Order Number 9009715

Enzyme expression and morphological maturation of myeloid cells by cancer-derived polypeptide factors

Bailey, Steven Clark, Ph.D.

City University of New York, 1989

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

ENZYME EXPRESSION AND MORPHOLOGICAL MATURATION OF MYELOID CELLS BY CANCER-DERIVED POLYPEPTIDE FACTORS

by

STEVEN CLARK BAILEY

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1989

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

6/2/89
Date

Chair of Examining Committee

June 2, 1989
Date

Executive Officer

Dr. Marion Orlowski

Dr. Sherwin Wilk

Dr. Jeffrey Lipton

Dr. George Weber

Supervisory Committee

The City University of New York

ABSTRACT

ENZYME EXPRESSION AND MORPHOLOGICAL MATURATION OF MYELOID CELLS BY CANCER-DERIVED POLYPEPTIDE FACTORS

BY

STEVEN CLARK BAILEY

ADVISOR: Professor Olga Greengard

Investigations were carried out on mammary carcinoma 5A (MC) with the objective of elucidating mechanisms underlying alterations in bone marrow function caused by non-hematopoietic malignancies. The progressive elevations in γ-glutamyltanspeptidase (GGT) and alkaline phosphatase (AP) concentrations resulting from subcutaneous MC transplantation to rats were found to be reproducible in normal bone marrow cells by incubation with serum from MC hosts (MC-serum). A bioassay system in vitro could thus be established for the first time to examine the nature and provenance of blood-borne factors hypothesized to be responsible for enzymic abnormalities in "uninvolved" tissues of cancer hosts. The GGT and AP inducing factor(s), a heat-stable, α-chymotrypsin sensitive, 60,000 molecular weight polypeptide, was found to be also elaborated by the carcinoma in vitro, and preparations with 200,000 times higher specific activity than in the

MC-serum were obtained from the MC-conditioned medium (MC-CM).

Nuclear hypersegmentation, found in 30-50% of the mature blood granulocytes of MC-bearing rats, was also evoked in bone marrow from normal animals: after 48 hr incubation with MC serum or MC-CM preparations (but not with colony stimulation factors, CSFs now shown to induce both GGT and AP expression) half the mature neutrophils were hypersegmented. There were also decreases in the % of myelobasts, promyelocytes, myelocytes, and metamyelocytes, with an increase in that of mature neutrophils. MC-CM exerted differentiational effects and growth inhibition in the WEHI-3 mouse myelomonocytic cell line and in primary culture of rat Shay leukemia cell, increased 40-fold the GGT content of the Shay cells, and stimulated AP expressions in leukemic cells from human subjects.

These investigations 1) demonstrate the elaboration by the mammary carcinoma of a polypeptide promoting the maturation of normal and leukemic myeloid cells, 2) describe the first enzymic response of bone marrow to CSFs, 3) introduce a new experimental system for the study of biochemical and morphological granulocyte maturation, and 4) provide evidence that neutrophil hypersegmentation can arise from the action of tumor-elaborated polypeptides on normal non-mitotic myeloid cells in the bone marrow.

ACKNOWLEDGEMENTS

I would like to thank all the people who with their help and support made this thesis possible. While their have been many who have played important parts, I can only mention those who have had starring roles and I dedicate this thesis them.

To my thesis advisor, Dr. Olga Greengard. Her ideas and advise form the basis of all of this work. Her patience with me is perhaps the greatest of all involved. Over the past 5 years, Olga has molded and formed this impetuous youngster into the scientist that I am today. She allowed me to explore enough avenues and yet kept me with my eye on the "prize". To her I will be forever indebted.

To my wife, Margie, who stood beside me throughout and held my hand during the "birth". Without her support, both literally and figuratively, I feel that it would have been impossible to carry this project to its completion. Her love and patience was the cornerstone. I love her eternally.

To my parents, Clark and Phyllis, whose love and ideals have served me well throughout my life. They supported and prodded me along the way, and have been there for me throughout all the good and bad times.

To my grandparents, especially my grandfather, Donald

P. Roehm, who have impressed upon me the value of education and that the thirst for knowledge is perhaps one of the noblest pursuits. My grandfather, throughout my life has been perhaps the strongest role model that I have had. His love of science was infectious and has formed the basis of my life.

To my laboratory partners, Drs. Jonathen Head, Sandra Russo, Ruth McChesney, Oya Tugal and Girija Bhargava, without whose help and support this thesis would barely be more than an idea. The atmosphere that they and Dr. Greengard created was very instrumental in the creative process which formulated the experiments contained herein.

To my fellow graduate students, Ron Buono, Melissa Anderson and the "Mud-Fudds", Drs. Dan Weiss and Saul Karpen, whose support was extremely appreciated.

To Dr. Samual Waxman, who provided much advise and support throughout my tenure at Mt. Sinai. He provided me with human bone marrow samples and shared his clinical expertise at a critical time in this thesis.

To the memory of Dr. Horace Hodes, whose friendship and brilliance will not be forgotten by me.

Boston, 1989

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

ABBREVIATIONS

AP.....Alkaline phosphatase
ACTH.....adrenocorticotrophic hormone
ADH.....antidiuretic hormone
AML.....acute myelogenous leukemia
AMP.....adenosine monophosphate
ATP.....adenosine Triphosphate
CFU-S.....colony forming unit- spleen
CML.....chronic myelogenous leukemia
CSA.....colony stimulating activity
CSF.....colony stimulating factor
CSF-L.....L-cell conditioned medium
CSF-GCT...giant cell tumor conditioned medium
DMF.....dimethyl formamide
DMSO.....dimethylsulfoxide
DNA.....deoxyribonucleic acid
DT.....doubling time
EGF.....epidermal growth factor
G-CSF.....granulocyte colony stimulation factor
GM-CSF....granulocyte/macrophage colony stimulation factor
GGT.....gamma-glutamyltranspeptidase
hCG.....human chorionic gonatotropin
h.....human
hr.....hour
hyperseg..hypersegmented
IL-3.....interleukin-3
MC.....mammary carcinoma
MC-CM.....mammary carcinoma-conditioned medium
MC-S.....mammary carcinoma bearing rat serum
MC-T.....mammary carcinoma extract
MDS.....myelodysplastic syndrome
mGM-CSF...murine GM-CSF
MW.....molecular weight
NBT.....nitro blue tetrazolium
PTH.....parathyroid hormone
RA.....retinoic acid
rhG-CSF...recombinant human G-CSF
TGF.....transforming growth factor
TK.....thymidine kinase
TPA.....tetradecanoyl phorbol acetate
Vit D_3....vitamin D_3
WBC.....white blood cell

Chapter I

BACKGROUND

Clinical studies on neoplastic disease have provided extensive evidence that the host organism can undergo profound alterations before the tumor is sizable or invades vital organs. It is often times these alterations, referred to as "paraneoplastic syndromes", that prove to be fatal. Ruddon (1987) estimates that some 70% of cancer patients experience one or more of these ill understood effects of tumors. They include anorexia, anemia, fever, leukocytosis, loss of immunocompetence, as well as neurological and endocrine problems. Functional abnormalities and tissue wastage or hyperplasia are often exhibited by organs anatomically distant from the tumor or its metastases. These observations led to the hypothesis that many of the changes in the host organism are brought about by substances released by tumors to the systemic circulation (Greenstein, 1947).

In subsequent years evidence has been obtained that paraneoplastic syndromes specifically associated with some tumors originating from neuroendocrine cells are attributable to the unique ability of these tumors to elaborate excessive amounts of known regulators such as ACTH, epinepherine, insulin, or serotonin (Liddle & Ball, 1973). However these tumors are rare. Much attention has focussed on effects which are common to the majority of

tumors especially on cachexia which accounts for 95% of the morbidity of cancer patients (Waterhouse, 1963). The associated wastage of body mass has been attributed to a loss of appetite (DeWys, 1974) as well as to the parasitic nature of tumors, i.e. to the thermodynamic cost of supporting a growing neoplastic mass (Morrison, 1971; Theologides, 1972). However, detailed studies on cancer patients led to the conclusion that body weight loss bears no necessary correlation to tumor burden or food intake, and that the problem lies in the utilization rather than merely in the availability of nutrients (Costa & Donaldson, 1979). The possible effect of cancer on enzymes regulating metabolic processes was thus of interest and, with the availability of many transplantable animal tumors causing cachexia-like effects, became the subject of extensive research. Essentially all the information about altered biochemical composition of host tissues originates from these investigations. A summary of the salient results is, therefore, the first section of the background review to be presented.

Enzymic changes in uninvolved tissues of cancer hosts.

Researchers in the late 1940's have noticed depressed liver catalase activity in animals with different types of tumors (Nakahara & Fukuoka, 1948; Lucke et.al., 1952; Adams & Roe, 1953). Nakahara postulated that this

depression was due to the elaboration of a toxic substance by cancer cells. They isolated a fraction of tumor tissue that when injected in normal rats caused diminished hepatic catalase activity. The active substance which they called "toxohormone" was water soluble, ethanol precipitable and thermostable. Parabiosis experiments (e.g. Lucke et.al., 1952) supported the postulate that a blood borne factor is responsible for the effect of tumors on the liver. However, the phenomenon could not be reproduced in vitro, and attempts that different investigators made to characterize the factor whereby tumor extracts evoke the enzyme changes in vivo led to contradictory results. The toxohormone research concept has thus been discarded, and non-specific consequences of tumor transplantation (such as bacterial infection, malnutrition, or stress-associated hormonal changes) have been suspected to be responsible for the depressed hepatic level of catalase and other enzymes.

In subsequent studies, the changes in host liver were found to be also manifested in increased levels of some enzymes, including those of the fetal isozyme of pyruvate kinase, and it became clear that the many enzymes involved were quite heterogeneous in terms of function, regulatory properties, and subcellular location. Nevertheless, with information eventually obtained about the biochemical composition of the liver during normal ontogeny, it was

possible to discern a common pattern behind the alterations in host livers (Greengard, 1971), namely a partial undifferentiation of their enzymic composition. For, "adult" enzymes (i.e. those emerging at late stages of ontogeny) were the only ones at diminished titers, while "fetal" enzymes (i.e. those decreasing in the course of normal development) were the ones showing inceased levels in the liver of animal (Herzfeld & Greengard, 1972; Suda et.al., 1966) and human subjects (Herzfeld et.al., 1980) with various extrahepatic cancers. A hepatic enzyme pattern akin to that in immature liver is unlikely to adequately serve the adult organism, and thus explains in part the metabolic abnormalities seen in cancer patients. The early onset of some of the hepatic enzyme changes (seen before the tumors are palpable) also has some diagnostic implications, in that tests of hepatic functions can provide a non-invasive means of revealing the presence of latent cancers. The consequences that relatively small quantitative enzymic changes can have is illustrated by observations on drug metabolism. In tumor-bearing rats with only a 30% reduction on the microsomal cytochrome-c reductase, the duration of hexobarbital hypnosis was appreciably longer than normal, and further loss of enzyme with increasing tumor size was paralleled by a progressive increase in sleeping time (Greengard, 1979). The hepatic enzymes and sleeping times reverted to

normal within a few days after surgical eradication of the tumor, except in rats where subsequent autopsy revealed tumor regrowth; thus the length of sleeping time revealed whether surgery was successful or failed to remove all cancer cells.

Organs of cancer hosts other than the liver have also been reported to exhibit enzymic changes. Thymidine kinase (TK) is elevated in the spleen, and blood cells of rats with subcutaneous lymphoma show an increase in TK and in pyroline 5-carboxylate reductase (Herzfeld & Greengard, 1977). More recently, it has been observed that transplantation of a rat mammary carcinoma resulted in striking increases in gamma glutamyl transpeptidase (GGT) and alkaline phosphatase (AP) in liver, hematopoietic tissue, lung, but not in kidney, and intestine (Koss & Greengard, 1982). In the bone marrow and the spleen this was attributable to the granulocytic elements, since granulocytes were the blood cells showing increase in number as well as GGT and AP content. In subsequent studies it became possible to reproduce for the first time <u>in vitro</u> enzymic changes caused by tumors in distant host tissues: stimulation of GGT and AP expression in bone marrow cells from normal animals was achieved by their incubation with serum from rats bearing mammary carcinoma 5A (Greengard et.al., 1984). The present investigations originate from the above studies on the effect of mammary

carcinoma on granulocytosis and induction of GGT and AP. Therefore some salient background information on these two enzymes is presented in the following paragraphs, and the proliferative effect of tumors with special reference to granulocytosis will be the subject of subsequent sections of the introduction.

Gamma-glutamyltranspeptidase (GGT) is a membrane bound glycoprotein that catalyzes the transfer of the gamma - glutamyl moiety of glutathione to the alpha amino group of a variety of amino acids or peptide acceptors, forming gamma-glutamyl peptides (Orlowski & Meister, 1970). It is the only known enzyme capable of initiating the breakdown of glutathione to its component amino acids (Meister & Tate, 1976; Abbott et.al., 1984). This is now considered part of what Meister has described as the Gamma-glutamyl cycle and this transfer of gamma-glutamyl groups is thought to be important in the transport of amino acids (Meister, 1973; Orlowski & Meister, 1970, Bridges & Meister, 1985; Vina et.al., 1983). It is noteworthy that expression of GGT is increased in tissues where adsorptive and secretory functions are performed such as kidney, pancreas, intestine, and mammary gland (Curthoys & Hughey, 1979; Orlowski et.al., 1974; Vina et.al., 1983). However Inoue et.al (1977) dispute its role as specific inhibitors of GGT failed to affect the rate of transport of certain amino acids.

The quantitative changes that GGT undergoes during biological processes shows striking tissue differences. One type of behavior is exhibited in tissues which, like kidney, intestine or pancreas, perform secretory or adsorptive functions important to the body as a whole. The concentration of GGT rises precipitously during the critical early postnatal period of functional maturation of these tissues. Its titers in normal fetal, and also neoplastic, variant of kidney or pancreas is much lower than in mature ones (Fujiwara et.al., 1982). Thus in these systems GGT is looked upon as a marker of "differentiation." The other type of behavior (on account of which it is sometimes referred to as a marker of "dedifferentiation") is exemplified by hepatic tissues: fetal or neonatal liver, and also hepatocellular carcinomas exhibit much higher GGT content than the cognate mature organ (Cheng et.al., 1978; Fujiwara et.al., 1982, Bodanszky et.al., 1980). This duality in behavior suggests that in the mature kidney, intestine or pancreas, GGT subserves a special differentiated function (requiring orders of magnitude of higher activity than elsewhere), and that it also subserves other processes (e.g. proliferation) that most cell types have to undergo at some time or another. Increases in the specific activity of GGT accompany many hyperplastic reaction, such as that of seminal vesicles (DeLap et.al., 1975), endometrium

(Tarachand & Eapen, 1982), and mammary glands (Pocius et.al., 1980) upon treatment with appropriate hormones, and of liver regenerating after partial hepatectomy (Cameron et.al., 1978).

Increased GGT activity was also observed in mitogen stimulated lymphocytes (Novogrodsky et.al., 1976). The involvement of GGT in these processes may be related to its role in amino acid transport or the breakdown of extracellular glutathione so that its component amino acids can be transported into the cell. Glutathione, which is essentially unable to cross the plasma membrane (Meister & Tate, 1976) is one of the major storage pools of cysteine in the body (Meister, 1984). In the case of L1210 cells both of these are essential substrates for cell division (Ishii et.al., 1985).

Alkaline phosphatase (AP) is probably one of the most extensively studied enzymes. It is capable of acting on a wide variety of substrates with phosphate esters, including proteins and nucleic acids. It is not clear, however, which of the many potential functions of AP are of major physiological importance, although it does appear to be essential for the transport of otherwise insoluble substrates by the removal of the phosphate group (McComb et.al., 1979). Isozymes of AP are useful markers in the diagnosis of some cancers (Balinsky, 1987), but again, there is no clear information on its involvement in tumor

growth or neoplastic transformation. AP often exhibits a localization not very different from GGT. Both are present in membranes important in transport, and it not entirely unexpected that GGT and AP would respond in concert to proliferative or differentiative changes.

Tumor-elaborated growth factors

Some of the other effects caused by neoplasms include increased mitotic frequency of hepatocytes, hyperplasia of bone marrow, as well as increases in lung weight accompanied by increases in DNA content (Greengard & Head, 1987). The underlying mechanism is not known but there is evidence that tumors are capable of secreting a variety of substances that can stimulate the proliferation of both normal and cancer cells. Transforming growth factors (TGFs) are being studied most extensively (Sherwin et.al., 1983; Nickell et.al., 1983). The biological role of these TGFs is thought not to lie in their tumorigenicity as their name would imply, but rather in providing a constant stimulus for cell proliferation (Roberts et.al., 1981). Some tumors are known to secrete abnormal amounts of the endocrine factors adrenal corticotrophic hormone (ACTH), antidiuretic hormone (ADH), human chorionic gonadotrophin (hCG), parathyroid hormone (PTH), or insulin-like substances. These give classical symptoms and often aid in the detection and differential diagnosis of the cancer

(Ruddon, 1987; Liddle & Ball, 1973). These cancers may overproduce more than one hormone resulting in a complex paraneoplastic syndrome (Rees et.al., 1974; Hirata et.al., 1976). Tumors of the pancreas are especially prone to this, secreting glucagon, gastrin and vasoactive intestinal peptide (Wynick et.al., 1988). Other tumor elaborated substances that have sparked research interest are the angiogenesis factors. These agents are capable of stimulating the formation of new blood vessels. One such substance angiogenin, found to be secreted by colon cancer cells, causes blood vessel development in both normal organs and tumors (Fett et.al., 1985). In addition, fibroblast growth factor and TGF β are also capable of being angiogenic (Bicknell & Vallee, 1988). The therapeutic potential of these as stimulators of microvascular vessel formation for tissue grafting or revascularization remains to be exploited.

Tumors of non-hematopoietic origins also can have effects on blood cell production. These range from anemia, thrombocytopenia, leukopenia to leukocytosis. Leukocytosis due to tumor growth is a well-documented phenomenon in both animals (Delmonte et.al., 1966; Liebelt et. al., 1974; Lee et.al., 1980; Thomas et.al., 1985) and man (Chen & Walz, 1958; Asano et.al., 1977). This leukocytosis is often quite pronounced, up to 500,000 WBC/mm^3 of blood. Since it is not attributable to

infection, it was postulated (Chen & Walz, 1958) to be due to a tumor-elaborated substance.

Delmonte et.al. (1966) isolated a granulocytosis promoting factor from a mouse mammary tumor which was a low molecular weight (<2000), heat stabile, protein-free micromolecule. They also found evidence for a high molecular weight (55,000) heat stable substance capable of causing granulocytosis. Robinson (1974) reported that the serum and urine of patients bearing granulocytosis inducing tumors contained bone marrow colony stimulating activity (CSA). Lipton and Sachs (1981) isolated 3 factors from Krebs ascites tumors. The two with 23,000 and 25,000 MW, respectively are capable of inducing granulocyte-macrophage colony formation in the spleen in vivo, and a 45,000 MW species capable of inducing leukemia cells to differentiate in vitro.

Kovacs et.al. (1985) reported that mice bearing lung carcinoma, exhibited an expansion of proliferative immature granulocytes and macrophages in the bone marrow. They also observed an increase in primitive erythroid progenitors with a decrease in more mature elements. This was borne out by the decrease in ^{59}Fe incorporation. All of these effects could be mimicked by injection of mice with tumor extracts. This confirmed earlier work by Lee et.al. (1980) that mice bearing carcinomas had increased numbers of progenitor cells in the marrow. Since these

tumors did not invade the bone marrow, they must have acted via substances secreted by these tumors, i.e. hematopoietic growth factor or as they are now known, colony stimulating factors (CSFs).

The present study involves such a granulocytosis producing tumor, which secretes a factor with maturational effects on normal and leukemic myeloid cells. For these reasons salient background information on blood cell production and CSFs is presented, as well as a short review on agents causing leukemic cell differentiation.

Granulopoiesis

The development of mature granulocytes from precursor stem cells is a complex phenomenon. Granulocytes arise from the bone marrow, which is one of the largest organs in the body and, in humans, the principal site of most blood cell formation. Hematopoiesis is extremely rapid as billions of red cells, platelets, and granulocytes are produced each day (Golde & Cline, 1977). The bone marrow is also responsible for monocyte and lymphocyte production. Studies over the last twenty years have shown that the differentiated end cells have finite life spans and are without the capacity of self renewal. Only in their immature state are they capable of proliferation. Continuous cell production thus requires the presence of stem cells capable of both self replication and

differentiation (Lajtha, 1979).

Stem cells constitute a pool of self-perpetuating cells ranging from multipotential or pluripotent to "committed" cells destined to be differentiated into a particular cell type. The multipotential stem cell is often given the designation CFU-S (colony forming unit-spleen). This term comes from the assay for these cells which consists of the intraperitoniel injection of bone marrow cells into irradiated mice and counting of the colonies formed in the spleen. During the first 4-5 days these cells remain undifferentiated indicating their pluripotent nature (Curry & Trentin, 1967). Later differentiation takes place resulting in the development of committed stem cells capable of terminal maturation.

Although little is known of the factors inducing pluripotent stem cell differentiation, the stromal environment of the marrow is thought to play a very important regulatory role. A well accepted view is that within the hematopoietic microenvironment there are protective "niches" which maintain the character of the stem cells and inhibit their response to differentiation stimuli, whereas outside of these "niches", cells are free to respond (Schofield, 1978; Dexter, 1987). It is also thought that some of the growth factors effecting the proliferation of stem cells are on the surface of the stromal cells, and that the stem cells must be attached to

the stroma in order to respond to the bound factor (Roberts et.al., 1987). The stromal extracellular matrix can, however, bind growth factors to present to their respective target cell also (Gordon et.al., 1987).

Committed stem cells have been extensively studied using the semisolid medium cell culture technique (Bradley & Metcalf, 1966). This technique revolutionized hematologic growth factor research. The cells are thought to respond to lineage specific growth factors resulting in the formation of terminally differentiated cells. It appears that each cell type has its own lineage specific growth factor, such as granulocyte-colony stimulation factor (G-CSF), macrophage-colony stimulating factor (M-CSF), erythropoietin (EPO), thrombopoietin (TPO), eosinophil-colony stimulating factor (EO-CSF). The stages that the different cell lineages go through as they differentiate toward their particular end cell has been fairly well characterized.

Neutrophil development

The stages of blood cell differentiation have been defined in the past by microscopic criteria alone, primarily by the appearance or disappearance of nuclei and of cytoplasmic elements recognizable by histological staining. Shilling (1911) has thus divided the development of the neutrophil into six morphological substages. This

staging, though arbitrary in the sense that differentiation is a continuous process, has provided a basis for analysis by modern methods. For example, ultrastructural studies describing the sequential acquisition of different cytoplasmic granules (Bainton et.al., 1971) and monoclonal antibodies detecting stage-characteristic surface proteins (Fitchen & Cline, 1979; Ross et.al., 1978; Griffin et.al., 1981) have permitted more sophisticated analysis of the process of neutrophil maturation.

Histochemical assay for enzymes has been another tool for discriminating not only between different blood cells but also between stages in the development of the same cell type. By using substrates which cleave to products capable of complexing to dyes, it has been possible to specifically stain cells of a particular lineage or maturational state (Ackerman, 1962; Phillips et.al., 1983).

The earliest stage of neutrophil maturation in which enzymes have proven useful as a classification criterion is the promyelocyte. Azurophilic granules, which are in abundance in the promyelocyte and represent its predominant structural feature, are characterized by richness in lysozomal enzymes. These include acid phosphatase, myeloperoxidase, indoxyesterase, arylsulfatase, and β-glucuronidase (Ackerman, 1964; Wetzel

et.al., 1967; Bainton & Farquhar, 1968a,b; Bainton et.al., 1971).

In immature neutrophils, enzymes involved in DNA synthesis are elevated indicating the proliferative status of these cells. DNA polymerases (Rabinowitz, 1966), as well as deoxycytidine kinase an enzyme in the salvage pathway of pyrimidine synthesis (Coleman, et.al., 1975), are elevated. Cytidine deaminase activity, on the other hand, is low in the immature granulocyte and increases with maturity.

The myelocyte is considered the last stage which is capable of proliferation (Bond et.al., 1959; Rubini et.al., 1960), and it is during and after this stage that the most extensive morphological alterations of the immature neutrophil occur. The nucleolus, present in the nuclei of both the myeloblast and promyelocyte, disappears, and another type of granule, in addition to the already present azurophilic granule, becomes evident. This second type of granule, or "specific" granule, stains slightly pinkish with the standard Romanovsky stains used in differential screening of both blood and bone marrrow. The most noteable change, enzymically, is the appearance of alkaline phosphatase (leukocyte alkaline phosphatase, LAP) which is associated with the specific granules (Bainton, 1975; Bendix-Hansen, 1987).

While the metamyelocte and band stages have not been

studied in terms of their enzyme content, much is known biochemically about the final stage, since it is fairly easy to separate the mature neutrophil from the other white blood cells and erythrocytes. The segmented nuclei render it morphologically distinct. In addition, the cytoplasm is much less basophilic than in progenitor cells, indicating a decrease in RNA content which is in accord with the non-proliferative status. The azurophilic granules are much less abundant than the specific granule. The former contain myeloperoxidase, α-mannosidase, β-galactosidase, and β-glucuronidase, and decreased arylsulfatase levels. The specific granules contain Mg^{2+} ATPase, collagenase, and high levels of alkaline phosphatase (Bainton & Farquhar, 1968a,b; Heyneman & Vercauteren, 1982). Ribonuclease, lysozyme and cathepsin appear to be distributed in both granules.

There have been some studies on cytoplasmic enzymes that are not associated with the granules. Lindena et.al. (1982) found that high levels of lactate dehydrogenase, glucose-6-phosphate dehydrogenase, adenylate kinase, and phosphohexose isomerase are quite specific to neutrophils. Neutrophils also have high cytidine deaminase activity (Coleman et.al., 1975) and elevated arginase activity (Tanaka & Valentine, 1960) resulting in a low level of arginine. Interestingly, glycerate-3-phosphatase dehydrogenase, a key enzyme in serine biosynthesis, is low

in neutrophils which appears to underly the serine requirement of these cells (Pizer & Regan, 1972). Alkaline phosphatase, for which a histochemical stain has been developed (Ackerman, 1962), is present in very high levels as compared to other leukocytes and is considered a specific marker for neutrophils (Wachstein, 1946; Wiltshaw & Moloney, 1955). In view of its considerably higher activity in the fully differentiated neutrophil than in the myelocyte and metamyelocte, maturational studies of bone marrow cells have used AP as an indicator of maturity of the neutrophil (Stewart, 1974; Bondue et.al., 1980; Sato et.al., 1985; Evans et.al., 1986). Although much of the AP is localized to the specific granules in human neutrophils, there are some indications that a plasma membrane component exists as well (Bretz & Baggiolini, 1974; Heyneman & Vercauteren, 1982).

In addition to morphologic, antigenic and enzymic changes, neutrophils exhibit functional alterations during differentiation. As they mature they become more deformable, adherent to surfaces, form psuedopods, ingest particles and become motile (Lichtmann & Weed, 1972). Studies by Glasser and Fiederlein (1987) indicate that acquisition of these functional characteristics is stepwise rather than simultaneous. The capacity for phagocytosis is acquired as early as the promyelocyte stage, and the increase in this capacity is paralleled by

expression of Fc receptor. Complement receptors are expressed at the myelocyte/ metamyelocte stage, and the ability for bacterial cell killing is also acquired at this point. NBT reduction, an indicator of the ability of superoxide generation and respiratory burst, is manifested late in development (band/segmented neutrophil). Chemotaxis is the last function acquired by the mature neutrophil.

Colony Stimulating Factors (CSF)

Much of the research on hematopoiesis has centered on the growth factors responsible for the proliferation of bone marrow cells. As was mentioned in the section on tumor induced leukocytosis, many researchers believed that secreted factors were responsible for the leukocytosis observed in some cancer patients, as well as for the day-to-day maintenance of the level of circulating blood cells by the bone marrow under normal conditions. These studies were facilitated by an *in vitro* cloning method developed independently by Pluznick and Sachs (1965) and Bradley and Metcalf (1966). The method consists of culturing marrow cells in semi-solid agar, where the granulocyte-macrophage progenitor cells proliferate and differentiate to form granulocyte and/or macrophage colonies. This process is totally dependent on the presence of polypeptides termed colony stimulating factors (CSF).

Colony stimulating activity (CSA) has been found in a wide variety of tissues and body fluids. These range from thymus and uterus (Sheriden & Stanley, 1971), kidney (Sheriden & Metcalf, 1973), spleen (Sheriden & Stanley, 1971), and embryo (Stanley et.al., 1971). Lung is also a potent source for CSA (Sheriden & Stanley, 1971; Bradley et.al., 1971). Virtually every tissue as well as body fluids like serum and urine contain CSFs (Stanley & Metcalf, 1969; Sheriden & Stanley, 1971). CSFs have been demonstrated in hematopoietic cell types as well. Macrophages are a rich source (Chervick & LoBuglio, 1972; Goldie & Cline, 1972; Shah et.al., 1977), as are mitogen stimulated lymphocytes (Parker & Metcalf, 1974).

CSF is secreted by a wide variety of cancers (Di Persio et.al., 1978) as well as by several tumor cell lines, such as mouse myelomonocytic leukemia (Williams et.al., 1978), sarcoma (Ohno et.al., 1978), T-lymphoma (Lusis & Golde, 1980), and pancreatic tumor cells (Wu et.al., 1979). Thus, while some CSFs are found in normal serum, it was from these other sources that most of the CSFs were initially purified. There are currently three well characterized CSFs: macrophage-colony stimulating factor (M-CSF or CSF-1), granulocyte-macrophage CSF (GM-CSF), and granulocyte-CSF (G-CSF). All are glycoproteins, and have been purified from human as well as mouse sources. These, and IL-3 which is sometimes called multi-

CSF, will be discussed in detail below.

Macrophage colony stimulating factor (CSF-1 or M-CSF) was the first CSF subclass to be clearly defined and seperated from other CSF's. It stimulates macrophage colonies primarily (Stanley, 1979) and has weak effects on granulocytes colony formation (Metcalf, 1984). It has been purified from human urine and mouse L cells and determined to possess a MW of 70,000 (Stanely et.al., 1975; Stanley & Heard, 1977). The molecule exists as a dimer of two 14,000 MW peptides which are heavily glycosylated. The dimer possesses biological activity while the monomers are without activity. Deglycoslation does not affect its biological activity (Das & Stanley, 1982).

Binding studies with M-CSF indicate that macrophages and their precursors are the main target cells. The receptor is a large membrane bound protein about 165,000 MW and exists in relative aboundance (Stanley & Guilbert, 1981; Morgan & Stanley, 1984). The M-CSF receptor has been sequenced and found to be structurally related, and possibly identical, to the c-fms oncogene product (Sherr et.al., 1985).

While M-CSF is a potent stimulator of murine macrophage formation, it is not a very effective

stimulator of human precursor cells (Kawasaki et.al., 1985; Nagata et.al., 1986). It may be that human M-CSF functions more as a cell survival and activating factor than as a proliferative stimulus (Clark & Kamen, 1987).

Granulocyte-Macrphage colony stimulating factor (GM-CSF) is probably the most extensively studied CSF. It promotes the proliferation of granulocyte and macrophage colonies, the ratio of which depends on its concentration in the culture (Burgess & Metcalf, 1977). It is detectable in almost all organs tested (Sheriden & Stanley, 1971). The cell types shown to be capable of its synthesis include T lymphocytes, macrophages, fibroblasts, endothelial and epithelial cells (Metcalf, 1987). Serum-free medium conditioned by lung tissue from endotoxin injected mice was the original source from which GM-CSF was purified (Burgess et.al., 1977). Using a six step procedure which has been modified to include HPLC steps (Burgess et.al., 1977; Sparrow et.al., 1985), two major species were isolated, 21,000 and 23,000 MW. Upon deglycosylation with endoglycosidase F, the MW of both dropped to 16,500, indicating a difference only in post translational modification with carbohydrate residues. GM-CSF is composed of 124 amino acids, with two glycosylation sites and two disulfide bridges. The latter may be of functional importance as β-mercaptoethanol destroys the

activity.

In contrast to the high number of CSF-1 receptors on macrophages, GM-CSF receptors are not abundant on their target cells (Walker & Burgess, 1985) which has posed some problems for purification. Crosslinking studies indicate that the receptor is about 51,000 MW (Metcalf, 1987).

The target cells of GM-CSF include not only G-M precursors, but also post-mitotic end cells of the G-M series. It acts to enhance the survival and activation of mature neutrophils, eosinophils, and macrophages (Begley et.al., 1986; Weisbart et.al., 1985; Lopez et.al., 1983; Grabstein et.al., 1986).

Granulocyte-Colony Stimulating Factor (G-CSF) is synthesized in most of the same tissues as GM-CSF (Nicola & Metcalf, 1981). As its name implies, it supports primarily the proliferation of neutrophils (Nagata et.al., 1986; Souza et.al., 1986). Other types of colonies seen in G-CSF supported cultures, may arise from indirect effects (Stanley et.al.,1986). It has been purified from both mouse (Nicola et.al., 1983) and human cell lines (Welte et.al., 1985), with an apparent MW of 25,000 and 18,000 respectively. Sequence analyses show that there is no homology with any of the other CSFs (Metcalf, 1987). The G-CSF molecule has disulfide bonds which are necessary for activity. It is very heat stabile, boiling does not

destroy its activity.

Receptor binding studies indicate specificity for the granulocyte monocyte populations. Receptor number, about 300 per cell, is as low as those for GM-CSF. Interestingly, this number rises with maturation: post-mitotic cells (metamyelocyte, bands, and segmented neutrophils) all express more receptors for G-CSF than their mitotic precursors (Nicola & Metcalf, 1985). The receptor has a MW of 150,000 (Metcalf, 1987). Perhaps the most interesting feature of G-CSF is the ability to bind to myelomonocytic leukemia cells and promote their differentiation (Nicola et.al., 1983; Souza et.al., 1986). In these earlier studies it was referred to as "differentiation factor."

G-CSF also acts on mature cells. In post-mitotic cells, it modulates functions such as cell motility, membrane receptor expression, phagocytosis, synthesis of biological mediators, and cytotoxicity (Burgess & Metcalf, 1980; Metcalf, 1986). It enhances the effect of fMLP, a chemotactic stimulus of neutrophils (Platzer & Kalden, 1987). Because of these effects, and the higher number of receptors on mature cells, G-CSF may well be less of a proliferative factor than as a modifier of specific functions.

Interleukin-3 (IL-3) or Multi-CSF is unique in that

only one known normal cell type is capable of producing it, the antigen or mitogen primed T lymphocyte (Metcalf, 1984). IL-3 is also secreted by some tumor cell lines, and capable of inducing proliferative effects in a broad range of cell types - granulocytes, macrophages, eosinophils, megakaryocytes, erythroid, multipotential, stem cells and mast cells (Goldwasser et.al., 1983; Ihle et.al., 1985).

IL-3 has been purified from conditioned medium of mitogen activated T lymphocytes based on its ability to induce the enzyme 20- hydroxy-steroid dehydrogenase in splenic lymphocytes (Ihle et.al., 1981; Ihle et.al., 1982). It has an apparent molecular weight of 23-28,000 MW depending on the source from which it is purified (Ihle et.al., 1982). From the sequence of 134 amino acids, one would predict a MW of 15,000 (Fung et.al., 1984; Yokota et.al., 1984) indicating a fair amount of glycosylation. Although GM -CSF and IL-3 stimulate cells of similar ancestry (Metcalf, 1985), they show no structural or sequence homology, and there is no crosscompetition for their respective receptors.

In the last few years, all of the CSF's have been cloned from mouse (m), human (h) or both sources. mGM-CSF was cloned by Gough et.al. (1984), mIL-3 by Fung et.al. (1984), hGM-CSF by Wong et.al. 1985, hCSF-1 by Kawasaki et.al. (1985) and Wong et.al. (1987). It has also been

possible to clone hG-CSF (Souza et.al., 1986) and hIL-3 (Yang et.al., 1986). Very little sequence homology exists. No homology was found between CSFs and other growth factors or oncogenes. With the advent of recombinant technology, CSFs have become available in large quantities. This has allowed researchers to investigate the in vivo effects. Infusion of G-CSF in normal monkeys results in a dramatic leukocytosis with total white cell counts of 50,000 per mm^3 (Clark & Kamen, 1987). In experiments with monkeys undergoing bone marrow transplantation, GM-CSF enhanced the graft regrowth (Clark & Kamen, 1987). GM-CSF injected mice also exhibited higher levels of neutrophils and eosinophils, and macrophages exhibited increased mitotic activity and phagocytosis (Metcalf, et.al., 1987).

INDUCTION OF DIFFERENTIATION AND LEUKEMIA THERAPY

As described earlier, the hematopoietic system consists of three compartments. The first is a self renewing pluripotent stem cell compartment. The second consists of cells already commited to a particular lineage, and the final compartment is that of the mature endstage cells which are incapable of self renewal and possess specialized functions.

Acute myelocytic leukemia (AML) often arises from the neoplastic transformation at the puripotent stem cell,

leading to a block in maturation to a recognizable myeloblast or promyelocyte (Koeffler, 1983). The same is true for chronic myelogenous leukemia (CML) patient in blast crisis. The transformed cells fail to cease multiplying and soon replace the normal progenitors and fill the marrow. In CML the cells of all myeloid lineages are derived from a single clone (Failkow et.al., 1977). In AML the situation is more complex. In some patients only the granulocytic cells belong to the leukemic clone and in other granulocytic as well as erythroid cells may be involved (Messner & Griffin, 1986). In either case, the patient becomes appreciably vulnerable to infections as the leukemic cell thus released to the blood do not possess the functional capacity of mature neutrophils.

Treatment of leukemias has historically centered on the use of cytotoxic agents to destroy the rapidly dividing leukemic blast. This has been highly successful for childhood lymphocytic leukemias. Unfortunately it has not been effective for myelocytic leukemias. This may be in part due to the fact that a considerable fraction of the blasts are in the resting stage (G_0) or the G_1 phase (Gavosto, 1973; Clarkson & Fried, 1971). Another problem with this therapy is the significant morbidity due to the therapy itself (Riess et.al., 1986).

A new concept of cancer therapy has been developing in recent years. It arose from studies on hematopoietic

cell lines indicating that malignancy may not be an irreversible state. The first evidence that a neoplastic stem cell could be chemically induced to differentiate was obtained by studies on leukemic cell lines in vitro. Metcalf et.al. (1969) reported that murine myelomonocytic leukemia cells (WEHI-3B D+) in agar culture could form colonies of mature granulocytes under the influence of CSF. This was soon followed by reports from here at the Mount Sinai Medical Center that DMSO, a polar-planar solvent, could induce Friend murine erythroleukemia cells to begin hemoglobin synthesis, a marker of differentiation (Friend et.al., 1971).

Since that time, a number of murine and human myeloid cell lines have been developed to study differentiation. In addition to providing models in which to test the efficacy of agents of possible use for differentiation therapy, these inducible cell lines have also provided the researcher with homogeneous sources of material for the study of normal hematopoiesis. For most of the changes that occur during the chemical induction of differentiation are the same as those seen during regular maturation. They include morphological changes, generation of superoxide, phagocytosis, increases in Fc and complement receptors, and motility and chemotaxis (Newberger et.al., 1979, Wallace et.al., 1987). Another parameter of maturity, but not frequently measured, is the

increase in the production of leukotrienes seen in HL-60 cells induced with either DMSO or retinoic acid (Imaizumi & Breitman, 1987).

It has been possible to isolate human cell lines that appear to be blocked at different stages of maturation. ML1 and ML3 are myelomonoblastic (Takeda et.al., 1981), KG1 (Koeffler et.al., 1980) and U937 (Sundstrom & Nilsson, 1976) are monoblastic, while HL-60 appear to possess characteristics of promyelocytes (Collins et.al., 1977). This has allowed some dissection of the maturational stages and comparisons of the differential sensitivity of these stages to various differentiation inducing drugs (Collins et.al., 1980; Schwartz & Sartorelli, 1982; Matsui et.al., 1984; Koeffler et.al., 1980; Takeda et.al., 1981).

One of the most studied cell lines has been the HL-60 line. This human promyelocytic leukemia is capable of differentiating to either macrophages and granulocytes. Treatment with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and 1,25 dihydroxycholecalciferal (Vit D_3) induces HL-60 cells to differentiate into macrophages (Rovera et.al., 1979; Koeffler et.al., 1981; Murao et.al., 1983; Matsui et.al., 1984). Polar-planar compounds like dimethylsulfoxide (DMSO) and dimethylformamide (DMF) cause these cells to mature into granulocytes (Collins et.al., 1978; Breitman et.al., 1980); similar effects are exerted by some purine and pyrimidine analogs as well as

chemotherapeutic agents like actinomycin D and methotrexate (McPortland et.al., 1974; Bodner et.al., 1980; Lotem & Sachs, 1980).

HL-60 differentiation induced by TPA occurs with a fairly well characterized set of events. 95% of the cells become adherent and display macrophage-like morphology along with the synthesis of nonspecific acid esterase. Other changes include the development of pseudopodia (40%), and phagocytosis (60%). Nitroblue tetrazolium reduction also occurs but may not be as dramatic as in normal granulocyte maturation. Enzyme changes indicative of macrophage differentiation in human leukemic cell lines consist of increases in lysozyme, β-glucuronidase and acid phosphatase (Rovera et.al., 1979; Teritto & Koeffler, 1981).

Another class of compound known to induce differentiation toward macrophages are the teleocidins. These are naturally occurring indole alkaloids and are also potent tumor promoters. While they are distinctive from the phorbol esters which are also potent tumor promoters, they appear to inhibit the binding of phorbol esters to their cell surface receptor, protein kinase C (Huberman et.al., 1982).

Several physiological compounds appear to offer promise as differentiation agents. Incubation of HL-60 cells with trans retinoic acid (RA) causes granuloctye

differentiation as indicated by increased O_2^- production, NBT reduction, positive chloroacetate esterase staining and increase in the hexose monophophate shunt (Breitman, 1982; Hemmi & Breitman, 1984). WEHI-3B cells are also differentiated toward granulocytes by RA (Gamba-Vitalo et.al., 1986). Retinoic acid also appears to be promising for use in myelodysplastic syndrome, but no clear results have been obtained with myeloid leukemias (Koeffler, 1983). Vit D_3 has also been used to induce HL-60 cells. The path of differentiation appears different from that in the case of RA as these cells progress toward monocytes (Miyaura et.al., 1981; Koeffler, 1983).

Cancer cells other than leukemic blood cells also appear to be capable of differentiation by many of the same agents. Embryonal carcinoma, neuroblastoma, melanoma, and a variety of carcinoma cell lines all can be induced to mature with agents like TPA, RA, DMSO, DMF, and agents capable of raising their levels of cAMP (Reiss et.al., 1986).

The clinical usefulness of the above discussed compounds as leukemia chemotherapeutic agents are questionable for several reasons. The dangers of using phorbol esters like TPA, is that they are extremely potent tumor promoters (Hirakawa et.al., 1982) and imflammatory agents, and are also known to induce lymphoblastic

leukemia in mice (Armuth, 1976). In addition TPA in low concentrations actually increases in vitro the growth of leukemic cells isolated from patients (Koeffler, 1981; Pegoraro et.al., 1981), and at concentrations capable of blocking the proliferation of HL-60 cells these agents inhibit normal myeloid proliferation (Griffin et.al., 1983). Such undesirable side effects and toxicity are also associated with other classes of chemical inducers. Moreover, their differentiational effect on cell lines is not necessarily exerted on leukemic cells in vivo or even in vitro. For example, despite the effectiveness of DMSO, HMBA (another polar-planar compound) or Actinomycin D in cell cultures, they failed to differentiate peripheral blast cells of acute myelocytic leukemia (AML) patients (Koeffler, 1983; Forbes et.al., 1981).

Physiological regulators of hematopoiesis in vivo are less likely to exert undesireable side effects and prohibitive toxicity, and there has been much interest recently in their possible differentiation enforcing effects on leukemic cells. The first studies in this connection showed that the myeloid leukemia cell line M1 (Ichikawa, 1969) and R453, a Rauscher leukemia virus induced cell line developed by Ichikawa (1976), were caused to form macrophages or granulocytes by incubation with sera from mice injected with endotoxin, (Kasukabe et.al.,1979), as well as with 2 other preparations from

this animal: spleen extracts (Ichikawa, 1969) and peritoneal macrophages conditioned medium (Ichikawa et.al., 1976). All three preparations,have been found to be rich in hematopoietic growth factors. Similar results were obtained by Metcalf and coworkers using agar cultures of two murine myelomonocytic leukemia cell lines (WEHI-3B D+ and M1). These cells normally produce compact colonies, but in the presence of ES (serum from endotoxin treated mice) colonies were diffuse with fewer cells and upon microscopic and histologic examination, these cells were found to be differentiated granulocytes (Metcalf et.al., 1969; Metcalf, 1980; Burgess & Metcalf, 1980). The active agent in serum, after partial purification, was designated G-CSF as stimulation of predominately granulocyte colony formation was its effect on normal bone marrow agar cultures (Nicola et.al., 1983).

Around this same time another differentiation activity was being isolated based on its ability to differentiate mouse M1 cells (Lotem & Sachs, 1980) as measured by lysozyme production. Maeda et.al. (1977) identified a factor in mouse embryo cells which, designated D-factor, was capable of inducing granulocte differentiation of M1 cells as judged by assay of induced phagocytosis of latex beads, Fc and complement receptors, as well as disperse colony formation. A D-factor was also isolated from ascites tumor cell conditioned medium

(Tomida et.al., 1984). These factors are all much higher molecular weight glycoproteins (62-45Kd) than G-CSF (24-25Kd). The difference may be due to extensive N-linked glycosylation. When the sugar moieties are removed, the molecular weight becomes approximately that of G-CSF. This argues for their identity (Nicola et.al., 1983).

In vitro studies of the effect of G-CSF on peripheral neutrophils from patients with myelodysplastic syndrome (MDS), a preleukemic condition, and from CML patients, showed that G-CSF was able to correct functional deficiencies in experimentally stimulated O_2^- production as well as alkaline phosphatase content (Yuo et.al., 1987) indicating a possible clinical usefulness for this compound. Recombinant human GM-CSF has been used successfully in clinical trials to stimulate leukocytosis in patients with myelosuppression due to chemotherapy (Antman et.al., 1988). It might thus decrease the morbidity due to infection in patients undergoing chemotherapy as well as allow a dose escalation of these chemotherapeutic agents.

It is possible that while these differentiation inducing polypeptides may not be entirely satisfactory for leukemia therapy, their use in conjunction with traditional cytotoxic agents may be of advantage. For example, if these agents can drive the G_0 and G_1 leukemic blasts into the proliferating pool one may be able to

shorten the course of cytotoxic drug treatment needed to kill them. Agents which promote terminal differentiation without influencing proliferation may be equally useful, since, in patients where the leukemic blast can progress to the committed stage, they could assure the production of functionally differentiated myeloid cells.

Chapter II

METHODS

Animals

Adult male Fischer rats, weighing 175-250g were used in most experiments for both tumor transplantantation and as normal control rats. Mammary carcinoma 5A was propagated by serial subcutaneous transplantation in the flank of these animals. The biochemical and growth characteristics of the tumor remained stable for many transplant generations. Mice used in some of the experiments were adult females of the C57Bl/6 strain.

Blood from normal and tumor bearing rats was drawn by heart puncture with a sterile syringe. Blood was allowed to clot and centrifuged for collection of serum. For blood cell studies, the blood was collected in a heparinized syringe to prevent clotting.

Epidermal Growth Factor (EGF), insulin, hydrocortisone, and concanavalin A were purchased from Sigma Chemical Company, St. Louis, MO., and Transforming Growth Factor-β from Collaborative Research, Waltham, MA. CSF-1 (macrophage-colony stimulation factor) and L-cell conditioned medium were a generous gift of Dr. E. Richard Stanley, Albert Einstein School of Medicine; CSF-2τ (murine granulocyte/macrophage-CSF) and mIL-3 were purchased from Genzyme Corp., Boston, MA. Giant Cell Tumor

conditioned medium was purchased from GIBCO Laboratories, Grand Island, NY. Recombinant human granulocyte-CSF (G-CSF) was purchased from AmGEN corporation, Thousand Oaks, CA.

Separation of Leukocytes

Heparinized whole blood was collected by heart puncture and mixed with 5 parts of 1.5% Dextran in isotonic saline. Red cells were allowed to settle 45 minutes, and the leukocyte enriched top layer was collected and washed with sterile isotonic saline. Cells were pelleted by centrifugation for 10 min at 2000 rpm. The pellet was dispersed in cold hypotonic saline (0.2% NaCl) for 2 minutes to lyse the red blood cells, after which 1.8% NaCl was added to make the solution isotonic. This leukocyte suspension was used for enzyme analysis or, in the case of leukemic peripheral blood, for incubation in liquid culture. In some cases granulocytes and lymphocytes were separated by the method of Boyum (1968). This involved using a Percoll density gradient. Percoll, obtained from Pharmacia, was mixed 9 parts to 1 part 9% NaCl to obtain a an osmolality of 300 mOs/kg H_2O. The pH was adjusted to 7.2 with 1N HCl. Aliquots of this preparation mixed with a 0.9% NaCl solution (65:35 v/v) consisted the dilute Percoll solution. Whole blood was diluted with 4 volumes of 0.9% NaCl; 20 mls were carefully

layered on 15mls of the diluted Percoll solution and centrifuged at 300 X g for 40 minutes at room temperature in a swinging bucket rotor of an IEC centrifuge. Pasteur pipets were used to collect the upper lymphocyte layer and the granulocytes sitting on the top of the sedimented RBCs. Both fractions were washed by suspension in 0.9% NaCl and recentrifuged. Contaminating RBCs were lysed with 2ml ice cold H_2O for 30 seconds; after the osmolality was restored by the addition of 2ml 1.8% NaCl, cells were centrifuged down and resuspended in 0.9% NaCl. one aliquot was used for total and differential WBC counts, and the remainder was used for enzyme assays.

Routine Biochemical Assays

The assays of gamma-glutamyltranspeptidase (GGT) and alkaline phosphatase (AP), a minor modification of those of Seymour & Peters (1977), are based on the fluorimetric determination of the released 2-naphthylamine (from gamma-glutamylnaphthylamide using glycylglycine as an acceptor) and 4-me-umbelliferone (from its phosphate), respectively. With the optimal concentrations of the substrate and cofactors used, activity remained maximal during the incubation period, and was proportional to the amount of enzyme added. Protein was assayed by the method of Lowry et.al. (1951).

Rat Bone Marrow Incubation System

Femurs were dissected from normal healthy male rats and cleaned well prior to removal of the bone marrow. With a syringe fitted with a 21 gauge needle, 10 ml of Minimal Essential Medium (MEM) was flushed through each femur. The bone marrow cells were washed twice with 0.9% NaCl solution, and resuspended in 3-5ml of MEM. Cell number was counted in a 0.1ml aliquot diluted with 9 volumes of 3% acetic acid. Each 12 X 75 mm sterile snap-cap tube contained 1-2 X 10^6 cells, 0.125 ml (i.e. 10%) of the indicated sera, 20ug gentamycin, and MEM in a total volume of 1.25 ml. After incubation at 37°C and 5% CO_2 for 24-48 hours cells were harvested and washed 3X with 0.9% NaCl. The sedimented cells were resuspended in 0.25ml saline for enzyme assay and histology.

Enzyme concentration, expressed as units (nmol or 0.1 nmol/ min/ 10^6 cells) were averages for three tubes incubated without (control) and with (experimental) the indicated CSF or MC preparations.

Mouse Bone Marrow Colony Forming Assay

The bone marrow colony forming assay was based on the method of Bradley & Metcalf (1966). Bone marrow cells were obtained from alcohol cleaned femurs of female C57Bl/6J mice in a manner similar to the rat liquid bone marrow assay. Cells were flushed from the clipped femurs with

0.5ml Dulbecco's Modified Eagles Medium (DMEM) using a 1cc tuberculin syringe and dispersed in a 35mm tissue culture dish. After a series of sterile saline washes, cells were resuspended in DMEM. Cells, 0.1 X10^6 cells per ml, were cultured without a feeder layer in 0.3% agar in DMEM containing 15% heat inactivated fetal calf serum and 10% normal rat serum (modified from Bradley and Metcalf, 1966 and Metcalf, 1980) in 35 mm plastic petri dishes. Cultures were incubated for 7 days at 37^{o}C and 5% CO_2. Colonies were counted with an inverted microscope at 40X magnification.

Leukemic Cell Culture

WEHI-3B D+ cell line, obtained from Dr.Malcolm Moore of Memorial Sloan Kettering Institute, was grown in Dulbecco's modified Eagle's medium containing 100U/ml penicillin/streptomycin, 2mM Glutamine, and 10% Fetal Bovine Serum (heat-inactivated at 56^{o}C for 30 minutes). Cells were plated at a density of 100,000/ ml and incubated at 37^{o}C in a humidified atmosphere with 5% CO_2. Cells were allowed to grow for 2-3 days to a density of about 2 X 10^6 cells/ ml at which time they were split back to 100,000/ ml.

HL-60 cells, obtained from Dr. William Scher of Mount SInai Medical Center were grown in RPMI-1640 with glutamine , penicillin/streptomycin and 10% heat-

inactivated fetal bovine serum added. They were plated at a density of approximately 200,000 cells/ ml and incubated at 37^{o}C and 5% CO_2.

Shay Chloroleukemia cells were provided by Dr A. Yunis from the University of Miami Medical Center, Miami, Florida. Cells were propagated as a solid tumor obtained by injection of 5 X 10^6 cells subcutaneously to 100-g Long-Evans rats. Tumors were transplanted every 2 weeks. They were excised in a sterile environment and dispersed by aspiration of the minced tumor tissue in Dulbecco's MEM containing Pen/Strep and Fungizone with a 20 guage needle. Cells were plated in the presence of 10% heat-inactivated fetal bovine serum and 10% heat-inactivated horse serum at a cell density of 100,000 cells/ ml.

Cell Staining and Differential Counting

Blood cells were smeared on glass slides for differential staining. Bone marrow smears were prepared from cells suspended in isotonic saline, with 10% serum added to minimize cell breakage, by means of a cytocentrifuge (Shandon Scientific Co., Sewickley PA.).

Cells were examined by light microscopy after May-Grunwald Giemsa staining. The criteria used for the identification of the various cell types were those of Wintrobe (1961), and Joffey et.al. (1965) as modified for the rat by Sanderson & Phillips (1981). Neutrophils

exhibiting greater than than 6 segments or lobes were considered "hypersegmented". As in previous studies (Lindenbaum & Nath, 1980) a nuclear structure was considered to be a distinct segment if it appeared to be clearly separate from the rest of the nucleus or connected by a thin chromatin thread.

To identify the cell types composing the myeloid colonies, some of the agar cultures were stained for esterases specific for certain myeloid lineages. Phillips et.al. (1983) developed a cytochemical technique which facilitates identification of the types of cell aggregates which proliferate in semi-solid agar cultures of bone marrow. Instead of having to remove colonies from the plate for separate typing, his sequential staining method permits distinction of monocytic, neutrophilic and eosinophilic cells in the same preparation. Staining for α-naphthol acetate esterase and for naphthol AS-D-chloroacetate esterase distinguishes between monocytes and neutrophilic granulocytes, respectively, while Luxol fast blue reveals eosinophils. Briefly, agar cultures were fixed with 2 ml of a citrate buffered acetone-methanol solution for 20 minutes. After removal of the fixative, agar gels were floated in distilled water to rinse and air dried. Cells were stained with twice the recommended substrate concentrations for α-naphthyl acetate esterase

and naphthol AS-D chloroacetate esterase to identify monocytes and granulocytes, repectively. The substrates were α-naphthol acetate and naphthol AS-D chloroacetate which is coupled to fast blue RR salt and fast Corinth respectively. Excess stain was removed by rinsing and floating agar discs in distilled water; they were mounted on 75 X 50 mm slides and air dried.

Cells were also stained histochemically for AP and GGT. Leukocyte alkaline phosphatase was demonstrated in citrate buffered acetone fixed smears by the color development of the diazonium salt (Fast Blue RR salt) coupled naphthol AS-MX as a result of the enzymatic cleavage of naphthol AS-MX phosphate (Ackerman, 1962). Histochemical demonstration of γ-glutamyltranspeptidase was performed as described by Ruttenberg et.al. (1969). The substrate, γ-glutamyl-4-methoxy-2- naphthylamide, was cleaved by GGT, the glutamyl group being accepted by glycylglycine and the 4-methoxy -2- naphthylamine coupled to the azo dye Fast Blue BBN. After rinses in saline the azodye conjugate is chelated with a cupric sulfate solution. Both AP and GGT stains were counterstained in methyl blue for nuclear contrast.

Protein Purification Techniques

Mammary Carcinoma (MC) extract preparation: homogenates

of MC were made in 4 volumes of 0.15 M KCl from which particulate material was removed by centrifugation at 100,000 X g for 30 minutes.

MC- conditioned medium (MC-CM): 1-5 mm^3 tumor pieces were incubated in calcium- and magnesium-free Eagle's minimal essential medium (0.5g MC per 10 ml of medium) for 2 days, which was followed by centrifugation at 100,000 X g for 30 minutes and filter sterilized.

Dialysis: preparations were dialyzed against 0.06M Tris-HCl buffer at pH 7.4 using Mr 50,000 cutoff dialysis tubing.

DEAE-Cellulose Chromotography:DEAE-cellulose gel was equilibrated in 0.06M Tris-HCl pH 7.4 and poured into a column with a bed of 3.0 X 18 cm. The run through fractions showing absorption at 280 nm were dialyzed against distilled H_2O and lyophilized. Bound proteins were eluted with a linear gradient of 0.0 to 0.3 M NaCl in Tris- HCl buffer at pH 7.4.in a total volume of 300 ml at a flow rate of 20 ml/ hr., and 6.6-ml fractions were collected.

G-100 Sephadex Chromatography: Active fractions from the DEAE column were dialzyed against 0.06M Tris-HCl, pH 7.4 and applied to a G-100 Sephadex column (2.5 X 80 cm) and run with a pressure head of 70 cm. The eluting buffer was 0.06M Tris-HCl, pH 7.4. Fractions were collected (5ml) and

dialyzed against H_2O. The void volume was determined using Blue Dextran 200 and molecular weight markers were run to calibrate the column.

Hydroxylapatite Chromatography: Hydroxylapatite (Biogel HTP; Bio-rad Laboratories, Rockville Centre, NY) columns (2.5 X 3 cm), were equilibrated with 1mM sodium phosphate buffer, pH 7.0. Active fractions from the G-100 Sephadex column, dialyzed against 1mM sodium phosphate buffer, pH 7.0, were loaded onto this column which was then washed extensively with 1mM phosphate buffer. Bound material was eluted with 15, 50, and 200 mM phosphate buffer, pH 7.0, and dialyzed against H_2O for assay.

Concanavalin A-Sepharose Chromatography: MC serum (5 ml) was dialyzed (Mr 50,000 cutoff dialysis tubing) against 0.2 M sodium acetate buffer, pH 5.0, and applied to a concanavalin A-Sepharose column (1.5 X 20 cm) equlibrated in the same buffer. Unabsorbed proteins were washed through the column with the above buffer before eluting the bound material with methyl α-D-glucopyranoside (0.5 M). The absorbed and non-absorbed fractions were pooled separately, dialyzed against distilled water and lyophilized. The lyopholized protein fractions were reconstituted in medium and tested in the *in vitro* bone marrow system.

Blue Sepharose Chromatography: Preparations were dialysed against 0.05 M Tris-HCl buffer, pH 8.0, containing 0.05M

NaCl, and applied to a Blue Sepharose CL-6B column equilibrated in the same buffer. Blue Separose contains a dye, Cibacon Blue F-3 G-A, which has a high affinity for serum albumin. Unbound proteins were eluted from the column with the equlibration buffer.

Treatment with proteolytic enzymes and mercaptoethanol: MC-protein preparations were incubated with Protease (Sigma P 4531; 4 units/mg protein), derived from streptomyces griseus and attached to insoluble agarose. After 3 hours at 37°C, Protease was removed by centrifugation through 0.45u Centrex microfilters. Incubation with trypsin (30:1), at 37°C for 2 hours; this was followed by a 30 minute incubation with soybean trypsin inhibitor (0.5 ml/mg Trypsin) attached to insoluble agarose, which was then removed by centrifugation. Sensitivity to α-chymotrypsin (Sigma C9134) was tested by incubation with 4 units/ mg protein for 4 hours at 37°C; α-chymotrypsin attached to beaded agarose, was then removed by centrifugation.

Sensitivity to mercaptoethanol was tested by diluting MC-serum or MC-CM to a concentration of 1mg/ml and 0.1mg/ml protein, respectively, and incubating with 100 mM 5-mercaptoethanol for 1 hour at 23°C.

Chapter III

CHARACTERIZATION OF A MAMMARY CARCINOMA ELABORATED FACTOR STIMULATING τ-GLUTAMYLTRANSPEPTIDASE EXPRESSION IN BONE MARROW CELLS

Introduction

Investigations concerned with the systemic effects of neoplasms suggested that tumor-elaborated blood-borne factors (rather than nutritional or hormonal changes) are responsible for many of the alterations noted in the enzymic composition of "uninvolved" organs of the host (Costa, 1977). Much of the work has focussed on the liver, where many of the enzymes change their levels toward that found in the immature liver of both animals and man (Herzfeld & Greengard, 1972; Herzfeld & Greengard, 1977, Herzfeld et.al., 1980, Suda et.al., 1966). This explains many of the altered liver functions seen in patients bearing extrahepatic neoplasms (Stoaniemi et.al., 1977; Greengard, 1979).

Other organs are also affected in tumor-bearing animals. Enzymic changes have been observed in the spleen (Greengard & Lempert, 1972) and kidneys (Wu, 1973). Koss and Greengard (1982) has studied two non tissue specific enzymes τ-glutamyltranspeptidase (GGT) and alkaline phosphatase (AP), in animals bearing a number of different transplantable tumors. The spleen, bone marrow, and

peripheral leukocytes of mammary carcinoma 5A (MC) bearing rats exhibited increased AP and GGT levels, while the kidney and intestine showed no response.

These animals, and also rats bearing ascites tumor 4A, exhibited granulocytosis. Resection of the tumors normalized the granulocyte counts as well as the GGT and AP concentrations. This reversibility suggested that the alterations were not due to cancerous invasion of the hematopoietic tissues, but probably to a secreted, blood-borne factor.

Attempts were made, therefore, to design an in vitro system in which the enzyme changes observed in the tumor-bearing animals could be reproduced. It was found that bone marrow cells of normal rats could be maintained for up to 3 days in liquid cultures containing normal serum with no change in GGT and AP concentrations, but that these concentrations rise after 18-72 hour incubation with sera from from MC tumor bearing rats. Serum from animals bearing only a 1 g tumor had a detectable effect, but efficacy increased with increasing tumor size (up to 40 g).

The serum factor inducing the enzymes in vitro appeared to be a non-dialyzable, acid-stable, ethanol-HCl soluble molecule (Greengard et.al., 1984). The main purpose of the following studies was to obtain more information about the factor.

RESULTS

The previously described liquid culture system of rat bone marrow cells, in which serum from MC hosts (MC serum) stimulated GGT expression (Greengard et.al., 1984) was used as a test system in the present studies. The incubation time was standardized at 48 hours, and aliquots of the same bone marrow suspension incubated with 10% normal rat serum (which maintains the AP and GGT content for at least 3 days) served as controls. Table III-1, confirming previous results, shows the lower values in the control tubes (line 1) than in those incubated with MC serum. GGT was of primary interest since no induction of this enzyme in vitro have hitherto been identified. Thus, while AP was also measured in most cases, values for GGT only will be presented in some cases.

After (as well as before) incubation, 90% of the GGT resides in the particulate fraction of bone marrow homogenates (Greengard et.al., 1984); whole homogenates were thus used for the enzyme measurements. The results of these measurements (i.e. catalytic rates per 10^6 cells at optimal substrate and cofactor fortification) will be referred to as "concentration" or "content" so as to reserve the term "activity" for the ability of MC-preparations to raise GGT and AP (nmolx10/min/10^6 cell for GGT and nmol/min/10^6 cells for AP) above that of control values. The concentration increments (as illustrated with

TABLE III-1 Effect of MC Serum on the Enzyme Content of Incubated Normal Rat Bone Marrow Cells.

	GGT	AP
Incubation with:	units/million cells	
Normal Serum	0.15±0.03(7)	2.25±0.35(7)
MC-Serum	0.45±0.05(7)	11.73±1.99(7)

10^6 cells were incubated for 48 hr in minimal essential medium containing 10% serum from normal or tumor bearing rats. units=nmol/min

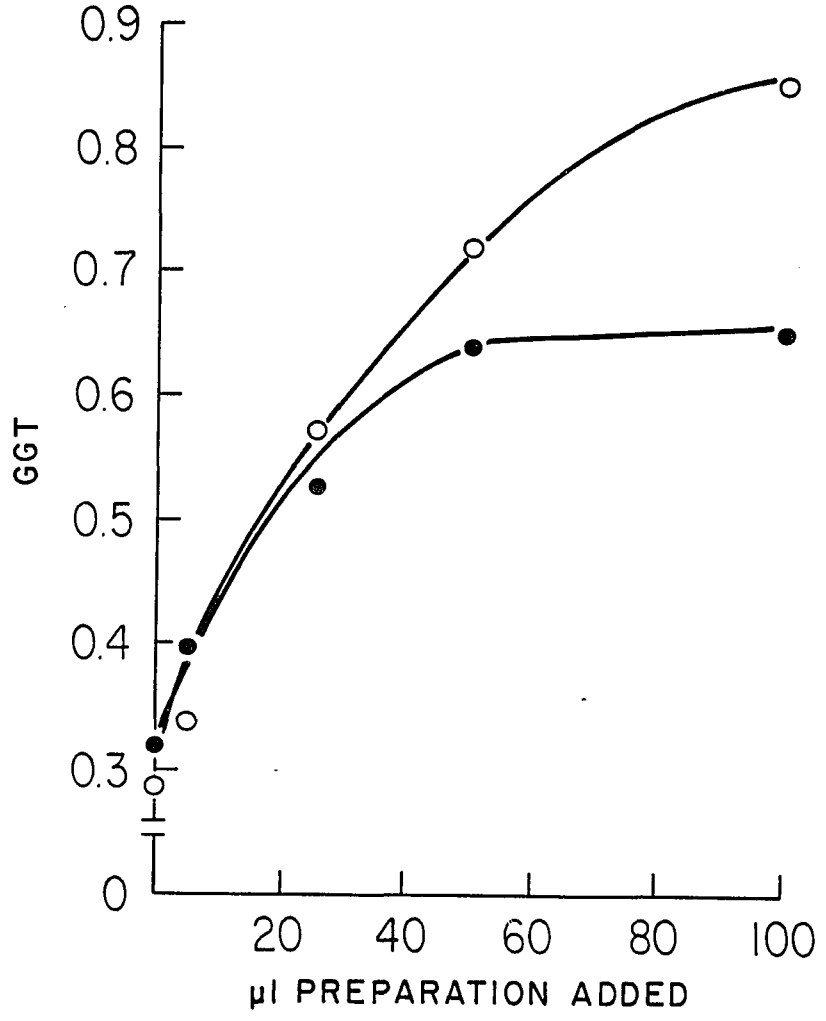


Figure III-1. The dose dependent stimulation of GGT expression in vitro. GGT, nmol/min/10^6 cells (ordinate), was measured in the standard liquid culture of rat bone marrow after incubation with varying amounts (abscissa) of MC-serum (●) or MC-conditioned medium (○); these 2 preparations contained 55 and 0.027 mg protein per ml, respectively. Points are means of 3-4 determinations with Standard Deviations less than 10% of the mean.

2 examples in Figure III-1) were proportional at first to the amounts of any active preparation added and then declined to variable extents. In every assay, therefore, several different amounts of the preparations were added, and their activity (in units-G or units-A, referring to GGT and AP induction, respectively) was determined from the initial, near linear portion of the dose response curves thus obtained. Depending on the size of the carcinoma at the time of bleeding of the animal, the serum activity per mg protein varied from 1-5 units-G and 2-7 units-A.

In order to characterize the active component in the MC serum, chemical and thermal sensitivities and chromatographic behavior were studied. The first column of Table III-2 shows that upon treatment with nonspecific protease, 70-90% of the MC-serum's GGT inducing activity was lost. No loss was incurred by treatment with trypsin or mercaptoethanol or by heating at 100^{o}C for 1 hour. When applying concanavalin A- Sepharose affinity chromatography, 79% of the acive protein passed through the column unabsorbed indicating the lack of glucoside and mannoside moieties. It may also be seen that the activity of MC-serum resides in the M_r 50,000 Amicon filter retained material. Exhaustive dialysis of the serum using a M_r 50,000 cutoff tubing resulted in no loss of activity. Binding to serum albumin was excluded by the

Table III-2. Stability and other characteristics of the active factor in the MC-serum.

		Activity[a] % of total units-G	
		MC-serum	MC-conditioned medium
After treatment[b] with protease		25±5	0
"	with trypsin	121±31	85±13
"	with mercaptoethanol	110±26	--
"	100°C for 30 min	100±19	75±12
Con A-Sepharose[b] column chromatography	unadsorbed	79;82	95;100
	adsorbed	18;13	5;2
50K cut-off filtration[c]	retentate	110±16	95
	filtrate	6±5	8±6

[a]Results in the standard bone marrow test system, as percent of total units-G, refer to the active DEAE fraction (see Figure III-2) of the MC-serum (first 4 numbers in column 1) or to the unfractioned MC-serum (last 4 numbers in column 1), or to MC-conditioned medium (2nd column). Values are means (±S.D.) of 3 determinations or are single results from 2 separate experiments.

[b]For details see Chapter II.

[c]Centricon microconcentrators (Amicon Corp., Danvers, MA) were used.

very small loss of activity when the serum was passed through Cibacron Blue-Sepharose columns.

DEAE-cellulose chromatography of the MC-serum showed that the active protein was negatively charged at pH 7.4. The results of gradient elution in Figure III-2 (representing 1 of 3 closely agreeing experiments) show that essentially all the activity and 25% of the protein of the original serum was eluted from the DEAE columns at 0.11-0.14M NaCl concentrations.

Chromatography on G-100 Sephadex column in the presence of markers with known MW indicated that the active protein was approximately 60,000 (see Figure III-3, referring to 1 of 3 different preparations yielding essentially identical results).

Not only MC-serum but also extracts of the carcinoma itself (MC-extract) stimulate GGT and AP expression by rat bone marrow cells in liquid culture. MC-extract consisted of homogenates of MC tumors in 4 volumes of 0.15M KCl from which particulate material was removed by a centrifugation at 100,000 X g for 30 min. The activity of the MC extract showed similar behavior on DEAE cellulose and G-100 column chromatography as did MC serum, but its specific activity was not of a higher order of magnitude. Efforts were made, therefore, to identify a richer source of the factor. To this end, MC-conditioned medium was prepared by incubation of 1 to 5 mm^3 pieces of MC in calcium- and magnesium-free

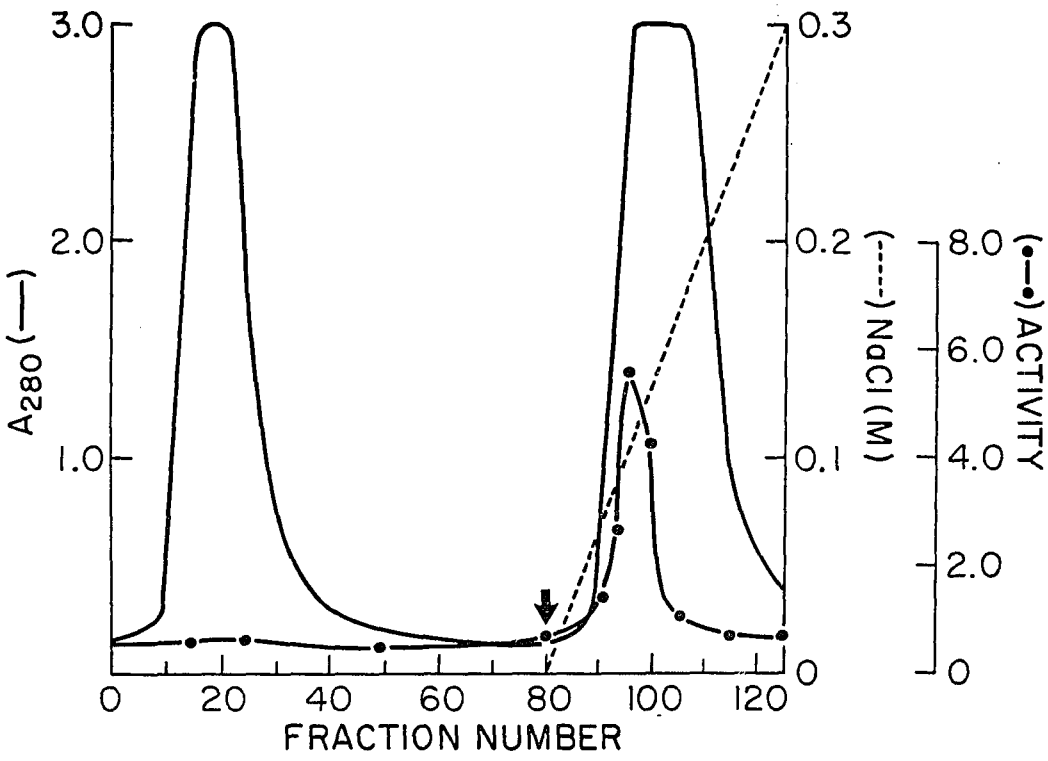


Figure III-2. Chromatography on DEAE-cellulose columns. MC-serum, after extensive dialysis against 0.06 M Tris-HCl buffer at pH 7.4 in 50K cut-off dialysis tubing, was applied to a DEAE-cellulose column (3.0 x 30 cm) equilibrated with the same buffer. After collecting the run through fractions, the proteins bound to the column were eluted with a linear gradient (see arrow and right ordinate) of 0.0 to 0.3 M NaCl in the equilibration buffer in a total volume of 300 ml at a flow rate of 20.0 ml per hour. Each fraction volume was 6.6 ml. Solid line refers to UV absorbance at 280 nm; activity units (based on the GGT response evoked in the liquid bone marrow culture) are shown by solid line with closed circles.

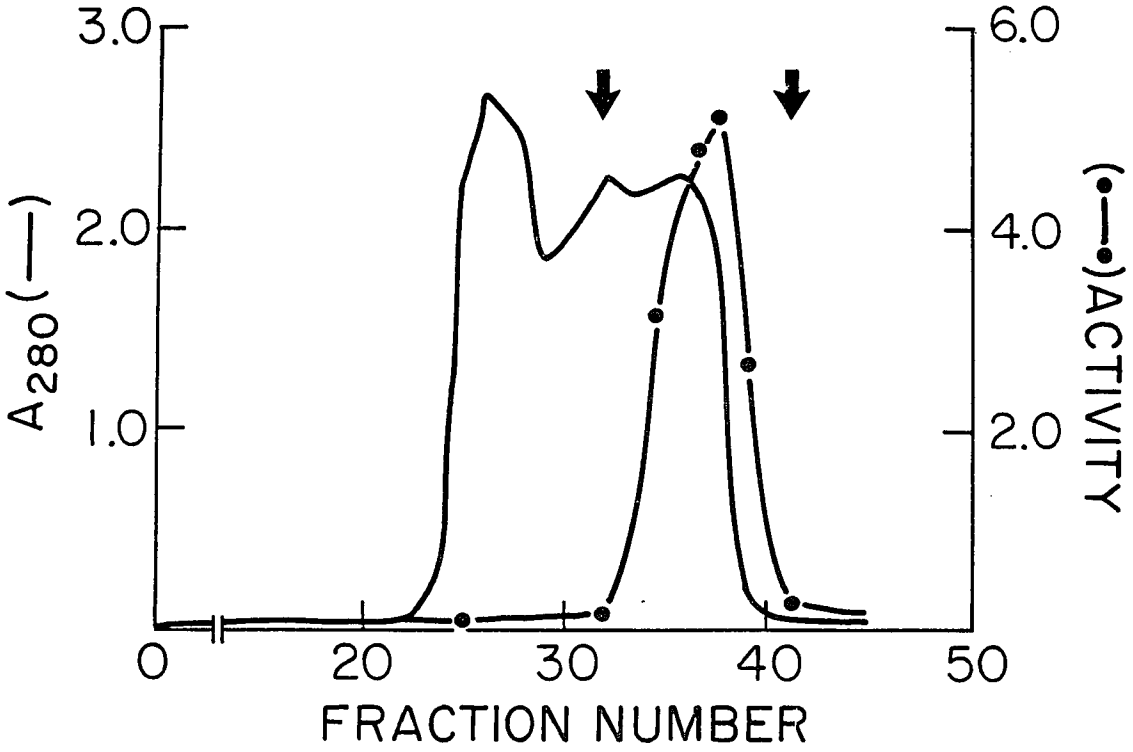


Figure III-3. Sephadex G-100 fractionation. Active fractions from DEAE-cellulose chromatography (see Figure III-1) of MC-serum were pooled, dialyzed against 0.06 M Tris-HCl buffer pH 7.4, placed on a Sephadex G-100 column (2.5 x 80 cm) and run with a pressure head of 70 cm using 0.06 M Tris-HCl buffer pH 7.4 (containing 0.01% azide) for elution. Fractions of 5.0 ml were collected. Molecular weight markers, bovine serum albumin (68,000) and ovalbumin (43,000) were run on the same column and the position of their elution is indicated by arrows. UV absorbance at 280 nm and activity (units-G) are illustrated as in Figure III-2.

Eagles minimal essential medium (0.5g MC per 10 ml medium) for 2 days, which was followed by a 100,000 X g centrifugation for 30 min and filter sterilization of the supernatant.

The results in Table III-3 demonstrate the considerable ability of the carcinoma to produce active protein in vitro. Endogenous activity (see MC-extract) was only 307±47 units-G / g, whereas 17,000 units-G / g tumor were found in the medium after 2-4 days of incubation (Table 3, column 3). It may also be seen from Table 3 that the amount of total protein released per mg tumor was only 5.90 mg/g. Therefore the specific activity (units-G/mg protein) of the conditioned medium (MC-CM) was very high (3060) compared to that (6.6) in extracts of freshly excised MC.

The activity (see Table III-2, column 2) was again found to reside in the higher than 50,000 MW fraction, to be unadsorbed on concanavalin A affinity column, and its sensitivity to proteolytic enzymes and heat was also similar to that of MC-serum (see Table III-2) or MC-extract (not shown). MC-CM was indistinguishable from MC-serum (and MC-extract) in terms of elution patterns from DEAE-cellulose and G-100 Sephadex columns. Application of the active fractions of MC-CM thus obtained to hydroxylapatite columns (see typical run in Figure III-4) yielded preparations 40-60 times higher specific activity,

Table III-3. Production of the active protein in vitro as compared to that endogenously present in MC.

preparations[a]	total protein mg/g tumor	units-G/ g tumor	units-G/ mg protein
MC Extract (3)	47±2	307±47	6.6±0.8
MC-CM	4.55	13,200	2,900
	6.00	16,300	2,720
	6.00	21,400	3,570
Mean	5.90±0.84	17,000±4,140	3060±448

[a]From each of 3 MC tumors, extracts (see mean±SD) and conditioned media (see individual values and mean±SD) were prepared and added to standard liquid culture.

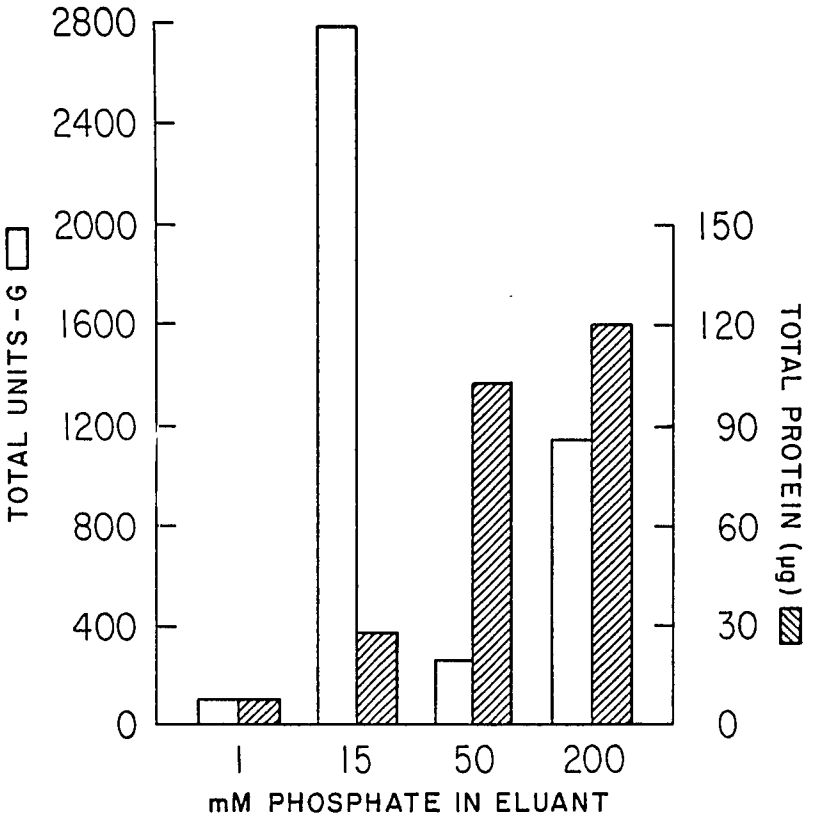


Figure III-4. Hydroxylapatite chromatography of MC-conditioned medium preparations after purification step 3.

for sixty to 70% of the total recovered activity, but very little of the total protein, was in the fraction eluting at 15mM phosphate concentration. A significant portion of the remaining activity (together with a large portion of the total protein) was eluted at 200mM (rather than 1 or 50mM) phosphate concentration (Figure III-4). The activity of the MC-serum preparations showed the same biphasic elution pattern. However due to the higher protein content of the 15mM phosphate eluent, the increase in specific activity (over that in the active G-100 column fraction) was very small, so that only in the case of MC-CM was hydroxylapatite chromatography a useful purification step.

The results of sequential application of the purification steps to the different sources of the MC factor are shown in Table III-4. As seen in column 1, the yield for MC-serum (probably due to an inhibitor present in the crude serum) was over 100%, whereas 60-75% of the initial activity of the MC-extract and MC-CM was lost through the same two chromatography steps. At this stage (see column 3) the active fractions from MC-serum and MC-extract showed specific activities of 40 and 560 respectively, whereas 34,000 was the value for MC-CM. Also, hydroxylapatite chromatography achieved a further significant purification in the case of MC-CM, so that the final specific activity (in the 15mM phosphate eluant) was 185,000. In view of the molecular weight of 60,000,

Table III-4. Purification of the MC protein.

Preparations[a]	Total Activity (units-G)	Total Protein (mg)	Specific Activity (units-G/ mg protein)	Fold Purification
MC-serum	9,600	6,460	1.5	
DEAE-cellulose	13,200	1,560	8.5	5.6
G-100 Sephadex	9,850	244	40	27
MC-extract	6,030	1,010	6.0	
DEAE-cellulose	4,030	62.0	65	11
G-100 Sephadex	2,420	4.3	560	93
MC-CM	21,600	7.06	3,060	
DEAE-cellulose	11,400	0.98	11,600	3.8
G-100 Sephadex	5,800	0.17	34,100	11
Hydroxylapatite 15 mM phosphate	1,850	0.010	185,000	60

[a]The sequence of purification steps, and the fractions from chromatography columns (illustrated in Figures III-2 to 4), were as described under Chapter II.

Table III-5. The AP response of bone marrow cells.

	units-A[a] (total)	units-A (specific)	Fold Purification	units-A/ units-G
MC-serum	25,600	3.96		2.64
DEAE-cellulose	23,100	14.8	3.7	1.74
G-100 Sephadex	8,640	35.3	8.9	0.88
MC-CM	13,200	1,870		0.611
DEAE-cellulose	6,810	6,950	3.7	0.599
G-100 Sephadex	2,000	11,800	6.3	0.346
Hydroxylapatite 15 mM phosphate	648	64,800	44	0.350

[a]Results refer to measurement of units-A of the same preparations whose units-G and protein contents are shown in Table III-4.

this means that the MC protein exerts significant activity at less than 100 pM concentration.

In the experiments of Table III-4, the effects of the treatments on AP-inducing ability of the MC-serum and MC-CM preparations were the same as those shown for their GGT inducing ability. In DEAE cellulose, G-100, or hydroxylapatite chromatography, the two activities were concentrated in the same fractions. However in the case of MC-serum, 66% of the total AP-inducing activity was lost, and the ratio of units-A to units-G decreased from 2.46 to 0.88 (see first part of Table III-5). In the case of MC-CM, this decrease, as well as the initial ratio was much lower: the units-A:units-G ratio was unchanged in the first chromatography step, decreased from 0.599 to 0.346 in the next one, and remained unchanged in hydroxylapatite chromatography (last part of Table III-5).

The GGT and AP response of rat bone marrow cells was also evoked by CSFs; (see Table III-6 and Chapter IV). Additional growth factors and hormones were therefore, tested for possible enzyme induction in the same system. TGF-β was without effect. The plant-derived mitogen, concanavalin A, did evoke the GGT response; however, unlike the CSFs or MC-preparations, it was without effect on AP. It may also be seen from Table III-6 that EGF, insulin, and hydrocortisone had no effect and did not potentiate the activity of the MC serum.

Table III-6. The effect of various growth factors on bone marrow GGT and AP.

	Activity	
Addition[a]	Units-G	Units-A
CSF-1	10.0±2.3	7.8±2.1
CSF-2γ (mGM-CSF)	10.5±1.2	4.7±1.8
CSF-GCT	9.8±1.9	5.2±2.0
TGF-β	0	0
Concanavalin A	11.9±1.5	0
Insulin	0	0
HC	0	0
EGF	0	0
MC serum	9.6±1.9	16.0±2.9
" + insulin	8.4±2.0	18.1±3.6
" + HC	7.0±1.8	19.5±3.1
" + EGF	9.6±2.2	17.6±1.9

[a]The standard normal bone marrow liquid culture was incubated for 48 hrs. The amounts of CSF-1, CSF-2γ , CSF-GCT, TGF-β and concanavalin A were 4 ng, 45 ng, 321 ug, 2 ng and 12.5 ug, respectively. When indicated, insulin (5 ug), HC (hydrocortisone, 0.45 ug) or EGF (1.0 ug) was added without or with MC-serum (25ul, containing 1.9 mg total protein). Values are means ± S.D. of 3-5 determinations.

DISCUSSION

In view of the association of hormone-induced hyperplasia in target tissues *in vivo* (mammary gland, endometrium, seminal vesicles, etc.) with elevated GGT content (e.g. Tarachand & Eapen, 1982, Vina et.al., 1983), the elevation in the bone marrow of MC-bearing rats (Koss & Greengard, 1982) may have been due to the stimulation by this carcinoma of the release of hormones or growth factors from endocrine (or other) tissues of the host. However, this is not the case. The active factor is elaborated by the carcinoma itself. MC-CM (or MC-extracts) also stimulated GGT expression in normal rat bone marrow cells *in vitro*, and the protein responsible for this appears to be identical to the active factor in MC serum.

The high capacity of the carcinoma for synthesis of MC-protein *in vitro* is indicated by the fact that the units of activity released to the medium in 2-4 days (17,000/ g tumor) were far in excess of its content prior to incubation (307 units/g tumor)(see Table III-3). Due perhaps to a lower rate of production *in vivo*, or to a faster degradation or uptake by target tissues, this factor is a very minor constituent of the MC-serum. The small amount of total protein released by MC *in vitro* is another reason why conditioned medium yielded preparations with several order of magnitude higher

specific activity than did the MC-serum. Amounts of these preparations containing 90 fmol protein were sufficient to evoke a significant GGT response.

It was necessary to consider the possibility that the MC protein may be identical to one of the known CSFs, since we found that several of these (see Chapter IV and Table III-6) can also stimulate GGT expression in the liquid culture system. The trypsin insensitivity of one of them, CSF-1 (Stanley & Metcalf, 1971), also appears to be a characteristic of MC protein since 85 ± 13% of its activity was recovered after trypsin treatment (Table III-2). However, several other properties differ from CSF-1 as well as from other CSFs. Concanavalin A column chromatography, for example, resulted in no loss of activity from MC-CM, and only 13-18% of the activity of crude MC-serum remained on the column. Its molecular weight (approximately 60,000) is different from than that of CSF-1 (a 70,000 MW dimer) (Stanley & Heard, 1977), CSF-2τ, mGM-CSF (35,000) (Prestidge et.al., 1984), G-CSF (25,000) (Nicola et.al., 1983), or GM-CSF (23,000) (Burgess et.al., 1977). Also, 50% of the activity of CSF-1 and G-CSF is lost in 30 min at 90^{o}C and 70^{o}C, respectively (Stanley & Metcalf, 1969, Nicola & Metcalf, 1984), whereas 75 ±12% of the activity of MC- protein was present after boiling for 30 min (Table 2). Stability to mercaptoethanol further contrasts it from CSF-1 (Stanley &

Heard, 1977), and elution from DEAE column at 110-140mM NaCl distinguishes it from GM-CSF and CSF-2τ which elute at 50 (Burgess et.al., 1977) and 75 mM NaCl (Prestidge et.al., 1984) concentrations, respectively.

Although specific growth factors or hormones elaborated by animal tissues (cancerous or normal) have not been previously reported to induce GGT in any cell type in vitro, increases in this enzyme were found to follow stimulation of lymphocyte DNA synthesis by plant-derived mitogens (Novogrodsky et.al., 1976). It is likely, therefore that the GGT reponse to a mitogen now tested (concanavalin A; see Table III-6) is attributable to the lymphocytic cells present in the unfractionated bone marrow suspension. This phenomenon, however, is qualitatively different from the effect of the MC protein and of the CSFs which (a) act on myeloid cells (Koss & Greengard, 1982, and Burgess & Metcalf, 1980) and which (b) evoke the AP as well as the GGT response in the rat bone marrow system.

While a portion of the AP-inducing ability of the MC-serum may be attributable to a possibly unstable factor of unknown nature, most of it co-chromatographed with the GGT inducing protein and was present in preparations with the highest specific activity for GGT induction (e.g. 123,000 times higher than MC serum, see first and last lines in Table III-4). The possibility that we are dealing

with 2 factors in MC-CM could not be excluded. However, highly purified CSF-1 and recombinant human G-CSF (see Chapter IV) also stimulated both GGT and AP expression in rat bone marrow cells. Thus, while no common regulator of these two different enzymes have previously been identified, it is clear that a single polypeptide can initiate events leading to the induction of GGT and AP.

Chapter IV

ENHANCEMENT OF γ-GLUTAMYLTRANSPEPTIDASE EXPRESSION IN BONE MARROW CELLS BY COLONY STIMULATION FACTORS

INTRODUCTION

Mechanisms regulating myelopoiesis, the importance of which is evident from the many situations (e.g. blood loss and infections) where the bone marrow is called upon to produce large numbers of granulocytes, are as yet incompletely understood. Expectations of gaining insight to these mechanisms has been a major motivation for extensive investigations in the last 15 years on a series of glycoprotein colony stimulating factors (CSFs) which act upon neutrophil and monocyte precursors and which were found to be elaborated by a variety of normal (as well as cancer) tissues (Metcalf, 1986). Recently these investigations culminated in the cloning of the cDNA for several murine and human CSFs (Seiff et.al., 1985; Wong et.al., 1985). There is thus precise knowledge about their chemical structure, and the nature of the cells to which they bind (Guilbert & Stanley, 1980). Their interaction with specific cell surface receptors (Nicola & Metcalf, 1985; Walker et.al., 1985) has also been studied. However, as recently summarized by Metcalf (1985), "There is only fragmentary information on the biological events occurring when CSFs stimulate cells to pass through the cell cycle

and divide." There is indeed no knowledge, for example, about changes in cellular enzymic composition that follow the impact of CSFs in vitro or in vivo. The possibility of identifying such a change emerged from observations on rats carrying the granulocytosis inducing mammary carcinoma 5A. In the tumor bearing rat the onset of granulocytosis is preceded by increases in the cancer-free bone marrow's γ-glutamyltranspeptidase (GGT) and alkaline phosphatase (AP) concentration (Koss & Greengard, 1982). These increases are reproducible in normal bone marrow cells in vitro by incubation with the host's serum for 1-3 days (Greengard et.al., 1984) and are attributable to a carcinoma elaborated protein (see preceding Chapter). The following studies show that several CSFs exert similar effects on GGT and AP.

RESULTS

The colony stimulating factors used in these studies were from a variety of sources. Giant cell tumor conditioned medium (CSF-GCT), medium harvested from a continous culture of a human fibrous histiocytoma lung metastasis and containing human granulocyte/macrophage - CSF, GM-CSF (Dipersio et.al., 1978) was obtained commercially. Purified CSF-1, a stimulator of macrophage colony formation, and L-cell conditioned medium (CSF-L (a source of CSF-1), were generously supplied by E. Richard Stanley of the Albert Einstein School of Medicine (Bronx, NY). Interleukin-3 (IL-3, also known as multi-CSF), murine(m) GM-CSF (CSF-2τ) isolated from supernates of a PHA stimulated murine T-cell lymphoma (LBR-335A4) culture (Prestidge et.al., 1984) and recombinant human granulocyte-CSF (rhG-CSF) were obtained commercially.

The three MC- preparations derived from rats bearing the mammary carcinoma 5A (MC) and described in the previous Chapter were: host serum (MC-S), extract of the MC tumor (MC-T), and MC conditioned medium (MC-CM). Previous studies with the liquid culture of normal rat bone marrow cells have shown that GGT and AP content increases significantly as early as 24 hours after incubation with MC-serum and reaches a maximum at 72 hours (Greengard et.al., 1984). The effect of the other MC

preparation (studied in the previous Chapter) and of the CSFs with enzyme inducing capacity showed a similar time course. The incubation time, therefore, was standardized to 48 hours. Normal rat serum (10%), which maintains the cells' original GGT and AP activity was added to all tubes with the exception of those containing 10% MC-serum.

It may be seen from Figure IV-1 that GGT expression is stimulated in a dose dependent manner, not only by MC preparations but also by purified CSF-2τ (mGM-CSF) and CSF-1. The same was true for L-cell conditioned medium containing CSF-1 (Guilbert & Stanley, 1980) and human giant cell tumor conditioned medium, rich in GM-CSF (DiPersio et.al., 1978). Il-3, on the other hand was without effect (Figure IV-1).

The AP concentrations of the bone marrow cells, measured in each experiment of Figure IV-1, also showed several-fold increases upon incubation with the above preparations (except IL-3), and the shapes of the dose-response curves (Figure IV-2) resembled closely those in Figure IV-1. The multiplicative potential of the CSFs (and of the other preparations including IL-3) is not evident in this short-term liquid culture, nor was there any significant cell death or loss of viability. Thus, 100±10% of the cells added to each test tube at time 0 was recovered after the 48h of incubation with (or without) the CSF or MC preparations studied. The colony stimulating

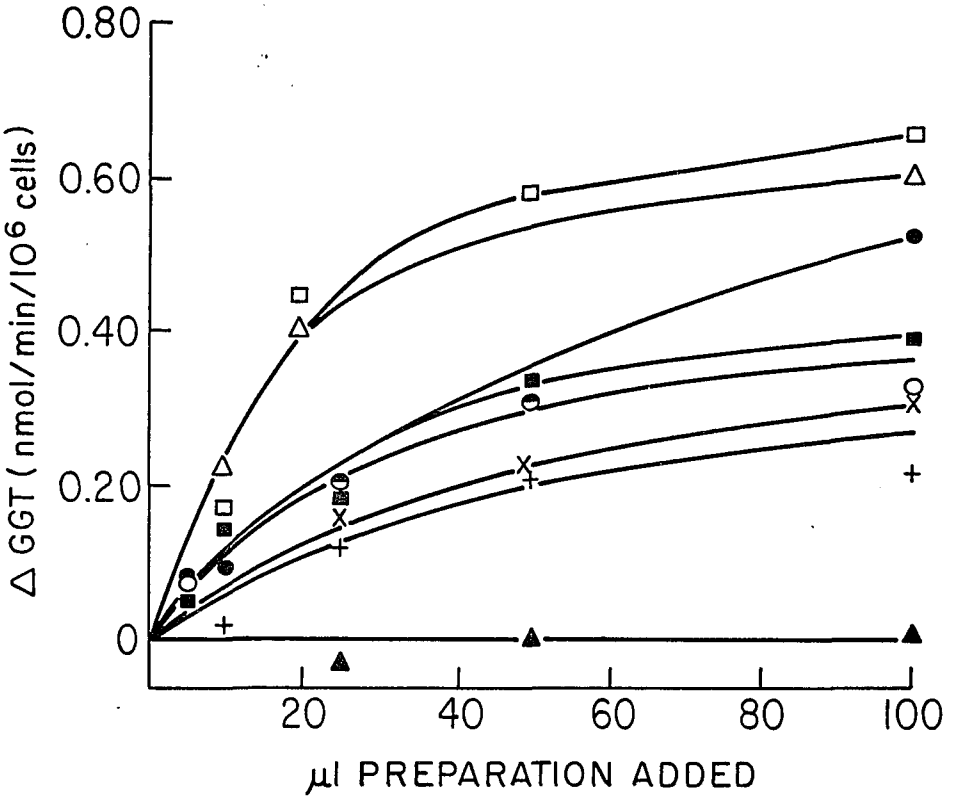


FIGURE IV-1. Stimulated GGT expression in 48 hr normal bone marrow liquid culture by CSFs and MC preparations. The protein content of CSF-1 (+), CSF-2 γ (▣), L-cell conditioned medium (CSF-L, X), IL-3 (▲), MC conditioned medium (MC-CM, □), giant cell conditioned medium (CSF-GCT, ◍), MC tumor extract (MC-T, Δ) and MC serum (MC-S, ○) were 0.040×10^{-3}, 0.454×10^{-3}, 2.5×10^{-3}, 0.5×10^{-3}, 0.05, 3.21, 12.5 and 60 ug/ul, respectively.

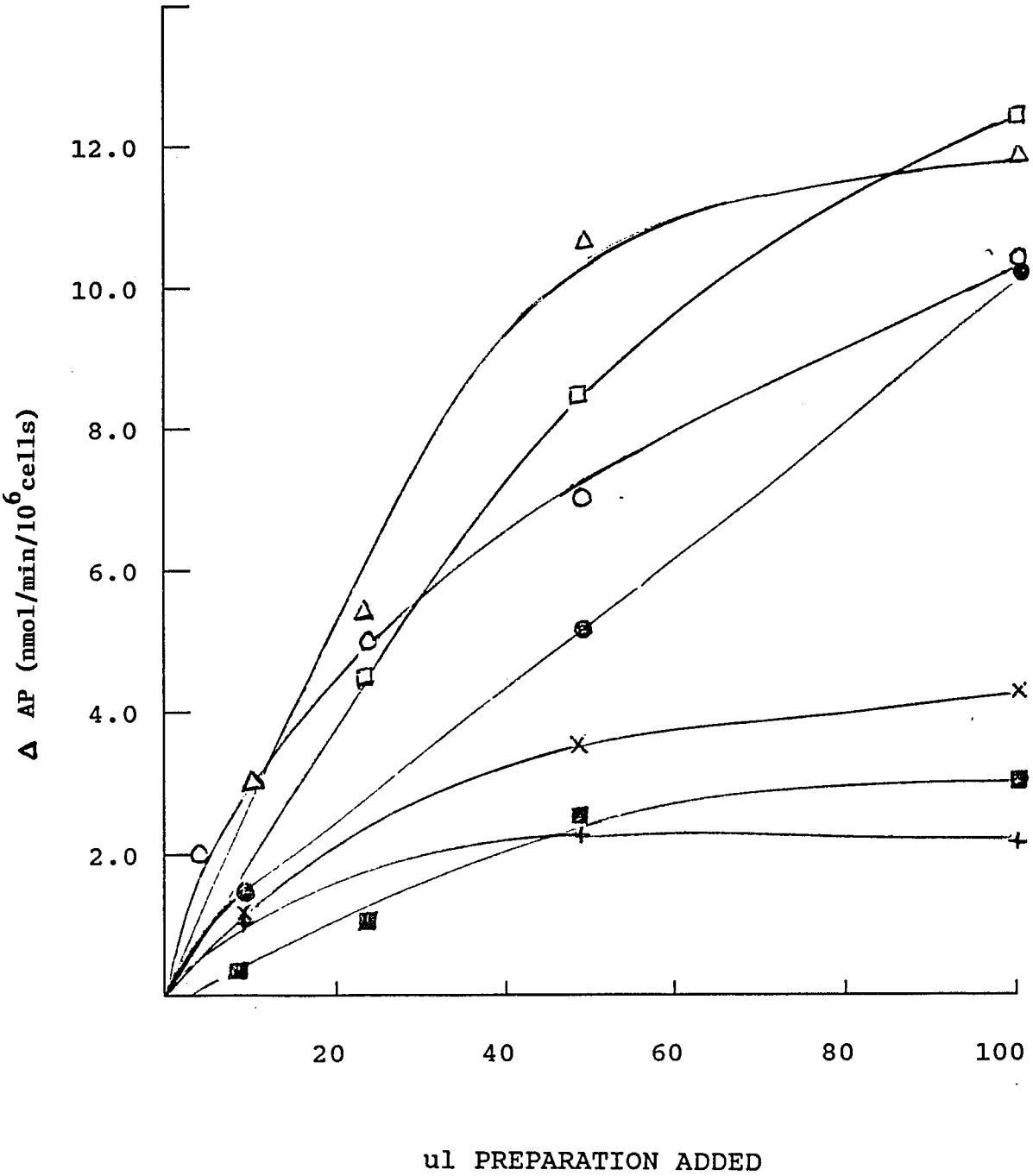


FIGURE IV-2. Stimulated AP expression in 48 hr normal bone marrow liquid culture by CSFs and MC preparations. For symbols see Figure IV-1.

effect of these preparations was determined in agar cultures of mouse bone marrow, the standard test system for rodent and human CSFs. Serum from MC-bearing rats was previously shown to be active in this system, and the same was now found to be true for MC-extract and MC-CM. The curves in Figure IV-3 illustrate the dependence of the stimulation on the amount of the MC and the CSF preparations.

Experiments in which the mouse bone marrow agar culture colonies were stained for identification of cell type showed (Table IV-1) that CSF-1 (as found previously (Stanley & Heard, 1977)) is primarily a macrophage CSF, and that the percentages of granulocyte, macrophage and mixed colonies in the presence of GM-CSFs (CSF-2τ and CSF-GCT) were not unlike those obtained with the MC preparations. Although IL-3 promotes the growth of a variety of cell types, granulocyte and macrophage colonies were found to appear predominantly in the second week of its incubation with bone marrow agar cultures (Ihle et.al., 1985). Accordingly, while non-myeloid cell colonies represented 36% of total, myeloid cell colonies were in the majority (64%, Table IV-1).

In order to obtain the dose-response curves in Figures IV-1,2,3, each preparation (except for MC-serum) was diluted to an appropriate degree (see widely varying protein contents in Figure IV-1), so that increases of

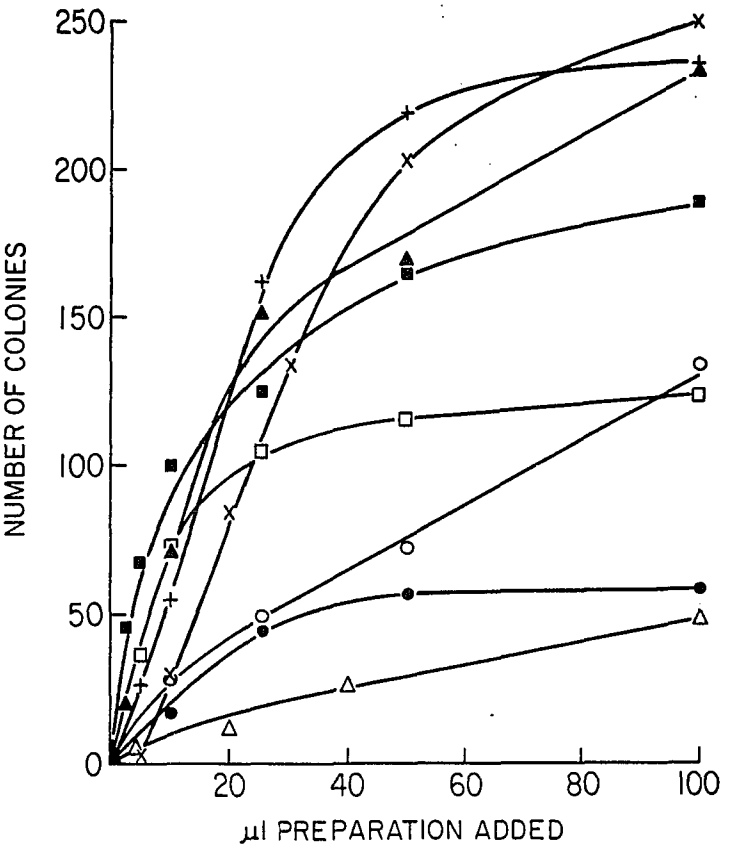


FIGURE IV-3. Stimulation of colony formation in 7 day bone marrrow agar culture by CSFs and MC preparations. For abbreviations and symbols see Figure IV-1 legend. Protein contents of CSF-1, CSF-2γ, CSF-L, IL-3, MC-CM, CSF-GCT, MC-T and MC-S were 0.040 x 10^{-3}, 0.227 x 10^{-3}, 0.250 x 10^{-3}, 0.500 x 10^{-3}, 0.100, 0.321, 1.25 and 60 ug/ul, respectively.

TABLE IV-1. Types of mouse bone marrow agar culture colonies generated by CSFs and MC preparations

Preparations tested[a]	Granulocyte colonies as percent of total	Macrophage	Mixed
CSF-1	13	44	43
CSF-2γ (mGM-CSF)	26	40	34
CSF-L	--	--	--
IL-3	20	27	17 [b]
MC-CM	32	34	33
CSF-GCT	30	33	37
MC-T	23	38	38
MC-S	16	44	44

[a]For abbreviations see Figure IV-1, legend.

[b]36% of colonies not of myeloid origin.

enzyme expression with increasing volume added does not reveal at a glance the wide variation in potency among the different preparations. Quantification of colony stimulating activity was based (as is the standard practice, e.g. Burgess et.al., 1977) on the initial portion of the dose-response curves where 20-200 is the range of total colony number. From this, we calculated the specific activity of the preparation, i.e. total number of colonies they generate per milligram protein. Similarly, the initial, near-linear portion of curves depicting the dose-dependent rise of GGT and AP was the basis of quantifying the activity that the different preparations (per mg protein exerted). In Table IV-2, they are listed in the order of increasing colony stimulating activity. It may be seen that the identical order is obtained by listing the preparation (with the exception of IL-3) according to specific activity manifested in the stimulation of GGT and AP expression of bone marrow cells in the liquid culture.

In terms of number of colonies of all types, specific activities varied over 6 orders of magnitude, and variations in terms of GGT (or AP) response evoked were almost as wide. For this reason, it is not immediately apparent that not only is there a rank order but also a quantitative correlation between the activities in the two systems. Figure IV-4 illustrates, however, that if colony

TABLE IV-2. Comparison of colony stimulating and enzyme inducing activities.

Preparations tested	Activity per mg protein of preparations[a]		
	Agar culture colonies	Induction in liquid culture	
		GGT	AP
CSF-1	$138{,}000 \times 10^3$	$1{,}000 \times 10^3$	$1{,}200 \times 10^3$
CSF-2 γ (GM-CSF)	$81{,}000 \times 10^3$	140×10^3	130×10^3
CSF-L	$17{,}000 \times 10^3$	22×10^3	31×10^3
IL-3	$16{,}000 \times 10^3$	not detectable	
MC-CM	72×10^3	4.5×10^3	1.8×10^3
CSF-GCT	6.2×10^3	31	56
MC-T	680	18	26
MC-S	47	2.0	4.6

[a]Values were calculated from dose response curves (see text and Figures IV-1,2

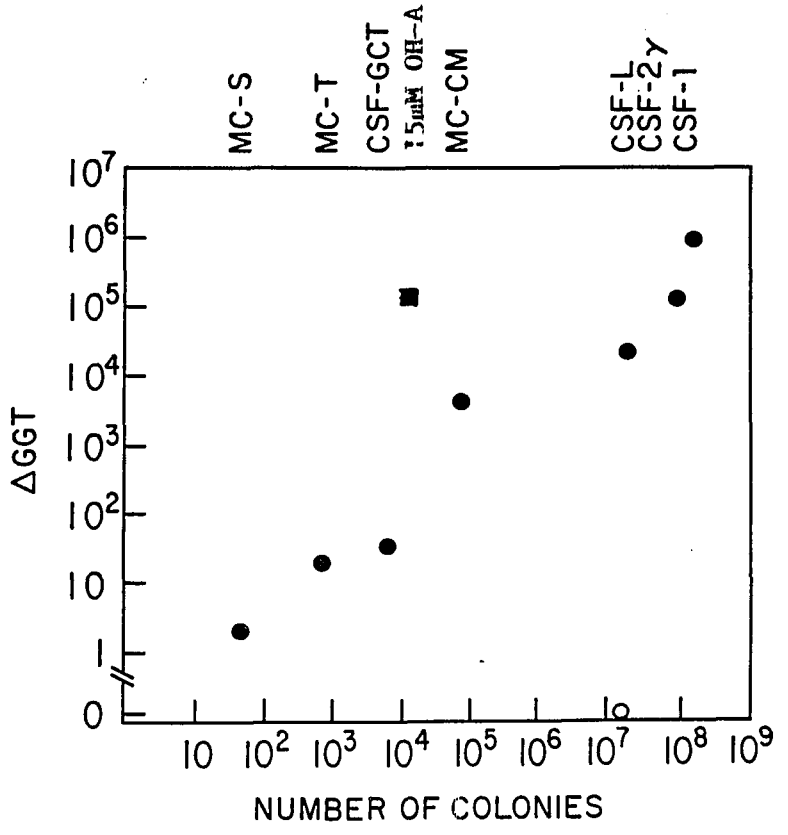


FIGURE IV-4. Correlation between ability to stimulate GGT expression and colony formation. The specific activities in the liquid BM culture (ordinate) and agar culture (abscissa) systems are based on results in Table 1. Each solid circle refers to a preparation identified on the top of the figure with abbreviations listed in Fig IV-1 legend. The Pearson correlation coefficient (not including results on IL-3, open circle) was $r=0.976$, $p<.001$.

stimulating activity is plotted against the same preparation's activity as judged by stimulation of GGT expression in the liquid culture (note log coordinate), then the points (see closed circles) fall close to a straight line. The Pearson parametric correlation coefficient was 0.976 ($p<.001$). The correlation of colony stimulating activity to AP induction was equally significant ($r=0.985$, $p<.001$).

These quantitative correlations did not extend to the purified preparation of the MC protein, i.e. to the 15mM phosphate eluent of hydroxyapatite columns (see Chapter III). With a GGT inducing activity 120,000 times higher than in MC-serum) ammounting to 185,000 units-G per mg protein, its colony stimulating activity was still considerable (13,000 colonies per mg protein), however, the point (square) depicting these results in Figure IV-5 was well above the regression line. Thus, the ratio of enzyme inducing to proliferative effect of the MC protein appears to be higher than that of the CSFs. The results are consistent with the possibility that the portion of the colony stimulating activity of crude MC-serum or MC-CM is due in part to CSFs, especially since CSFs are known to be elaborated by a large variety of cancers and normal cells, and are present in serum.

After the establishment of the relationship between the colony stimulating activity and the induction of both

GGT and AP by CSFs, recombinant human G-CSF became available. As seen in Fig. 5 there is an increase in both GGT and AP concentration in the normal rat bone marrow with increasing dose of rhG-CSF added to the liquid culture system. The relationship of these specific activities (0.9×10^6 and 4.4×10^6 units for GGT and AP induction, respectively) to that in terms of colony stimulation by rhG-CSF (1.0×10^6 units) fitted the relationship found for the other CSF and MC preparations within the 95% confidence limits. With the new point added to the data in Fig. 5, the regression line and its significance thus remained similar, $r=.910$ $p<.005$.

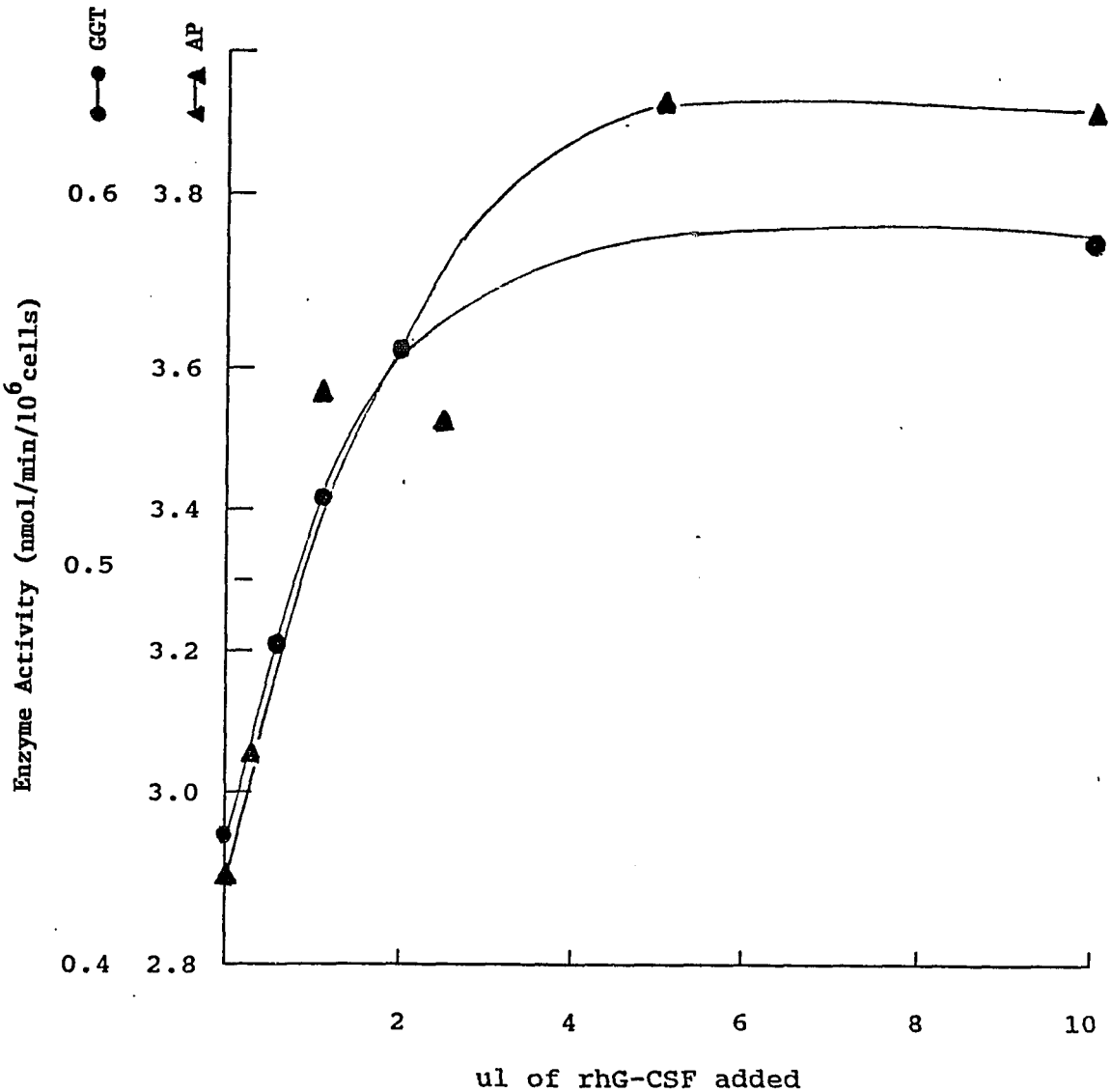


FIGURE IV-5 Stimulated GGT (●) and AP (▲) expression in 48 hr normal bone marrow liquid culture by rhG-CSF.

DISCUSSION

The mammary carcinoma elaborated protein which was found to induce GGT expression in normal bone marrow cells has not yet been purified to homogeneity, so that no direct proof could be obtained that the colony stimulating activity of the MC preparations is attributable to the same protein. In the case of the highly purified or cloned CSFs, however, the colony stimulating proteins themselves must have been responsible for the induction of GGT (and AP) expression. That the same is true for the CSF-GCT and L-cell conditioned medium (CSF-L) is strongly suggested by the fact that the results on these conform to the same quantitative correlation between these two activities as do the purified CSFs (Table IV-2, Figure IV-4). In the course of purification of the MC protein the ratio of enzyme inducing to colony stimulating activity increased and became higher than the ratio for the different CSFs. This finding is of potential importance in connection with the differentiational effect of MC protein on leukemic cells (Chapter VI). For it implies a lesser ability to stimulate myeloblast proliferation and thus an advantage, in terms of leukemia therapy, over differentiation-enforcing CSFs.

Origin from cancer tissues or transformed cells does not appear to be a necessary criterion of GGT and AP

inducing ability, since the CSF-1 used here (Stanley & Heard, 1977) has also been isolated from normal mouse tissues (Sheridan & Stanley, 1971) and human urine (Das et.al., 1981). Nor is this ability associated with growth stimulatory activity in general since IL-3, at least, failed to evoke the enzyme response in rat (Figure IV-1) bone marrow cells. This difference may be related to the fact that, while committed precursors of the myeloid lineage are the targets of CSFs (Burgess & Metcalf, 1980), IL-3 (which is a lymphokine stimulating T cell differentiation (Ihle et.al., 1981)), also acts on the pluripotent stem cell (Ihle et.al., 1985).

The significance of the present results on GGT and AP lies in the fact that no enzymic changes have previously been shown to be caused by purified G and/ or M-CSFs. Even for crude preparations, only one such change seems to have beeen reported, namely , aminopeptidase expression (revealed by histochemical staining) in response to L-cell conditioned medium (Mergenthaler et.al., 1982). The liquid culture method employed in that study (n.b. the use of hydrophilic surface dishes permitting cell adherence) favored macrophage differentiation and survival, so that the aminopeptidase positivity which was detectable only after 10 days of incubation could be explained by the fact that the cells present at this time (with the death of 87% of the total added) were all mature macrophages

(Mergenthaler, 1982). This type of explanation does not appear to be applicable to the enzymic changes obtained in the present system by 48 hour of incubation with CSFs. For (see Chapter V) no signs of morphological maturation accompanied the mGM-CSF induced rise in GGT and AP. Furthermore, no change in cell number could be detected in the liquid culture system during incubation with CSFs. The possibility does exist that the enzyme induction they caused constitute a biochemical prelude to proliferation.

On the other hand, it is conceivable that increased enzyme expression is unrelated to proliferation since CSFs are now known to exert several effects on mature white blood cells. CSF-1 stimulates secretion of plasminogen activator and prostaglandins from macrophages (Stanley, 1981). G-CSF has been reported to promote fMLP receptor acquisition (Platzer et.al., 1985), both GM-CSF and G-CSF are capable of activating neutrophils to produce superoxide and become cytotoxic to other cells (Weisbart et.al., 1985; Platzer et.al., 1986) Therefore, a possible relation of these functional changes to the herein described enzyme induction can not be excluded. On account of its unusual properties, GGT is of particular interest in this connection: it is a plasma membrane bound enzyme with the ability to act on extracellular substrate (Novogrodsky et.al., 1976), it is thought to play an important role in amino acid transport into cells (Griffin

et.al., 1979; Orlowski et.al., 1974; Vina et.al., 1983) and it is the only enzyme capable of initiating the hydrolysis of glutathione (Curthoys & Hughey, 1979)

Identification of the cell types subject to the newly discovered enzymic effects of CSFs, and quantitative comparison of specific binding of these factors to the surface receptors of these cells, awaits flowcytometric sorting and analysis of distinct bone marrow cell subpopulations. The results obtained in these studies, however, have some immediate practical significance. For the incubation in the short term liquid culture system and measurement of GGT could serve to screen body fluids or tumor extracts for the several types of CSFs active in this system. It may also offer a convenient assay useful in the purification of these factors.

Chapter V

NEUTROPHIL MATURATION AND HYPERSEGMENTATION PROMOTED IN NORMAL BONE MARROW BY A CARCINOMA-ELABORATED PROTEIN FACTOR

Introduction

Granulocytosis of varying degrees is a frequent accompanyment of non-hematopoietic malignancies in man (Robinson, 1974) as well as in experimental animals with non-metatastic tumors of various cell types (Asano et.al., 1980, Dunning & Reich, 1943, Delmonte et.al., 1966, Thomas et.al., 1985). This phenomenon, often referred to as "leukemoid response" has in several cases been noted to be associated with multiple hematologic abnormalities. In mice with a transplanted tumor, the accumulation of normal mature granulocytes was found to be accompanied by an increased percent of myeloid precursors in the bone marrow and by the release of some of the immature cells in to the circulation. An interesting observation made by Delmonte et.al. (1966) in their study on the excessive production of mature granulocytes in mice carrying mammary adenocarcinoma CE1460 was that the additional cells released to the blood were not all normal myeloid precursors, but that some exhibited various abnormalities of nuclear or cytoplasmic morphology and included hypersegmented neutrophils. Murine mammary cancer

BALB/cfC3H was the subject of another animal study by Thomas et.al. (1985), which mentioned that hypersegmented neutrophils (known to occur in some human cancer subjects as well as in megaloblastic anemia (O'Grady, 1984)) were present in mice implanted with this granulocytosis inducing tumor. The occurrence of hypersegmented neutrophils of rats carrying mammary carcinoma 5A (MC) was thus not too surprising. However, since the majority of blood neutrophils rather than an occasional one was now found to exhibit obvious hypersegmentation, MC-bearing rats appeared to constitute a unique animal model for study of this abnormality.

Investigations described in this chapter thus began with microscopic and biochemical examination of bone marrow and blood cells from MC-bearing rats. The subsequent, similar examination of bone marrow cells from normal (tumor-free) animals which have been incubated with the MC serum or with MC-conditioned medium showed that a protein factor(s) in these preparations promotes the differentiation of granulocyte precursors, as well as the formation of a large number of hypersegmented neutrophils <u>in vitro</u>. A general conclusion from these studies is that neutrophil hypersegmentation in vivo can arise from direct action of tumor-elaborated, blood-borne polypeptide factors on normal myeloid cells in the bone marrow.

RESULTS

A striking previously noted alteration that subcutaneous MC transplantation caused in the host's hematopoietic tissues in vivo, consisted of a progressive increase in the blood granulocyte fraction; this was accompanied by a concomitant rise in the GGT and AP content (per cell) of this fraction as well as of the bone marrow (Table V-1). Studies of the effect of MC in vivo were now extended to more detailed microscopic examination of myeloid cells in the blood and bone marrow. Most of the experiments were carried out in rats with 27-36g tumors, i.e. at a stage when granulocytosis (see Table V-2) and the above mentioned enzyme changes are pronounced but when the animals are still devoid of any other signs of ill health.

The results showed that their blood was devoid of immature cells, that the number of eosinophilic granulocytes (with normal mature appearance) was as low as in control rats, and that the 20 times higher than normal value for the total number of granulocytes per ml was thus attributable to an increase in mature neutrophils. However, many of these neutrophils showed striking hypersegmentation; the appearance of such a cell, as compared to that of the typical rat neutrophil is illustrated in Figs V-1A and B. This abnormal nuclear

TABLE V-1

γ-Glutamyltranspeptidase and Alkaline Phosphatase Activity in Hematopoietic Tissues OF Normal and tumor-bearing Rats

	γ-Glutamyltranspeptidase		Alkaline Phosphatase	
	Control	Tumor-bearing	Control	Tumor-bearing
	units/g			
BM	0.55±0.07(5)	1.90±0.45(3)	5.7±1.9(4)	63.0±41.0(3)
	milliunits/ml blood			
WBC	1.17±0.35(7)	25.7±18.6(6)	8.2±2.7(8)	227±140(8)
	milliunits/million cells			
WBC	0.29±0.05(7)	3.33±1.93(6)	2.1±0.9(8)	29.1±20.8(8)

BM, bone marrow; WBC, white blood cells; units=micromoles / min.

morphology characterized almost a third of the neutrophils, i.e. 7.65 million of the total of 19.79 million per ml blood, in rats with 27-36g tumors. The first part of Table V-2 which presents these mean values also includes results on two rats which show that the appearance of hypersegmented neutrophils is not a late event, in that their titer is already considerable (1.54 million per ml) when the tumor repesents only about 1-2% of the total body mass and when granulocytosis is not yet severe.

The second part of Table V-2 shows that in the bone marrow of the experimental rats no hypersegmented neutrophils could be detected at either early or late stages of tumor growth. The microscopic appearance of the nine other cell types listed in Table V-2 was normal, and their percentages showed no statistically significant differences from control values. Elevated GGT and AP content, reported previously (Koss & Greengard, 1982) and confirmed here, was thus the only reflection of the altered functional state of the bone marrow of MC-bearing rats.

These enzyme elevations have been shown to be reproducible in liquid culture of normal bone marrow cells by 48 hr incubation with the MC-hosts' serum (MC-serum) or MC-conditioned medium (see Chapter III, and see also Table V-7). The present microscopic studies on bone marrow were

Table V-2 Effects of mammary carcinoma in vivo

	Normal Rat	MC Rat 3-5g tumor	MC Rat 27-36g tumor
Blood[a]		number[b]/ml blood X 10^6	
Neutrophil, segmented	0.8±0.4	1.88	12.1±2.7
Neutrophil, hypersegmented	0.0	1.54	7.6±1.2
Bone Marrow		percent of all myeloid cells	
Myeloblast	2.0±0.6	1.4	1.4±0.6
Promyelocyte	5.5±2.0	2.2	4.3±1.0
Neutrophilic Myelocyte	9.6±2.6	9.4	12.4±3.4
Neutrophilic Metamyelocyte	23.8±1.0	23.4	29.0±6.8
Band Form	37.2±3.7	42.1	39.1±6.4
Segmented Neutrophil	11.1±1.9	14.4	7.4±2.4
Hypersegmented Neutrophil	0.0	0.0	0.0
Eosinophilic Myelocyte	2.0±0.6	2.0	1.9±1.0
Eosinophilic Metamyelocyte	6.4±1.8	3.0	3.2±0.4
Mature Eosinophil	2.0±1.0	1.4	1.1±0.5

[a]Blood and bone marrow were from rats without (Normal) and with (MC) tumors of the indicated size.
[b]Values± and without SD are means of results on three rats, and average results on 2 rats, respectively.

extended, therefore, to the search for possible morphological changes occurring during such incubations.

In the liquid culture of bone marrow, preservation of cell integrity requires the presence of 10% normal rat serum (or MC-serum). Comparison with unincubated samples of the same bone marrow suspension showed that after 2 days of incubation with normal serum there was some shift towards a more differentiated cell type distribution (see first column of Table V-3 with that of Table V-2). However, 48 hr incubation with MC-serum resulted in a further shift to the right in the neutrophilic myeloid population. There was a decrease in the percent of myeloblasts, promyelocytes, myelocytes, metamyelocytes, and band forms (Table V-3). This was accompanied by a 2-fold rise in the percentage of all mature neutrophils (both segmented and hypersegmented). The number of hypersegmented neutrophils represented 25.7% of all myeloid cells, while this percentage was negligible after incubation with normal serum. There was no significant change in the percentage of eosinophils, although the increase in the mature form with decreases in its precursors (seen also after incubation with MC-CM) may be looked upon as a consistent, slight shift to the right.

Before describing the similar though somewhat more striking effect of small amount of MC-conditioned medium it should be noted that the portion of this medium that

Table V-3 Myeloid maturation and neutrophil hypersegmentation in liquid culture of normal bone marrow cells.

	control	MC-serum	MC-CM	MC-CM (24hr)
cell type[a]	percent of all myeloid cells[b]			
1. Myeloblast	1.1±0.7	0.6±1.2	0.3±0.7	0.5
2. Promyeloblast	2.4±2.1	1.7±0.8	1.5±1.0	1.2
3. Neutrophilic Myelocyte	3.0±1.4	3.0±1.1	2.4±0.7	3.9
4. Neutrophilic Metamyelocyte	14.3±5.9	8.6±3.3	7.3±2.7[c]	14.3
1+2+3+4	22.8±8.8	13.7±5.6	11.6±2.3[d]	19.9
5. Band forms	39.4±6.2	16.2±3.3[d]	8.4±3.7[d]	51.0
6. Segmented Neutrophils	27.3±11	39.1±9.1	35.4±5.2	15.0
7. Hypersegmented Neutrophils	0.5±0.4	25.7±7.5[d]	35.6±8.0[d]	6.5
6+7	27.8±10.4	64.2±5.7[d]	71.6±6.6[d]	21.5
8. Eosinophilic Myelocyte	1.0±1.1	0.0	0.0	0.5
9. Eosinophilic Metamyelocyte	6.3±3.3	3.1±1.4	2.6±0.6[c]	4.7
10. Mature Eosinophils	2.2±2.1	2.2±1.6	3.7±2.7	2.1

[a]Cell types were counted after 48hr (or 24hr, column 4) incubation of liquid bone marrow culture in the presence of 10% MC-serum or MC- conditioned medium (MC-CM) plus 10% normal rat serum.
[b]Values are means of results on 5 (±SD) or 2 (no SD) experiments.
[c,d]Differences from values under "control" were significant, $p<.05$ and $p<.001$, respectively.

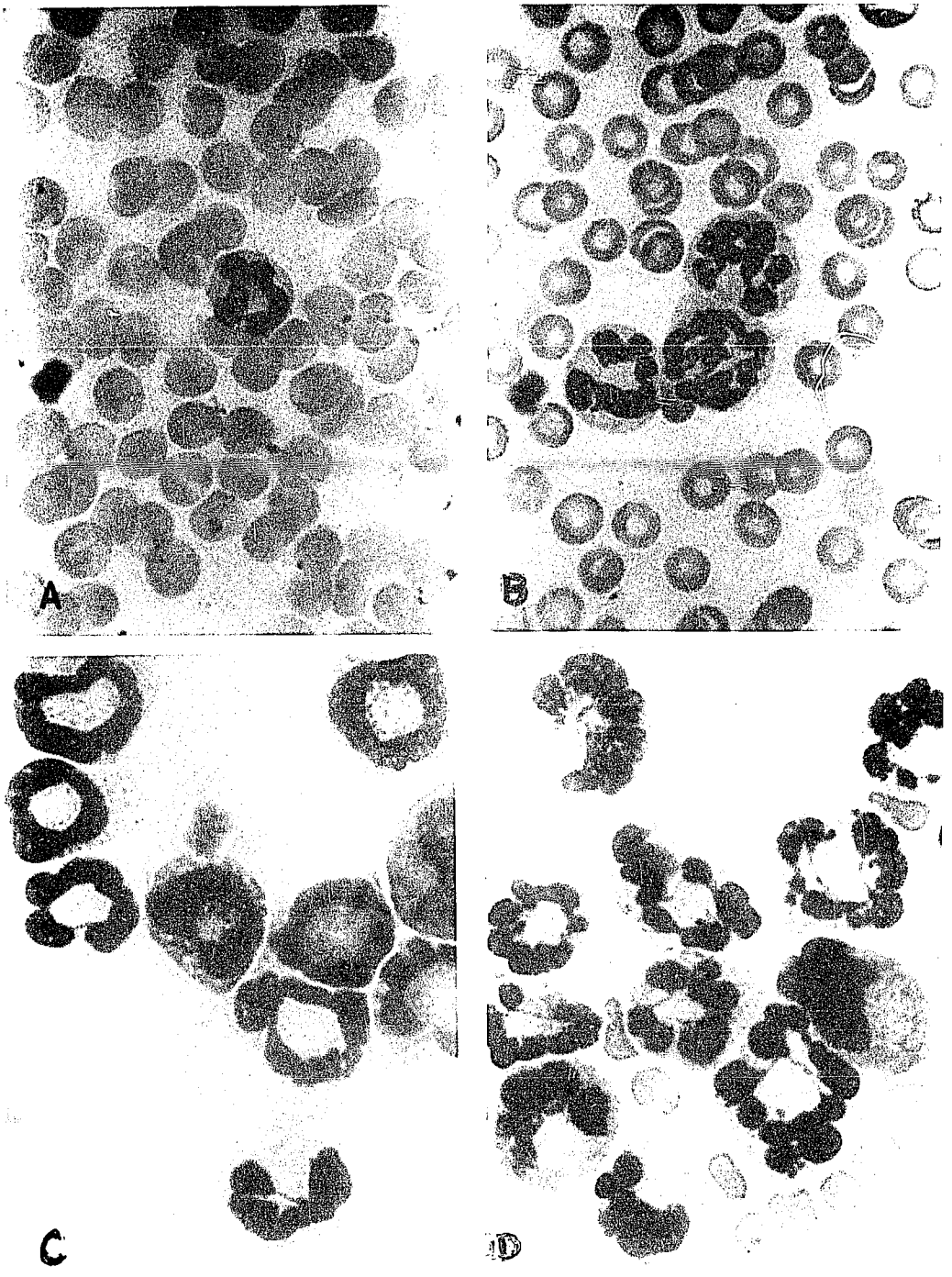


Figure V-1. Normal and hypersegmented rat neutrophils. Typical normal and hypersegmented neutrophils in the circulation of control (A) and MC-bearing (B) rats, and in liquid culture of normal bone marrow cells after 48 hours incubation without (C) and with (D) MC-CM are shown.

passes through 50K Amicon filters (being devoid of any activity) was discarded, so that the (extensively dialysed) retentate of this filtration is what is referred to here as MC-CM. It contained 10 microgram protein per 100ul. In tubes incubated for 48 hr with this amount of MC-CM (plus normal serum), as compared to normal serum alone (Table 3), there was a significant decrease in the percents of metamyelocytes, of band forms, and of the sum of myeloblasts, promyelocytes, myelocytes, and metamyelocytes. All mature neutrophils (lines 6 plus 7 in Table V-3) showed an over 2-fold increase. The number of hypersegmented neutrophils amounted to 50% of mature neutrophils or 35.6% of all myeloid cells. These percentages were already striking, 44 and 6.5 , by the 24th hr of incubation with MC-CM. Total cell number, however, remained unchanged; the number added to each tube, 1.44 million, was essentially the same after 48 hour incubation with MC-CM (1.37 ± 0.09 million) or with normal serum alone (1.41 ± 0.11 million). In both cases, at least 95 % of all cells were viable as judged by Trypan Blue exclusion.

The microscopic appearance of these hypersegmented cells, and of control neutrophils (i.e. those in aliquots of the same bone marrow incubated with normal serum alone) are shown in Fig V-1 D and C. Histochemical tests were also carried out in order to identify the cell types

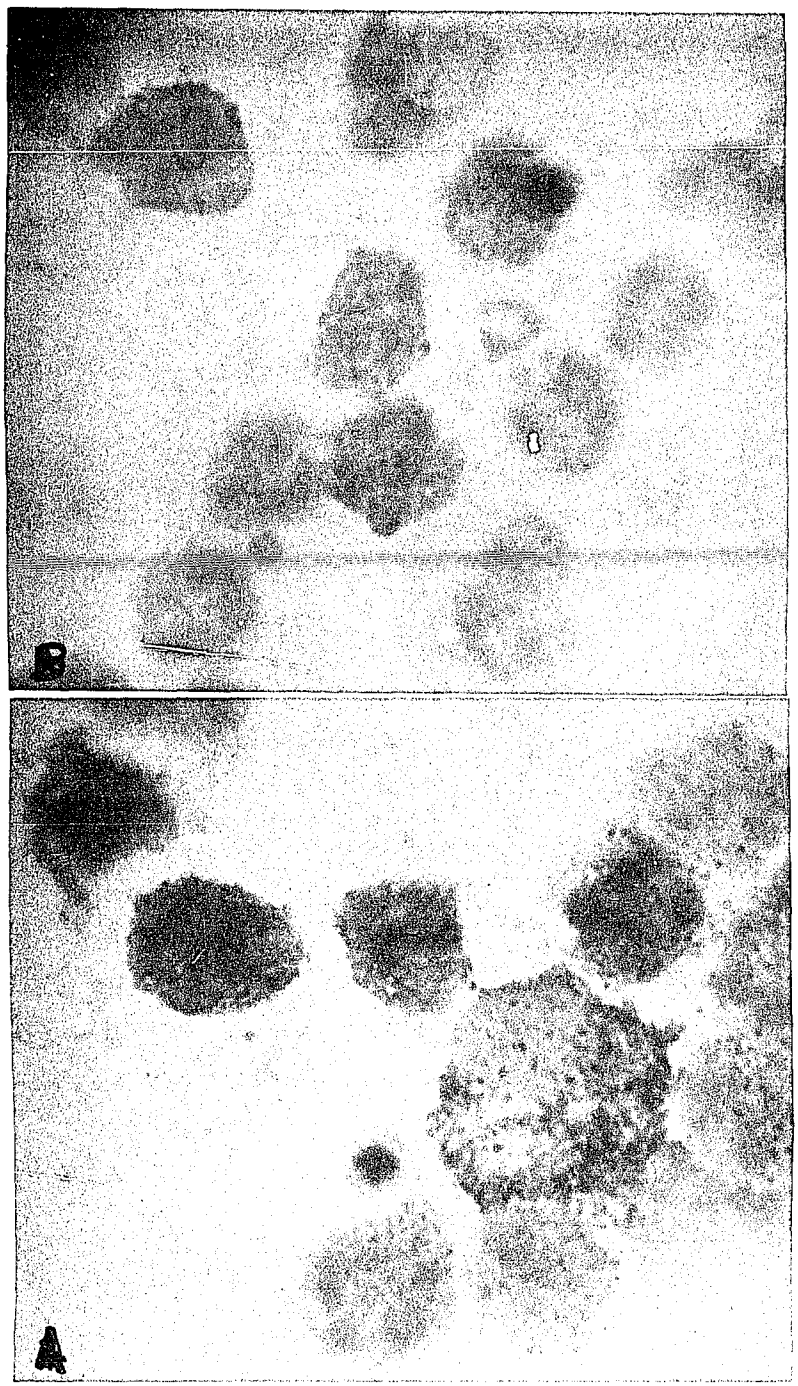


Figure V-2. Incubated bone marrow cells stained with histochemical stains for GGT and AP (see Chapter II). (A) and (B) are representative fields of GGT stained bone marrow cells incubated with or without MC-CM, respectively. (C) and (D) are representative fields of AP stained bone marrow cells incubated with or without MC-CM, respectively.

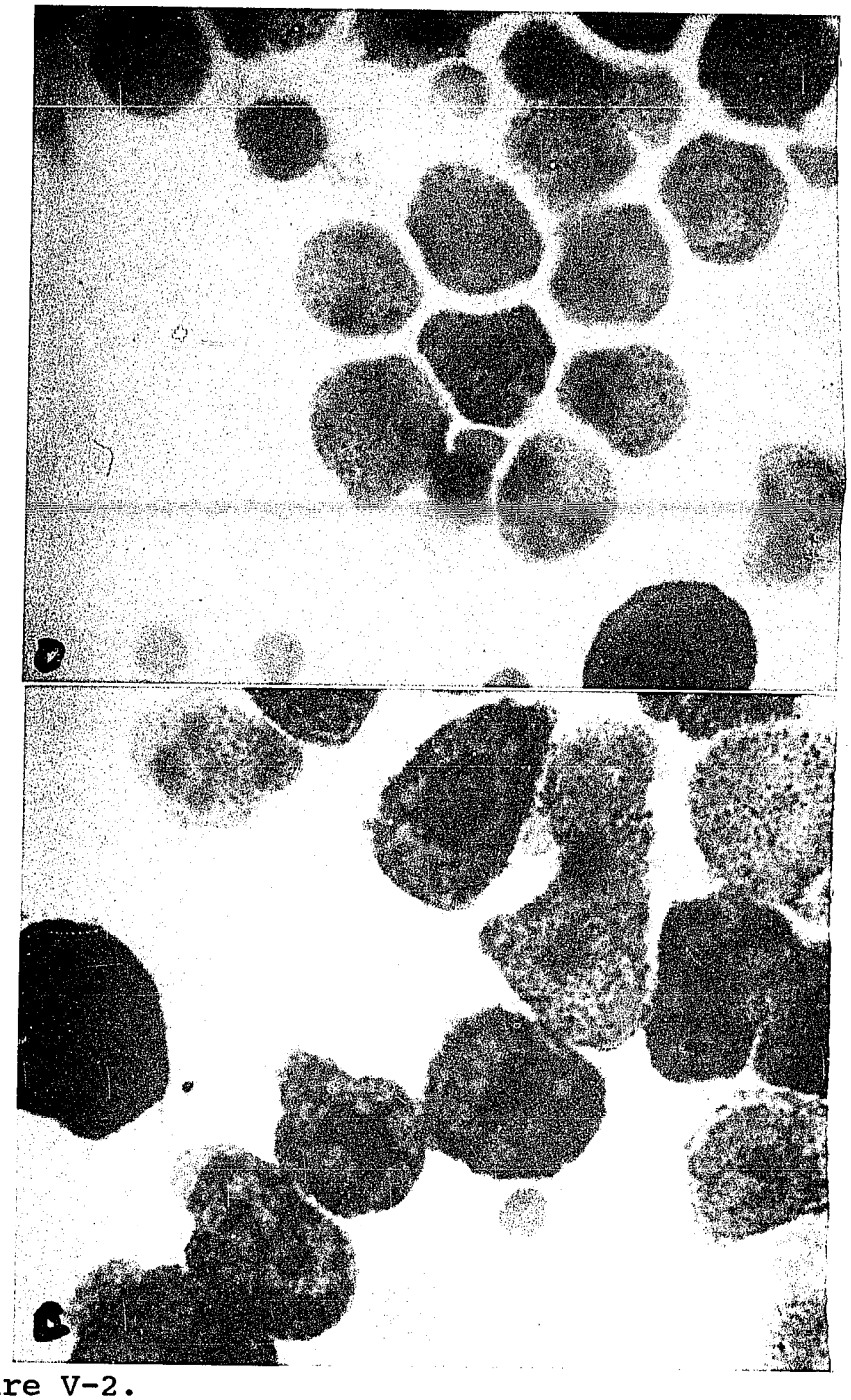


Figure V-2.

Table V-4 Effect of treatment[a] with proteolytic enzymes on the activity of MC-CM.

	control	MC-CM	Protease treated MC-CM	α-chymotrypsin treated MC-CM
cell types[b]		percent of all myeloid cells		
Band forms	30.0	4.0	24.3	16.2
Neutrophil	52.2	50.4	64.8	65.1
Hypersegmented Neutrophil	1.6	32.8	2.7	6.3

[a]For procedure see Chapter II.
[b]Differential counts were carried out on portions of the same normal bone marrow cells incubated in the presence of normal serum without (control) and with untreated or enzyme-treated MC-CM.

TABLE V-5 Effect of treatment[a] with proteolytic enzymes on the GGT and AP-inducing activity of MC-CM.

	GGT	AP
	units/ million cells	
treatment		
Normal rat serum	0.35±0.01	12.65±0.50
MC-CM	1.49±0.15	31.21±2.45
Protease treated MC-CM	0.41±0.05	13.10±1.30
α-chymotrypsin treated MC-CM	0.45±0.03	13.27±0.41

[a]For procedures see Chapter II.
units=nmols/min

Table V-6 Effects of different amounts of MC-CM preparations on neutrophil maturation.

Additions (ul)		% of myeloid cells		
		Band	Mature Neutrophil Segmented	Hypersegmented
none		26.4	57.0	2.5
MC-CM	100	0.6	89.0	52.0
	50	6.0	84.0	38.0
	25	11.0	80.0	13.0
MC-CM-A	100	1.8	91.0	39.0
	50	1.0	86.0	35.0
	25	18.0	70.0	9.0
	10	24.0	64.0	2.0
MC-CM-B	100	5.5	78.5	29.1
	25	11.5	77.5	19.4

Aliquots of normal bone marrow cell suspension were incubated for 48 hour with MC-CM, MC-CM-A and MC-CM-B obtained as described in the text. Protein concentrations were 150, 100, and 0.05ug per 100ul, respectively.

responsible for the increase in GGT and AP concentration. For both enzymes, appreciably heavier staining than in the controls (i.e. cells incubated without MC-CM) was seen in mature neutrophils only. The segemented (rather than hypersegmented) population showed the increase in GGT activity (Fig V-2A and Fig. 2B), whereas the increased AP expression was evident in both types of neutrophils (Fig V-2C and D).

Experiments in which the most striking effects shown in Table V-3 were reexamined showed that, while native MC-CM reduced the percent of band forms from 30 to 4 (see Table V-4), this percentage remained high upon incubation of aliquots of the same bone marrow suspension with MC-CM treated with either protease or α-chymotrypsin (23.3 and 16.2 respectively). Simarily, the percent of all mature neutrophils showed a smaller increase (from 53.8 to 67.5 and 71.4) in response to treated MC-CM than native MC-CM (83.2). More strikingly, less than 10% of all mature neutrophils were hypersegmented while 60% exhibited this abnormality after incubation with untreated MC-CM. Measurement of GGT and AP in the same experiment showed that the enzyme-inducing potential of MC-CM was also lost by protease and α-chymotrypsin treatment (see Table V-5).

Studies under Chapter III showed that when applying MC-CM to G-100 Sephadex columns, and eluting with 0.1M Tris-HCl, pH 7.4, the GGT and AP inducing activity is

concentrated in the approximately 60,000 Mr protein fraction. Repeating these procedures, we now found that the same fraction, referred to in Table V-6 as MC-CM-A, was also the most active in terms of evoking morphological maturation. A significant portion of the original total activity which was lost during chromatography was partially recovered by a second elution of the column, with 0.1M Tris containing 2M NaCl, pH 7.4. The dialysed eluant, referred to as MC-CM-B contained 10ng protein per 100ul and its specific activity in terms of both neutrophil maturation and hypersegmentation (Table V-6) was much higher than that of MC-CM-A. Table V-6 also illustrates that the effects of these partially purified preparations, as well as of MC-CM, are dose dependent. Further purification is required, of course, to determine whether we are dealing with a single protein factor. In the meantime it was of interest to begin to determine whether the ability to induce hypersegmentation <u>in vitro</u> is unique to the MC- elaborated factor(s).

Results presented in Chapter III indicated that the MC protein evoking the GGT and AP response in normal bone marrow in liquid culture is not identical to any of the extensively studied colony stimulating factors (CSFs). Nevertheless, the same enzymic responses were also evoked by L-cell conditioned medium (CSF-L, rich in CSF-1, a stimulator of monocyte/macrophage colonies), human giant

cell conditioned medium (CSF-GCT, rich in granulocyte/macrophage-CSF, GM-CSF), purified murine GM-CSF, and the same was found to be true for recombinant human granulocyte-CSF (rhG-CSF) (see Chapter IV). It was, therefore, of interest to test whether these factors, and also IL-3 which had no effect on GGT or AP (see Chapter IV), would also cause neutrophil hypersegmentation or other morphological changes in the liquid rat bone marrow culture system. The amounts of the CSF preparations used were higher than required for maximal stimulation of GGT and AP induction shown in Table V-7. These amounts (and those of IL-3) were also higher than required for maximal colony stimulation in agar cultures of mouse or rat bone marrow (see Chapter IV), although in the liquid culture this proliferative effect was not expected to be evident. Indeed, as in the case of MC-CM, the total cell number remained constant.

It may be seen in Table V-8 that IL-3 had no significant effect on any of the cell type distribution parameters, but that the same was true for mGM-CSF which, in the contrast to IL-3, did evoke GGT and AP response. The other CSFs caused a significant increase in the percent of mature neutrophils with an accompanying decrease in band forms, although unlike in the case of MC-CM (see Table V-3) there was no significant decrease in metamyelocytes and in the sum of the first four immature

cell types listed. It may also be noted that the numbers of hypersegmented neutrophils, though showing an increase over the essentially zero control value, represented only 3-12 percent of all myeloid cells and 5-18 percent of all mature neutrophils. They were thus considerably lower than the corresponding values, 35 and 50-60, percent for hypersegmented neutrophils after 48 hr incubation with MC-CM (Tables V-3,4,8).

Table V-7 Stimulation of enzyme expression by MC and CSF preparations *in vitro*.

	BM Liquid Culture	
	GGT	AP
Preparation added[a]	nmol/min/106 cells	
None	0.34±0.01	2.41±0.20
MC-serum	1.30±0.09	9.18±4.10
MC-CM	1.03±0.10	8.90±0.06
rhG-CSF	0.56±0.05	4.60±0.50
CSF-GCT (hGM-CSF)	1.32±0.19	7.61±2.00
mGM-CSF (CSF-2γ)	1.39±0.12	7.10±1.80
CSF-1 (CSF-L)	1.34±0.23	10.21±2.10

[a]Normal serum (10%) was present in cultures referred to on horizontal lines 1,3,4.
[b]Values are means of results of 3-5 cultures (±SD) or the average of results of 2 cultures (no SD).

Table V-8 Myeloid maturation and neutrophil hypersegmentation in liquid culture of normal bone marrow cells in the presence of MC-CM and CSF preparations.

cell type[a]	control	MC-CM	rhG-CSF	CSF-GCT (hGM-CSF)	CSF-L (CSF-1)	mGM-CSF (CSF-2 γ)	IL-3
			percent of all myeloid cells[b]				
1) Myeloblast	0.8±.8	0.4±0.8	0.3±0.4	0.0	1.6±0.7	1.3±1.2	0.0
2) Promyelocyte	2.9±1.8	1.4±1.0	1.2±0.7	0.9±1.1	2.6±1.4	2.6±0.9	0.7±1.2
3) Neutrophilic myelocyte	7.5±6.5	3.6±2.3	3.6±1.7	2.8±0.9	3.3±0.8	5.6±3.9	3.1±2.7
4) Neutrophilic Metamyelocyte	15.7±7.0	7.7±3.0	7.4±2.1	8.0±3.5	8.8±1.2	10.3±4.0	11.5±4.1
1)+2)+3)+4)	26.9±13.4	13.1±2.4	13.6±2.8	11.6±3.3	15.1±3.6	19.7±7.9	15.3±6.8
5) Band form	34.6±11.3	7.8±5.1[c]	14.1±3.1[c]	8.5±3.4[c]	16.2±3.8[c]	30.2±3.2	25.8±4.2
6) Segmented neutrophil	34.2±8.9	37.2±8.2	61.6±7.3[c]	63.4±6.3[c]	54.4±4.8[d]	38.8±12.0	48.2±7.0
7) Hyperseg. neutrophil	0.5±0.4	31.2±9.3[c]	7.8±2.7[c]	12.2±2.7[c]	3.5±1.3[d]	1.2±0.6	3.7±2.0
6)+7)	34.6±8.6	68.4±5.2[c]	75.6±5.3[c]	66.9±3.6[c]	59.8±5.4[d]	39.9±12.0	51.9±9.2

[a] Cell types were counted after 48 hour incubation of bone marrow cells in the presence of CSFs and 10% normal serum.

[b] Values are means of results of 5 experiments (±SD).

[c,d] Differences from the values under the "control" were significant, $p < .001$, $p < .005$, respectively.

DISCUSSION

Clinical studies have shown that neutrophil hypersegmentation, a sensitive and sometimes only indication of megaloblastic anemia (Hattersley & Engels, 1974), is attributable to B12 or folate deficiency in 90% of cases (O'Grady, 1984). Thus, even though no other sign of megaloblastic anemia was present in MC-bearing rats, the suspicion naturally falls on vitamin deficiency as a possible cause of the neutrophil hypersegmentation. In view of the occurrence of neutrophil hypersegmentation in some leukemic patients (O'Grady, 1984), cancerous involvement of the rat's bone marrow (though not apparent) is another concievable explanation. However, no such secondary effects of prolonged tumor growth need be invoked here, since bone marrow from normal, tumor-free rats (and cultured in folate supplemented medium) gave rise to hypersegmented neutrophils when incubated with with MC-serum. Nor can this finding be attributed to possible deficiencies of MC-serum with respect to some unknown substances (present in normal serum) necessary for normal nuclear morphology in vitro . For, despite the presence of 10% normal serum, MC-conditioned medium, or nanomole quantities of a protein thereof, also evoked the formation of hypersegmented neutrophils and in numbers (e.g. 50% of all mature neutrophils) as striking as those

seen in the blood of MC-bearing rats. The present studies thus demonstrate that the hypersegmentation in vivo can be explained by the action of a MC-elaborated protein on normal myeloid cells in bone marrow, and suggest that blood borne polypeptide factors with such an action may be responsible for some of the previously noted instances of neutrophils hypersegmentation in human cancer subjects.

The MC-elaborated protein appears to be an effective differentiation inducing agent in myeloid cells; 48 hr incubation, during which no cell multiplication occurred, resulted in a consistent shift to the right, i.e. an increase in differentiated neutrophils at the expense of their immature precursors. Incubation of this system with CSFs was of interest, for their action on immature myeloid cells leads to the formation of colonies of fully differentiated granulocytes and macrophages but, as stated in a recent review by Metcalf (1985), it is uncertain whether CSFs have the capacity to directly influence the events of maturation or whether these events inevitably follow those of cell division. The present results with rhG-CSF, hGM-CSF and CSF-1 suggest the possibility of a direct influence since, even though the decrease in percent of immature cells did not reach statistical significance, the increase in mature neutrophils was significant (Table V-8).

Stimulation of GGT and AP expression by MC serum and

MC-CM in the liquid bone marrow culture system, is attributable to a protein with an apparent MW of 60,000 (and with chemical properties indicating non-identity to CSFs) (see Chapter III). Although a protein fraction in this MW range also caused neutrophil maturation and hypersegmentation, it remains to be determined whether the same or another α-chymotrypsin sensitive protein are responsibile for these newly discovered effects. It seems clear, however, that increased GGT and AP activity is not a consequence of, and is not necessarily accompanied by, morphological differentiation or hypersegmentation. For example, the maturational effect of mGM-CSF in the present liquid culture system stopped short at inducing these enzymes. Also, the number of hypersegmented neutrophils after incubation with the CSFs was either negligible or much lower than in the case of the MC factor, even when both the GGT and AP activities rose to similar levels.

The cellular lesion underlying neutrophil hypersegmentation in megaloblastic anemia is thought to occur at a relatively early stage in granulopoiesis, since abnormal nuclear morphology has been seen not only in mature neutrophils (that are released from the bone marrow as soon as they are formed) but also in immature myeloid cells that may still undergo one or two cell divisions (Nath & Lindenbaum, 1979). The reversibility of this abnormality as well as of the neutropenia in megaloblastic

anemia by B12 and folate supplementation (Nath & Lindenbaum, 1979), and the occurrence of hypersegmentation not only in vitamin deficiencies but also in leukemic subjects and during cytotoxic drug treatment (Laslo & Rundles, 1977), were consistent with the hypothesis attributing neutrophil hypersegmentation to deranged DNA synthesis and inadequate granulopoiesis (Wickramasinghe, 1972), Perille et.al., 1967). That neither of these is necessary, however, for hypersegmentation to occur is indicated by the present results. Signs of ineffective granulopoiesis are not present in MC-bearing rats. On the contrary, they exhibit neutrophilia, the circulation is devoid of immature cells, and there are no light microscopically detectable abnormalities in the bone marrow. This in itself does not disprove a possible effect of the MC-elaborated factor on DNA synthesis. However, such an effect could not play a significant role in the hypersegmentation that this factor evokes in bone marrow from normal (tumor free) animals in liquid culture where no detectable cell multiplication occurs. Also, as seen from the percent distribution of members of the myeloid series in Table V-3, most of the neutrophils were derived not from the mitotic precursors but from relatively mature cells, mainly myelocytes and bands.

It appears, therefore, that in MC-bearing rats, and perhaps in some human cancer subjects, too, neutrophil

hypersegmentation is attributable not to a defect in synthesis of DNA but to a rearrangement of preexisting DNA. The chemical and fine structural changes associated with this abnormality have important implications to the more fundamental problem of the incompletely understood process of normal nuclear segmentation. Thus, with the herein described experimental system, which permits for the first time the study of neutrophil hypersegmentation *in vitro*, one may also gain new insights into physiological mechanisms whereby the unique nuclear morphology of normal neutrophils is acquired during differentiation.

Chapter VI

EFFECTS OF THE MC PROTEINS ON LEUKEMIC CELLS

Introduction

The maturational effect of the MC-elaborated protein on normal rat bone marrow cells has been presented in the preceding chapters. With as little as 2.5 ng of MC protein over 70% of myeloid cells of the bone marrow became mature neutrophils. This raised the possibility that the MC-factor may also exert a differentiational effect on leukemic myeloid cells. The experimental approach to testing this possibility was based to a large extent on previous investigations of the effects of CSFs on leukemic cell lines.

One such cell line, WEHI-3B, was obtained when mineral oil injection was found to cause myelomonocytic leukemia in BALB/c mice; the leukemia cells were propagated *in vitro* and cloned (Warner et.al., 1969; Metcalf et.al., 1969). The WEHI-3B cells were cultured in semisolid agar where they were found to form compact colonies typical of blast-like immature cells. A subclone named WEHI-3B D+ was isolated which seemed capable of differentiating. For, in the presence of serum from endotoxin treated mice, these cells gave rise to some disperse, sparsely populated colonies (Metcalf, 1980). Since cells isolated from these colonies were no longer blast-like, but possessed

morphological features indicative of maturing neutrophils and macrophages (Metcalf & Nicola, 1982), disperse colony formation on agar is considered to denote differentiation. WEHI-3B D+ cells (in agar as well as liquid culture) were therefore used to test the MC protein for differentiational activity.

A human cell line, HL-60 was another test system used. Cells of this line are morphologically similar to promyelocytes and are capable of differentiating toward granulocytes when exposed to polar-planar compounds like DMSO (Collins et.al., 1980; Breitman et.al., 1980). Vitamin D_3 and TPA causes them to differentiate toward macrophages (Koeffler et. al., 1981).

In order to test for differentiational changes in primary cultures rather than immortalized cell lines, rats with Shay chloroleukemia were used. It can be propagated by subcutaneous implantation giving rise to a tumor characterized by a loose array of homogeneous polygonal myeloblastic cells with large nuclei and small cytoplasms, containing few mitochondria. Intravenous injection of 5 X 10^6 dissociated tumor cells induces an acute leukemia capable of killing the host within 14 days (Shay et.al., 1955). The leukemic cells can also be carried in liquid culture without the loss of tumorigenicity of the parent line (Yunis et.al., 1975) In addition, the cells placed in diffusion chambers and implanted in rats have been found

to undergo morphological changes suggesting a capacity for differentiation (Jimenez & Yunis, 1987).

RESULTS

In order to examine the possible response of murine and human leukemic cells to the rat mammary carcinoma elaborated protein(s) it was necessary to obtain evidence for its ability to act on cells from species other than rat. Studies in Chapter IV have already shown that MC serum and MC-CM are capable of stimulating colony formation in normal mouse bone marrow agar culture, but whether they are also capable of evoking the enzymic responses remained to be tested. Normal mouse bone marrow cells were, therefore, incubated in the standard liquid culture system. The basal GGT content (Table VI-1, first line) which remained unchanged during 48 hour incubation with normal serum, was about one fifth that in normal rat bone marrow cells and showed a dose dependent response to MC serum and MC-CM. The results in Table VI-1 refer to the amount of MC-serum and MC-CM which evoke maximal increases in GGT, and it may be seen that these increases, 3-and 9-fold, respectively, were similar to those evoked by CSF-GCT and CSF-2τ(mGM-CSF). On the other hand, the AP response seen in rat bone marrow cells was not evoked by the MC preparations, nor by any of the CSFs including mouse derived GM-CSF, Table VI-1.

We also tested the effects of MC-CM on normal human bone marrow as well as on bone marrow cells and peripheral

Table VI-1. Enzymic effect of the MC elaborated protein and CSFs in mouse bone marrow liquid culture.[a]

	GGT	AP
	nmoles/min/10^6 cells	
Control	0.06 ± 0.01 (3)	0.16 ± 0.05 (3)
MC serum	0.22 ± 0.04 (3)	0.12 ± 0.02 (3)
MC-CM	0.52 ± 0.06 (3)	0.14 ± 0.02 (3)
CSF-GCT[b]	0.52 ± 0.02 (3)	0.16 ± 0.02 (3)
CSF-2γ[c]	0.23 ± 0.05 (3)	0.17 ± 0.01 (3)

[a]The liquid culture medium and preparation of mouse bone marrow was the same as described for the standard rat bone marrow system under Chapter II. Concentration of preparations used gave maximal GGT induction in cultured rat bone marrow cells.

[b]GM-CSF and G-CSF from a human giant cell tumor.

[c]mouse GM-CSF.

blood leukocytes from myeloid leukemia subjects. The same liquid culture system was used but replacing normal rat serum with heat-inactivated fetal calf serum. MC-CM was capable of inducing AP expression but without any increase in GGT (Table VI-2). The effect on AP was especially pronounced in the leukemic cells, showing 3-to 7-fold increases. Therefore it seemed reasonable to test MC-CM for differentiational effects on human as well as murine leukemic cell lines.

Studies on WEHI-3B Cells

The WEHI-3B D+ cell line, obtained from Dr. Malcolm Moore of the Sloan-Kettering Cancer Center, was grown in Dulbecco's Modified Eagle Medium with Penicillin/Streptomycin 100U/ml, 2Mm Glutamine, and 10% Fetal Bovine Serum, heat inactivated for 30 min at 56°C. Cells were plated at a concentration of 100,000 cells/ml and incubated at 37°C in a humidified atmosphere with 5% CO_2. After 2 or 3 days of incubation, cultures were split back to a concentration of 100,000 cells/ml again, thus providing a ready supply of cells in the logarithmic phase of growth.

The agar culture differentiation assays were performed according to the method of Metcalf (1980). Briefly, approximately 500 cells were mixed in 1 ml of Dulbecco's MEM with 0.3% sterilized Bactoagar. This warm

Table VI-2 The Enzymic Effect of 2 Day Incubation of Human Myeloid Cells with MC-CM.

	AP		GGT	
	nmol/min/10^6 cells			
Human subjects	Control	MC-CM	Control	MC-CM
Normal Bone Marrow				
H.O.	0.28	0.87	0.36	0.46
R.B.	0.23	0.37	0.84	0.70
M.S.	0.37	0.43	0.28	0.20
Leukemic bone marrow				
L.S.	0.03	0.17	1.02	0.90
Leukemic leukocytes				
A.R.	0.32	1.11	0.14	0.17
L.S.	0.12	0.88	0.62	0.66
E.E.	0.01	0.06	0.19	0.25
E.E	0.04	0.19	0.25	0.25

Peripheral blood and bone marrow from subjects with AML and control subjects was collected in heparinized tubes. Diagnosis was based on clinical, hematologic, cytochemical criteria.
These values represent means of results of two dishes.

TABLE VI-3 The Effect of Different MC-CM Concentrations on WEHI-3B D+ Cells in Agar Culture

MC-CM added (ul)	% DISPERSED	TOTAL NO. OF COLONIES
0.00	21.5±9.9(4)	206±28(4)
0.05	40.3	119
0.10	40.8	122.5
0.25	47.4	121.5
0.50	50.4	127.5
1.00	54.6	112
2.50	78.0	123.5
5.00	79.7	140.5
25.00	98.4	150

values represent means of results on two cultures or 4 (see SD).

FIGURE VI-1A WEHI-3B D+ COMPACT COLONY FORMED IN CONTROL CULTURES

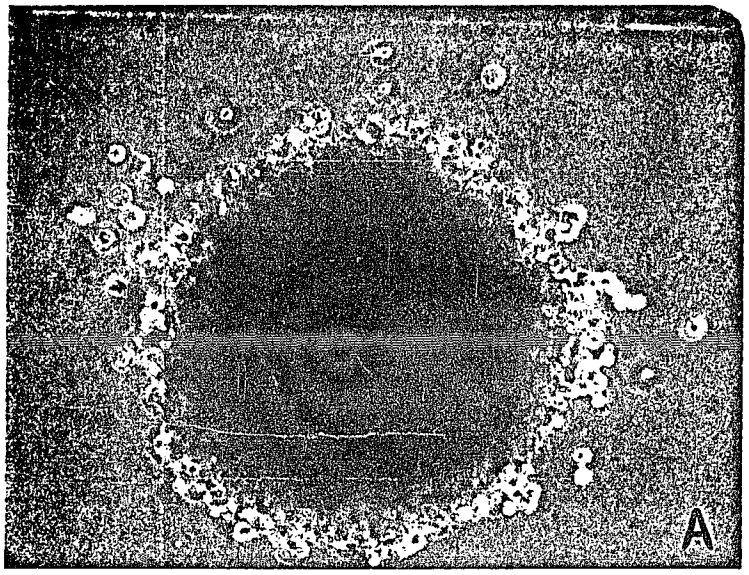
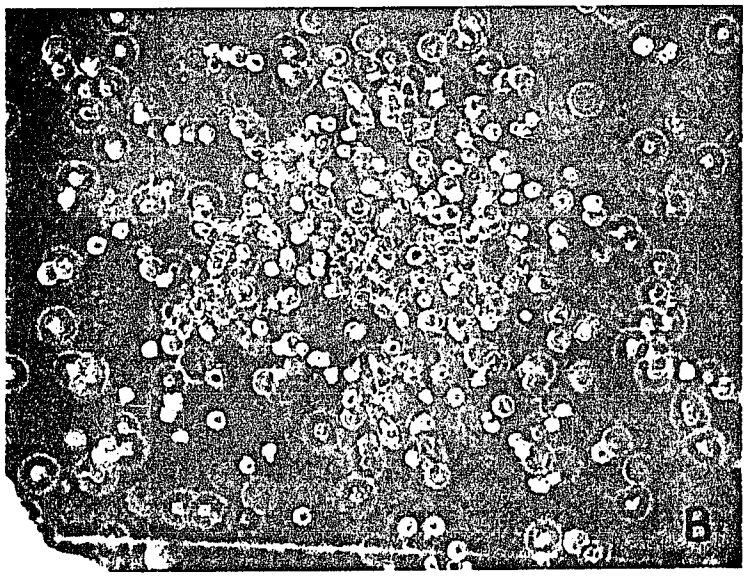


FIGURE VI-1B WEHI-3B D+ DISPERSE COLONY FORMED IN CULTURES TREATED WITH MC-CM



suspension was added to 35mm Corning petri tissue culture dishes containing various concentrations of MC-CM and mixed well before it hardened. These dishes were incubated for 7 days under the above conditions. Colonies were then counted with an inverted microscope and categorized as to compact or diffuse phenotype.

The first column of Table VI-3 shows that with increasing amounts of MC-CM more colonies showed the dispersed phenotype. In control cultures 26.2 % were disperse, though not dramatically disperse. At 0.05ul of MC-CM added, 40.3% were differentiated, and at 25ul all colonies were differentiated. An example of the compact colonies found in control cultures is presented in Figure VI-1a, while Figure VI-1b exemplifies a disperse (differentiated) colony. Results of the colony formation experiments were reproducible, mean colony numbers in 7 control cultures were 215 ± 30, while the number in the presence of 100 ul of MC-CM, 157 ± 16 (8), was significantly lower ($p<.005$). An effect was already evident at 0.05 ul of MC-CM (smaller amounts were not tested). The percents of dispersed colonies was also quite consistent; 20.4 ± 9.1 was the percent in control cultures, while MC-CM treated cultures with 97.2 ± 1.3% consisted exclusively of differentiated colonies. Again the difference was highly significant ($p<.005$). The smaller total number of cells within each disperse colony

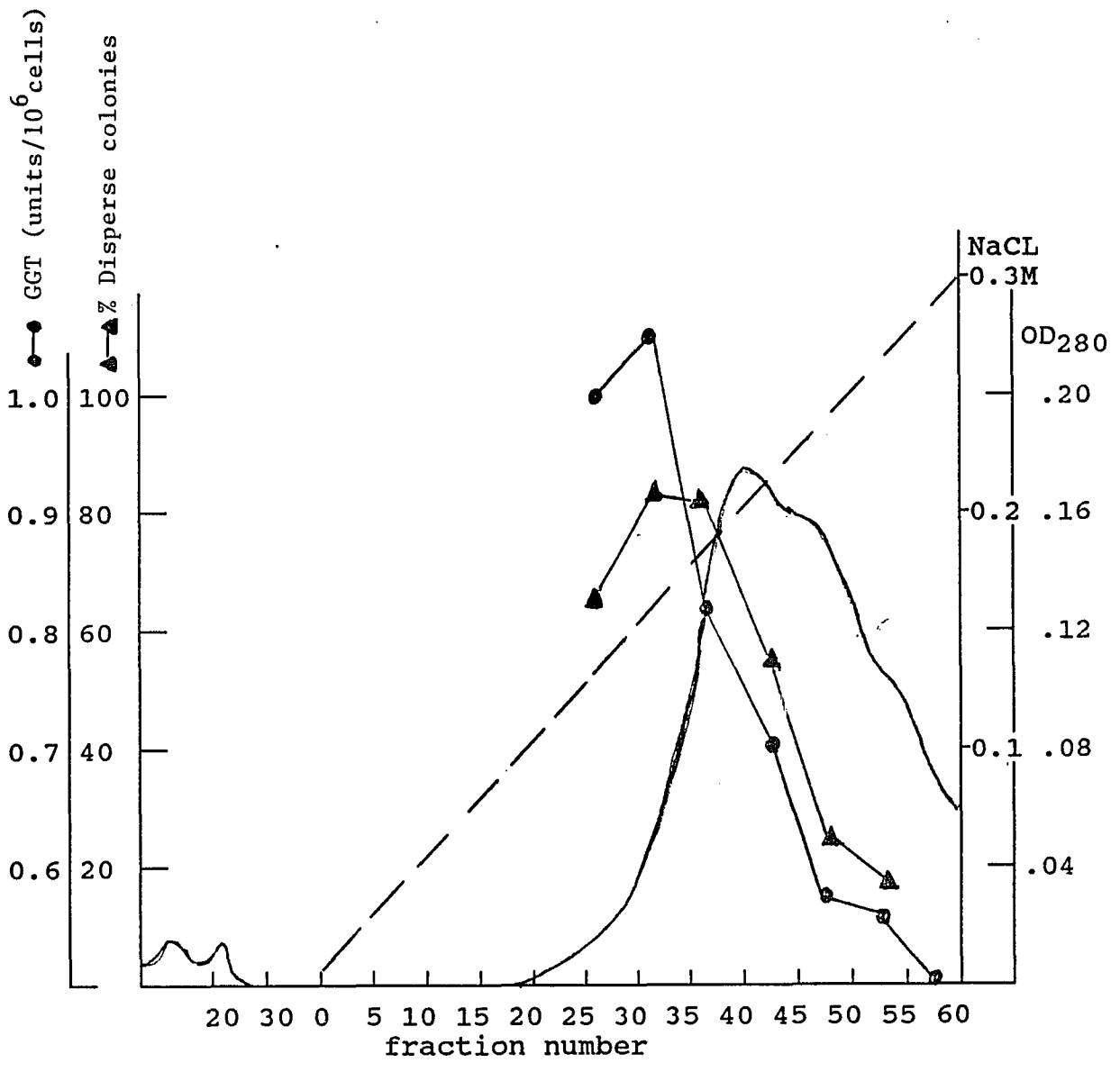


FIGURE VI-2 DEAE Cellulose chromatography of MC-CM. Superimposed are GGT induction (●), % disperse colony formation (▲), and OD_{280} nm (-). The eluting NaCl concentration is indicated by the broken diagonal line.

than within the compact colonies (see Figure VI-1) is a further indication of a decrease in proliferative potential resulting from MC-CM treatment.

Fractions of MC-CM obtained by DEAE cellulose chromatography were assayed for both differentiational activity (i.e. % of dispersed colonies formed) in leukemic cells and GGT induction in normal rat bone marrow cells. As depicted in Figure VI-2, the two activities are eluted at approximately the same salt concentration. Preliminary results are thus consistent with the possibility that the two activities are attributable to a single factor.

GGT and AP could not be measured in agar cultures. Liquid culture of WEHI-3B was therefore used to test for enzyme inducing activity,as well as for the determination of the effect of MC-CM on multiplicative rate. After 2 or 3 days of culture, cells were reseeded at approximately 100,000 cells/ml and incubated with or without MC-CM. The harvested cells were counted, washed and aliquots were taken for enzyme assays and cytospin preparations were obtained for morphological assessment.

It may be seen from Table VI-4 that 2 days of incubation with MC-CM was sufficient to cause an almost two fold increase in GGT content, and that similar, statistically significant, increases were found on subsequent days. Analysis of AP content did not reveal any significant increase in MC-CM treated cultures. This is

TABLE VI-4 GGT Content of WEHI-3B D+ Cells Treated With MC-CM

DAYS	TREATMENT	GGT % OF CONTROL		
2	Control	100	±25	(16)
	MC-CM	189	±96	(15)**
3	Control	100	±7	(3)
	MC-CM	142	±8	(3)**
4	Control	100	±35	(7)
	MC-CM	163	±71	(7)*
5	Control	100	±15	(7)
	MC-CM	202	±85	(7)*

Values are means ±SD of results (nmol/min/10^6 cells as percent of control) on the number of cultures shown in parentheses.

* $p<.05$

** $p<.005$

probably due to the murine origins of the WEHI-3B cells, since MC-CM was without effect on AP in mouse bone marrow cells even though it stimulated GGT expression (Table VI-4). Cell Doubling Time (DT), an indication of proliferative potential, was calculated from the formula:

$$DT = \frac{\ln(t)}{\ln(V_o / V^t)}$$

where t= time of incubation and V_o and V_t are the cell numbers at time o and time t, respectively. In the WEHI-3B D+ cells treated with MC-CM there was an increase in DT from that of control cultures, on each day and this was to reach significance on day 5 (see TABLE VI-5).

HL-60 cell experiments

HL-60 cells were grown in RPMI-1640 medium supplemented with glutamine, penicillin and streptomycin. 10% of Heat Inactivated Fetal Calf Serum was added. Cells were plated at concentrations of 200,000 cells per ml and incubated for 2 days at 37^{o}C and 5% CO_2.

The enzyme levels of GGT and AP showed little change on day 2. However, on day 4 there was a significant increase of the AP content of MC-treated cells (586% of control; $p<.05$), and an increase in GGT (though not statistically

TABLE VI-5 Change in Doubling Time (DT) of WEHI-3B D+ Cells in the Presence and Absence of MC-CM

DAYS TREATED	CONTROL	MC-CM
	DT (HRS)	
	mean ± SD	
2	12.5±1.7(14)	13.9±4.0(16)
3	14.5±0.5(4)	15.2±0.5(4)
4	13.6±5.1(7)	19.5±3.9(7)
5	13.6±0.7(7)	15.4±2.1(7)*

* $p<.05$

TABLE VI-6 Effect of MC-CM on HL-60 Cell Line Doubling Time and Enzyme Content

DAYS	TREATMENT	DT	GGT	AP
		HRS	% OF CONTROL	
2	CONTROL	19.1 ± 1.5(3)	100 ± 22(3)	100 ± 20(3)
	MC-CM	19.5 ± 1.2(3)	116 ± 7(3)	94 ± 12(3)
4	CONTROL	18.5 ± 3.8(3)	100 ± 25(3)	100 ± 27(3)
	MC-CM	20.8 ± 3.3(3)	136 ± 29(3)	586 ± 294(3)*

For expression of results see Table VI-4.

* $p<.05$.

significant) was also apparent (Table VI-6). The doubling time of MC-CM treated cells on the second day of incubation was virtually identical to the controls, while on the fourth day it was longer (Table VI-6), although the statistical significance of this difference was not demonstrated.

Shay Chloroleukemia studies

The Shay tumor were removed and dispersed into Dulbecco's Modified Eagle medium, with glutamine, penicillin and streptomycin added, and grown under the following conditions. Cells were plated at a concentration of 100,000 cells/ml in medium containing 10% heat inactivated fetal Bovine Serum and 10% heat inactivated Horse Serum. After 2-3 days incubation cells were split back to their starting concentration.

The induction of GGT in these cells was very striking. As early as day 2 of incubation with MC-CM, the values were 30 times above control. On subsequent days the level of GGT increased even more, to approximately 40 times of control (Table VI-7). All increases were highly significant ($p < 0.005$).

AP induction did not mirror the induction of GGT, in that AP was increased only on day 4. This increase, to 241±63% of control was highly significant ($p < 0.005$).

Doubling time (measured as in the case of WEHI-3B

cells) became longer on the 3rd day of incubation with MC-CM (16 hours vs 14 hours for controls). On the fourth day, too, the difference was highly significant ($p < 0.005$) (see Table VI-8).

Morphological changes consisted of a small decrease in the nuclear to cytoplasmic ratio, a quality seen in maturing myeloid cells. The blue staining with May-Grunwald Giemsa of the cytoplasm became less intense (indicating a tendency toward the "neutrality" of mature cells). In a few cases, macrophage-like cells appeared with their multicolored cytoplasms indicating phagocytosis of debris. The nuclei of some of the MC-CM treated cells exhibited segmentation, but with nucleoli still present. Other cells exhibited doughnut shaped nuclei reminiscent of rat and murine metamyeloctes. Changes in chromatin pattern were evident; some cells showed strands of chromatin rather than the "silky" immature chromatin patterns seen in the blasts. In addition, some mature macrophages and segmented neutrophils appeared during incubation with MC-CM.

TABLE VI-7 Effect of MC-CM on the Enzyme Content of Shay Chloroleukemia Cells

DAYS	TREATMENT	GGT	AP
		% OF CONTROL	
2	CONTROL	100 ± 16(8)	100 ± 14(8)
	MC-CM	3147 ± 1440(8)*	118 ± 40(8)
3	CONTROL	100 ± 5(3)	100 ± 12(3)
	MC-CM	4267 ± 284(3)*	75 ± 8(3)
4	CONTROL	100 ± 4(4)	100 ± 15(4)
	MC-CM	3997 ± 782(4)*	241 ± 63(4)*

For expression of results see Table VI-4

* $p<.005$

TABLE 8 Effect of MC-CM on Doubling Time of Shay Chloroleukemia Cells

DAYS	TREATMENT	DOUBLING TIME (HRS) mean ± SD
2	CONTROL	13.8 ± 1.6(7)
	MC-CM	14.0 ± 2.2(7)
3	CONTROL	14.0 ± 0.3(3)
	MC-CM	16.5 ± 0.2(3)*
4	CONTROL	11.1 ± 0.1(3)
	MC-CM	17.6 ± 1.3(4)*

* $p<.005$

DISCUSSION

Since mouse bone marrow agar cultures constitute the standard test system for the proliferative effects of CSFs, and since it was of interest to determine whether the enzymic responses to the protein elaborated by the rat MC were species specific, some of the preparations were tested on mouse and human bone marrow cells in liquid culture.In human bone marrow cells MC-serum and MC-CM were unable to stimulate GGT expression but did evoke the AP response. In mouse bone marrow cells, on the other hand, they stimulated GGT but not AP expression (and the same was true for the CSFs, see Table VI-1). It thus appears that the protein factor(s) is capable of acting on mouse, human as well as rat bone marrow cells, but whether it induces GGT or AP or both depends on the species of the responding cells.

While HL-60 cells failed to show any significant change in proliferation and little change in enzyme content (with the exception of AP on Day 4), WEHI-3B and rat chloroleukemia cells exhibited appreciable differentiational changes in response to MC-CM. In the case of WEHI-3B D+ cells this was indicated by the predominance of disperse (differentiated) colonies over compact (characteristic of blast-like cells) colonies in agar culture. In the liquid culture, the presence of MC-CM

resulted in increased doubling times which, taken together with the decrease in numbers of colonies formed in agar culture as well as fewer cells per colony, indicating a loss of proliferative potential, provided additional evidence of maturation.

GGT content has not been previously used as an indicator of maturity in blood cells. However GGT as well as AP induction has been found to accompany differentiation in melanoma and breast cancer cells induced with sodium butyrate and DMSO (Nordenberg et.al., 1987; Wasserman et.al., 1987). AP has been used as a marker of maturity in myeloid cells as it is known to increase during the differentiation of myeloid progenitors to the mature neutrophil (Sato et.al, 1985; Evans et.al., 1986). Thus the induction of GGT in liquid culture of WEHI-3B cells (Table VI-4), and of AP in leukocytes from patients with myeloid leukemias (Table VI-2) are indicative of the differentiational effect of MC-CM. In Shay leukemia cells morphological as well as chemical changes were clearly evident and the enzyme inductions were much more pronounced. Incubation with MC-CM for just 2 days resulted in a 40 fold increase in GGT content, and AP expression began to increase on the fourth day. The acquisition of certain structural characteristics of maturing myeloid cells, (such as large cytoplasmic relative to nuclear volume) and the appearance of cells

with nuclear segmentation, constituted evidence for the ability of MC-CM to induce morphological differentiation.

While other criteria (such as NBT reduction, esterase and peroxidase content, and expression of lineage specific surface antigens) might prove useful in further characterizing the state of differentiation of leukemic cells (Glasser & Fiederlein, 1987; Olsson et.al., 1984; Cooper et.al., 1982), the decrease in proliferative potential in both liquid and agar colony assays, the diffuse colony formation, GGT and AP induction and the morphological changes in leukemic cells exposed to MC-CM all provide good evidence of differentiation.

Initial chromatographic studies are consistent with the hypothesis that the differentiational effects of MC-CM on leukemic cells and the enzyme induction it causes in normal rat bone marrow are attributable to the same protein factor. However it has not been purified to homogeneity. Future purification could be based on differentiated colony formation in agar culture by WEHI cells and growth inhibition of the same cells in liquid culture. Alternatively, the MC-preparations can be tested for morphological differentiation or GGT expression in Shay leukemic cells. It would seem that the reliance on biochemical criteria alone (a practice in many studies on HL-60 cells, for example) is inadequate, and does not

necessarily imply structural differentiation. Conversely, the acquisition by leukemic cells of the morphological characteristics of their normal counterparts does not prove full differentiation, for defects in enzymic composition (though not revealed by microscopy or histochemistry) may still persist and preclude the acquisition of normal metabolic functions and growth properties.

An important finding in these experiments is the discovery of a model system for the study of the GGT induction. The Shay chloroleukemia provides a homogeneous population of cells, whereas isolation of specific cells from the bone marrow is not easy. In addition, while normal bone marrow cells can be used for short term experiments only, Shay cells can be propagated in continuous cultures for a long period. Most importantly, no other system in vitro showed such a striking GGT response. The 30 fold increase during only 2 days of incubation with MC-CM renders Shay leukemia cells uniquely suitable for study of the mechanisms of GGT induction. This cell population, especially in view of its homogeneity, should also facilitate characterization of the receptor of the MC protein.

Chapter VII

GENERAL DISCUSSION

The first evidence for multiple bichemical alterations in "uninvolved organs of cancer hosts was obtained by Jessie Greenstein in the early 1940's (Greenstein, 1942) in the course of studies on rats carrying subcutaneously implanted hematomas. They showed that deficiency in catalase, arginase, d-aminoacid oxidase and riboflavin was characteristic not only of the hepatomas but that these constituents were also at diminished levels in the host's (cancer-free) liver. Similar observations were then made on hosts of various non-hepatic cancers, and indications have also been obtained for the increased proliferation of normal cells adjacent to or distant from growing tumors. Greenstein interpreted these phenomena as "a generalized, systemic 'cancerousness' of the organs of the tumor bearing animals, so that the liver, for example, of such animals take on certain neoplastic features", and proposed that tumors secrete hormone-like substances which can exert an influence on normal tissues (Greenstein, 1942).

These ideas catalyzed many subsequent investigations which eventually branched into two separate lines of research with quite different foci. One, focussing on the substances whereby cancer cells evoke a proliferative response, has succeeded (with the aid of bioassays in

vitro) in the isolation and chemical characterization of many polypeptide growth factors and in defining their target cell selectivity; it also made major advances in elucidating the interaction of these factors with specific cell surface receptors, but the ensuing changes in the cells' enzymic composition and their role in the proliferative mechanism are essentially unknown. The other line of research, in contrast, specified numerous enzymic abnormalities in "uninvolved tissues" of cancer hosts which underlie their metabolic functions; it also obtained circumstantial evidence that systemic factors evoke some of the biochemical abnormalities, however, the chemical nature and origin of such factors remained unexplored. The significance of the present investigations lies in linking together these two separate areas of research.

Although investigations on cancer hosts detected biochemical abnormalities in several host tissues, the liver is the organ in which the largest number of enzymes were found to undergo changes in amount or isoenzyme pattern. Many of these changes are harbingers rather than consequences of the physiological deterioration of cancer hosts. For decreases and increases in catalysts of important hepatic functions were found to be of early onset, detectable before the subcutaneously transplantable tumor attains palpable size, and long before the onset of metastatic disease or any sign of ill health such as

cachexia. Observations on normal rats parabiosed with tumor bearing ones or treated with their serum, attested to the humoral mediation of the hepatic abnormalities. However, such experiments in vivo do not disclose whether these abnormalities were brought about by tumor factors (a) acting on the liver directly; or (b) via changes (endocrine, immunological, etc.) they caused elsewhere in the organism. That a system in vitro is still not available for study of such questions in the liver is due to the difficulty of maintaining the biochemical integrity of hepatocytes in culture long enough for the expected alterations in enzyme concentration to occur. The possibility for an alternative system emerged from studies on the effect of MC transplantation on the GGT and AP content of hematopoietic tissues. For it was noted that in normal bone marrow cells the levels of these enzymes is preserved for at least 3 days in liquid culture and, while none of the previously known enzymic abnormalities in any tissue have been reproduced in vivo, a significant GGT and AP response in this culture could be evoked by the addition of serum from MC-bearing rats. To determine the chemical nature and origin of the enzyme inducing factor was thus the initial aim of the present investigations.

The induction of GGT and AP was found to be attributable to a protein(s) with an apparent MW of 60,000. Since MC-extracts also had some inducing activity,

production of the factor *in vitro* was attempted by incubating fine MC minces in serum-free medium. The conditioned medium thus obtained after a short (3 day) incubation period contained orders of magnitude more GGT and AP inducing activity than present in the tumor endogenously. The potency resided in the over 50,000 Mr fraction, further indicating that the presence of the active factor in MC serum is the resultant of elaboration and release by the tumor. Comparison of the activities of preparations partially purified from MC serum and MC-CM indeed showed that they were indistinguishable in terms of apparent molecular weight, chromatographic mobilities as well as stability to heat and proteolytic enzymes. However, MC-CM was a richer source, yielding preparations with 2000 times higher specific activities than did MC serum.

Simultaneously with the purification studies, efforts were made to further characterize the normal bone marrow culture system, to test the MC-protein for additional effects, and to compare its actions with that of known growth factors.

This led to the identification of the first enzymic change so far found to be caused by CSFs: we showed that several well characterised, highly purified (including recombinant) CSFs also stimulate GGT and AP expression in the liquid bone marrow culture system. Hyperplastic

responses *in vivo* evoked, for example, by steroid hormones in their target tissues (Tarachand & Eapen, 1982) are known to be associated with increased GGT activity. However, the nature of this association is not clear, since no animal derived hormones or growth factors have been found to induce GGT expression *in vitro* in any cell type. The present results indicate that, in normal myeloid cells at least, increased GGT activity is not contingent on prior cell proliferation but is, nevertheless, related to the colony stimulating potential of CSFs. For the GGT inducing activity of various CSFs (per mg protein) in the liquid culture where no cell proliferation occurs bore a highly significant, quantitative correlation to the specific activity of the same CSFs as judged by the number of colonies formed in the standard agar culture system (Figure IV-4). A practical significance of this observation is that it offers an alternative method for screening for CSFs. While the currently used agar culture assay requires at least 7 days, the GGT and AP response in liquid culture is detectable by 24 hours. Also colony counting is a somewhat subjective, laborious procedure, whereas simple, sensitive and automated methods can be used for objective assessment of GGT and AP activity.

There has been much debate in the literature as to whether the CSFs have direct maturational effects or whether these events inevitably follow those of cell

division (Metcalf, 1985). The liquid culture system we employ does not appear to allow any significant proliferation, thus providing a unique opportunity to test the CSFs for their ability to cause morphological differentiation in normal bone marrow myeloid cells. While the % of immature cells failed to decrease to a statistically significant extent, there was a significant increase in mature neutrophils in cultures incubated with rhG-CSF and CSF-1. The similar effects of one of the GM-CSF preparation (GCT) must be due to a recently demonstrated (Erikson-Miller et.al., 1988) contamination with G-CSF, since no morphological maturation was caused by the more highly purified GM-CSF (see CSF-2τ, Table V-8).

As shown previously (Koss & Greengard, 1982) and confirmed here, in the blood of MC-bearing rats only the granulocytes show increased titers and stimulated GGT and AP expression. The effect of MC <u>in vivo</u> thus appears to be exerted on the granulocytic compartment of the bone marrow. The present histochemical tests indicate that after MC transplantation, or incubation of normal bone marrow with MC preparations, the mature neutrophilic cells are the ones with increased GGT and AP activity. More importantly, the percentage of these cells increased dramatically in the <u>in vitro</u> system and, unlike in the case of CSFs, there was also a significant decrease in

immature precursors.

The maturation effect of MC-CM was also apparent in leukemic myeloid cells. It caused the myelomonocytic cell line WEHI-3B D+ to form differentiated cell colonies on agar culture, decreased multiplicative rate and, as also shown in liquid culture of the same cells, stimulated GGT expression. The murine origin of these cells must be the reason for the absence of a concomitant AP response, for this enzyme was also not induced in normal mouse bone marrow. In human bone marrow, on the other hand, MC-CM induced AP but not GGT and the same was true for the human leukemic cell line HL-60. The ability of MC-CM to also offset the AP deficiency of blood myeloid cells from leukemic subjects (Table VI-2) is of interest, because AP is often used as a marker of neutrophil maturity (Sato et.al., 1985; Evans et.al., 1986), and because it supports the postulate that the differentiational effect of the MC protein is not restricted to established cell lines. Indeed, in primary culture of Shay leukemic cells it inhibited proliferation with acquisition of some morphological features indicative of differentiation. The induction of both GGT and AP was in accord with the species (rat) of the Shay cells; however, the GGT response- a much greater increase (40-fold) than in any other system studied in the past- was more striking. Thus, the homogeneous population of Shay cells offers an

excellent model for detailed study of the action of the MC protein and isolation of its receptor.

There has been much interest recently in the possible usefulness of differentiation-enforcing CSFs in leukemia therapy (Nicola et.al., 1985; Souza et.al., 1986; Clark & Kamen, 1987). They also stimulate, however, the proliferation of myeloblasts (leukemic as well as normal) (Clark & Kamen, 1987; Platzer & Kalden, 1987), and investigators caution that one needs to weigh differentiational effects against the proliferative potential of CSFs. The danger might be less serious in the case of factors like MC protein. For, in partially purified preparations (Figure IV-4), the ratio of enzyme inducing to colony stimulating activity was appreciably higher than the ratio between the two activities in CSFs as well causing a decrease in proliferation of leukemic cells both in liquid and agar assays. By the same token the promise that CSFs have in the treatment of neutropenia would not extend to the MC protein with its relatively low proliferation-inducing potential.

The granulocytopoietic effects of previously studied cancers has been reported to be associated with the appearance of a myeloid subpopulation with various abnormalities in cytoplasmic or nuclear morphology and with presence in the blood of immature cells as well as a few hypersegmented neutrophils. The syndrome associated

with MC appears to different. The morphology and relative numbers of members of the myeloid series in the bone marrow is normal, and the circulation is devoid of immature cells. However, among the increased number of neutrophils 30-50% exhibit nuclear hypersegmentation. It is thought (although direct evidence is lacking) that abnormal DNA synthesis underlies the occurrence of neutrophil hypersegmentation in cancer patients as well as in megaloblastic anemia. However pre-existing rather than newly synthesized DNA is implicated by the present studies showing the reproducibility of this abnormality in liquid cultures of bone marrow cells from normal animals. For no significant cell multiplication can occur in such liquid cultures (hence the use of semi-solid culture of normal bone marrow in growth factor studies), and yet 50% of the mature neutrophils exhibited obvious hypersegmentation after 48 hour incubation in MC serum or MC-CM. As judged by chromatographic mobility and elution pattern, apparent MW, and stability to heat and proteolysis, the same MC protein may be responsible for hypersegmentation and for GGT (and AP) induction. It may be noted, however, that hypersegmentation is a more specific event, in that it was negligible during incubation with CSFs even though they evoked the GGT and AP response.

The results clearly indicate that the MC tumor is secreting a factor capable of maturing myeloid leukemia

cells as indicated by the diffuse colony formation. It causes a decrease in proliferation of leukemia cells (decreased size and number of colonies and increased doubling time in liquid culture). Cultures of normal rat bone marrow incubated with MC-factor exhibit a clear shift toward more mature forms which would also indicate the presence of a differentiational activity. It is now clear that several different criteria and test systems need to be used in purification studies in order to determine whether the remarkable ability of the MC- preparations to cause granulocyte maturation, neutrophil hypersegmentation as well as differentiation in leukemic myeloid cells is attributable to a single protein. It is also of interest to determine the relationship of this activity to the enzyme induction observed in both normal and leukemic cells. The results observed with CSFs would indicate that a single factor is capable of both enzyme induction and maturation.

The significance of these investigations lies in part in the introduction of new experimental systems. The first bioassay system <u>in vitro</u> described here for characterizing factors responsible for the enzymic alterations in "uninvolved" tissues of cancer hosts proved to have multiple use, permitting, for example, study of normal myeloid cell differentiation in the absence of multiplication. We not only found that the Shay leukemia

tumor constitutes a good source for uniform cells with reproducible properties for studying in primary culture the mode of action of factors like the MC protein but also that, in view of the inducibility of uniquely high GGT levels, it offers a valuable system for elucidating the mechanisms in the regulation of GGT synthesis. The mammary carcinoma 5A (MC) has long been employed in research on cancer, biochemistry, growth, and host effects, but its usefulness to a hematological area of research became apparent only in the course of the present studies. First, we observed an effect of MC in vivo - extensive neutrophil hypersegmentation - a phenomenon for which no appropriate animal model has been available. Second, we could reproduce the hypersegmentation in a short term culture of bone marrow from normal animals; this not only allows identification of the nature and mode of action of substances causing abnormal segmentation, but might also help to elucidate mechanisms wherby the unique nuclear morphology of normal neutrophils is aquired during their maturation in the bone marrow of the disease-free organism.

BIBLIOGRAPHY

Abbott, W.A., Bridges, R.J., Meister, A. J. Biol. Chem. 259: 15393 (1984).

Ackerman, G.A. Lab. Invest. 11: 563 (1962).

Ackerman, G.A. Ann N.Y. Acad. Sci. 113: 537 (1964).

Adams, D.H., and Roe, F.J. Br. J. Cancer 7: 509 (1953).

Antman, K.S., Griffin, J.D., Elias, A., Socinski, M.A., Ryan, L., Cannitra, S.A., Oette, D., Whitley, M., Frei III, E., Schnipper, L.E. New Engl. J. Med. 319: 593 (1988).

Armuth, V.L. Br. J. Cancer 34: 516 (1976).

Asano, S., Urabe, A., Okabe, T., Sato, N., Kondo, Y., Ueyama, Y., Chiba, S., Ohsawa, N., Kosaka, K. Blood 49: 845 (1977).

Asano, S., Sato, N., Mori, M., Ohsawa, N., Kosaka, K., Ueyama, Y. Br. J. Cancer 41: 689 (1980).

Bainton, D.F. Br. J. Hematol. 29: 17 (1975).

Bainton, D.F. and Farquhar, M.G. J. Cell Biol. 39: 286 (1968a).

Bainton, D,F. and Farquhar, M.G. J. Cell Biol. 39: 299 (1968b).

Bainton, D.F., Ullyot, J.L., Farquhar, M.G. J.Exp. Medicine 134: 907 (1971).

Balinsky, D. In Cancer Markers; Diagnostic and Developmental Significance (S. Sell, ed.) Clifton : Humana Press, (1987).

Begley, C.G., Lopez, A.F., Nicola, Warren, D.J., Vadas, M.A., Sanderson, C.J., Metcalf, D. Blood 68: 162 (1986).

Bendix-Hansen, K. Br. J. Hematol. 65: 127 (1987).

Bicknell, R. and Vallee, B.L. Proc. Nat. Acad. Sci. USA 85: 5961 (1988).

Bodanszky, H., Greengard, O., Den, T. Enzyme 25: 97 (1980).

Bodner, A., Tsai, S., Ting, R., Collins, S., Gallo, R.

Leukemia Res. 4: 151 (1980)

Bond, V.P., Fliedner, T.M., Cronkite, E.P., Rubini, J.R., Brecher, G., Schork, P.K. Acta Haematol. 21: 1 (1959). Bondue, H., Machin, D., Stryckmans, P.A. Scand. J. Haematol. 24: 51 (1980).

Boyum, A. Scand. J. clin. Lab. Invest. 21: 77 (1968).

Bradley, T.R. and Metcalf, D. Aust. J. exptl. Biol. Med. 44: 287 (1966).

Bradley, T.R., Stanley, E.R., Sumner, M.A. Aust. J. Exp. Biol. Med. Sci. 49: 595 (1971).

Breitman, T.R. In Expression od Differentiated Functions of Cancer Cells (Revoltella, R.F., ed.) New York: Raven press, (1982).

Breitman, , T.R., Selonick, S.E., Collins, S.J. Proc. Nat. Acad. Sci. USA. 77: 2936 (1980).

Bretz, U., and Baggiolini, M. J. Cell Biol. 63: 251 (1974).

Bridges, R.J. and Meister, A. J. Biol. Chem. 260: 7304 (1985).

Burgess, A.W., and Metcalf, D. In Experimental Hematology Today (S.J. Baum and G.D. Ledney,eds.) Berlin: Springer-Verlag (1977).

Burgess, A.W. and Metcalf, D. Blood 56: 947 (1980).

Burgess, A.W., Camarkis, J., Metcalf, D. J. Biol. Chem. 252: 1998 (1977).

Cameron, R., Keller, J., Kolin,A., Malkin, A., Farber, D. Cancer Res. 38: 823 (1978)

Chen, H.P. and Walz, D.V. Am. J. Clin. Pathol. 29: 345 (1958).

Cheng, S., Nassar, K., Levy, D. FEBS Letters 85: 310 (1978).

Chervick, P.A., and Lu Buglio, A.F. Science 178: 164 (1972).

Clark, S.C., Kamen, R. Science 236: 1229 (1987).

Clarkson, B. and Fried, J. Med. Clin. North Am. 55: 561

(1971).

Coleman, C.N., Stoller, R.G., Chabner, B.A. Blood 46: 791 (1975).

Collins, S.J., Gallo, R.C., Gallagher, R.E. Nature 270: 347 (1977).
Collins, S.J., Ruscetti, F.W., Gallagher, R.E., Gallo, R.C. Proc. Nat. Acad. Sci. USA. 75: 2458 (1978).

Collins, S.J., Bodner, A., Ting, R., Gallo, R.C. Int. J. Cancer 25:213 (1980).

Cooper, P.C., Metcalf, D., Burgess, A.W. Leukemia Res. 6: 313 (1982).

Costa, G. Cancer Res. 37: 2327 (1977).

Costa, G. and Donaldson, S.S. New Engl. J. Med. 300: 1471 (1979).

Curry, J.L. and Trentin, J.J. Dev. Biol. 15: 395 (1967).

Curthoys, N.P. and Hughey, R. Enzyme 24: 383 (1979).

Das, S.K., Stanley, E.R. J. Biol. Chem. 257: 13679 (1982).

Das, S.K., Stanley, E.R., Guibert, L.J., Forman, L.W. Blood 58: 630 (1981).

DeLap, L.W., Tate, S.S., Meister, A. Life Sciences 16: 691 (1975).

Delmonte, L., Liebelt, A.G., Liebelt, R.A. Cancer Res. 26: 149 (1966).

DeWys, W. In Clinical Oncology, 4th ed., ed. by P. Rubin. Rochester: American Cancer Society, (1974).

Dexter, T.M. J. Cell Science 88: 1 (1987).

Dipersio, , J.F., Brennan, J.K., Lichtman, M.A., Speiser, B.L. Blood 51: 507 (1978).

Dunning, W.F. and Reich, C. Cancer Res. 3: 266 (1943).

Erickson-Miller, C.L., Abboud, C.N., Brennan, J.K. Exp. Hematol. 16: 184 (1988).

Evans, W.H., Wilson, S.M., Mage, M.G. Leukemia Res. 10: 429 (1986).

Failkow, P.J., Jacobson,, R.J., Papayannopoulou, T. Am. J. Med. 63: 125 (1977).

Fett, J.W., Strydom, D.J., Bethune, J.L., Riordan, J.F., Vallee, B.L. Biochemistry 24: 5480 (1985).

Fitchen, J.H. and Cline, M.J. Blood 53: 794 (1979).

Forbes, P., Dobbie, D., Powles, R., Alexander, P. In Haematology and Blood Transfusion, vol 26 (Gallo, R., Graf, R., Mannnweiler, K., Winkler, K, eds.) Berlin: Springer-Verlag, (1981).

Friend, C., Scher, W., Holland, J.G., Stato, T. Proc. Nat. Acad. Sci. USA 68: 378 (1971).

Fujiwara, K., Katyal, S.L., Lombardi, B. Enzyme 27: 114 (1982).

Fung, M.C., Hapel, A.J., Ymer, S., Cohen, D.R., Johnson, R.N., Cambell, H.D., Young, I.G. Nature 307: 315 (1984).

Gambo-Vitalo, C., Blair, O.C., Keyes, S.R., Sartorelli, A.C. Cancer Res. 46: 1189 (1986).

Gavosto, F. Bibl. Haematalogica 39: 968 (1973).

Glasser, L. and Fiederlein, R.L. Blood 69: 937 (1987).

Golde, D.W. and Cline, M.J. J. Clin. Invest. 51: 2981 (1972).

Golde, D.W. and Cline, M.J. In Hematology, second edition (W.Williams, E.Beutler, J. Erslev, R.W. Rundles, eds.) New York:McGraw-Hill (1977).

Goldwasser, E., Ihle, J.N., Prystowsky, M.D., Rich, J., Van Zant, G. In Normal and Neoplastic Hematopoiesis eds. D.W. Golde and P.A. Marks.New Yok: Alan R. Liss, Inc. (1983).

Gordon, M.Y., Riley, G.P., Watt, S.M., Greaves, M.F. Nature, London 326: 403 (1987).

Gough, N.M., Gough, J., Metcalf, D., Kelso, A., Grail, D., Nicola, N.A., Burgess, A.W., Dunn, A.R. Nature 309: 763 (1984).

Grabstein, K.H., Urdal, D., Tushinski, R.J., Morchizuki, D.Y., Price, V.L., Cantrell, M.A., Gillis, S., Conlon, P.J. Science 232: 506 (1986).

Greengard, O. Essays Biochem. 7: 159 (1971).

Greengard, O. Biochem. Pharmacol. 28: 2569 (1979).

Greengard, O. and Head, J.F. In Subcellular Pathology of Systemic Disease (T.J. Peters, ed.) London: Chapman and Hall, (1987).

Greengard, O. and Lempert, N. Enzyme 14: 229 (1972).

Greengard, O., Head, J.F., Koss, B., Manton, M. Cancer Res. 44: 472 (1984).
Greenstein, J.P. J. Nat. Cancer Inst. 2: 525 (1942).

Greenstein, J.P. Biochemistry of Cancer. New York: Academic Press, Inc. (1947).

Griffin, J.D., Ritz, J., Nadler, L.M., Schlossman, S.F. J. Clin Invest. 68: 932 (1981).

Griffin, J., Beveridge, R., Schlossman, S. Leukemia Res. 1: 43 (1983).

Griffin, O.W., Bridges, R.J., Meister, A. Proc. Nat. Acad. Sci. USA. 76: 6319 (1979).

Guilbert, L.J. and Stanley, E.R. J. Cell Biol. 85: 153 (1980).

Hattersley, P.G. and Engels, J.L. West. J. Med. 121: 179 (1974).

Hemmi, H. and Breitman, T.R. Developments in Cancer Chemotherapy (Glazer, R.I., ed.) Boca Raton: CRC Press, (1984).

Herzfeld, A. and Greengard, O. Cancer Res. 32: 1826 (1972).

Herzfeld, A. and Greengard, O. Cancer Res. 37: 231 (1977).

Herzfeld, A., Greengard, O., Mcdermott, W.V. Cancer 45: 2383 (1980).

Heyneman, R.A., Vercauteren, R.E. Enzyme 27: 141 (1982).

Hirakawa, T., Kakumaga, T., Fujiki, H., Sugimura, T. Science 216: 527 (1982).

Hirata, Y., Matsukura, S., Imura, H., Yakura, T., Ihjima, S., Nagase, C., Itoh, M. Cancer 38: 2575 (1976).

Huberman, E., Braslowsky, G., Callahan, M., Fijika, H. Carcinogenesis 3: 111 (1982).

Ichikawa, Y. J. Cell Physiol. 74: 223 (1969).

Ichikawa, Y, Maeda, M., Horiuchi, M. Int. J. Cancer 17: 789 (1976).

Ihle, J.N., L. Rebar, Keller, J., Lee, J.C., Hapel, A. Immunol. Rev. 98: 101 (1981).

Ihle, J.N., Keller, J., Henderson, L., Klein, F., Palaszyniski, E. J. Immunol. 129: 2431 (1982).

Ihle, J.N. , Keller, J., Rein, A., Cleveland, J. Rapp, U. In Cancer Cells 3/ growth factors and transformation Cold Spring Harbor: Cold Spring Harbor Laboratory.(1985).

Imaizumi, M. and Breitman, T. Eur. J. Haematol. 38: 289 (1987).

Inoue, M., Horiuchi, S., Morino, Y. Eur. J. Biochem. 78: 609 (1977).

Ishii, T., Todoroki, T., Takada, A., Sugita, Y. Cell Structure and Function 10: 89 (1985).

Jimenez, J.J. and Yunis, A.A. Science 238: 1278 (1987).

Joffey, J.M., Hudson, G., Osmond, D.G. J. Anatom. 99: 841 (1965).

Kasukabebe, T., Honuma, Y., Hozumi, M. Gann 70: 119 (1979).

Kawasaki, E.S., Ladner, M.B., Wang, A.M., Van Arsdell, J., Warren, M.K., Coyne, M.Y., Schwickart, D.L., Lee, M., Wison, K.J., Boosman, A., Stanley, E.R., Ralph. P., Mark, D.F. Science 230: 291 (1985).

Koeffler, H.P. Blood 62: 709 (1983).

Koeffler, H.P. and Golde, D.W. Blood 56: 344 (1980).

Koeffler, H.P., Billing, R., Sparkes, R.S., Golde, D.W. Blood 56: 265 (1980).

Koeffler, H.P., Bar-Eli, M., Territo, M. Cancer Res. 41: 919 (1981).

Koss, B. and Greengard, O. Cancer Res. 42: 2146 (1982).

Kovacs, C.J., Emma, D.A., Evans, M.J., Johnke, R.M., Scarantino, C.W Cell Tissue Kin. 18: 235 (1985).

Lajtha, L.G. Blood Cells 5: 447 (1979).

Laslo, J. and Rundles, R.W. In Hematology (W.J. Williams, E. Beutler, A.J. Erslev, Rundles, R.W, eds.) New York: McGraw-Hill (1977).

Lee, M.Y., Sperlin, A., Dale, D.C. Exp. Hematol. 8: 249 (1980).

Lichtman, M.A. and Weed, R.L. Blood 39: 301 (1972).

Liddle, G. W. and Ball, J.H. In Cancer Medicine, ed. J.F. Holland and E. Frei III. Philadelphia: Lea & Feginger, (1973).

Liebelt, R.A., Gehring, G., Delmonte, L., Schuster, G., Leibelt, A.G. Ann. N.Y. Acad. Sci 230: 547 (1974).

Lindena, J., Sommereld, U., Hopfel, C., Wolkersdorfer, R., Trautschold, I. Enzyme 29: 229 (1982).

Lindenbaum, J. and Nath, B. Br. J. Haematol. 44: 511 (1980).

Lipton, J.H., Sachs L. Biochim. Biophys. Acta 673: 552 (1981).

Lopez, A.F., Nicola, N.A., Metcalf, D., Burgess, A.W., Battye, F., Sewell, W., Vadas, M.A. J. Immunol. 131:2983 (1983).

Lotem, H. and Sachs, L. Int. J. Cancer 25: 561 (1980).

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. J. Biol. Chem. 193: 265 (1951).

Lucke, B., Berwick, M., Zeckwer, I. J. Nat. Cancer Inst. 13: 681 (1952).

Lusis, A.J. and Golde, D.W. J. Supramol. Struct. Suppl. 4: 41 (1980).

Maeda, M., Horiuchi, M., Numa, S., Ichikawa, Y. Gann 68: 435 (1977).

Matsui, T., Nakao, Y., Kobayashi, N., Kishihara, M., Ishizuka, S., Watanabe, S., Fujita, T. Int. J. Cancer 33: 193 (1984).

McComb, R.B., Bowere, G., Rosen, S., (eds.) In Alkaline Phosphatase, New York: Plenum Press (1979).

McPortland, R.P., Wang, M.C., Block, A., Weinfeld, H. Cancer Res. 34: 3107 (1974).

Meister, A. Science 180: 33 (1973).

Meister, A. Fed. Proc. 43: 3031 (1984).

Meister, A. and Tate, S.S. Ann. Rev. Biochem 45: 559 (1976).

Mergenthaler, H.G., Dormer, P., Staber, F.G., Hultner, L. Exp. Hematol. 10: 789 (1982).

Messner, H.A. and Griffin, J.D. Clinics in Haematology 15: 641 (1986).

Metcalf, D.W. Int. J. Cancer 25: 225 (1980).

Metcalf, D. Hematopoeitic Colony Stimulating Factors. New York: Elsevier (1984).

Metcalf, D. Cell 43:5 (1985).

Metcalf, D. Blood 67: 257 (1986).

Metcalf, D. In Control of Animal Cell Proliferation, Vol II, (A.L. Boynton and H.L. Leffert,eds.) Orlando: Academic Press,Inc., (1987).

Metcalf, D. and Nicola, N.A. Int. J. Cancer 30: 773 (1982).

Metcalf, D., Moore, M.A.S., Warner, N.L. J. Nat. Cancer Inst. 43: 983 (1969).

Metcalf, D., Begley, C.G., Williamson, D.J., Nice, E.C., De Lamarter, J., Mermod, J.-J., Thatcher, D., Schmidt, A. Exp. Hematol. 15: 1 (1987).

Miyaura,C., Abe, E., Kunbayashi, T., Tanaka, H., Konno, K., Nishi, Y., Suda, T. Biochem. Biophys. Res. Commun. 102: 937 (1981).

Morgan, C.J. and Stanley, E.R. Biochem. Biophys. Res. Commun. 119: 35 (1984).

Morrison, S.D. Cancer Res., 31: 98 (1971).

Murao, S.-I., Gemmel, M.A., Callahan, M.F., Anderson, N.L., Huberman, E. Cancer Res. 43: 4989 (1983).

Nagata, S., Tsuchiya, M., Asano, S., Kaziro, Y., Kubota, N., Oheda, M., Nomura, H., Ono, M. Nature 319: 415 (1986).

Nakahara, W. and Fukuoka, F. Japan Med. J. 1: 271 (1948).

Nath, B.J. and Lindenbaum, J. Ann. Int. Med. 90: 757 (1979).

Nickell, K.A., Halper, J., Moses, H.L. Cancer Res. 43: 1966 (1983).

Nicola, N.A. and Metcalf, D. J. Cell. Physiol. 109: 253 (1981).

Nicola, N.A. and Metcalf, D. In Genes and Cancer (J.M. Bishop, M. Greaves, J.D. Rowley, eds.) New York: Alan R. Liss, (1984).

Nicola, N.A. and Metcalf, D. J. Cell. Physiol. 124: 313 (1985).

Nicola, N.A., Begley, C.G., Metcalf, D. Nature 314: 625 (1985)

Nicola, N.A., Metcalf, D., Matsuumoto, M., Johnson, G.R. J. Biol. Chem. 258: 9017 (1983).

Nordenburg, J., Wasserman, L., Peled, A., Malik, Z., Stenzel, K.H., Novogrodsky, A. Br. J. Cancer 55: 493 (1987).

Novogrodsky, A., Tate, S.S., Meister, A. Proc. Nat. Acad. Sci. USA 73: 2414 (1976).

Newburger, P.E., Chovaniec, M.E., Greenberger, J.S., Cohen, H.J. J. Cell Biol. 82: 315 (1979).

O'Grady, L.F. In Laboratory Hematology (Koepke, J.A., ed.) New York: Churchill Livingstone (1984).

Ohno, T., Seki, M., Shikita, M. Blood 51: 911 (1978).

Olsson, I.L., Sarngadharan, M.G., Breitman, T.R., Gallo, R.C. Blood 63: 510 (1984).

Orlowski, M. and Meister, A. Proc. Nat. Acad. Sci USA 67: 1248 (1970).

Orlowski, M., Sessa, G., Green, J.P. Science 184: 66 (1974).

Parker, J.W. and Metcalf, D. J. Immunol. 112: 502 (1974).

Pegararo, L., Bagnara, G., Bonsi, L., Biogini, G., Garabino, G., Pagliardi, G. Cancer Res. 41: 5049 (1981).

Perille, P.E., Kaplan, S.S., Finch, S.C. New Engl. J. Med. 277: 10 (1967).

Phillips, P.G., Chikkappa, G., Brinson, P.S. Exp. Hematol. 11: 10 (1983).

Pizer, L.I. and Regan, J. Nat. Canc. Inst. 48: 1857 (1972).

Platzer,E. and Kalden, J.R. Blut 54: 129 (1987).
Platzer, E., Welte, K., Gabrilove, J.L., Lu, L., Harris, P., Mertelsmann, R., Moore, M.A.S. J. Exp. Med. 162: 1788 (1985).

Platzer, E., Oez, S., Welte, K., Sendler, A., Gabrilove, J.L., Mertelsmann, R., Moore, M.A.S. Immunobiology 172: 185 (1986).

Pluznick, D.N. and Sachs, L. J. cell. comp. Physiol. 66:319 (1965).

Pocius, P.A., Baumrucker, C.R., McNamara, J.P, Bauman, D.E. Biochem J. 188: 565 (1980).

Prestidge, R.L., Watson, J.D., Urdal, D., Mochizuki, D., Conlon, R., Gillis, S. J. Immun. 133: 293 (1984).

Rabinowitz, Y. Blood 27: 470 (1966).

Rees, L.H., Bloomfeild, G.A., Rees, G.M., Corrin, B., Franks, L.M., Ratcliffe, J.G. J.Clin. Endocrinol. Metab. 38: 1090 (1974).

Reiss, M., Gamba-Vitalo, C., Sartorelli, A.C. Cancer Treatment Reports 70: 201 (1986).

Roberts, A.B., Lamb, L.C., Newton, D.L., Sporn, M.B., DeLarco, J.E., Todaro, G.J. Proc. Nat. Acad. Sci. USA 78: 5339 (1981).

Roberts, R.A., Spooncer, E., Parkinson, K., Lord, B.I., Allen, T.D., Dexter, T.M. J. Cell Physiol. 132: 203 (1987).

Robinson, W.A. Ann. N.Y. Acad. Sci. 230: 212 (1974).

Ross, G.A., Jarowski, J.A., Rabellino, E.M., Winchester, R.J. J.Exp. Medicine 47: 730 (1978).

Rovera, G., Santoli, D., Damansky, C. Proc. Nat. Acad. Sci. USA. 76: 2779 (1979).

Rubini, J.R., Cronkite, E.P., Bond, V.P., Fliedner, T. J.Clin. Invest. 39: 909 (1960).

Ruddon, R.W. Cancer Biology, 2nd edition, New York: Oxford University Press (1987).

Ruttenburg, A.M., Hawkyu, K., Fischbein, J.W., Hanker, J.S., Wasserkrug, H.L., Seligman, A.M. J. Histochem. and Cytochem. 17: 517 (1969).

Sanderson, J.H., Phillips, C.E. An Atlas of Laboratory Animal Haematology. Oxford: Clarendon Press, (1981).
Sato, N., Asano, S., Mori, M., Ueyama, Y., Sugano, K., Ohsawa, N., Takaku, F. J. Cell. Physiol. 124: 255 (1985).

Schofield, R. Blood Cells 4: 7 (1978).

Schwartz, E.L. and Sartorelli, A.C. Cancer Res. 42: 2651 (1982).
Seymour, C.A. and Peters, T.J. Clin. sci. mol. Med. 52: 229 (1977).

Shah, R.G., Caporale, L.H., Moore, M.A.S. Blood 50: 811 (1977).

Shay, H., Gruenstein, H., Harris, G. Acta Haematologica 14: 337 (1955).

Sheriden, J.W. and Stanley, E.R. J.Cell Physiol. 78: 451 (1971).

Sheriden, J.W. and Metcalf, D. J. Cell Physiol. 81: 11 (1973).

Sherr, C.J., Rettenmeir, C.W., Succa, R., Roussel, M.F., Look, A.T., Stanley, E.R. Cell 41: 665 (1985).

Sherwin, S.A., Twardzik, D.R., Bohn, W.H., Cockley, K.D., Todaro, G.J. Cancer Res. 43: 403 (1983).

Shilling, V. Deutsch med Wchnschr 37: 1159 (1911).

Sieff, C.A., Emerson, S.G., Donahue, R.E., Nathan, D.G., Wang, E.A., Wong, G.G., Clark, S.C. Science 230: 1171

(1985).

Souza, L.M., Boone, T.C., Gabrilove, J., Lai, P.H., Zsebo, K.M., Murdock, D.C., Chazin, V.R., Bruszewski, Lu, H., Chen, K.K., Barendt, J., Platzer, E., Moore, M.A.S., Mertlesmann, R., Welte, K. Science 232: 61, (1986).

Sparrow, L.G., Metcalf. D., Hunkapillar, M.W., Hood, L.E.,

Burgess, A.W. Proc. Nat. Acad. Sci., USA. 82: 292 (1985).

Stanley, E.R. Proc. Nat. Acad. Sci. USA. 76: 2969 (1979).

Stanley, E.R. In The Lymphokines (Hadden and Stewart, eds.) Clifton : Humana Press, (1981).

Stanley, E.R. and Guilbert, L.J. J. Immunol. Methods 42: 253 (1981).

Stanley, E.R. and Heard, P.M. J. Biol. Chem. 252: 4305 (1977).

Stanley, E.R. and Metcalf, D. Aust. J. Exp. Biol. Med. Sci. 47: 467 (1969).

Stanley, E.R., Bradley, T.R., Sumner, M.A. J. Cell. Physiol. 78: 301 (1971).

Stanley, E.R., Hansen, G., Woodcook, J., Metcalf Fed. Proc. 34: 2272 (1975).

Stanley, E.R., Bartucci, A., Patinkin, D., Rosendaal, M., Bradley, T.J. Cell 45: 667 (1986).

Stewart, C.A. Pathology 6: 287 (1974).

Stoaniemi, E.A., Pelkonen, R.O., Mokka, R.E., Huttenen, R., Viljakainen, E. Eur. J. Clin. Invest. 7: 269 (1977).

Suda, M. Tanaka, T., Sue, F., Harano, Y., Morima, H. Gann Monograph 1: 127 (1966).

Sundstrom, C. and Nilsson, K. Int. J. Cancer 7: 565 (1976).

Takeda, K., Minowada, J., Bloch, A. Cancer Reseach 42: 5152 (1981).

Tanaka, K. R. and Valentine, W.N. J. Lab.Clin. Med. 56: 754 (1960).

Tarachand, U. and Eapen, J. FEBS Letters 141: 210 (1982).

Territo, M. and Koeffler, H.P. Br. J. Haematol. 47: 479 (1981).

Theologides, A. Cancer 29: 484 (1972).

Thomas, E., Smith, D.C., Lee, M.Y., Rosse, C. Cancer Res. 45: 5840 (1985).

Tomida, M., Yamamoto-Yamaguchi, Hozumi, M. FEBS Letters 178: 291 (1984).

Vina, J., Puertes, I., Montoro, J., Vina, J.R. FEBS Letters 159: 119 (1983).

Wachstein, M. J. Lab. clin. Med. 31: 1 (1946).

Walker, F. and Burgess, A.W. EMBO J. 4: 933 (1985).

Walker, F., Nicola, N.A., Metcalf, D., Burgess, A.W. Cell 43: 269 (1985).

Wallace, P.J., Packman, C.H., Lichtman, M.A. Exp. Hematol. 15: 34 (1987).

Warner, N.L., Moore, M.A.S., Metcalf, D. J. Nat. Cancer Inst. 43: 963 (1969).

Wasserman, L., Nordenburg, J., Beery, E., Deutsch, A.A., Novogrodsky, A. Expl. Cell Biol. 55: 189 (1987).

Waterhouse, C. J. Chron. Dis. 16: 637 (1963).

Weisbart, R.H., Golde, D.W., Clark, S.C., Wong, G.G., Gasson, J. C. Nature 314: 361 (1985).

Welte, K., Platzer, E., Lu, L., Gabrilove, J.L., Levi, E., Mertelsmann, R., Moore, M.A.S. Proc. Nat. Acad. Sci. USA. 82: 1526 (1985).

Wetzel, B.K., Spicer, S.S., Horn, R.G. J. Histochem. Cytochem. 15: 311 (1967).

Wickramasinghe, S.N. Br. J. Haematol. 22: 111 (1972).

Williams, N., Eger, R.R., Moore, M.A.S., Mendelsohn, N. Differentiation 11: 59 (1978).

Wiltshaw, E. and Maloney, W.C. Blood 10: 1120 (1955).

Wintrobe, M.M. Clinical Hematology. Philadelphia: Lea and Febinger, (1961).

Wong, G.G., Witek, J., Temple, P.A., Wilkens, K.M., Leary, A.C., Luxenburg, D.P., Jones, S.S., Brown, E.C., Kay, R.M., Orr, E.C., Shoemaker, C., Golde, D.W., Kaufman, R.J., Hewick, R.M., Wang, E.A., Clark, S.C. Science 228: 810 (1985).

Wong, G.G, Temple, P.A., Leary, A.C., Witek-Giannotti, J.S., Yang, Y.-C., Ciaretta, K.B., Chung, M., Murtha, P., Kriz, R., Kaufman, R.J., Ferenz, C.R., Sibley, B.S., Turner, K.J., Hewick, R.M., Clark, S.C., Yanai, N., Yokota, H., Yamada, M., Saito, M., Motoyoshi, K., Takaku, S. Science 235: 1504 (1987).

Wu, C. Int. J. Cancer 11: 438 (1973).

Wu, M.-C., Cini, J.K., Yunis, A.A. J. Biol. Chem. 254: 6226 (1979).

Wynick, D., Williams, S.J., Bloom, S.R. New Engl. J. Med. 319: 605 (1988).

Yang, Y.C., Ciarletta, A.B., Temple, P.A., Chung, M., Kovacic, S., Witek-Giannotti, J.S., Leary, A.C., Kriz, R., Donahue, R.E., Wong, G.G., Clark, S.C. Cell 47: 3 (1986).

Yokota, T., Lee, F., Rennick, D., Hall, C., Arai, N., Mosmann, T., Nabel, I.G., Cantor, H., Arai, K. Proc. Nat. Acad. Sci. USA. 81: 107 (1984).

Yunis, A.A., Arimura, G.K., Haines, H.G., Ratzan, R.J., Gross, M.A. Cancer Res. 35: 337 (1975).

Yuo,A., Kitagawa, S., Okabe, T., Urabe, A., Komatsu, Y., Itoh, S., Takaku, F. Blood 70: 404 (1987).