Negative Regulators of Hematopoiesis from Normal and Leukemic Granulocytes

by

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B. Sc. The University of British Columbia, 1990

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

Department of Microbiology and Immunology

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

February, 1996

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ABSTRACT

Hematopoiesis is controlled by a dynamic equilibrium between positive and negative growth regulatory signals. Initially, much investigation focused on the positive regulatory signals. The importance of the negative regulators in maintaining the tightly controlled limits on cell numbers seen *in vivo* is now being appreciated. This thesis describes research into the role of negative regulation in normal and leukemic hematopoiesis. The proteins believed to be responsible for the inhibitory activity of two crude neutrophil preparations, one from patients suffering from chronic myeloid leukemia (CML) and one from normal donors, have been identified. Limited characterization of these activities on normal and leukemic hematopoiesis has also been performed.

Previous work in our laboratory described an activity derived from immunoaffinity enriched cell lysates of patients with chronic or acute myeloid leukemia (CML or AML). This material demonstrated significant inhibition of growth of committed progenitor cells (especially CFU-GM) from normal individuals (both human and murine) but did not inhibit equivalent colonies from samples taken from patients with CML. Preliminary attempts to characterize this material (referred to previously as CAMAL) isolated the active constituent to within a 30 to 35 kDa molecular size fraction.

Further purification of this activity was undertaken. Using reverse phase high pressure liquid chromatography (rpHPLC) to fractionate the immunoaffinity enriched material, the inhibitory activity was found to elute exclusively in a single rpHPLC fraction corresponding to the leading portion of the peak corresponding to the 29 to 37 kDa serine protease homologue azurocidin/CAP37. The main portion of the azurocidin peak was found to have no inhibitory activity. Two-dimensional gel analysis of the active part of the peak and a reference of azurocidin (isolated from normal azurophilic granules) showed no entity distinct from the higher molecular weight glycoforms of azurocidin.

As azurocidin is found within normal neutrophils, we purified this molecule from this source. Only one of six azurocidin preparations from normal donors was found to contain inhibitory activity on normal CFU-GM.

Recently, we have devoted our efforts to extend the identification and characterization of a myelopoietic inhibitory activity originally described by Böyum and colleagues from normal neutrophils (147). Using density gradient fractionation of neutrophil lysates, we localized the activity to the cytosol and the specific granules fraction. Using sub-fractionation of granulocytes, ammonium sulfate and heat precipitation coupled with size exclusion and anion exchange chromatography, we have purified the molecule responsible for the inhibitory activity on myeloid progenitors to a single, silver stained band of approximately 15 kDa. Identification of this material as cytidine deaminase (CD) was established by Western blot analysis, the use of recombinant cytidine deaminase and blockage of the inhibitory activity by the known inhibitor of CD activity tetrahydrouridine (THU). Normal human, murine and CML progenitors were equally susceptible to the inhibitory activity of this molecule. In addition the proliferation and clonogenicity of various leukemic cell lines was also inhibited. Interestingly, even at high concentrations (> 60 ng/ml) of pure recombinant CD, no more than a 70% inhibition of myeloid colony formation was seen.

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ACKNOWLEDGEMENT

I would like to thank Dr. Julia Levy for allowing me to perform this work in her laboratory. Dr. Levy provides the opportunity to succeed at the highest levels and shows by example that it is possible. I would also like to thank Dr. Charles Dowding for his friendship, wise words and for teaching me the subtleties of hematology and science. Drs. Stephen Yip and Mark Curry my friends and compatriots in arms. Thank-you for your help and camaraderie over the years, I couldn't have done it without you. Thanks are also in order for the entire Levy lab, past and present, for their friendship and assistance.

To Mom, Dad and Joyce

Without your love and support this work would not have been possible.

CHAPTER 1 - INTRODUCTION

1.1 Introductory Remarks

Cancer involves the loss of proliferative growth control. This may occur as a result of over-expression of positive growth factors which override inherent control mechanisms. An alternative possibility is that the negative growth control mechanisms have been lost. With the concept that the accumulation of multiple mutations result in the development of cancer, a combination of the two mechanisms is also possible. Initial investigations in cancer research were focused on the positive regulation of cell growth through the oncogenes and growth factors. More recently, the importance of negative regulatory factors such as products of tumor suppressor genes and other factors known to induce growth arrest, differentiation or apoptosis, has been appreciated.

This thesis describes work performed over the last four years investigating the role of negative regulation in normal and leukemic hematopoiesis. The proteins responsible for the inhibitory activity of two crude neutrophil preparations, one from patients suffering from chronic myeloid leukemia (CML), and one from normal donors, have been identified. Limited characterization of these activities on normal and leukemic hematopoiesis has also been studied.

This introduction covers material important for the appreciation of the research reported herein. This is not meant to be an exhaustive review of each subject. Examples of many of the proteins and mechanisms involved in the development, control and function of hematopoiesis are presented such that a basic level of understanding of the field pertaining to this work may be attained.

1.2 Hematopoiesis

i) Hematopoiesis

Hematopoiesis is an intricate and dynamic process occurring initially in mammalian development in the embryonic yolk sac. With time, the site of hematopoiesis moves sequentially to the fetal liver, spleen and finally the adult bone marrow (1). Most of the mature cells produced by this system have a short life-span, consisting of a few hours to a few days, and must be replaced continuously throughout life. In fact, approximately 3.7 x 10^{11} new hematopoietic cells are needed daily to maintain the steady-state demands of the average human (2).

The pluripotent stem cell is the seminal element of the hematopoietic process. It is through the proliferation and differentiation of this cell that all blood cells are derived. A delicate balance between self-renewal, differentiation and quiescence is maintained in the stem cell pool by a complex network of interactions. As these cells divide, they can self-renew or differentiate, giving rise to more mature cells which progressively lose the capacity for self renewal while gaining the ability to perform more specialized tasks. During the later stages of this process, an exponential expansion of increasingly mature cells from lineage restricted precursor cells results in the production of the massive cell numbers required on a daily basis. The factors which regulate the hematopoietic system, by maintaining this equilibrium, have been under intense investigation over the past few decades.

Hematopoiesis gives rise to two major cell lineages. The lymphoid lineage, which generates both T and B cells, and the myeloid lineage which leads to the development of erythrocytes, platelets, macrophages and granulocytes. The granulocyte is a classification of cells which includes the neutrophils, eosinophils and basophils. These mature lymphoid

and myeloid cells have specialized roles in the hemopoietic system. T and B cells play a critical role in cellular and humoral immunity respectively. Erythrocytes are involved in oxygen and carbon dioxide transport. Platelets mediate the hemostatic mechanisms and the granulocytes and macrophages act as general scavengers and accessory cells responding against invading organisms and their by-products. Despite the wide ranging roles of these cells, all are originally derived from the original pool of pluripotent stem cells.

Proof of the existence of the hemopoietic stem cell was provided by the seminal work of Till and McCulloch in the early 1960's. Using bone marrow transplantation from histocompatible donor mice, they were able to rescue lethally irradiated recipients from the hematopoietic ablative effect of radiation (3). Further investigation of the recipient mice revealed that their spleens developed macroscopically visible, multilineage colonies on their surface (4). Cells from these spleen colonies (colony forming unit spleen, CFU-S) were able to reconstitute other lethally irradiated recipients, thus forming more colonies on the spleens of these animals, an indication of self renewal (5). Of considerable importance is the fact that with the use of these techniques, Till and McCulloch developed an assay to enumerate the primitive hematopoietic CFU-S cells from the donor marrow (the CFU-S assay). In addition, these observations, allowed them to define the characteristics of the hematopoietic stem cell which are still widely held today: (1) the stem cell is pluripotent with the capacity to give rise to all of the different mature cells; (2) it is capable of extensive proliferation; (3) it is capable of self renewal; (4) it is responsive to its environment and; (5) the cell forms other cells that progressively lose their capacity for self renewal and become increasingly committed to produce a certain end stage cell (6). This last characteristic is based on results performed by plating bone marrow cells in a semi-solid medium and monitoring the development of colonies (the *in vitro* colony assay).

The advent of the *in vitro* colony assay in the mid 1960's was one of the most important advances in hematopoiesis research. In this assay, bone marrow progenitor cells are suspended in agar or methylcellulose in the presence of medium, serum and a source of

growth factors (will be discussed in the next section) and their clonal development monitored (7, 8). In these experiments, the resulting colonies were comprised of mature and maturing granulocytes and macrophages. When attempts were made to replate these cells in a secondary colony assay, no colonies were seen. This was the first indication that these more committed cells lacked the ability for self renewal.

With advances in culture techniques, researchers have been able to obtain colonies from bone marrow containing all myeloid cell types (9, 10). The progenitor cell which gives rise to a colony type is named by the type(s) of cell(s) it produces. In this case, because the colonies are composed of cells from all of the different myeloid types, the progenitor cell is referred to as the colony forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte (CFU-GEMM, also known as CFC-Mix). Attempts at replating cells from CFU-GEMM colonies have been met with mixed results. This indicates that some of the cells in this heterogeneous population may be able to self renew. However, the CFU-GEMM does not self renew to the same extent as the CFU-S; nor does it give rise to the lymphoid, T and B cells. This may be an indication that the optimal culture conditions have yet to be found in which the CFU-GEMM population may be able to do these specialized tasks. An alternative and more probable explanation is that the CFU-GEMM represents a more mature, more committed myeloid subtype, derived from the CFU-S, which has a greater capacity for self renewal. Again, recent work has shown that the CFU-S is a heterogenous population containing some multipotent stem cells as well as more mature cells which have lost the ability to fully re-establish hematopoiesis (11). Therefore, an even more primitive population of stem cells than the CFU-S is believed to exist. This population has self renewal capacity and the ability to give rise to the lymphoid stem cell which can produce either T or B cells after differentiation in lymphoid tissues. However, despite all of the *in vivo* evidence for such a cell, only recently has an *in vitro* culture system been developed which supports both myeloid and lymphoid cell production (12, 13).

Critical to erythropoieisis *in vitro* is the addition of the hormone erythropoietin (EPO) (14). Two colony types emerge from murine cultures containing this hormone. The first, colony forming unit-erythroid (CFU-E) appears within 2-4 days and is small, 8-16 cells. The second, burst forming unit-erythroid (BFU-E) appears within 7 days and consists of aggregates of the CFU-E (15).

A flow chart outlining the hierarchical organization of hematopoiesis is provided in figure 1. This figure is separated into three cellular compartments. The stem cell compartment contains primitive cells capable of self-renewal and a relatively low degree of commitment. It is important to note that these cellular populations are heterogeneous, this point is made in the figure, with the overlapping of the circles indicating the populations. The second compartment comprises committed progenitors cells which grow, divide and further differentiate to give the cells which make up the end stage compartment. These cells have specialized functional tasks and for the most part have lost their ability to divide. It is the myeloid cellular population of the second compartment which are being investigated in the conventional *in vitro* colony assay.

The majority of the early experiments reported here were performed using mice or murine bone marrow. The results using human bone marrow have yielded similar results with minor variations. For example, the human CFU-GEMM and BFU-E appear after day 14 rather than day 7. Of course, the CFU-S assay of Till and McCulloch cannot be performed in humans, therefore other assays were developed to look at cells which are more primitive than the CFU-GEMM in humans.

The long-term culture system was first described by Dexter et al (16). This technique was developed to model much of the environment present within the bone marrow *in vivo* by plating hematopoietic cells over a pre-established bone marrow stroma. As seen *in vivo*, mature cells in this system die and are replenished by the primitive progenitors present. Sutherland, Ploemacher and colleagues have used this culture system to develop an assay to quantify the presence of a primitive cell type called the long-term

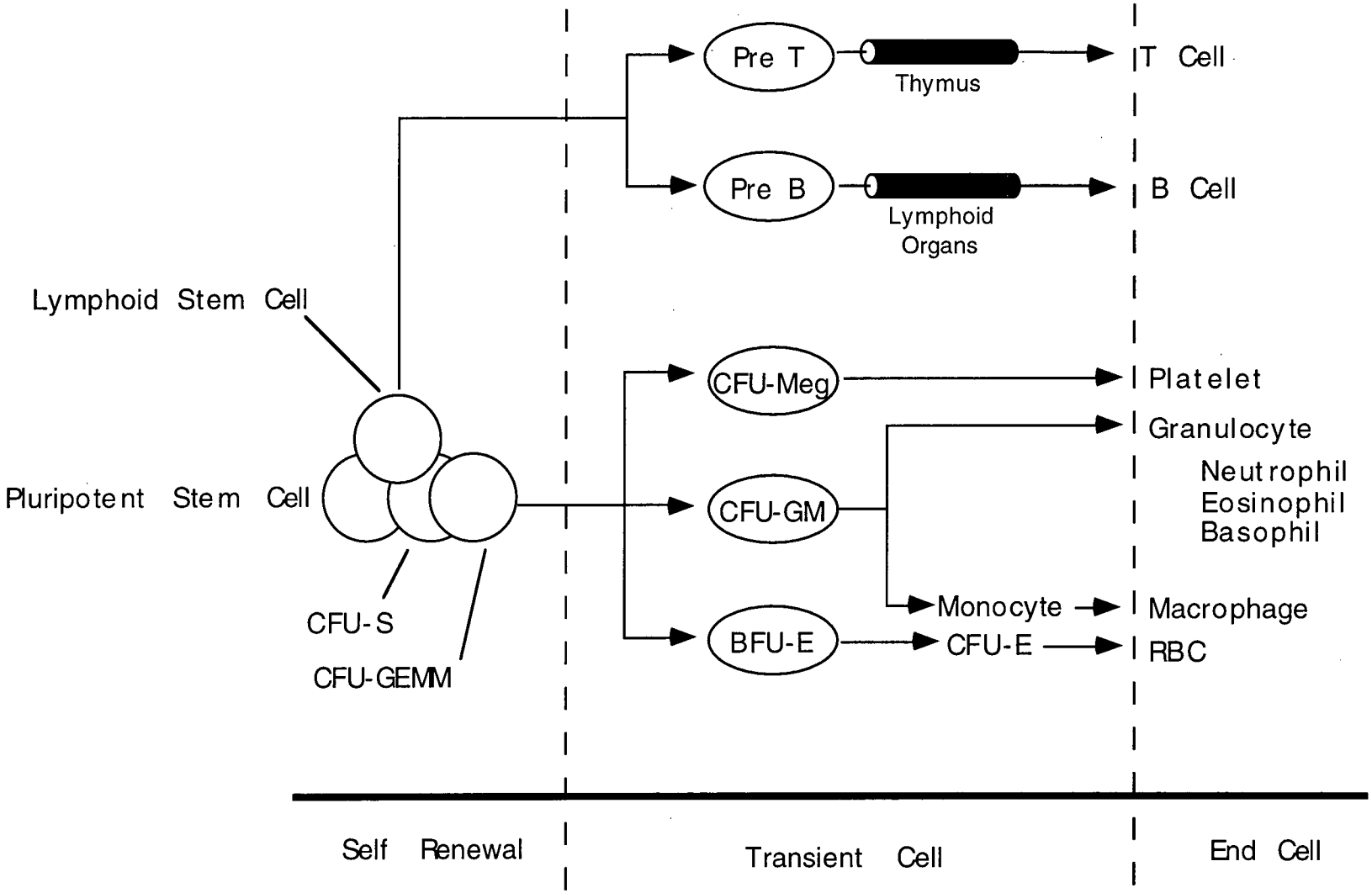


Figure 1 **Schematic diagram showing the organization of normal hematopoiesis**

culture initiating cell (LTC-IC) (17, 18). On a weekly basis, cultures are either fed or sacrificed and plated in a conventional colony assay or the development of cobblestone areas are monitored (areas of active granulopoiesis). Using the conventional colony assay, the development of colonies reflects the number of primitive progenitors initially plated in the long-term culture. As the long-term culture ages the mature colonies seen in the colony assay are derived from an increasingly primitive cell population originally present in the system. After five weeks, in culture, this primitive cell which gives rise to colonies is referred to as the long-term culture initiating cell (LTC-IC) (19). It is possible to quantitate the number of LTC-IC present during initial plating by limiting dilution analysis (18). Where the LTC-IC in humans is in the hierarchy of hematopoiesis with respect to the CFU-S in mice has yet to be determined, and difficult to ascertain because this culture system looked exclusively at myelopoiesis. Recently, the Vancouver group (Eaves et al) used a combination of the Whitlock/Witte culture system for B cell lymphopoiesis in conjunction with their culture system for myelopoiesis. With this system they were able to measure the presence of LTC-IC which has myeloid and lymphoid potential (LTC-IC_{ML}) (20).

Another method of looking at primitive human hematopoiesis is the xenogeneic transplant. To date, engraftment of human progenitors into sheep fetuses (21) and transplants into severe combined immunodeficient (SCID) mice have been investigated (22). Attempts at quantifying the frequency and efficiency of human hematopoiesis have met with limited success; however, long term human hematopoiesis has been seen in these animals.

In addition to defining the stem cell, the progenitor cells and the hierarchical organization of hematopoiesis, the *in vitro* colony assay as well as those assays looking at primitive hematopoiesis have demonstrated the importance of the regulation of this process. The next sections will describe some of the ways in which the hematopoietic system is known to be regulated.

ii) Positive regulators of hematopoiesis

Historically, erythropoietin (EPO) was the first recognized regulator of hematopoietic cells. It was discovered in 1906 in the serum of rabbits made anemic by bleeding (23). Despite not being purified until 1977 (24) the activity of impure preparations was studied extensively throughout the 1950's and 1960's (25).

It took another 60 years before the next hemopoietic regulators, the colony stimulating factors (CSF's), were discovered. The development of the colony assay was critical to their discovery. In order for cells to grow *in vitro* and produce colonies feeder cells (8), tissue fragments (7) or medium conditioned by different tissues (26) had to be present. Furthermore, the number and size of the resulting colonies correlated with the amount of this material which was added.

The first of the CSF's to be purified was only able to stimulate the proliferation of murine macrophage colonies and was isolated out of human urine (27). It was found to be a 45 kDa glycoprotein and, as it was the first of the CSF's to be discovered, it was named CSF-1 but is now commonly referred to as M-CSF (macrophage colony stimulating factor).

Concurrently, the medium conditioned by the lung tissue of mice was found to stimulate the production of both granulocytes and macrophages in the colony assay (28). Subsequent purification of the molecule responsible followed (29) and it was given the name GM-CSF (granulocyte-macrophage colony stimulating factor).

Nicola and co-workers identified from human placenta conditioned medium a factor which stimulated the growth of granulocytic colonies (30). They were later successful at purifying a 25 kDa factor out of mouse lung conditioned medium which had these properties and thus called it granulocyte colony stimulating factor (G-CSF) (31).

Initial stimulants used in the colony assay were only able to induce the production of granulocyte and macrophage colonies. However with the use of the growth supernatant

of the murine myelomonocytic cell line WEHI-3B or the mitogen stimulated spleen cell conditioned medium and as mentioned earlier, EPO, the production of erythroid, megakaryocytic and mixed colonies became possible. Attempts at purifying the factor responsible for the stimulation of the various colony types out of the WEHI-3B conditioned medium identified a 23-28 kDa factor which, because of its ability to give rise to multipotential colonies, was called multipotential CSF (Multi-CSF) (32). The properties of Multi-CSF resembled the activated T-Cell product first described by Clark-Lewis and Schrader, called persisting cell stimulating factor (PSF) (33) and those of interleukin-3 (IL-3) (34). Subsequently, these three factors were shown to be identical (35).

Researchers have now successfully cloned and expressed the cDNA of all four murine and human CSF's. Recombinant expression of these molecules have allowed for the unequivocal assignment of many of their *in vitro* activities and the initiation of *in vivo* studies. Due to their ability to elevate white blood cell counts in primates without toxicity, all four CSF's have entered into human, clinical trials and are at various stages of this process.

Since the discovery of the colony stimulating factors, at least 15 other molecules have been identified as having positive regulatory function, at least *in vitro,* on hematopoiesis. This introduction is not meant to be an exhaustive review of the myriad of actions and interactions of all of these cytokines. Some of the key players in this process are presented with a goal of reaching a basic understanding of this increasingly complex field. It is now possible to separate these growth factors into three different categories based on the hierarchical stage at which they act and the degree of lineage specificity they demonstrate: (1) late-acting lineage-specific factors; (2) intermediate-acting lineage-non-specific factors; (3) factors involved with cycling of dormant progenitors (36). Figure 2 gives a schematic representation of hematopoiesis and the stages at which the various growth factors are thought to mediate their effect. Despite these classifications, growth factors are known to act synergistically on a wide range of cell types producing pleiotropic

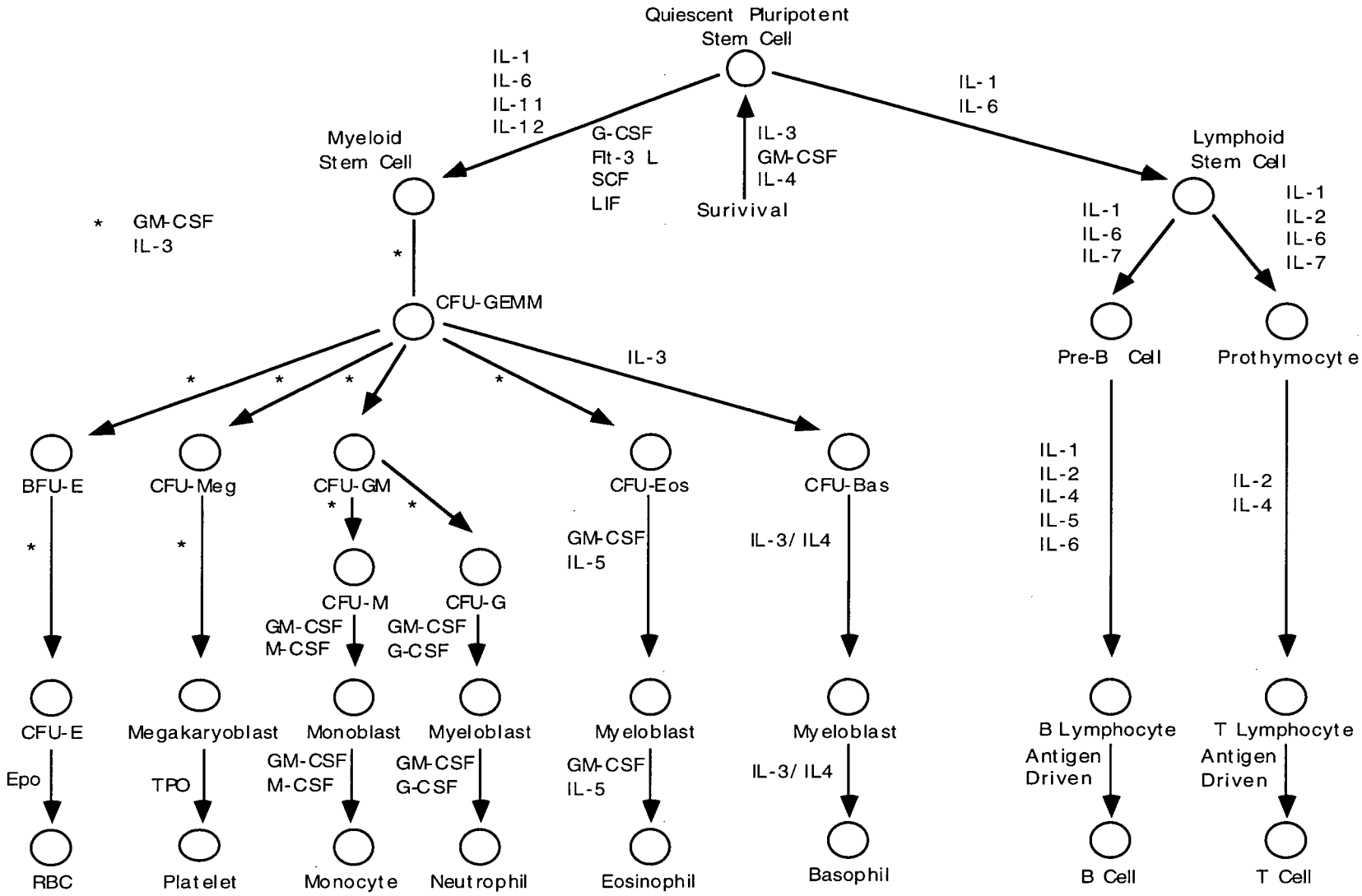


Figure 2 **Site of action of some positive hematopoietic regulators**
Asterisk (*) indicates GM-CSF and IL-3.

and overlapping effects. Thus, this type of classification may be an oversimplification of the interactions occurring *in vivo* but at least give some idea of the properties of these molecules.

Members of the late-acting lineage-specific factors include M-CSF, EPO, G-CSF, IL-5 and the newly identified thrombopoietin (TPO). All of these factors support the proliferation and maturation of committed progenitors. EPO acts on late stage erythropoiesis, IL-5, M-CSF and G-CSF promote eosinophil, macrophage and neutrophil development respectively. Despite acting on the committed progenitors giving rise to the neutrophil, G-CSF is also thought to act synergistically with other factors on the very early pool of progenitors known to be quiescent (37).

The intermediate-acting lineage-non-specific factors support the proliferation of multipotential progenitors after they enter the cell cycle. They are not known to act on the late stage, committed progenitors with the exception of basophil/mast cell development. These factors include IL-3, GM-CSF and IL-4.

IL-1, IL-6, IL-11, IL-12, G-CSF and stem cell factor (SCF) act synergistically with IL-3 to support colony formation of dormant primitive progenitors. These factors can also act synergistically with the intermediate-acting factors GM-CSF and, with the exception of IL-12 and SCF, IL-4. The leukemia inhibitory factor (LIF) was also found to trigger quiescent stem cells to enter the cell cycle. Flt-3 ligand is another factor which has recently been cloned from human (38) and murine (39) cells. It has been shown to synergize with IL-3 and GM-CSF and to have similar effects as SCF but does not stimulate erythropoiesis (40).

The bone marrow is known to provide an environment conducive to the production of the various hematopoietic cells. One of the ways that it accomplishes these tasks is through the binding of growth factors to the surface of the bone marrow's stromal cells. Stromal cells get their name from the Greek work stroma which means mattress. These cells can bind, concentrate and possibly present the growth factors via sugars found on

their surface (41). In fact, some growth factors such as M-CSF and SCF can exist in a membrane bound form and require cell contact in order to mediate their activities (42). The importance of stromal interactions are demonstrated *in vitro* in the aforementioned long-term culture where bone marrow cells are plated over a pre-formed stromal cell layer.

The way these growth factors are classically believed to act is through paracrine activation of their target cells. This involves the discharge of the factors in soluble form by secreting cells and the subsequent binding to cell surface receptors present on target cells. When the secreting cells stimulate themselves, the process is known as autocrine activation. Differential expression of cell surface receptors for these cytokines is an important regulatory mechanism employed by the hematopoietic system. The susceptibility of a target cell to the actions of a particular cytokine is dependent on the expression of the appropriate receptor which, is in turn dependant upon the cellular microenvironment, lineage restriction and stage of differentiation.

Two broad classes of receptors exist for the cytokines mentioned above. The first are able to mediate intra-cellular signals via a cytoplasmic tyrosine kinase domain which phosphorylates proteins on tyrosine residues upon receptor engagement with its ligand. Members of this group, called the receptor tyrosine kinases (RTK's) include c-Fms also known as the M-CSF receptor, Flt3 or Flk2 which are the receptors for the Flt3 ligand and the receptor SCF which also goes by the names steel, kit, mast cell growth factor receptor and SCF receptor (43). They are identifiable not only by their intrinsic kinase activity but also by the presence of five immunoglobulin-like domains in their extra-cellular region.

The second class of receptor is called the cytokine receptor superfamily. These receptors are characterized by the lack of tyrosine kinase activity and the presence of two conserved extracellular domains. These domains include, four cysteine residues which give structurally important disulphide bonds and the presence of a pentapeptide sequence, WSXWS, (where W is aspartic acid, S is serine and X is any amino acid) adjacent to the membrane which is also thought to have structural importance (44).

Both receptor families are thought to only send signals when two or more receptor subunits are brought together after ligand binding. In the case of the RTK's, homodimers are formed and in the case of the cytokine receptor superfamily, both homodimers and heteromers (both dimers and trimers) are formed.

When RTK's dimerize, they phosphorylate each other, thereby launching a cascade of signalling events. These events are regulated, to a large extent, by the associations of other proteins with the phosphorylated tyrosines on the cytoplasmic tail of the dimerized RTK's. These associations are mediated by a domain contained by various proteins known as the src kinase homology 2 domain (SH2). Through these SH2 interactions, a number of signalling proteins concentrate at the cytoplasmic tail of the receptor. What results is a complex web of interactions ultimately leading to a cellular response. This response is dependent upon the nature and state of the cell which also governs the number and types of signalling molecules and receptors present.

The cytokine receptor superfamily can be broken down on the basis of interactions resulting from ligand binding. The members of the first subfamily include the receptors for EPO, G-CSF and TPO. These receptors all consist of a single chain and form homodimers after activation. All of the other subfamilies consist of at least two subunits. The first subunit is responsible for ligand binding but this is a low affinity interaction. Upon ligand binding another subunit, which is shared amongst the members of a particular subfamily, dimerizes with the first subunit thereby enhancing the affinity of the initial interaction while sending the appropriate intracellular signals. It is important to note that signalling does not occur without the proper dimer being formed. Members of the second subfamily include the receptors for GM-CSF, IL-3 and IL-5 which share a common β subunit refereed to as β_c. The third subfamily share a common receptor subunit referred to as gp-130. Members of this family include receptors for IL's 6, 11 and 12 as well as LIF. The fourth subfamily again share a common subunit referred to as the γ_c receptor chain but in some cases (such

as with IL-2) may have two unique receptors. Members of this family include the receptors for the IL's 2, 4, 7, 9, and 15. The physiological importance of the γ_c receptor subunit was demonstrated when mutations in the gene encoding the receptor were shown to be the cause of X-linked severe combined immunodeficiency (45).

As the members of the cytokine receptor superfamily lack intrinsic kinase activity, they must associate with molecules which have the capacity to propagate the signal initiated by ligand binding. Critical for these associations is a conserved region found within the cytoplasmic domain of these receptors referred to as box1 and box 2 motifs. These motifs govern the interactions with a family of cytoplasmic tyrosine kinases referred to as the Janus (Roman god of gates and doorways) kinases (Jak's). Members of this family of kinases include Jak's 1-3 as well as Tyk 2. Receptor dimerization results in the activation of the appropriate Jak(s) resulting in auto and cross phosphorylation which further activate these kinases (46). These phosphorylations may lead to many of the same interactions seen with the RTK's. However, a family of substrates for Jak's has been found which results directly in gene transcription. This family is known as signal transducers and activators of transcription Stat. When Stat's are phosphorylated by the Jak's, they form homo and heterodimers resulting in their activation and translocation to the nucleus. Once in the nucleus, they can bind to specific DNA sequences thereby initiating the transcription of various genes (45).

iii) Negative regulators of hematopoiesis

As seen in the previous section, many well characterized molecules exist which have a role in inducing proliferation in the hematopoietic system. Intuitively, one would assume that a parallel system would have evolved to keep the effects of these molecules in check in order to maintain the relatively tight range of cell numbers and types seen in the

hematopoietic system. In fact, recent work has shown several methods that this system uses to negatively regulate proliferation. The presence of soluble receptors which are secreted as truncated versions of the membrane bound form or have been proteolytically cleaved from the cells expressing them have the capacity to bind to their ligand extra-cellularly. This reduces the number of ligand molecules available for binding to membrane bound receptors. Many examples of soluble receptors have been found including the receptors of IL-2, 4 and 7 (47). The system also uses one of the first regulatory mechanisms to be discovered called negative feedback. This is one of the most simple yet elegant control mechanisms which exists in biology. It involves an end product, in this case a cell or a cytokine, and the release of a factor by accumulating daughter cells or accumulation of the cytokine. These factors negatively regulate the cell which originally gave rise to the daughter cell or cytokine. Examples of such control mechanism exist in the development of platelets (48), monocytes/macrophages (49, 50) and maintenance of quiescence of primitive progenitors (51). Another regulatory method used by the system is the binding of cells to the bone marrow stroma. Verfaillie and colleagues have shown that interactions between the integrin adhesion molecules of progenitor cells and their stromal ligands results in the inhibition of proliferation (52, 53). Others have shown that negative signals may be sent by inhibitory cytokines such as macrophage inflammatory protein 1α (MIP-1α) and transforming growth factor β (TGF-β), bound to the bone marrow stroma (54-56). Molecules like acidic isoferritins have ferroxidase activity, the ability to convert iron from its ferrous form (Fe^{2+}) to its ferric form (Fe^{3+}). Transferrin is an iron transport protein which makes iron available to cycling cells which require iron for their metabolism. Transferrin binds iron in its ferric form and in order for it to transfer iron into cells it has to be in its ferrous form. Acidic isoferritins ensure that the iron is in its ferric form so it cannot be released and transferred to the needy cells thereby suppressing proliferation (57). It seems as though this activity affects early as well as the more committed progenitors and is

identical to another activity known as leukemia inhibitory activity (LIA) (58). Finally, and possibly most importantly, a cytokine system of soluble inhibitory molecules acting on membrane bound receptors exists.

Despite the rapid growth in understanding of the positive regulatory cytokines, the knowledge of the inhibitory cytokines have been relatively slow to follow. To date many hematopoietic inhibitory activities have been isolated, some of which have been purified and cloned. These molecules may act directly by decreasing the proliferative capabilities of the target cell or may act indirectly by silencing the effects of stimulatory cytokines. When acting directly on target cells these cytokines may work by cytostatic, differentiative, or cytotoxic mechanisms. As with positive regulatory molecules, these cytokines have multifunctional activities and may even enhance proliferation depending on the target cell and microenvironment. Members of this growing list of "inhibitory" factors include the interferons (IFN's), tumor necrosis factors (TNF's), TGF-β's, the chemokine family, the peptide inhibitors, lactoferrin and the prostaglandins (PG) E_1 and E_2.

The IFN's which include α and β (type 1) and γ (type 2) were originally identified by their antiviral activities. Since then, their inhibitory effects on hematopoiesis have been discovered (59). IFN-γ is also known to synergize with other factors such as IL-3 and GM-CSF to stimulate hematopoiesis (60, 61). Two receptors exist for the IFN's, a type 1 and type 2 receptor. Both receptors are members of the cytokine receptor superfamily and signal through independent JAK/STAT pathways (62). They are thought to exert their inhibitory effect by inducing programmed cell death (also known as apoptosis) (63-65).

The TNF's (α and β) were originally discovered in the serum of mice primed with bacillus Calmette-Guerin subsequently challenged with endotoxin (66). They are primarily produced by activated macrophages and are know to have stimulatory activity on normal $CD34^+$ progenitors in the presence of IL-3 and/or GM-CSF (67) and inhibitory activity on

myeloid colony formation (68). The pleiotropic effects of TNF-α are mediated by two distinct receptors called TNFR-p55 and TNFR-p75 which are part of the nerve growth factor (NGF) receptor family (69). The way in which this cytokine is believed to mediate its inhibitory activity is either through cytotoxicity (63, 64, 70) or by down regulation of cytokine receptors such as that for stem cell factor (71, 72).

The TGF-β's are a family of five multifunctional proteins which regulate cell growth and differentiation. The prototype of this family, TGF-β1, can synergize with GM-CSF to enhance the proliferation of more committed progenitors (73) and can inhibit the proliferation of primitive progenitors regardless of the cytokines present (74). Furthermore, TGF-β1 was shown to mediate its effects on primitive progenitors directly. The mechanisms by which its inhibitory activities are mediated are now emerging. It is becoming apparent that this platelet derived factor can induce differentiation (75), cause apoptosis (76) and evoke cytostatic mechanisms (77) to inhibit the proliferation of primitive cells. Of considerable interest is the reversible effects of the cytostatic response. It has been shown that TGF-β1 maintains cells in G_o/ G_1 (quiescence) by preventing the phosphorylation of the tumor suppressor gene, retinoblastoma (pRb). A more detailed explanation of this mechanism will be presented in the next section on the cell cycle.

The chemokines represent a superfamily of low molecular weight proteins with four conserved cysteine motifs. The superfamily can be subdivided on the basis of the first two cysteines being separated by another amino acid (C-X-C) or not (C-C). Members of the C-X-C family include IL-8, the macrophage inflammatory protein 2α (MIP-2α), interferon inducible protein 10 (IP10) and platelet factor 4 (PF4). The C-C have as members, MIP-1α and the monocyte chemotactic and activating factor (MCAF). The chemokines are known to be involved in leukocyte activation and attraction. However, all of the chemokines listed

above are also known to have myelopoietic inhibitory effects (78). MIP-1α is the most extensively investigated chemokine in this regard. It has inhibitory activity only on primitive progenitors and there is evidence to suggest that it may play a role in the maintenance of stem cell quiescence (79).

It has been over 20 years since Paukovitz and Laerum first described a low-molecular weight inhibitor of granulopoiesis (80). Since then they have been able to isolate the pentapeptide responsible for the activity and synthesize it. Referred to as pEEDCK (or HP5B), it has the following amino acid sequence (pGlu-Glu-Asp-Cys-Lys). It has been shown to be a general and reversible inhibitor of primitive stem cells and progenitors of the myeloid and erythroid lineages in both humans and mice (81, 82). Interestingly, a dimer of this molecule was found to have enhancing activity on hematopoiesis (83). The tetrapeptide AcSDKP, also known as seraspenide, was first isolated from fetal calf bone marrow (84) but is now obtained by chemical synthesis (80). Like pEEDCK, it can inhibit primitive cells as well as the myeloid and erythroid lineages. However, AcSDKP unlike pEEDCK, has profound inhibitory effects on normal progenitors while having no effect on leukemic (CML) cells (85).

Lactoferrin, like the acidic isoferritins, is an iron binding protein. However, it mediates its inhibitory effects on hematopoiesis indirectly by inhibiting CSF production from monocytes and macrophages. It is found in the specific granules of normal neutrophils and has an inhibitory role on granulopoiesis. As a result, it has been postulated that it may be involved in a negative feedback regulation of granulopoiesis (86).

PGE_1 and E_2 have a direct inhibitory effect on myeloid progenitors with the CFU-M progenitors being the most susceptible to its inhibitory effect followed by the CFU-GM and finally the CFU-G. Interestingly, erythroid progenitors are insensitive to the inhibitory effects of these molecules (57).

In addition to the well characterized molecules whose effects have been studied in pure or recombinant form, several crude activities have been identified out of cellular

extracts which also have inhibitory activity. In 1978 Broxmeyer and colleagues presented evidence of a granulopoietic inhibitory activity out of leukemic cells (87). Olofsson and Olsson extended these initial observations and found that medium conditioned by low density AML and CML bone marrow or peripheral blood cells had a reversible inhibitory activity on normal but not leukemic granulopoiesis (88). Preliminary attempts have been made to characterize this activity biochemically (89), however, the molecule responsible for this activity has not been identified and the activity of homogeneously purified material has yet to be investigated.

Two other crude preparations with inhibitory effects on myelopoiesis exist. This thesis involves the further characterization and purification of the molecules responsible for these two activities. As these activities are of particular relevance to this thesis, they will be discussed separately in the last two sections of this introductory chapter under the heading *Background and summary of this research.*

1.3 The Cell Cycle

i) Control of the Cell cycle

Life in the mammalian cell involves a constant decision between proliferation, differentiation, growth arrest and of course death. The factors which govern these decisions revolve around the cellular engine known as the cell cycle. The cell cycle is broken down into four phases named the synthesis phase (S) where DNA replication occurs, the G_2 phase which is the post-synthetic, pre-mitotic gap, the mitotic phase (M) where the cell undergoes mitotic division and the G_1 phase which is the post-mitotic, pre-synthetic gap. Mechanisms controlling the progression through the cell cycle have evolved

to ensure that initiation of a cell cycle event is dependent on the successful completion of the previous cell cycle event. These controls exist as checkpoints in both G_1 and G_2 and govern the decisions of a cell to either continue through the cell cycle, undergo growth arrest or undergo apoptotic death. Loss of function of the proteins regulating these checkpoints have been associated with the development of various cancers.

In general, cells respond to extra-cellular growth signals during the G_1 phase of the cell cycle. As mentioned in the previous section, various soluble factors are known to exert a growth regulatory function on target cells. These factors both negatively and positively induce cells to progress to a substage of G_1 known as the "Restriction Point" (R). Once cells reach this point, they are committed to complete one cell cycle even in the absence of mitogenic factors as long as survival factors are present. By binding to their receptors on the target cell surface, these factors initiate a cascade of signalling events. In some cases these signals are known to modify the expression or assembly of heterodimeric enzyme complexes formed by regulatory subunits known as cyclins and catalytic subunits known as cyclin dependent kinases (CDK's). These complexes are formed at various stages and are responsible for the sequential and controlled progression through the cell cycle.

The first complex activated after cells enter the cell cycle in G_1 from their growth arrested, quiescent state (also referred to as G_0) are those composed of the D type cyclins and either CDK4 or CDK6 depending on the type of cell (90). It is believed that this complex is important in mid to late G_1. Interestingly, its levels drop immediately after growth factor removal. Cyclin E is synthesized after cyclin D and its activated complex, which is formed upon association with CDK2, peaks in activity late in G_1 and is necessary for progression into S phase. Upon entry into the S phase, cyclin E is degraded allowing CDK2 to associate with the next important cyclin, cyclin A. Cyclin A also binds to Cdc 2 (another CDK) and the activity of this complex peaks in G_2 before being degraded suddenly. The liberated Cdc 2 is thus allowed to bind to cyclin B forming the Maturation Promoting Factor (MPF) which is critical for entry into the M phase. Finally cyclin B

degradation is necessary for exit of the M phase. Other cyclin/CDK complexes are known to exist but their role in controlling the cell cycle are less defined.

The most widely studied substrate for the serine/threonine kinase activity of the resulting cyclin/CDK complexes is the retinoblastoma protein (pRb). pRb mutations have been associated with cancers such as childhood eye tumors, small cell lung and bladder tumors. In its activated, hypophosphorylated state, pRb binds a transcription factor known as E2F. Late in G_1 pRb is thought to get phosphorylated by the D type cyclin complexes resulting in the release of E2F. E2F then associates with another protein known as DP-1 and this heterodimer acts as a transcription factor activating a host of S phase genes. TGF-β1 is believed to maintain cells in G_0/G_1 by preventing the phosphorylation of pRb possibly by inhibiting the activity of cyclin/CDK complexes (77). Recent results suggest that there is a growing family of pRb, E2F and cyclin/CDK complex proteins which may be regulated in this fashion and may be controlling the G_0/G_1 transition.

As TGF-β1 has been found to inhibit the function of the cyclin/CDK complex, other molecules are also known to regulate the activity of these complexes. These molecules, known as CDK inhibitors (CKI's), bind to the heterodimers thereby inactivating them. Examples of CKI's include $p21^{CIP1}$ whose expression is induced during terminal differentiation and is induced by a molecule known as p53. p53 is a transcription factor which is involved in negative growth control. Like pRb, mutations of this molecule is associated with a large number of different cancers including myeloid leukemias. Another CKI $p27^{KIP1}$, is present in quiescent cells but it's levels drop upon growth factor stimulation. Other CKI's include $p57^{KIP2}$, $p15^{INK4b/MTS2}$, $p16^{INK4bMTS1}$, p18 and p19.

ii) Apoptosis

Homeostasis in mammalian cells is controlled not only by the proliferation and differentiation of cells but also by cell death. It is believed that all cells have the ability to initiate a program of cell suicide termed apoptosis. Apoptosis is characterized by nuclear condensation as endonucleases are activated and break down the DNA into fragments. Cytoplasmic condensation is also initiated as endogenous proteases break down the cytoskeleton resulting in cell shrinkage and membrane blebbing. Eventually, apoptotic bodies of membrane bound DNA fragments are formed and the decaying cell is rapidly phagocytosed by macrophages or neighboring cells. Necrosis is a pathological form of cell death distinguishable from apoptosis because it results from acute injury and is characterized by membrane swelling and lysis. Many intracellular and extracellular signals are capable of initiating or inhibiting apoptosis. Factors which initiate apoptosis include cellular damage, low concentration of survival factors (essential growth factors), nutrient deprivation and negative regulatory factors (TNF-α, TGF-β, IFN-α). It is believed by some that apoptosis is linked to the cell cycle (91, 92).

Diseases attributed to a lack of apoptosis include viral infections, various cancers and autoimmune disorders. An increase in apoptosis is responsible for the pathology seen in AIDS, neurodegenerative disorders, myelodysplastic syndrome, toxin induced liver disease and ischemic injury (93). Some researchers believe that apoptosis is a default pathway and that if the proper factors are not present then it is invoked (94).

1.4 The Neutrophil

i) Characteristics of the neutrophil

The neutrophilic granule is the most abundant leukocyte, making up approximately 60% of all white blood cells. The half-life of neutrophils in the circulation is only 8-20 hours. As a result, most of the bone marrow's resources are dedicated to producing the 0.9 x 10^9 neutrophils per kilogram per day in the average human. There are 4 x 10^6 mature neutrophils per millilitre of blood as well as a considerable amount in the bone marrow. It is believed that the rapid increase in circulating neutrophils (up to ten fold) seen after infection is due to release of this population.

The neutrophil is known to play a critical role in non-specific immunity, rapidly responding to bacterial and fungal infections. Acting as the first line of defense, these cells respond quickly and with great force against invading pathogens. Neutrophils are thus highly motile, responding to a variety of chemotactic signals, and are equipped with a potent armory of cytotoxic mechanisms.

The mature neutrophil contains a variety of modularly organized cytoplasmic granules. These granules contain the cytotoxic molecules necessary for its ability to neutralize invading pathogens. There are a number of different granule types which differ in their contents and the developmental stage of their appearance. These granules are known as the azurophilic (primary), specific (secondary), gelatinase-containing (tertiary) and the secretory vesicles (95). An important component of the azurophilic granules which is important in typing the neutrophil is myeloperoxidase which is identified with the peroxidase staining. Circulating neutrophils are normally found in a non-activated state. Upon pathogenic or physiologic stimulation, these cells become primed for full scale activation. This priming process involves the fusion of some types of these granules to the

plasma membrane thereby increasing the level of various receptors on the cell surface. At this point, the cells are in a state of readiness and are capable of full scale activation upon engagement of their surface receptors (96).

Neutrophils kill their target pathogens by a process known as phagocytosis. They usually recognize foreign cells because they are coated with proteins known as opsonins (which include antibodies, complement fragments, acute phase proteins). Neutrophils which have receptors for the opsonins, bind to the invading pathogens thereby initiating a cascade of signalling events such as that seen with cytokine mediated stimulation. The cell, now in an activated state, engulfs the invading body by the process of phagocytosis and activates its armament of anti-bacterial mechanisms. In addition, activated granulocytes are know to degranulate and exocytose their granulocyte contents allowing them to act on pathogens extracellularly and to inform other immune cells of the infection.

There are two mechanisms by which neutrophils kill bacteria. One is oxygen dependent and involves the activation of a plasma membrane bound enzyme called NADPH oxidase. The other is oxygen independent and involves the fusion of the granules with the phagosome created by the engulfed foreign body thereby releasing the toxic granule contents (97).

ii) Development of neutrophils

As indicated in the "Control of hematopoiesis" section of this introduction, all hematopoietic cells originate from a common precursor known as the pluripotent stem-cell. As this cell divides, it can self-renew to give two pluripotent stem-cells or it can differentiate to give cells which have increasing commitment to a particular hematopoietic lineage while gradually losing their self-renewal potential. The first identifiable cells of the neutrophil lineage are the myeloblasts. On the basis of morphological analysis, peroxidase

staining, light and electron microscopy, it is possible to identify those myeloblast derived cells which are increasing in maturity. These include, in order of increasing maturity, the promyelocyte, myelocyte, metamyelocyte, band cell, and segmented mature neutrophil (98).

The myeloblasts are relatively undifferentiated cells which have a large nucleus but almost no cytoplasmic granules. The rough endoplasmic reticulum and golgi apparatus stain peroxidase positive, an indication that the myeloperoxidase enzyme is just being formed. Myeloblasts are capable of proliferation.

The promyelocytes have large numbers of peroxidase positive granules. They are termed azurophilic because they stain purple with azure dyes. These granules are also known as primary because they are the first granules to appear. Again, the rough endoplasmic reticulum and golgi apparatus stain peroxidase positive indicating that this is a major developmental stage for incorporation of this enzyme. Promyelocytes are capable of proliferation.

The myelocytes are characterized by their accumulation of peroxidase negative granules. These granules are also known as specific or secondary. The azurophilic granules stop being made in this cell type which has very limited proliferative potential.

The metamyelocytes, band cells and segmented cells have all lost their ability to divide. The metamyelocytes, like the myelocytes, accumulate the peroxidase negative granules. The band and segmented cells on the other hand accumulate the gelatinase positive, tertiary granules (95). A final albumin containing granule type known as the secretory vesicles is also apparent in these cells. It was once believed that the mature neutrophil could no longer produce protein but it now seems that upon stimulation it can produce some cytokines. For example, lipopolysaccharide stimulation of neutrophils results in the production of TNF-α and IL-8, and prolonged exposures to G-CSF (>3 hours) results in the expression of IFN-α (98). These proteins are known to mediate

inhibitory activities on hematopoiesis but because of the prolonged exposures necessary for the production of these factors, the physiological relevance of their production by the neutrophil is in question.

iii) Neutrophilic granules and their constituents

It is believed that the neutrophilic granules represent a continuum of granules. They are distinguished on the basis of their constituents as well as their differing densities. Borregaard et. al. have suggested that the components of a particular granule represent the population of proteins produced at a particular phase of development of the neutrophil. This hypothesis would account for the presence of the overlap seen with some granule constituents. In this section, the different proteins found within the granules are discussed.

The azurophilic granules are the most dense granules and are the first granules to get produced, initially appearing in the promyelocyte (99). Proteins found within the azurophilic granules include myeloperoxidase, bactericidal permeability inducing factor (BPI, also known as CAP 57), the bactericidal serine proteases and their homologues, the defensins and lysozyme (97).

Myeloperoxidase is the most prominent protein found within the azurophilic granule. Most of the identification of the stages of neutrophil development were made possible because of a cell staining technique exploiting the enzymatic activity of this molecule. It is green and thus purified azurophilic granules have a distinctive greenish tinge. It mediates its cytotoxic function through the oxygen dependent mechanism.

BPI is a 55-60 kDa protein with bactericidal activity against gram negative bacterial strains with defective or short-chain lipopolysaccharide. A 25 kDa fragment of this molecule retains the bactericidal activity. BPI has maximal bactericidal activity at neutral pH and is inhibited by millimolar concentrations of magnesium and calcium (100).

The serine proteases are the most thoroughly understood family of enzymes as a result of their extensive examination over the past 40 years. These enzymes are so named due to a common catalytic mechanism characterized by the presence of a peculiarly reactive serine residue in their active site. This serine (Ser-195) is the critical component of the active site's catalytic triad which also contains histidine-57 (His-57) and aspartate-102 (Asp-102) (101). Serine proteases are regulated in a variety of ways. They are initially secreted in an inactive, "zymogenic" form which is activated by proteolytic cleavage. They are regulated by inhibitors present in plasma, and have differing activities dependant upon their glycosylation (102) and microenvironment, being active only in neutral and slightly basic conditions.

Various serine proteases have interesting hematologic functions. These include the cytotoxic T cell associated granzymes which play a role in antigen-specific cytotoxicity (103). Clotting factors are involved in the activation of zymogens and cells. An example is the activation of platelets by thrombin. The actions of thrombin are mediated via the cleavage of the amino terminus of its receptor, thereby creating a new terminus which acts as a tethered ligand and activates the receptor (104).

The neutrophilic granule-associated serine proteases are of particular interest in the etiology of myeloid leukemias. These enzymes are normally sequestered, binding to the inner matrix of the granule. They are released into the phagocytic vacuoles during phagocytosis where they digest foreign protein and antibody complexes. With cell death, degranulation and leakage from the phagocytic vacuole, the contents of these granules are released to the cell's exterior. Once these proteins are in the extracellular environment it is believed that some may act as chemotactic factors, while others are believed to be involved in the activation or inactivation of plasma hormones. The presence of these enzymes extracellularly is particularly dangerous when the physiological serine protease inhibitors normally present in the plasma are in too low a concentration (105). Such could be the case

with chronic myeloid leukaemia, where massive neutrophil turnover might result in the release of relatively large amounts of these enzymes.

Elastase and myeloblastin are serine proteases which are found within the azurophilic granules of neutrophils. Elastase is a glycoprotein which can rapidly degrade connective tissue and has been implicated in the onset of pulmonary emphysema, a condition characterized by a low concentration of its inhibitor (106). Elastase is a highly cationic, glycosylated, serine protease. It can degrade proteins on bacterial cell walls and it is known to potentiate lysozyme and cathepsin G mediated bactericidal effects by a non-enzymatic mechanism. Down-regulation of myeloblastin, using treatment of HL60 cells (a promyelocytic cell line) with anti-sense oligonucleotides to inhibit translation of the message, results in growth arrest and differentiation (107). Therefore, it is conceivable that a specific inhibitor of myeloblastin activity could promote proliferation of myelopoiesis.

Azurocidin (also known as CAP 37) is a bactericidal azurophilic granule protein which lacks serine protease activity due to substitutions of two of the three members of the catalytic triad. The critical serine is substituted by a glycine and the histidine is substituted with a serine. Azurocidin was recently cloned and found to have 44% homology with neutrophil elastase, 3 N-linked glycosylation sites and 8 intramolecular cysteines (108, 109). It has a molecular mass which ranges from 29 to 37 kDa due to differential glycosylation and is quite basic. In addition to its potent bactericidal effects on gram negative bacteria it is also chemotactic for monocytes (110). Despite its lack of serine protease activity, azurocidin has an active site with 65% homology with that of elastase. Azurocidin, Myeloblastin and Elastase are organized as a single locus on the terminus of the short arm of chromosome 19, revealing the fact that the three probably evolved from the same gene (111). Cathepsin G is a differentially glycosylated chymotrypsin-like serine protease with toxic effects on gram positive and gram negative bacteria as well as fungi. Interestingly, enzymatically inactive cathepsin G is known to retain these bactericidal effects (100).

The defensins are a family of basic peptides of 29-43 kDa. These molecules mediate their effect by inducing membrane permeabilization. They have bactericidal effects on a wide range of gram positive and gram negative bacteria, many fungi and are capable of inactivating several enveloped viruses (112).

Lysozyme is found in both the azurophilic and specific granules. Originally described by Flemming in 1922 it is effective in killing gram positive bacteria. It is a glycolytic enzyme and has a molecular mass of 15 kDa.

The peroxidase negative granules which include the specific and gelatinase-containing granules as well as the secretory vesicles, contain various receptors. These granules fuse more readily with the plasma membrane than the other granules and as they contain various receptors are probably involved in priming of the neutrophil. The only major bactericidal protein in these granules is lactoferrin which is found in the specific granules.

Lactoferrin is a 78 kDa glycoprotein that belongs to the transferrin family of iron binding proteins. It is only found within specific granules and it has mainly bacteriostatic rather than bactericidal activity. This molecule is also known to have inhibitory activity on hematopoiesis (86).

The contents of neutrophilic granules could prove to be important in the regulation of hematopoiesis. As mentioned, the neutrophil is the most abundant white blood cell. Upon activation, the contents of its granules may be excreted into the extracellular space. The potential effect of these factors should not be underestimated.

1.5 Leukemia

i) General classification and characteristics

The clinical features and classification of leukemias, are presented in this section. As this thesis investigates the negative regulatory mechanisms in normal and leukemic myelopoiesis, a focus will be placed on the description of myeloid leukemias.

Leukemias are blood-borne malignancies which are believed to arise from a primary neoplastic lesion in a cell with a capacity for self renewal. These cells have a heritable inability to regulate the processes of proliferation, terminal differentiation and/or programmed cell death. The type of lesion and the stage of development at which the malignant transformation occurs accounts for the phenotypes of the different leukemias.

The initial classification of leukemias is dependent upon the aggressiveness of each disease's onset. In acute leukemias there is a rapid proliferation of abnormal blast cells leading to early mortality if left untreated. By contrast, chronic leukemias have a slow but insidious onset, allowing people suffering from these diseases to survive for up to 5 years when left untreated. Leukemias are further classified on the basis of their lineage commitment, myeloid, lymphoid or mixed. As a result there are four main classifications for leukemia; acute myeloid or lymphoid leukemia (AML and ALL respectively) where there is an accumulation of either myeloid or lymphoid blast cells and chronic myeloid or lymphoid leukemia (CML and CLL respectively) where there is an accumulation of a more differentiated myeloid or lymphoid cell.

The incidence of leukemias is approximately 10 cases per year per 100 000 people; of these, half are acute and half are chronic. Amongst the acute leukemias approximately half are AML and half ALL. By contrast CLL is four times more prevalent than CML. Acute leukemias are the most common malignancy found in children accounting for one

half to one third of all pediatric cancers. In adults, leukemias constitute only 4% of all malignancies. AML comprises 20% of childhood leukemias but is more common in the adult population increasing in incidence with age. ALL can occur at any age but has a maximum incidence in children between the ages of 2 and 6 (113). Like ALL, CML can be seen at any age but has a median age of incidence of 50 years (114). CLL is rarely seen in individuals below the age of 40.

ii) AML

Acute myeloid leukemia (AML) represents a class of leukemia in which there is an expansion of a myeloid progenitor at a certain stage of maturity. The French American and British (FAB) classification system differentiates the type of AML based on the progenitor cell affected. FAB-M1 is AML without maturation, M2 with some maturation. FAB-M3, is an acute promyelocytic leukemia, M4 an acute myelomonocytic leukemia and M5 an acute monocytic leukemia. FAB-M6 is an acute erythroleukemia, and finally M7 is an acute megakaryoblastic leukemia. Many genetic lesions associated with AML have been discovered (Table 1) and their possible role in the development of the disorders is now being elucidated (115).

Typical lesions include translocations (t), inversions (inv) and deletions (del). In the descriptions of these lesions, information is given on the chromosomes affected as well as the areas on these chromosomes that are involved (eg. t(9;11)(p22;q23)) is a translocation between the short arm (p) of chromosome 9 at position 22 to the long arm (q) of chromosome 11 at position 23). These lesions may mediate their effect by causing the loss of function of a certain gene or due to altered expression or activity of a given product.

Table 1 Genetic lesions associated with AML

Genetic Lesion	AML Subtype(s)	Genes Involved
t(8;21)(q22;q22)	M1-M2-M4	AML1/ETO
inv(16)(p13q22) t(16;16)(p13;q22)	M4-M4Eo	CBFβ/MYH11
t(15;17)(q21;q11)	M3	PML/RARα
t(11;17)(q23;q11)	M3-like	PLZF/RARα
del 11(q23) t(9;11)(p22;q23) t(11;19)(q23;p13)	M4-M5	MLL MLL/AF9 MLL/ENL
inv(3)(q21;q23) t(3;3)(q21;q26)	M1	EVI1
t(6;9)(q21;q26)	AML	DEK/CAN

In virtually all M3 AML, there is a translocation from the PML gene on chromosome 15 to the retinoic acid receptor α (RARα) gene on chromosome 17 (116). Both of these genes encode DNA binding transcription factors. This translocation results in arrested differentiation and accumulation of the promyelocyte. In most cases, treatment of patients with this disease with all-trans retinoic acid results in complete clinical remission. The other genetic lesions have yet to yield such direct connections with useful therapies. They are however interesting as they provide information of the control mechanisms which are important in normal hematopoiesis and may prove to be a better way of classifying patients for therapeutic response and prognosis.

iii) CML

CML was the first disease to be associated with a chromosomal abnormality. More than 95% of patients presenting with this disease have the Philadelphia chromosome which results from the translocation of a truncated Abl gene from chromosome 9 to the break-

point cluster region (Bcr) of chromosome 22. The resulting fusion may incorporate sequences from the first one, two or three exons of Bcr. If only the first exon is included, a 190 kDa fusion product is formed. This 190 kDa fusion product is found in 5% of childhood and 20% of adults afflicted with ALL. If the second or third Bcr exons are incorporated, a 210 kDa protein is formed ($p210^{Bcr\text{-}Abl}$) (117). It is this protein which is associated with the development of CML. In CML, the $p210^{Bcr\text{-}Abl}$ fusion product, a protein with enhanced tyrosine kinase activity, is believed to play a major role in the onset of the disease. The Philadelphia chromosome has been found in all hematopoietic lineages in patients with this malignancy establishing the fact that CML is a stem cell disease.

The chronic phase in CML is characterized by the expansion of the entire granulocyte series and an accumulation of a relatively normal neutrophil. A differential leukocyte count shows an increased myelocyte and neutrophil count with lesser numbers of metamyelocytes (113). This phase is thought of as being a relatively benign "preleukemia" which responds well to treatment and lasts from 2 to 5 years. The disease then progresses to an accelerated phase lasting 6 to 18 months, where there is increasing resistance to chemotherapy, proliferation, and maturation arrest. The final development of CML is blastic transformation where two-thirds of cases evolve into an AML and one-third evolve into an acute lymphocytic leukaemia (ALL). The conversion to the accelerated and blastic phases is accompanied by further genetic changes. The three phases of CML supports the idea that leukemias arise as a result of a multi-step process involving the accumulation of several different genetic lesions (118).

The proto-oncogene c-Abl is the cellular homologue of the transforming v-Abl gene of the Abelson murine leukemia virus. It's product is a member of the src family of protein tyrosine kinase and is localized in the nucleus. Although the role of Abl is unclear, there is some evidence to suggest that it is involved in control of the cell cycle (119). It's carboxyl terminal contains two distinct regions which can bind F-actin (120) and DNA (121) respectively. During metaphase, c-Abl hyperphosphorylation results in the loss of its ability

to bind DNA. Recently, c-Abl has been found to complex with the pRb protein *in vitro* (122, 123). The c-Abl gene was linked to myelopoiesis by experiments involving antisense treatment of bone marrow CD34+ with anti-c-Abl oligodeoxynucleotides. This treatment prevented the entry of these cells into S phase and inhibited their differentiation into CFU-GM (124).

Juxtaposition of the Bcr gene to the truncated Abl gene in the Philadelphia chromosome results in an increase in the intrinsic tyrosine kinase activity of Abl. When transfected into cells, Bcr-Abl causes cellular transformation, growth factor receptor independent cell growth and can induce a CML-like illness in mice when introduced into their bone marrow (125).

As mentioned previously, when RTK's are stimulated by their ligand, they dimerize and phosphorylate themselves and/or each other. Intracellular signaling molecules with an SH2 domain can bind to the cytoplasmic tail of the receptor at phosphorylated tyrosines, thereby launching a cascade of signalling events. When cells are stimulated with SCF, it is believed that two SH2 containing molecules , Shc and Grb2, associate with its receptor. Upon phosphorylation, it is believed that Shc associates with Grb2 which associates with another protein Sos which is a guanine nucleotide exchange factor. This complex can then promote the conversion of an inactive, GDP bound Ras to an active, GTP bound Ras. Active Ras can then target the Raf kinase to the cell membrane allowing for a cascade of signaling events to be sent through molecules such as Mek and MAP kinase. This ultimately results in continued proliferation (126).

Mutations resulting in constitutive activation of Ras appear to be a common feature of all myeloid malignancies with the exception of Bcr-Abl positive CML. One of the ways in which Bcr-Abl is believed to mediate its transforming effect is by mimicking the effects of activated, phosphorylated receptors such as that for SCF (127). As it can autophosphorylate, it is believed that Bcr-Abl can bind to Shc and Grb2 constitutively thereby continuously activating the Ras pathway through Sos. As a result, no Ras

mutations are necessary for transformation as Ras is constitutively active. In support of this data, recent results have confirmed the genetic requirement for Ras in the transformation of fibroblasts and hemopoietic cells with Bcr-Abl (128).

Oncogenic Abl mutants are also known to transform by Ras independent pathways. These include through association with JAK/STAT signalling (129) and also by interacting with the cell cycle molecules cyclin D/pRb (123, 130).

It has been widely accepted that the selective outgrowth of the leukemic clone in CML is not due to a faster progression through the cell cycle (131). Therefore, these leukemias produce an environment which is inhibitory to normal myelopoiesis while enhancing the growth of the leukemic clone. Despite the present understanding of the positive and negative regulators of hematopoiesis, their role in the onset and progression of myeloid leukemias is still poorly understood.

iv) Treatment of CML

CML was first treated in 1856 using arsenicals. From the 1900's to the 1950's radiation therapy was the treatment of choice. In the 1950's the oral alkylating agents began being used. The most successful alkylating agent initially used to treat CML was a drug called busulfan. Busulfan has the advantage of being inexpensive, but it has pulmonary toxicity and, as it affects primitive bone marrow progenitors, the leukocyte counts take 10-14 days to drop and the drug's effects persist for a similar period after being discontinued. Hydroxyurea, a drug developed in the 1960's, although more costly, was found to have more rapid control of leukocyte counts and no pulmonary toxicity. To date, for many hematologists, these are the treatments of choice. They are effective in controlling the signs and symptoms in greater than 90% of the patients treated at diagnosis (132). However,

these patients develop some resistance to treatment and invariably progress into an accelerated and eventually a blast phase.

Two relatively new discoveries have revolutionized the treatment of CML. Once thought to be an incurable disease, it is now possible to cure two thirds of the patients undergoing allogeneic bone marrow transplantation (BMT) (133). However, there is a 30% mortality rate attributed to patients undergoing this treatment due for the most part to graft-versus host disease (GVHD), a condition where the donor immune cells see the recipient tissues as foreign and mount an immune response against them. In addition, approximately 70% of patients are not eligible for allogeneic BMT due to age or lack of an HLA matched donor. In some of these patients, treatment with IFN-α has been shown to induce a complete cytogenic response. In a fraction of patients, these responses have endured and correlated with increased survival (132).

As some patients do not respond to IFN-α treatment, and with those that do there is still the presence of residual disease, alternative treatments need to be explored. As it is well established that a pool of normal ($p210^{Bcr-Abl}$ negative) cells exist in patients suffering from CML, one of the areas believed to hold some promise for a future treatment is autologous BMT. This procedure has the advantages of not needing a matched donor and that GVHD is not a problem. However the obvious problem with autologous BMT is the need to eradicate all or enough of the leukemic cells in the harvested marrow to get a prolonged response. Some of the techniques that have been used to purge autologous marrow of leukemic cells include Bcr-Abl directed therapies (eg. anti-sense oligonucleotides, ribozymes, inhibitors of the enzymatic activity), *ex vivo* expansion/selection of the residual normal cells, cytotoxic therapies (photodynamic therapy, IFN-α, Mafosfamide/4HC) and the use of molecules called chemoprotectants (MIP-1α and the peptide inhibitors) which selectively and reversibly inhibit normal cell proliferation while allowing leukemic cells to

continue their proliferation thereby maintaining their susceptibility to the cycle specific purging agents.

Despite these limited successes, therapy for CML is very much in its infancy. There still remains a vast majority of patients who lack effective treatment for their condition. Hopefully it will one day be possible to "cure" the remaining 80% of patients who either fail to respond to allogeneic BMT or are not suitable for this treatment.

1.6 Background and Summary of this Research

i) Common Antigen of Myeloid Acute Leukemia (CAMAL)

In the late 1980's, work performed in this laboratory identified an activity in peripheral blood lysates from patients with CML which had inhibitory activity on myelopoiesis. This work was performed with immunoaffinity enriched material using an antibody thought to be leukemia specific. Originally, leukemia specific antibodies were isolated from the serum of rabbits co-injected with AML peripheral blood leukocyte (PBL) lysates and antiserum previously raised against normal PBL lysates. As the anti-normal PBL sera masked the normal epitopes, antibodies unique to leukemic determinants from the AML sample were raised preferentially. The resulting anti-sera was then passed repeatedly over a column made with a normal PBL lysate so as to remove antibodies reacting to normal determinants. The fall-through from this column was then tested for activity against PBL lysates from normal donors and patients with either AML or CLL in an enzyme-linked immunosorbant assay (ELISA). The sera was found to react only against the AML PBL lysates indicating that AML specific antigens might exist and that this antiserum might react against these epitopes (134).

In order to increase the specificity of the system, another anti-serum was raised against a better defined AML antigen (135). Normal and AML PBL lysates were applied separately to an immunoaffinity column made with antibodies against normal PBL lysates. The resulting fall throughs were separated by SDS-PAGE and components of the gels specific to the AML PBL lysate were eluted from the gel and tested for reactivity using the original anti-AML serum. The most immunoreactive component was used to immunize subsequent rabbits. In fluorescence activated cell sorting (FACS) studies, anti-serum from these animals proved to react preferentially against BM and PBL samples from patients suffering from both AML and CML when compared with comparable samples from normal donors and patients suffering from lymphoid disorders (135, 136). This data indicated that the second anti-serum was directed to an antigen only common to myeloid leukemias.

More AML-specific antigens were purified using this technique and material eluted from the gels was used to make monoclonal antibodies. As this was a common antigen of myeloid acute leukemias (CAMAL), the resulting monoclonal antibody (mAb) was named CAMAL-1 (137). FACS analysis showed that the CAMAL-1 mAb had the same specificity for myeloid leukemia cells as the rabbit anti-serum but the fluorescence detected in the analysis was less intense (137).

In an attempt to determine the sub-cellular localization of the CAMAL antigen the immunoperoxidase slide test was used to investigate rabbit anti-CAMAL and CAMAL-1 staining of BM and PBL from patients with myeloid and lymphoid leukemias as well as comparable cells from normal donors (138). As expected immunoperoxidase staining with both sources of antibody was specific to myeloid leukemia cells. Interestingly, staining with CAMAL-1 was localized to the cytoplasm and that of rabbit anti-CAMAL to the plasma membrane. In addition a lower percentage of the myeloid leukemia cells were detected by CAMAL-1 indicating that possibly other myeloid leukemia antigens exist. This would explain the weak fluorescence staining with CAMAL-1 in the FACS analysis as compared to rabbit anti-CAMAL. For PBL samples from patients with CML in chronic

phase, 7/7 samples were CAMAL-1 positive with a mean percentage of positive cells of 19.3 ± 2.6 and a range of 6.5 to 25%.

As CAMAL-1 staining was restricted to myeloid leukemia cells and because the amount of staining was variable, an extensive double blind study was undertaken to determine whether there was a relationship between CAMAL-1 staining and the stage of disease in AML. Interestingly, this correlation proved to be correct. Patients in remission and those having undergone BMT, whose CAMAL-1 values remained low or decreased were found to be less likely than those with higher or increasing values, to relapse (139). A three year follow-up study on the CAMAL-1 values of AML patients undergoing chemotherapy showed that the lower CAMAL-1 value group, post-chemotherapy, had a much greater disease free survival (140). Taken together, this data suggests that CAMAL-1 reactivity correlates with prognosis and that its antigen could play a role in the progression of the disease.

As the presence of the CAMAL-1 antigen correlated with prognosis in myeloid leukemias, studies were initiated to investigate the effect of the CAMAL-1 antigen on hematopoiesis. CML PBL lysates were passed over immunoaffinity columns made with the CAMAL-1 antibody. After extensive washing, material eluting off of the column was run on an SDS-PAGE gel and tested for activity on normal and myeloid leukemia cells in a standard CFU-GM colony assay (141). SDS-PAGE analysis of the immunoaffinity enriched material revealed two populations of protein, one at approximately 65 kDa and the other at 30 kDa. Western blot analysis showed that the rabbit anti-CAMAL antibody only recognized the 65 kDa population (142). The colony assay revealed that this crude material, had no or in some cases a stimulatory effect on leukemic colony formation and had an inhibitory effect on normal CFU-GM. It was hypothesized that this selective inhibitory activity on normal hematopoiesis might give the leukemic clone a growth advantage over its normal counterparts.

Further characterization of the activity of the crude immunoaffinity enriched material indicated that it had inhibitory activity on murine colony formation and inhibited the ability of donor, murine bone marrow cells to form CFU-S in irradiated recipients (143). In addition to the inhibitory activity on normal human and murine progenitor cells, this material was also found to stimulate leukemic colony formation (144).

Preliminary attempts to biochemically characterize the molecule(s) responsible for the activity have shown that the activity lies within the population of proteins at approximately 30 to 35 kDa. N-terminal amino acid sequencing of this population revealed a sequence highly homologous with serine proteases (145). Subsequently, it was shown that serine protease inhibitors were able to block the inhibitory activity of the crude preparations (146). As this population was not recognized by the rabbit anti-CAMAL antibody, it is possible that the antigen and biological activity are mediated by distinct proteins.

The first part of the research reported here carries on from these initial observations. The specific objectives and results are summarized in the last section of this introduction.

ii) Boyum's GRE

In 1976, Böyum and colleagues published a study identifying an inhibitor derived from the granulocytes of normal donors and individuals with CML. In these experiments, they used diffusion chambers implanted within the peritoneum of mice and injected murine bone marrow alone or in the presence of normal or leukemic granulocytes. After seven days they assayed the resulting cell mixture for cell number and colony forming ability. They found that both normal, and to a lesser extent, leukemic granulocytes had an inhibitory activity on granulopoiesis (147). Further characterization of this activity revealed that it could be found in granulocyte conditioned medium or granulocyte lysates and was

given the name granulocyte extract (148). In addition, in this study it was found to be serum dependent and its effects could be reversed by washing the cells up to five hours after incubation with the cells.

Subsequent work has shown that dependent upon the culture conditions, crude granulocyte extracts can mediate inhibitory or enhancing effects (149) and thymidine was shown to be critical for the granulocyte derived inhibitory activity (150).

The second part of the thesis research carries on from these initial observations. The specific objectives and results are summarized in the next section.

iii) Summary of the research objectives and results of this thesis

The objectives of this thesis were to purify and identify molecules with inhibitory activity on myelopoiesis and to determine what role if any they play in the development of CML. The initial activity that was chosen for analysis was shown to have a specific inhibitory effect on normal but not CML myelopoiesis. This activity, known as CAMAL was derived from immunoaffinity preparations from PBL lysates of patients suffering from CML.

Chapter 3 describes the use of reverse phase HPLC (rpHPLC) in purifying the molecule responsible for the selective inhibitory activity. Furthermore data are presented, through rpHPLC and two-dimensional electrophoresis, that the molecule responsible for this inhibitory activity may be a glycoform of the normal neutrophil protein, azurocidin.

As this protein was a normal neutrophil protein, the possibility that it was a normal regulator of myelopoiesis was investigated (chapter 4). In this chapter the purification of azurocidin from normal neutrophils is described. Only one normal azurocidin preparation out of six was found to have inhibitory activity on normal myelopoiesis. It was therefore postulated that it was a particular glycoform of azurocidin which mediated the inhibitory

activity and that this form of the molecule was more prevalent in the neutrophils of patients with CML.

Due to the difficulties in working with a molecule requiring proper glycosylation for activity, a decision was made to focus our efforts on the characterization and purification of another crude extract known to inhibit myelopoiesis. Using Böyum and colleagues' initial observations, we confirmed the presence of a myelopoietic inhibitory activity on normal progenitor cells in normal and leukemic (CML) neutrophils. Chapter 5 involves the characterization and localization of the molecule(s) responsible for these inhibitory activities on normal and leukemic myelopoiesis. Evidence in this chapter suggests that normally derived material had an equal effect on normal and leukemic (CML) myelopoiesis. Localization studies showed that in normal neutrophils the inhibitory activity was localized to the specific granule and cytosolic fractions. By contrast, in leukemic neutrophils, the azurophilic granule fraction also contained inhibitory activity. Evidence was also provided that the inhibitory activity of the normal cytosolic material was acting on a fairly committed progenitor cell population.

In chapter 6 the purification of the protein responsible for the inhibitory activity of the normal cytosolic material is presented. Using Western blot analysis and the use of recombinant material, it was possible to identify cytidine deaminase (CD) as the molecule responsible for the inhibitory activity. Preliminary experiments presented in this chapter also suggest that it may be acting by arresting cells in the S phase of the cell cycle.

Therefore, in this thesis two molecules with putative inhibitory activity on normal myelopoiesis have been purified and identified. One activity, that believed to be due to specific glycoforms of azurocidin, is found almost exclusively in leukemic cells and seems to have a selective inhibitory activity on normal myelopoietic progenitors. The other, that due to CD, is found in both normal and leukemic cells and seems to have equivalent effects on both normal and leukemic progenitors.

CHAPTER 2 - MATERIALS AND METHODS

2.1 Antibodies and Immunoaffinity Enrichment

i) Antigen production

The antigen preparations used in the production of the CAMAL-1 and K18 mAb's have been described in detail elsewhere (145). Briefly, AML PBL membrane extracts adsorbed of all of their normal PBL membrane components were separated by non-denaturing SDS-PAGE. Protein bands reacting with a rabbit anti-AML antibody in an ELISA were pooled brought to 50% with Freund's adjuvent and injected sub-cutaneously into 2.5 kg New Zealand White female rabbits. Polyclonal sera was obtained after clotting blood from animals injected 4-6 times with antigen.

ii) Antibody production

The CAMAL-1 and K18 mAb, both of the IgG_1 subclass, were derived from fusions of NS-1 myeloma cells with Balb/c splenocytes from mice immunized with the appropriate antigen using the method of Oi and Herzenberg. Cell fusion and cloning were performed by Ms. Herma Neyndorff at the facilities of QLT PhotoTherapeutics. The CAMAL-1 mAb was raised against antigen prepared as outlined in the previous section. Material eluted from CAMAL-1 immunoaffinity columns ranged in size from 16 to 68 kDa. The K18 mAb was raised to 30 to 35 kDa material eluted off the CAMAL-1 column which was found to be responsible for the inhibitory activity on normal progenitor cells *in vitro* (151).

Hybridomas were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% FCS. Cells were cryopreserved at $3x10^6$ cells/ml in 90% FCS and 10% DMSO (Sigma, Tissue culture grade). 1 to 1.5 ml of this mixture was added to cryovials (Nunc) and this was left overnight at -70°C before being submerged in liquid nitrogen.

Balb/c female mice (Charles River) between the ages of 6 weeks and 1 year were primed with an intraperitoneal (ip) injection of 500 µl of pristane (2,6,10,14-tetramethylpentadecane, Aldrich). After 5-14 days, ascites was prepared by injecting 2-5 $x10^6$ of thawed hybridoma cells ip. 7-21 days after hybridoma injection, ascites was obtained by draining the distended peritoneum using a 16 gauge needle. This procedure was performed a maximum of 3 times before euthanization. Upon collection, the ascites was centrifuged for 10 minutes at 5000 G in a silencer in order to remove fibrin clots.

The rabbit anti-CD antibody was provided by Dr. Momparler (Hopitale St. Justine, Montreal Canada). It was raised to a peptide corresponding to the last 15 amino acids of the C-terminus of cytidine deaminase.

iii) Antibody purification

Antibody purifications were initially performed using hydroxylapatite (HA) chromatography. Columns were prepared with 5 ml of hydrated HA was prepared for every ml of ascites. Ascites was diluted 1:1 with HA binding buffer (0.01 M sodium phosphate, ph 6.8) and applied to the HA column equilibrated with binding buffer at a flow rate of 0.5 ml/min. The column was then washed with binding buffer until absorbance at 280 nm (A_{280}) was $\leq$ 0.05. Non-Ig protein was eluted from the column with 0.12 M sodium phosphate pH 6.8 until again A_{280} was $\leq$ 0.05. Ig was then eluted from the column with 0.3 M sodium phosphate pH 6.8. One large peak resulted, this was concentrated and its purity and activity determined by SDS-PAGE and ELISA respectively.

Subsequent purifications were performed using either the Protein A MAPS II kit (Bior-Rad) or a goat anti-mouse Ig column (Hyclone). For the MAPS II kit, ascites was diluted 1:1 with binding buffer and applied to the column equilibrated with 5 volumes of binding buffer. The column was then washed with 15 volumes of binding buffer and Ig eluted with 5 ml of elution buffer into a tube containing 320 µl of 1M Tris HCl pH 9 per ml of elution buffer. For the Hyclone kit, ascites was diluted 1:2 in PBS and passed over a goat anti-mouse Ig column at 0.5 ml/min. The column was washed until A_{280} was ≤ 0.05 and then Ig was eluted using 0.1 M HCl. The eluate was once again neutralized with 1 M Tris HCl pH 9. For both kits, the eluates were concentrated and analyzed by SDS-PAGE and ELISA.

iv) Immunoaffinity columns

Affi-Gel 10 activated affinity agarose (Bio-Rad) was used as the support for the immunoaffinity columns. The beads were prepared as per the manufacturer's instructions. Briefly, the Affi-Gel 10 beads were washed with ddH_2O at 4°C and the pelleted moist gel cake transferred to a 15 ml polypropylene tube. Purified antibody at 10 mg/ml was dialyzed against 0.1 M HEPES (Sigma) pH 6.5 and added to the moist gel cake at a volumetric ratio of 1:1. This mixture was rocked gently for 4 hours at 4°C. A_{280} readings were taken before and after incubation in order to insure that coupling was greater than 80%. The beads had the supernatant removed and any active esters remaining were blocked by resuspension in 1 M ethanolamine HCl pH 8 for 1 h at 4°C. This suspension was then transferred to a column and washed with 5 volumes of PBS. When not in use the columns were stored at 4°C in PBS with 0.2% sodium azide.

v) Leukemia cell lysis and enrichment

Cells used for protein purification in this study were obtained from cryopreserved apheresis samples from untreated patients newly diagnosed (within one month) with CML at the Hammersmith hospital, London, England. Briefly, these cells were thawed and mixed with one tenth the volume of 10 X lysis buffer (200 mM Tris-HCl, pH 7.5; 1.5 M NaCl, 10.0 mM ethylenediaminetetracetic acid (EDTA), 10.0% Triton X-100, and 5% NaDOC(sodium deoxycholate)) containing 2.0 KU/ml of pancreatic DNAse I (Sigma Chemical Company, St. Louis, MO). This mixture was stirred at room temperature for 1-2 h and then at 4°C overnight (all subsequent immunopurification steps were performed at 4°C). This cell lysate was then centrifuged at 15000 rpm for 30 min in a Sorvall SS34 rotor in order to remove insoluble material. The resulting supernatant was then passed over a K18 immunoaffinity column at a flow rate of 0.5-0.8 ml/min. The column was then washed with a 100 x bed volume of PBS, and the bound protein eluted with a 2.5 x bed volume of 0.1 M HCl. The eluate was then immediately neutralized with 1.5 M tris-HCl, pH 7.5 before being concentrated by centrifugation in a Centricon-10 microconcentrator (Amicon Corp., Danvers, MA) having a molecular mass cut-off of 10 kDa according to the manufacturer's instructions.

2.2 Chromatography

i) Reverse phase HPLC

Concentrated, immunoaffinity enriched leukemic cell extracts were filtered through a 0.22 mm filter unit, had a tenth volume of 1% TFA (Tri-fluoro acetic acid, Pierce, Rockford, IL) added to them, and were then applied to a wide pore (250 x 4 mm) C4 reverse phase column (Vydac, The Separations Group, Mojave, CA) that had been pre-equilibrated in 0.1% TFA. Proteins were eluted at 1 ml/min using an acetonitrile gradient of 2-95% over 75 minutes

(see figure legends for details) and monitored for absorbance at 210 nm. Fractions were collected every 30 seconds (or as indicated in the figure legend), dried in a Speed-vac concentrator (Savant Instruments, Farmingdale, NY) and washed once with 100 µl of 0.1% glacial acetic acid before being dried again. Bovine serum albumin (BSA, Boehringer Mannheim, Mannheim, Germany), at a final concentration of 0.02% was added to samples used in biological assays before being dried in order to prevent denaturation. Protein concentrations were determined by comparing their integrated peak areas obtained to those obtained from a known quantity of the control proteins, Cathepsin G and Elastase (Calbiochem, La Jolla, CA).

ii) Size exclusion chromatography

All size exclusion chromatography was performed using a Superose 12 column (Pharmacia). Sample in a volume of 200 µl was applied to the column. The choice of buffer was determined by the next chromatographic step in order to exchange buffers. The column was run for 60 minutes at 0.5 ml/min. Unless otherwise indicated, 2.0 ml fractions (4 minutes) were collected. During long periods of inactivity the column was stored in 0.02% sodium azide (Fisher) as a preservative.

iii) Ion exchange chromatography

Two types of ion exchange columns were used for protein purification, DE52 (Whatman) and MonoQ (Pharmacia). All separations were run on a Pharmacia FPLC at 4°C.

For DE52 separations, the loading and washing buffer (Buffer A) consisted of 20 mM NaCl, 20 mM Tris Acetate pH 7.6, 0.1 mM EDTA, 1 mM DTT. Samples in relaxation buffer

(see nitrogen cavitation on page 52) were diluted 5 fold in Buffer A minus NaCl and loaded onto a DE52 column pre-equilabrated with Buffer A at a flow rate of 0.4 ml/min. The column was then washed with 20 ml of Buffer A. Bound protein was eluted with a linear gradient to 100% Buffer B (identical to Buffer A except with 500 mM NaCl) over 100 ml. 3 ml fractions were collected, concentrated in a 10 kDa cut-off concentrator unit (Millipore) and assayed for biological activity on NMBM by colony assay (see hematopoietic progenitor cells assays, page 55). In order to prevent bacterial growth during periods of inactivity, the column was stored in 0.2% Benzalkonium Chloride (Sigma).

For MonoQ separations, Buffer A consisted of 20 mM Tris pH 7.5, 20 mM KCl, 2.5 mM DTT. Samples derived from Superose 12 separations performed with MonoQ Buffer A were injected into a 10 ml Superloop (Pharmacia) which was used to load the sample onto the column. After the sample was loaded the column was washed with 20 ml of Buffer A. Bound protein was eluted with a linear gradient to 100 % MonoQ Buffer B (identical to Buffer A except with 500 mM KCl) over 25 ml. 2 ml fractions were collected and concentrated as above before further analyses. 20% ethanol was used as a column preservative.

2.3 Protein Analyses

i) Quantitation of protein by the BCA method

BSA standards were prepared in the 10 to 100 µg/ml range. 5 µl of the appropriate concentration of standard was plated in triplicate into 1/2 area 96 well plates (Costar) which had all of the wells in the periphery filled with 100 µl of ddH_2O. 5 µl of diluted samples was added to wells in triplicate. BCA solution (Sigma) was then added to 4% $CuSO_4 \bullet 5H_2O$ (Sigma) in the ratio of 50:1 and 100 µl of this solution was added to each well. The covers were then

sealed to the plates using parafilm and the plates incubated for 12-16 hours at 37°C. Plates were removed from the incubator and allowed to come to room temperature and then read at 545 nm in a Molecular Dynamics plate reader. A standard curve was plotted and an equation for the resulting line was determined. The concentration of protein present within the sample wells were calculated using the equation for the standard curve. Dilutions of the same sample found to be in the linear area of the standard curve were averaged and used.

ii) One dimension SDS PAGE

One dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 1.0 mm thick slab gels using the discontinuous buffer system originally described by Laemmli (152). Briefly, the separating gel solution consisting of 375 mM Tris, pH 8.8, 0.05 % APS, 0.05% TEMED and the appropriate concentration of 30% acrylamide/0.8% PDA (see figure legend for the final acrylamide concentrations of gels in question) was poured between the plates, overlayed with ddH_2O saturated iso-butanol and allowed to polymerize. The butanol solution was then removed and a stacking gel comprising of 125 mM Tris pH 6.8, 0.05 % APS, 0.1% TEMED and 4% acrylamide/PDA was poured on top of the separating gel, a sample comb inserted before polymerization. Samples or molecular weight standards (low molecular weight silver stain standards, Bio-Rad) were diluted in sample buffer (2% SDS, 10% glycerol, 125 mM Tris pH 6.8, 0.01% bromophenol blue) with or without 10 mM DTT (indicated in the figure legend) and loaded into rinsed sample wells. Gels were then electrophoresed in running buffer (25 mM Tris pH 8.3, 192 mM glycine and 0.1% SDS) at 20 mA constant current per gel through the stack and 30 mA constant current per gel through the separating gel until the indicator dye, bromophenol blue, was within 1 cm from the bottom of the gel. Unless indicated otherwise, all gel reagents were obtained from Bio-Rad.

iii) Two dimensional NEPHGE

Two dimensional non-equilibrium pH gradient electrophoresis (NEPHGE) SDS-PAGE was performed using the Bio-Rad mini-Protean II 2-D gel system by the method first described by O'Farrell (153) in the first dimension. Briefly the gel solution (2.75 g Urea (Millipore), 650 µl 30% acrylamide/0.8% PDA, 2% CHAPS (Sigma), 360 µl ampholines (pH 3-10 ampholines, Pharmacia) per 5 ml) was incubated at 37°C to allow the urea to dissolve. This solution was then filtered and de-gassed for 15 minutes. 5 µl of 10% APS and 3.5 µl of TEMED was then added and the solution drawn into capillary tubes before being allowed to polymerize for 90 minutes. The top of the gels were then rinsed and then had 50 µl of overlay buffer (4.8 g urea, 0.3 g CHAPS, 500 µl ampholines, 0.1543 g DTT per 10 ml) added to them. Samples were then reconstituted in sample buffer (same as overlay buffer except 5.89 g urea) and layered on top of the gel. De-gassed anolyte 20 mM NaOH was then poured into the electrophoresis tank. The electrophoresis unit containing the capillary gels was then placed into the tank and de-gassed catholyte (10 mM H_3PO_4) poured into the unit such that the anolyte and catholyte never mix. The gels were then run at 500 V for 10 minutes and 750 V for 98 minutes at constant voltage. Gels were then extruded directly into a long well in a one dimensional SDS-PAGE gel and run as described in the previous section.

iv) Silver staining of polyacrylamide gels

After completion of electrophoresis gels to be silver stained were fixed in 10% acetic acid/50% methanol for at least 30 minutes. They were then microwaved for 1 minute in a solution of 10% acetic acid/10% methanol. After a 5 minute incubation with ddH_2O the gels were microwaved for 1 minute in $5X10^{-5}$ M DTT. The gels were then left in 0.1% $AgNO_3$

(Sigma) for 15 minutes. After a 30 second rinse with ddH_2O the gels were developed with 3% Na_2CO_3/0.037% formaldehyde (BDH) until the desired contrast was obtained. The reaction was stopped by bringing the solution to 115 mM citric acid.

v) Western blot analysis

Western blot analyses were performed using the Bio-Rad transblot apparatus and the method first described by Towbin . Briefly, after SDS-PAGE, the gel, as well as 2 sheets of 3 MM paper (Whatman) and 1 sheet of transfer membrane (either nitrocellulose (Schleicher & Schull) or Immobilon-P (PVDF, Millipore)) cut to the size of the gel, were equilibrated in 4°C transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). The gel was then placed on one of the 3 MM sheets, the transfer membrane layered on top and the other 3 MM sheet on top of this. This arrangement was mounted into the transfer unit such that the gel was closest to the negative pole and the transfer membrane closest to the positive pole. The electrophoretic tank was then filled with transfer buffer, the cooling unit inserted and the blot was performed at 100 V (constant voltage) with constant stirring.

Upon transfer, non-specific antibody binding to the membrane was prevented by blocking >2 h with blocking buffer 4% milk powder in tris-buffered saline with 0.1% Tween 20(TBS-T, 8 g NaCl, 0.2g KCl, 3 g Tris pH 7.4 per litre). The blot was then incubated for 2 h with the primary antibody (1:1250 dilution for rabbit anti-cytidine deaminase antibody) in 0.5% milk in TBS-T. The blot was then rinsed once briefly, once for 15 min and twice for 5 min with TBS-T. The membrane was then incubated with Protein-A-horseradish peroxidase (Amersham) for 45 min. The blot was then rinsed as before and placed in the ECL reagent (Amersham) for 1 minute and the blots were used to expose X-ray film. Typical exposures ranged between 30 seconds to 10 minutes.

2.4 Granulocyte Subfractionation

i) Granulocyte harvest and nitrogen cavitation

Granulocyte subfractionation was performed by the method first described by Borregaard and colleagues (154). Briefly, 450-500 ml of blood (one unit) was collected from normal healthy individuals into 600 ml blood pack containing citrate phosphate dextrose (1.66 g sodium citrate dihydrate, 1.61 g dextrose monohydrate, 188 mg citric acid, 140 mg monobasic sodium phosphate monohydrate) as an anticoagulant (Baxter). Blood was then mixed 1:1 with a 2% Dextran T-500 solution in PBS and the red cells allowed to settle for 1 hour at room temperature. The white blood cell rich supernatant was collected into 250 ml polypropylene tubes (Corning) and spun at 200 g for 12 minutes at 4°C. The cell pellet was washed in cold PBS and the cells resuspended in 30 ml of PBS in a 50 ml polypropylene tube (Falcon). 20 ml of Ficoll Hypaque (Pharmacia) was then layered under the 30 ml cell suspension 390 g for 40 minutes. Mononuclear cells layered on top of the Ficoll were collected, washed and used in colony assays (see hemopoietic progenitor assays, page 55). The more dense granulocytes with contaminating red blood cells which have passed through the Ficoll and pelleted at the bottom of the tube were washed once in PBS. The residual red blood cells were then removed by two rounds of hypotonic lysis. The cell pellet was first resuspended in ice cold ddH_2O for 30 seconds and isotonicity restored by addition of an equal volume of 1.8% NaCl. After pelleting, the cells were resuspended in ice cold 0.2% NaCl for 30 seconds and again isotonicity restored by adding an equal volume of 1.6% NaCl. The resulting cell mixture which is comprised of greater than 95% granulocytes was then resuspended in 5 mM DFP (Aldrich) in order to inactivate proteases, for 5 minutes on ice. Cells were washed and resuspended in 10 ml of relaxation buffer (100 mM KCl, 3 mM NaCl, 1 mM $ATPNa_2$, 3.5 mM $MgCl_2$, 10 mM PIPES pH 7.2). Granulocyte yields per unit of blood ranged from $0.5\text{-}1.5X10^9$.

Leukemic granulocytes were derived with consent from samples of peripheral blood collected by Drs. Michael Barnett and Nöel Buskard (Vancouver Hospital) of patients suffering from CML at presentation. 30-40 ml of blood was collected into heparanized tubes. Samples were diluted 4 fold and separated by Ficoll centrifugation as outlined above. The resulting pellet had a large red blood cell contamination and required 4 rounds of hypotonic lysis with ddH_2O. Cells were then again resuspended in 10 ml of relaxation buffer. Granulocyte yields from patients with CML ranged from $3\text{-}4X10^9$ per preparation.

Granulocyte suspensions in relaxation buffer, in a volume of 10-15 ml, were placed in a 50 ml polypropylene tube cut off at the 25 ml mark with a 1/2" stir bar in it. The tube was then packed in ice and placed into a modified nitrogen cavitation apparatus (Parr). During constant stirring the cells were pressurized to 375 psi over 5 minutes in nitrogen gas (Medigas). Cells were then left to equilibrate for 20 minutes. The pressure was then released slowly such that approximately 1 drop per second of the resulting cell lysate fell into a 13 ml polystyrene tube (Falcon) containing 200 µl of 62.5 mM EGTA for every 10 ml of lysate. Nuclei and intact cells were then pelleted for 10 minutes at 450 g at 4°C. The resulting supernatant containing intact granules, plasma membrane and cytosol was referred to as the post-cavitate supernatant and used to test for biological activity and as the starting material for further separations.

ii) Discontinuous Percoll gradient

Two Percoll solutions were prepared with a 1 tenth volume of 10X relaxation buffer containing 12.5 mM EGTA and ddH_2O such that the final densities were 1.12 g/ml and 1.05 g/ml. pH of the Percoll solutions was adjusted to 6.8 with 2 M HCl. 10 ml of the post-cavitate supernatant was on top of a discontinuous Percoll (Pharmacia) gradient consisting of 14 ml of the 1.12 g/ml Percoll solution layered under 14 ml of the 1.05 g/ml Percoll solution in a 40 ml

polycarbonate tube (Nalgene). The post-cavitate supernatant was then separated by centrifugation at 20 000 rpm (48 000 g) for 15 minutes at 4°C in a Sorvall SS34 rotor. After centrifugation 3 distinct bands were apparent. The bottom or alpha band corresponding to the azurophilic granules, the middle band, corresponding to the specific granules and the top band, corresponding to the plasma membrane. Layered on top of the plasma membrane band is the cytosolic fractions. The three bands and the cytosolic fractions were collected with a Pasteur pipette into Ultra-Clear ultracentrifuge tubes (Beckman) and spun in an SW41 ultracentrifuge rotor (Beckman) for 125 minutes at 35 000 rpm (180 000 g) at 4°C. After centrifugation the granule and plasma membrane fractions were found layered directly on a hard pellet of Percoll. This material was then collected in relaxation buffer containing relaxation buffer with 1.25 mM EGTA and frozen at -80°C. For the cytosolic fraction, pelleted material was discarded and the supernatant was frozen in aliquots at -80°C.

Granules and the vesicles within the plasma membrane were then disrupted with 7 rounds of freeze thawing in an ethanol/dry ice bath. Granule and plasma membranes were then pelleted for 30 minutes at maximum speed in an Eppendorf microfuge at 4°C. The supernatants were removed and the protein bound to the membranes was extracted with a 40 minute incubation in 500 µl of 50 mM glycine pH 2.0 at room temperature with periodic vortexing. Extracts were pelleted as before, and the supernatant neutralized with 1 M Tris.

2.5 Cellular Biology

i) Cells

Normal leukocytes for protein preparations and biological assays were obtained from the peripheral blood of consenting donors or the bone marrow of consenting patients undergoing cardiac surgery with Dr. Larry Burr (Vancouver Hospital). Leukemic leukocytes

were obtained from patients with untreated CML at presentation from Drs. Noel Buskard and Michael Barnett. Apheresis samples from individuals with CML were obtained from Dr. Charles Dowding (Systemix). Granulocytes and mononuclear cells were prepared by density separation using Ficoll-Hypaque (Pharmacia) as described above in the granulocyte harvest and nitrogen cavitation section (see page 52).

Normal murine bone marrow was obtained from DBA2/J mice over 12 weeks of age. Briefly, femurs from these animals were harvested, the ends cut off and using a 25 gauge needle, the bone marrow was extruded as a plug. The plug containing the hemopoietic progenitors was resuspended in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% serum and cells were washed twice before being reconstituted in the appropriate media.

Cell lines used in this study included the promyelocytic leukemia cell lines U937, NB4 and HL60. EM2 and K562 are both derived from patients with CML in blast crisis and KG1 which is from a patient with histiocytic leukemia. All cell lines were grown in RPMI media supplemented with 10% CPSR3 (fetal calf serum replacement, Sigma) with the exception of HL60 and KG1 which were grown in DMEM/10% CPSR3 and IMDM/10% CPSR3 respectively.

All cells were counted in a Spiers/Levy eosinophil counter using an equal volume of 7% Trypan blue in PBS as a measure of cell viability. For primary cells, 2% acetic acid was incorporated into the Trypan blue solution in order to lyse red cells.

ii) Culture supernatants for progenitor cell assays

For human progenitor cell assays two types of culture supernatants were used to stimulate growth. Initially, the growth supernatant of the human bladder cell carcinoma line 5637 was used. Briefly, cultures were initiated in 50 ml of DMEM supplemented with 10% FCS in T-175 flasks (Nunc) at 37°C and 5% CO_2. The adherent cells were grown to

confluency, the media removed and replaced with fresh DMEM/10% FCS. This was grown for 10 days, the media removed and centrifuged at 1000 g for 20 minutes in order to remove any cellular debris. The culture supernatant was filtered sterilized with a 0.45 μm acrodisc filter (Gelman), aliquoted and frozen at -70°C.

Due to the inconsistencies found with this growth media, an alternative source, PHA-LCM, was used. Blood from normal consenting donors was collected as described in the granulocyte harvest and nitrogen cavitation section (see page 52) aliquoted into 50 ml polypropylene tubes and left to settle for 90 minutes. Buffy coats (upper, yellow, opaque layer of cells) were removed and spun at 400 g and washed once in IMDM. Cells were then resuspended in IMDM supplemented with 10% FCS and 1% (v/v) of 1 mg/ml PHA (Sigma) at a concentration of $1x10^6$ cells/ml. Cells were cultured as before in T-175 flasks at 37°C and 5% CO_2 for 7 days. Cell suspensions were then removed and the cells pelleted at 400 g. The supernatant containing the stimulatory media was then spun at 1000 g for 20 minutes, filtered through a 0.45 μm acrodisc filter, aliquots made and frozen at -70°C.

For murine progenitor cell assays, PWM-SCCM (Stem Cell Technologies) was used as a source of growth factors

iii) Hemopoietic progenitor cell assays

Human colony assays were initially performed by the method of Messner and colleagues (155). Briefly, mononuclear cells derived as indicated above were plated with or without test samples in 1% methyl cellulose (Fluka), 30% FCS, 7.5% day 10 5637 supernatant (156) in IMDM. Cells from normal peripheral blood were plated at $5x10^5$ cells/ml, whereas cells from CML peripheral blood and normal human bone marrow were plated at $1 x10^5$. Using a 16 gauge needle, 1 ml of the above mixture was plated into 35 mm dishes (Nunc) in triplicate. Two of these dishes and one dish containing 5 ml of ddH_2O were placed into a 100

mm petri dish (Fisher). Plates were incubated for 14 days at 37°C and 5% CO_2 before being scored on an inverted phase contrast microscope (Zeiss). Clusters of cells greater than or equal to 50 cells were considered to be a colony. In later studies, 0.3% agar, 30% FCS, 10% PHA-LCM in CMRL 1066 media was used for the semi-solid mixture.

Murine progenitor cell assays were performed according to the method of Metcalf (157). Briefly, bone marrow cells from the femurs of 4-6 week old male DBA/2J (Jackson Laboratories) mice, were treated as above, with the exception that these cells were plated in IMDM with 20% heat inactivated fetal calf serum (FCS, Sigma), 10% BSA (Boehringer Mannheim), 2.5% pokeweed mitogen-stimulated spleen cell conditioned medium (SCCM, Terry Fox Laboratories Media Services,Vancouver) and 0.3% Agar Noble (Difco), and incubated for 7 days rather than 14 before scoring. Where indicated, SCCM was used at sub-optimal levels (0.14% rather than 2.5%) and supplemented with recombinant human granulocyte colony stimulating factor (rhG-CSF, Sigma) at a concentration of 1 ng/ml in order to stimulate murine CFU-G formation (158).

iv) Long-term marrow cultures

Long-term marrow cultures were performed as described by Eaves and colleagues (19). Briefly normal bone marrow mononuclear cells were plated at a density of $2x10^6$cells/ml in long-term culture medium (LTCM, Stem Cell technologies) in Corning 6 well plates. Cultures were grown for 7 days at 37°C and 5% CO_2, after which they were incubated at 33°C and 5% CO_2. Cultures were fed weekly by removing half of the supernatant medium containing cells and replacing it with fresh medium.

v) MTT assay for cellular proliferation

The outer 2 wells of a Costar 1/2 area 96 well plate are filled with 100 µl of ddH_2O. Cell lines at a concentration of 2 X 10^5 cells/ml and primary mononuclear cells at a concentration of 2 X 10^6 cells/ml in CMRL1066 media (media may vary depending on the experiment, see figure legend) containing 7.5% CPSR3 (FCS for primary cells) and with or without 2.4 X 10^{-4} M Thymidine (Gibco). 50 µl of the cell suspension (or media alone as a background control) was added in quadruplicate to wells. Factor or media alone in a volume of 25 µl was then added to each well. Plates were then incubated for 3 days at 37°C in a humidified incubator with 5% CO_2. After the incubation period, wells had 5 µl of 5 mg/ml MTT (in PBS, Sigma) and left at 37°C and 5% CO_2 for 3 hours. Wells then had 75 µl of 0.04 M HCl in isopropanol (HPLC grade, Fisher) using a 12 channel multi-well pipettor and mixed vigorously. Plates were then read at 595 nm in a Molecular Dynamics plate reader. Values reported have had the background control readings subtracted from them.

2.6 Purification of cytidine deaminase

i) Ammonium sulphate and heat precipitation

The initial steps in the purification of cytidine deaminase were first described by Momparler and Laliberte. Briefly, cytosolic fractions of granulocytes, prepared as described (see section 2.4 granulocyte subfractionation, page 52), were brought up to 40% v/v saturated ammonium sulphate (SAS, 76 g/100 ml) by dropwise addition through a 23 gauge needle attached to an appropriately sized syringe with constant stirring at 4°C. This solution was left to

precipitate for at least 2 hours at which time the solution was put into 1.7 ml epitubes and centrifuged at top speed in an Eppendorf microfuge at 4°C for 30 minutes. The supernatant was removed and brought up to 55% SAS as before and left overnight to precipitate. Again this slurry was spun as before and the supernatant removed. The pellet was then suspended in 200 µl of MonoQ Buffer A (see ion exchange chromatograph page 47) and incubated in a water bath at 75°C for 12 minutes. The heat precipitated protein was removed by centrifugation as before and the supernatant was used for Superose 12 size exclusion chromatography (see page 47). Active Superose 12 fractions were subjected to either rpHPLC or MonoQ ion exchange chromatography as described on page 47.

ii) Recombinant cytidine deaminase

Recombinant cytidine deaminase was a generous gift of Prof. Alberto Vita (University of Camerino, Camerino, Italy).

CHAPTER 3 - PURIFICATION AND IDENTIFICATION OF AN IMMUNOAFFINITY ENRICHED SELECTIVE INHIBITOR OF HEMOPOIESIS FROM CML PBL

3.1 Introduction

Chronic myelogenous leukemia (CML) is a clonal, myeloproliferative stem cell disorder in which there is an insidious accumulation of neutrophilic granulocytes and their progenitors in the peripheral blood and bone marrow. The way in which the leukemic clone is established is poorly understood. It is generally accepted that a pool of normal stem cells remain in patients with CML (118, 159, 160). In addition, the malignant cells are not believed to progress through the cell cycle faster than their normal counterparts (161, 162). Considering these points, a possible mechanism for the growth advantage of the leukemic cells may be through the release of factors which are inhibitory to the normal pool of hematopoietic progenitor cells, an idea supported by studies performed by Broxmeyer and colleagues and Olofsson and Olsson (87, 89). Ongoing investigations in our laboratory have focused on the characterization of a distinct inhibitory factor suspected to be secreted by acute myelogenous leukemia (AML) and CML cells (Chapter 1). This material, previously referred to as CAMAL (143-145), was obtained through immunoaffinity chromatography of extracts from leukemic peripheral blood cells. Through the use of size exclusion chromatography and preparative gel electrophoresis, it has been previously shown that a 30-35 kDa component of this immunoaffinity enriched material inhibited normal human (142, 151) and murine (143, 151) CFU-GM (colony forming unit granulocyte macrophage) formation equally. In contrast to the inhibitory activity on normal myeloid progenitors, this material enhanced the formation of CML CFU-GM (144, 145). Previous attempts to obtain N-terminal amino acid sequence of the 30 kDa active component revealed a sequence subsequently found to be identical to that of azurocidin (also known as

CAP37 and hHBP) (142, 145). Azurocidin is a basic, proteolytically inactive serine protease homologue, with bactericidal effects and chemotactic activity on monocytes (163-166). It has a molecular mass which ranges from 29 to 37 kDa due to differential glycosylation and is abundant in the azurophilic granules of neutrophilic granulocytes (110, 164, 165).

3.2 Objective

To clarify the previously established link of the selective inhibitory activity with azurocidin. This was performed by separating crude immunoaffinity enriched preparations using rpHPLC, identification was then made by elution time and profile as well as two dimensional electrophoresis.

3.3 Results

i) Separation of immunoaffinity enriched material by rpHPLC

Leukemic cells from patients with both AML and CML have been shown to release factors which have a negative effect on hemopoiesis. Using immunoaffinity separation of crude leukemic blood lysates, we have previously shown the presence of inhibitory activity corresponding to material of approximately 30 kDa (145). In figure 3, a typical SDS-PAGE separation of an immunoaffinity preparation can be seen. Due to the presence of many impurities, it was apparent that the immnuoaffinity enriched material had to be further purified in order to identify the molecule(s) responsible for the activity. Previous attempts to purify the molecule responsible for the activity to homogeneity by ion exchange and size exclusion chromatography were unsuccessful (142). Despite this, attempts were made to sequence

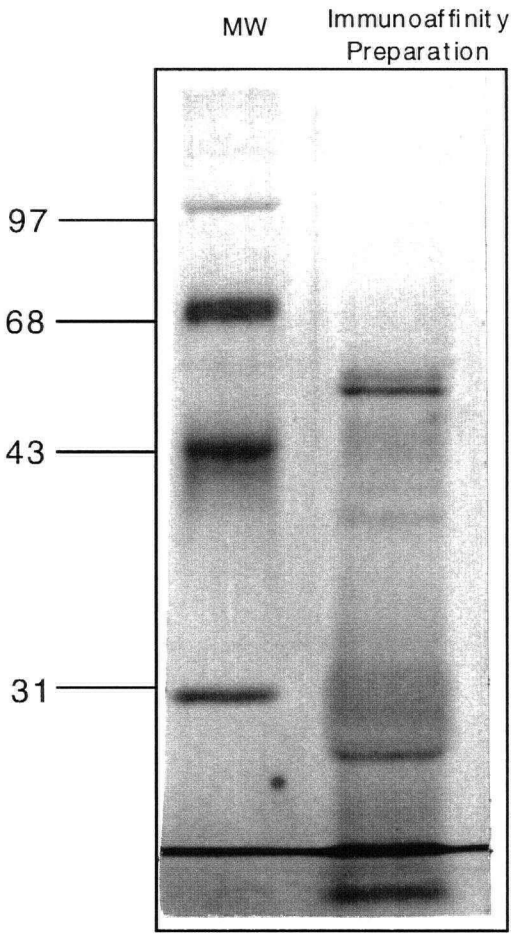


Figure 3 SDS-PAGE analysis of crude immunoaffinity enriched material

12% SDS PAGE of an immunoaffinity enriched preparation visualized by silver staining.

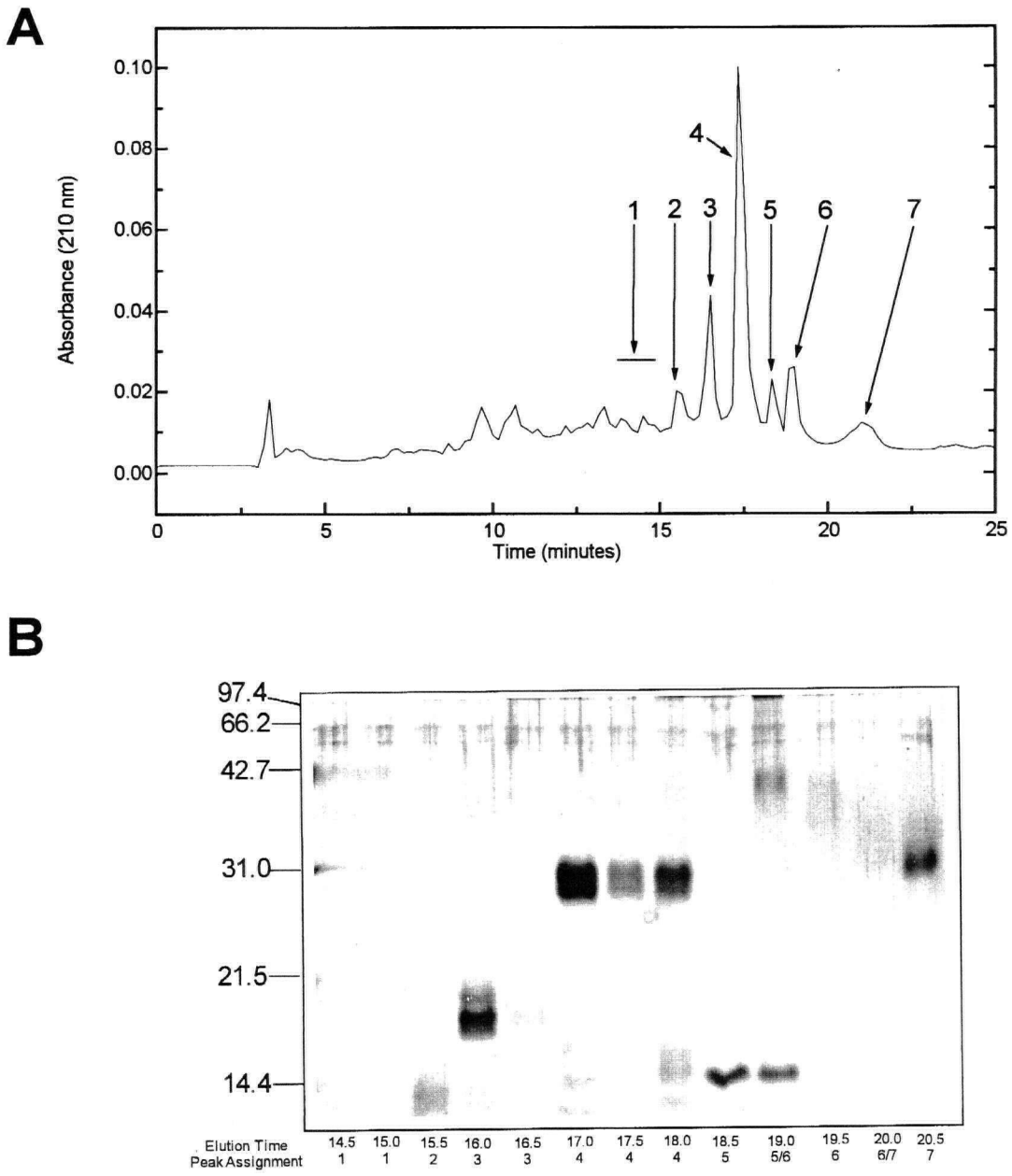


Figure 4 **Analysis of immunoaffinity enriched material**

(**A**) rpHPLC separation of the immunoaffinity enriched material seen in figure 3 using a C_4 column and monitoring absorbance at 210 nm. Peaks were numbered for identification and further analysis. The major component of each peak was determined as indicated in the text and are as follows: 1. Unknown; 2. Defensins; 3. Eosinophil cationic protein; 4. Cathepsin G; 5. Major Basic Protein; 6. Lysozyme; 7. Azurocidin. This figure shows an elution profile of a linear acetonitrile gradient of 2-38% for the first 12 minutes and 0.67%/minute thereafter. 0.5 minute fractions were collected for further analyses. (**B**) 12%, silver stained, SDS-PAGE analysis of the different rpHPLC fractions.

immunoaffinity enriched material further purified by size exclusion chromatography. Material corresponding to approximately 30 kDa was sequenced and this sequence was subsequently determined to be that of azurocidin. Due to the presence of contaminating protein at approximately 30 kDa we could not ensure that another, 30 kDa protein, whose sequence was N-terminally blocked was not mediating the inhibitory activity. Therefore, reverse phase HPLC (rpHPLC), which is based on hydrophobic interaction of protein with a carbon matrix, was used to separate the crude immunoaffinity preparations. Figure 4a shows a typical rpHPLC chromatogram (one of four) of immunoaffinity enriched material. In light of the fact that azurocidin, which is localized to the azurophilic neutrophil granules, was found in active, size exclusion material, this chromatogram was compared to those of normal azurophilic granule preparations (163, 164). The similarity between the normal azurophilic granule and immunoaffinity enriched chromatograms allowed for the putative identification of a number of these peaks (see Figure 4 legend). Figure 4b shows the SDS-PAGE analysis of the various rpHPLC fractions. Since an equal proportion by volume of each fraction was loaded on the gel, this figure gives information on both the quantity and size of the proteins in the various fractions. With an idea of the quantity, the relative activity of the fractions may be assessed. The molecular mass determinations of the various peak fractions allowed for the confirmation of the peak designations outlined in the legend of figure 4a. As a further means of confirmation, the fraction corresponding to peak 4 which was designated, as cathepsin G, demonstrated proteolytic activity on appropriate peptide substrates, whereas peak 7 designated as azurocidin, had no activity on a panel of peptide substrates, consistent with it being proteolytically inactive (142).

ii) Determination of the peak of interest

Individual rpHPLC fractions from figure 4a were tested for their effect on normal murine bone marrow progenitor cells at a final concentration of 800 ng/ml (figure 5a). As can be seen, most of the inhibitory activity is contained in the pooled 19.5-20 minute fraction which elicited 70% inhibition of the normal CFU-GM. SDS-PAGE analysis (figure 4b) confirmed the relative elution times of the peaks seen in figure 4a and indicated that the 19.5-20 minute fraction aligned with the trailing edge of the lysozyme peak (17 kDa band in figure 4b, 19.5 minute fraction) and the leading edge of the azurocidin peak (faint 35 kDa band in figure 4b, 20 minute fraction). A titration of the pooled 19.5-20 minute fraction is shown in figure 5b. In this assay, a maximum inhibition of approximately 65% was seen at a concentration of 800 ng/ml which titrated down to 0% at a concentration of 1.3 ng/ml indicating the existence of a dose dependent relationship. The 20.5 minute fraction, corresponding to the bulk of the azurocidin peak (figure 4b) can be seen to have very little inhibitory activity at 800 ng/ml (figure 5a).

In order to determine whether lysozyme was responsible for the observed activity, lysozyme was tested and shown to have no activity even at concentrations of 1 mg/ml (data not shown). Therefore it was clear that the activity lay in the leading edge of the peak corresponding to azurocidin . Subsequent experiments focused on further analysis of this peak.

iii) Identification of azurocidin as the molecule responsible for the activity

A number of different immunoaffinity enriched preparations were analyzed by rpHPLC and a preparation devoid of lysozyme with a large azurocidin peak was chosen for further analysis (figure 6). In case any residual lysozyme was present, the acetonitrile gradient was

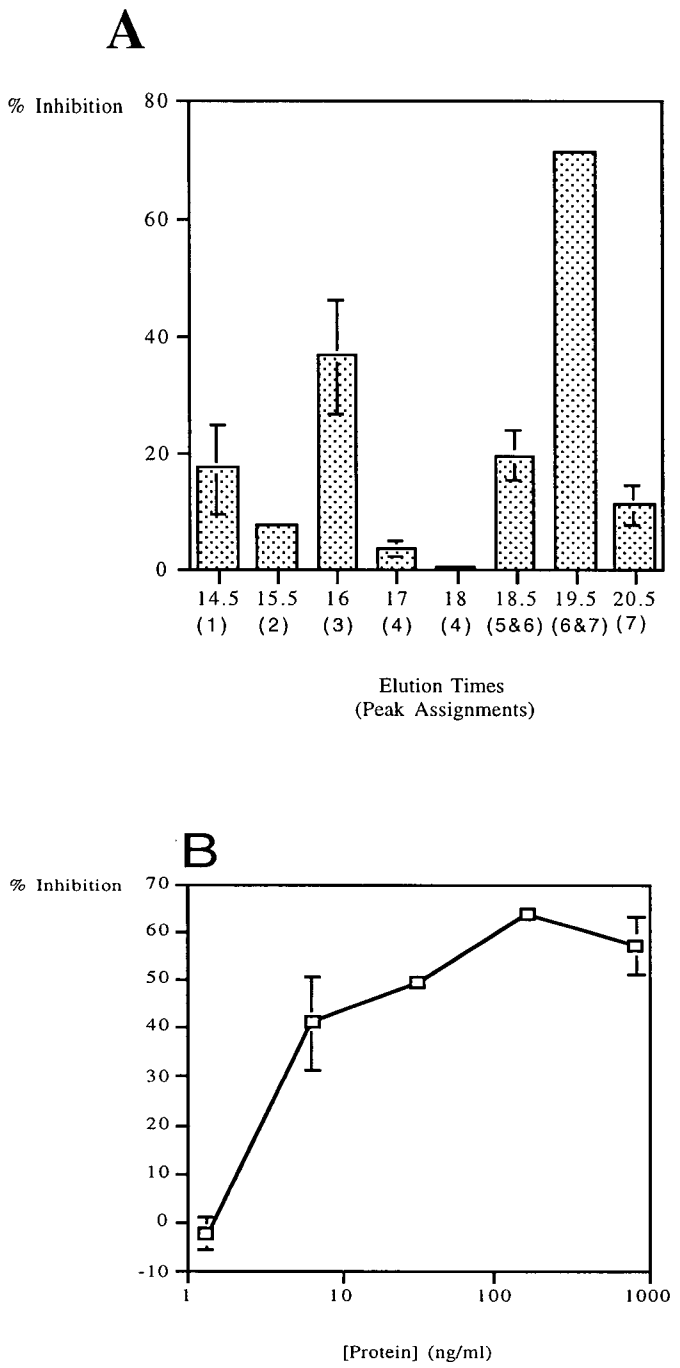


Figure 5 **Identification of the inhibitory component of the immunoaffinity enriched material**

(**A**) Inhibitory activity of the various rpHPLC fractions (figure 4), at a concentration of 800 ng/ml on day 7 murine CFU-GM colonies as compared to IMDM only controls (control colony numbers were 76 ± 3.54). (**B**) Inhibitory activity of the 19.5-20.0 minute rpHPLC fraction (fig. 2) on day 7 murine CFU-GM formation from 1.3 to 800 ng/ml (control colony numbers were 43 ± 0).(Representative data from 3 experiments are presented)

adjusted in an attempt to further separate it from azurocidin (figure 6 legend). This resulted in the 24.5 minute elution of azurocidin rather than the previously observed elution at 20.5 minutes (figure 6 compared to figure 4a). The peak of interest was collected into two fractions, 1 and 2, representing the front third and back two thirds of the azurocidin peak (figure 6 inset). In order to confirm our suspicion that the inhibitory activity eluted in the leading edge of the azurocidin peak, the two fractions were assayed for their effects on colony formation. Since it has been previously shown that it was the granulocytic progenitors which were most sensitive to the inhibitory effects of the immunoaffinity enriched material (145), the assay involved the addition of rhGCSF (1 ng/ml) to bone marrow cells in culture stimulated with sub-optimal levels of SCCM (0.14% rather than 2.5%) in order to enhance granulocytic colony growth. As can be seen in figure 7a, fraction 1 retained all of the activity while fraction 2 displayed none whatsoever over the concentration range tested (0.65-8 ug/ml). In contrast, fraction 1 completely abolished colony formation at the highest concentration tested (8 ug/ml). From these data it was evident that the inhibitory activity lay in the leading shoulder of the azurocidin peak and that the bulk of azurocidin had no activity.

These same fractions were further examined for their effects on CML colony formation (figure 7b), since the activity described previously was not inhibitory to malignant progenitors (145). In these experiments, our earlier observations were confirmed in that neither fraction showed any inhibitory activity, even at the highest concentrations tested (6.7 mg/ml). In fact, there appeared to be a 30% enhancement of colony formation at these concentrations.

Because of the non-Gaussian configuration of the azurocidin peak (figure 6 inset), the possibility was considered that components other than azurocidin could be eluting at this time. In order to determine whether the active fraction (fraction 1) contained a unique protein(s), two- dimensional (2-D) NEPHGE electrophoresis was performed. Fraction 2 material and control protein were analyzed in an identical manner (figure 8). Figure 8a shows a 2-D separation of the active component of the peak, which migrates at 32 kDa in the basic end and trails up to 42 kDa in the acidic end of the gel. In contrast, fraction 2, contains the bulk of the

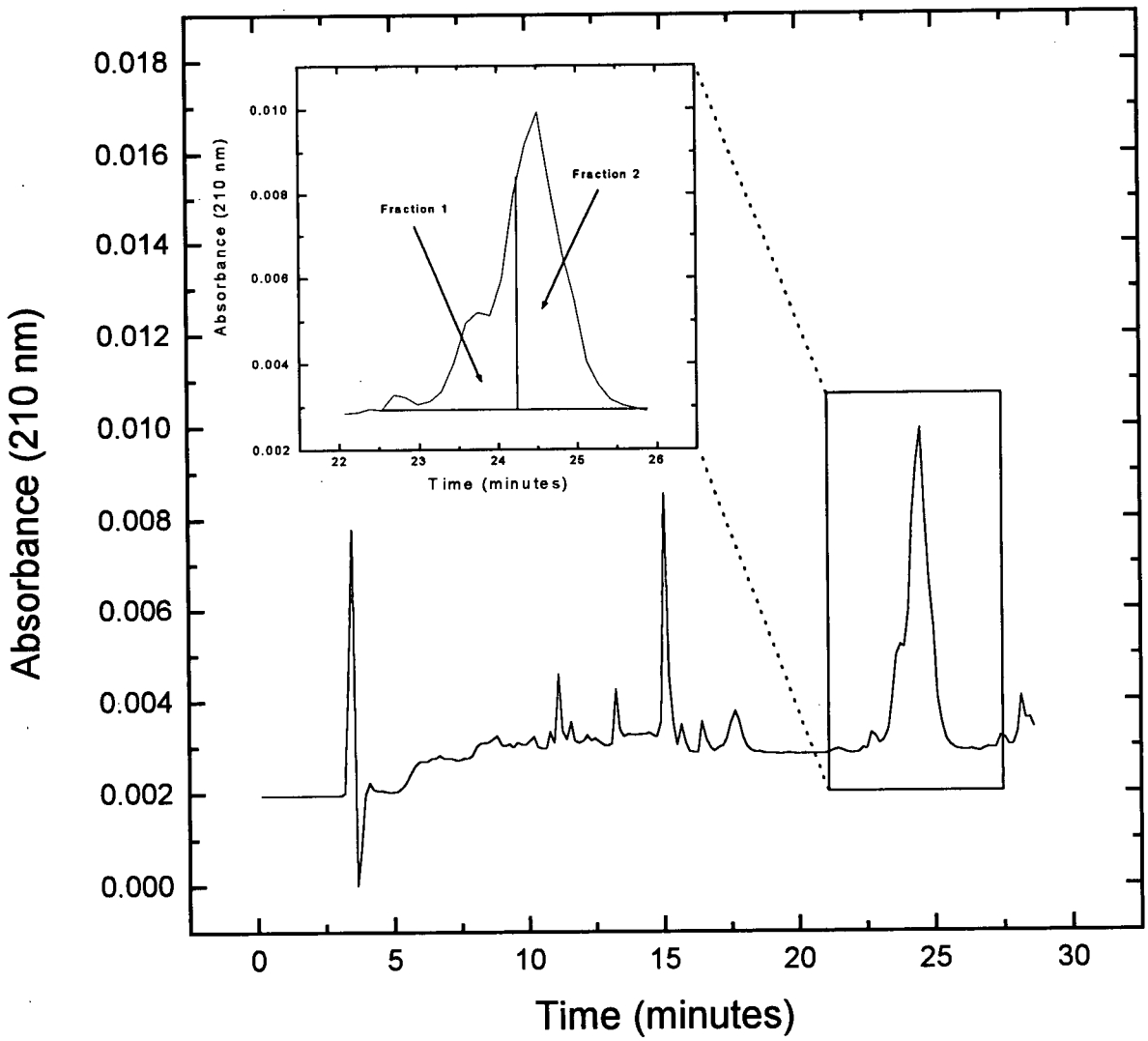


Figure 6 **rpHPLC separation of another immunoaffinity enriched sample indicating fractions 1 and 2 (inset)**

A linear acetonitrile gradient of 1%/minute was used for this separation. Fractions were collected by hand into fractions 1 and 2 so as to collect all of the shoulder material (see inset) into fraction 1.

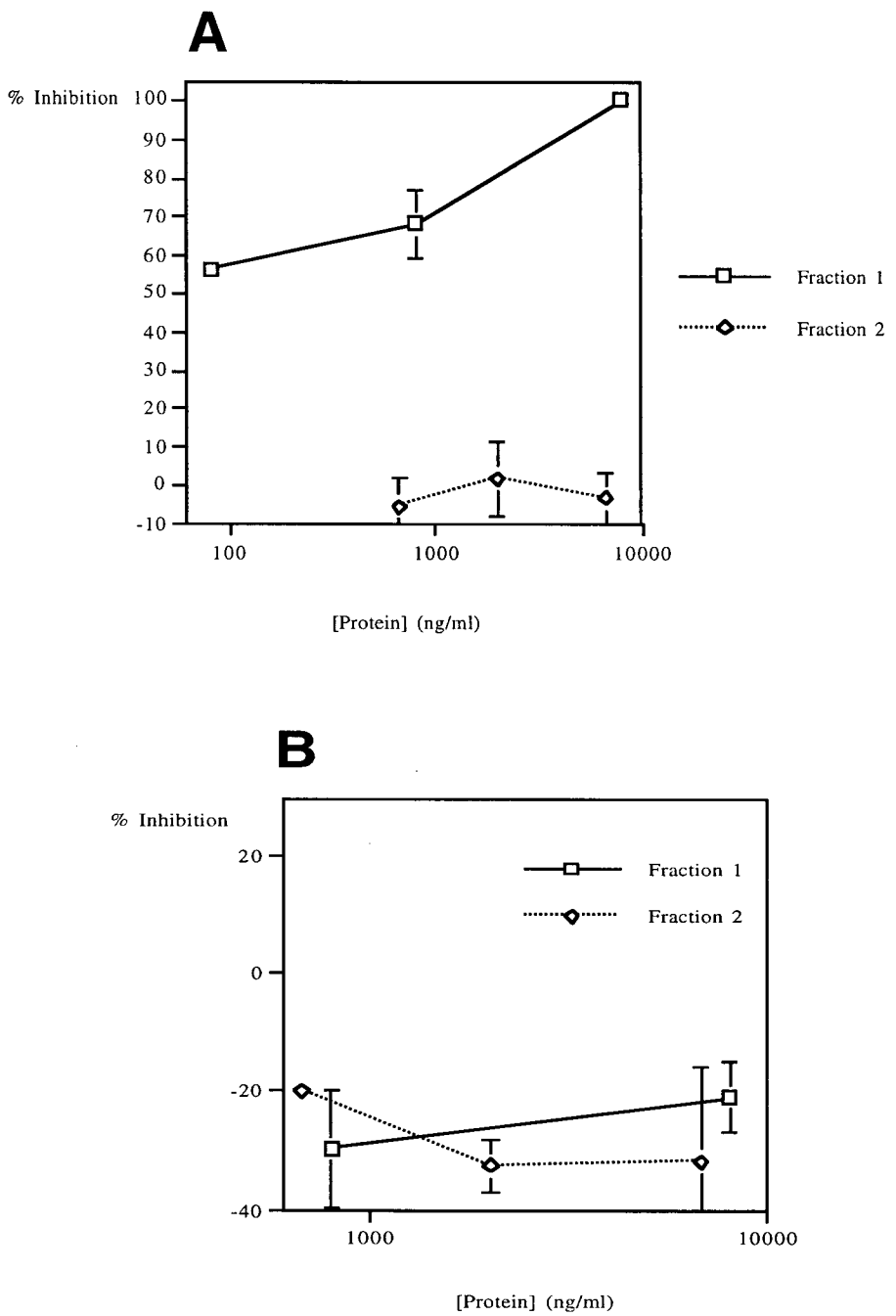


Figure 7 Biological activity of fractions 1 and 2 from figure 6

(**A**) Comparison of the effects of varying concentrations of fractions 1 and 2 on day 7 murine CFU-GM formation. (**B**) Comparison of the effects of varying concentrations of fractions 1 and 2 on day 14 CML CFU-GM formation.

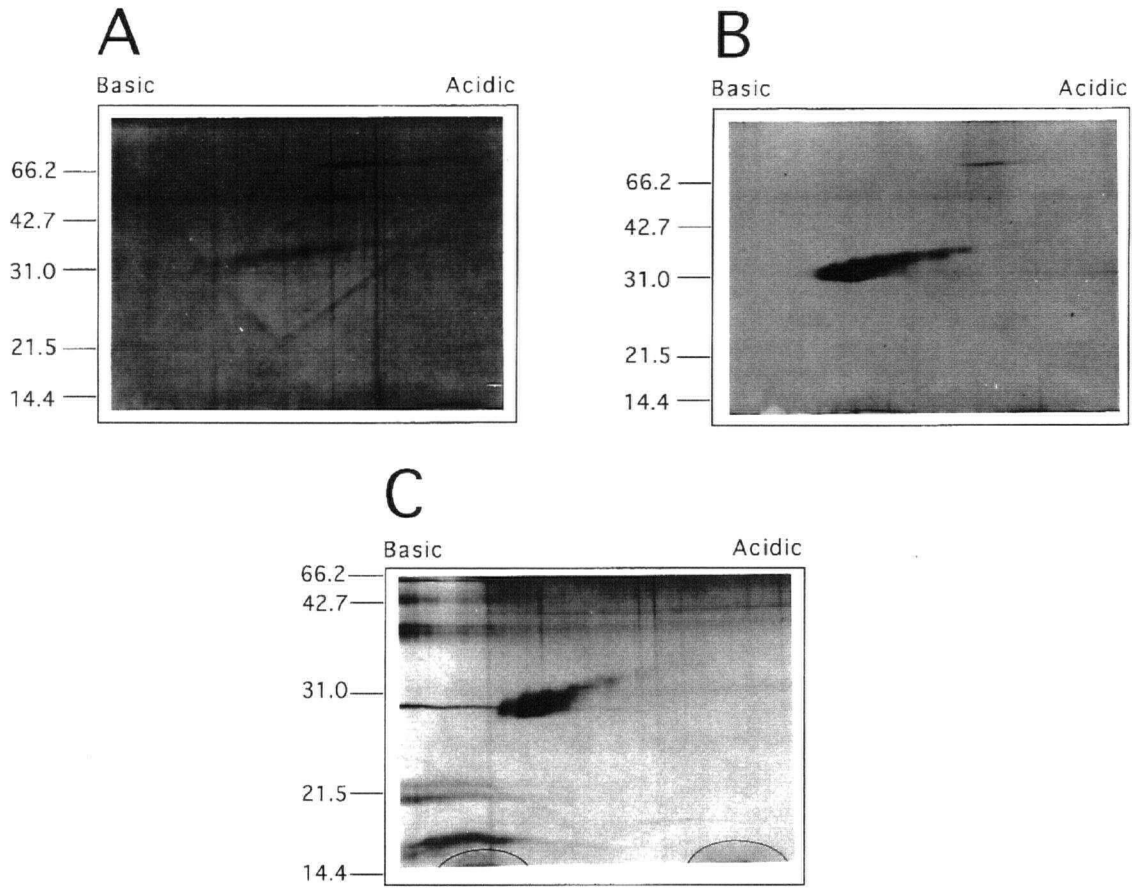


Figure 8 **2-D NEPHGE, silver stained analysis of fraction 1 and 2 material from figure 6 and control proteins**

(**A**) 2-D analysis of fraction 1. (**B**) 2-D analysis of fraction 2. (**C**) 2-D analysis of control lysozyme and azurocidin.

protein (figure 8b), is less streaky and ranges from 31 kDa to 35 kDa. The control proteins in figure 8c correspond to Calbiochem lysozyme at 17 kDa in the basic end and to normal azurocidin (obtained from the neutrophilic granules of a normal human donor) at 32 kDa which is slightly more acidic. All three gels in this figure were run simultaneously and the figures show identical fields of the gels. NEPHGE electrophoresis was chosen for this analysis because of it's ability to resolve basic proteins such as those found within azurophilic granules (we are able to resolve proteins of the same approximate molecular mass with iso-electric points 0.5 pH units apart).

3.4 Discussion

In this chapter we have extended the characterization and identification of an activity derived from myeloid leukemia cells which mediates the selective inhibition of normal myeloid progenitors described in earlier reports (143-145, 151). With the use of rpHPLC, immunoaffinity enriched material equivalent to that used in previous reports was found to contain a number of the constituents of azurophilic granules (figure 4a). When the original N-terminal amino acid sequence (142, 145) was found to be identical to that of azurocidin, investigation of the related literature revealed the similarity between the chromatograms of azurophilic granule proteins and our immunoaffinity enriched material. Using the chromatographic procedure described by Gabay, Wilde and co-workers (142, 145, 163, 164) SDS-PAGE analysis and the detection of protease activities of relevant peaks we were able to assign identities to most of the rpHPLC peaks (figure 4a legend).

In order to assess the biological activity of the various rpHPLC fractions, their effect on murine CFU-GM colony formation was tested (figure 5a). Only one fraction, that which aligned with the leading portion of the azurocidin peak, showed significant inhibitory activity. No other fraction tested was found to have comparable activity. Therefore investigations

focused on this fraction. A titration of this material (figure 5b) showed that there was a dose response in inhibitory activity from 0% at 1.3 ng/ml to a plateau of approximately 60% between concentrations of 160 ng/ml and 800 ng/ml. Previous experiments using partially enriched materials had indicated the susceptibility of neutrophilic progenitors to this activity (145). In light of this observation, the plateau of inhibition in all probability represented an effect in which the more vulnerable neutrophilic progenitors were completely inhibited, leaving behind the more resistant monocytic progenitors.

Electrophoretic analysis of the pooled 19.5-20 minute fractions established the presence of 17 kDa material (figure 4b, 19.5 minute fraction) and a weakly stained band of higher molecular mass material at 35 kDa (figure 4b, 20 minute fraction). The 17 kDa material was identified as lysozyme by N-terminal amino acid sequence analysis (142). The 35 kDa material represents the leading edge of the rpHPLC azurocidin peak (figure 4a and 4b, 20.5 minute fraction). Lysozyme was ruled out as a candidate for the observed activity for a number of reasons: at 17 kDa it is considerably smaller than the previously reported 30 kDa molecular mass for the active material (145); commercially available lysozyme was tested for inhibitory activity in the colony inhibition assay and was found to have no effect even at concentrations of 1 mg/ml; 18.5 and 19 minute fractions from figure 4b had a considerably higher concentration of lysozyme and yet had almost no activity (figure 5a), and active fraction 1 material (figure 7a) does not contain any lysozyme as seen by 2-D gel analysis (figure 8a).

Immunoaffinity enriched material with a large azurocidin peak, containing a leading edge shoulder, was chosen for further analysis (figure 6a). The peak was divided into two fractions (fractions 1 and 2) and the entire shoulder was collected into fraction 1. Fractions 1 and 2, were tested for biological activity on murine CFU-G and CML CFU-GM (figures 7a and 7b). In this instance rhGCSF was used in the murine progenitor assay in order to increase its sensitivity, since previous studies showed that granulocytic progenitors were particularly susceptible to the inhibitory activity. Fraction 1, the shoulder of the azurocidin peak, showed dramatic inhibitory activity on the G-CSF stimulated colonies, resulting in complete inhibition

at 8 mg/ml. In contrast, fraction 2, the bulk of the azurocidin peak, had no effect on colony formation even at concentrations of 6.7 ug/ml. When tested on CML progenitor cells (figure 7b), neither fractions 1 or 2 showed any inhibitory activity on colony formation. In fact, a 30% enhancement of colony formation was seen, corresponding well with previous findings with immunoaffinity enriched material (144).

Despite repeated attempts using different acetonitrile gradients, we have been unable to achieve a separation of the shoulder from the bulk of the peak. Therefore, these two fractions were subjected to 2-D NEPHGE electrophoresis, which separates proteins on the basis of iso-electric point in the first dimension and molecular mass in the second. NEPHGE electrophoresis was chosen for the first dimension because of its ability to separate basic proteins, such as those known to be present within azurophilic granules (eg. lysozyme, defensins, cathepsin G and azurocidin) (153). Fraction 2 material (figure 8b) appeared as a spot centered at 32 kDa in the basic end which tailed up to 36 kDa in the acidic end, whereas fraction 1 material (figure 8a) started at 34 kDa and tailed up in a thin line to 41 kDa further into the acidic end. As seen previously (figure 4b, 20 minute fraction vs 20.5 minute fraction), the earlier eluting, active material was found to be of a higher molecular weight. A faint, streaky spot at approximately 67 kDa can be seen in both figure 8a and 8b. This may be due to residual albumin (which elutes in the rpHPLC system used in the general area of azurocidin, unpublished observation), and may be used as a reference point to compare the two gels. The control azurocidin in figure 8c looks identical to fraction 2 (figure 8b) except that it trails higher and appears to be essentially identical to the shoulder material separated from the bulk azurocidin as fraction 1. Thus fractions 1 and 2 combined provide a 2-D pattern similar if not identical to normal azurocidin.

In this chapter I have defined a selective inhibitory activity on normal myeloid progenitor cells prepared from the peripheral blood of patients suffering from CML. This selective activity was found by rpHPLC to co-elute exclusively with the leading edge of the azurocidin peak and by 2-D gel analysis to co-migrate with higher molecular weight

glycoforms of this serine protease homologue. The remainder of the peak, containing the bulk of azurocidin, had no activity on colony formation. Our findings can be explained in one of two ways. It is possible that specific glycoforms of azurocidin mediate the inhibitory activity. Alternatively, an as yet uncharacterized protein which co-elutes and co-migrates with these glycoforms of azurocidin may be responsible. Azurocidin has 3 N-linked glycosylation sites and has various glycoforms accounting for its variability in electrophoretic mobility (29 to 37 kDa) (110, 165). Recent studies have shown that the biological activity of molecules such as tissue-type plasminogen activator and erythropoietin (important in hemostasis and hematopoiesis) are very sensitive to differences in N-linked glycosylation (167, 168). These observations set a precedent which support the possibility that this selective activity may be due to glycoforms of azurocidin. Alternatively, the inhibitory activity of the immunoaffinity enriched material was found in earlier studies to be completely abrogated with the use of serine protease inhibitors (146) which should have no effect on the inactive serine protease homologue, azurocidin. Since these studies were performed on materials known to contain several serine proteases, these results are difficult to unify with the present data using rigorously purified materials. If these inhibitors were found to abolish activity of the purified material, this would support the possibility that an as yet uncharacterized protein is responsible for the observed activity.

It is probable that the molecule responsible for this selective inhibition is also present in normal azurophilic granules and may be involved in a receptor mediated down-regulation of normal granulopoiesis. This possibility is supported by the observation that the inhibitory activity of the immunoaffinity enriched material can be elicited by a relatively short (45 minutes) incubation of progenitor cells with active material prior to washing and culturing the treated cells in progenitor assays (143, 144). Evidence is provided here that the granulocyte progenitors of patients with CML are insensitive to this inhibitory activity *in vitro*. This supports the possibility that dysfunctional regulatory elements in the myelocytic cells of

patients with CML allow them to escape this activity and may serve to exacerbate the leukemic condition.

The next chapter addresses the possibility that this activity is a normal regulator of myelopoiesis by analyzing the activity of azurocidin purified from the azurophilic granules of normal neutrophilic granulocytes.

The neutrophilic granule-associated proteins may play a role in the maintenance of myeloid leukemias. With cell death and leakage from the phagocytic vacuole, the contents of these granules are released to the extracellular environment. One report suggests that 89% of azurophilic granule contents are released from neutrophils upon phagocytosis of *Staphylococcus aureus* (169). Considering the massive neutrophil turnover that exists in patients with CML, there could be the release of relatively large amounts of these proteins. With the insensitivity of the CML progenitors to this activity, this turnover could result in the establishment of an environment which provides a distinct growth advantage for the leukemic clone. If this were proven to be the case, blocking its activity might result in the prolongation of remission for patients suffering with this debilitating disease.

CHAPTER 4 - PURIFICATION AND ANALYSIS OF AZUROCIDIN FROM NORMAL INDIVIDUALS

4.1 Introduction

Normal hemopoiesis involves a delicate balance between positive and negative regulation. Due to the large numbers of the neutrophilic granulocytes, this balance is particularly important in maintaining the level of monocytic and neutrophilic progenitors within the relatively fixed numbers seen *in vivo*. The knowledge of negative regulators has expanded over the last 10 years and now there are several molecules known to mediate the restraint inherent in the hemopoietic system including Mip-1α, the peptide inhibitors, TGF-β, TNF-α, and the interferons (see chapter 1 for review).

The previous chapter suggested that specific glycoforms of azurocidin from patients with CML were possibly responsible for the selective inhibitory activity on normal granulocytic progenitors. In view of the fact that this is a normal molecule found within the azurophilic granules of neutrophils, it was considered unlikely that in CML, a mutation had occurred within the azurocidin sequence resulting in a molecule with this selective inhibitory activity. Furthermore, although the CAMAL-1 mAb reacts only with myeloid leukemia antigens, it does not react with the population of protein at 30 kDa which is thought to be responsible for the activity (142). This is an indication that the activity may also be found in normal cells and that in leukemic PBL lysates that it may be co-precipitating with the CAMAL-1 mAb specific antigens. Therefore, in this chapter the possibility that normally derived azurocidin is a member of the growing family of negative regulators was investigated.

4.2 Objective

In chapter 3 evidence was provided for the possibility that higher molecular weight glycoforms of azurocidin from leukemic cells (CML) were responsible for the selective inhibitory activity on normal progenitor cells. The objective of this chapter was to determine if normal cells produced higher molecular weight glycoforms of azurocidin capable of mediating the same effect.

4.3 Results

i) Granulocyte fractionation

The neutrophilic granulocyte is a highly compartmentalized and modular cell with a range of different granules containing the various molecules which mediate its host defense and chemotactic properties (170). We exploited this compartmentalization to purify the azurophilic granules which exclusively have as one of their constituents azurocidin. Borregaard and colleagues have elegantly shown that it is possible to purify the different granule populations of the neutrophil on the basis of their different densities (171).

Initially, neutrophils were purified from peripheral blood using Dextran mediated red blood cell (RBC) sedimentation, Ficoll separation of the leukocyte rich supernatant and hypotonic lysis of residual RBC's. Figure 9 shows the Giemsa staining of a neutrophil preparation. Microscopic analysis of these preparations has revealed that over 95% of the cells remaining after this procedure have the polymorphonuclear phenotype characteristic of the neutrophilic granulocyte. This preparation was then subjected to nitrogen cavitation in order to lyse the cellular membrane while maintaining the integrity of the granules. This allowed for the separation of the different granules by a discontinuous Percoll gradient as depicted in figure 10.

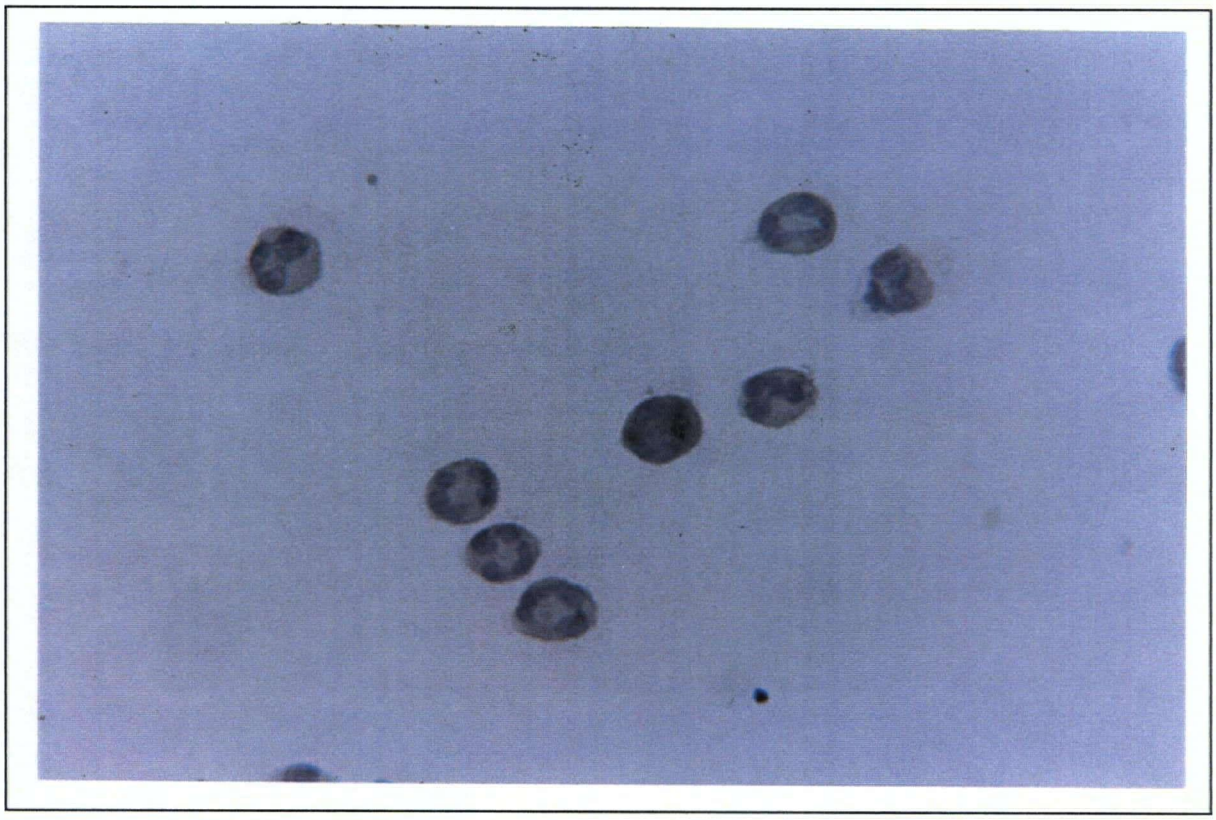


Figure 9 **Giemsa staining of neutrophil preparations**

Normal peripheral blood with the majority of RBC's removed by Dextran sedimentation had mononuclear cells removed by Ficoll separation. Residual RBC's were then lysed hypotonically. The resulting neutrophil preparation was cytospun and stained.

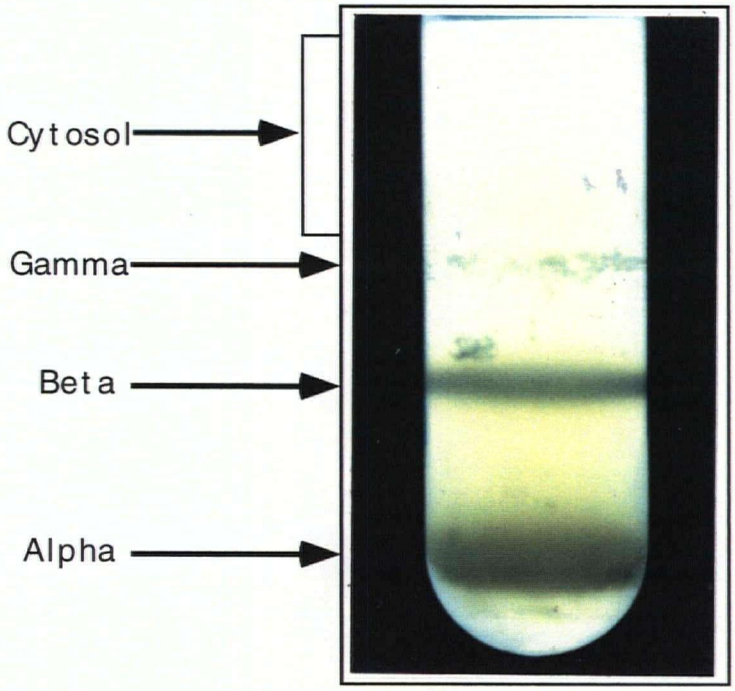


Figure 10 Discontinuous Percoll density gradient fractionation

Soluble supernatant material from a cavitate lysis of normal neutrophils was separated on a 1.05/1.12 g/ml discontinuous Percoll gradient yielding the indicated fractions. The α fraction contains alpha granules, the β fraction contains specific granules and the γ fraction contains the plasma membrane. As indicated, the cytosolic components are layered above the γ fraction.

From this figure, four fractions are apparent; a stratum of the least dense cytosol layered on top of a pack of cytosol (the gamma fraction), and distinct bands corresponding to the increasingly dense specific granules (the beta fraction) and azurophilic granules (the alpha fraction). The specific and azurophilic granules were distinguishable by colour, with the former being brownish and the latter, due to the presence of myeloperoxidase, appearing green. In addition, the consistency of the packed granules differed, with the specific granules adhering strongly to one another whereas the azurophilic granules did not.

ii) Biochemical analysis of azurophilic granule material

Using a Pasteur pipette to collect the azurophilic granule band, coupled with ultracentrifugation to remove the Percoll, it was possible to obtain a homogenous population of granules (171). This granule preparation was then lysed with seven rounds of freeze thawing with intermittent 30 second pulses of sonication. Protein adhering to the azurophilic membrane was then removed by a 40 minute extraction in 50 mM glycine pH 2.0 coupled with periodic mixing. This preparation was centrifuged at 14000 G for 30 minutes and the resulting supernatant was separated by rpHPLC as seen in figure 11a. It is important to note that as in figure 6 (chapter 3), a different acetonitrile gradient was used in this fractionation compared to that in figure 4 (chapter 3, compare figure legends 4a, 6 and 11a). This was done in an effort to further separate the lysozyme and azurocidin peaks. This resulted in an elution profile almost identical to that of figure 6, with azurocidin eluting at approximately 26 minutes rather than the previously observed 20.5 minutes (figure 4).

SDS-PAGE analysis of the rpHPLC fractions from figure 11a can be seen in figure 11b. As before, the characteristic azurocidin streak of material ranging from 29 to 37 kDa is apparent in the 27 minute fraction. In the 25 minute (figure 11b) fraction a hint of material at approximately 17 kDa can be seen which corresponds to the lysozyme peak eluting just prior to

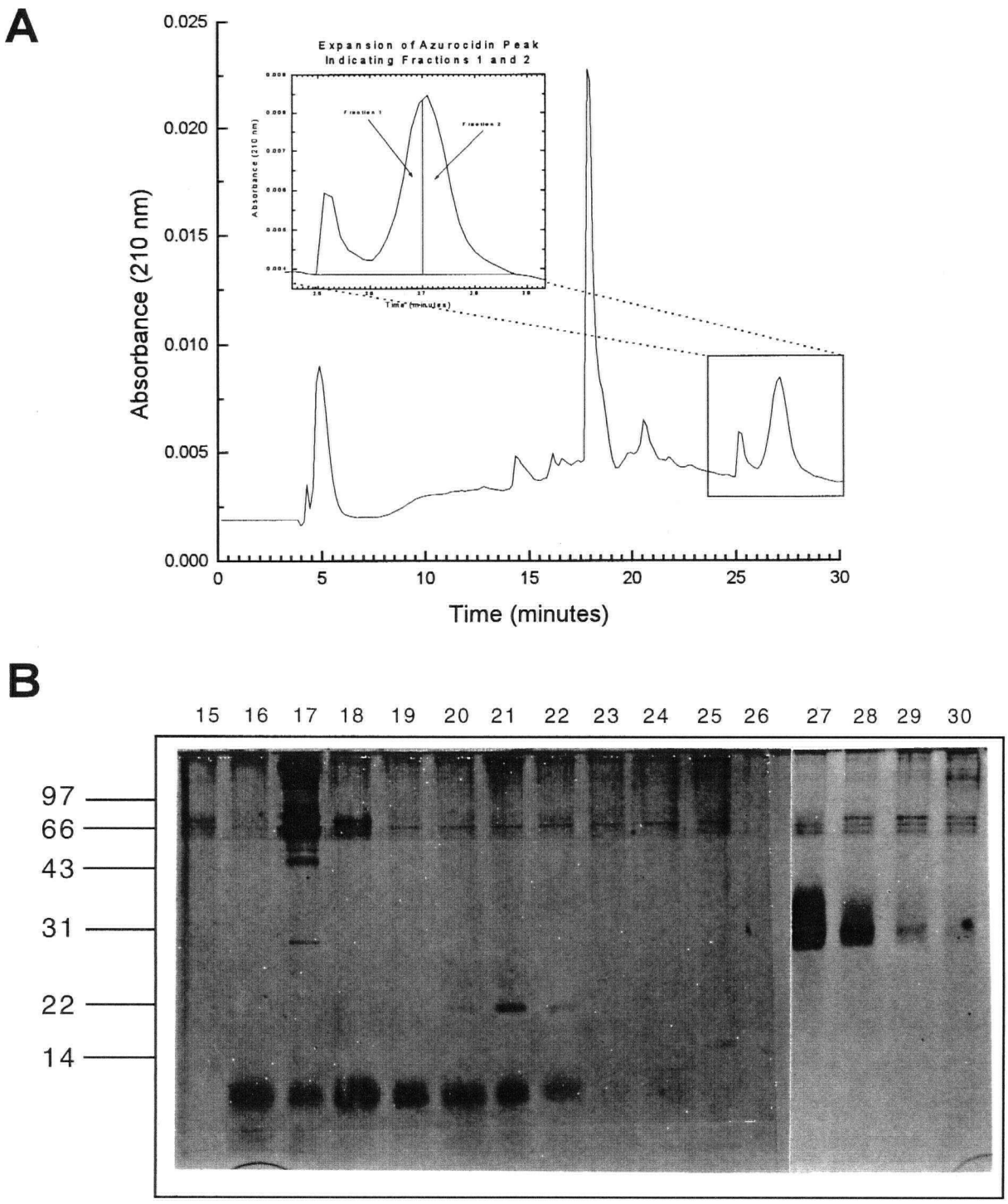


Figure 11 Analysis of alpha granule extracts from normal neutrophils

(**A**) rpHPLC separation of alpha granule extract (see figure 10) using a C_4 column and monitoring absorbance at 210 nm. Inset is an expansion of the azurocidin peak indicating fractions 1 and 2. (**B**) 12%, silver stained, SDS-PAGE analysis of the different rpHPLC fractions.

azurocidin (see figure 11a inset) indicating that the change in the acetonitrile gradient was not able to completely resolve the two peaks.

iii) Biological activity of purified azurocidin

The chromatograms of immunoaffinity enriched material from leukemic lysates seen in the previous chapter demonstrated the non-Gaussian appearance of the azurocidin peak. Figure 11a shows once again that the azurocidin peak of this normal preparation was not perfectly symmetrical. As the selective activity described in the previous chapter was only found in the leading edge of the azurocidin peak, this peak was separated into the leading shoulder (fraction 1) and the bulk of the peak (fraction 2) as indicated in the inset of figure 11a. Figure 12a shows the effect of a titration of fraction 1 and 2 material on normal murine CFU-GM. As described in chapter 3, all of the inhibitory activity on murine CFU-GM was found within fraction 1. Interestingly 200 ng/ml of the shoulder material (fraction 1) was enough to mediate 100% inhibition. Whereas with the leukemic, fraction 1 material 800 ng/ml only elicited 65-70% inhibition. The activity of normal fraction 1 material resulted in approximately 20% at 2 ng/ml. Fraction 2 material, on the other hand, had no effect on murine CFU-GM, even at concentrations up to 1.3 ug/ml. As before, no inhibitory activity was mediated by either fraction on CML CFU-GM (figure 12b). These findings are in keeping with those of the previous chapter suggesting that specific glycoforms eluting in the leading edge of the azurocidin peak alone mediated a selective inhibitory activity on normal CFU-GM while having enhancing effect on CML CFU-GM.

In an attempt to further characterize the activity of the normal material, an effort was made to obtain further preparations with activity. Figure 13a shows the rpHPLC chromatogram of a subsequent normal azurophilic granule extract. This separation was run under the same conditions as that in shown figure 4a (chapter 3) because of the lack of success we had in

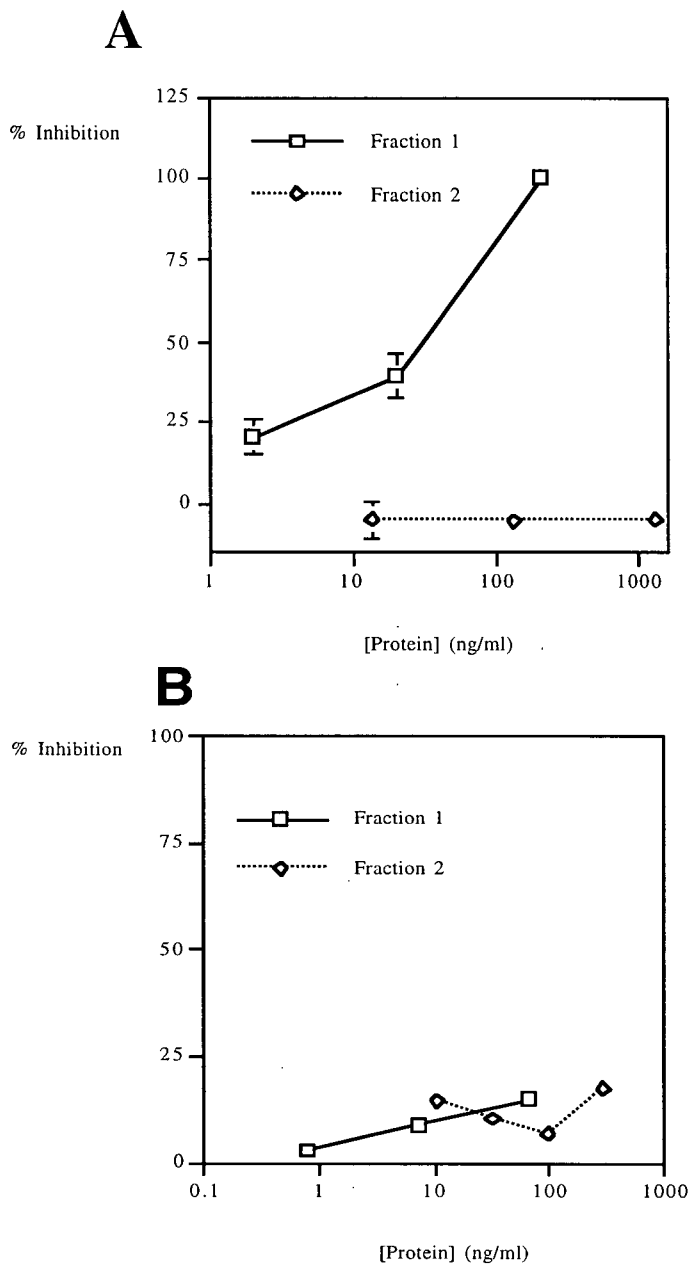


Figure 12 Biological activity of fractions 1 and 2 from figure 11

(**A**) Comparison of the effects of varying concentrations of fractions 1 and 2 on day 7 murine CFU-GM formation. (**B**) Comparison of the effects of varying concentrations of fractions 1 and 2 on day 14 CML CFU-GM formation. Error bars indicate SEM. (Data from one experiment is shown)

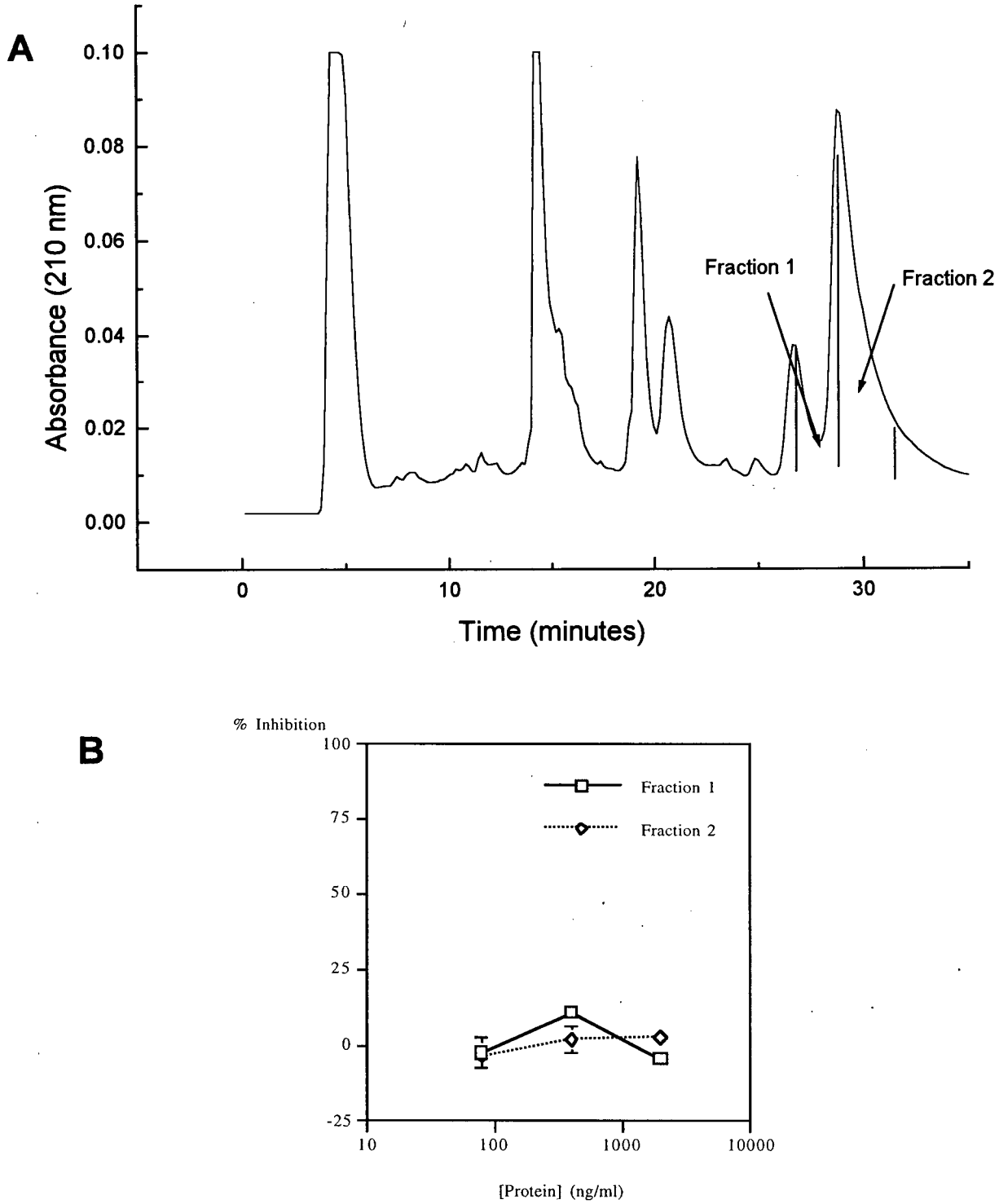


Figure 13 Analysis of another normal azurocidin preparation

(A) rpHPLC separation of alpha granule extract (see figure 10) using a C_4 column and monitoring absorbance at 210 nm. Inset is an expansion of the azurocidin peak indicating fractions 1 and 2. **(B)** Comparison of the effects of varying concentrations of fractions 1 and 2 on day 7 murine CFU-GM formation. Error bars indicate SEM. (Representative of 5 other separations)

separating the lysozyme and azurocidin peaks with the altered gradient (figure 11a). As expected, due to the use of an identical acetonitrile gradient, a very similar chromatogram to that of figure 4a was seen with lysozyme eluting just prior to azurocidin which eluted at approximately 20.5 minutes.

In contrast to other preparations, the non-Gaussian shape of the azurocidin peak was not observed. In any case, the shoulder of the azurocidin peak (fraction 1) and the bulk of the peak (fraction 2) were compared for their activity on normal murine CFU-GM (figure 13b). Surprisingly, no inhibitory activity can be seen in either fraction. Furthermore, as table 2 indicates, the subsequent five normal azurocidin purifications had no inhibitory activity whatsoever on normal hemopoiesis.

Table 2 Effect of various normal azurocidin preparations

Summary of inhibitory activity mediated by various normal azurocidin preparations on normal murine bone marrow derived CFU-GM				
Preparation Number	Concentration of Azurocidin Shoulder (ng/ml)	% Inhibition ±SEM	Concentration of Azurocidin (ng/ml)	% Inhibition ±SEM
1	4000	8.2 ± 1.5	4000	4.1 ± 5.6
1	800	-21.3 ± 8.6	800	3.2 ± 5.2
1	32	6.4 ± 3.1	160	9.8 ± 6.4
2	2000	1.9 ± 0.6	1000	-3.7 ± 1.5
2	400	6.1 ± 3.2	50	13.1 ± 5.8
2	20	-2.8 ± 1.4	5	-3.7 ± 3.6
3	2000	-4.7 ± 2.2	2000	2.3 ± 3.1
3	400	10.1 ± 2.4	400	1.4 ± 4.5
3	80	-3.0 ± 5.0	80	-3.8 ± 1.2
4 & 5*	1000	-5.3 ± 4.3	NT	NT
4 & 5*	200	-18.0 ± 5.8	NT	NT
4 & 5*	40	-8.5 ± 3.1	NT	NT

*Pool of two preparations testing a pooled fraction containing azurocidin and its shoulder

NT not tested alone, pooled with the shoulder

4.4 Discussion

In this chapter, we have demonstrated the purification of neutrophil derived azurophilic granules using nitrogen cavitation and Percoll density gradient centrifugation. After rpHPLC separation of the contents of azurophilic granules it was possible to purify azurocidin. The previous chapter provided evidence that the specific glycoforms of azurocidin from leukemic immunoaffinity preparations mediated a selective inhibitory activity on normal myelopoiesis while having enhancing effect on leukemic (CML) progenitors. The initial azurocidin purification presented in this chapter demonstrated similar if not identical activity to that seen previously with the leukemic preparations. Subsequent azurocidin preparations from azurophilic granules of normal neutrophils, despite having similar, even identical rpHPLC chromatograms and SDS-PAGE analyses, failed to have any biological activity on normal hemopoiesis. Therefore, from the data presented in this chapter, it is unclear whether or not normal neutrophils contain the selective inhibitory activity described in chapter 3. However it is clear that equivalent treatment of normal material does not consistently yield material with activity. It is possible that the normal activity is more labile than the leukemic activity and thus more susceptible to inactivation by the organic solvents used during rpHPLC and the lyophylization of the samples after the chromatographic procedure. There are many examples of molecules which are affected by these harsh treatments. It is also conceivable that the activity is simply less prevalent in normal cells. As malignant cells are known to mediate abnormal protein glycosylation (172, 173), transformed neutrophils with the Bcr-Abl translocation may produce more of the active glycoforms of the molecule. Alternatively, these cells may have fewer ways of inactivating the activity (eg. protease deficiencies). Another, although highly unlikely possibility is that an unknown leukemia specific molecule exists which co-elutes with

the active glycoforms of azurocidin and co-migrate with it in two dimensional electrophoresis and has the ability to mediate the previously described activity. In order to rationalize this possibility one would also have to assume that a toxic substance was in the one sample tested from the first normal preparation or that the donor, whose granulocyte numbers were within normal levels, was not exactly normal.

In light of the profound difficulties in obtaining reproducible activity from normal cells, and because the goal of this research was to identify negative regulators of hematopoiesis, further work focused on the purification, identification and characterization of a previously described crude inhibitory activity from normal neutrophils originally described by Böyum and colleagues.

CHAPTER 5 - LOCALIZATION AND CHARACTERIZATION OF HEMOPOIETIC INHIBITORY ACTIVITY FROM NORMAL AND LEUKEMIC GRANULOCYTES

5.1 Introduction

As alluded to previously, hemopoiesis involves a delicate balance between positive and negative signals. Dr. Leo Sachs, in 1969, first proposed the idea of a negative feedback mechanism in the maintenance of neutrophil numbers (174). Since then several investigators have provided evidence to support this theory. Broxmeyer et al provided evidence over 15 years ago for the neutrophil specific granule protein lactoferrin, mediating inhibitory effects on myeloid progenitors (86). Böyum and colleagues described, in 1976 an inhibitory activity on normal human and murine myelopoietic progenitors derived from crude lysates of normal and leukemic (CML) granulocytes (147).

In light of the problems described in the previous chapter in obtaining consistent inhibitory activity out of the azurophilic granules of normal neutrophil preparations, we decided to focus our efforts on extending the characterization and purification of the molecule responsible for the activity first described by Böyum. This activity was chosen because at that time, it was not well characterized and all analyses were performed using crude neutrophil lysates. Lysates from both normal and leukemic (CML) neutrophils were found to have inhibitory activity on both murine and human CFU-GM (148). These studies showed that other neutrophil derived inhibitors such as lactoferrin or the hemoregulatory peptide first described by Paukovits (82) were not responsible for the observed activity. In addition, thymidine was found to be an important co-factor for the effects of the crude lysates (150). The possibility that this activity might be the same as that described in our previous work was also considered.

5.2 Objective

The objective of this chapter was to repeat Böyum's initial observations, to extend the characterization of this activity and, by using the techniques introduced in the previous chapter, to determine the subcellular localization of the molecule responsible.

5.3 Results

i) Effect of a crude granulocyte extract on normal human and murine CFU-GM formation

In order to determine if neutrophils did in fact contain an inhibitory activity on myelopoiesis, a cellular preparation (performed as described previously, figure 9 chapter 4) was subjected to hypotonic lysis. The resulting soluble fraction was used to test for biological activity in co-culture experiments on normal human and murine progenitor cells (figure 14). All biological assays in this and the following chapter were performed using CMRL medium with additives in the presence of thymidine at 1.6×10^{-4} M. The use of this medium and, most importantly, the thymidine was critical in order to observe the activity described by Böyum (150). Despite reports of the inhibitory activity of high thymidine concentrations (175, 176), the concentration used had no effect on clonogenicity. It is important to note that, due to the high level of contaminating proteins and most probably contaminating activities, the X axis label for figures demonstrating biological activity in this chapter are in granulocyte equivalents/ml. For the biochemical analyses of the following chapter, all X axis values are reported in ng/ml. This view of the concentration of material will also facilitate the interpretation of the physiological relevance of the activity. As can be seen, human and murine progenitors are equally susceptible to an inhibitory activity present in crude neutrophil lysates. From figure 14, the ED_{50} (dose at which 50% of the maximal effect is reached) is at approximately 10^5 granulocyte equivalents/ ml. This is slightly less active than the results

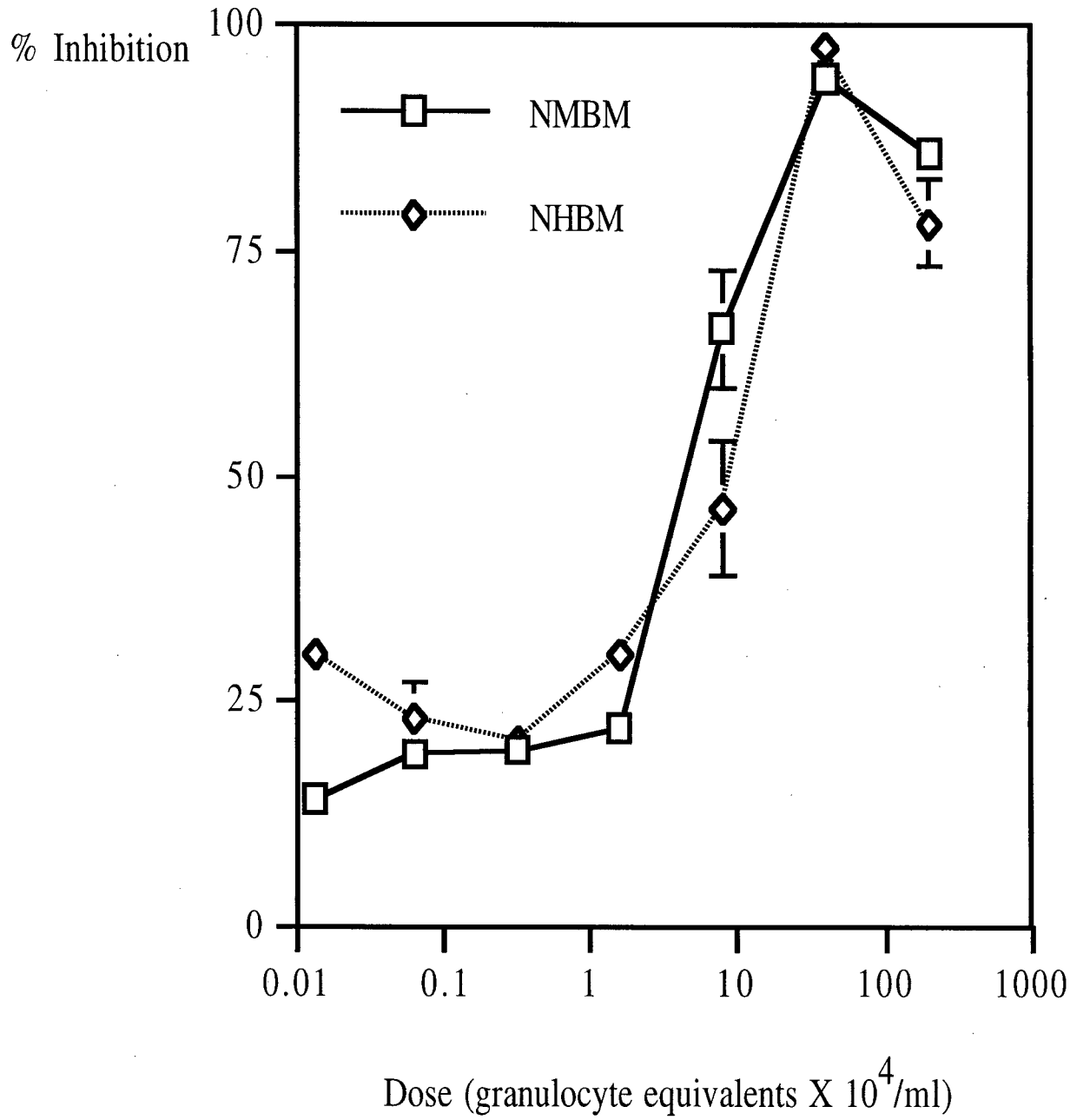


Figure 14 Effect of a normal neutrophil extract on normal human and normal murine bone marrow progenitor cells

Comparison of the effect of a crude neutrophil extract on day 7 normal murine and day 14 normal human bone marrow CFU-GM. Error bars represent SEM. (Pooled data from 3 experiments performed in triplicate)

presented by Böyum and colleagues who found that crude GRE yielded and ED_{50} of approximately half this value (148). Although this is not a large difference, small changes in thymidine concentration and the use of different colony stimulants may account for the observed differences.

ii) Comparison of the effect of the crude granulocyte extract on normal human vs. CML CFU-GM and cell line clonogenicity

A reason for the possible outgrowth of the leukemic clone in CML may be that leukemic progenitors are insensitive to the normal negative regulatory signals which exist in the hemopoietic system. In figure 15, the susceptibility of CML progenitors is compared to those of a normal human donor. As seen with the murine progenitors, no significant differences in sensitivity to the crude granulocyte lysate of normal donors can be seen. The colonies resulting from the CML progenitors were not tested by karyotypic analysis or PCR in order to ensure that they have originated from the leukemic clone. However, in view of the fact that in CML the majority of progenitors are of leukemic origin and that the leukemic progenitors are plated at a tenth of the concentration of normal progenitors, it is unlikely that more than five colonies were of a normal phenotype. Therefore this figure gives an idea of the overall reaction of CML peripheral blood progenitors to the crude activity of a neutrophil lysate. Any difference between the susceptibility of normal and leukemic progenitors would still be apparent even if a few normal colonies were present.

Figure 16 shows the activity of normal crude neutrophil extract material at a concentration of $5x10^5$ granulocyte equivalents/ml on the clonogenicity of a variety of factor independent, myeloid cell lines. It is evident that all of the cell lines tested were susceptible to the inhibitory activity of the extract. Interestingly, only EM2 (a cell line derived from a patient with CML in blast crisis) and HL60 (a promyelocytic cell line) were inhibited to the same extent as the primary CML cells. There is no evidence in the literature that this crude material

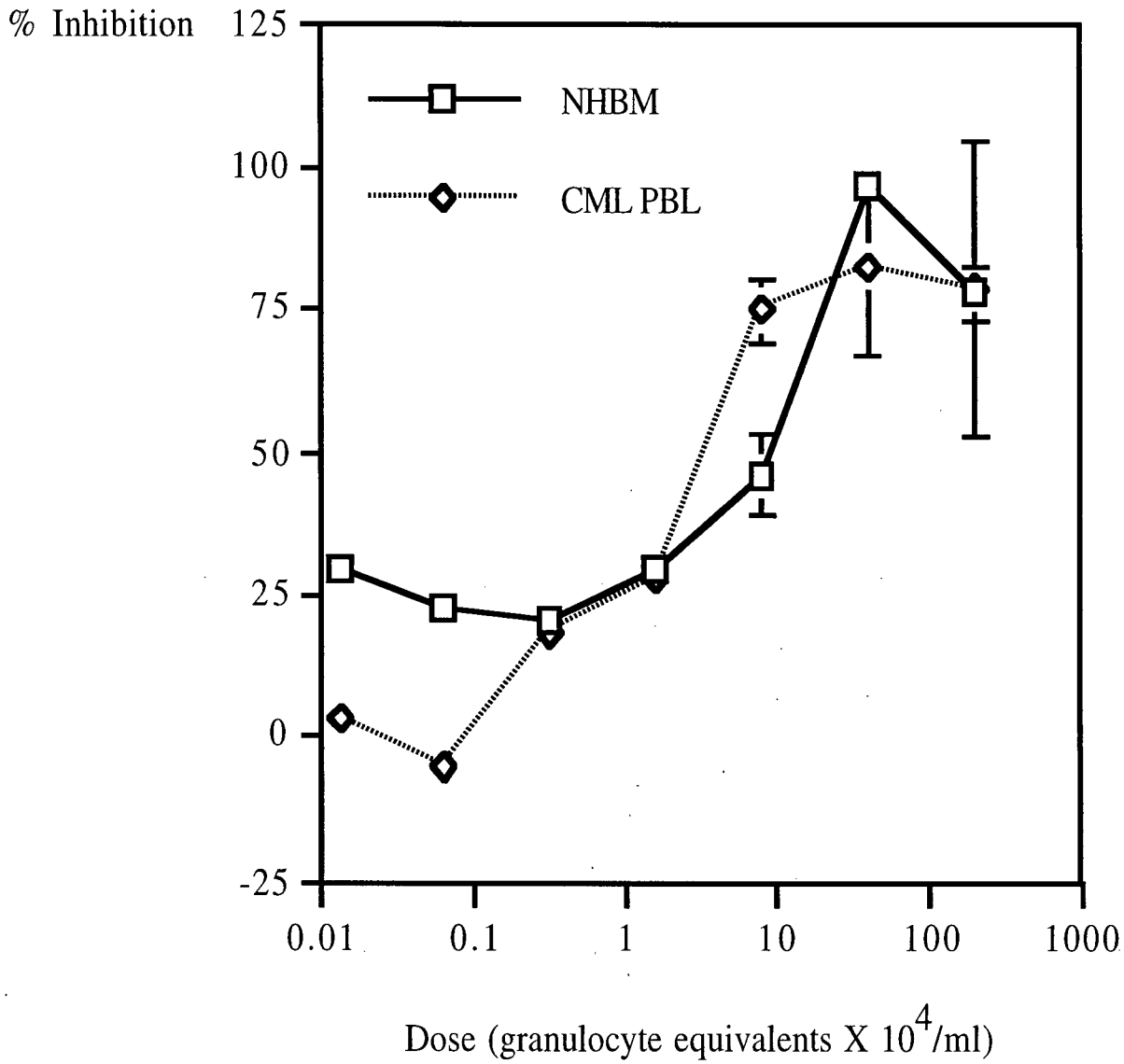


Figure 15 Susceptibility of CML and normal hemopoietic progenitors to the inhibitory activity of a crude extract from normal neutrophils

Comparison of the effect of a crude neutrophil extract on day 14 CML PBL and normal human bone marrow CFU-GM. Error bars represent SEM. (Pooled from 2 experiments performed in triplicate)

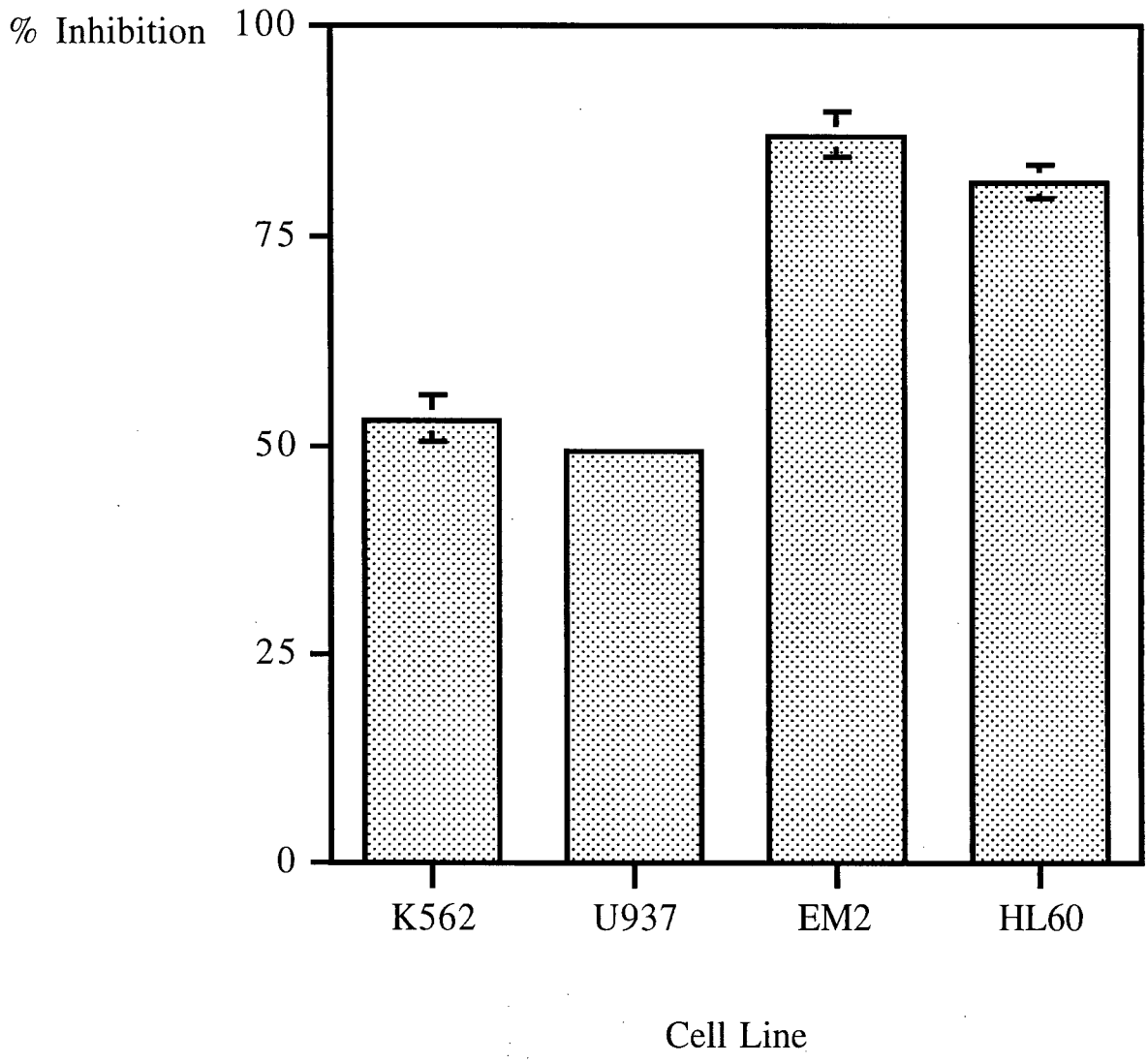


Figure 16 Effect of a crude neutrophil extract on the clonogenicity of various leukemic cell lines

Comparison of the effect of a crude neutrophil extract on the colony formation of K562 (Erythroblastoid cell line from a patient with CML), U937 (Promyelocytic leukemia cell line), EM2 (Myeloblastic cell line from a patient with CML), HL60 (Promyelocytic leukemia cell line). Error bars represent SEM. (Representative of 2 experiments performed in triplicate)

has never been tested for activity on primary CML cells or leukemic cell lines. These results do not support the hypothesis that the leukemic clone is in part established due to the insensitivity of leukemic progenitors to the normal inhibitory activity. In addition, this marks the first evidence that the Böyum activity differs from the selective inhibitory activity described in the previous results chapters.

iii) Effect of a crude neutrophil extract on cell line proliferation

Figure 17 shows the effect of a normal neutrophil extract on the proliferation of various factor independent myeloid and lymphoid lines using the proliferative MTT assay. Interestingly, the T-cell lymphoma line, Jurkat, seemed to be most inhibited. As seen in figure 16, all of the myeloid lines were sensitive to the activity. It is important to note that in this MTT experiment, a media alone control was not used to blank the samples. In subsequent MTT analyses this was performed and as a result greater differences between tests and controls were observed.

iv) Localization of crude inhibitory activity within normal and leukemic neutrophils

In order to determine the subcellular localization of the crude inhibitory activity, normal neutrophil preparations were subjected to nitrogen cavitation lysis coupled with Percoll density gradient separation of the lysate as described in the previous chapter. The various fractions obtained (figure 10) were then assayed for activity on murine myelopoiesis. Figure 18a shows a comparison of the activity of the neutrophil hypotonic lysate to the nitrogen cavitation lysate. As expected, both lysates had equivalent activity on colony formation. Figure 18b shows the activity of the subcellular neutrophil fractions on colony formation. As can be seen, the majority of the activity was contained within the gamma fraction corresponding to the plasma membrane and cytosol. Some activity was also contained within the beta fraction which is

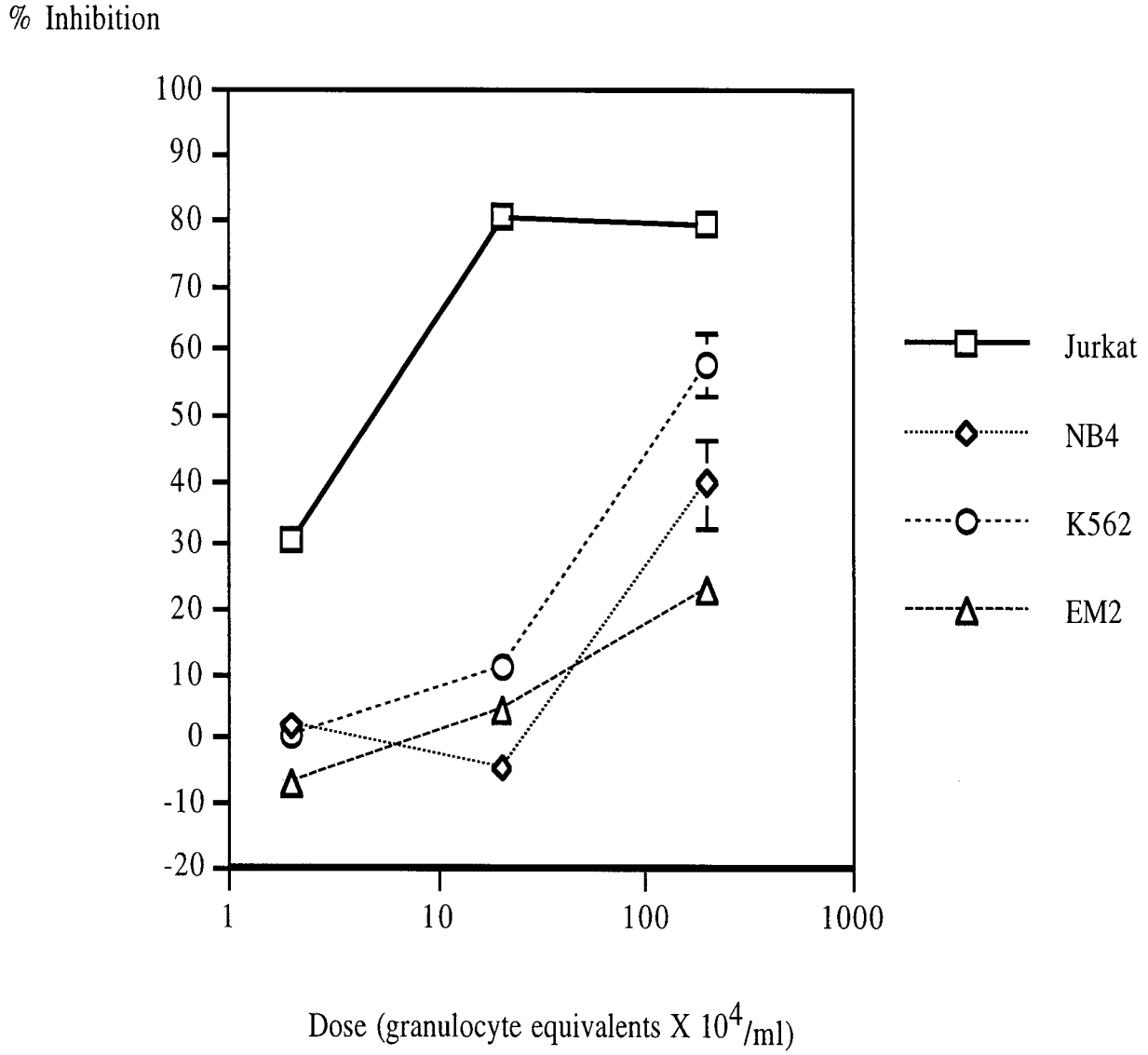


Figure 17 Effect of a crude neutrophil extract on the proliferation of various cell lines

Comparison of a three day treatment with a crude granulocyte extract on the proliferation of NB4 (promyelocytic leukemia cell line), K562, EM2 and Jurkat (T-cell lymphoma line) as measured by MTT. Error bars represent SEM.

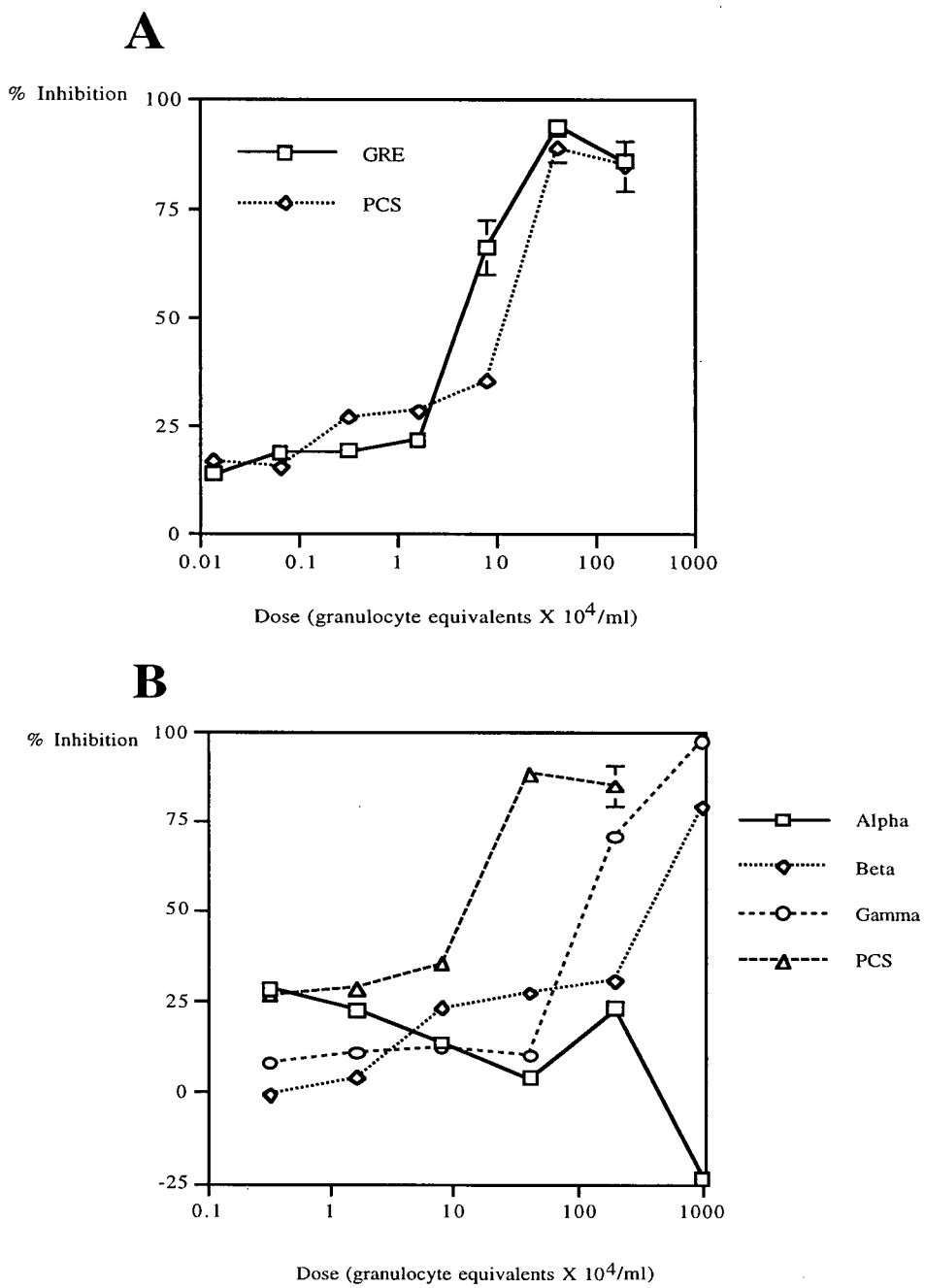


Figure 18 Localization of the inhibitory activity within normal neutrophils

(**A**) Comparison of the activity of neutrophils lysed by nitrogen cavitation or hypotonic lysis on day 7 normal murine CFU-GM formation. (**B**) Inhibitory activity of the post cavitate supernatant (PCS) subfractions (see figure 10) on day 7 normal murine CFU-GM formation. Error bars represent SEM. (Pooled data from 2 experiments performed in triplicate)

comprised of the specific granules. The alpha fraction corresponding to the azurophilic granules which contain the active glycoforms of azurocidin contained no inhibitory activity and thus provide further evidence that this activity is not the same as that described in chapters 3 and 4. Nevertheless, there is still a possibility that due to contaminating enhancing activities present within the azurophilic granule fraction that any inhibitory activity could be masked. This hypothesis was not investigated.

Differences in the localization of the activity within CML neutrophils could account for different physiological effects existing in patients with this disorder. Due to the number of neutrophils present within these patients, the release of even weak activities could have far reaching ramifications. In figure 19 inhibitory activity of subcellular fractions derived from neutrophil preparations of patients with CML are compared to those from normal neutrophils. The result of the Percoll density gradient subfractionation of the leukemic neutrophils was identical to that of the normal neutrophil preparations (see figure 10). Figure 19 shows that the leukemic neutrophils do contain the crude activity (CML, PCS activity) a fact which is consistent with the observations of Böyum and colleagues (148). Furthermore, this figure demonstrates that the localization of inhibitory activity differs from that of normal cells. In the leukemic neutrophils, the alpha, azurophilic granule fraction contains a considerable amount of activity, whereas in normal neutrophils, this fraction does not (figure 18b). Chapter 3 demonstrated how glycoforms of azurocidin, out of neutrophils of patients with CML, were believed to mediate an inhibitory activity on normal CFU-GM. This protein is localized to the azurophilic granules and thus this data is in keeping with the inhibitory activity of the azurophilic granule fraction seen in figure 19. Furthermore, in chapter 4 the majority of normal azurocidin preparations were found not to have inhibitory activity thus explaining the lack of inhibitory activity of the azurophilic fraction from normal neutrophils.

In order to determine if leukemic cell lines contained the crude inhibitory activity found within normal and leukemic neutrophils, a number of cell line lysates were tested for biological activity. These cell lines, which were all of a myeloid, blastic phenotype, included K562,

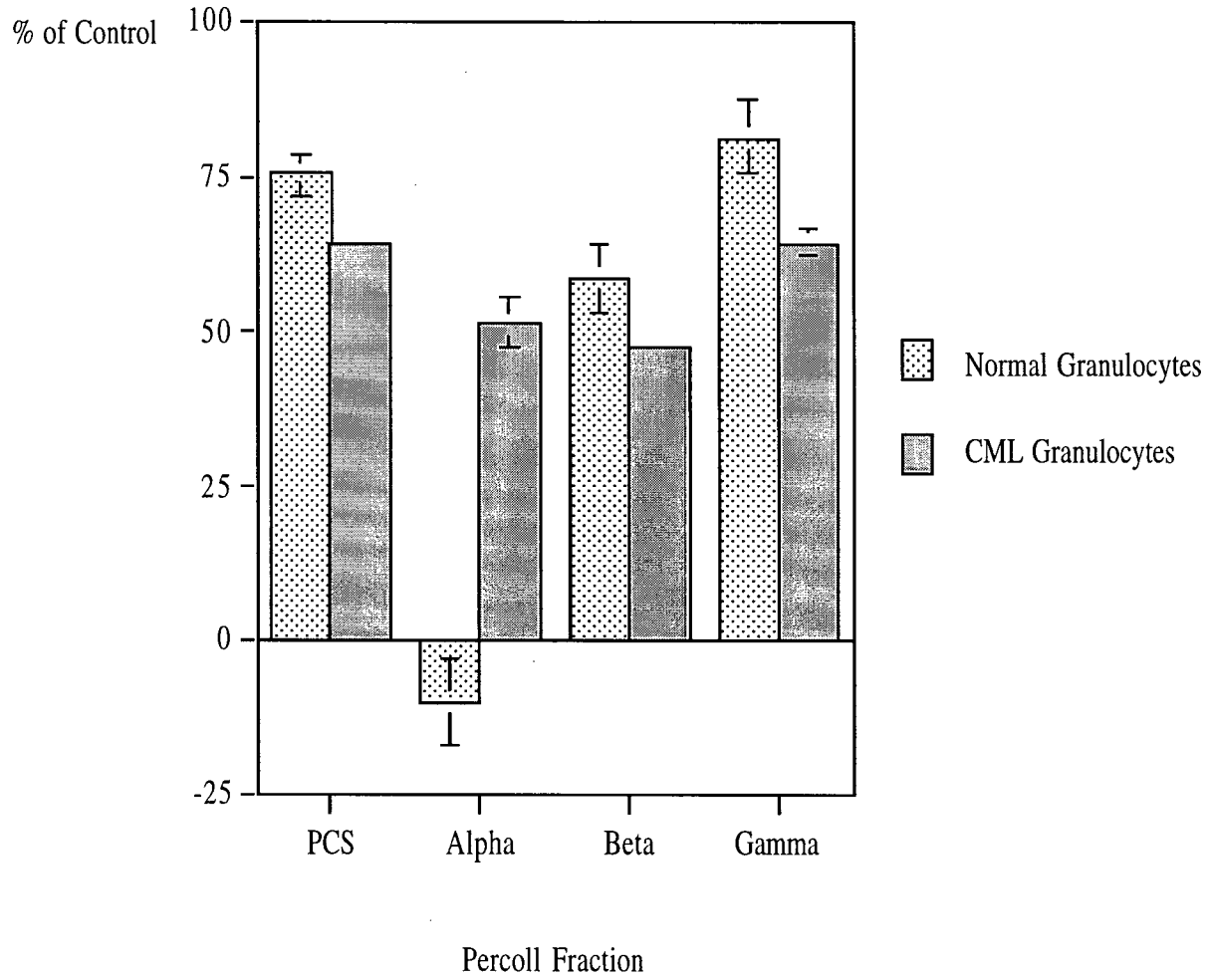


Figure 19 Comparison of the localization of the inhibitory activity in normal versus CML neutrophils

Effect of PCS (post cavitate supernatant) subfractions from both normal and CML neutrophils on day 7 normal murine CFU-GM. All fractions were tested at 1 x 10^7 granulocyte equivalents/ml). Error bars represent SEM. (Pooled data from 2 experiments performed in triplicate)

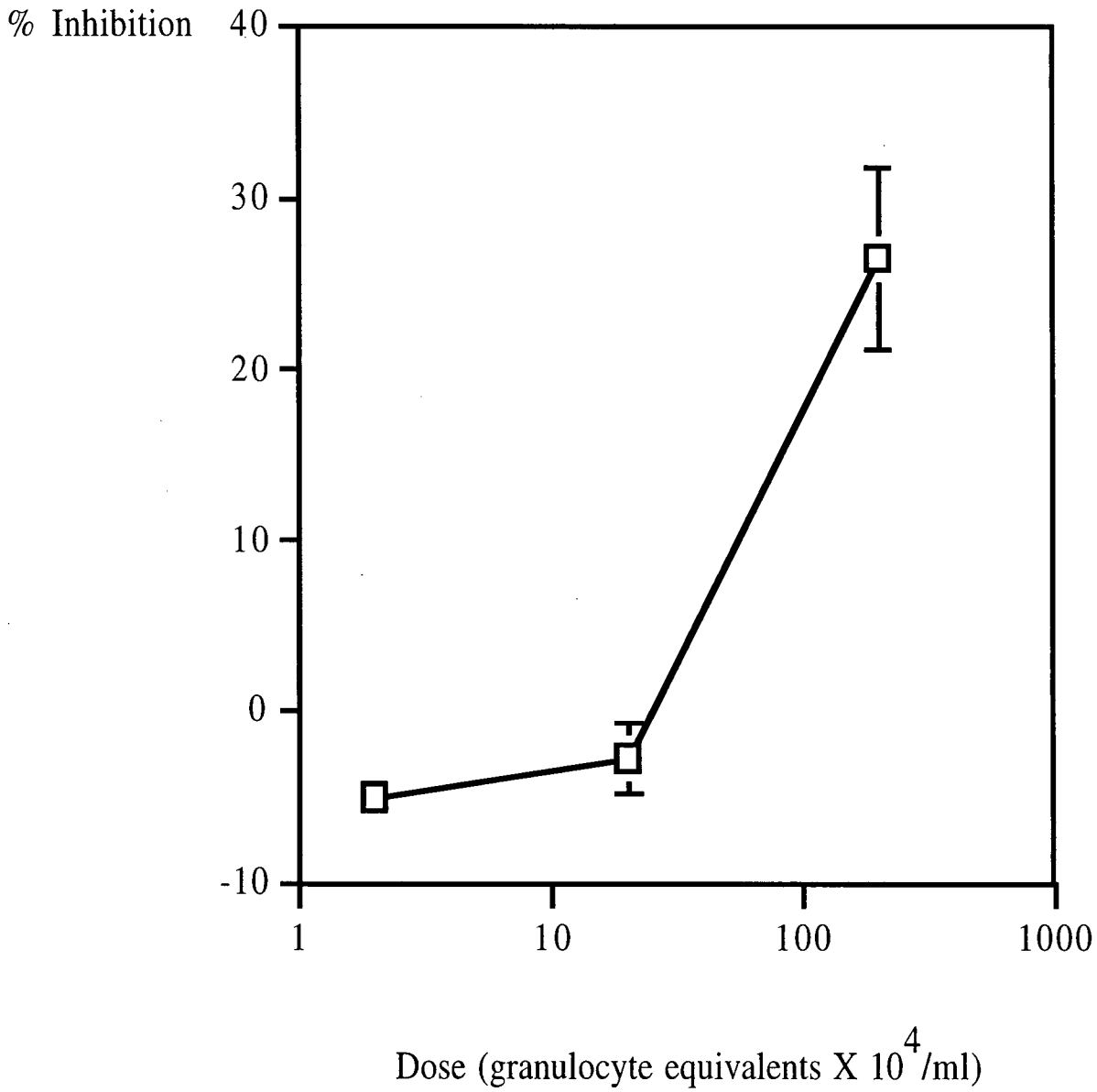


Figure 20 Effect of a crude KG1a extract on K562 proliferation

Inhibitory effect of a three day treatment with a crude KG1a (histiocytic leukemia cell line) extract on the proliferation of K562 as measured by MTT. Error bars represent SEM. (Representative of 2 experiments)

HL60, NB4, and KG1a. Interestingly, of all the lines tested, only KG1a produced any inhibitory activity (figure 20). As can be seen the highest dose, 2 X 10^6 cell equivalents/ml only yielded 30% inhibition of K562 proliferation whereas at this concentration normal neutrophil lysates mediate a 70% inhibition (Data not shown). These results are not surprising in light of the immaturity of the cell lines tested, many of the proteins present within mature myeloid cells would not be synthesized in these cells. In view of these results, the most active gamma (cytosol and plasma membrane) fraction of normal neutrophils was used to further investigate this activity.

v) Biological activity of normal gamma Percoll fraction material

From figure 18 it is apparent that the majority of the inhibitory activity contained within neutrophils is found within the cytosolic and plasma membrane fraction (on a per gram basis). Therefore, all further characterization and purification studies used this as the starting material. Figure 21 compares the activity of this material on normal human versus CML progenitor cell colony formation. At higher concentrations of material, normal and leukemic progenitor cells are equally susceptible to the inhibitory activity. However, at lower concentrations, below 6 x 10^5 granulocyte equivalents/ml normal progenitors appear to be less sensitive than their leukemic counterparts. As this effect does not diminish with decreasing dosage, it is difficult to draw any conclusions regarding this apparant difference. In any case, from these results, it is clear that mature progenitor cells are sensitive to an inhibitory activity found within the gamma Percoll fraction. The next section addresses the susceptibility of primitive progenitors from normal donors to this activity.

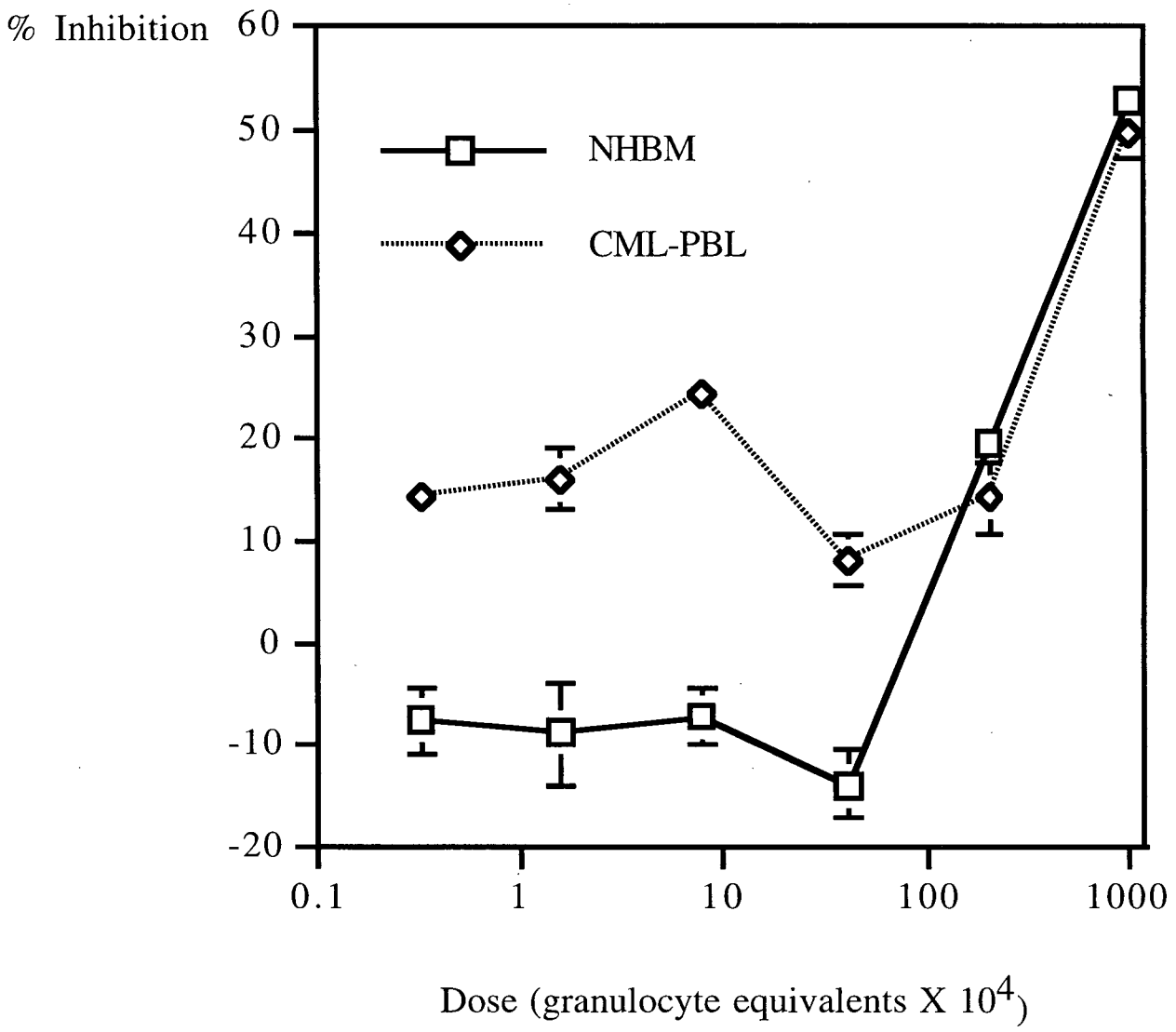


Figure 21 Effect of the gamma Percoll fraction from normal neutrophils on normal and CML hemopoietic progenitors

Comparison of the inhibitory activity of the gamma Percoll fraction (figure 10) on day 14 CML and normal human CFU-GM. Error bars represent SEM. (Pooled data from 2 experiments done in triplicate)

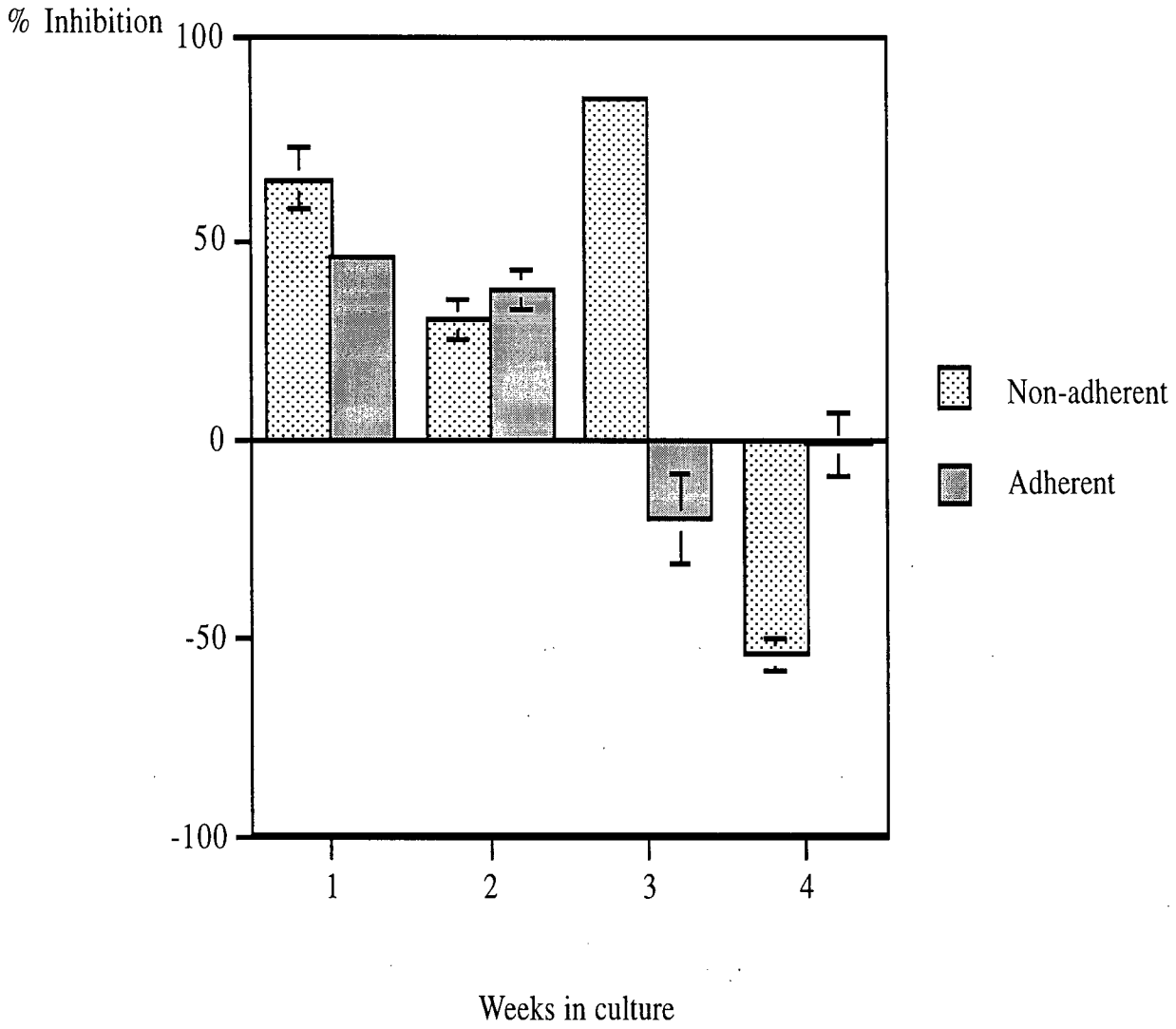


Figure 22 Effect of the gamma Percoll fraction from normal neutrophils on normal, primitive progenitor cells

Effect of an overnight treatment of normal bone marrow progenitor cells with the normal gamma Percoll fraction on the ability to maintain long term myelopoiesis in an *in vitro* long-term culture assay. Error bars represent SEM. (Data from one experiment is shown)

vi) Effect of gamma Percoll material on normal, primitive progenitor cells by long-term culture

The long-term culture system involves the culture of bone marrow cells on top of irradiated stroma comprised mainly of fibroblasts and adipocytes. Because of the presence of various culture factors and interactions with the stroma, stem cells grow, divide, differentiate and die in a manner thought to be analogous to *in vivo* hemopoiesis (19). Cultures are analyzed by a traditional colony assay on a weekly basis. As the initial stromal cultures age, the effect of a treatment on a progressively more primitive progenitor cell can be investigated. In figure 22 the effect of an overnight treatment by the gamma fraction on primitive myelopoiesis is observed. Two cell populations were investigated. The non-adherent fraction comprises the cells in the culture supernatant as opposed to the adherent fraction found associated with the stromal layer. As can be seen, the adherent fraction becomes less susceptible to the inhibitory activity with time in stromal culture. All of the inhibitory activity on this population is lost by three weeks in culture. By contrast the non-adherent population had greatest susceptibility to the activity at three weeks. At four weeks an enhancement of colony formation was seen. Therefore, it appeared that the more primitive progenitors of both populations were insensitive to the inhibitory activity of the gamma Percoll fraction of normal neutrophils.

vii) The inhibitory activity of the gamma Percoll fraction is protein mediated

Due to the crude nature of the material containing the activity, it was not evident whether or not this was a protein mediated effect. Recent evidence suggests that leukotrienes and lipoxins, lipoxygenase products formed by leukocytes and neutrophils, can inhibit CFU-GM formation. Therefore, it was important to establish whether or not the observed effect was protein mediated. Figure 23 shows the result of various treatments on the biological activity of the gamma Percoll fraction. Both a one hour 80°C incubation and protease digestion of the

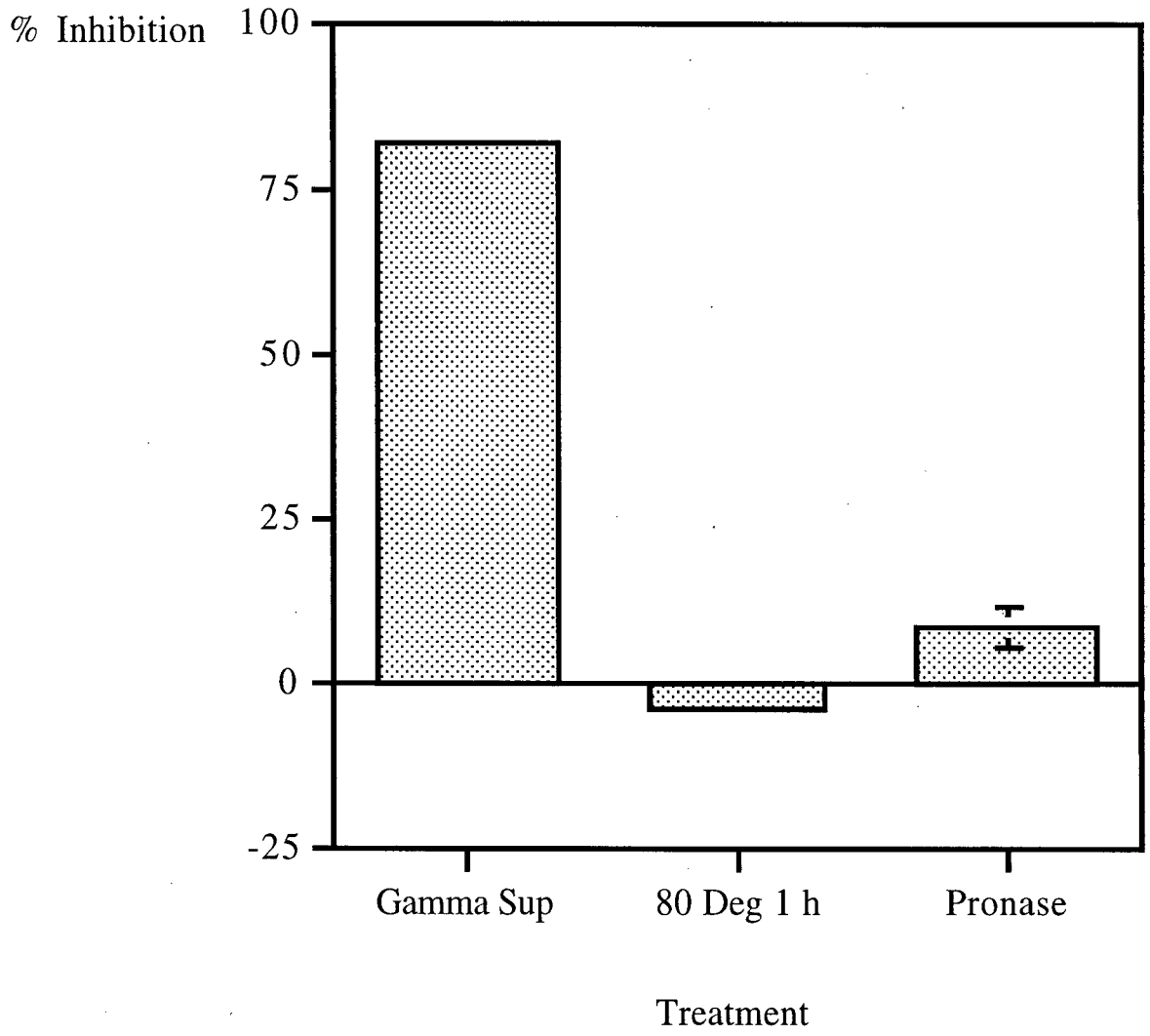


Figure 23 Effect of various treatments on the inhibitory activity of the normal gamma Percoll fraction

Normal gamma Percoll fraction at 3 μg/ml (final concentration) was either incubated at 80°C or treated with 18 mg of pronase/agarose (1:3 E/S) at 37°C. After 1 hour, the samples were centrifuged and supernatants used to treat normal murine bone marrow and day 7 CFU-GM formation was monitored. Error bars represent SEM. (Data from one experiment)

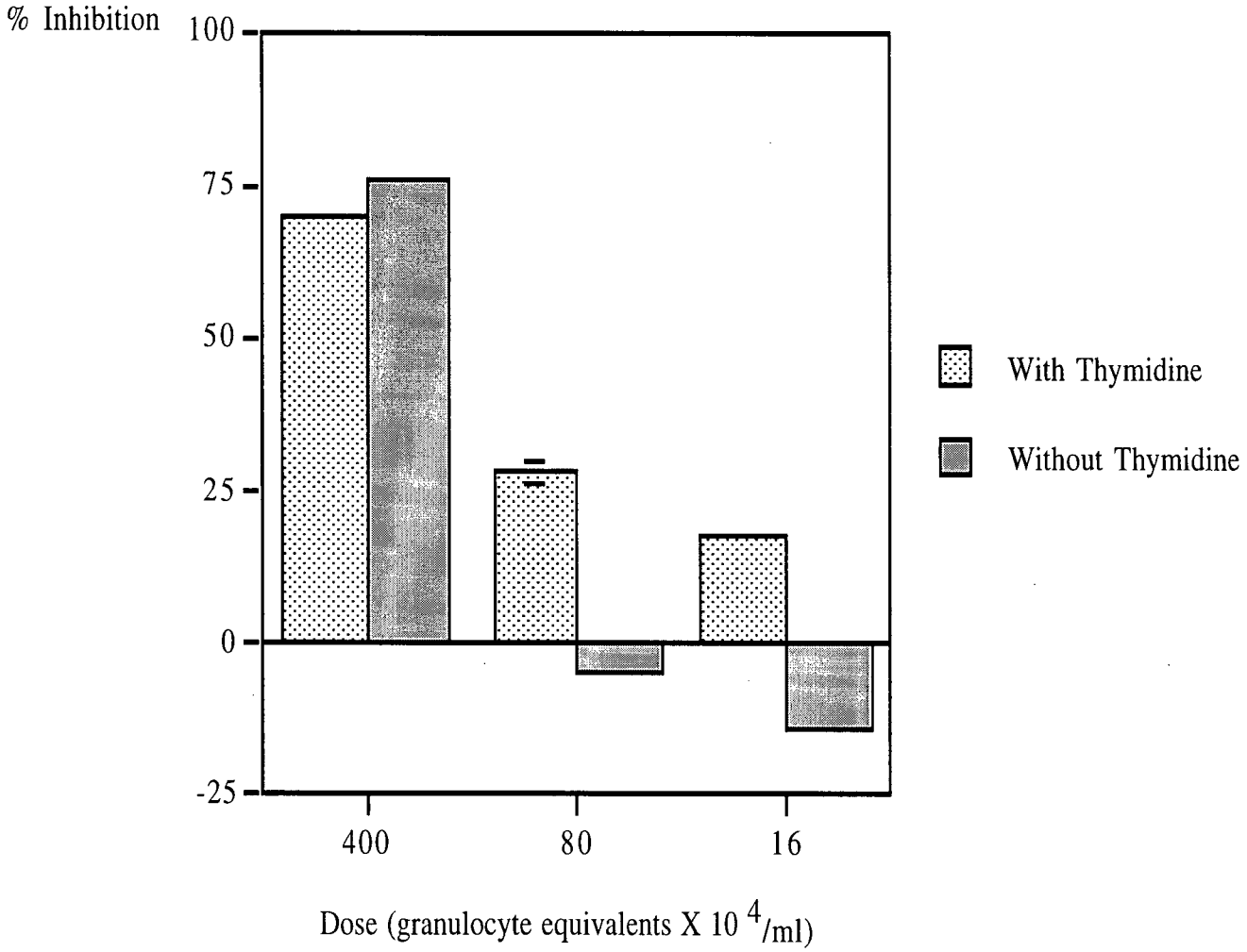


Figure 24 Effect of thymidine on normal neutrophil derived inhibitory activity on K562 proliferation

Gamma Percoll fraction material was incubated with K562 cells with or without the presence of 180 μM thymidine for 3 days. Proliferation was then determined using the MTT assay. Error bars represent SEM. (Representative of 2 experiments done in quadruplicate)

gamma Percoll fraction resulted in a complete loss of activity. These experiments clearly indicate that the activity is mediated by a protein. The purification strategy outlined in chapter 6 reflects this result.

viii) Investigation of the thymidine dependence of the gamma Percoll fraction

Böyum and colleagues published a paper in 1988 suggesting that the inhibitory activity originally observed was thymidine dependent (150). In order to establish that our activity was the same as his, and in order to confirm his previous observations, we investigated the role of this nucleotide in the inhibitory activity of the gamma Percoll fraction. Figure 24 demonstrates the importance of thymidine to the activity. At the highest dose of the gamma Percoll fraction (4 x 10^6 granulocyte equivalents/ml), thymidine is not critical for the activity. At concentrations of 8 x 10^5 granulocyte equivalents/ml and below, the presence of thymidine was critical for the inhibitory activity.

ix) THU a known inhibitor of cytidine deaminase abrogates neutrophilic inhibitory activity

During the course of this investigation, Böyum and colleagues presented evidence that cytidine deaminase (CD) might be the molecule responsible for the activity (177). Using semi-purified material, they demonstrated that they could knock out inhibitory activity with the use of the known specific inhibitor of CD, 3,4,5,6-tetrahydrouridine (THU). Furthermore, they showed that this preparation could inactivate the chemotherapeutic drug 1-β-D-arabinofuranosylcytosine (ARA-C), a known effect of CD. The only known effect of CD is to deaminate the cytosine analogue chemotherapeutic agents such as ARA-C as well as cytidine (CR), deoxycytidine (CdR), uridine (UR) and deoxyuridine (UdR). There is no known effect

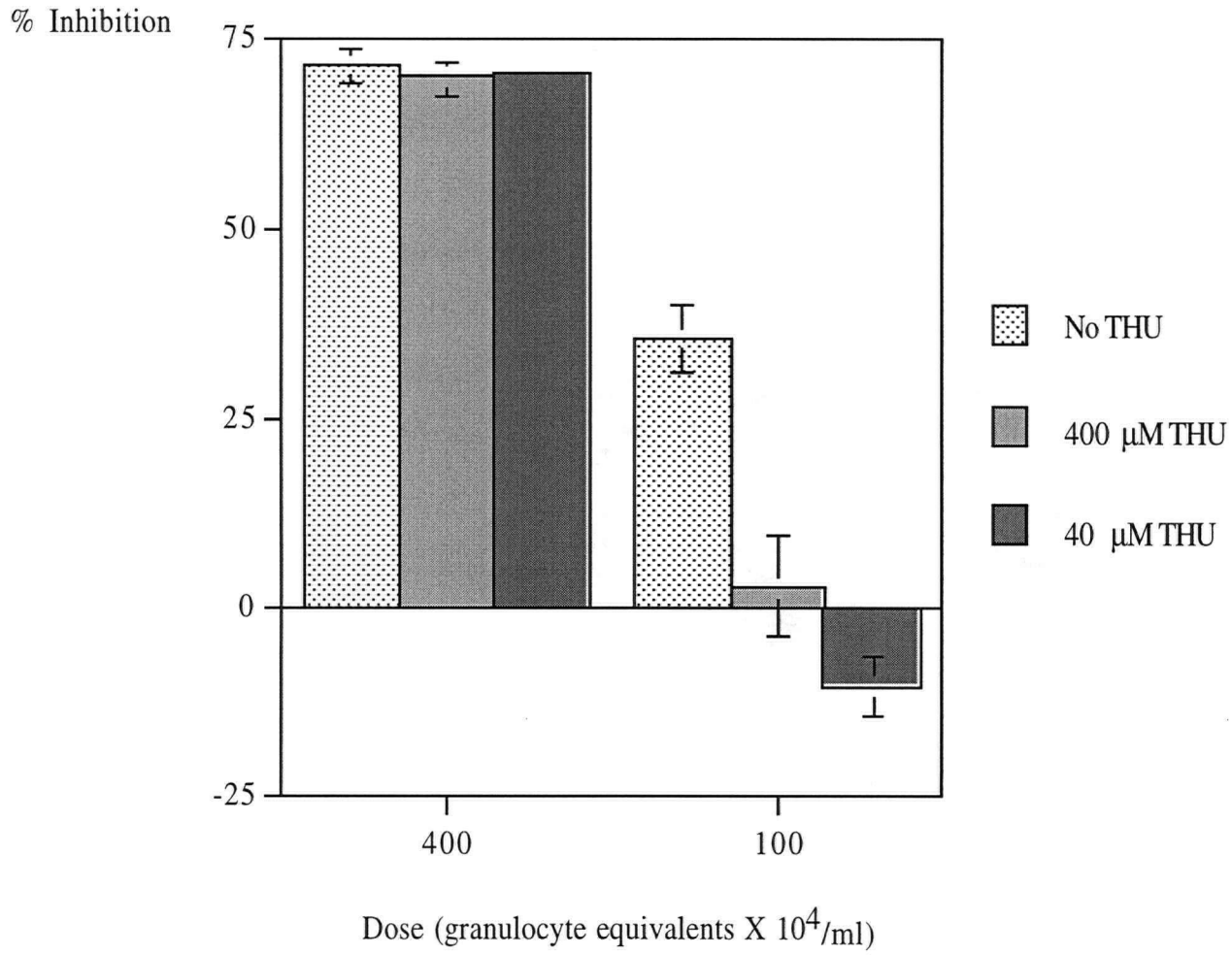


Figure 25 Effect of THU on normal neutrophil gamma Percoll fraction mediated inhibition of K562 proliferation

Gamma Percoll fraction material was incubated with K562 cells with or without the presence of tetrahydrouridine (THU), a specific inhibitor of cytidine deaminase activity, for 3 days. Proliferation was then determined using the MTT assay. Error bars represent SEM. (Representative of 2 experiments performed in quadruplicate)

of CD on thymidine. Therefore, in an effort to repeat Böyum's initial observations and again to ensure that we were working with the same activity, we investigated the effect of a THU treatment on the biological activity of the gamma Percoll fraction. In figure 25 it is apparent that THU did not interfere with the antiproliferative effect of the high dose of the extract on K562 cells. In contrast at lower gamma Percoll fraction doses (1 x 10^6 granulocyte equivalents/ml) treatments of 400 μM and 40 μM of THU result in the complete loss of inhibitory activity.

5.4 Discussion

In this chapter we have shown that normal and leukemic (CML) neutrophils do contain a myelopoietic inhibitory activity. Crude preparations from normal neutrophils were tested for activity on a variety of normal human, murine and leukemic progenitors as well as various cell lines. These experiments were performed with the understanding that the preparations were crude and that it was likely that both competing and additive activities were present. However, they provided data on the effect of the crude lysates and provided an introduction to an interesting biological phenomenon. Figure 14 showed that murine progenitors were equally susceptible to the activity as normal human progenitors. The ability of this activity to cross the species barrier may reflect the fundamental importance of this activity in the regulation of myelopoiesis. It would be interesting to determine if murine neutrophils also contained this inhibitory activity.

Figure 15 demonstrates that CML progenitors are also equally susceptible to the inhibitory activity of crude neutrophil lysates. In fact, all of the myeloid cell lines tested were susceptible to this activity. Interestingly, both proliferation (figure 17) and clonogenicity (figure 16) of the cell lines were affected. Unlike the inhibitory activity of chapters 3 and 4, this activity does not appear to be selective for normal progenitor cells.

The localization of the crude inhibitory activity in subcellular fractions was found to differ between normal and leukemic neutrophils. In normal neutrophils, inhibitory activity was detected within the beta and gamma Percoll fractions of normal neutrophils which correspond to the specific granule and cytosolic, plasma membrane fractions respectively (figure 18). By contrast, in leukemic neutrophils, all Percoll fractions, including the azurophilic granules contained a significant amount of inhibitory activity (figure 19). This is interesting in light of the results of chapters 2 and 3 which suggest that the specific glycoforms of azurocidin, a molecule found within the azurophilic granule fraction of neutrophils, mediated an inhibitory activity. In addition, the lack of activity within the azurophilic granule fraction of normal neutrophils supports the difficulties we had (chapter 3) in consistently obtaining inhibitory activity from the azurocidin containing fraction. As mentioned earlier, the activity of the crude neutrophil lysates described in this chapter did not appear to have any of the selectivity of the previously investigated activity. Therefore, it is not plausible that this activity could account for the specific outgrowth of the leukemic clone. In any case, it appears likely that this activity plays a role in the negative regulation of hemopoiesis.

As the cytosolic, plasma membrane fraction of normal neutrophils contained both the majority of activity and the majority of protein, this fraction was analyzed for further activity. Although the beta or specific granule fraction contained inhibitory activity, it is also known to contain lactoferrin a previously described inhibitor of myelopoiesis (86). Therefore, to avoid the complications of a contaminating activity, this fraction was avoided.

In an attempt to find a reliable and consistent source of active material a number of myeloid cell line lysates were analyzed for activity. Figure 20 shows the limited activity of the lysate of KG1a, a histiocytic leukemia cell line. This was the only myeloid line which showed any activity. The KG1a lysate, although active, had only approximately 25% of the activity (required four times the material to produce the same level of inhibition of K562 proliferation) of the normal neutrophil lysate demonstrated in figure 24. Because all of these lines have a very immature phenotype, it is probable that the gene encoding the molecule responsible for the

inhibitory activity is not activated until a later stage of development. As there was not the same extent of activity in KG1a lysates as the gamma fraction of neutrophils, the latter was used for further analyses.

Figure 21 demonstrates that both normal human and CML progenitors are equally susceptible to the inhibitory activity of the gamma Percoll material at higher concentrations, an observation consistent with those made with the crude lysate material (figure 15). However at lower concentrations, below 6×10^5 granulocyte equivalents/ml, the CML progenitors seemed to be more sensitive to the inhibitory activity. As this effect does not titrate, it is unclear whether this reflects a different baseline or true sensitivity to the inhibitory activity. Regardless, our observations at higher concentrations do not support the hypothesis that this activity plays a role in the selective outgrowth of the leukemic clone in CML. In this study only the granulocytic and monocytic progenitors were monitored. In depth analysis would have to be carried out to determine whether some leukemic progenitor types were more or less susceptible than their normal counterparts.

Many regulators of hemopoiesis are known to have pleiotropic effects depending on the target cell lineage, stage of differentiation, immediate microenvironment and the presence of other cytokines (57, 178). For example, TGFβ is known to inhibit primitive progenitors while enhancing more committed progenitors (77). In an attempt to determine if more primitive progenitors were more or less susceptible to this inhibitory activity, gamma Percoll material was used to treat bone marrow cells plated in a long-term culture experiment. In this study, both the adherent and non-adherent populations were investigated. Recent evidence has suggested that inhibitory signals are transmitted to cells which adhere to stroma via the $\alpha_4\beta_5$ integrin molecule (53, 179, 180). These cells may respond differently to treatment by various factors. It is apparent from figure 22 that the adherent population was susceptible for only the first two weeks of culture. On the other hand the non-adherent fraction was maximally susceptible for three weeks, but at 4 weeks clonogenicity in this fraction was enhanced. As

mentioned earlier, these were short, one time treatments with the gamma Percoll fraction just prior to the initiation of the long-term cultures. Signals sent by stromal interaction would only be felt by the cells after they have been treated. The fact that the non-adherent population remains more susceptible for a longer period of time may represent some overlap of the negative signals. Regardless, it seems as though progenitors after 4 weeks in culture are no longer affected by the single treatment. As the cells analyzed after 8 weeks in culture are generally some of the most primitive cells, this suggests that cells at the myelocyte/promyelocyte stage may be the targets of the inhibitory activity. This is in keeping with the possibility that this activity works as a negative regulator being produced by an end-stage cell to inhibit its production by progenitor cells. By acting on a fairly committed progenitor population, the effects of the inhibitory activity on neutrophil numbers would be seen soon after release and it's effects would not endure for long.

In light of recent evidence that non-protein inhibitors of hemopoiesis exist within neutrophils, it was important to establish whether or not the observed activity was protein mediated. The gamma Percoll fraction was subjected to heat denaturation (80°C for one hour) and protease digestion (pronase digestion for one hour) and the result on the biological activity on murine progenitor cells was observed (figure 23). Consistent with the activity being protein mediated, both treatments resulted in a complete inactivation of the inhibitory activity.

Amidst some controversy, Böyum and colleagues published some data indicating that their inhibitor was dependent on the presence of thymidine (149, 150). In order to assess the thymidine dependence of our activity and to further prove that we were working with the same activity, the effect of the gamma Percoll fraction was assessed with and without the addition of exogenous thymidine. From figure 24 it is clear that the presence of thymidine is only required for the inhibitory activity of gamma Percoll material at concentrations of 8×10^5 cell equivalents/ml and below. At higher concentrations, exogenous thymidine was not necessary for the activity. This is an indication that two or more inhibitors are present and that at the higher concentrations a thymidine independent activity is active.

During the course of this work, Böyum published a paper providing evidence for cytidine deaminase being the molecule responsible for the inhibitory activity (177). Again, in an attempt to show that we were working with the same inhibitor, we tested the effect of THU, a known inhibitor of cytidine deaminase activity on the inhibitory activity of the gamma Percoll fraction. As seen previously, at high concentrations of the gamma Percoll fraction, THU was not able to block the inhibitory activity whereas at lower concentrations both 40 and 400 μM resulted in a complete abrogation of the inhibitory activity. This result also supports the possibility that at least one additional inhibitor is present in this crude preparation.

In the next chapter the purification of one of the molecules responsible for the inhibitory activity of the crude gamma Percoll fraction will be discussed.

CHAPTER 6 - PURIFICATION AND IDENTIFICATION OF CYTIDINE DEAMINASE AS ONE OF THE INHIBITORS PRESENT

6.1 Introduction

In the previous chapter, the characterization of myelopoietic inhibitory activities from normal and leukemic (CML) neutrophils was described. These activities have been localized to the specific granule and cytosol/plasma membrane fraction of normal neutrophils. In addition to these two fractions, the azurophilic granules of CML neutrophils was also found to contain an inhibitory activity. These activities could prove to be important players in the normal regulation of myelopoiesis. Furthermore, despite the fact that CML and normal progenitors were equally susceptible to the inhibitory activity of the crude preparations and the gamma Percoll fraction, there was still a possibility that a distinct inhibitory activity from the azurophilic or specific granule fraction could have selective inhibitory activity on normal and not CML progenitors, thereby exacerbating the leukemic condition. To date, the molecules responsible for these activities have yet to be successfully purified. However, Broxmeyer and colleagues have provided evidence that the specific granule-associated protein, lactoferrin, may mediate inhibitory activity by limiting the release of cytokines such as G and GM-CSF (86). In light of the fact that a known inhibitory activity was present within the specific granule fraction, a decision was made to pursue the purification of the molecule responsible for the activity within the gamma Percoll fraction of normal neutrophil lysates. Results from the previous chapter have shown that this activity was both protein mediated and thymidine dependent. During the course of this work, evidence was published by Böyum et al suggesting that cytidine deaminase (CD) might play a role in the inhibitory activity of crude neutrophil lysates (177). This fact was corroborated in the previous chapter when THU, a known inhibitor of CD,

abrogated the inhibitory activity of the gamma Percoll fraction (figure 25). In this chapter we pursued the purification and characterization of the molecule responsible for the activity found within the gamma fraction. Our results provide proof that CD can, by itself, mediate a myelopoietic inhibitory activity. These results will allow for the further characterization of this novel activity and provide further evidence for negative feedback regulation in myelopoiesis.

6.2 Objective

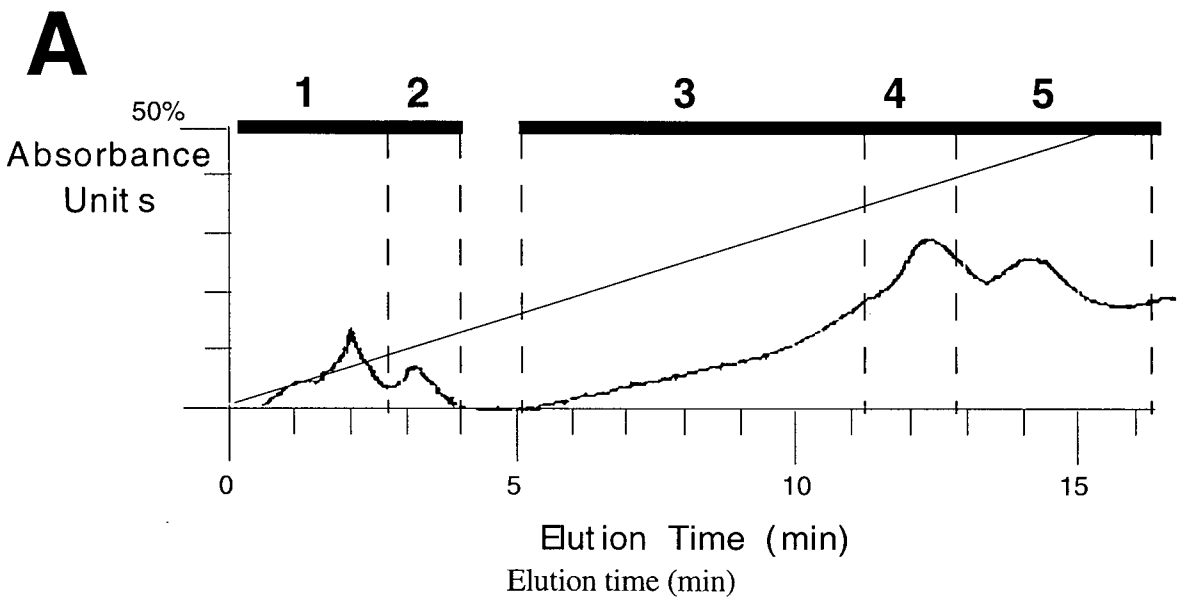
The objective of this chapter was to purify to homogeneity and to identify one of the molecules responsible for the inhibitory activity found within the plasma membrane, cytosolic fraction of normal neutrophils.

6.3 Results

i) DE52 Chromatography of the cytoplasmic fraction

In the previous chapter, evidence was provided for the presence of inhibitory activities in crude neutrophil lysates. In normal neutrophils these activities were partitioned between the beta (specific granule) and gamma (cytosol/plasma membrane) Percoll fractions. Because the specific granule fraction was known to contain an inhibitor of myelopoiesis, lactoferrin, the purification of the molecule responsible for the activity out of the gamma Percoll fraction was pursued.

Figure 26a shows a DE52, anion exchange separation of the gamma Percoll material. Fractions were collected as indicated and these fractions were tested for their effect on murine CFU-GM. From figure 26b it can be seen that there was increasing inhibitory activity in the



Elution time (min)

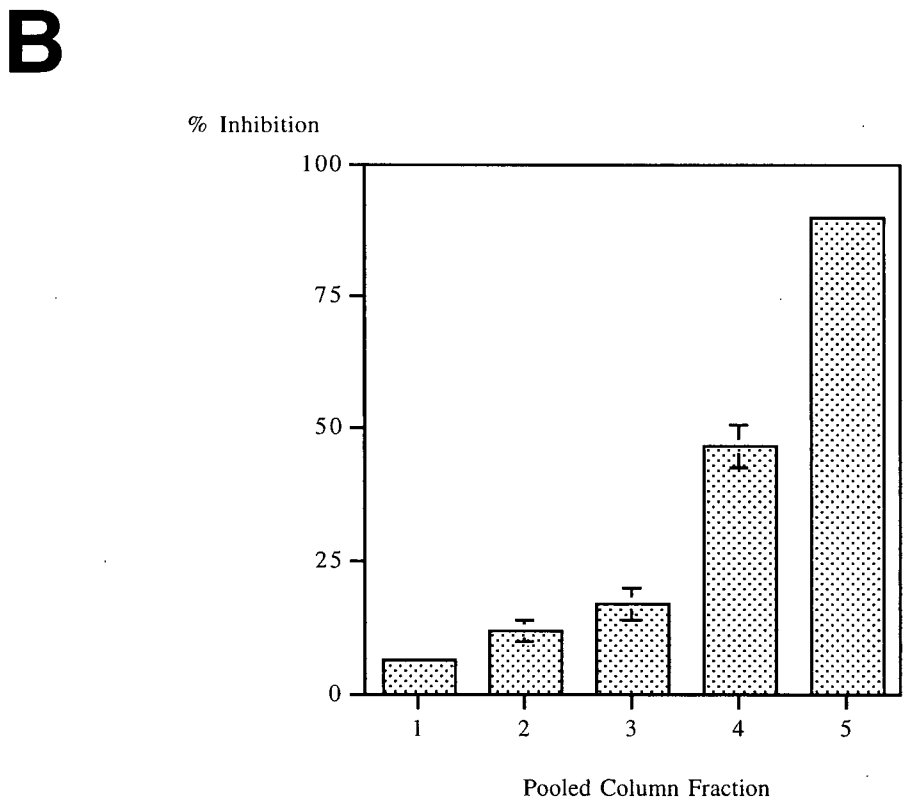


Figure 26 DE52 ion exchange chromatography of the gamma Percoll fraction

(**A**) DE52 anion exchange chromatographic separation of gamma Percoll fraction material. Fractions were pooled as indicated in the figure. (**B**) Effect of the DE52 fractions from **A** on murine CFU-GM formation. Error bars represent SEM. Control colony numbers were 183.5 ± 7.14. (Representative of 2 separations)

later eluting fractions. This reached a maximum of approximately 85% in the last fraction collected, fraction 5. SDS-PAGE analysis of the fractions demonstrated that each contained a number of bands (data not shown). Therefore, it was apparent that further purification was needed. Size exclusion separation of the most active DE52 fraction, fraction 5 was subsequently undertaken.

ii) Superose 12 size exclusion chromatography

Figure 27a shows the Superose 12, size exclusion separation of DE52 fraction 5. These Superose 12 fractions were pooled as indicated and tested for biological activity on murine CFU-GM (figure 27b). All of the inhibitory activity was contained within pooled fraction 3 material (corresponding to fractions 6 and 7). When compared with known standards, this elution time corresponded to material of approximately 55 kDa. As with the DE52 fractions, analysis of the SDS-PAGE analysis of the Superose 12 fractions revealed the presence of multiple proteins (data not shown).

During the course of the purification and characterization of the gamma Percoll fraction material, Dr. Böyum and colleagues published a paper providing evidence that cytidine deaminase (CD) was responsible for the activity out of crude neutrophil lysates (177). CD is known to be localized in the cytoplasm of neutrophils, a component of the gamma Percoll fraction (181). As a result, a purification strategy was devised based on the possibility that CD was mediating the inhibitory activity of this material. Momparler and colleague recently purified and cloned CD from human placenta (182). The purification and identification scheme that was subsequently used incorporated some of Momparler's ideas. A flow chart of the procedures is shown in figure 28.

Initially, one unit of normal peripheral blood was enriched for white blood cells by Dextran mediated sedimentation of erythrocytes. The leukocyte rich supernatant was then

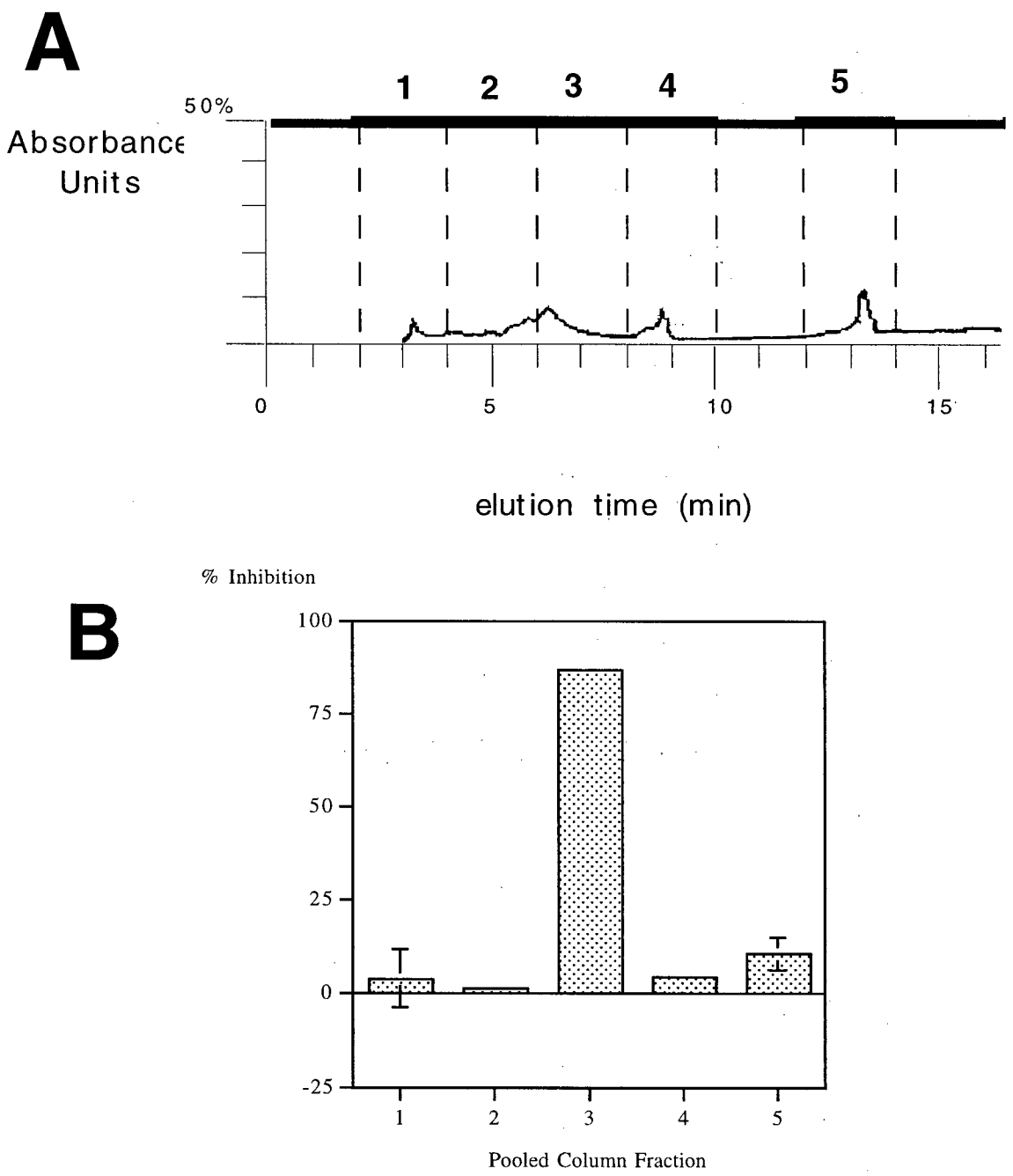


Figure 27 Size exclusion chromatography of DE52 fraction 5

(**A**) The most active DE52 fraction, fraction 5 from figure 26, was separated by Superose 12, size exclusion chromatography. Fractions were pooled as indicated in the figure. (**B**) Effect of the DE52 fractions from **A** on murine CFU-GM formation. Error bars represent SEM. Control colony numbers were 183.5 ± 7.14. (Representative of 2 separations)

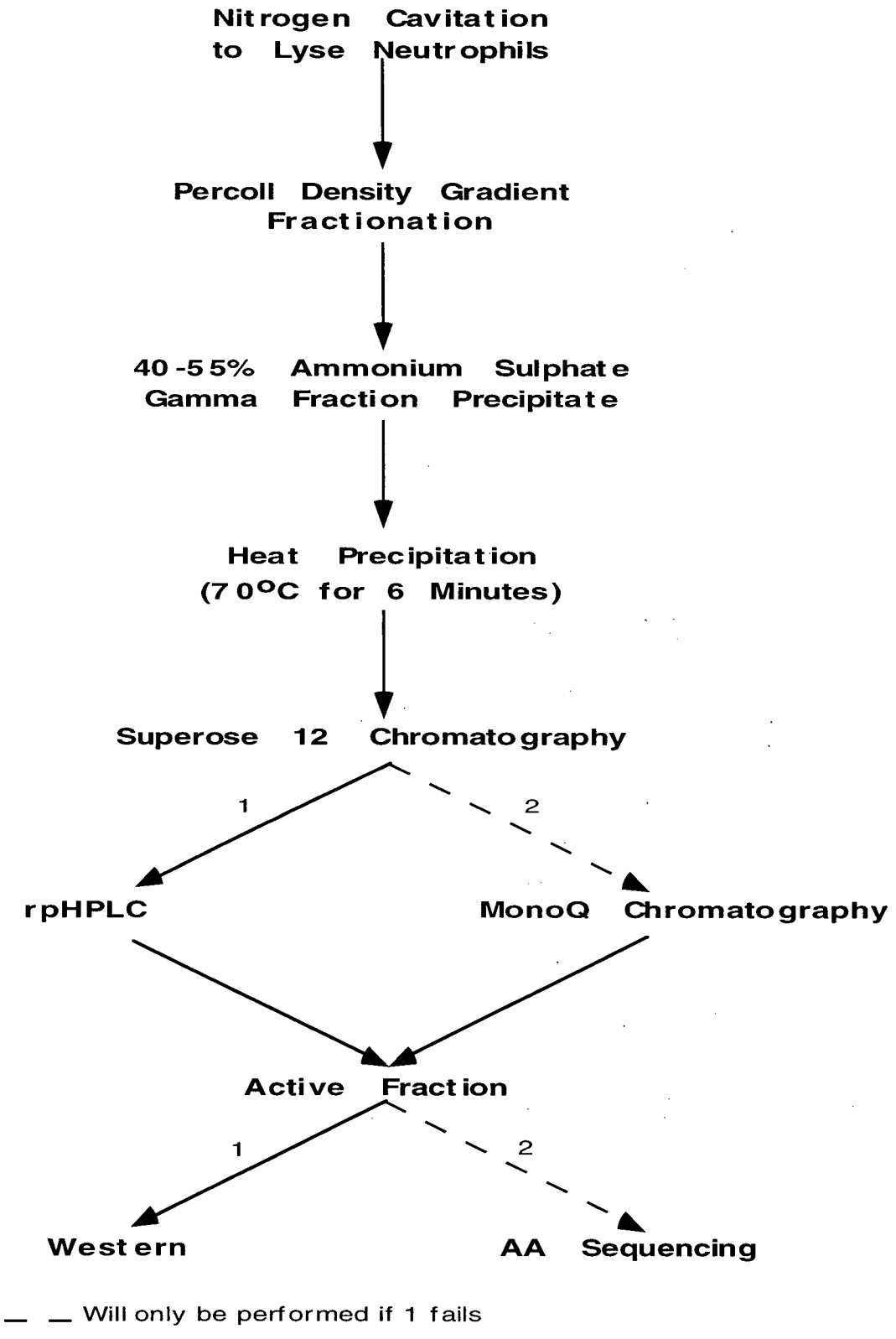


Figure 28 Flow chart outlining the purification strategy

layered onto the cellular separation medium, Ficoll, and centrifuged. The neutrophils, being more dense than the mononuclear cells, were allowed to pass through the Ficoll leaving a relatively pure pellet of cells. Residual RBC's were then removed by hypotonic lysis leaving approximately 1 x 10^9 neutrophils over 95% pure (figure 9). These cells were then lysed by nitrogen cavitation. The resulting lysate was separated by a discontinuous Percoll gradient yielding the alpha (azurophilic granule), beta (specific granule) and gamma (cytosol and plasma membrane) fractions (see figure 10). The gamma fraction was then brought to 40% saturated ammonium sulphate and the precipitate discarded. The resulting supernatant was brought to 55% ammonium sulphate and the supernatant was discarded. The 55% saturated ammonium sulphate precipitate was reconstituted in MonoQ buffer A and was then treated at 70°C for 6 minutes. This material was centrifuged, the pellet discarded, and the supernatant subjected to Superose 12, size exclusion chromatography.

Figure 29a shows the result of a size exclusion separation of the ammonium sulphate and heat treated gamma Percoll material. A very similar chromatogram to that resulting from size exclusion separation of the active DE52 material (figure 27a) was seen, except for the fact that the sensitivity of the detector is five fold more sensitive in figure 29a. There are a cluster of peaks from fractions 5 to 7 and more distinct peaks in fractions 9 and 13. Figure 29a has a peak in fraction 11 which does not appear in figure 27a. Figure 29b shows the inhibitory activity of the various Superose 12 fractions. It is obvious that all of the activity is retained within fractions 6 and 7. This corresponds to material of approximately 55 kDa. This is consistent with the size of the homotetramer in which CD exists in its native conformation which would be approximately 60 kDa. SDS-PAGE analysis of fractions 5 to 9 are shown in figure 30. In this figure, there is a major band at 15 kDa (putatively designated as CD) in fraction 6 with a number of higher molecular mass contaminants a small amount of the 15 kDa band in fraction 7 which has two prominent slower migrating and two prominent faster migrating contaminating bands. Interestingly, the two lower bands are also present in fraction 8

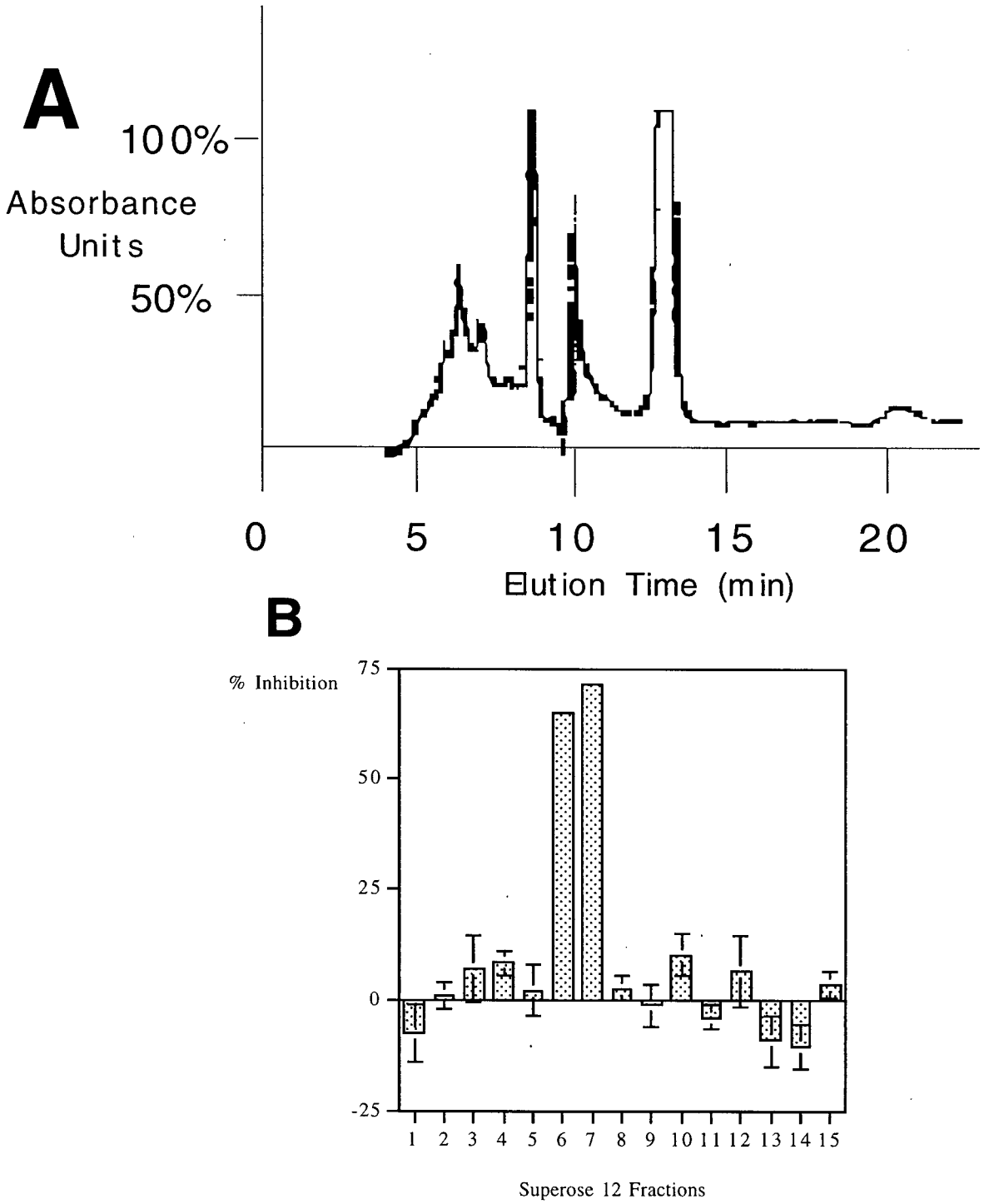


Figure 29 Size exclusion chromatography of active precipitated material

(**A**) Size exclusion chromatographic separation of ammonium sulphate precipitated neutrophil cytosol after heat treatment using a Superose 12 column. (**B**) Effect of the Superose 12 fractions from **A** on murine CFU-GM formation. Error bars represent SEM. Control colony numbers were 124.33 ± 5.90. (Representative of 2 separations)

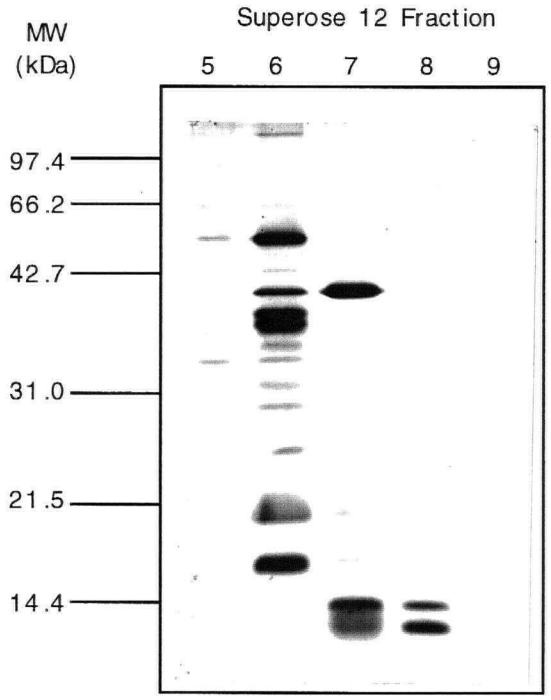


Figure 30 SDS-PAGE analysis of Superose 12 fractions

12% silver stained gel analysis of the various Superose 12 fractions from figure 29.

which has no inhibitory activity. Therefore, these bands do not appear to be responsible for the activity. Also of interest is the fact that despite having more of the 15 kDa material, fraction 6 did not inhibit colony formation more than fraction 7. In any case, further biochemical separation was necessary to purify the molecule responsible for the activity to homogeneity. As the schema in figure 28 has indicated, the next approach used rpHPLC.

iii) Reverse phase HPLC

As both DE52 anion exchange and Superose 12 size exclusion did not fully purify the molecule responsible for the activity, rpHPLC was used alone or in concert with the purification scheme outlined in figure 28 in an attempt to purify the molecule responsible for the myelopoietic inhibitory activity to homogeneity. rpHPLC separates proteins based on their interaction with a hydrophobic matrix. Hydrophobic proteins in an aqueous phase will adhere to the hydrophobic matrix. Proteins are then eluted with an increasing concentration of acetonitrile which both alters the conformation and provides an increasingly organic mobile phase thus allowing the proteins to be eluted off the column. Figure 31a shows an rpHPLC chromatographic separation of gamma Percoll material. The resulting fractions were tested for their effect on the proliferation of K562 cells (figure 31b). It was apparent from these data that the majority of the activity lay within fractions 25-28. An expanded chromatogram of these fractions can be seen in the inset of figure 31a. Based on the activity profile of the fractions, it was believed that the smaller peak centred at 26.5 minutes, which is in between the two larger peaks at 25.5 and 28.5 minutes, contained the active material. SDS PAGE analysis of the rpHPLC fractions indicated that the fractions were again quite impure (data not shown).

At this point Dr. Böyum's data implicating CD as the molecule responsible for the activity became available (177). Therefore, we separated the active, pooled fraction 6 and 7, material from Superose 12 separations of ammonium sulphate and heat precipitated gamma

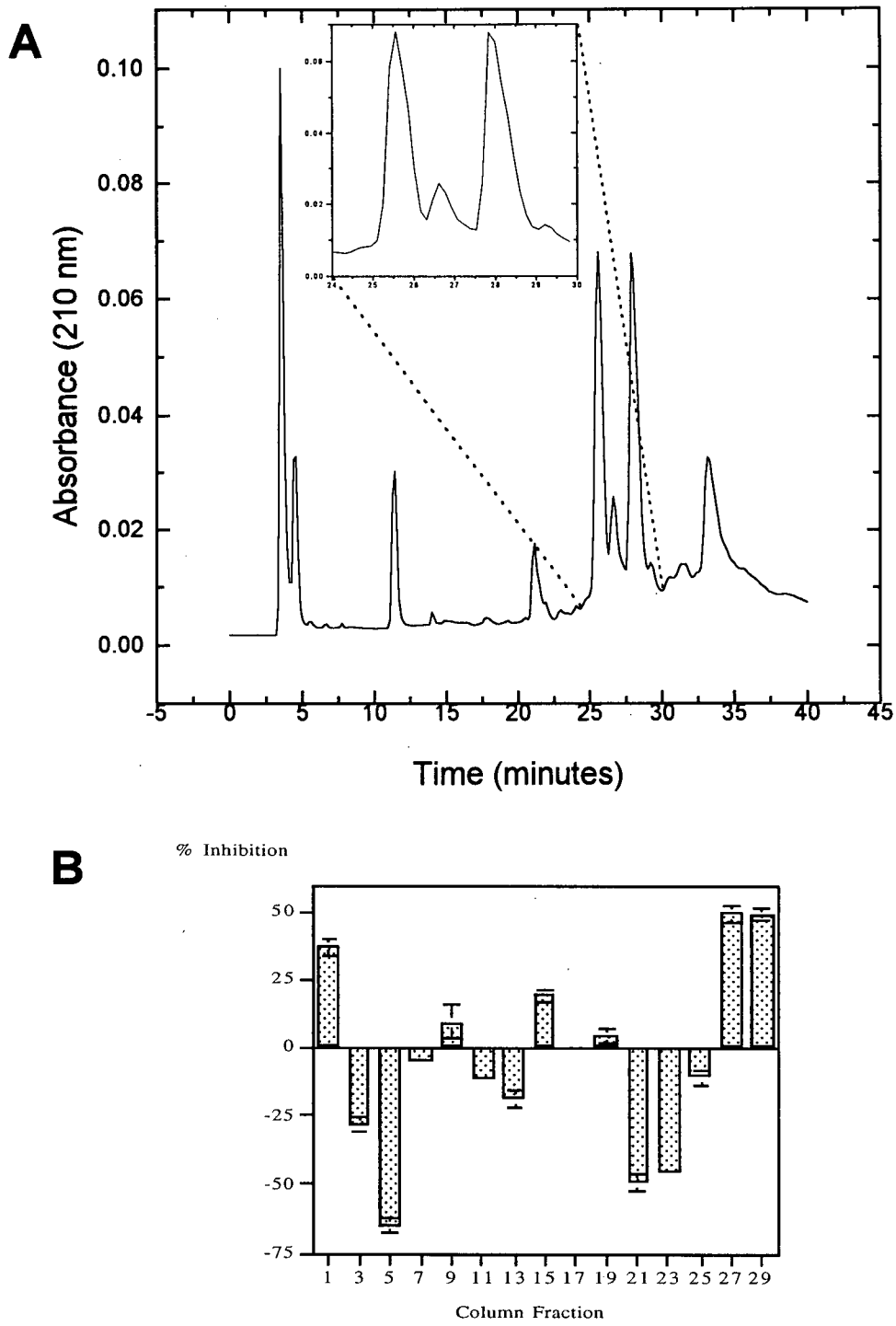


Figure 31 rpHPLC separation of gamma Percoll material

(**A**) rpHPLC separation of gamma Percoll fraction material (figure 10) using a C_4 column and monitoring absorbance at 210 nm. (**B**) One minute fractions from **A** were collected and every second fraction tested for effect on the proliferation of K562 using the MTT assay. Error bars represent SEM. (Representative of 2 experiments)

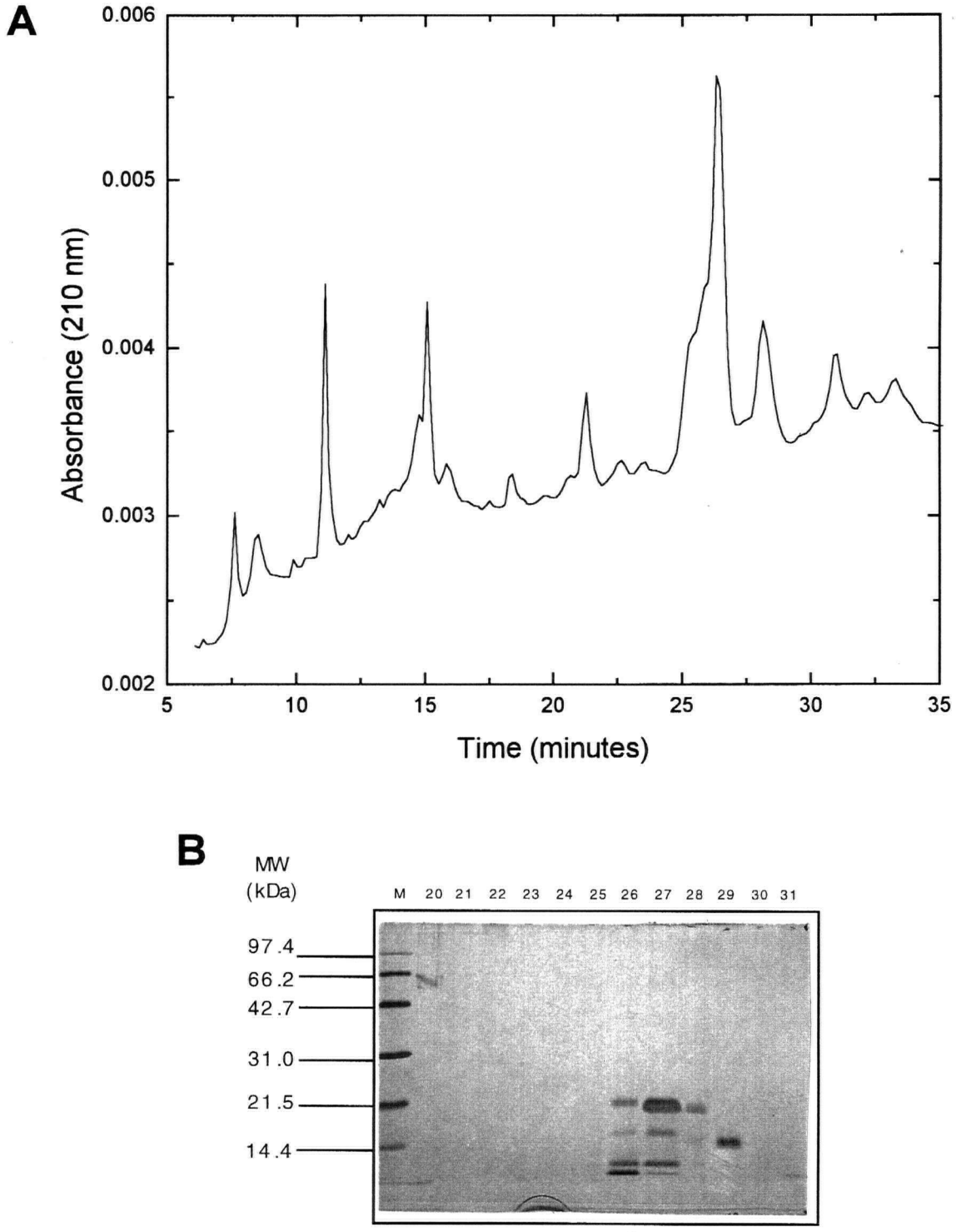


Figure 32 rpHPLC separation of active Superose 12 material

(**A**) rpHPLC separation of Superose 12 fractions 6 and 7 from figure 29 using a C_4 column and monitoring absorbance at 210 nm. (**B**) 12% silver stained SDS-PAGE analysis of indicated rpHPLC fractions from **A**.

Percoll material by rpHPLC. The resulting chromatogram is shown in figure 32a. Interestingly, the most prominent peak in this chromatogram appears at approximately 27 minutes which is in agreement with the results of figure 31a. SDS-PAGE analysis of these fractions in figure 32b revealed 5 bands, one at approximately 21 kDa, two at approximately 15 kDa (lanes 26-28 and lane 29), and two below 15 kDa. It was obvious that rpHPLC was unable to fully separate the proteins present in the activity-containing fraction. Therefore, following the schema presented in figure 28, we decided to use MonoQ anion exchange rather than rpHPLC to separate pooled fraction 6 and 7 material from the size exclusion column.

iv) MonoQ anion exchange chromatography

As rpHPLC did not permit separation of the proteins present in the active Superose 12 fractions, MonoQ anion exchange chromatography was used in its place. Figure 33a shows the activity of the MonoQ fractions on murine CFU-GM. It is apparent that all of the inhibitory activity is found in fractions 5-7, with the majority of the activity in fraction 6 yielding almost 75% inhibition. SDS-PAGE analysis of MonoQ fractions 6-8 presented in figure 33b shows a single silver stained band of approximately 15 kDa in fraction 6 and a line of artifactual staining in all fractions at approximately 40 kDa. Therefore, we have managed to purify a molecule with a molecular mass consistent with that of monomeric CD (15 kDa) from the cytosolic and plasma membrane fraction of normal neutrophils which inhibits murine CFU-GM. Despite the similarity of the molecular weight, further information was needed to prove that CD was responsible for the observed activity. It is important to note that the activity was extremely labile after the size exclusion chromatography step. Even in the presence of 2.5 mM DTT, which is known to stabilize the activity, the activity did not last 24 hours. Therefore, all purifications and subsequent experiments had to be performed on the same day.

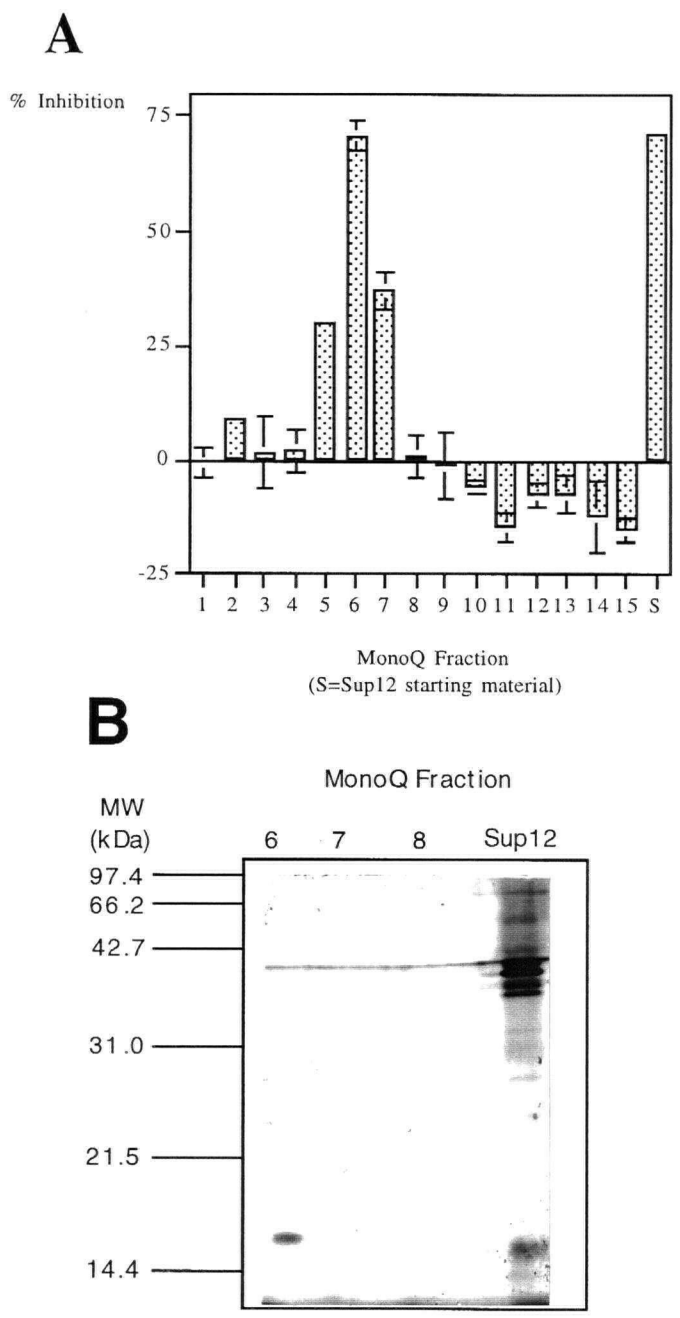


Figure 33 Anion exchange separation of Superose 12 purified material

(A) Pooled, active Superose 12 fractions 6 and 7 (see figure 29) were separated on a MonoQ ion exchange column and the effect of the fractions on murine CFU-GM was analyzed. **(B)** 12% SDS PAGE analysis of selected MonoQ fractions from **A**. Molecular mass of molecular weight markers are provided on the left in kDa's. (Representative of 3 separations)

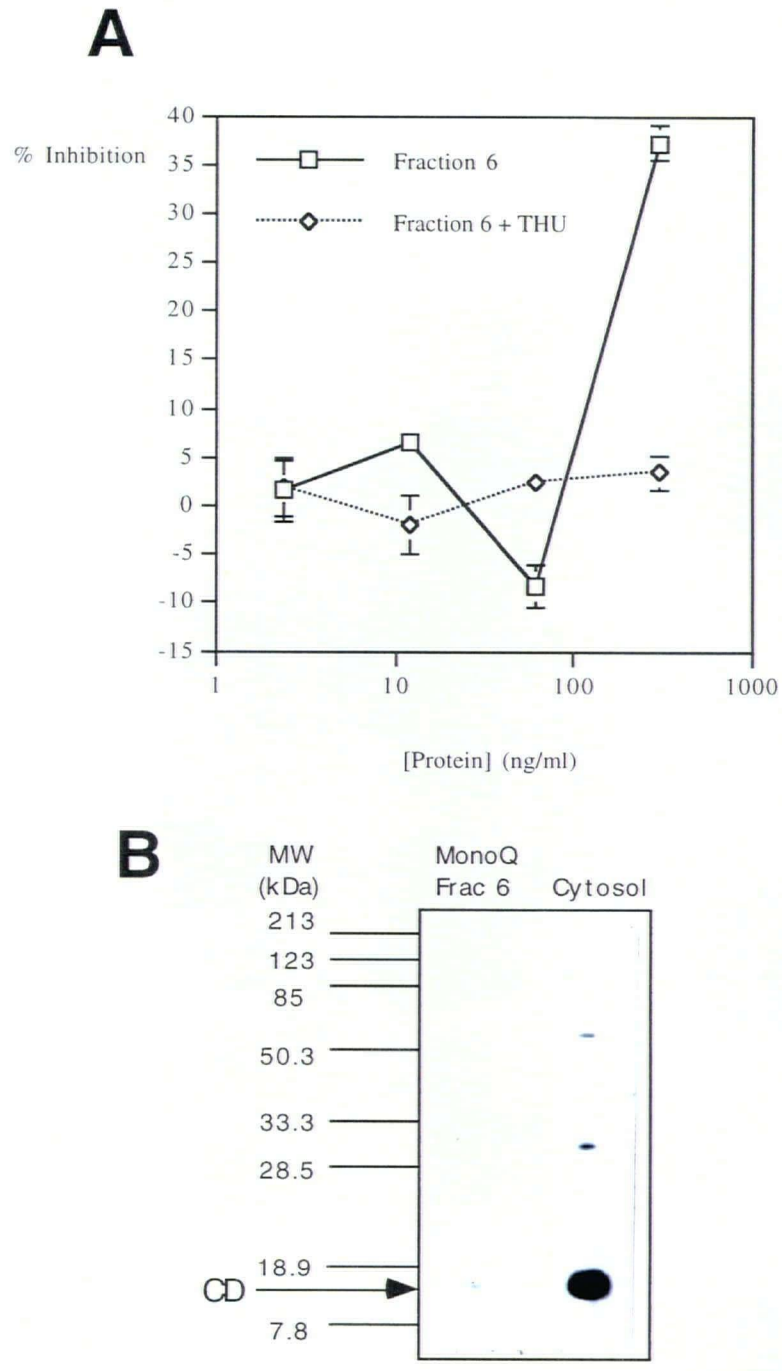


Figure 34 Identification of cytidine deaminase as the molecule responsible for the inhibitory activity

(**A**) Effect of THU, a known inhibitor of cytidine deaminase activity, on the inhibitory activity of MonoQ fraction 6 material. (**B**) Western blot analysis of MonoQ fraction 6 material from figure 33a using an antibody raised against the N-terminus of cytidine deaminase. 35 ug of cytosolic material and 3 ng of fraction 6 material was used in the Western Blot.

v) CD is responsible for the observed inhibitory activity

In the previous chapter THU, a known inhibitor of CD, was shown to abrogate the inhibitory activity of crude gamma Percoll material. Figure 34b shows how THU can block the inhibitory effects of MonoQ fraction 6. In the presence of THU this fraction has no inhibitory activity. However when THU is not present, inhibitory activity can be detected. Only 35% inhibition is seen at the highest concentration of fraction 6 in this experiment. This is due most probably to the labile nature of this activity. As mentioned in the previous section, it was very difficult to maintain the activity of the purified material. Despite the fact that THU abrogated the inhibitory activity and that fraction 6 contained a protein of the same size as CD, it was still possible that another molecule of the same size could be susceptible to the effects of THU. Therefore, a Western blot analysis was performed on fraction 6 material using a polyclonal rabbit antiserum raised against a synthetic peptide corresponding to the N-terminus of CD (gift of Dr. Momparler). As can be seen in figure 34b, a single 15 kDa band appears in the lane containing fraction 6 material. The lane containing neutrophil lysate also had a significant 15 kDa band but there was some cross reactivity with some other high molecular mass proteins. Pre-immune sera from the same rabbit did not react to either lanes of material (data not shown).

vi) Specific activity and fold purification

A table indicating the specific activity and fold purification of the inhibitory activity at each stage of the purification is presented in table 3. For this purpose, the specific activity was defined as the amount of material, in nanograms, needed to elicit 50% of the maximal effect, which of the purified material is approximately 75% inhibition in a CFU-GM assay on normal murine bone marrow. This table does give some information about the effectiveness of the

purification strategy used but is also somewhat misleading. As alluded to earlier, there is probably more than one inhibitor in the crude lysate which would give misleadingly low specific activities (the lower the specific activity, the greater the activity of the material). In addition, as mentioned, the labile nature of purified CD may result in the need for much greater amounts of material at later stages of the purification due to the denaturation of some of the active material.

Table 3 Purification table for CD

Summary of the specific activity and fold purification following each purification step		
Sample	**Amount for 50% Effect (ng)**	**Fold Purification**
Neutrophil Lysate	1030	N/A
Gamma Percoll Fraction	750	1.4
40-55% NH_4SO_4 Precipitate	110	9.4
Heat Precipitate Supernatant	80	12.9
Superose 12 Fractions 6 & 7	25	41.2

vii) Recombinant CD has the observed activity

In order to provide further proof that CD is at least partly responsible for the inhibitory activity of the neutrophil lysates, recombinant CD (a generous gift of Dr. Alberto Vita) was tested for its effect on murine CFU-GM. Figure 35 demonstrates that the recombinant material has inhibitory activity in the range between 60 ng/ml and 2.4 ng/ml. Interestingly, a plateau of inhibitory activity of approximately 70% is reached when concentrations above 60 ng/ml are used (data not shown). At concentrations higher than 1 μg/ml, the recombinant material begins

to lose its effectivity, suggesting that this material does not act by a cytotoxic mechanism. THU a known inhibitor of CD was previously shown to partly abrogate the inhibitory activity of crude neutrophil lysates (figure 25) and completely abolish the activity of the purified material (figure 34b). In this figure, THU can be seen to completely abrogate the activity of the recombinant material.

viii) CD is not responsible for the inhibitory activity of all neutrophil fractions

In the previous chapter, evidence was provided for the localization of inhibitory activity to the beta and gamma fractions of normal neutrophils as well as the alpha fraction of the leukemic (CML) neutrophils (figure 19). In order to assess whether or not CD was present in these fractions, Western blot analyses were performed on the various fractions from both normal and leukemic neutrophils (figure 36). In order to get an idea of the proportional amount of CD in each fraction, an equal amount of protein (3 μg) was loaded into each well. As expected, these results clearly demonstrated the presence of an approximately 15 kDa band corresponding to CD in the lysates of both normal and leukemic neutrophils. However, only the gamma fraction from both normal and leukemic neutrophils contained this band. Interestingly, a higher molecular band of approximately 60 kDa is apparent in both the normal neutrophil lysate lane and the leukemic gamma fraction. This 60 kDa band may correspond to the homotetrameric form of the molecule which is found in its native state. In any case, from these data, it is apparent that CD can only be found in the gamma, plasma membrane and cytosolic fraction of normal and leukemic neutrophil lysates. Therefore, another molecule(s) must be mediating the inhibitory activity seen in the beta fraction in normal and the alpha as well as beta fractions in the leukemic neutrophil preparations.

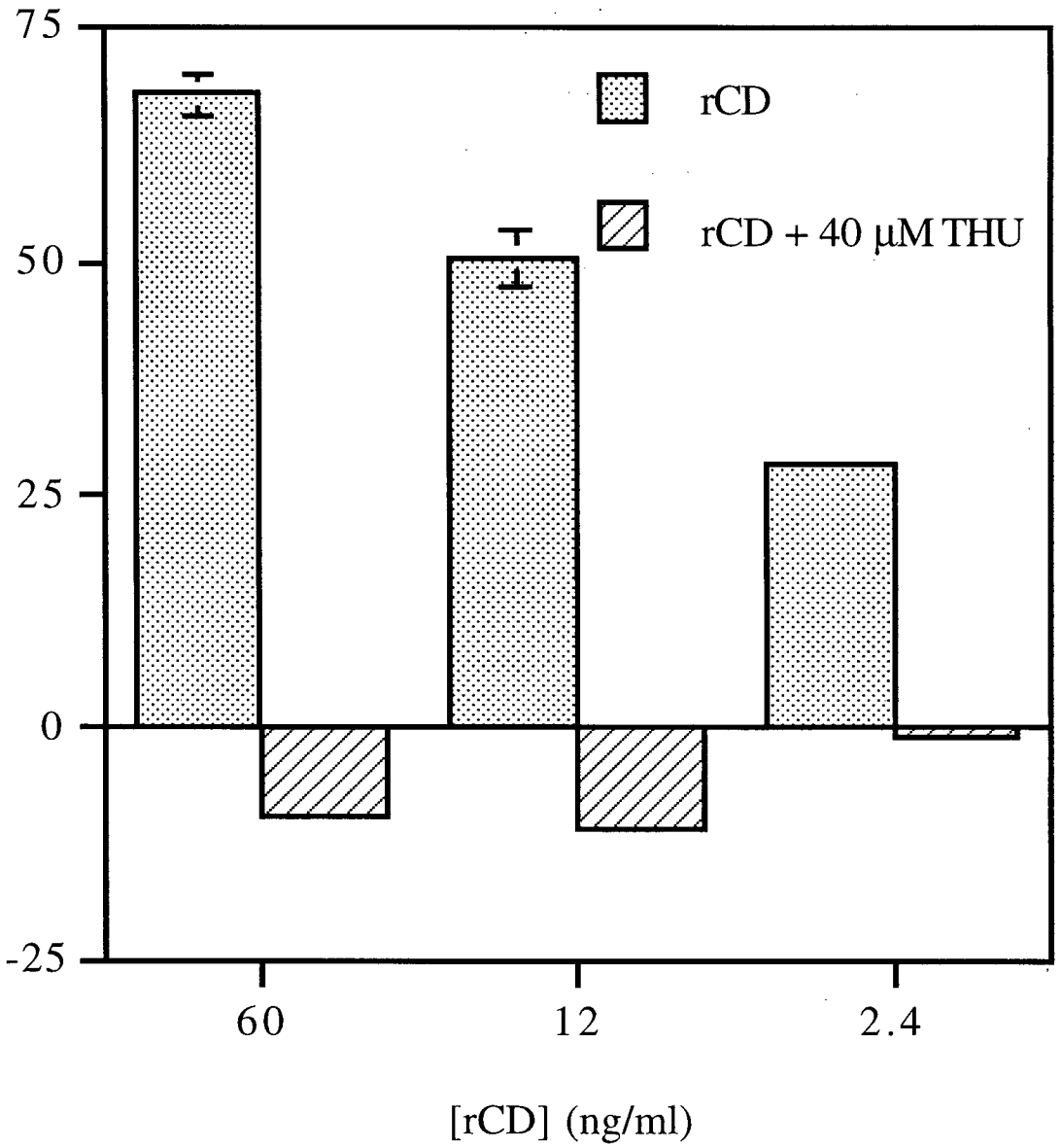


Figure 35 Effect of recombinant CD on murine CFU-GM

Inhibitory activity of recombinant CD on day 7 murine CFU-GM with or without the presence of THU, a known inhibitor of CD activity. Error bars represent SEM. Control colony numbers were 97.83 ± 2.81. (Representative data from 3 experiments performed in triplicate)

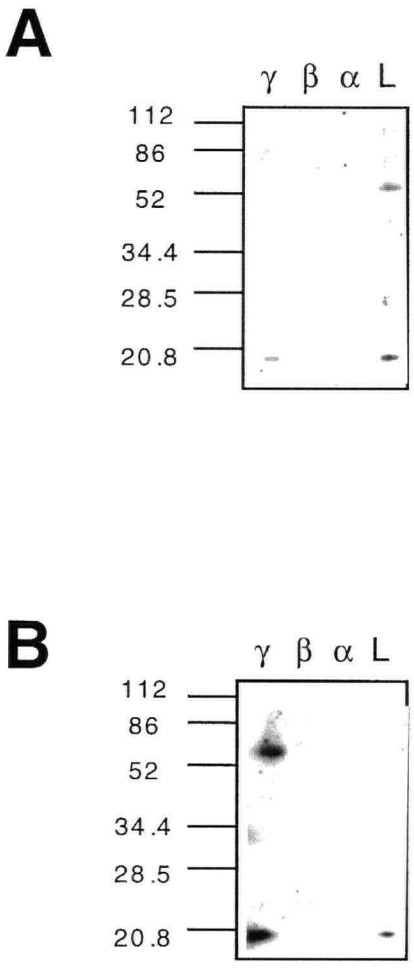


Figure 36 Anti-CD Western blot analysis of the various Percoll fractions

(A) Neutrophil lysate (L) and the various Percoll fractions from a normal preparation were run on a 12% SDS-PAGE gel, bloted and probed with an antibody specific to cytidine deaminase. **(B)** As in **A** except the lysate and resulting fractions were derived from a patient with CML. 3 ug of protein was added per lane.

6.4 Discussion

In this chapter, we have provided further evidence for the possibility that CD is a negative regulator of hemopoiesis. Initial attempts to purify the molecule responsible for the activity out of the gamma Percoll fraction using DE52 anion exchange chromatography and Superose 12 size exclusion chromatography were unsuccessful. However, these studies did reveal that the activity could withstand these chromatographic procedures and revealed the molecular mass of the cytoplasmic and plasma membrane derived activity. Figure 27b shows how all of the activity lay in the Superose 12 fractions 6 and 7 (pooled fraction 3). When this elution time was compared to that of known standards, this corresponded to material of approximately 55 kDa.

Concurrently, experiments were performed to see if rpHPLC was able to adequately separate the proteins present within the gamma Percoll fraction. Again, the fractions containing the activity had numerous contaminating proteins. However, as before, these experiments showed that the activity was retained after the chromatographic procedure. In addition, information on the elution time of active material using this extremely reproducible procedure was obtained.

At this time, Dr. Böyum and colleagues published a paper providing evidence for the possibility that neutrophil derived CD had inhibitory activity on myelopoiesis (177). CD is a 15 kDa enzyme which normally exists as a tetramer with a native molecular mass of approximately 60 kDa. It is known to catalyze the conversion of cytidine and 2-deoxycytidine to uridine and 2-deoxyuridine respectively. Furthermore, it can inactivate many chemotherapeutic agents including Ara-C and 5-Azacytidine. To date it has been found in human tissues such as the placenta, liver and neutrophil. Recently, the CD gene has been mapped to chromosome 1 band p35-p36.2 (183). Aberrations in this region have been associated with human malignancies such as neuroblastoma and AML (184). In the neutrophil

CD is known to be localized in the cytoplasm (181). It has no known physiological role in the neutrophil.

Since we were looking at a negative regulator of myelopoiesis from the cytoplasm of neutrophils, which had a native molecular mass consistent with that of CD we decided to determine whether or not our activity could be mediated by this molecule. A preliminary experiment was performed investigating the effect of THU, a competitive inhibitor of CD activity on the ability of the gamma Percoll fraction to inhibit murine CFU-GM (figure 25). At high concentration of the crude material, (4 x 10^6 granulocyte equivalents/ml) THU had no effect. However, at lower dosages, (1 x 10^6 granulocyte equivalents/ml) THU did knock out the activity indicating that CD may be important for at least some of the inhibitory activity found in the gamma Percoll fraction.

In light of this result and the findings of Böyum, we decided to base our purification strategy on the possibility that CD could be mediating the observed affect. A purification scheme was developed incorporating some of the procedures cited in a recent publication by Momparler and Laliberte who purified and cloned CD from human placenta (182). This purification scheme can be seen in figure 28. After ammonium sulphate and heat precipitation of the crude gamma Percoll fraction, size exclusion chromatography was performed as before. The resulting chromatogram in figure 29a is very similar to the previous size exclusion chromatogram of active DE52 material (figure 27a). In addition, the activity in both separations lay within fractions 6 and 7 corresponding to material of approximately 55 kDa. Despite this nice sharp peak in the activity profile, the SDS-PAGE analysis of the fractions revealed the presence of multiple other proteins. When the intensity of the band at the expected molecular mass of CD is analyzed, it becomes apparent that fraction 6 contains a much greater amount of CD than does fraction 7 (figure 29b). This brings into question why this difference in amount of CD did not result in a significant difference in amount of activity. We believe that this is due to the fact that even with fraction 7 material there is enough CD present to achieve a maximal effect of approximately 70% inhibition. Even if one goes higher in the dosage, it is not possible

to go over this maximum. This may indicate that only a certain cellular population may be affected. Due to similar findings with cell lines (unpublished observation) which are relatively homogenous, it is possible that cycling status of the cell is important. As multiple proteins were present in the activity containing fractions, the purification scheme of figure 28 was followed and an attempt was made to separate these proteins by rpHPLC.

The rpHPLC chromatogram seen in figure 32a reveals a prominent 26 minute peak. This is in keeping with the separation of the crude material which demonstrated that the majority of the activity was contained within this fraction. However, there was still the presence of 5 bands of contaminating protein (figure 32b). Following the scheme in figure 28, MonoQ anion exchange chromatography was used to separate the proteins present within the active Superose 12 fraction.

Proteins were loaded onto the MonoQ column in a low salt buffer and eluted with an increasing gradient of salt concentration. The chromatograms of the MonoQ separations are not shown because the high salt concentrations of the eluting buffer absorb substantially at the monitoring wavelength (280 nm) and thus the protein signal is obscured. In any case, it was possible to obtain data on the effect of the fractions on murine CFU-GM. All of the activity off of the MonoQ column was contained within fractions 5-7 (figure 33a). SDS-PAGE analysis of these fractions reveals a single band of 15 kDa in the lane corresponding to fraction 6. A faint band was also seen in the original gel at the same molecular mass in fraction 7 material but this did not show up in the photograph. Again, a maximum of approximately 70% inhibition was seen in fraction 6 off of the MonoQ column. This may be further evidence that a maximum of 70% inhibition is attainable with this activity in this assay, or it may simply be due to the fact that not enough of fraction 6 material was present. When an attempt was made to determine the effect of a titration of fraction 6 material on murine CFU-GM, a maximum of 35% inhibition was observed (figure 34a). This is most probably due to the loss of activity during purification. Regardless, we have succeeded in purifying a molecule to apparent homogeneity which has inhibitory activity on CFU-GM. Despite the fact that this molecule had the same molecular

mass as CD, further proof was needed in order to implicate it as the one responsible for this activity.

Figure 34a clearly shows that THU, a competitive inhibitor of CD activity, completely blocks the inhibitory activity of the purified MonoQ fraction 6 material. Furthermore, when Western blot analysis was performed on this material a single band of approximately 15 kDa was seen. This information allowed us to conclude that CD is an inhibitor of CFU-GM.

In an attempt to monitor the effectiveness of the purification strategy performed in this study, table 3 outlines the fold purification after each purification step. A final purification of 41 fold was achieved. This figure is not very dramatic due most probably to the fact that many inhibitors were present initially in the crude lysate and that CD became increasingly susceptible to denaturation after each subsequent purification step.

Figure 35 demonstrates the effects of recombinant CD on murine CFU-GM. This information provides further proof that CD is at least one of the inhibitors present.

In chapter 5, we demonstrated how inhibitory activity in normal neutrophils could only be found in the specific granule (beta Percoll) fraction and the cytoplasm/plasma membrane (gamma Percoll) fraction. By contrast, leukemic neutrophils were found to contain an inhibitory activity in their azurophilic granules (alpha Percoll fraction) as well (figure 19). In order to determine if CD may be mediating the effect seen in the alpha fraction in CML neutrophils and the beta fraction in both normal and CML neutrophils, Western blot analyses were performed on these fractions (figure 36). Interestingly, only the gamma fraction of both normal and leukemic neutrophils had CD present. Therefore other inhibitory molecules must be responsible for the activity found in the other fractions. Candidates include, lactoferrin from specific granules (86), the peptide inhibitor of Paukovits et al (81) and as mentioned in chapters 3 and 4 of this thesis, active glycoforms of azurocidin from azurophilic granules.

In this chapter, we have shown that neutrophil derived CD is a negative regulator of myelopoiesis. This provides further evidence for the presence of a negative feedback mechanism in myelopoiesis involving this molecule. Upon death or excitation, CD may be

released from neutrophils, thereby mediating a down regulatory signal on those cells which produce it. We have preliminary evidence which suggests that an inhibitory activity is released from purified neutrophils stimulated with PMA (unpublished observation). In light of the fact that Yu et al have noted an increase in the release of CD from neutrophils stimulated with FMLP (185), CD is likely to be at least one of the mediators of this effect. As mentioned, the CD gene has been mapped to an area commonly affected in AML. It is possible that disruption of this activity may result in the loss of this inhibitory activity and contribute to the establishment of the leukemic clone in this collection of disorders.

As CD is known to deaminate cytidine and deoxycytidine, it may be mediating its effect by depleting the pool of these nucleotides which are essential for cellular growth and proliferation. Evidence presented in the previous chapter (figure 24) as well as Böyum and colleagues (150) has indicated that thymidine is necessary for the activity of semi-purified material. Although, CD is not known to act on thymidine, this nucleotide is a competitive inhibitor of CD's activity on deoxycytidine (186). Thymidine may be acting to increase the levels of deoxythymidine triphosphate (dTTP) thereby decreasing the activity of ribonucleotide reductase (176, 187) resulting in the decreased conversion of cytidine to deoxycytidine. Depletion of deoxycytidine may result from deamination by CD and the reduction of its formation due to the presence of thymidine resulting in a synergistic inhibitory effect. Alternatively, the presence of thymidine in the active site of CD may allow for the binding of CD to a receptor at the cell surface thereby inducing a negative regulatory signal to be initiated. Yet another possibility, is that CD is acting on deoxycytidine or thymidine resulting in the formation of a cytotoxic or cytostatic product.

Many questions remain regarding the mechanism of action of this inhibitory activity as well as the cells affected by it. Using the recombinant material, a more in depth analysis is possible. The following chapter goes on to investigate in greater depth, the possibile mechanisms of action and proposes some future directions for research dealing with this interesting and relevant activity.

CHAPTER 7 - DISCUSSION

7.1 Overview of Results

i) CAMAL

Studies undertaken in this laboratory a decade ago to define leukemia specific antigens in myeloid malignancies resulted in the development of a leukemia specific mAb (CAMAL-1) (137). Using an immunoperoxidase slide test it was demonstrated that CAMAL-1 bound, in a prognostic fashion, to cells of patients with AML in remission (139, 140). PBL's from patients with CML were also recognized by this antibody (138). Since it was expressed extensively prior to clinical relapse, it was hypothesized that the antigen could be involved in the establishment and outgrowth of the leukemic cells in the myeloid malignancies. Immunoaffinity columns made with CAMAL-1 were used to enrich whole blood cell lysates from patients with myeloid leukemias for the antigen recognized in the immunoperoxidase slide tests (141). These immunoaffinity enriched preparations were also found to have inhibitory effects on normal CFU-GM colony formation while not affecting equivalent cells from patients with myeloid leukemias. These extracts were composed of two general protein populations, one centered at approximately 60 kDa and the other at approximately 30 kDa. It was subsequently determined that the 30 kDa population was responsible for the observed activity. N-terminal amino acid sequence analysis of the 30 kDa population identified a 22 amino acid peptide with 59% homology with serine proteases (142). Further attempts to characterize the activity have indicated that it has inhibitory activity on murine progenitors (143) and that it may enhance leukemic myelopoiesis (144).

The initial focus of this study was to purify and identify the molecule responsible for the inhibitory activity from these immunoaffinity enriched preparations (chapter 3). The

approach taken to solve this problem was to separate active material by rpHPLC and to test the fractions for activity in the conventional colony assay. Concurrently, another graduate student in the laboratory (Joan Shellard) was using a molecular biological approach to identify the molecule containing the N-terminal peptide. During the course of this investigation, the 22 amino acid peptide was found to correspond to the N-terminus of a protein known as azurocidin (108, 109). This protein is a differentially glycosylated, cationic, heparin binding protein (166). Despite sharing a high degree of homology with serine proteases (188), it is inactive due to mutations in the region known to be critical for activity in these molecules. It is one of the major 30 kDa proteins found within the azurophilic granules of normal neutrophils (189).

Our initial rpHPLC chromatographic separations of immunoaffinity enriched preparations revealed the presence of many protein peaks (figure 4a). Due to the impurity of the immunoaffinity preparations, the amino acid sequence data was put into question. Was azurocidin the molecule responsible for the activity or just present within the crude activity containing preparation? In order to answer this question it was necessary to purify to homogeneity the molecule responsible for the activity.

As the immunoaffinity enriched material was derived from patients with CML, a condition characterized by the expansion of the neutrophil, and because azurocidin is a neutrophilic protein, we compared our rpHPLC chromatograms with those from normal neutrophil extracts (164, 189). Interestingly, the chromatograms were very similar. This similarity along with SDS-PAGE analyses (figure 4b) and limited protease activity data (142), allowed for the identification of many of the peaks (figure 4 legend).

Analysis of the inhibitory activity on murine CFU-GM of rpHPLC derived fractions showed that the activity lay exclusively within the fraction corresponding to the leading edge of the azurocidin peak. The remainder of the peak, which corresponds to the majority of protein, had no inhibitory activity. When tested on CML CFU-GM, no inhibitory activity was seen by either fraction. The leading edge of the azurocidin peak was separated by 2-D gel

electrophoresis, and was indistinguishable from glycoforms of azuoricidin (figure 8). There is still the possibility that another inhibitory molecule could co-purify with these glycoforms of azurocidin and mediate the observed activity. However given that this molecule would have to co-elute in rpHPLC and co-migrate in 2-D electrophoresis this is unlikely. Therefore, from these data it was concluded that the specific glycoforms of azurocidin are responsible for the selective inhibitory activity seen in the immunoaffinity enriched preparations.

Although the CAMAL-1 mAb was found to be leukemia specific, it is possible that a normal molecule associated with a leukemia specific molecule would be co-enriched in immunoaffinity preparations made using this mAb. Since azurocidin is normally found in neutrophils, this was a considered reasonable.

In chapter 4 work investigating the possibility that this activity was found in normal azurocidin preparations was reported. Only one of six preparations was found to have the selective inhibitory activity reported in chapter 3. In this preparation, the activity was again only found in the leading edge of the azurocidin peak. As seen previously, CML progenitors were insensitive to the inhibitory activity contained in this fraction. These results suggest that the glycoforms of azurocidin involved in the selective inhibitory activity are rarely present in the neutrophils of normal donors.

The characterization of the glycosylation involved in maintaining the observed activity is particularly difficult, therefore a decision was made to focus our efforts on the characterization, purification and identification of the molecule responsible for the inhibitory activity of another crude preparation.

ii) Boyum's GRE

Over twenty years ago, Boyum and colleagues published a paper identifying a myelopoietic inhibitory activity out of normal and leukemic (CML) neutrophils (147). This

activity was found to be mediated by a molecule between 30 and 60 kDa (149) and to be thymidine dependent (150). In chapter 6 further characterization of this inhibitory activity was presented. These studies showed that normal murine and human, as well as CML, CFU-GM were equally susceptible to the inhibitory activity of the crude extract (figures 14 and 15). Furthermore, a variety of different myeloid and lymphoid leukemic cell lines were found to be susceptible to the inhibitory activity of this preparation (figure 17). Through subcellular fractionation of neutrophils, the inhibitory activity was localized to the specific granules and cytosolic fraction of normal neutrophils (figure 18). Leukemic (CML) neutrophils, in addition to the specific granule and cytosolic fractions, also had inhibitory activity in their azurophilic granule fraction (figure 19). As we had identified a myelopoietic inhibitor from the azurophilic granules of leukemic neutrophils (chapter 3), and an inhibitor localized to the specific granules had already been identified (86), the purification and identification of the molecule responsible for the inhibitory activity in the cytosolic fraction of normal neutrophils was pursued.

Normal and CML progenitors were equally susceptible to the inhibitory effects of the cytosolic fraction (figure 21). When this material was used to treat normal bone marrow cells in a long-term culture assay, the more primitive progenitors were found to be insensitive to its effects (figure 22). The importance of thymidine to the cytosolic activity was investigated and thymidine dependent and independent activities were found (figure 24). This is an indication that at least two inhibitors are present within this fraction. When subjected to heat denaturation or protease digestion, this fraction lost its activity. Therefore, the activity is obviously protein mediated.

During the course of this investigation, evidence emerged suggesting that neutrophil derived cytidine deaminase (CD) may be partly responsible for the inhibitory activity of neutrophil lysates (177). CD catalyzes the hydrolytic deamination of cytidine and deoxycytidine to uridine and deoxyuridine, respectively. It can also deaminate the chemotherapeutic agents cytosine arabinoside and 5-azacytidine resulting in a loss of their cytotoxic activity (190). In an effort to confirm this observation, experiments were performed looking at the effect of a

known inhibitor of CD activity, THU, on the inhibitory activity of the cytosolic fraction. As with the thymidine dependence studies, THU sensitive and insensitive inhibitory activities were present within this fraction (figure 25) which again suggested that two or more inhibitory molecules were present in this material.

The purification and identification of CD as one of the molecules present within the cytosolic fraction is described in chapter 6. Before the information regarding CD became available, the cytosolic fraction was separated using various chromatographic procedures including; DE52 ion exchange chromatography, size exclusion chromatography and rpHPLC. These techniques, in the combinations used, did not purify the molecule responsible for the activity to homogeneity. However, they did give information about the size of the native molecule (approximately 50 kDa) and showed that these techniques yielded active material. After the data implicating CD was published, a purification scheme was developed (figure 28) based on the recent purification of this molecule from human placenta (182). Using differential ammonium sulphate precipitation, heat precipitation, size exclusion and anion exchange chromatography, it was possible to purify the molecule responsible for the inhibitory activity to apparent homogeneity (figure 33b). Antibodies raised against a CD peptide were shown to recognize this single protein band through Western blot analysis (figure 34). Investigation of this purification scheme reveals that only a 40 fold purification was obtained. A possible reason for such a low fold purification value is that other inhibitory proteins contributed to the initial specific activity of the cytosolic fraction. Alternatively, synergistic or additive affects of these molecules with CD could account for such an increase. Another possibility is that the inhibitory activity was lost during the purification procedure due to denaturation. It was observed that the activity was very labile and that if left at 4°C for even 24 hours it was lost.

Further proof that CD is one of the molecules responsible for the observed activity comes from the inhibitory activity of recombinant CD on CFU-GM (figure 35). Recombinant CD was found to mediate no more than 70% inhibition of colony formation. This plateau continued until 60 ng/ml. At concentrations below this, the activity titrated out with only 30%

inhibition seen at 2.4 ng/ml. At higher concentrations (greater than 1 μg/ml), the recombinant material started to lose its inhibitory activity. A possible reason for this plateau is presented with the proposed mechanism of action of CD in the following section.

To determine if CD mediated the inhibitory activities of the beta specific granules in normal and leukemic neutrophils and the azurophilic granules in leukemic neutrophils, Western blot analyses were performed on these samples using the anti-CD antibody. These results show that CD is only present within the cytoplasmic fractions of both normal and leukemic neutrophils (figure 36). Other molecules such as active glycoforms of azurocidin in leukemic, azurophilic granules and lactoferrin from normal and leukemic specific granules are candidates for the inhibitory activities out of these fractions.

7.2 Speculation on the Mechanism of Action of these Molecules

i) CAMAL

Direct contact between progenitor cells and bone marrow stroma is known to be important for negative growth regulation (53, 191). These negative regulatory signals may be due to the presence of stroma bound inhibitory cytokines such as TGF-β or MIP-1α. Alternatively, they may be sent by engagement of integrins on the progenitor cell surface by their stroma bound ligands (52). Integrins are a family of activation dependent adhesion molecules. Binding to their ligand is governed by an increased avidity rather than increased expression (192). Once thought to only mediate adhesion, they are now believed to play a role in signal transduction (193). Normal progenitors adhere to bone marrow stroma through a variety of molecules including the $\alpha_4\beta_1$ (VLA-4) and $\alpha_5\beta_1$ (VLA-5) integrin adhesion

molecules. $\alpha_4\beta_1$ binds to the vascular cell adhesion molecule (VCAM) and the heparin binding domain of fibronectin which are both found on the stromal cell surface. Therefore, it can be thought of as a heparin like receptor molecule. $\alpha_5\beta_1$ recognizes the RGD (arginine-glycine-aspartic acid) sequence of fibronectin (193). CML progenitors have been shown to have impaired binding to stroma (194). Dowding et al showed that IFN-α treated CML stromal cultures had enhanced progenitor cell adhesion (195). Recent studies suggest that this increase in adhesion is due to release of MIP-1α by the IFN-α treated stroma and the resulting restoration of $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrin binding (196).

Azurocidin is a heparin binding, cationic chemotactic protein which has been found to act as a survival factor for long-term cultures of monocytes. It also causes confluent fibroblast and endothelial cell layers to contract and induces the expression of the integrin binding molecule thrombospondin (166). Its ability to bind heparin is governed by four glycosaminoglycan (gag) recognition sites believed to be on the surface of the protein (197). It has three surface localized N-linked glycosylation sites which are believed to be in proximity to the gag recognition sequences (165). This indicates that differential glycosylation of this molecule may affect its ability to bind heparin. The chemotactic effect on monocytes was found to be mediated by a heparin like receptor (197). Since the effects of the immunoaffinity enriched material are mediated within 45 minutes of treatment (151), it is probable that its effects on target cells is through a membrane bound receptor.

A possible mechanism by which azurocidin may inhibit normal progenitor cells is by affecting integrin engagement at the cell surface. This may occur in one of three ways. It may bind directly to a heparin like integrin receptor (such as $\alpha_4\beta_1$) resulting in down regulatory signals. Alternatively, it may act indirectly by inducing the activation of integrin molecules such that they can bind to their ligand. This may occur through engagement of a completely

unrelated receptor or it may activate the integrin molecule directly by binding to its surface and exposing cryptic ligand-binding sites (192).

Despite having normal levels of the $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrin molecules, CML cells have reduced adhesion to stromal layers (179, 194). This is likely due to perturbations in their intracellular signaling machinery which ultimately lead to inactive integrin conformations.

MIP-1α (78, 198) and TGF-β (54, 199, 200) have been shown to have heterogeneous effects on CML progenitor cells. Some researchers have shown no effect, some an inhibitory effect and some have shown enhancement. These experiments reveal the heterogeneity of this disorder which may be due to the stage of the disease or previous treatment.

Our data suggests that specific glycoforms of azurocidin may have differential effects on normal and CML CFU-GM. CML CFU-GM may not be susceptible to the inhibitory effects of this molecule due to inactivation of its integrin molecules.

ii) Cytidine deaminase

The majority of pyrimidine nucleotides are formed using the *de novo* biosynthesis pathway (reaction 1, figure 37a). This pathway involves the conversion of ribonucleotides to their corresponding deoxynucleotide using the enzyme ribonucleotide reductase (201). The less common salvage pathway involves the phosphorylation of the deoxynucleosides to form the appropriate deoxynucleotide (reaction 2, figure 37a). A detailed view of pyrimidine biosynthesis is presented in figure 37b. This figure illustrates how the deoxynucleotide, deoxythymidine-triphosphate (dTTP) is formed in the *de novo* pathway from the ribonucleotides uridine diphosphate (UDP) and cytidine diphosphate (CDP). The resulting deoxynucleotides (dUDP and dCDP respectively) are then dephosphorylated to form dUMP and dCMP. dCMP can undergo further conversion to give dUMP. dUMP can then get converted to deoxythymidine monophosphate (dTMP) which is phosphorylated to produce

dTTP. dTTP is then ready for incorporation into DNA. Formation of deoxycytidine triphosphate (dCTP) in the *de novo* pathway is through the phosphorylation of dCDP (202).

This figure also illustrates how exogenous pools of thymidine (dTdR) and deoxycytidine (dCdR) can be converted to dTMP and dCMP respectively by the nucleoside kinases of the salvage pathway (reactions 2a and 2b).

Thymidine (dTdR) has been used for decades to synchronize cells *in vitro* in S phase (176). Prolonged exposure of cells to thymidine in the mM range has been found to be toxic (187). The mechanism believed to be responsible for this toxicity involves the decrease in dCTP pools. Exogenous dTdR gets converted to dTMP by thymidine kinase of the salvage pathway. Eventually, dTTP levels increase and through a negative feedback mechanism (175), this results in the inhibition of ribonucleotide reductase (reaction 1, figure 37b), and thymidine kinase (reaction 2a, figure37b). Inhibition of ribonucleotide reductase results in the decreased formation of dCTP and dTTP. As a result, dCTP levels drop and the cells arrest in S phase. It is possible to rescue cells with exogenous dCdR through the salvage pathway conversion of dCdR to dCMP (reaction 2b, figure 37b) which eventually increases the dCTP levels (203).

CD is known to deaminate cytidine (CdR) and deoxycytidine (dCdR) to uridine (UdR) and deoxyuridine (dUdR) respectively. The most probable mechanism for the CD mediated effect in our system is through the depletion of the exogenous dCdR pools present in the fetal calf serum and medium. Cells were cultured in medium containing 160 μM thymidine. No effect on proliferation or clonogenicity was seen at this concentration. This is likely due to the rescue by dCdR present in the fetal calf serum and the medium. When CD is present, it can convert dCdR to dUdR (reaction 3, figure 37) resulting in decreased levels of dCdR for DNA synthesis, which leads to arrested cellular proliferation.

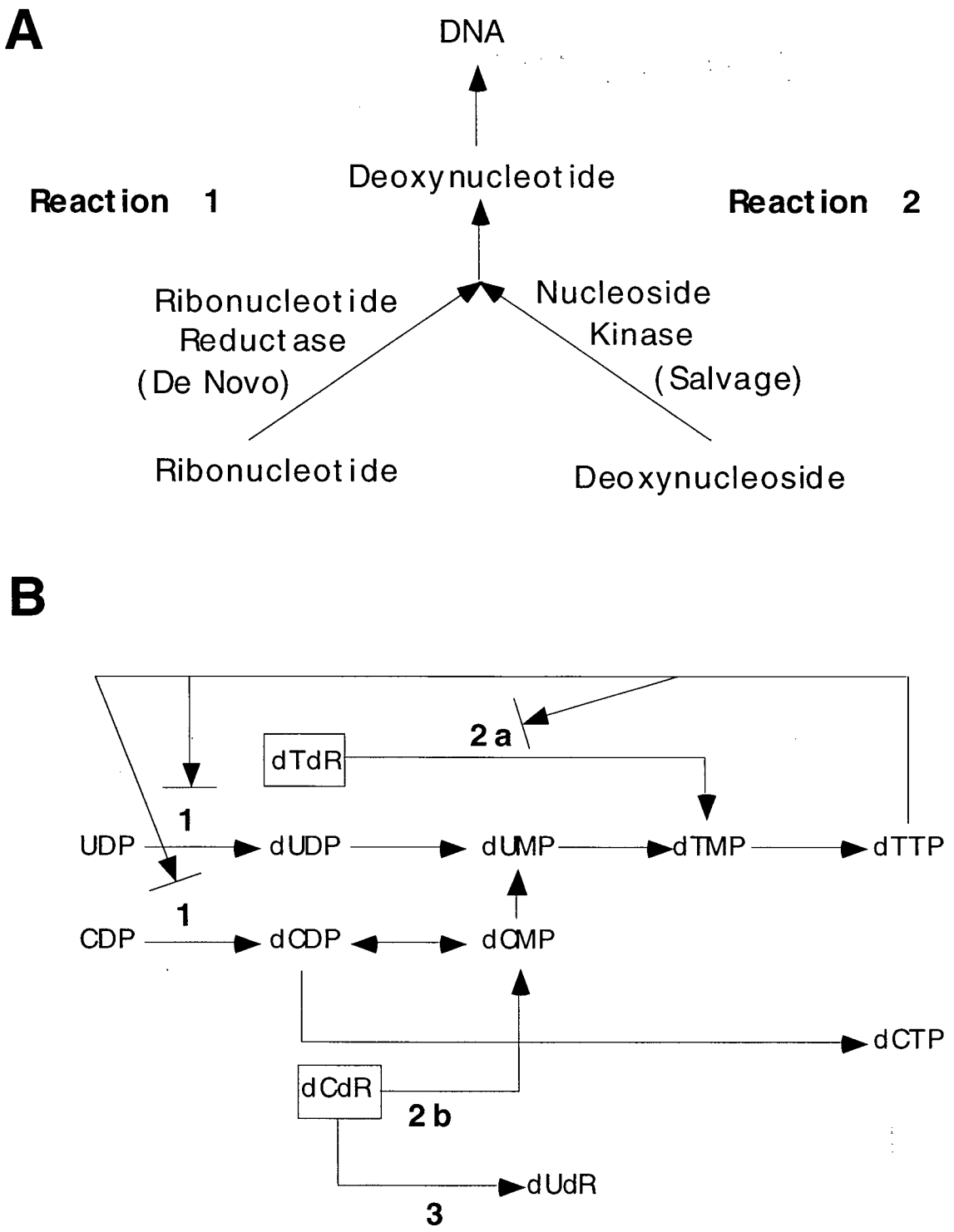


Figure 37 Outline of pyrimidine nucleotide biosynthesis

(A) Involvement of the *de novo* and salvage pathways of DNA synthesis. **(B)** Regulation of pyrimidine biosynthesis. Blocked arrows indicate negative feedback, reaction 1 is mediated by ribonucleotide reductase, 2 by the nucleoside kinases and 3 by cytidine deaminase.

A possible reason for the plateau of 70% inhibition of colony formation by CD is that thymidine concentrations were not sufficient to completely block the *de novo* production of dCTP. Therefore, although all salvage pathway production of dCTP would be stopped by CD treatment, some cells could escape CD's inhibitory activity due to dCTP production by the alternative *de novo* pathway. If this were the case, higher concentrations of thymidine would result in greater inhibitory activity.

All of the experiments in this study were performed *in vitro. In vivo,* CD is known to be released by activated neutrophils (185). However, median thymidine concentrations in human plasma are approximately two orders of magnitude lower than those used in the above experiments (204). A thorough investigation has yet to be performed on the optimal thymidine concentration needed for inhibitory activity. If high thymidine concentrations prove to be necessary for CD activity, it may have an inhibitory activity in bone marrow microenvironments with high thymidine concentration. These thymidine concentrations could fluctuate and act to regulate the activity of CD. One way in which thymidine concentrations could increase is through its release from macrophages in the bone marrow (205) clearing the extruded nuclei of normoblasts during erythroid development (206).

7.3 Future Directions and General Conclusions

i) CAMAL

The first part of this study focused on the purification and identification of the molecule responsible for the inhibitory activity of immunoaffinity enriched preparations from patients with CML. The molecule responsible for this activity was found to co-elute in rpHPLC and co-migrate in 2D electrophoretic gels with certain glycoforms of the normal neutrophilic protein azurocidin. These purified preparations were found to inhibit normal myelopoiesis while

having no effect on equivalent progenitors from patients with CML. When this molecule was purified from normal neutrophils only one of six preparations had the activity seen in the leukemic cells.

The mechanism by which the leukemic clone is established in CML is poorly understood. However, it is widely accepted that CML progenitors do not progress through the cell cycle faster than their normal counterparts (131). In addition, it does not appear to be due to an increased sensitivity to stimulatory factors (207, 208). It is possible that insensitivity to inhibitory factors could play a role in the establishment of the disease. This appears to be the case with active glycoforms of azurocidin. Insensitivity to its inhibitory effects could play a role in the development of an environment conducive to leukemic hematopoiesis. It is hoped that after its physiological role is established that treatments targeting its actions may one day result in the reversal of the dominance gained by the leukemic clone.

In this chapter, a mechanism for the action of active glycoforms has been proposed. Experiments to prove this hypothesis could include the following. Perform glycosidase treatment on active azurocidin preparations to clarify the role of this post-translational modification on the activity of the molecule. Perform integrin blocking studies to identify the importance of these molecules in the susceptibility to the actions of azurocidin. Determine if cells treated with azurocidin are inhibited by cytotoxic or cytostatic mechanisms. Perform greater numbers of CML susceptibility experiments to determine whether the enhancement of leukemic myelopoiesis seen with the crude material is mediated by the same molecule as the inhibitory activity.

ii) Cytidine deaminase

The second part of this research focused on the characterization, purification and identification of the molecule responsible for the myelopoietic inhibitory activity first described

by Boyum and colleagues. This activity was localized to the cytoplasmic fraction of both normal and leukemic neutrophils. It was found to be thymidine dependent and normal murine and human as well as CML progenitors were equally susceptible to the inhibitory activity. The molecule responsible for the inhibitory activity was purified to homogeneity and identified as cytidine deaminase (CD) by Western blot analysis. This was further confirmed by the inhibitory effect of recombinant CD on myelopoietic progenitors.

In this chapter, a mechanism of action for CD's effects on myelopoiesis has been proposed. Experiments which would determine its validity are presented here. A thorough analysis of the thymidine dependence of the recombinant material should be performed. This has been done with the purified material but careful experiments need to be repeated with recombinant CD (rCD) to ensure that this nucleotide is necessary for the activity and if so in what concentrations. Perform time course analysis of the susceptibility of cells to rCD treatment. Treat cells with rCD in the presence of optimal thymidine concentrations for the optimal time and then add deoxycytidine and THU. If the proposed mechanism is correct, cultures without deoxyctidine will be inhibited and those with deoxycytidine will be rescued and thus unaffected.

Despite the appeal of the previously mentioned mechanism, others may exist. CD may mediate its effects via a membrane bound receptor. An experiment to test this hypothesis would be to perform proliferation assays on cells using a trans-well insert. In this system, the cells would not be able to come into contact with rCD and if the inhibitory activity was still seen, this would provide evidence against this mechanism. Yet another mechanism would be that CD is acting on thymidine to produce a toxic product. An experiment testing this possibility would involve treatment of thymidine containing medium with rCD, removal of rCD by filter concentration and treatment of cells with the non-rCD containing fraction. If this fraction retains the activity, a case can be made for this mechanism.

As the majority of the characterization of the activity of this molecule was performed using impure material, it is important to repeat this work using the recombinant protein. This

includes, the susceptibility of normal human, murine and CML progenitors to the activity as well as the long-term culture experiments. Other important experiments would include testing for the effects in multi-potential colony assays to determine if a certain progenitor type is affected. Analysis of the cycling status of treated cells as well as cytotoxicity assays to determine if a cytotoxic or cytostatic mechanism was at play. Finally, *in vivo* experiments would help to determine if CD is a physiological regulator of myelopoiesis. Initial studies could involve intravenous injection of CD and bone marrow transplants of treated cells. More sophisticated experiments would include the use of gene knock-out mice.

Several studies have shown that thymidine can synergistically enhance the activity of chemotherapeutic agents both *in vitro* and *in vivo* (176). However, it has been difficult to attain the plasma thymidine concentrations needed for effectivity. If CD was shown to complement thymidine mediated toxicity, rCD could be used to lower the dosages of thymidine needed *in vivo* and also enhance its activity *in vitro.*

ARA-C is one of the most commonly used drugs for the treatment of AML (209). In patients with AML, a pre-treatment regimen of thymidine and rCD could starve the rapidly dividing leukemic cells of dCTP. Subsequent treatment of these patients with ARA-C, which is known to mediate its cytotoxicity by incorporation into DNA in place of dCTP, would result in increased incorporation of dCTP into DNA and increased cytotoxicity. As CD is known to inactivate ARA-C, a CD inhibitor such as THU may have to be administered along with ARA-C. Regardless, due to the promising clinical implications of such a treatment, experiments investigating these possibilities should be explored.

iii) General conclusions

In this work we have purified and identified two molecules from human neutrophils believed to have myelopoietic inhibitory activity. Considering the massive number of these

cells produced daily, the physiological importance of these activities could prove to be significant.

The activity attributed to the active glycoforms of azurocidin is interesting in that it is derived almost exclusively from patients with CML and inhibits normal but not leukemic myelopoiesis. Due to the enormous numbers of neutrophils in these patients, release of this activity could result in a growth advantage for the leukemic clone. Therapies targeting the effects of this molecule could help to manage the levels of circulating myelocytes.

Regulation of myelopoiesis by CD involves the most elegant and basic control mechanisms which exist in biology. As a neutrophil derived factor acting to suppress the growth of myeloid cells, CD may be involved in the negative feedback regulation of the myelopoietic system. If the proposed mechanism turns out to be correct, it mediates its effects by starving cells of the nutrients they need in order to divide. Although not as intricate as cytokine mediated regulation, it may be more permanent and relevant. Regulated by its release and the local thymidine concentration, this molecule may act as a general regulator of hematopoisis.

The potential exists for the use of CD alone or in combination with other chemotherapeutic agents in bone marrow purging protocols for patients undergoing autologous transplantation and as a possible treatment *in vivo* for various malignancies.

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