs ascites cells showed no detectable carbohydrate. The secretion of MGI-1 and MGI-2 was also not inhibited by inhibitors of glycosylation. Although the presence of carbohydrate, if present, may be valuable in aiding purification of MGI [24—26,48], our results indicate that both MGI-1 and MGI-2 are capable of functioning in the absence of carbohydrate. Neither MGI-1 or MGI-2 showed plasminogen activator activity, or were affected by protease inhibitors added to the assay for these activities. The higher sensitivity of MGI-2 than of MGI-1 to treatment with proteases suggests that the level of cellular proteases may play a role in regulating the relative amounts of MGI-1 and MGI-2 that are present in vivo.

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