

PURIFICATION OF MACROPHAGE DEACTIVATING FACTOR

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Macrophages ($M\phi$)¹ can use products of the respiratory burst to kill tumor cells and microbial pathogens and to regulate the function of host cells (1). In turn, each class of target cells can regulate the capacity of macrophages to undergo a respiratory burst (2-11). Most studies of regulation of macrophage respiratory burst capacity have focussed on its enhancement (12). Much less is known about macrophage deactivating factors.

After culture with tumor cells, mouse peritoneal macrophages could no longer undergo a respiratory burst (13). The phenomenon could be reproduced with medium conditioned by a wide variety of murine (6, 14) and human (15) tumor cells and some nonmalignant cells (6). Macrophages incubated in tumor cell-conditioned medium (TCM) were markedly suppressed in their production of superoxide and hydrogen peroxide in response to phorbol diesters, yeast cell walls, toxoplasma trophozoites, or leishmania promastigotes (6, 7), in their killing of toxoplasma and leishmania (7, 16), and in their expression of Ia antigens in response to rIFN γ (17). We use the term "MDF" to refer to the macrophage-deactivating factor(s) responsible for the above effects.

Suppression of the respiratory burst in TCM-treated macrophages was attributed to the increased K_m of their superoxide-producing oxidase for NADPH (18). Subsequently, TGF- β 1 and TGF- β 2 were also found to suppress macrophage respiratory burst capacity (8). However, these cytokines did not increase the K_m of the oxidase for NADPH, and antibodies that neutralized suppression of the respiratory burst by TGF- β did not prevent suppression of the respiratory burst by MDF (19). Recently, calcitonin gene-related peptide (CGRP) (9) and IL-4 (10, 11) were reported to suppress the respiratory burst capacity of monocytes.

Below we describe the purification from TCM of an acid-stable polypeptide of \sim 13 kD associated with potent MDF activity. This molecule appears to be distinct from TGF- β , CGRP, and IL-4.

Materials and Methods

Bioassay for MDF. Activated macrophages ($M\phi$) were washed from the peritoneal cavities of female CD1 mice (Charles River Laboratories, Wilmington, MA) with α MEM (KC Bio-

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¹ *Abbreviations used in this paper:* CGRP, calcitonin gene-related peptide; MDF, macrophage deactivating factor; $M\phi$, macrophages; RP, reversed phase; TCM, tumor cell-conditioned medium; TFA, trifluoroacetic acid.

logicals, Lenexa, KS) 3–4 d after injection of 1 ml autoclaved sodium caseinate (practical grade; Eastman Kodak Co., Rochester, NY) in normal saline, as described (20). The cells were centrifuged (1,070 g, 10 min, 4°C) and resuspended in α MEM containing 10% heat-inactivated horse serum (HyClone Laboratories, Logan, UT) with 100 U/ml penicillin and 100 μ g/ml streptomycin. This medium will be referred to as M-10. 2×10^5 cells in 25 μ l were added to each well of 96-well plates (Costar Data Packaging, Cambridge, MA) containing test samples and horse serum, such that the final volume was 135–150 μ l and the final horse serum concentration was 10%. The cells were incubated overnight in 5% CO₂ at 37°C and assayed for H₂O₂ release as described (20). In brief, the plates were washed in saline and the adherent cells incubated in Krebs-Ringer phosphate buffer with glucose and 100 ng/ml PMA as a trigger of the respiratory burst. The oxidation of the fluorescent indicator scopoletin by H₂O₂, catalyzed by horseradish peroxidase, was monitored fluorometrically for 45–90 min, the cell protein in the same wells was measured spectrophotometrically, and the nmol H₂O₂ released per milligram adherent cell protein calculated as reported (20). A range of volumes of MDF-containing fractions was tested; the volume of test sample required for 50% inhibition of H₂O₂-releasing capacity was determined by interpolation and defined as one unit. Units per milliliter of test sample were divided by milligrams of protein per milliliter of test sample to give the specific activity of MDF. Specific activity is \sim 10-fold higher when macrophages are incubated in MDF for 48 rather than 24 h (6, 8); the shorter assay was used for convenience.

TCM. P815 mouse mastocytoma cells (American Type Culture Collection, Rockville, MD) were cultured at an initial density of 1.5×10^6 /ml in α -MEM. After 48 h, the medium was centrifuged (5,225 g, 30 min, 4°C) and the supernatant was sterilized by filtration (0.45 μ Millex-HA; Millipore, Bedford, MA) and stored at 4°C.

Concentration of MDF Activity from TCM. Three methods were used alternatively as indicated in Results. (a) Solid ammonium sulfate was slowly added to TCM at 4°C with constant stirring, maintaining the pH at 7.0 with NaOH until 70% saturation (wt/wt). The suspension was stirred for 4–6 h and the precipitate collected by centrifugation (12,100 g, 30 min, 4°C). The pellet was dissolved in 20 mM phosphate buffer pH 7.2 and dialyzed extensively against the same and finally against PBS (Gibco Laboratories, Grand Island, NY). The dialysate was centrifuged (12,100 g, 10 min, 4°C) and the supernatant filter-sterilized and stored at –20°C. (b) TCM was concentrated by ultrafiltration on either YM-300, YM-100, YM-10, or YM-5 membranes (Amicon Corp., Lexington, MA) under N₂ at 4°C with slow stirring. Filtrate and retentate were both reserved. (c) TCM was extracted with acid ethanol according to the method of Roberts et al. (21) with minor modifications. Briefly, 1 vol TCM or the retentate obtained upon ultrafiltration through YM100 or YM5 membranes was mixed overnight at 4°C with 3 vol of 0.23 N HCl in 95% ethanol (in some experiments this contained 86 mg/liter of PMSF). The acid-insoluble proteins were discarded after centrifugation (5,225 g, 10 min, 4°C) and the pH of the supernatant adjusted to 3–4 with ammonium hydroxide. The acid-soluble proteins were further concentrated by the addition of 2 vol anhydrous ether (Baker Laboratories, Phillipsburg, NJ) and 1 vol ethanol to 1 vol of supernatant. After 24 h at –20°C, precipitated proteins were collected by centrifugation (5,225 g, 20 min, 4°C) and the supernatant discarded. The precipitate was dissolved in 1 M acetic acid and dialyzed against the same overnight at 4°C. Insoluble material was discarded and the supernatant lyophilized (Savant Instrument Co., Farmingdale, NY) and stored at –20°C.

Sephacryl S400 Chromatography. MDF concentrated by methods *a* or *b* was fractionated on a 1.5 \times 50 cm column of S-400 (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with PBS at 4°C at a flow rate of 10 ml/h. 4-ml fractions were collected and aliquots assayed for MDF activity, protein content and polypeptide migration on SDS-PAGE. The same three analyses were conducted on fractions from each of the following chromatographic procedures.

Biogel P60 Chromatography. Lyophilized MDF extracted by method *c* was dissolved in 1 M acetic acid. The precipitate was removed by centrifugation and the supernatant was further clarified by filtration (0.45 μ m) and applied on a 1.5 \times 100 cm column of Biogel P60 (Biorad Laboratories, Richmond, CA) equilibrated with 1 M acetic acid at room temperature. 6-ml fractions were eluted in 1 M acetic acid at a flow rate of 20 ml/h.

Ion-Exchange Chromatography. Active fractions from the Biogel P60 column were pooled, dried, and reconstituted in 10 mM histidine HCl buffer, pH 6.0, containing 1 mM sodium azide and 0.5 mM PMSF (buffer A) and applied on a Mono Q column (Pharmacia Fine Chemicals, HR 5/5) equilibrated with buffer A flowing at 0.25 ml/min. Bound proteins were eluted in 0.5 ml fractions over 30 min with a linear gradient of NaCl increasing to 1 M in buffer A.

Reversed-Phase HPLC. MDF from the Mono Q step was applied to a 4.6 mm ID \times 25 cm (5 μ m pore) diphenyl RP column (Vydac, Hesperia, CA) equilibrated with 0.1% TFA (Pierce Chemical Co., Rockford, IL) at a flow rate of 1 ml/min. Bound proteins were eluted over 30 min in an increasing gradient of acetonitrile in 0.1% TFA. The elution profile was monitored at 214 and 280 nm and 1-ml fractions were collected, dried, and reconstituted in 0.1% TFA. The bioactive fraction was further purified by two sequential fractionations on a RP C4 column (4.6 \times 50 mm, 6.5 μ m pore) (Synchrom, Lafayette, IN) equilibrated with 0.1% TFA (phase A). Phase B was 100% acetonitrile in 0.1% TFA. Elution was monitored at 214 and 280 nm and 1-ml fractions were collected.

Electrophoresis and Immunoblotting. SDS-PAGE (15 \times 17 \times 0.08 cm gels) was carried out by the method of Laemmli (22). The proteins were fixed in 30% methanol and 10% acetic acid and visualized by silver staining with minor modifications of the method of Morrissey (23). For immunoblots, the unfixed proteins, including pure porcine platelet TGF- β 1 (R & D Systems, Minneapolis, MN), pure recombinant human TGF- β 1 (a gift of M. Narachi; Amgen, Inc., Thousand Oaks, CA), and pure rat CGRP (Peninsula Laboratories, Belmont, CA) were transferred to nitrocellulose (0.22 μ m, Schleicher & Schuell, Keene, NH) according to the method of Towbin et al. (24) as modified (25). The transfer buffer contained 0.1% SDS in 40 mM glycine, 25 mM Tris, 20% methanol pH 8.3. After transfer, proteins were visualized with amido black or Ponceau red and blocked overnight in blocking buffer (5% nonfat dry milk in 20 mM Tris-buffered saline, pH 7.5 containing 0.2% Tween 20) at 4°C. The blots were incubated with turkey anti-TGF β 1 IgG, nonimmune turkey IgG (kind gifts of M. Sporn; National Cancer Institute, Bethesda, MD), purified rabbit IgG antibody to ubiquitin and ubiquitin-protein conjugates (a kind gift of A. Haas; Medical College of Wisconsin, Milwaukee, WI), a rabbit antiserum and a mouse mAb to retroviral p15E (kind gifts of Dr. George Cianciolo, Duke University, Durham, NC), or nonimmune rabbit IgG for 2-3 h at 37°C. The blots were washed three times with blocking buffer and then incubated in rabbit IgG anti-turkey IgG (Zymed Laboratories, San Francisco, CA) or goat IgG anti-rabbit IgG (Boehringer Mannheim, Indianapolis, IN) coupled to alkaline phosphatase. Immune complexes were visualized after reaction with indolyl phosphate and nitroblue tetrazolium (United States Biochemical Co., Cleveland, OH) in veronal acetate buffer pH 9.6.

Measurement of Protein Concentration. Protein was determined by the method of Lowry et al. (26) with BSA as the standard. The concentration of protein in MDF fractions of highest purity was determined by amino acid analysis after hydrolysis in 6 N HCl (kindly performed by M. O. McGinley and M. F. Rohde; Amgen).

Control of Bacterial LPS Contamination. The water used to prepare all reagents was purified by reverse osmosis and redistilled in glass shortly before use. Glassware was baked for 4 h at 180°C. In some cases, LPS was removed from chromatographic columns by treatment with 0.1 N NaOH (27). LPS contamination of MDF fractions, culture media, and assay reagents was monitored by a chromogenic limulus amoebocyte lysate assay (Whittaker MA Bio-products, Walkersville, MD) with a sensitivity of \sim 10 pg/ml. Test samples containing $>$ 0.3 ng/ml LPS were discarded.

Results

Distribution of MDF in Both High and Low M_w Fractions of TCM. MDF was recovered quantitatively in the supernatant after centrifugation of TCM at 100,000 g (not shown). This suggested that MDF was not comprised of viruses or membrane vesicles (28). MDF was only partially inactivated by 2:1 chloroform/methanol, and no ac-

tivity was recovered in the organic phase (not shown), suggesting that a lipid did not account for the bioactivity. Association of MDF with a restricted set of proteins was indicated by its quantitative recovery in the material remaining in solution in 40% ammonium sulfate but appearing in the precipitate at 70%. When MDF was precipitated in 70% ammonium sulfate, redissolved, and fractionated on a Sephacryl S-400 column, activity eluted both in the void volume ($M_r > 450,000$) and in late fractions ($M_r \sim 12-25,000$) (Fig. 1). MDF-rich fractions from the void volume of S-400 columns stained positively on SDS-PAGE by the Alcian blue method for proteoglycans and negatively charged glycoproteins (29). Likewise, ultrafiltration of TCM on membranes with nominal M_r cut-offs of 300,000 or 100,000 distributed MDF almost equally to the retentates and filtrates (not shown). These observations suggested that MDF might exist in TCM both as a low- M_r form and in association with high- M_r glycoproteins or proteoglycans. We therefore sought a method to dissociate MDF from other proteins.

Acid-Ethanol Extraction of MDF. We took advantage of the facts that MDF is stable (18) under the acidic conditions used to activate latent TGF- β (30) and that acid-ethanol extraction and ether precipitation have been used to purify several cytokines, including TGF- β (21). These procedures concentrated MDF with quantitative recovery and a 24-fold increase in specific activity (Table I), far surpassing results with ammonium sulfate precipitation or ultrafiltration (not shown).

Gel Filtration. When MDF had been extracted in acid-ethanol, precipitated in ether, and dissolved in 1 M acetic acid, it consistently eluted from Biogel P60 in a broad peak between ubiquitin (8,700 M_r) and chymotrypsinogen (25,000 M_r); occasionally a minor portion of activity eluted with higher M_r materials (Fig. 2 A). On SDS-PAGE under reducing conditions (Fig. 2 B), very few polypeptides were demonstrable in the active fractions; species migrating below 14,000 M_r predominated. All active fractions also contained a species migrating at 66,000 M_r . Fractions from the leading edge of the bioactive peak contained a 24,000 M_r species, but this was much less abundant in the second half of the peak. Fractions that contained large amounts of the 24,000 M_r species were devoid of MDF activity. No spe-

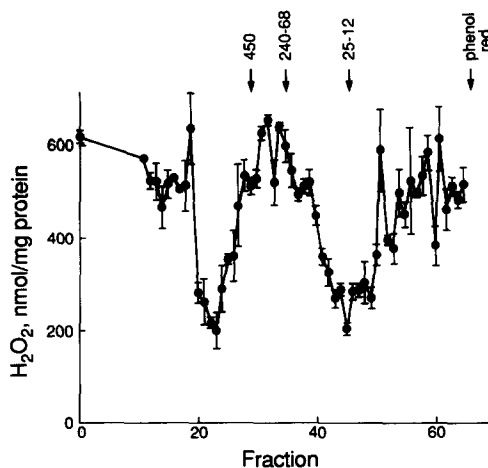


FIGURE 1. Fractionation of MDF on Sephacryl S-400. The 70% ammonium sulfate precipitate of TCM was redissolved and dialyzed against PBS and eluted from the column with PBS at 10 ml/min. 4-ml fractions were collected, from which 100 μ l aliquots were tested, revealing MDF bioactivity in the void volume as well as in low M_r fractions. Arrows at the top mark the elution of ferritin, catalase, BSA, chymotrypsinogen, RNase, cytochrome *c*, and phenol red ($M_r \times 10^3$ shown). In this and subsequent figures, fraction 0 represents control medium; results are means \pm SEM for triplicate wells in which activated macrophages were incubated with test samples for 24 h and washed before H_2O_2 release was measured over 90 min after the addition of PMA, unless otherwise indicated; where error bars are not visible, they fall within the symbol.

TABLE I
Summary of Purification of MDF

Stage	Specific activity <i>U/mg protein</i> [*]	Recovery % [†]	Fold purification
TCM	9.4 ± 0.8 (7)	100	1.0
Extract [‡]	222 ± 21 (4)	106 ± 11	23.7
Gel filtration [§]	1,008 ± 174 (4)	46 ± 8	108
Anion exchange [¶]	1,860 (2)	45	198
RPHPLC (diphenyl)	6,060 (2)	14	647
RPHPLC (C4) ^{**}	57,534 ± 3,318 (3) ^{‡‡}	2	6,140

* Mean ± SEM for number of batches of TCM shown in parentheses. Seven batches were processed through gel filtration and three through RPHPLC. Results are shown for fewer fractionations in cases where MDF activity was not measured in enough doses to permit assignment of units, or protein was not measured.

† With reference to TCM.

‡ Acid-ethanol extraction and ether precipitation.

§ Biogel P60.

¶ Mono Q.

** Two fractionations in tandem.

‡‡ Corrected for estimated 50% albumin contamination.

cies migrated differentially under reducing or nonreducing conditions (not shown). Despite the extensive purification afforded by this step, as evidenced by SDS-PAGE, specific activity increased only 4.6-fold (Table I). This raised the possibility that MDF might be partially inactivated by fractionation, either by denaturation or by separation from a cofactor. The concentration-response curve for the bioactive fractions was not affected by the addition of pooled inactive fractions (data not shown), militating against the latter possibility.

Ion-Exchange Chromatography. Active fractions from the Biogel step were applied to a Mono Q column. MDF activity was recovered in the flow-through and first few included fractions, while most of the contaminating species, including albumin, were retained, along with unidentified materials that absorbed strongly at 254 nm (Fig. 3) but not at 280 nm (not shown). This suggested that MDF was cationic at pH 6. However, MDF could not be recovered from either of the cation exchange columns tested (polyaspartate and sulfonic acid Synchronapak, not shown). Despite the elimination of most of the remaining protein, the Mono Q fractionation afforded only a 1.8-fold enrichment in specific activity. All the MDF activity applied to the column was recovered. An activity that enhanced macrophage H₂O₂-releasing capacity was contained in the last two fractions from the Mono Q column whether MDF was loaded (Fig. 3) or not (not shown), and thus represents an artefact.

RPHPLC. MDF from the Mono Q step eluted from a diphenyl RP column in a single fraction at 54 ± 3% acetonitrile (Fig. 4 A). Analysis of this fraction on a reduced Phastsystem minigel (Pharmacia) revealed a single band at 11–13,000 M_r (Fig. 4 B). However, a large 10–25% gradient gel (see Materials and Methods) resolved this fraction into four bands, and revealed a fifth at 66,000 M_r (Fig. 4 C). The bands in the 11,000–13,000 M_r region were electroblotted for gas-phase amino acid sequencing. Only three major sequences were obtained, corresponding to ubiquitin,

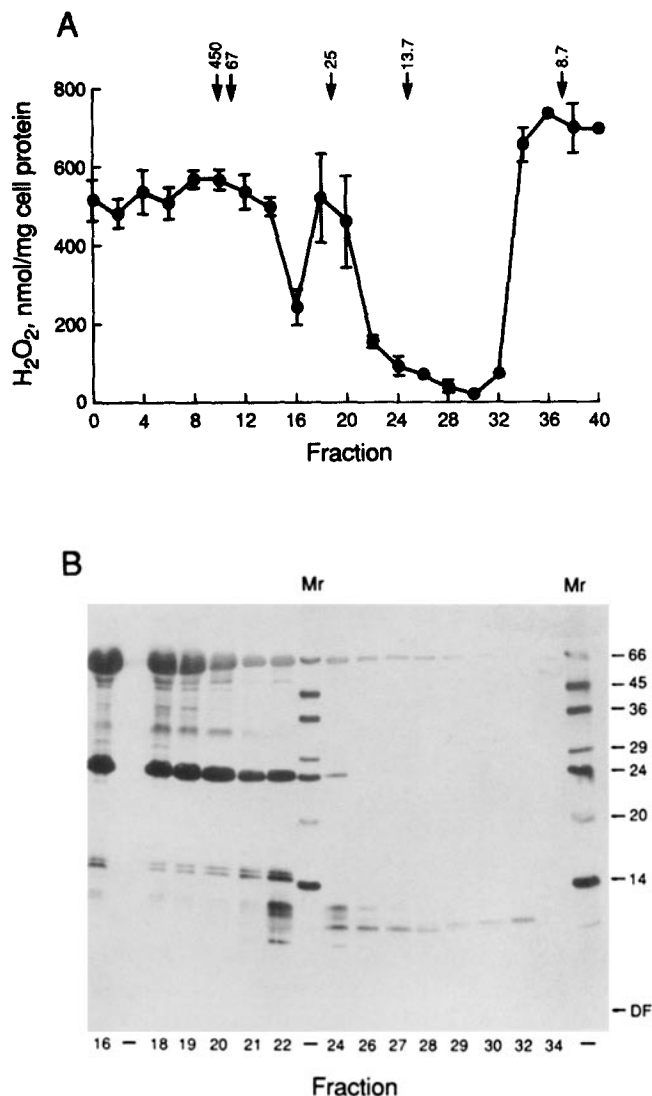


FIGURE 2. Elution of MDF from Biogel P60. The starting material was TCM that had been extracted with acid-ethanol and precipitated in ether. 4-ml fractions were collected. (A) Bioactivity. Arrows at the top mark the elution of ferritin, bovine serum albumin, chymotrypsinogen, RNase, and ubiquitin (M_r indicated in kDa). (B) SDS-PAGE (silver-stained 10–25% gradient gel run under reducing conditions) of the indicated fractions. Lanes marked M_r contained standards, as designated at the right ($\times 10^{-3}$). DF, dye front.

β 2-microglobulin, and a COOH-terminal fragment of albumin (Simal, S., M. McGinley, M. Rohde, M. Narachi, and C. Nathan, unpublished observations). This fractionation increased specific activity 3.3-fold, with recovery of 31% of the activity loaded.

MDF from the diphenyl RPHPLC was fractionated twice by C4 RPHPLC. In the first fractionation, bioactivity eluted in a single peak at $50 \pm 2\%$ acetonitrile (Fig. 5), while species absorbing at 280 and 214 nm eluted in multiple peaks (not shown). As revealed by SDS-PAGE, the first C4 fractionation separated the bioactive species in the 13,000 M_r range (fractions 49 and 50) from more abundant, inactive species of similar M_r (fractions 46–48, Fig. 5, *inset*). In a second C4 fractionation, the single peak of bioactivity (Fig. 6 A) corresponded to the sole absorbance

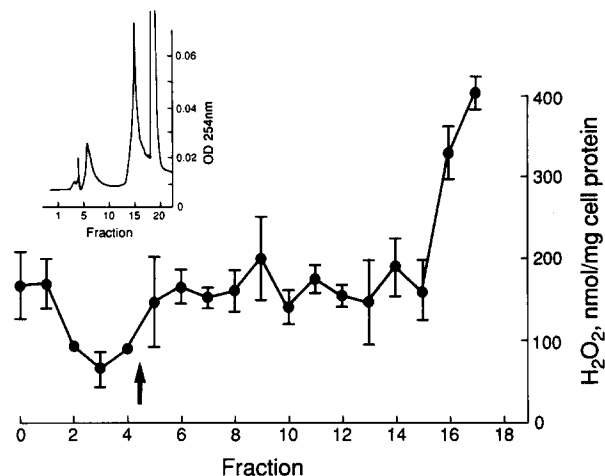


FIGURE 3. Elution of MDF from Mono Q anion-exchange column. The bioactive material from the Biogel P60 fractionation shown in Fig. 2 was lyophilized and reconstituted in 2 ml 10 mM histidine pH 6. 500 μ l was applied to the anion-exchange column and eluted at 0.25 ml/min in 10 mM histidine pH 6. From the time marked by the arrow, a gradient was developed of 0–1 M NaCl in 10 mM histidine pH 6. Fractions of 0.5 ml were collected, from which 25 μ l were assayed for ability to suppress macrophage H_2O_2 release after a 24-h incubation, measured at 45 min after the addition of PMA. (Inset) Elution of proteins monitored by absorbance at 254 nm.

peak (Fig. 6 B). Silver-stained SDS-PAGE revealed one band (sometimes appearing as a doublet) at $\sim 13,000 M_r$ in the midst of a diffuse, faintly stained zone (Fig. 6 C). The tandem C4 fractionations increased the specific activity of MDF 9.5-fold and permitted recovery of 14% of the activity loaded, with a yield of $\sim 1 \mu$ g protein/100 ml starting material.

The purification sequence described above was completed with three batches of TCM. Two preparations contained a band at $66,000 M_r$ whose intensity on silver staining was closely comparable to that of the $\sim 13,000 M_r$ band, even though the $66,000 M_r$ band was barely visible in photographs (Fig. 6 C). In the third run, the staining intensity of the $66,000 M_r$ band was only $\sim 5\%$ of that of the $\sim 13,000 M_r$ band. For both bands, the SDS-PAGE pattern was the same under reducing and nonreducing conditions. Both bands and no additional species were revealed on SDS-PAGE by autoradiography of the final product after iodination by the chloramine T method. To detect carbohydrate, we oxidized the gel-bound proteins with periodate, coupled vicinal aldehydes to digoxigenin via a hydrazide linkage, and stained with an alkaline phosphatase-conjugated antidigoxigenin mAb, using a commercially available kit (Boehringer-Mannheim). The $66,000 M_r$ band was visualized by this method; the $\sim 13,000 M_r$ band was not (gels not shown).

When 4 μ g of the final bioactive fraction was placed directly in the sequenator, one clearcut sequence was obtained, corresponding to the 20 NH_2 -terminal residues of albumin. When 1.7 μ g of this preparation was subjected to SDS-PAGE and the $13,000 M_r$ region electroblotted to a polydivinylfluoride membrane, no sequence could be obtained (Srimal, S., M. McGinley, M. Rohde, M. Narachi, and C. Nathan, unpublished observations). Thus, MDF was contaminated with albumin, and its own NH_2 terminus appeared to be blocked.

Fractionation of Control Medium. Serum-containing M-10 medium in which no tumor cells had been grown was extracted with acid-ethanol, precipitated with ether, and fractionated on Biogel P60 and diphenyl RPHPLC exactly as for MDF. No MDF activity could be detected (not shown).

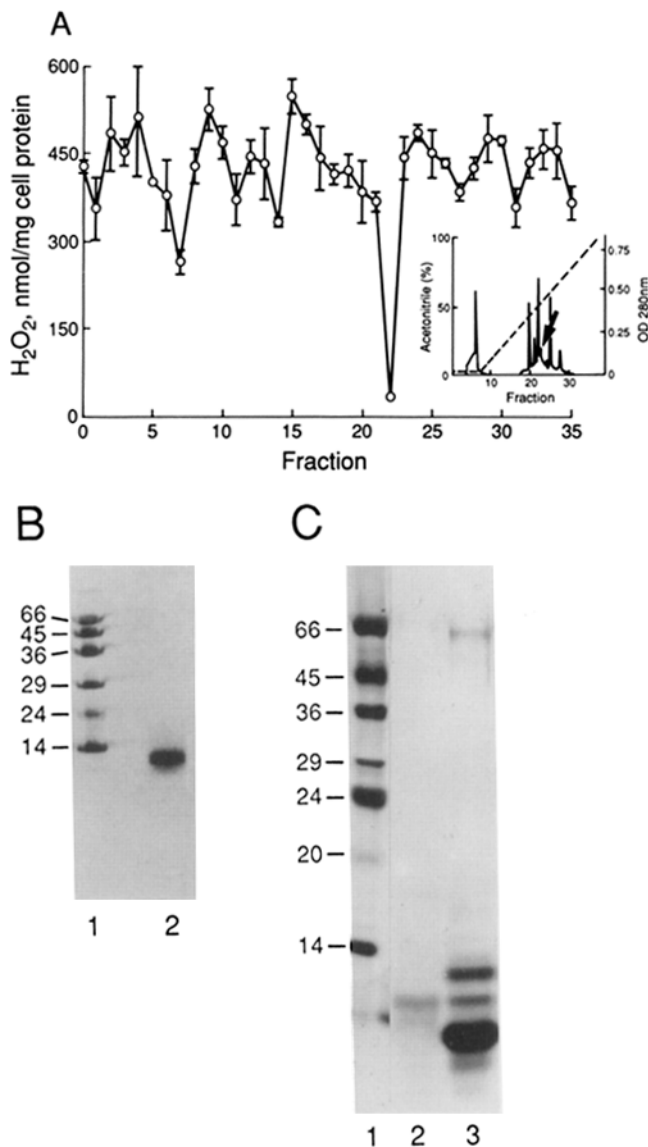


FIGURE 4. Fractionation of MDF by RPHPLC. (A) Bioactive material from the Mono Q column was applied to a diphenyl RP column and eluted at 1 ml/min with 0.1% TFA and a linear gradient of 0-100% acetonitrile as indicated by the dashed line in the inset. 1-ml fractions were collected, from which 25- μ l aliquots were assayed. The bioactive fraction is marked by an arrow on the absorbance profile (solid line) in the inset. (B) SDS-PAGE of bioactive fraction (lane 2) on 9-25% gradient Phastsystem Minigel (Pharmacia Fine Chemicals, $0.43 \times 0.5 \times 0.04$ cm) run under reducing conditions and stained with Coomassie blue. The appearance was the same when the gel was stained with silver. (Lane 1) M_r markers ($\times 10^{-3}$). (C) SDS-PAGE of bioactive fraction (lane 3) and an inactive fraction eluting one minute earlier (lane 2), using a $15 \times 17 \times 0.08$ cm 10-25% gel run under reducing conditions and stained with silver. (Lane 1) M_r markers ($\times 10^{-3}$).

Immunoblotting and Immunoneutralization. Western blot with turkey anti-TGF- β antibody 121 was positive when as little as 1 ng pure recombinant human TGF- β 1 was subjected to SDS-PAGE and electrophoretic transfer to nitrocellulose. However, this antibody failed to blot the protein transferred when 80 ng (4.6 U in the 24-h assay) of MDF was electrophoresed (not shown), suggesting TGF- β was not present in excess of $\sim 1.3\%$. Since ~ 15 pg TGF- β 1 constitutes 1 U in the 24-h bioassay for MDF (8), 0.09% contamination of MDF with TGF- β 1 could account for the observed bioactivity and escape detection by Western blot. Accordingly, the most highly purified MDF fraction was incubated in the fluid phase with 64 μ g/ml of antibody 121. Under

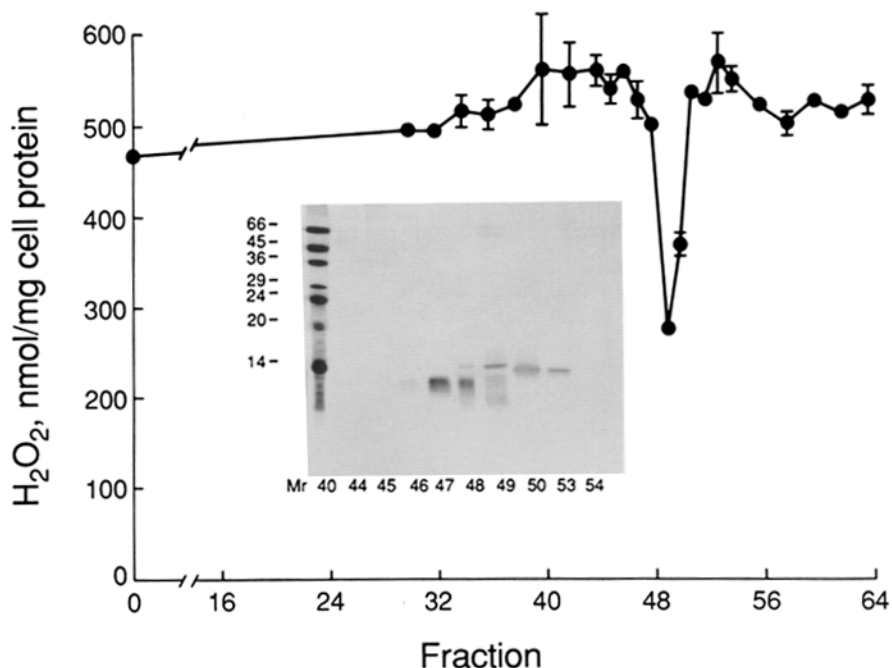


FIGURE 5. RPHPLC fractionation of MDF on a C4 column, using the bioactive peak from the diphenyl-C4 RPHPLC. (*Main figure*) Bioactivity profile. Aliquots of 25 μ l were tested from 1-ml fractions. The gap in the graph corresponds to fractions that were not tested. (*Inset*) Silver-stained, reducing 9–20% SDS-PAGE of indicated fractions. Note, only fractions 49 and 50 were bioactive. M_r standards are indicated ($\times 10^{-3}$).

these conditions, the H₂O₂ release-suppressing activity of 10 ng/ml authentic TGF- β 1 was completely neutralized. However, the bioactivity of purified MDF was not affected (Table II). Likewise, polyclonal and monoclonal antibodies to P15E, a virus-derived, monocyte-inhibitory peptide (4), did not inhibit MDF (not shown). An antibody that reacts with ubiquitin and ubiquitin-protein conjugates (31) immunoblotted a species within the MDF-active fraction from the diphenyl RPHPLC step, but no longer reacted with MDF purified on the C4 column. Finally, an antibody that Western-blotted rat CGRP (4,500 M_r) gave no signal with MDF, and authentic rat CGRP lacked activity in the MDF assay (not shown).

Discussion

MDF was purified 6,140-fold from P815 cell-conditioned medium, yielding a single peak on RPHPLC and a single major band on SDS-PAGE at $\sim 13,000 M_r$. No NH₂-terminal amino acid sequence could be obtained from this band in either the fluid or solid phase, suggesting that the NH₂ terminus of MDF may be blocked. Efforts are underway to derive amino acid sequence from fragments of MDF. Proof that the $\sim 13,000 M_r$ species is MDF will require the generation of antibodies and recombinant proteins corresponding to such sequences. Until this evidence is in hand,

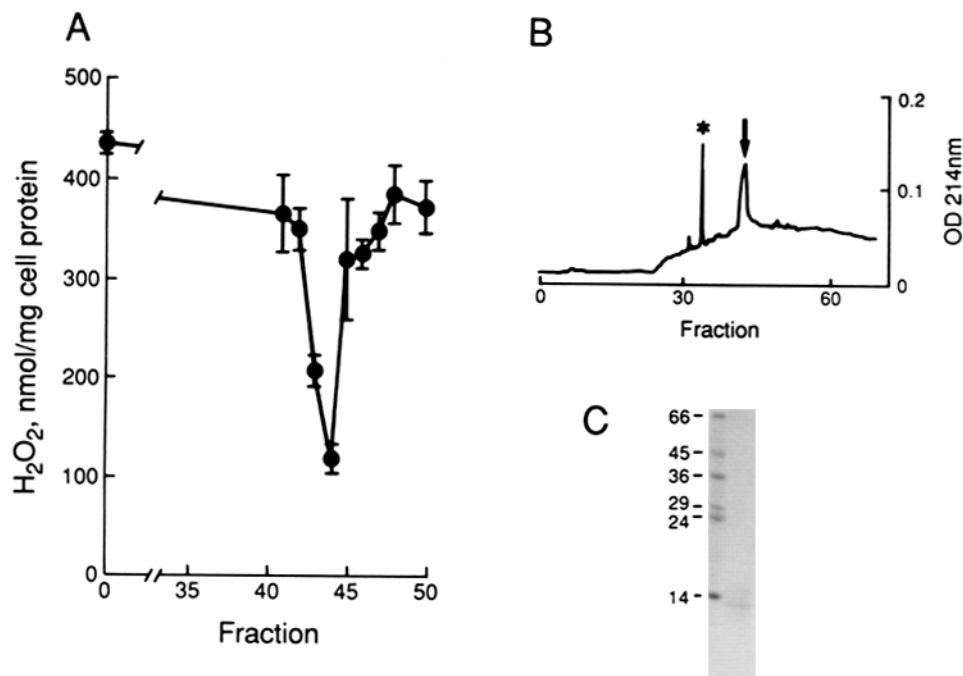


FIGURE 6. Repeat RPHPLC fractionation of MDF on a C4 column, using the bioactive peak from the first C4 column. (A) Bioactivity profile, assayed as in legend to Fig. 5. In both runs, MDF eluted at $50 \pm 2\%$ acetonitrile. (B) Elution profile at 1 ml/min, collecting 1-ml fractions and monitoring absorbance at 214 nm. The column was eluted with 0.1% TFA diluted with acetonitrile to give the indicated percent acetonitrile at the end of each interval: 0–10 min, 0%; 11–39 min, 45%; 40–49 min, 55%; 50–65 min, 65%; 66–70 min, 100%. The asterisk indicates an absorbance peak seen with buffer alone. The arrow marks the peak corresponding to MDF bioactivity. (C) SDS-PAGE of 40 ng of protein from the bioactive peak.

it cannot be excluded that MDF may be a molecule undetected by SDS-PAGE at the present level of abundance.

The final preparations contained albumin in amounts varying from $\sim 5\%$ to $\sim 50\%$, as identified by its migration on SDS-PAGE at 66,000 M_r and by its NH_2 -terminal amino acid sequence. There appeared to be a high affinity between MDF and albumin, such that albumin persisted through all seven steps, including three reversed-phase separations, used to purify MDF. We could only separate MDF from albumin by elution from polyacrylamide gels. However, gel eluates caused artefacts in the bioassay, and MDF activity could not be unequivocally demonstrated. The tendency of MDF to associate with higher M_r proteins was also evident in the starting material, which contained as much bioactivity in fractions of $>450,000 M_r$ as in fractions of $\sim 12\text{--}25,000 M_r$. This is reminiscent of the behavior of several growth factors. For example, TGF- β forms noncovalent complexes both with the remnant of its precursor and with α_2 -macroglobulin (32). Alternatively, the bioactivity in high M_r fractions could have been due to aggregates of MDF or to protein(s) other than MDF.

Purified MDF afforded 50% inhibition of macrophage peroxide-releasing capacity

TABLE II
Inability of Anti-TGF- β Antibody to Neutralize Purified MDF

Cytokine*	Antibody	H ₂ O ₂ release [†]
—	—	413 \pm 41
TGF- β 1 [‡]	pre-immune Ig [§]	93 \pm 7
	immune IgG [¶]	403 \pm 10
—	—	481 \pm 93
MDF**	—	333 \pm 22
	pre-immune IgG	276 \pm 12
	immune IgG	316 \pm 21

* Macrophages were incubated in M10 alone or with the indicated cytokines for 24 h, washed, and challenged with PMA.

[†] nmol/mg protein in 60 min (mean \pm SEM of triplicates).

[‡] 3.5 ng/ml of pure TGF- β 1 from porcine platelets.

[§] Purified by ammonium sulfate precipitation from turkey serum and included in the bioassay at 40 μ g/ml.

[¶] Purified turkey IgG no. 121, included in the bioassay at 64 μ g/ml, enough to neutralize completely 10 ng/ml TGF- β in this assay.

** Final preparation from second C4 column, diluted to 0.26 ng/ml to give partial inhibition to increase the chance of neutralizing inhibitory activity.

at \sim 10 nM (130 ng/ml) in the overnight assay used for most of the experiments, or at \sim 1 nM when the incubation with macrophages was extended to 48 h. This potency is similar to that of several cytokines in bioassays that use primary, unselected populations of indicator cells, rather than clones of transformed cells selected for their sensitivity. Biogel P60 gel filtration and Mono Q anion exchange, the first two chromatographic steps, eliminated the vast majority of the protein in TCM, yet afforded disproportionately small increases in specific activity. This raises the possibility that MDF may have been partly denatured as MDF was separated from most of the higher M_r materials with which it was associated in TCM (Fig. 1). If so, the specific activity of MDF in its native state may be higher than after purification by the methods used here.

Nonetheless, the fact that purified MDF was \sim 1,000-fold less potent than pure rTGF- β 1 at suppressing macrophage peroxidase-releasing capacity (8) raised the possibilities that MDF may be TGF- β or may act by inducing TGF- β during the bioassay. TGF- β was not detected at any stage of the purification. Specifically, we did not observe a polypeptide migrating at 24,000 M_r under nonreducing conditions and at 12,500 M_r under reducing conditions; the NH₂-terminal amino acid sequence of TGF- β ; a species reacting with antibody to TGF- β on Western blot; or most important, a bioactivity neutralizable with rabbit anti-TGF- β antibody LC14- β 1 (19) or turkey anti-TGF- β antibody 121. Antibody LC14- β 1 neutralizes both TGF- β 1 and TGF- β 2. Antibody 121 is specific for TGF- β 1; turkey antibodies specific for TGF- β 2 also failed to inhibit MDF (not shown). All these antibodies completely neutralized authentic TGF- β s when the antibodies were added directly to the MDF assay. Finally, TCM and TGF- β appeared to suppress the macrophage respiratory burst by distinct mechanisms (19). Production of recombinant MDF and antibodies to recombinant MDF or MDF-encoded peptides will be necessary to exclude a contribution

of TGF- β . Determination of the MDF sequence will address the further possibility that MDF may be a member of the TGF- β superfamily (32).

MDF appears distinct from polypeptides other than TGF- β that have been reported to affect respiratory burst capacity of mononuclear phagocytes. M_r distinguishes MDF in its $\sim 13,000 M_r$ form from α_2 -macroglobulin-protease complexes ($\sim 750,000 M_r$) (33), although we cannot exclude a protease that acquires MDF activity upon associating with α_2 -macroglobulin in the serum used for the bioassay. MDF appears to be smaller than IL-4 (20,000 M_r) (10, 11) and larger than CGRP (4,500 M_r) (9). Moreover, IL-4 (34) and CGRP were inactive in our MDF assay. In contrast to the studies in which IL-4 and CGRP were found to suppress the respiratory burst, we used tissue macrophages rather than blood monocytes, and murine rather than human cells. We added IL-4 or CGRP after the cells were activated, rather than during the activation process. These differences may account for the divergent findings.

TCM derived from P815 cells has served as the starting material for several attempts to purify factors that suppress lymphocyte proliferation or antibody secretion. Cornelius and Normann (35) reviewed these efforts, and noted that the findings of most such studies are consistent with their own evidence that the inhibitory material is a lipid bound to proteins of $>150,000 M_r$. Such factors are probably unrelated to MDF.

In our earlier work, the lack of toxicity of TCM was evident from both the reversibility (6) and selectivity of its actions. Thus, there was no decrease in the ability of TCM-treated macrophages to adhere; phagocytize; secrete plasminogen activator, lysozyme, or arachidonate; catabolize hydrogen peroxide; or take up glucose (6, 18). Nor were TCM-treated macrophages deficient in their specific content or activity of cytochrome b_{558} , NADPH, NADP, catalase, glutathione, glutathione peroxidase, glutathione disulfide reductase, myeloperoxidase, or protein kinase C, or in their ability to translocate protein kinase C from a soluble to a particulate fraction (6, 18, 19). Likewise, in the present study, suppression of the respiratory burst by purified MDF was not due to toxicity. The adherent cell protein content in wells containing macrophages treated with purified MDF was no less than in control wells, and MDF-treated macrophages appeared well spread by phase-contrast microscopy.

Purification of MDF was not possible without the appreciation that traces of LPS can both induce (36) and block (3) macrophage activation. Several years of effort were expended on chromatographic procedures that did not yield consistent results until we monitored LPS routinely and excluded it rigorously.

Purification of MDF will help us learn if MDF shares any of the actions of TGF- β (32) besides suppression of the macrophage respiratory burst. Likewise, purification of MDF brings us closer to testing the hypotheses that some nonmalignant cells may contain MDF, and that MDF may play a role in regulating inflammation.

Summary

Macrophage deactivation factor (MDF) in P815 tumor cell-conditioned medium was assayed by its suppression of the ability of activated mouse peritoneal macrophages to release hydrogen peroxide. MDF displayed properties of a soluble protein(s) associated with both low (8-25,000) and high ($>450,000$) M_r fractions. MDF was purified 6,140-fold by a seven-step procedure: extraction with acid-ethanol; precipitation with ether; and fractionation on gel filtration, anion-exchange, diphenyl reversed-

phase and C4 reversed-phase HPLC columns, the last column twice. The final preparation contained two species: (a) a $\sim 13,000 M_r$ band on reducing or nonreducing SDS-PAGE and on autoradiograms after radioiodination with chloramine T, and (b) a $66,000 M_r$ species ranging from $\sim 5\%$ to $\sim 50\%$ of the protein detectable by silver stain. The $66,000 M_r$ species was identified as albumin from its NH_2 -terminal amino acid sequence. However, no amino acid sequence could be obtained for the $\sim 13,000 M_r$ species, either in fluid phase or after electroelution of the corresponding SDS-PAGE band. Thus, $\sim 13,000 M_r$ MDF associates tightly with albumin through a variety of separation techniques, and may have a blocked NH_2 terminus. Purified MDF afforded 50% inhibition of activated macrophage H_2O_2 releasing capacity at a concentration of 1-10 nM. Separation of MDF from most higher M_r moieties was associated with disproportionately small increases in specific activity, suggesting MDF might be partially inactivated by purification. As purified, MDF was $\sim 1,000$ -fold less potent at deactivating macrophages than $\text{TGF-}\beta$. Antibodies that neutralized the macrophage-deactivating effect of $\text{TGF-}\beta$ did not inhibit deactivation by MDF.

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