(International Equipment Company). Purified bovine albumin was used in all process optimization studies. Substitution of HSA for bovine albumin has no impact on currently measured neohemocyte properties.

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Five male rats [200 to 250 g; Cr1:CD® (SD)BR] were maintained under pentobarbital anesthesia for the duration of the study. Each rat received a 50 percent transfusion of a 25 percent neohemocyte suspension. Transfusions consisted of 2-ml withdrawals and replacements from a jugular vein catheter until each animal's red blood cell hematocrit level was equal to, or less than 50 percent of, its original value. The 25 percent suspension of NHC had the properties listed in Table 1. Immediately after the last 2-ml blood exchange, each rat was injected through the same jugular vein catheter with 0.2 ml of a second batch of NHC, which was identical to the first in all respects except that it was prepared from a Hb solution to which a tracer amount of ^{14}C-labeled sucrose had been added; all sucrose was encapsulated at the time of injection. Urine was collected quantitatively from urinary catheters implanted in each subject for the duration of the study. Blood samples were taken at 0.25, 0.5, 1, 2, 4, 6, and 8 hours after transfusion. There was neither a loss of encapsulated sucrose nor a significant increase in methemoglobin formation for NHC stored for 5 days at 4°C. An apparent half-life was calculated from a nonlinear least-squares fit to the log of the blood concentration values with equal weights given to each value.

Intravenously administered sucrose, like inulin, is rapidly cleared from circulation, $t_{1/2} < 10$ minutes [T. M. Allen and J. M. Everest, *J. Pharmacol. Exp. Therap.* **226**, 539 (1983)], by glomerular filtration and is excreted quantitatively in urine. Because free sucrose is cleared from blood much faster than encapsulated sucrose, the amount of ^{14}C in the blood reflects the amount of circulating, encapsulated sucrose.

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The one rat that showed signs of moderate acute focal hepatic necrosis on day 1 after transfusion (Table 2) had the highest clinical chemistry values in each category listed. The SGOT and SGPT values were 3.1 and 2.9 times the mean control values, respectively.

The vascular retention times of liposomes having the same composition as these NHC increase with decreasing diameter for a dose of constant surface area [R. M. Abra and C. A. Hunt, *Biochim. Biophys. Acta* **666**, 493 (1981)]. However, the maximum encapsulated volume per mole of lipid decreases with decreasing diameter. Increased intravascular retention times are therefore possible. Neither the membrane composition nor the size of the NHC has been optimized for a specific set of desired functional properties in vivo, because there may not be a single set of preferred properties. For example, the degree to which increases in intravascular retention of NHC would contribute to improved efficacy may depend on the intended function of the NHC [A. G. Greenburg, G. W. Peskin, D. B. Hoyt, W. Y. Moores, *Crit. Care Med.* **10**, 266 (1982)].

Rats transfused with normal saline died before the hematocrit value was reduced to 5 percent of the initial values. The amount of Hb in the red blood cells of each group in Fig. 3 after transfusion was less than 1 g/dl, too low to sustain life. Death would be expected if the total effective amount of Hb (based on a-v O_2 delivery) dropped below a critical value higher than 1 g/dl. Rats in groups A and B died, presumably, because the effective amount of Hb in their blood dropped below this critical value. A similar explanation may account for the deaths of three of five rats in the group transfused with NHC. Death would be expected if the total amount of effective Hb fell below the critical level as a result of neohemocyte clearance or oxidation of Hb to methemoglobin in vivo. Survival would only occur if the combination of the rising amount of Hb in the red blood cells and declining amounts of Hb in the NHC maintained the total effective amount of Hb above the critical value.

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Now that these specifications have been met, it is desirable to create a more demanding list of specifications. For example, one may set a minimum on the rate of methemoglobin formation in vivo. We had no control over this process, and its rate may be a major factor governing the functional lifetime of NHC as they are currently formulated. Meeting new specifications may require coencapsulating agents such as methemoglobin reductase along with its required cofactors. Another more rigorous specification may be the mean concentration of Hb within NHC. In preliminary studies we have increased the mean concentration of encapsulated Hb from the current value of 15.8 g/dl to as much as 28.6 g/dl. Significantly more demanding specifications will require reoptimization of the procedure.

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16. The two preparations of NHC had identical lipid compositions. One batch included tracer amounts of ^{3}H-dipalmitoylphosphatidylcholine, and the other included tracer amounts of ^{14}C-cholesterol. We assumed that the combined average recovery of these two markers was identical to the average total lipid recovery.
17. Vigorous mixing of either peroxide-free diethyl ether or TCTFE with purified Hb for up to 1 hour did not result in degradation of Hb as measured by precipitate, methemoglobin, or hemichrome formation. The primary independent variables governing emulsion stability are particle size of the dispersed phase, surfactant concentration and properties, differences in surface tension, viscosity and density of the two phases, and osmolality of the aqueous phase. Of these, only the last two could be varied in this procedure. The use of TCTFE made it possible to match the densities of the volatile and aqueous phases, a step that is essential for subsequent success. If detectable coalescence occurred within the 5 minutes after 5 minutes of vigorous shaking, additional ether or TCTFE was added. The mixture was placed in screw-cap bottles such that the air space was minimized, and then the bottles were shaken for 30 minutes on an industrial paint shaker (Red Devil). If excess methemoglobin or hemichromes were present, lipid-protein aggregates with intended membrane lipids could form. If the combined amount of these two proteins exceeded 5 percent of the total Hb content, the yield of NHC was reduced.
18. We thank the Drug Delivery Group at the University of California at San Francisco for helpful discussions and F. DeVenuto and G. Greenburg for supplying samples of purified human Hb. This work was supported in part by DHHS grant RO1-GM-24612, by the U.S. Army Medical Research and Development Command, contract DAMD17-79C-9045, and funds provided by FMC Corporation. Part of this work was in fulfillment of degree requirements for R.R.B. A preliminary report of an earlier procedure was presented as a paper before the Academy of Pharmaceutical Sciences, San Antonio, Texas, November 1980. A report of preliminary results from experiments performed in vivo was presented as a paper before the International Symposium on Blood Substitutes, San Francisco, Calif., September 1982 [C. A. Hunt and R. R. Burnette, *Prog. Clin. Biol. Res.* **122**, 59 (1983)].

25 February 1985; accepted 22 August 1985

Biosynthesis and Secretion of Proatrial Natriuretic Factor by Cultured Rat Cardiocytes

Abstract. Rat atrial natriuretic factor (ANF) is translated as a 152–amino acid precursor preproANF. PreproANF is converted to the 126–amino acid proANF, the storage form of ANF in the atria. ANF isolated from the blood is approximately 25 amino acids long. It is demonstrated here that rat cardiocytes in culture store and secrete proANF. Incubation of proANF with serum produced a smaller ANF peptide. PreproANF seems to be processed to proANF in the atria, and proANF appears to be released into the blood, where it is converted by a protease to a smaller peptide.

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Atrial natriuretic factor (ANF) is a peptide hormone with potent diuretic and natriuretic properties that probably plays an important role in controlling blood pressure (*1*, *2*). ANF is released from the atria in response to an increase in blood volume (*3*). Understanding the mechanisms that control release of this peptide from the atria is critical to understanding the mechanisms that control blood pressure. One approach to identifying these mechanisms is to study the peptide's biosynthesis. Analysis of the ANF gene and messenger RNA (mRNA) suggests that rodent ANF is translated as a 152–amino acid precursor, preproANF (Fig. 1) (*4*–*7*). PreproANF has many structural features common to other peptide hormone precursors (*8*) and thus may be processed similarly. PreproANF is converted to a 126–amino acid form, proANF (Fig. 1), by removal of the amino terminal hydrophobic leader segment and two residues from the carboxyl terminus. ProANF is the storage form of ANF in the atria (*9*) and has less diuretic activity than smaller ANF-related peptides (*2*). The subsequent steps in the

maturation of proANF are incompletely defined. Smaller ANF peptides are found in extracts of rat atria (*2, 9–11*) and in plasma (*3, 12*). ANF in the plasma is approximately 25 amino acids long (*3*). All of these peptides are derived from proANF and share a 21–amino acid sequence near its carboxyl terminus. The nature and location of the enzyme that converts proANF to smaller peptides is unknown. We report here that proANF is stored and secreted from rat cardiocytes in culture and that proANF can be cleaved to a smaller peptide by a protease in the serum. These studies with cultured cardiocytes suggest that, in the intact animal, proANF may also be secreted from the atria and then cleaved by a protease in the blood.

Studies of the biosynthesis, processing, and secretion of peptide hormones have been facilitated by the availability of cultured cells in which biosynthetic intermediates can be radioactively labeled and identified by immunological methods (*8*). Because transformed lines of atrial cells do not exist, we used cell cultures derived from rodent hearts (*13*) to study synthesis and secretion of ANF. Cantin *et al.* (*14*) have demonstrated secretory granules in cultured atrial cardiocytes prepared by treating adult rat hearts with trypsin. Similar secretory granules found in atria contain ANF (*15*). To produce cardiocyte cultures enriched for atrial cells, sections of hearts (including both atria) from 1-day-old rats were treated with trypsin, and the dispersed cells were plated in culture medium (*16, 17*). Between 10 and 15 percent of the cells in the cardiocyte cultures were specifically stained (*18*) by antisera to synthetic ANF peptides (*19*) corresponding to residues 81 to 92 and 142 to 150 of preproANF (Fig. 1). Electron microscopic examination revealed that between 10 and 15 percent of the cultured cardiocytes also contained secretory granules.

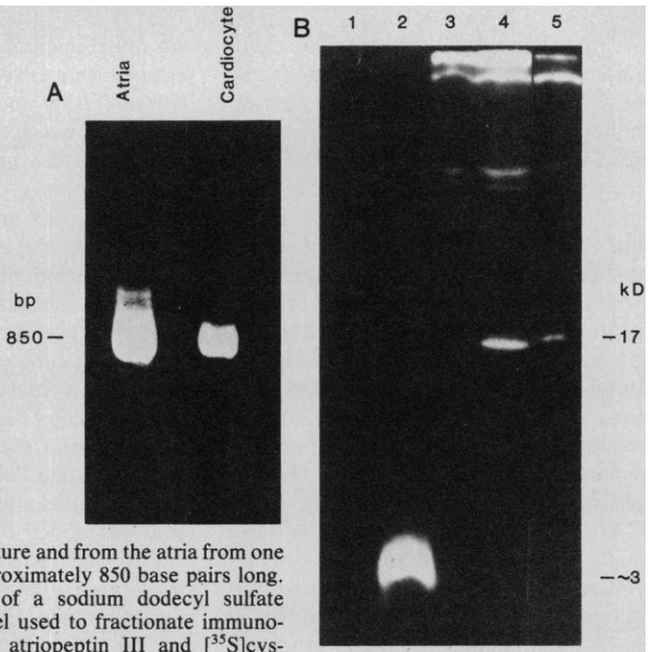

Fig. 2. Cultured cardiocytes contain ANF mRNA and synthesize ANF peptides. (A) An autoradiogram of Northern blot analysis of cardiocyte RNA demonstrates ANF mRNA. Atrial RNA (10 μg) and cardiocyte RNA (10 μg) were fractionated on a 1.3 percent formaldehyde-agarose gel, transferred to nitrocellulose paper, and hybridized to a ^{32}P-labeled Pst-I restriction fragment derived from a rat ANF cDNA (*4*). RNA was isolated from 1 × 10^6 cultured rat cardiocytes after 10 days in culture and from the atria from one rat. ANF mRNA is approximately 850 base pairs long. (B) An autoradiogram of a sodium dodecyl sulfate (SDS)-polyacrylamide gel used to fractionate immunoprecipitated ^{125}I-labeled atriopeptin III and [^{35}S]cysteine-labeled ANF peptides. Iodinated atriopeptin III was incubated with normal rabbit serum (lane 1) and antiserum to a synthetic peptide, preproANF residues 142 to 150 (lane 2). Culture medium from cardiocytes labeled with [^{35}S]cysteine (*17*) was incubated with normal rabbit serum (lane 3) and with antisera against both synthetic peptides, preproANF residues 142 to 150 (lane 4) and preproANF residues 81 to 92 (lane 5). Although other radiolabeled peptides are evident in lanes 3 to 5, only the 17-kD peptide is specifically precipitated by the ANF antisera but not by normal rabbit serum.

Fig. 1. The amino acid sequence of rat preproANF predicted by nucleotide sequence analysis of the rat complementary DNA (cDNA) (*4–7*). The structure of proANF, residues 25 to 150 (thin and bold underline), and atriopeptin III (Peninsula Laboratories), residues 127 to 150 (bold underline), are indicated. Synthetic peptides (*) corresponding to amino acid residues 81 to 92 and residues 142 to 150 were synthesized by the Merrifield solid-phase method, coupled to keyhole limpet hemocyanin, and used to immunize rabbits (*19*). Closed circles above the methionine residues indicate the residues at positions 13, 26, and 33 of the proANF molecule (Fig. 3). The amino acid residues are indicated in the single-letter amino acid code: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; and Y, tyrosine.

To determine the ability of the cultured cardiocytes to synthesize ANF, we examined their ANF mRNA and the ANF-related peptides they secreted into the culture media. The cardiocytes contain ANF mRNA for at least 10 days in culture (Fig. 2A). The ANF-related peptides that were released into the culture media were characterized by radiolabeling, immunoprecipitation, and fractionation on sodium dodecyl sulfate–polyacrylamide gels (Fig. 2B). A 17-kilodalton peptide was precipitated by antisera to ANF but not by normal rabbit serum. Only minute quantities of smaller ANF peptides were detected. Because antiserum to the preproANF peptide (residues 142 to 150) was able to immunoprecipitate a synthetic peptide related to ANF, atriopeptin III (Fig. 2B, lanes 1 and 2), and because atriopeptin III was not degraded by incubation with cardiocytes, we are convinced that small ANF peptides would have been detected had they been present. Based on these results, we conclude that the predominant secreted form of ANF was a 17-kD molecule rather than a smaller ANF molecule.

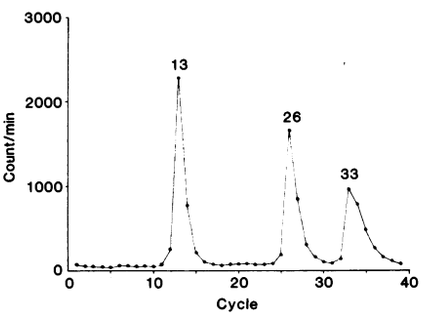

Fig. 3. Amino-terminal sequence analysis of the [^{35}S]methionine-labeled 17-kD peptide. The labeled ANF peptide was isolated from cardiocyte culture medium by immunoprecipitation and fractionation on an SDS–polyacrylamide gel (*17*). It was subjected to amino acid sequence analysis on a sequenator (Beckman 890C) in the presence of sperm whale apomyoglobin (10 nmol). The radioactivity released after each cycle of Edman degradation was measured by liquid scintillation spectrometry (count/min). The release of nonradioactive phenylthiohydantoin derivatives from the sperm whale apomyoglobin was measured to monitor the efficiency of Edman degradation. The location of the methionine residues is consistent with the structure of proANF (Fig. 1).

Three additional observations supported the idea that the 17-kD molecule was proANF. (i) The molecule was immunoprecipitated with two non–cross-reactive antisera to the preproANF peptides (Fig. 1 and lanes 4 and 5 in Fig. 2B). (ii) Nonradioactive atriopeptin III inhibited the binding of radiolabeled 17-kD molecule to antiserum to the preproANF peptide, residues 142 to 150. (iii) The location of methionine residues in the 17-kD molecule were determined (Fig. 3). Methionine residues were found at positions 13, 26, and 33 of the 17-kD molecule (Fig. 3) as predicted by the proANF sequence (Fig. 1). Although the sequence of the carboxyl terminus of the 17-kD molecule was not defined by these studies, the only ANF peptide isolated from rat atria that shares its amino terminal sequence with the 17-kD molecule is the 126-amino acid peptide, proANF. Furthermore, the 17-kD ANF peptide was found in cardiocyte extracts. Amino terminal sequence analysis of the ^{35}S-labeled 17-kD molecule isolated from cardiocyte extracts confirmed that this molecule is also proANF.

The advantage of a culture system in which the cells are producing the hormone is that one can determine the fate of radiolabeled precursors of the hormone (*8*). Cardiocytes were incubated with [^{35}S]cysteine for 10 minutes and were then incubated with nonradioactive cysteine for increasing time intervals (Fig. 4A). The 17-kD peptide was detectable in cardiocyte extracts after a 10-minute incubation with [^{35}S]cysteine. Thirty minutes after nonradioactive cysteine was added to cardiocyte cultures, radiolabeled proANF was detectable in culture media, and it steadily accumulated over 4 hours. During this interval, the quantity of labeled 17-kD peptide in cardiocyte extracts declined. There was no accumulation of smaller ^{35}S-labeled peptides in cells or culture media. Peptides of small size, which were occasionally immunoprecipitated in minute quantities, probably represent proteolytic degradation products of the 17-kD peptide generated during isolation.

Pulse-chase analysis of proANF in cardiocyte extracts and cardiocyte culture media shows that proANF is not converted to the circulating form of ANF in the cell culture system. These results suggest that proANF is cleaved to smaller ANF peptides in the blood rather than in the atria. To determine whether blood contains a protease that can convert proANF to smaller peptides, we incubated labeled cardiocyte culture medium containing proANF with rat serum (Fig. 4B), and the ANF-related peptides were isolated. The 17-kD protein was completely converted to a peptide of smaller molecular size, approximately that of the circulating form of ANF.

We showed that cardiocytes in culture synthesize and secrete proANF. We are convinced that the radiolabeled proANF detected in the culture media resulted from secretion and was not due to leakage from damaged cells. Approximately 50 percent of the proANF synthesized during a 10-minute incubation with [^{35}S]cysteine was released into the culture medium during a subsequent 4-hour incubation. During this interval, there was no increase in damaged cells as detected with fluorescent spheres coated with antiserum specific for myosin, a sensitive assay for myocardial cell injury (*20*).

Other investigators have speculated that processing of proANF generates peptides lacking the 21 amino acid residues shared by the ANF peptides (*7*). These other peptides would have escaped detection in the experiments described here because they lacked cysteine residues or because they were not recognized by the antisera. Experiments with appropriately labeled proANF produced by the cardiocytes in culture should determine whether these other peptides are generated. Cultured cells will also be useful for studies directed toward identifying the signals that stimulate release of ANF from the atria.

Our observations suggest that cultured cardiocytes lack the enzyme or enzymes necessary to cleave proANF and that proANF is synthesized in the atria and released before its conversion to the circulating form of ANF. Whether this conversion depends on a specific serum enzyme or one that cleaves a variety of peptides is unknown.

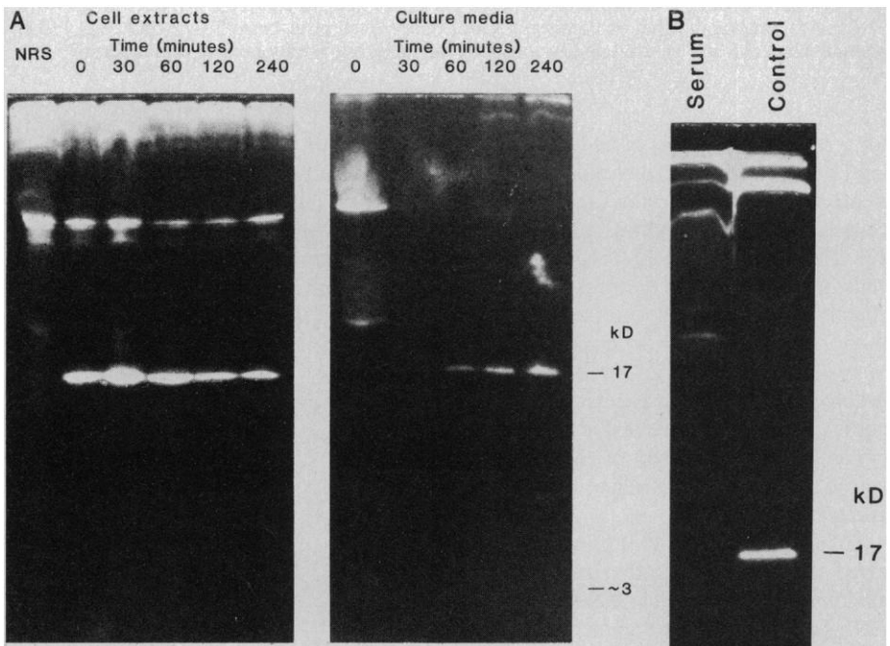

Fig. 4. Autoradiograms of SDS-polyacrylamide gels of pulse-labeled cardiocyte extracts and culture media (A) and of cardiocyte culture medium incubated with serum (B). (A) Five tissue culture wells, each containing approximately 5×10^5 cells, were incubated in RPMI 1640 without either cysteine or Nu-Serum for 30 minutes. The medium was replaced, and 200 μCi of [^{35}S]cysteine was added. After 10 minutes, the culture medium and cells from one culture were harvested (time 0). To the remaining cultures, "cold" cysteine was added to achieve a final concentration of 5 mM; culture medium and cells were harvested after 30, 60, 120, and 240 minutes of incubation. Harvested cells were lysed by the addition of 0.5*M* NaCl, 10 m*M* tris (*p*H 7.4), 0.5 percent Nonidet P-40, and protease inhibitors (*17*). For each time point, one-fifth of the cell extract or culture medium was incubated with antiserum to the preproANF peptide (residues 142 to 150). The cardiocyte extract that had been harvested at time 0 was also incubated with normal rabbit serum (NRS). (B) Equal volumes of [^{35}S]cysteine-labeled cardiocyte culture medium and rat serum were combined, incubated at 21°C for 60 minutes and at 37°C for 30 minutes, and immunoprecipitated with the antiserum to the preproANF peptide (residues 142 to 150) (Serum). ^{35}S-Labeled cardiocyte culture medium, treated with proteolytic inhibitors, was similarly immunoprecipitated (Control).

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17. Preparing cardiocyte cultures: 1-day-old rats were killed with ether anesthesia. Hearts were excised and transversely sectioned; sections containing atria were dispersed in 0.85 mg of trypsin per milliliter with 0.2 mg/ml EDTA, plated in Dulbecco's minimal essential medium without glutamine (Gibco), supplemented with 15 percent Nu-Serum (Collaborative Research) and 0.6 mg/ml thymidine, and placed at 37°C in a 5 percent CO_2 atmosphere (*16*). Ten neonatal rats yielded 0.5×10^7 to 1×10^7 cells. Radiolabeling cardiocytes: cultured cardiocytes were incubated in 1 ml of RPMI 1640 (Gibco) without either cysteine or Nu-Serum for 30 minutes. The culture medium was replaced, and 100 μCi of [^{35}S]cysteine (1000 Ci/mmol) was added. After a 3-hour incubation, the medium was harvested and centrifuged to remove cellular material. Nonidet P-40, Tris *p*H 7.4, aprotinin, and phenylmethylsulfonyl fluoride (PMSF) were added to the culture medium to yield final concentrations of 0.5 percent, 10 m*M*, 100 Kallikrein inactivated units (KIU) per milliliter, and 0.5 m*M*, respectively. Immunoprecipitation of proANF: immunoprecipitations were performed in phosphate-buffered saline with 0.1 percent Nonidet P-40, 100 μg of bovine serum albumin per milliliter, 100 KIU/ml aprotinin, and PMSF 0.5 m*M*. Atriopeptin III (Fig. 1), iodinated by the chloramine-T method [W. M. Hunter and F. C. Greenwood, *Nature (London)* **194**, 495 (1962)], or culture medium from [^{35}S]cysteine-labeled cardiocytes were incubated with antiserum for 1 hour at 21°C and 3 hours at 4°C. Antigen-antibody complexes were precipitated by incubation with goat antiserum to rabbit gammaglobulin at 4°C for 2 hours [S. G. Rockson, C. J. Homcy, E. Haber, *Circ. Res.* **46**, 808 (1980)]. The precipitates were solubilized and boiled in 3 percent sodium dodecyl sulfate (SDS) and 5 percent β-mercaptoethanol. Proteins were electrophoresed on a 17 percent polyacrylamide gel containing SDS; gels were dried and analyzed by autoradiography. The molecular sizes of the radiolabeled peptides fractionated on these gels were estimated by comparison with the electrophoretic mobility of protein standards (Bethesda Research Laboratories).
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Human Recombinant Granulocyte-Macrophage Colony-Stimulating Factor: A Multilineage Hematopoietin

Abstract. *Human recombinant granulocyte-macrophage colony–stimulating factor (GM-CSF) was tested for its ability to induce colony formation in human bone marrow that had been enriched for progenitor cells. In addition to its expected granulocyte-monocyte colony-stimulating activity, the recombinant GM-CSF had burst-promoting activity for erythroid burst-forming units and also stimulated colonies derived from multipotent (mixed) progenitors. In contrast, recombinant erythroid-potentiating activity did not stimulate erythroid progenitors. The experiments prove that human GM-CSF has multilineage colony-stimulating activity.*

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The survival, proliferation, and terminal differentiation of hematopoietic progenitors in vitro depends on cellular hematopoietic growth factors, also known as colony-stimulating factors (CSF's) (*1*). These factors are usually classified by the types of mature cells found in the colonies to which they give rise in response to the differentiation process they stimulate. Two murine factors have been purified to homogeneity, and their genes have been cloned and sequenced. One, interleukin-3 (IL-3) (*2*), influences multipotent (that is, mixed) progenitors and those with restricted lineage. This single factor has burst-promoting activity (BPA) for immature erythroid burst-forming units (BFU-E) and also stimulates the formation of granulocyte-macrophage colonies. A second murine factor, granulocyte-macrophage colony-stimulating factor (GM-CSF) (*3*), is required for the growth of granulocyte-macrophage colonies (CFU-GM). Both of these nonhomologous murine factors are now thought to be derived from T lymphocytes, but the full range of cells capable of their expression has not been determined.

Little is known about the biochemistry and biological activities of highly purified human CSF's. Both BPA and GM-CSF are produced by various human cell types, including T-lymphocytes (*4*) and monocytes (*5*). Until recently, neither factor had been purified to homogeneity, but studies of partially purified factors have not convincingly demonstrated that they have separate activities (*6*). The human GM-CSF gene was recently cloned from a Mo cell expression library (*7*). This human gene shows approximately 60 percent homology with its murine counterpart. We now report that recombinant human GM-CSF not only stimulates granulocyte-macrophage formation, but also has burst-promoting activity and stimulates the formation of multipotent colonies containing granulocytes, erythroid cells, monocytes, and occasionally, megakaryocytes.

To obtain complementary DNA (cDNA) clones that express biologically active human GM-CSF, we prepared cDNA's from membrane-bound messenger RNA's of lectin-stimulated Mo cells, constructed cDNA libraries in expression vectors, and screened the resulting plasmid pools by transient expression in monkey COS-1 cells (*7*). One of the plasmid vectors positive for GM-CSF in the screening assay was introduced by DNA transfection into a large number of COS-1 cells, which were then allowed to condition medium in the absence of serum. The recombinant GM-CSF produced in this manner had physical properties virtually identical to those of the natural protein obtained from Mo cell–conditioned medium (Mo-CM) (*7*) and was purified by gel filtration followed by reverse-phase high-pressure liquid chromatography (HPLC). The purified protein migrated as a single heterogeneous band with a molecular mass of 18 to 24 kD when analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and was estimated to be over 95 percent pure. The specific activity of the purified protein was 1×10^7 to 4×10^7 units per milligram in an agar bone marrow CFU-GM assay. (One unit is defined as the amount of GM-CSF that stimulates the formation of one colony per 10^4 cells above the background level when CSF is below saturation level.)