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Induction of Macrophage Tumoricidal Activity by Granulocyte-Macrophage Colony-Stimulating Factor

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Monocytes are a subpopulation of peripheral blood leukocytes, which when appropriately activated by the regulatory hormones of the immune system, are capable of becoming macrophages—potent effector cells for immune response to tumors and parasites. A complementary DNA for the T lymphocyte–derived lymphokine, granulocyte-macrophage colony-stimulating factor (GM-CSF), has been cloned, and recombinant GM-CSF protein has been expressed in yeast and purified to homogeneity. This purified human recombinant GM-CSF stimulated peripheral blood monocytes in vitro to become cytotoxic for the malignant melanoma cell line A375. Another T cell–derived lymphokine, γ-interferon (IFN-γ), also stimulated peripheral blood monocytes to become tumoricidal against this malignant cell line. When IFN-γ activates monocytes to become tumoricidal, additional stimulation by exogenously added lipopolysaccharide is required. No such exogenous signals were required for the activation of monocytes by GM-CSF.

MACROPHAGES ARE A HETEROGENEOUS population of cells involved in many aspects of immunity. Macrophages are required accessory cells in the development of specific immune responses mediated by T and B cells [reviewed in (*1*)]. In addition, macrophages themselves mediate nonspecific effector functions in the resistance to and eradication of microorganisms and neoplastic disease (*2*). The expression of these functions in vitro requires stimulation of monocytes or macrophages with an activating agent (*3, 4*). Substances capable of activating monocytes and macrophages include bacterial products such as lipopolysaccharide (LPS) or peptidoglycan (*3*) and, in addition, lymphokines secreted by activated T lymphocytes (*4*). Recent studies of such T cell–derived lymphokines have focused primarily on γ-interferon (IFN-γ). Treatment of macrophages in vitro with IFN-γ results in increased microbicidal activity, expression of cell surface antigens, and increased secretory activity (*5*). IFN-γ stimulates nonspecific tumoricidal activity of macrophages when LPS is present as an additional signal. Thus IFN-γ acts to prime the macrophage, making it more sensitive to the triggering effects of suboptimal amounts of LPS (*6*).

Whether molecules other than IFN-γ have the capacity to induce tumoricidal activity in macrophages has not been determined. Analysis of crude supernatants of cloned T cells or T-cell hybridomas (*7*) has generally failed to separate IFN-γ from any other lymphokines that activate macrophages to become tumoricidal. Although studies with antisera and monoclonal antibodies to IFN-γ have confirmed that IFN-γ activates macrophages, the existence of additional macrophage-activating lymphokines was not shown (*8*). However, investigators using biochemical fractionation of lymphokine preparations have been able to demonstrate the stimulation of macrophages to become tumoricidal by partially purified fractions devoid of detectable IFN (*9*).

Recombinant DNA technology combined with cellular bioassays in vitro has led to the cloning of the complementary DNA (cDNA) of several lymphokines, including IFN-γ (*10*). The availability of recombinant IFN-γ has confirmed the previous reports of the macrophage-activating properties of this lymphokine and the requirement for a second signal, such as LPS, to induce nonspecific tumoricidal activity (*8, 11*).

Recently, we (*12*) and others (*13*) reported cloning a cDNA for the human T cell–derived lymphokine granulocyte-macrophage colony-stimulating factor (GM-CSF). We report that purified recombinant human GM-CSF was capable of stimulating human peripheral blood monocytes to become tumoricidal against the human malignant melanoma cell line A375. GM-CSF cDNA was

Table 1. Differential requirement for LPS triggering of macrophage tumoricidal activity induced by GM-CSF and IFN-γ. Purified peripheral blood monocytes were prepared and cultured as described in Fig. 2 in the presence of lipopolysaccharide (LPS-W, Difco Laboratories), purified natural IFN-γ (Meloy Laboratories) or purified recombinant GM-CSF. After 24 hours, the culture medium was replaced, and [^{125}I]iododeoxyuridine-labeled A375 target cells were added. After an additional 72 hours, residual adherent A375 cells were harvested and counted. Units of IFN-γ are determined with a virus plaque reduction assay and comparison with an NIH international IFN-γ standard. GM-CSF units were determined as described by Cantrell *et al.* (*12*).

Lymphokine	Percent cytotoxicity with LPS (ng/ml) at				
	0	10.0	1.0	0.1	0.01
None	0	84	83	0	0
IFN-γ					
100 U/ml	0			40	
10 U/ml	0			28	
1 U/ml	0			25	
GM-CSF (500 CFU-C/ml)	63				

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cloned from a cDNA library prepared from messenger RNA (mRNA) of mitogen-stimulated peripheral blood T lymphocytes (*12*). The GM-CSF cDNA was expressed in yeast, with the α-factor promoter and leader sequences directing synthesis and secretion. The GM-CSF protein was purified from yeast supernatant by sequential reversed-phase high-performance liquid chromatography (HPLC) with a solvent system previously described (*14*). The resultant purified GM-CSF protein was analyzed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) as shown in Fig. 1. The two bands of GM-CSF represent glycosylated and nonglycosylated forms (*15*). This material was found to be free of endotoxin (less than 10 pg/ml) and was shown to induce peripheral blood monocytes to express tumoricidal activity against A375 target cells (Fig. 2A). Murine interleukin-3, produced in the same yeast expression system as GM-CSF, was found to be negative in this assay. Homogeneous human interleukin-2 did not induce cytolytic activity in these cultures, indicating that neither T cells nor NK cells were responsible for the observed activity. In addition, monocytes activated by GM-CSF were also able to lyse several other tumor targets, including a murine melanoma and a human bladder carcinoma. The same GM-CSF preparation was also assayed for the stimulation of human bone marrow cells to grow as granulocyte and macrophage colonies (Fig. 2B).

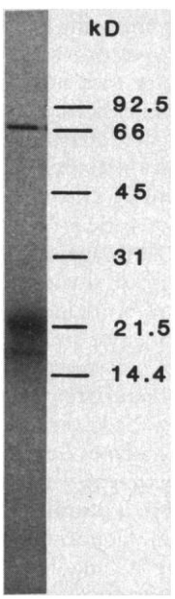

Fig. 1. Polyacrylamide gel electrophoresis of recombinant GM-CSF. Human GM-CSF cDNA was cloned and expressed in yeast as reported (*12*), with the yeast α-factor promoter and leader sequences used to direct secretion. GM-CSF was purified from yeast supernatant by HPLC by means of a solvent system previously described (*14*). The peak fraction, used for biologic assays in Fig. 2 and Table 1 was analyzed by SDS-PAGE and silver stained. This fraction contains 10 μg of protein per milliliter, with a specific activity of 5×10^5 CFU-C (colonies containing ≥50 cells) per microgram. Two GM-CSF bands (21.5 kilodaltons and 16 kilodaltons) represent glycosylated and nonglycosylated forms. The 66-kilodalton band is an artifact of the gel and not a contaminant of the preparation.

The capacity of GM-CSF to activate monocytes to lyse A375 target cells was independent of stimulation with suboptimal amounts of LPS. This was in contrast to the capacity of IFN-γ to activate monocytes, which required exogenous addition of suboptimal amounts of LPS in order to achieve lysis of tumor target cells (Table 1). Thus, GM-CSF, unlike IFN-γ, is capable of activating monocytes to become tumoricidal without any additional signal. Furthermore, LPS did not potentiate cytolytic activity induced by suboptimal amounts of GM-CSF.

Colony-stimulating factors are identified by their ability to stimulate the in vitro proliferation of hematopoietic cells from progenitors. The growth of different types of hematopoietic colonies in semisolid agar from committed progenitor cells is absolutely dependent on the presence of CSF's. Various lineages of differentiated cells arise in the presence of distinct CSF's.

The growth-promoting effects of these factors on hematopoietic precursors and mature cells was thought to be their primary function. However, it is clear that CSF's also affect survival and differentiation. Earlier studies have indicated that GM-CSF can induce antibody-dependent cellular cytotoxicity (*16*) and the killing of schistosomula by neutrophils and eosinophils (*17*), and the intracellular killing of *Leishmania* by macrophages (*18*). We have demonstrated that GM-CSF in vitro can potentiate antibody responses by activating an accessory function of murine splenic macrophages (*19*). CSF-1 has also been shown to induce antitumor activity in murine macrophage cultures (*20*).

The identification of CSF as the active moiety in these assays has generally depended on the biochemical purification of CSF with the factor active in the particular assay being studied. The purification of homogeneous protein from culture supernatants is difficult and may not provide sufficient material for verification of its biologic activities. By using homogeneous protein produced by yeast from a recombinant cDNA clone, we demonstrated a previously unreported biologic property of GM-CSF.

The apparent differences in the activation of monocytes by IFN-γ and GM-CSF suggest that distinct mechanisms or perhaps different monocyte subpopulations may be involved. One model (*21*) indicates that IFN-γ acts via the Ca^{2+}-dependent induction of protein kinase C activity, which may be further augmented by a diacylglycerol-like action of LPS in the cell membrane. The macrophage-CSF receptor has been shown to be identical to the c-*fms* proto-oncogene, which is a tyrosine kinase (*22*). If the GM-CSF receptor is also a

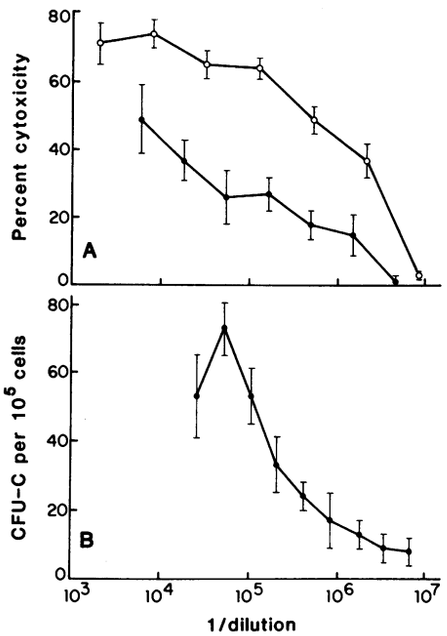

Fig. 2. (A) Peripheral blood monocyte activation by GM-CSF for tumor cytotoxicity in vitro. Human peripheral blood monocytes from two different donors (○ and ●) were prepared from Ficoll-Hypaque–purified peripheral blood leukocytes by Percoll density gradient centrifugation (*23*). These cells were allowed to adhere in 96-well culture plates for 1 hour in RPMI with 5% fetal bovine serum. After three washings to remove nonadherent cells, the adherent population was found to be greater than 95% pure monocytes as judged by Wright-Giemsa stain. After treatment of purified monocytes in vitro for 24 hours with the indicated dilution of purified recombinant GM-CSF, the culture medium was replaced and [^{125}I]iododeoxyuridine-labeled A375 target cells were added for an additional 72 hours at which time the residual adherent A375 cells were harvested and counted (*24*). Earlier studies (*24*) showed that more than 90% of the ^{125}I present in the supernatants of these cultures is in soluble form and the rest is associated with dead cells and debris. The GM-CSF used contained 10 μg of protein per milliliter, with a specific activity of 5×10^5 CFU-C/μg. Percentage cytotoxicity was calculated as [1 − (counts per minute in target cells cultured with activated monocytes/counts per minute in target cells cultured with control monocytes)] × 100. (B) Stimulation of granulocyte-macrophage colony formation by GM-CSF. Human bone marrow cells were purified by Percoll density gradient centrifugation and were cultured at a concentration of 10^5 cells per milliliter of semisolid agar (*12*) containing the indicated dilution of purified recombinant GM-CSF. After culture for 14 days, CFU-C were counted. Data are expressed as the mean of triplicate cultures ± SD.

tyrosine kinase, then IFN-γ and GM-CSF would have different pathways of action.

The regulation of macrophage activation by GM-CSF provides a mechanism whereby T lymphocytes, in response to antigen, may regulate a nonspecific effector function of macrophages in the absence of any requirement for an exogenous signal provided by bacterial products. This may therefore represent an important pathway of antitumor defense.

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The Neuroendocrine Thymus: Coexistence of Oxytocin and Neurophysin in the Human Thymus

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Immunoreactive oxytocin and neurophysin were identified and measured by radioimmunoassay in human thymus extracts. Serial dilutions of extracts paralleled the appropriate standard curves. Thymus-extracted oxytocin and neurophysin eluted in the same positions as reference preparations on Sephadex G-75. Authenticity of oxytocin was confirmed by biological assay and high-performance liquid chromatography analysis. In most instances, thymus contents of oxytocin and neurophysin were far greater than those expected from known circulating concentrations and declined with increasing age. The molar ratio of oxytocin to neurophysin in thymus was similar to that found in the hypothalamo-neurohypophyseal system, which strongly suggested with the other data a local synthesis of oxytocin. These findings indicate the presence of neurohypophyseal peptides in the human thymus and further support the concept of a neuroendocrine function integrated in an immune structure.

RECENT STUDIES HAVE PROVIDED evidence of reciprocal interactions between the neuroendocrine and immune systems (*1*). In this respect, thymic hormones, besides their known immunological properties, have been found to modulate some important hypothalamo-hypophyseal functions (*2*). In the thymus, distinct cell populations have been identified through the use of monoclonal antibody A_2B_5, which recognizes a complex ganglioside expressed on the membrane of neurons and neural crest-derived and neuropeptide-secreting endocrine cells (*3*). The thymus contains mesenchymal cells, probably of neural crest origin; the development of thymic epithelium may depend on induction by neural crest mesenchyme (*4*). To our knowledge, the presence of neurohormones in the thymus has not been systematically investigated until now.

Oxytocin is a 1000-dalton nonapeptide synthesized in hypothalamic magnocellular neurons as a large molecular weight precursor which is then cleaved during axonal transport into oxytocin and a 10,000-dalton oxytocin-related carrier protein, neurophysin (*5*). The main physiological actions of oxytocin in mammals are the stimulation of milk ejection and uterus contractions. Oxytocin and neurophysin are widely distributed in the central nervous system (*6*) and in peripheral organs such as ovary, testis, and adrenal medulla (*7*). These data led us to investigate the presence of oxytocin and neurophysin in the human thymus.

Thymus tissues were obtained from six patients undergoing cardiovascular or thoracic surgery for different reasons (Table 1): therapeutic thymectomy for myasthenia gravis (patient 1), coronary bypass (patients 2 and 3), anterior mediastinal mass (patient 4), and congenital heart disease (patient 5). One thymus was excised at autopsy of a newborn who died after acute respiratory distress (patient 6). Care was taken to obtain thymuses from patients in early childhood to advanced adult age; no other selection method was used. In most cases, thymic fragments were histologically analyzed (Table 1). The anterior mediastinal mass was found to be a thymoma (patient 4). In patient 1, further routine immunohistological analyses revealed a normal distribution of lymphoid phenotypes Leu-1 to Leu-4.

Thymic specimens were weighed, finely minced in a dish on crushed ice, and homogenized by sonication in 0.4*M* acetic acid at 4°C (1 ml per 100 mg of tissue). Homogenates were centrifuged at 13,000*g* for 20 minutes at 4°C; then supernatants were separated and filtered to remove insoluble particles. When mentioned further, the term "crude extract" will refer to this preparation step. An additional extraction procedure was carried out before we measured immunoreactive or biological activities and subjected the extract to high-performance liquid

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