The EMBO Journal vol.3 no.13 pp.3191 – 3197, 1984

Purification and characterization of cMGF, a novel chicken myelomonocytic growth factor

Achim Leutz, Hartmut Beug and Thomas Graf

European Molecular Biology Laboratory, Meyerhofstrasse 1, D-6900 Heidelberg, FRG

Communicated by L.Philipson

We describe the purification of a novel hematopoietic growth factor from conditioned medium of a transformed macrophage cell line. The factor, termed chicken myelomonocytic growth factor (cMGF) stimulates the growth of chicken myeloblasts transformed by *myb* oncogene-containing retroviruses and induces the formation of macrophage colonies in uninfected chick bone marrow cultures. The biological activity of the factor is destroyed by trypsin and by reducing reagents but not by SDS. Analysis of crude conditioned medium on non-reducing SDS gels reveals two active species of cMGF with mol. wts. of 23 and 27 kd. Incubation of radioiodinated partially purified cMGF with myeloblasts demonstrates the specific binding of 23- and 27-kd components under non-reducing, and 25- and 29-kd components under reducing conditions. Glycosylation inhibition experiments indicate that the larger molecules represent glycosylated forms of a single protein moiety. The 27-kd species has been purified to homogeneity (80 000-fold enrichment) and exerts its half maximal activity at 2 x 10^{-12} M and its maximal activity at 3 x 10^{-11} M. Antibodies prepared to purified cMGF completely neutralize the growth-stimulating activity of the factor.

Key words: avian hematopoietic cells/growth factor/oncogenes

Introduction

Hematopoietic precursor cells require specific growth factors which support their survival, growth and differentiation (Burgess *et al.*, 1980; Iscove *et al.*, 1982; Metcalf, 1981). A number of mammalian hematopoietic growth factors have been identified either by seeding bone marrow cells into semi-solid medium containing the factor to be tested (Burgess *et al.*, 1977; Stanley and Heard, 1977; Nicola *et al.*, 1983) or by using factor-dependent cell lines (Bazill *et al.*, 1983). Some of these factors are lineage specific while others are not (for review, see Dexter, 1984). Hematopoietic growth factors can be used to investigate the regulation of growth and differentiation, to identify possibly lineage-specific receptors and to delineate relationships to cellular oncogenes.

A particularly well-defined system to study the role of oncogenes and growth factors in hematopoietic differentiation are acute avian leukemia viruses that can be used to transform bone marrow cells by specific oncogenes *in vitro*. Depending on the viral oncogene introduced by the virus, transformed diploid cell strains resembling erythroid, myeloid or lymphoid progenitors can be obtained (for review, see Graf and Beug, 1978; Beug *et al.*, 1982b). While transformed erythroid or lymphoid cells grow in standard media containing bovine serum, myelomonocytic cells transformed by *myb*-oncogene containing E26 virus and avian myeloblastosis virus (AMV) are dependent on the addition to such media of a growth factor activity present in conditioned medium from chick-spleen cells stimulated with Concanavalin A (Con A-SCM, Beug *et al.*, 1982a).

The E26 virus contains the *ets* oncogene in addition to *myb* (Leprince *et al.*, 1983; Nunn *et al.*, 1983) and causes a mixed erythroleukemia-myeloblastosis, in which the erythroid component predominates (Sotirov, 1981; Moscovici *et al.*, 1981; Radke *et al.*, 1982). It also transforms erythroid or myeloid cells *in vitro*, depending on the assay conditions used (Graf *et al.*, 1979; Radke *et al.*, 1982; Moscovici *et al.*, 1983). AMV on the other hand contains the *myb* oncogene only (for review, see Graf and Stehelin, 1982) and does not affect the erythroid lineage (Radke *et al.*, 1982), suggesting that the *myb* gene is sufficient for myeloblast transformation.

In the absence of a functional *myb* oncogene product AMV- and E26-transformed myeloblasts apparently behave like normal macrophage progenitors, since cells infected with temperature-sensitive mutants of these viruses can be induced to differentiate into macrophages when shifted from permissive to non-permissive temperature (Moscovici *et al.*, 1983; Beug *et al.*, in preparation). The differentiated cells lose their growth factor responsiveness and synthesize their own growth factor (Beug *et al.*, in preparation). This observation together with the findings that the expression of the putative transforming protein of E26, p135 gag-myb-ets, is induced in factor-treated myeloblasts (Beug *et al.*, 1982a) and that E26 myeloblasts are relieved of their factor dependence when superinfected with retroviruses carrying oncogenes of the *src* family (Adkins *et al.*, 1984) prompted us to isolate and characterize the factor referred to as chicken myelomonocytic growth factor (cMGF). Here we describe the purification of cMGF, its biochemical and preliminary biological characterization and the production of antibodies that neutralize its growth-promoting activity.

Results

Production of cMGF in serum-free cultures

ConA-SCM was unsuitable as a source for purification of cMGF because of its high serum protein content. We therefore screened a number of avian cell lines for their ability to produce cMGF under serum-free conditions in the presence or absence of bacterial lipopolysaccharide (LPS). Factor activities comparable with those present in ConA-SCM were found in LPS-stimulated cultures of HD11 cells, an established line of chicken macrophages transformed by the *myc*-containing MC29 virus (Beug *et al.*, 1979). To determine the optimal conditions for cMGF production, supernatants of HD11 cells were harvested at different times after seeding the cells in serum-free medium, with or without LPS. As shown in Figure 1, LPS-stimulated cultures produced detectable levels of cMGF within 3 h and a maximum of factor activity at 20 – 24 h. The observation that only insignificant amounts of intracellular cMGF activity could be found in untreated

HD11 cells indicates that LPS induces a rapid *de novo* synthesis of cMGF rather than a release of stored factor (data not shown).

Purification of cMGF

To obtain sufficient material for purification purposes, HD11 cells were grown in large-scale cultures until they reached the desired cell density, and then transferred to LPS-containing, serum-free medium. Conditioned medium was collected from the cultures 24 h later. The purification scheme developed (for details, see Materials and methods) consisted of the following steps: (i) concentration of conditioned medium by ultrafiltration; (ii) ion-exchange chromatography; (iii) hydrophobic interaction chromatography; (iv) size exclusion chromatography; (v) hydroxylapatite chromatography; and (vi) reverse phase h.p.l.c.

Table I and Figure 2 show the results of a typical purification. Factor was eluted stepwise from both the ion-exchange and the hydrophobic interaction columns since this procedure yielded a single peak of biological activity while elution using a gradient led to a distribution of activity, into 2–3 peaks. Size exclusion chromatography resulted in a symmetrical peak of biological activity in the mol. wt. region of 20–30 kd; in addition, a small peak was detected in the 45–55 kd region. Chromatography of the 20–30 kd peak on hydroxylapatite using a potassium phosphate gradient yielded a minor peak at 15–20 mM and a major peak (containing >80% of the biological activity) at 35–55 mM phosphate. This latter material, termed HT-Peak II cMGF, was further purified by h.p.l.c.-reverse phase chromatography, giving rise to a single peak of biological activity which eluted at high acetonitrile concentrations (Figure 3).

Analysis of h.p.l.c. fractions by SDS-PAGE and silver staining indicated that fractions with biological activity contained a major protein band at mol. wt. ~29 000 and minor contaminant proteins corresponding to those eluted at lower acetonitrile concentrations. These contaminating proteins were removed by subjecting the biologically active fractions to a second round of chromatography on the same h.p.l.c. column, yielding a single protein peak containing all of the biological activity (insert in Figure 3). All fractions corresponding to this peak migrated as a single band of ~29 kd in silver-stained polyacrylamide gels under reducing conditions (Figure 4).

Sensitivity of cMGF to trypsin, SDS and reducing agents

We tested the sensitivity of partially purified cMGF towards

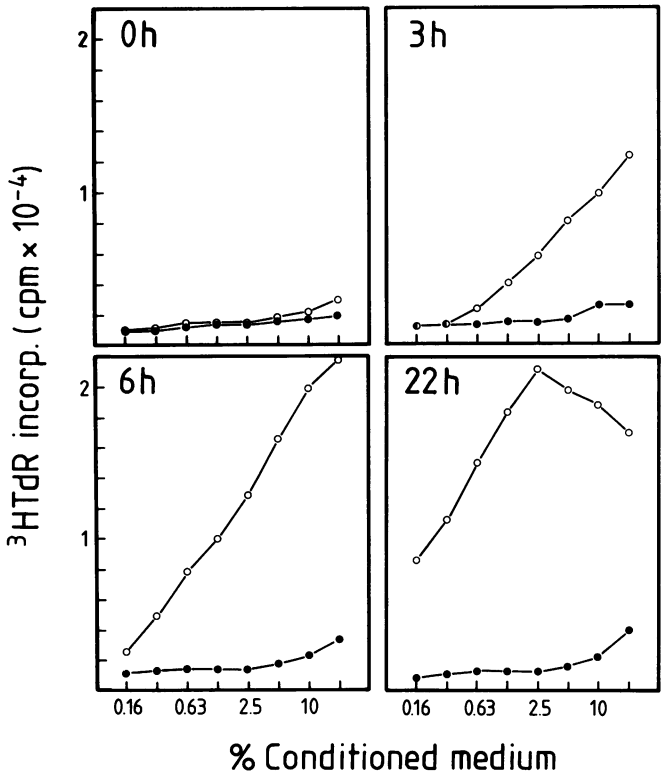

Fig. 1. Kinetics of cMGF production in LPS-stimulated and unstimulated HD11 cell cultures. Conditioned medium from HD11 cells (2 x 10^6 cells/ml) was harvested after the times indicated, centrifuged to remove cells and debris and then titrated on E26-transformed myeloblasts in the cMGF assay. (○), HD11 conditioned medium with LPS (5 μg/ml); (●), HD11 conditioned medium without LPS.

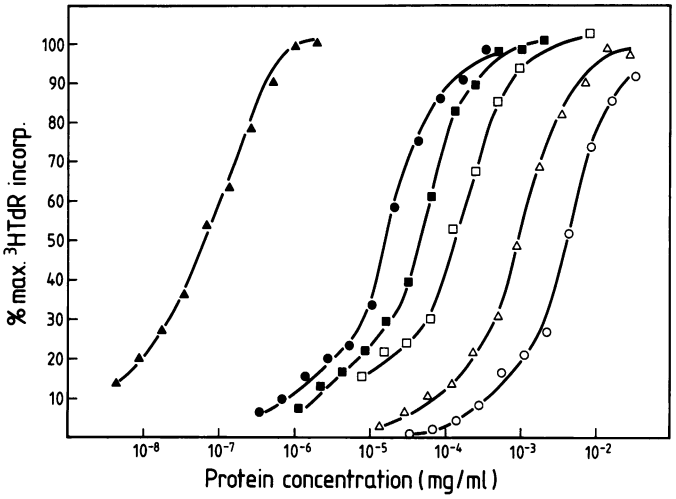

Fig. 2. Biological activity of cMGF at various levels of purification. Various cMGF containing fractions were tested in 2-fold serial dilutions on E26-transformed myeloblasts using the cMGF assay. (○), concentrated conditioned medium; (△), DEAE ion-exchange chromatography; (□), phenyl-Sepharose chromatography; (■), size exclusion chromatography; (●), hydroxylapatite chromatography; (▲), reverse phase h.p.l.c. chromatography step.

Table I. Summary of cMGF purification

Step	Protein (mg)	Activity (units)	Specific activity (units/mg x 10^{-3})	ED_{50} (ng/ml)	Purification -fold	Overall %
1. Conditioned medium	2200	4.9 x 10^5	0.22	4500	1	100
2. Ion-exchange chromatography	300	4.3 x 10^5	1.43	700	6.4	88
3. Hydrophobic interaction chromatography	135	4.8 x 10^5	3.56	280	16	98
4. Size exclusion chromatography	20	4.0 x 10^5	20	50	90	82
5. Hydroxylapatite chromatography	2.25	2.2 x 10^5	97	10	450	45
6. Reverse phase h.p.l.c.	0.0075	1.4 x 10^5	18 200	0.055	81 800	28

trypsin, SDS and reducing agents. Trypsin completely destroyed the biological activity, while no significant reduction was observed in untreated or trypsin inhibitor-treated controls. Concentrations of SDS between 0.1% and 5% had no effect on the biological activity of cMGF, while 0.1 M mercaptoethanol or dithiothreitol completely destroyed it (see Materials and methods; and data not shown).

To determine whether cMGF contains intra- or intermolecular disulfide bridges, h.p.l.c.-purified cMGF was run on polyacrylamide gels under reducing and non-reducing conditions and then silver stained. In a parallel lane cMGF dissolved in SDS-PAGE sample buffer lacking reducing agents was electrophoresed and then fractionated using a gel slicer. The biological activity eluted from the gel fractions aligned precisely with the silver-stained band (at 27 kd) in the lane run under non-reducing conditions. Under reducing conditions the protein band became sharper and migrated at an apparent mol. wt. of 29 kd (Figure 4). These results suggest that cMGF is a single chain polypeptide with intramolecular disulfide bridges.

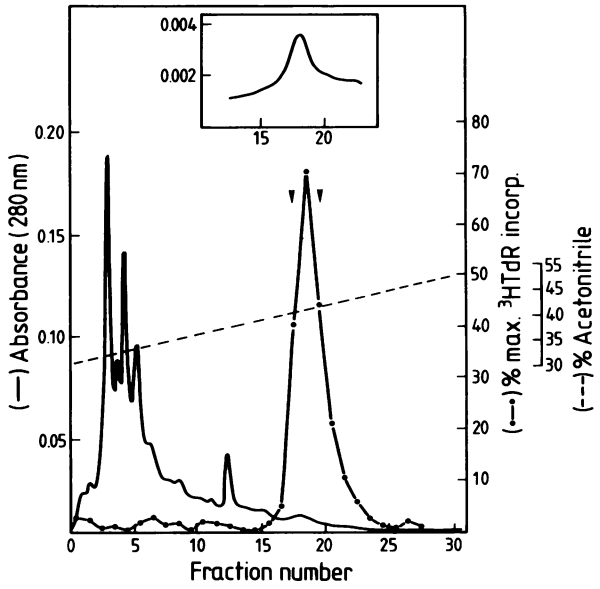

Fig. 3. Reverse phase h.p.l.c. chromatography. Fractions between arrowheads were pooled, freeze-dried and re-chromatographed under identical conditions on the same column. Insert shows the elution profile of the re-chromatography.

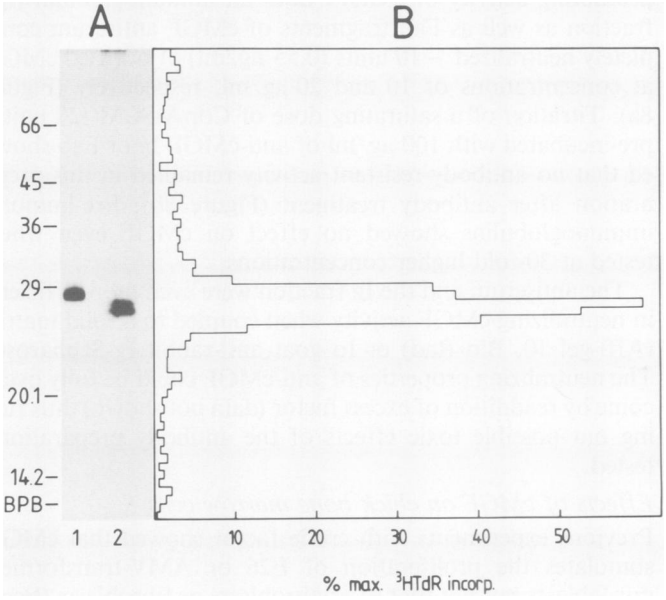

Fig. 4. SDS-PAGE of purified cMGF. Fractions 17, 18, 19 and 20 of the h.p.l.c. re-chromatography were pooled. Aliquots were then freeze-dried and subjected to SDS-PAGE. **(A)** Silver staining of ~100 ng cMGF separated under reducing **(lane 1)** and non-reducing **(lane 2)** conditions. **(B)** Biological activity eluted from gel slices of a parallel lane in the same gel, containing non-reduced, h.p.l.c.-purified cMGF. Mol. wts. are indicated in kd, BPB = bromphenol blue.

Binding of purified cMGF to transformed myeloblasts

Purified cMGF was iodinated using a chloramine-T procedure and then incubated with cMGF-depleted E26-transformed myeloblasts. These cells bound a 29 kd protein when electrophoresed under reducing conditions (Figure 5). Binding of this protein could be completely inhibited by pre-incubation with unlabelled crude, partially purified or purified cMGF as well as with ConA-SCM. In contrast, an erythroblast cell line transformed by avian erythroblastosis virus showed no specific binding of [^{125}I]cMGF (data not shown).

Glycosylated forms of cMGF

Purified cMGF corresponds to one major activity peak present in crude HD11 conditioned medium on non-reducing SDS-PAGE. We investigated whether HD11 cells produce several molecular forms of cMGF, or whether they produce different growth factors with similar biological activity. When supernatants from LPS-stimulated HD11 cells were analysed on SDS gels as above, major peaks of activity were seen in the 23 kd and the 25–30 kd regions. An additional broad peak of 35–50 kd was observed containing < 1/20 of

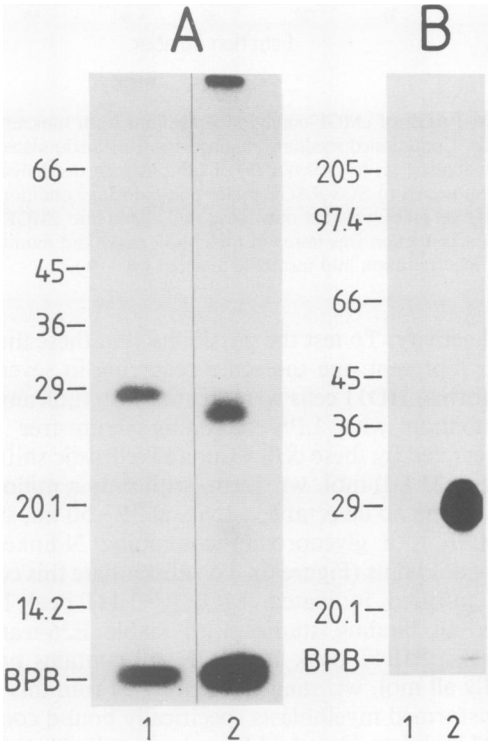

Fig. 5. SDS-PAGE of [^{125}I]cMGF and binding of [^{125}I]cMGF to E26 myeloblasts. **(A)** [^{125}I]cMGF analysed by SDS-PAGE and autoradiography under reducing **(lane 1)** and non-reducing conditions **(lane 2)**. **(B)** [^{125}I]cMGF bound to cMGF-depleted, E26-transformed myeloblasts in the presence **(lane 1)** or absence **(lane 2)** of cold cMGF. Mol. wts. are indicated in kd, BPB = bromphenol blue.

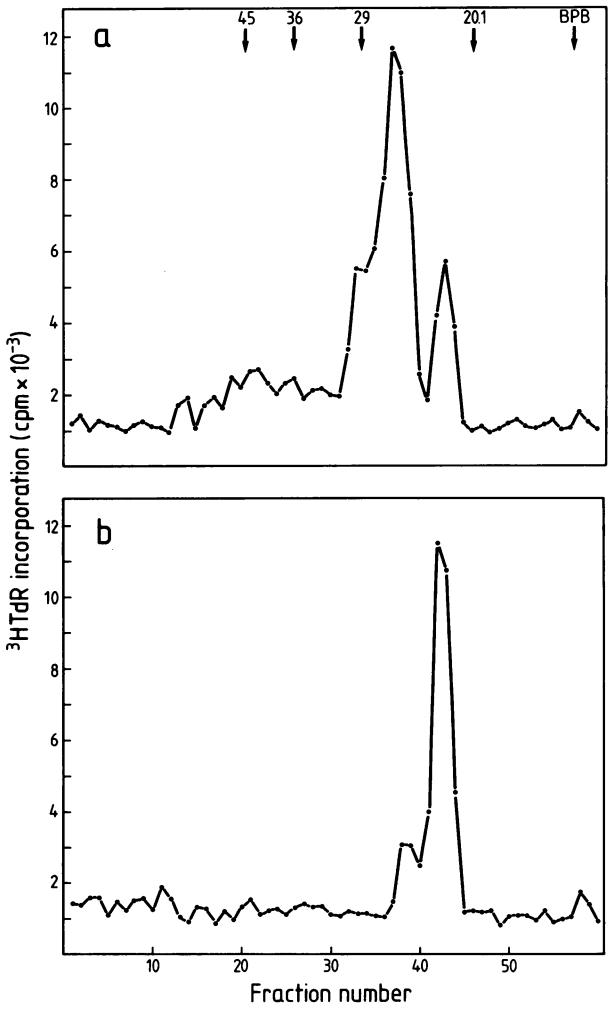

Fig. 6. SDS-PAGE of cMGF-conditioned medium from tunicamycin-treated cells. Conditioned medium obtained from LPS-stimulated HD11 cells in the absence **(a)** or presence **(b)** of tunicamycin was dialysed, freeze-dried and subjected to SDS-PAGE under non-reducing conditions. Appropriate gel lanes were cut out, sliced and eluted into cMGF assay medium. Each fraction was tested in the cMGF assay and monitored for thymidine incorporation into insoluble material (● – ●).

the total activity. To test the possibility that these three factor activities represent one molecule occurring in several glycosylated forms, HD11 cells were treated with tunicamycin during incubation with LPS-containing serum-free medium. cMGF secreted by these cells exhibited a drastic shift towards the lowest (23 kd) mol. wt. form, with only a minor peak at 25 – 27 kd and no detectable activity at 35 – 50 kd, suggesting that cMGF is a glycoprotein containing N-linked carbohydrate side chains (Figure 6). To substantiate this conclusion partially purified, iodinated cMGF ([^{125}I]HT peak II cMGF) was used in binding studies with viable E26-transformed myoblasts. [^{125}I]HT peak II cMGF still contains proteins in essentially all mol. wt. ranges (Figure 7). From this material, E26-transformed myeloblasts specifically bound components of 25 and 29 kd or 23 and 27 kd, when analysed under reducing or non-reducing conditions, respectively. Moreover, the biological activity of cold HT peak II cMGF electrophoresed under non-reducing conditions and eluted from a parallel lane aligned precisely with the positions of the labelled 23-and 27-kd bands.

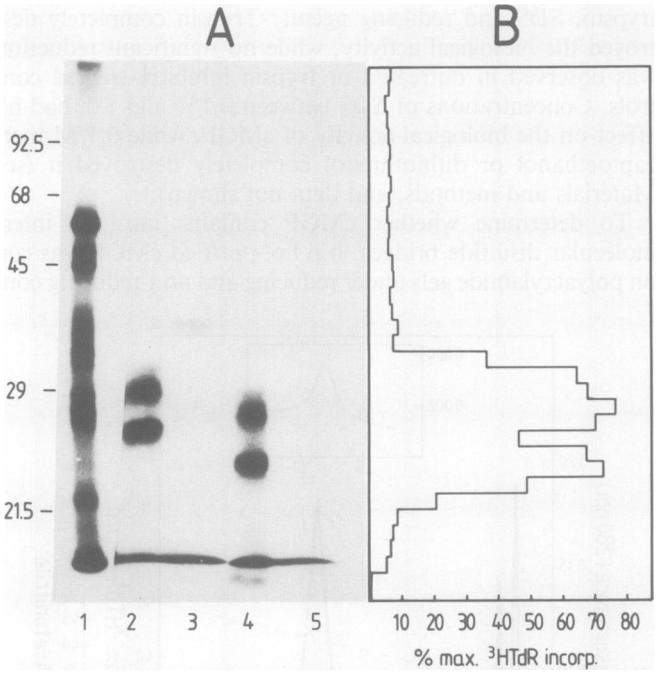

Fig. 7. Specific binding of different molecular forms of cMGF to E26 myeloblasts. **(A)** Iodinated HT peak II material **(lane 1)** was pre-incubated with SDS (see Materials and methods) and then bound to cMGF-depleted viable E26-transformed myeloblasts in the presence **(lanes 3** and **5)** or absence **(lanes 2** and **4)** of cold HT peak II cMGF. **Lanes 1, 4** and **5,** non-reducing conditions; **lanes 2** and **3,** reducing conditions. **(B)** Biological activity eluted from gel slices of a parallel lane in the same polyacrylamide gel receiving non-reduced, cold HT peak II material.

Antibodies to cMGF

The availability of pure cMGF in substantial quantities raised the possibility of producing antibodies against it. We succeeded in obtaining rabbit antisera that neutralized the growth-promoting activity of cMGF. Both an immunoglobulin (Ig) fraction as well as Fab fragments of cMGF antiserum completely neutralized ~10 units (0.55 ng/ml) of purified cMGF at concentrations of 10 and 20 μg/ml, respectively (Figure 8a). Titration of a saturating dose of ConA-SCM (20 units) pre-incubated with 100 μg/ml of anti-cMGF Ig or Fab showed that no antibody-resistant activity remained in this preparation after antibody treatment (Figure 8b). Pre-immune immunoglobulins showed no effect on cMGF even when tested at 30-fold higher concentrations.

The antiserum and the Ig fraction were even more efficient in neutralizing cMGF activity when coupled to a solid matrix (Affi-gel 10, Bio Rad) or to goat anti-rabbit Ig-Sepharose. The neutralizing properties of anti-cMGF could be fully overcome by readdition of excess factor (data not shown) thus ruling out possible toxic effects of the antibody preparations tested.

Effects of cMGF on chick bone marrow cells

Previous experiments with crude factor showed that cMGF stimulates the proliferation of E26 or AMV-transformed myeloblasts but not that of erythroblasts or fibroblasts (Beug *et al.*, 1982a). Similar results were also obtained with purified cMGF (data not shown). To determine whether or not the factor also stimulates the formation of hematopoietic colonies, chick bone marrow cells were seeded in semi-solid medium containing various concentrations of HT peak II cMGF. The cultures were incubated at 39°C and then

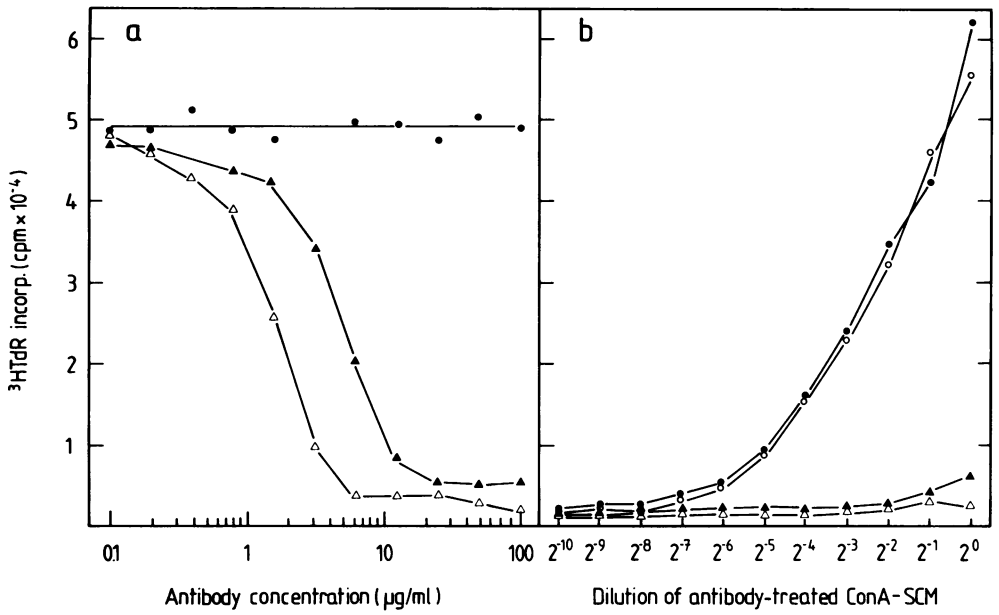

Fig. 8. Neutralisation of cMGF with specific antibodies. **(A)** Increasing amounts of immunoglobulin or PBS were pre-incubated with cMGF (10 units/ml; 0.55 ng/ml) and each sample tested in the cMGF assay. **(B)** Immunoglobulins (100 µg/ml) were pre-incubated with 15 units of ConA-SCM and then various dilutions tested in the cMGF assay. cMGF-containing samples were reacted with (△), anti-cMGF Ig; (▲), anti-cMGF-Fab; (●), pre-immune Ig or (○), PBS.

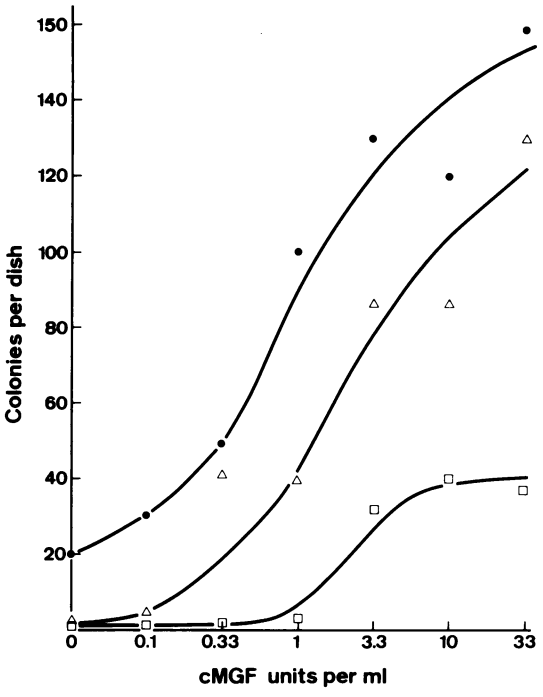

Fig. 9. Effect of partially purified cMGF on normal bone marrow cells. Bone marrow cells were prepared from a 1 week old chick, washed twice in growth medium and seeded in 35 mm dishes at various concentrations (● – ●, 1 x 10^5; △ – △, 5 x 10^4; □ – □, 2.5 x 10^4) in methylcellulose-containing medium supplemented with different concentrations of HT peak II cMGF. Colonies were scored 5 days after incubation at 37°C.

monitored for the numbers and types of colonies which grew after 5 days. As can be seen from Figure 9, cMGF stimulated the formation of colonies whose number was roughly proportional to the concentration of factor used. Examination of the cell types present in the colonies revealed that they consisted largely of macrophage-like cells. A minor proportion of granulocytes was also detected. When HT peak II cMGF was tested for its ability to replace erythropoietin under conditions where erythrocyte colonies can be obtained from virus-infected erythroblasts (Beug *et al.*, 1982c), the outcome was negative (data not shown).

Discussion

The growth factor purified from HD11 macrophages can completely replace ConA-SCM in its survival- and proliferation-promoting effects on E26-transformed myeloblasts and in its colony-stimulating effect on normal bone marrow cells. The finding that antibodies to cMGF from HD11 cells neutralize the growth factor activity present in ConA-SCM suggest that both activities are identical. The change in migration behaviour on SDS gels of purified cMGF run under reducing or non-reducing conditions indicates that cMGF consists of a single chain molecule that has a compact structure caused by intrachain disulfide bonds. This conformation is essential for its biological function since its activity is destroyed by reducing agents.

The finding of multiple species of biologically active material in crude conditioned medium can be explained by the presence of a single polypeptide glycosylated to different extents. The glycosylation inhibition experiments indicate that the protein moiety of cMGF has a mol. wt. of ~25 kd. We have, however, not ruled out the possibility of O-glycosidic linked residues, in which case the estimated mol. wt. of the polypeptide backbone might still be too high. The binding data obtained with partially purified cMGF substantiate the results obtained in the glycosylation inhibition experiments. That h.p.l.c.-purified cMGF is indistinguishable in its biological activity from HT peak II or crude cMGF suggests that the different cMGF forms do not mediate different signals.

The fact that our preparations of purified [^{125}I]cMGF contained large amounts of radioactivity present in low mol. wt. material (presumably representing iodinated polyethylene-

glycol) precluded an accurate determination of the specific binding of [^{125}I]cMGF to myeloblasts. Receptor numbers and their affinity could therefore not be determined. However, both partially and completely purified, iodinated cMGF preparations exhibited a competable and saturable binding to myeloblasts but not to erythroblasts (data not shown). This finding strongly indicates the existence of a specific high affinity receptor for cMGF present on the cell surface of myelomonocytic cells.

Our data suggest that cMGF is a hematopoietic growth factor since it was found to stimulate the outgrowth of macrophage-like colonies and possibly of granulocytes from normal bone marrow. No mixed erythroid/myeloid colonies have yet been found, indicating that the factor is not homologous to the mouse IL3 (BPA, multi CSF or HCGF) activity (Ihle *et al.*, 1983; Iscove *et al.*, 1982; Bazill *et al.*, 1983). A homology of cMGF with chicken IL2 (Schentzler *et al.*, 1983) is excluded because cMGF did not stimulate IL2-sensitive chick T-lymphoblasts (M.Schnetzler and R.Franklin, personal communication). Based on its mol. wt., cMGF appears also to be different from a macrophage colony stimulating activity present in the serum of endotoxin-stimulated chickens (Dodge *et al.*, 1980). The availability of purified cMGF and of neutralizing antiserum will help in revealing its relationship to other chicken hematopoietic growth factors. Molecular cloning and/or protein sequencing will be necessary to determine whether cMGF is related to any of the myeloid growth factors of mice and men (Burgess *et al.*, 1977; Stanley and Heard, 1977; Nicola *et al.*, 1983).

Materials and methods

Cells

Clones of myeloblasts transformed by AMV or E26 as well as macrophages transformed by MC29 virus were obtained as described by Beug *et al.* (1979) and propagated in standard growth medium; Dulbecco's minimal essential medium (DMEM) supplemented with 8% fetal calf serum (FCS), 2% chicken serum and 1% of a pre-tested ConA-SCM batch as a source of growth factor (Beug *et al.*, 1982a). AEV- or E26-transformed erythroblasts were obtained as described previously (Beug *et al.*, 1979; Radke *et al.*, 1982). For colony formation of normal cells, bone marrow cells were seeded in methylcellulose-containing medium (Graf *et al.*, 1981). Colonies were characterized by diluting the methocel with liquid medium, and staining cytospin preparations of the cells with a May Grünwald-Giemsa stain. The MC29 virus-transformed macrophage cell line HD11 (described earlier as LSCC-MC/MA1; Beug *et al.*, 1979) was cultured in standard growth medium without growth factor.

Assay for biological activity of cMGF

Test cells were washed twice in assay medium (DMEM plus 1.6% FCS and 0.4% chicken serum) and seeded in duplicate or triplicate samples in 96 well microtiter plates (NUNC) at 2 x 10^4 cells per 50 μl/well. After incubation for 2 days at 37°C, the cells were pulse-labelled for 2 h with 5 μl of a solution of [^{3}H]thymidine ([^{3}H]TdR, Amersham, ~30 Ci/mmol; 0.2 μCi/well) prepared in the same medium. The labelled cells were then collected onto glass fiber filters using a cell harvester (SKATRON, Norway) and insoluble radioactivity was measured by liquid scintillation counting. The variability between duplicate samples was found to be < 10%. One unit of cMGF activity was defined as the amount of growth factor activity/ml which gave half maximal [^{3}H]TdR incorporation in E26-transformed test cells.

Preparation of cMGF-containing conditioned medium

HD11 cells were expanded in roller bottles at 39°C. Then, 5–8 x 10^9 cells were washed four times in serum-free 'hybridoma medium' (Murakami *et al.*, 1982) and seeded in this medium at 2 x 10^6 cells/ml in 6000 cm^2 multitray units (NUNC). LPS from *Salmonella typhymurium* (Sigma) was added to a final concentration of 5 μg/ml. After 24 h the medium was harvested and centrifuged at 2000 *g* for 10 min and then at 70 000 *g* for 2 h. The supernatant was concentrated 50-fold by ultrafiltration (Amicon, PM-10 membrane) and dialysed at 4–8°C against 50 mM Tris-HCl pH 8 containing 50 mM NaCl (designated as 'Tris buffer'). A small protein precipitate which formed during concentration was removed by centrifugation.

Ion-exchange chromatography

Dialysed and concentrated conditioned medium was applied to a DEAE-cellulose column (Whatman, DE-52; 2.5 x 32 cm, flow-rate 50 ml/h), equilibrated in Tris buffer containing 100 mM NaCl. The column was washed with the same buffer and eluted with 600 mM NaCl in Tris buffer. The eluate was then concentrated to 30 ml and dialysed against Tris buffer containing 1 M $(NH_4)_2SO_4$. A small precipitate was removed by centrifugation.

Hydrophobic interaction chromatography

The material from ion-exchange chromatography was loaded on a phenyl-Sepharose 4B column (Pharmacia, 1.6 x 30 cm, flow-rate 25 ml/h) equilibrated with Tris buffer plus 1 M $(NH_4)_2SO_4$. After washing, the column was eluted with Tris buffer containing 50% ethyleneglycol (Merck). The eluate was concentrated to 10 ml and dialysed against Tris buffer containing 350 mM NaCl and 0.01% polyethyleneglycol 6000 (Serva).

Size exclusion chromatography

The material from phenyl-Sepharose was dialysed, concentrated to 2 ml, centrifuged to remove any precipitate and loaded on a gel filtration column (Bio Rad, Bio-gel, A-0.5m; 2.5 x 90 cm, flow-rate 25 ml/h), equilibrated in Tris buffer containing 300 mM NaCl. Fractions of 10 ml were collected and tested for biological activity. Active fractions were pooled and concentrated to 10 ml. Marker proteins (Serva, Dalton Standards MS-11) were run on the gel filtration column under the same conditions.

Hydroxylapatite chromatography

A hydroxylapatite column (Bio Rad, Bio-Gel HT, 1.6 x 20 cm, flow-rate 20 ml/h) was equilibrated with 5 mM potassium phosphate buffer pH 7. The concentrated material from size exclusion chromatography was dialysed against 5 mM potassium phosphate buffer pH 7.0 containing 0.01% polyethyleneglycol 6000 and applied to the column. The column was washed with 5 mM potassium phosphate buffer and protein was eluted with a linear gradient (gradient volume: 500 ml) of increasing phosphate concentration (high phosphate buffer: 100 mM potassium phosphate buffer pH 7.0). Fractions of 10 ml were collected and tested. Pooled activity fractions were concentrated to 10 ml, dialysed against 50 mM Tris-HCl pH 7.0, 100 mM NaCl and stored frozen in aliquots at −70°C.

Reverse phase chromatography

0.5–1.0 ml portions from hydroxylapatite chromatography were applied to an Ultrapore RPSC column (Altex, flow-rate 0.5 ml/min) connected to Beckman 112 solvent delivery module and a Beckman 116 absorbance detector with a 280 nm filter. The column was washed for 10 min with H_2O/ 0.1% trifluoracetic acid (TFA), developed with a 0–31.5% gradient of acetonitrile/0.1% TFA in 10 min and 31.5–49% in 30 min. 1 ml fractions were collected and aliquots of each fraction directly diluted into cMGF assay medium or freeze-dried for SDS polyacrylamide gel electrophoresis (PAGE).

Treatment of HT peak II material with protease, SDS and reducing reagents

20 μg of HT peak II with or without 1 μg of trasylol (Bayer, Leverkusen) was incubated with 0.1 μg trypsin, in a volume of 100 μl for 5 h at room temperature. The biological activity of the samples was then determined after appropriate dilution. In similar experiments various amounts of SDS (0.1–5%), 2-mercaptoethanol, 0.1 M final concentration or dithiothreitol (0.1 M) were added to aliquots of HT peak II cMGF. After incubation as above the samples were dialysed against phosphate-buffered saline (PBS) and the biological activity determined.

Tunicamycin treatment of HD11 cells

For glycosylation inhibition experiments, 5 μg/ml tunicamycin (Sigma) was added together with LPS (5 μg/ml) to HD11 cells grown in hybridoma medium. Supernatants were harvested after 8 h, centrifuged at 1000 *g* and then at 10 000 *g* to remove cells and debris. These supernatants (5 ml aliquots) were then dialysed against 50 mM ammonium carbonate buffer pH 8 and lyophilized. The dry residue was reconstituted in SDS-PAGE sample buffer lacking reducing reagents and subjected to SDS-gel electrophoresis without previous heating (Laemmli, 1970).

Iodination of cMGF

10 μg of partially purified cMGF and 1 μg of h.p.l.c.-purified cMGF were iodinated with 5 mCi or 2 mCi of carrier-free Na[^{125}I] (NEN), using a modified chloramine-T procedure in which dimethylsulfoxide and polyethyleneglycol 6000 are present in the reaction vessel (Stanley and Guilbert, 1981). Protein-bound radioactivity was separated by gel filtration on a G25 column (Pharmacia). Specific radioactivity was calculated to be ~3 x 10^5 c.p.m./ng protein for partially purified and h.p.l.c.-purified cMGF.

Binding of [^{125}I]cMGF to cultured cells

For binding experiments, cells were washed twice in DMEM plus 10% FCS and cultured in the absence of cMGF for 12–24 h. For binding experiments

cMGF-depleted cells were then washed twice, adjusted to the correct cell number (see below) and incubated at 37°C for 20 min. (For experiments with iodinated HT peak II cMGF, 1 x 10^7 cells/ml and 1 x 10^7 c.p.m. were used; with h.p.l.c.-purified [^{125}I]cMGF, 5 x 10^5 cells/ml and 1 x 10^6 c.p.m. were used.) To compete for [^{125}I]cMGF binding, a 50-fold excess of cold material was added to some samples. After the binding period cells were chilled, washed in DMEM at 4°C and centrifuged through a 5 ml cushion of ice cold FCS. Cells were then lysed in SDS-PAGE sample buffer with or without reducing reagents. For some experiments samples were incubated with 1% SDS for 1 h at 20°C, diluted ~20-fold with DMEM plus 10% FCS and dialysed overnight at 4°C against the same medium. Radioactivity was then adjusted to 1 x 10^6 c.p.m./ml and binding to cells performed as described above. Cell-bound radioactivity was analysed by SDS-PAGE and exposure of the dried gels to X-ray film (Kodak, XAR-5) at −70°C using an intensifying screen (Kodak, X-omatic regular).

Analysis of cMGF by SDS-PAGE

cMGF-containing samples were incubated with SDS-PAGE sample buffer for 1 h at room temperature, or 3 min at 95°C. Electrophoresis on 0.8 mm gels was carried out as described by Laemmli (1970). To reveal protein, gels were fixed for 2 h in 20% TCA , 50% methanol, and then silver stained according to the method of Ansorge (1983). For determination of cMGF activity, appropriate lanes were fractionated using a gel slicer and proteins were eluted from the slices by overnight incubation with 1 ml DMEM containing 2% FCS at 4−8°C. Aliquots of each fraction were tested for biological activity at dilutions ranging from 10^{-1} to 10^{-4}.

Protein assays

Protein was determined by the method of Peterson (1977) in all steps prior to the h.p.l.c. Protein concentration after h.p.l.c. purification was determined by integration of the peak area in the elution profile, assuming an average protein absorbance of 1.2 at 280 nm for a 1 mg/ml solution.

Antiserum against cMGF

A 12-month old outbred rabbit was immunized s.c. with 5 μg of h.p.l.c.-purified cMGF in PBS emulsified with 50% complete Freund's adjuvant. The rabbit was boostered twice with 2 μg cMGF in PBS plus 50% incomplete Freund's adjuvant and bled several times starting at 10 days after the second booster injection. An immunoglobulin fraction was obtained by adsorbing the antiserum to protein-A Sepharose (Pharmacia) and eluting with 4 M $MgCl_2$. The eluate was subsequently dialysed against PBS. Fab fragments were prepared from the immunoglobulin fraction according to the method of Porter (1959). Fab fragments were separated from undigested immunoglobulins and Fc fragments by extensive dialysis against water followed by chromatography on protein A-Sepharose.

Acknowledgements

The authors thank Drs M.Schnetzler and R.Franklin for chicken IL2-activity assays on cMGF, Dr R.Frank for help with h.p.l.c. separations, Drs N. Iscove, L.Guilbert and T.Burgess for discussions, Dr P.Kahn for suggestions and help with the manuscript, E.Kowenz and C.Walter for excellent technical assistance and B.Blanasch for typing.

References

Adkins,B., Leutz,A. and Graf,T. (1984) *Cell,* in press.
Ansorge,W. (1983) *Electrophoresis '82,* published by W.deGruyter & Co.
Bazill,C.W., Haynes,M., Garland,J. and Dexter,T.M. (1983) *Biochem. J.,* **210,** 747-759.
Beug,H., v.Kirchbach,A., Doederlein,G., Conscience,J.F. and Graf,T. (1979) *Cell,* **18,** 375-390.
Beug,H., Hayman,M.J. and Graf,T. (1982a) *EMBO J.,* **1,** 1069-1073.
Beug,H., Hayman,M.J. and Graf,T. (1982b) *Cancer Surv.,* **1,** 205-230.
Beug,H., Palmieri,S., Freudenstein,C., Zentgraf,H. and Graf,T. (1982c) *Cell,* **28,** 907-919.
Burgess,A.W., Camakaris,J. and Metcalf,D. (1977) *J. Biol. Chem.,* **252,** 1998-2003.
Burgess,A.T., Metcalf,D., Russell,S.H.M. and Nicola,N.A. (1980) *Biochem. J.,* **185,** 301-314.
Dexter,T.M. (1984) *Nature,* **309,** 746-747.
Dodge,W.H., Love,S.H., Bryant,D.L. and Mitchel,R.H. (1980) *Exp. Hematol.,* **8,** 395-403.
Graf,T. and Beug,H. (1978) *Biochim. Biophys. Acta,* **516,** 269-299.
Graf,T. and Stehelin,D. (1982) *Biochim. Biophys. Acta,* **651,** 245-271.
Graf,T., Oker-Blom,N., Todorov,T.G. and Beug,H. (1979) *Virology,* **99,** 431-436.
Graf,T., v.Kirchbach,A. and Beug,H. (1981) *Exp. Cell Res.,* **131,** 331-343.
Ihle,J.N., Keller,J., Oroszlan,S., Henderson,L.E., Copeland,T.D., Fitch,F., Prystowsky,M.B., Goldwasser,E., Schrader,J.W., Palaszyusky,E., Dy,M. and Lebel,B. (1983) *J. Immunol.,* **131,** 282-287.
Iscove,N.N., Roitsch,C.A., Williams,N. and Guilbert,L.J. (1982) *J. Cell. Physiol., (suppl.),* **1,** 65-78.
Laemmli,U.K. (1970) *Nature,* **227,** 680.
Leprince,D., Gegonne,A., Coll,I., deTaisne,C., Schneeberger,A., Lagrou,C. and Stehelin,D. (1983) *Nature,* **306,** 391-395.
Metcalf,D. (1981) in Baserga,R. (ed.), *Tissue Growth Factors,* Springer Verlag, Berlin/Heidelberg/NY, pp. 343-384.
Moscovici,C., Samarut,I., Gazzolo,L. and Moscovici,M.G. (1981) *Virology,* **113,** 765-768.
Moscovici,M.G. and Moscovici,C. (1983) *Proc. Natl. Acad. Sci. USA,* **80,** 1421-1425.
Murakami,H., Masui,H., Sato,G.S., Sueoka,N., Chow,T.P. and Kano-Sueoka,T. (1982) *Proc. Natl. Acad. Sci. USA,* **79,** 1158-1162.
Nicola,N.A., Metcalf,D., Matsumoto,M. and Johnson,G.R. (1983) *J. Biol. Chem.,* **258,** 9017-9023.
Nunn,F.E., Seeburg,P.N., Moscovici,C. and Duesberg,P.H. (1983) *Nature,* **306,** 391-395.
Peterson,G.L. (1977) *Anal. Biochem.,* **83,** 346-356.
Porter,R.R. (1959) *Biochem. J.,* **73,** 119-126.
Radke,K., Beug,H., Kornfeld,S. and Graf,T. (1982) *Cell,* **31,** 643-653.
Schnetzler,M., Oommen,A., Nowak,J.S. and Franklin,R.M. (1983) *Eur. J. Immunol.,* **13,** 560-566.
Sotirov,N. (1981) *J. Natl. Cancer Inst.,* **66,** 1143-1147.
Stanley,E.R. and Heard,P.M. (1977) *J. Biol. Chem.,* **252,** 4305-4312.
Stanley,E.R. and Guilbert,L.J. (1981) *J. Immunol. Methods.,* **42,** 253-283.

Received on 3 September 1984