CHARACTERIZATION OF THE EFFECTS OF P30-35 CAMAL ON NORMAL AND LEUKEMIC MYELOPOIESIS

by

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ABSTRACT

P30-35 CAMAL is a component of material immunoaffinity enriched from lysates of myeloid leukemia leucocytes using the monoclonal antibody CAMAL-1. Reactivity with CAMAL-1 is diagnostic of myeloid leukemias. CAMAL-1 enriched material inhibited myelopoiesis by normal progenitor cells *in vitro*. This study demonstrates that inhibition is associated with P30-35 CAMAL. Neutrophilic granulocyte colonies were preferentially inhibited by P30-35 CAMAL while higher concentrations affected all colony types. Non-adherent progenitor cell numbers were reduced in P30-35 CAMAL-treated long term cultures of normal marrow cells whereas adherent cell numbers were increased, suggesting a block in differentiation. In addition, colony formation by murine cells was inhibited by P30-35 CAMAL. As in cultures of human cells, neutrophilic granulocyte colonies were preferentially inhibited, indicating that downregulation of normal myelopoiesis by P30-35 CAMAL crosses species barriers, and might be an important regulatory mechanism.

Using highly enriched P30-35 CAMAL, a stimulatory effect on CML colony formation was defined. Stimulation of colony formation occurred at low and high concentrations of P30-35 CAMAL, but was reduced at intermediate concentrations. At low concentrations of P30-35 CAMAL primitive colonies were increased, whereas high concentrations affected all colony types. Colony formation by several myeloid leukemia-derived cell lines was increased by P30-35 CAMAL.

P30-35 CAMAL prepared using protease inhibitors lacked effects on colony formation. Alterations in normal and CML colony formation were similar whether cells were preincubated or cocultured with P30-35 CAMAL. Activity was retained in the supernatant of treated cells, indicating that effects were immediate and irreversible. Alterations in normal and CML colony formation were fully blocked using either phenyl methyl sulfonyl fluoride or a chloro-methyl ketone-linked peptide, both inhibitors of serine protease activity, suggesting that the alterations in myelopoiesis require serine protease activity. Experiments using characterized neutrophil proteins suggested protease activity in P30-35 CAMAL preparations which might be unique.

Reactivity of the monoclonal antibodies α -P30/35, raised against P30-35 CAMAL, and CAMAL-1 was compared. Both antibodies reacted more extensively with CML than with normal leucocytes, and α -P30/35 reacted with several myeloid leukemia-derived cell lines, suggesting that the antigen recognized by CAMAL-1 and the component which alters myelopoiesis might form an association.

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LIST OF ABBREVIATIONS

Α	Absorbence
AAPF pro-	Peptide with the sequence alanine-alanine-proline-phenylalanine (ala-ala-phe)
AAPV	Peptide with the sequence alanine-alanine-proline-valine (ala-ala-pro-val)
Ab	Antibody
abl	Protooncogene (c-abl); serine/threonine kinase
ABMT	Autologous bone marrow transplantation
AcSDKP	Tetrapeptide inhibitory to hematopoiesis
ALL	Acute lymphocytic leukemia
AML	Acute myeloid leukemia
ANLL	Acute non-lymphocytic leukemia
APF	Peptide with the sequence alanine-proline-phenylalanine (ala-pro-phe)
APL	Acute promyelocytic leukemia
Ara-C	Cytosine arabinoside
b	Basic
b2a2	Fusion gene joining bcr exon 2 to abl exon 2
b3a2	Fusion gene joining bcr exon 3 to abl exon 2
bas	Basophil
bcl	B cell leukemia
bcr	Breakpoint cluster region; serine/threonine kinase, GAP for rac
bcr-abl	Breakpoint cluster region/c-abl fusion gene, transcript or protein
BFU-E	Burst-forming unit-erythroid

bl-CFC	Blast colony-forming cell
BLV	Bovine leukosis virus
BM	Bone marrow
BMT	Bone marrow transplantation
BP	Binding protein
BPD	Benzoporphyrin derivative
BSA	Bovine serum albumin
Ca ²⁺	Calcium ion
CALLA	Common acute lymphoblastic leukemia antigen
CAM	Cellular adhesion molecule
CAMAL	Common antigen in myelogenous acute leukemia
cAMP	Cyclic adenosine monophosphate
CD	Cluster designation
cdc	Cell division cycle
CFC	Colony-forming cell
CMK	Chloro-methyl ketone
CML	Chronic myelogenous leukemia
CMV	Cytomegalovirus
CLL	Chronic lymphocytic leukemia
٥C	Degrees centigrade
CFU	Colony-forming unit
CFU-E	Colony-forming unit-erythroid
CFU-eo	Colony-forming unit-eosinophil
CFU-G	Colony-forming unit-granulocyte

CFU-GM	Colony-forming unit-granulocyte, macrophage
CFU-GEMM	Colony-forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte
CFU-S	Colony-forming unit-spleen
⁶⁰ Co	Radioactive cobalt-60
CO ₂	Carbon dioxide
CSF	Colony-stimulating factor
CSF-1	Colony-stimulating factor 1 (M-CSF)
d	Day
đ	Distilled
dd	Deionized, distilled
DAB	Diaminobenzidine (3,4,3',4'-tetra-aminobiphenyl hydrochoride)
DAG	Diacyl glycerol
DCC	Deleted in colorectal carcinoma
DIC	Differential interference contrast (microscopy)
DME	Dulbecco's modified Eagle's (medium)
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EBV	Epstein-Barr virus
ECM	Extracellular matrix
EDTA	ethylenediamine tetraacetic acid
EGF	Epidermal growth factor

ELISA	Enzyme-linked immunosorbent assay
еро	Erythropoietin
FAB	French-American-British classification of leukemias
FACS	Flourescence-activated cell sorting
FCS	Fetal calf serum
Fe ²⁺	Iron ion, ferrous form
Fe ³⁺	Iron ion, ferric form
FGF	Fibroblast growth factor
fms	Receptor for M-CSF or CSF-1 (c-fms); tyrosine kinase
fos	Proto-oncogene (c-fos); nuclear transcription factor
FPLC	Fast protein liquid chromatography
FPR	Peptide with the sequence phenylalanine-proline-arginine (phe-pro-arg)
FSH	Follicle stimulating hormone
fyn	src related kinase
g	Gravity
g	Gram
G	G protein
G ₀	Quiescent phase of cell growth cycle
G ₁	First growth phase of cell cycle, prior to DNA synthesis
G ₂	Second growth phase of cell cycle, after DNA synthesis
G6PD	Glucose-6-phosphate dehydrogenase
GAP	GTPase activating protein
G-CSF	Granulocyte colony-stimulating factor

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GGF	Peptide with the sequence glycine-glycine-phenylalanine (gly-gly-phe)
Gi	Inhibitory a subunit of G protein
GIA	Granulocytic inhibitory activity
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp	Glycoprotein
Gs	Stimulatory α subunit of G protein
GTP	Guanosine triphosphate
GVHD	Graft versus host disease
GVL	Graft versus leukemia
h	Hours
HA	High Affinity
4-HC	4-hydroperoxycyclophosphamide
HC1	Hydrochloric acid
HEL	Human erythrocytic leukemia
HEPES	N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
H-ferritin	Heavy chain subunit of acidic isoferritins
HILDA	Human interleukin for DA cells (LIF)
HLA	Human leucocyte associated antigen
HLH	Helix-loop-helix (nuclear transcription factors)
H_2O_2	Hydrogen peroxide
HOX	Homeobox
HP5b	Peptide inhibitory to hematopoiesis (pEEDCK, SP1)
HPLC	High performance liquid chromatography
HPP-CFC	High proliferative potential colony-forming cell

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HSA	Human serum albumin
HTLV	Human T-lymphotrophic virus
i	Inhibitory
ICAM	Inter-cellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP ₃	inositol triphosphate
jun	Proto-oncogene (c-jun); nuclear transcription factor
kDa	Kilodalton
kit	Receptor for Steel factor (c-kit)
1	Litre
LA	Low affinity
LAI	Leukemia associated inhibitor
LAK	Lymphokine-activated killer cell
lck	src related kinase
LCM	Leucocyte-conditioned medium
LIA	Leukemia inhibitory activity
LIF	Leukemia inhibitory factor (HILDA)
lin	Cell lineage-specific cell surface antigens
1N ₂	Liquid nitrogen
LPS	Lipopolysaccharide
LTCIC	Long-term culture initiating cell

LTMC	Long-term marrow culture
Μ	FAB classification for AML (M1 through M7)
Μ	Mitosis
Μ	Molar
mA	Milliamps
mAb	Monoclonal antibody
M-bcr	Major breakpoint cluster region (in P210 bcr-abl of CML)
m-bcr	Minor breakpoint cluster region (in P190 bcr-abl of ALL)
MC-540	Merocyanine-540
M-CSF	Macrophage colony-stimulating factor
mdr	Multidrug resistance
Meg	Megakaryocyte
mg	Milligram
MGF	Mast cell growth factor (Steel factor, stem cell factor)
MHC	Major histocompatibility complex
min	Minutes
MIP	Macrophage inflammatory protein
mM	Millimolar
ml	Millilitre
mRNA	Messenger ribonucleic acid
myb	Proto-oncogene (c-myb); nuclear transcription factor
myc	Proto-oncogene (c-myc); nuclear transcription factor
Ν	Normal
NA	Nitroanilide

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Na ₂ CO ₃	Sodium carbonate
NaDOC	Sodium deoxycholate
NBME	Normal bone marrow extract
NEP	Neutrophil endopeptidase
NF	Neurofibromatosis
ng	Nanogram
NGF	Nerve growth factor
NH_4	Ammonium
N terminus	Amino terminus
NH ₂ terminal	Amino terminal
NH ₄ Cl	Ammonium chloride
NK	Natural killer (cell)
OFP	Oncofetal protein
OSM	Oncostatin M
p	Chromosomal short arm
p	Protein
P	Protein
PAGE	Polyacrylamide gel electropheresis
PAI	Plasminogen activator inhibitor
PBL	Peripheral blood leucocytes
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
pEEDCK	Pentapeptide inhibitory to hematopoiesis (HP5b, SP1)

pg	Picogram
PGE	Prostaglandin E
Ph	Philadelphia chromosome
PHA	Phytohemagglutinin
phe	phenylalanine
PI	Phosphatidyl inositol
PIP ₂	Phosphatidylinositol phosphate
РКА	Protein kinase A
РКС	Protein kinase C
PMSF	Phenyl methyl sulfonyl fluoride
PWM	Pokeweed mitogen
q	Chromosomal long arm
QLT	Quadra Logic Technologies
r	Receptor
r .	Recombinant
rac	Protein with ras homology
ras	Proto-oncogene; tyrosine kinase
Rb	Retinoblastoma, tumour suppressor gene
RGD	Peptide sequence arginine-glycine-aspartate
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute (medium)
S	Stimulatory
S	DNA synthesis phase of cell cycle
SCA	Stem cell antigen

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SDS	Sodium dodecyl sulphate
SE	Standard error of the mean
sec	Seconds
SH	src homology region (SH2 or SH3)
SK&F	Smith Kline and French
SLF	Steel factor (Stem cell factor/SCF, Mast cell growth factor, MGF, c-kit ligand)
SP1	Peptide inhibitory to hematopoiesis (pEEDCK, HP5b)
src	Protooncogene
t	Chromosomal translocation
TGF	Transforming growth factor
TNF	Tumour necrosis factor
U	Unit
μg	Microgram
μ1	Microlitre
μΜ	Micrometer
VLA	Very light antigen

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CHAPTER 1

INTRODUCTION

1.1: Hematopoiesis

Current understanding of hematopoiesis is summarized in this section, with an emphasis on myelopoiesis. Included is a summary of current knowledge about the physiology of primitive stem cells and more committed progenitor cells, cytokines involved in hematopoiesis, and the role of the extracellular matrix in supporting the survival and regulating the proliferation and differentiation of hematopoietic cells. Culture systems for supporting and evaluating the survival, growth, and differentiation of hematopoietic progenitor and stem cells *in vitro* are described. Current knowledge about mechanisms of signal transduction are also summarized.

The process of hematopoiesis involves an exquisitely regulated balance between self-renewal and terminal differentiation. Mature cells, both erythrocytes and leucocytes, are constantly becoming senescent and must continually be removed and replaced. Neutrophilic granulocytes, for example, have a half-life in the circulation of a mere several hours (1). The removal and replacement of blood cells takes place on a massive scale; 3.7 X 10¹¹ are produced hourly (2). This is achieved through the maturation, or differentiation, of primitive pluripotent stem cells, which have high potential for self-

renewal, through a series of cell divisions. As the cells divide, they become progressively more differentiated, and progressively more restricted in their capacity for self-renewal. Regulation of the process of differentiation and maintenance of the stem cell pool is achieved through a complex network of interactions between the cells, their soluble products, and their immediate environment. The result is the transmission of both positive and negative regulatory signals to the cell nucleus via intercellular and intracellular pathways, the details of which are currently under intense investigation. Whether a cell will be induced to remain quiescent, or to proliferate and become terminally differentiated, its lineage of differentiation, and its state of activation are determined by the balance of signals acting on it.

1.1.1: Characteristics of stem and progenitor cells, assay systems, and terminology:

Classical assays demonstrating colony forming cells or progenitor cells were instigated by Till & McCulloch (3). In these studies, lethally irradiated mice were rescued with bone marrow from syngeneic donors, resulting in hematopoietic reconstitution, and in the formation of colonies of hematopoietic cells in the spleen. Examination of these colonies revealed that some were composed of one cell type, whereas others were composed of several; others still contained cell types of all the hematopoietic lineages. Since the mice were reconstituted with a limited number of cells, it was assumed that each colony arose from a single progenitor cell. This result was taken as evidence of the existence of a primitive pluripotent stem cell, capable of giving rise to all differentiated hematopoietic cell types, in addition to the existence of progenitor cells already committed to one lineage or another. These studies have since been repeated using retrovirally marked stem cells, confirming that the pluripotent stem cell does indeed exist (4).

Assay systems developed since provide convenient methods for studying stem and progenitor cells. Colonies of murine and human hematopoietic cells can be grown in vitro in agar or methylcellulose (4). In this way, factors which influence colony formation have been assessed, and characteristics of the progenitor cells themselves have been inferred from the characteristics of their progeny. A more physiological assay of hematopoiesis is the long-term marrow culture, or Dexter culture (5). Incubation of marrow cells under the appropriate conditions results in the formation of an adherent layer of stromal cells, consisting of fibroblasts, adipocytes, endothelial cells, and macrophages. These cells are in intimate contact with the primitive hematopoietic cells, and provide a microenvironment, thought to be similar to the microenvironment in the bone marrow, which supports the survival and maturation of hematopoietic cells until their release into the circulation. Interaction with the stroma is thought to maintain stem cell numbers and the production of progenitor cells committed to the various lineages (6). Contact with the stromal cells is required for maintenance of hematopoietic cells; if physically separated from the stroma, the hematopoietic cells die (2). Conditions in long-term cultures are evaluated by removing cells from culture supernatants at weekly intervals, and plating them in a colony assay. Hematopoiesis can be supported by preestablished allogeneic stromal layers, provided these are irradiated to prevent rejection reactions.

Colony assays were used to define the lineage commitment of progenitor cells. For example, when bone marrow cells are cultured for colony formation under permissive

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conditions, using rich culture medium containing a mixture of cytokines, several colony types result. Colonies of the myeloid lineage consisting of more than one cell type include granulocyte-macrophage colony-forming units or CFU-GM, and granulocyte, erythrocyte, macrophage, megakaryocyte colony forming units, or CFU-GEMM, but not other combinations of cell types. This is an indication that (neutrophilic) granulocytes and macrophages are more closely related to each other than to erythrocytes and megakaryocytes. The formation of single lineage colonies is also supported in these conditions; these include CFU-M (macrophage), which arises from a macrophage-colony forming cell or M-CFC. Also included are CFU-G (neutrophilic granulocyte), arising from a G-CFC, CFU-E (erythrocyte) and the more primitive and larger BFU-E (burst-forming unit-erythrocyte), and more rarely, CFU-Meg (megakaryocyte), CFU-eo (eosinophil), and CFU-bas (basophil). Infrequently in normals, and more frequently with leukemic specimens, colonies of immature blast cells will form, arising from bl-CFC. Colony assays and Dexter cultures support lymphopoiesis if required.

Recently it became evident that the pluripotent CFU-S (colony forming unit-spleen) of the spleen colony assay is not the most primitive hematopoietic cell type present *in vivo*. By transplanting spleen colonies to secondary irradiated recipients, it became clear that these animals could be reconstituted in the short term, but that long term engraftment did not occur, and conversely, that long-term reconstitution could be obtained without CFU-S (7). Hence, characteristics of yet more primitive cells have been investigated; these include the high proliferative potential colony forming cell or HPP-CFC (8), and the long term culture initiating cell (LTCIC, reference 9), which is capable of giving rise to Dexter

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cultures. By separating populations of bone marrow cells on the basis of physical characteristics such as light scatter (forward and perpendicular, reference 9) and wheat germ agglutinibility (10), or reactivity with dyes such as rhodamine 123 (9, 11, 12), or Hoechst 33342 (13), some of the characteristics of stem and progenitor cells have been defined. Progenitor cells committed to the various myeloid lineages express lineage specific markers (lin⁺) and the cell surface molecule cluster designation antigen 33 (CD33⁺). More primitive cells, including those committed to the myeloid lineage, are CD34⁺33⁺, whereas the most primitive hematopoietic cells are CD34⁺33⁻lin⁻, and have the appearance of small lymphocytes (9). Both the LTCIC and the HPP-CFC express CD34 but have no detectable expression of HLA-DR (9). CD34⁺ cells have also been shown to give rise to stromal cells of the bone marrow microenvironment (14). CD34 was recently cloned; the cDNA encodes a receptor-like 100 kDa transmembrane protein with no obvious homology to other known proteins (15). Two forms are generated by alternative splicing, which differ in the length of their intracytoplasmic domain. This finding could have implications regarding signal transduction by CD34 (16).

Headway has also been made into defining the nature of the cell-cell interactions in long-term cultures. Direct contact between stromal and hematopoietic cells is required for hematopoiesis to occur; in the absence of this contact, the hematopoietic cells rapidly die (2). Foci of hematopoietic cells form in regions covered by blanket cells, which have been shown to be endothelial cells (17). As these cells mature, they are released into the culture supernatant by passing through, rather than between, the blanket cells, presumably in the same manner as they are released from the bone marrow into the circulation (2). The precise signals that govern the adhesion, transport, and release of the hematopoietic cells are not clear, however, several molecules have been shown to be involved in adhesion. These include the proteoglycans of the extracellular matrix, specifically heparan sulfate (18), galactosyl and mannosyl residues on the cell surface glycoproteins of stromal cells, which recognize a 110 kDa membrane homing lectin on hematopoietic cells (19), and CD44, the homing receptor, which recognizes hyaluronate (20, 21). In addition, a 60kDa protein from bone marrow extracellular matrix (ECM), haemonectin, which mediates attachment of cells of the granulocytic lineage has been described (22). Other known components of the ECM include laminin, vitronectin, and type IV collagen (2). Antibodies directed toward classical ECM components such as fibronectin do not block adhesion, nor do peptides containing the adhesion sequence RGD (2). However, one group showed that the VLA-4 integrin receptor, expressed on day 12 CFU-S and cells which reconstitute hematopoiesis, attach to the C-terminal heparin-binding fragment of an alternatively spliced form of fibronectin (23). The same group demonstrated that neither type IV collagen nor laminin promoted attachment of day 12 CFU-S. Maturation of hematopoietic cells is associated with the loss of adhesion molecules, such as ICAM-1 (CD54, reference 24).

1.1.2: Colony-stimulating factors, interleukins, and other cytokines:

Original studies with colony assays were performed using conditioned media from various sources, providing a rich mixture of cytokines which support the growth of several colony types. Colony-stimulating and inhibiting activities have since been purified and characterized from these sources, as well as from the supernatants of cytokine-secreting cell lines, and many have been cloned. Their actions and interactions are being investigated by

the addition of recombinant factors to colony assays and to long term cultures, by their effects *in vivo*, and by over-expression in animals reconstituted with stem cells infected with retroviral constructs encoding cytokine cDNA. Cytokines that influence hematopoiesis are generally derived from a wide variety of sources, and have pleiotropic effects on a wide variety of cells. There is an extensive body of literature detailing the effects of these cytokines. Their effects are dependent on the state of the target cell; whether it is expressing the appropriate receptors, its cell lineage and stage of differentiation, the immediate microenvironment, and on which other cytokines are present (25, 26). Moreover, there is considerable overlap in the spectrum of activities of various cytokines (27). For these reasons, it is difficult to generalize about the biological effects of individual cytokines. The more well known effects and more interesting actions of the major factors known to influence hematopoiesis are summarized in Table 1. This list is by no means exhaustive.

1.1.3: Cytokines that are noteworthy in hematopoiesis:

Classical cytokines that are known to influence myelopoiesis include interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-1 (IL-1), as well as granulocyte colony-stimulating factor (G-CSF) and macrophage colony-stimulating factor (M-CSF). IL-3 supports the survival and proliferation of primitive colony forming cells (CFU-GEMM), and can support the survival of these cells in the absence of a stromal layer (88). It may act as a competence factor, stimulating the cells to leave G_0 and rendering them able to respond to other cytokines (28). GM-CSF supports
TABLE I. Summary of the more well known effects and more interesting actions of the interleukins, colony-stimulating factors, and other cytokines known to influence hematopoiesis.

		I						
CYTOKINE	OTHER NAMES	FEATURES	CHROMOSOMAL LOCATION	SOURCE	TARGETS, MAIN ACTIVITIES	RECEPTOR FEATURES	OTHER	REFERENCES
INTERLEUKINS								
П-1	II , $I \alpha = hemopoictin 1$	17-31kD	2g14	monocytes, neutrophils, endothelial, fibroblasts	enhances cytokine & receptor cypression, synergizes with CSFs, growth factor for hymphocyts, augments I cell response to antigen, endogenous pyrogen, induces production of acute phase proteins, radioprotective	87-100kD HA, 68-80kD LA, 3 irrrumoglobulin domains, trember of superfamily	transmembrane form of alpha, IL- Ircu is natural inhibitor, may be autocrine in AML, β is a 3 dimensional homologue of bPGF	28, 29, 30, 31, 32
11-2	T cell growth factor (TCGP)	14-17kD	4p	activated T	growth of T, B, & NK cells, antbody synthesis, cytokithe production	p55 Tac is low affinity, high affinity + p75 Tic, soluble forms of receptor, member of superfamily		29, 33, 34, 35
П.3	multi-CSF, persisting cell stimulating factor (PSP), hernatopoietic cell growth factor (HOZP), burst promoting activity (BPA), cosinophil-CSF, mast cell growth factor (MCGF)	15-30kD	5q23-31	T cells	strimulates growth of early progenitor cells and differentiation to all lineages	85, 135, 70 135 is member of superfamily, soluble form, shares bela subunit with GM-CSF, IL-5	solid-phase chemical symthesis of entire protein	28, 29, 31, 33, 34, 35, 36, 37
н 4	B cell stimulatory factor-1 (BSF-1)	20kD	5q23-32	CD4+ T	growth and activation of lymphocytes, especially B, increased class II expression on B	46kD LA, 114kD HA (includes p46), soluble form, member of superfarmity		28, 29, 31, 33, 34, 35
П.5	BCGF.II, T œll replacing factor (TRP), cosinophil differentiation factor (EDP)	45kD dimer	5q31-33	T cells	cosinophil growth and differentiation, also basophil, and B differentiation	50, 80, 130kDa b subunit shared with IL-3, GM-CSF, member of superfamily		28, 31, 35, 38
П-6	B cell stimulatory factor-2 (BSF-2)	22kD	7p21	monocytes, endothelial, T, B, fibroblasts, smooth muscle	B cell growth, supports hybridoma growth in vitro, synergizes with cryothenes on early progenitors, differentiation of murine monocytic leukernia M1, endogenous pyrogen, acute phase proteins	50, 60, 75, gp130	homology to OSM, LIF & G- CSF, receptor shares 130 kD subuit with LIF receptor, soluble form of receptor, receptor member of superfamily	29, 31, 34, 35, 39, 40
пл		17kD	8q12-13	T cells	B cell growth & differentiation, T cell functions	soluble form, member of superfamily		31, 33, 34, 35, 41
8-11	neutrophil chemotactic factor (NCP), neutrophil activating peptide (NAP)	10kD	4	monocytes, endothelial	chemotactic for neutrophils			31, 33, 42
6-11	mast cell growth enhancing activity (MEA)	40kD	5q31-35	T cells	enh ance s epo-dependent colony formation of BFU-E			31, 43, 44
П10		18kD			inhibits cytokine synthesis by monocytes, downregulates HLA class II expression, stimulates mast culls & progenitors, growth & differentiation of B		homologous to HBV gene BCRFI	41, 45, 46, 47, 48
П-11	•	23kD			acute phase proteins, synergizes with IL3 on carrly progenitors, and megakaryocy te progenitors, enhances Ab responses			39, 49, 50, 51
п12	cytotoxic lymphocyte maturation factor (CLMF)				maturation of CTL			52

COLONY STIMULATING								
GM-CSF	CSF-a	14-35kD	5q23-31	T cells, endothelial, fibroblasts, smooth muscle	growth & differentiation of neutrophils & noncorycat, alse ossitophils & basophils, activation of mature cull functions, decrease in noutrophil migration	85, 84kDa is member of superfamily, soluble form	induces differentiation of HL60, autocrine stirmulation in some AML, transgenic mice have many lesions	6, 28, 30, 31, 33, 34, 35, 37, 38, 53, 54, 55, 56
M.CSF	CSF-1	70-90kD disulfide-linked dimer	1p13-21	monocytes, neutrophils, endothelial, fibroblasts, srmooth muscle, preadipocytes	growth & differentiation of monocytes, activation of mature cell functions including phagocytosis, chemotaxis, ADCC	c-fins 105, 150, Y kinase domán, 5q23-34	гпау be autocrine in AML MS, structural homology to SLP	6, 28, 31, 33, <i>57</i>
G-CSF	CSF-ß	20kD	17q11-21	monocytes, neutrophils, endothelial, fibroblasts	growth & differentiation of G, activation of mature cell functions, including ordative burst, chemotaxis in response to stimuli, synergizes with IL-3 on carly progenitors	90, 150, member of superfamily	2 forms by differential splicing differ by 3 armino acids - longer less active, related to OSM, LIF, IL-6	6, 29, 31, 33, 35, 56, 58, 59
ery thropoietin		32-35kD	7q21	kidney	growth & differentiation of erythroid progenitors	55, 100, 85, 65, superfamily	prevents apoptosis of crythroid progenitors	31, 33, 34, 35
OTHER CYTOKINES		-						
Steel factor (SLF)	stern œll factor (SCP), kit-ligand, mast œll growth factor (MGF)	1840		fibroblasts	syncrgizes with CSFs on early stem cells and later progenitors, increases self- renewal, mast cell production	c-kit, product of W locus, tyrosine kinase domain	product of Sueel locus, homology to M-CSF, transmembrane form has putative proteolytic cleavage site	31, 60, 61, 62
Turnour necrosis factor (TNF)-α	cachectin	17kD	6 p 23	monocytes, neutrophils, endothelial, fibroblasts, mast cells	endogenous pyrogen, acute phase proteins, cachevia, septic shock, cubances primitive colonics, suppresses committed colonics	55, 75, distinct receptors, soluble form, NGF receptor homology	within MHC locus between class III & class I, transmembrane form	63, 64, 65
β-HNL	lymphotoxin	18kD	6p23	T cells	induces early response genes such as fos, jun, cytokines, receptors, bone resorption		within MHC locus	31, 65, 66
Transforming growth factor (TGP)-α						·	EGF family, transmembrane form, juxtacrine stimulation	67, 68
TGP-ß		25 kD homo or hetero-dimer	5 genes, separate chromosomes	platelets, normal strornal layers	regulates cytokines, carly response genes, suppresses primitive colonies, enhances lineage-committed colonies	•	·	69
Type I interferon (ifn-α and ifn-β)		18kD	cluster on 9p: 20 alpha genes, 1 beta	monocytes (a), fibroblasts (b), other	growth inhibitory, increased expression of Class I MHC, differentiation & activation of NK cells		genes lack introns	29, 70
Type II interferon (ifn-y)		21-24kD homo- dimer	13	T cells, NK cells	growth inhibitory, activation of monocytes, endothelial cells, NK cells, increased class I & II		induces apoptosis in effector T cells	29, 70, 71, 72
Nerve growth factor (NGP)		27kD hetero- dimer		fibroblasts, neurons, other	differentiation, activation, viability of eosinophils, neutrophils, monocytes, platelets, neurons	trk + p75 NGFR TNF receptor homology	phosphorylation of MAP kinase	73, 74, 75, 76
Basic Fibroblast growth factor (bFGF)			5q31-33	monocytes	mitogen for stromal cells, competence factor, induces MCSP, angiogenesis, neurotropic	multigene family includes int-2, hst/ks	3 dimensional homologue of Π 1β, heparin-binding	28, 77, 78, 79

conkernia nhibitory factor LIF)	human interleukin for DA cells (HILDA), differentiation inducing activity (DIA), D-factor, cholinergic differentiation factor (CDF)	58kD disulfide linked	22412	monocytes, endothelial, fibroblasts, T cells	maintains embryonal stem cells & hematopoietic stem cell viability, differentiation of murine leukernia M1, possible role in cachexia	2 subunit gp130 signal converter	related to OSM, G-CSF, and IL- 6, maintains stern cells during transfection for gene therapy (adenosine dearninase protocol)	31, 58, 80, 81, 82, 83, 84, 85, 86
Oncostatin M		28kD	33	activated monocytes and T cells	differentiation of M1, neuronal differentiation, acute phase proteins	receptor is gp130 of LIF receptor, also binds complete 2 subunit LIF	related to LJP, G-CSF, and IL-6	31, 58, 86, 87
latelet-derived growth factor PDGP)				platelets, monocytes, endothelial, fibroblasts	synergizes with IL-3 and GM-CSF on early progenitor cells, enhances growth of B progenitors & stromal cells	on chromosome 5	· .	62

colony formation of cells that are slightly more differentiated (CFU-GM). Still more differentiated CFU respond to single lineage factors such as G-CSF or M-CSF. The progressive responsiveness of more differentiated cells to different cytokines or sets of cytokines is achieved through sequential expression of receptors for the appropriate factors (6). A hierarchy of receptor expression exists, a phenomenon that has been referred to as receptor trans-downmodulation. For example, exposure of progenitor cells to IL-3 results in the downmodulation of receptors for GM-CSF, M-CSF, and G-CSF, whereas exposure to GM-CSF results in downmodulation of the M-CSF and G-CSF receptors (89). Receptor upregulation can also occur, for example, exposure of cells to IL-1 results in the expression of receptors for many cytokines (30). Cytokines also influence the expression of other cytokines. As one of numerous examples, IL-1 potentiates the response of progenitor cells to IL-3 and GM-CSF. This potentiation is mediated at least in part by upregulation of the transcription of the genes for various cytokines by accessory cells in addition to the upregulation of receptor expression by target cells (28). In addition to the trans-regulation of cytokines by other cytokines, some factors are capable of upregulating their own expression; these include IL-1, IL-6, tumour necrosis factor- α (TNF- α), and GM-CSF (90, 91). Thus, progenitor cells exhibit a degree of plasticity in their response to cytokines, depending on conditions in their immediate environment. The lineage of differentiation is thought not to be a predetermined property of primitive progenitors cells. but rather is determined by the balance of factors present (92). Many cascades of cytokine action are currently being defined in vitro and in vivo. Undoubtedly, common themes and patterns will emerge. In addition to their complex interactive effects, some cytokines, for example G-CSF, have been shown to exist in more than one form which differ by several amino acids, and which may show differences in activity (59).

A cytokine that has generated considerable interest is the recently cloned Steel factor (SLF). SLF is the product of the Steel locus (62, 93). Mutations at this locus result in the Steel phenotype in mice, a phenotype that includes severe macrocytic anemia in addition to germ cell defects, white spotting, and mast cell deficiency. This phenotype is similar to that seen in W mutants. The receptor for SLF is *c-kit*, a transmembrane protein with intrinsic tyrosine kinase activity, and the product of the W locus (61, 94). SLF is expressed by fibroblasts and other cells in a transmembrane form, although a soluble form exists (95). C-kit is expressed by hematopoietic cells (96, 97). SLF potentiates the response of a variety of progenitor cells to a variety of cytokines (98). SLF has been reported to increase the size of colonies in response to several factors (99), and to increase the secondary plating efficiency of colonies stimulated by IL-3, an indication that the selfrenewal capacity of these cells is upregulated (60). This finding has implications for the expansion of progenitor cell pools for bone marrow transplantation and in the treatment of neutropenia. SLF also potentiates the development of mast cells (100), hence one of its many names, mast cell growth factor, or MGF. Although widely accepted that signals transduced through the SLF/c-kit pathway have profound effects on hematopoiesis, the work of some groups has shown that the presence of wild-type SLF (101) or c-kit (102) is not strictly required for normal hematopoiesis in some circumstances. The numbers of hematopoietic stem cells were found to increase during the fetal development of SI/SI mice, which are homozygous for mutations in the Steel gene (101). Similarly, human subjects with *c-kit* mutations resulting in null alleles were found to lack hematological abnormalities (102). These results do not exclude the possibility that compensatory pathways may be operative in these situations.

Classically, cytokines have been thought of as soluble intercellular messengers produced by stromal and hematopoietic cells which exert their effect on binding to a transmembrane receptor on a nearby cell (paracrine regulation, reference 103). Cytokines are known to exert their effect in the bone marrow microenvironment at least in part through binding to cell surface proteoglycans of the extracellular matrix, which protects them from degradation, and facilitates their presentation to hematopoietic cells in local inductive microenvironments (2, 4, 104, 105, 106). Recently, several soluble cytokines have been shown to be derived by proteolysis from transmembrane precusors. These include IL-1 (107), TNF- α (64), transforming growth factor- α (TGF- α , reference 68), and SLF (95). The transmembrane forms of these cytokines may bind receptors on adjacent cells, which in some cases has been found to result in signal transduction (108), including activation of receptor kinase activity (109), and activation of proliferation. This phenomenon has been referred to as juxtacrine regulation. (67). Moreover, clustering of adhesion molecules such as integrins, a process that occurs during the formation of adhesive contacts, was shown to result in protein tyrosine phosphorylation (110). Thus, hematopoiesis appears to be mediated via two tiers of control; one through cell contact, and the second through the release of and response to soluble factors. The signals required for normal steady-state hematopoiesis may be regulated by cell-cell contacts, whereas soluble cytokines may mediate intercellular signals during periods of stress (28, 111). In addition to the existence of transmembrane cytokines, soluble forms of receptors for several factors have been described. These include the receptors for interleukins 2 through 8, GM-CSF, interferon-y (ifn-y) and TNF- α (34, 35). The soluble forms of these receptors may act as

physiological downregulators of cytokine activity by binding excess cytokine (34, 35, 72). Alternatively, they may act as ligands for transmembrane cytokines (112).

In addition to their effects on local hematopoiesis, several cytokines are known to exert systemic effects. These include IL-1, TNF- α , IL-6, and IL-11, all of which induce the production of acute phase proteins by the liver in response to inflammation and infection (29, 39). IL-1 and TNF- α induce fever by acting on the temperature control centre in the hypothalamus, (1, 29) and TNF- α , or cachexin, induces cachexia, a wasting syndrome in which a "futile" glycolytic pathway is induced in muscle and adipose tissue, resulting in the rapid turnover of these tissues (113). TNF- α is also involved in the induction of septic shock (63).

Analysis of the gene structure of several cloned cytokines and their receptors has resulted in their classification into subsets. Many cytokine receptors have incorporated commonly used structural motifs such as immunoglobulin and fibronectin-like domains (34). Some receptors have intrinsic tyrosine kinase activity; these include the SLF receptor *c-kit*, and the CSF-1/M-CSF receptor, *c-fms* (96, 114). Others, which are members of a hematopoietic growth factor receptor superfamily, have no kinase domain. Included in this family are the receptors for interleukins 2 (β chain), 3, 4, 5, 6, 7, GM-CSF, G-CSF, and erythropoietin (34, 35). These receptors are thought to transduce signals via interaction with non-receptor kinases, similar to the interaction of the *src* -related kinase *lck* with CD4 or CD8 of T cells (115, 116). Protein kinases and phosphatases are known to be involved in erythropoietin-mediated signal transduction (117). A potential candidate for the kinase involved in signal transduction from these receptors is the *src* related *c-abl*. Proliferative stimulation of cells through the receptor of this family results in the translocation of a cytoplasmic calmodulin binding protein to the nucleus (118). The members of this family all have a trp-ser-X-trp-ser motif in a section of the protein which is located just outside the cell membrane (27, 34, 35). The significance of these findings is unclear. The receptors for some cytokines, for example G-CSF, have been shown to exist in more than one form which differ by several amino acids, and which show differences in activity (119).

With still other cytokines, it has become clear on expression of recombinant protein that additional receptor subunits are required for high affinity binding of ligand. The receptors for IL-3, GM-CSF, and IL-5 all share a common β subunit (37, 38). This provides an additional mechanism for regulating the actions of these factors, as they must compete for possibly limiting concentrations of the β -subunit in order for high affinity binding of ligand to occur. It also indicates that several cytokines presumably activate common signal transduction pathways, and provides a mechanism by which the same cytokine could mediate its pleiotropic effects; different effects may be mediated via the interaction of the receptor with different β subunits. As a second example of this type of control, the signal converter for the leukemia inhibitory factor (LIF) receptor, gp130, also acts as a signal converter for IL-6, and is the receptor for oncostatin M (OSM, references 85, 87). Moreover, the α subunits for the LIF and IL-6 receptors show considerable structural homology, as do LIF, IL-6, and G-CSF themselves (58).

Although the gene products are unrelated by amino acid sequence, the genes for many cytokines and their receptors are closely linked by chromosomal location, an indication that they may share common modes of regulation (28, 33, 53). The genes encoding many of the colony-stimulating factors and their receptors are located on the long arm of chromosome 5. The 5q- syndrome, in which regions of this chromosome are deleted, involves hematological abnormalities and is a common chromosomal abnormality in AML secondary to therapy (30). The genes for GM-CSF and IL-3 are adjacent and are thought to be functionally linked (6). Moreover, an element common to the upstream regions of the genes encoding murine GM-CSF, IL-4 and IL-5 has been identified (120).

Production of cytokines has been noted in some acute leukemias, and it has been suggested that these may stimulate leukemic cell growth in an autocrine manner. Retroviral gene transfer of cytokine genes into progenitor cells, however, has produced nonneoplastic expansions of cell populations, an indication that autocrine cytokine production may aid in expansion of the leukemic cells but is likely not the primary lesion (6, 121).

Clinical trials have been undertaken using erythropoietin (epo), G-CSF, GM-CSF, IL-3, M-CSF, IL-1, IL-3, IL-4 and IL-6 for a diverse series of indications, including acceleration of recovery from myelosuppression following chemotherapy or bone marrow transplantation for the treatment of leukemia and other disorders (27).

1.1.4: Signal transduction:

Oncogenesis results from the disruption of signal transduction pathways. This disruption can take place at any step along a signalling pathway, from alteration of the structure or expression of a growth factor or its receptor, to alteration of a cytoplasmic

signal transducer, to alterations of nuclear factors involved in transcriptional control (122). More than 100 oncogenes have been identified (123); a complete discussion is beyond the scope of this paper. This section gives a brief overview of current understanding of signal transduction, as an indication of the steps at which leukemogenic changes could potentially occur.

Headway has been made in recent years in elucidating the pathways by which signals for cellular activation and division are transmitted from cell surface transmembrane receptors to the nucleus, although many aspects of various pathways, and the interactions between pathways, remain unclear. What is clear is that phosphorylation and dephosphorylation of protein substrates are crucial events in controlling the activity of cellular signal transducing proteins. This is especially true of phosphorylation and dephosphorylation of tyrosine residues, although serine and threonine residues are also involved. The alteration of activity of these proteins by phosphorylation and dephosphorylation ultimately results in the alteration of the activity or DNA binding state of nuclear transcription factors or tumour suppressor genes, and consequently the pattern of transcription and expression of sets of genes regulated by these factors is altered.

Many membrane receptors have intrinsic tyrosine kinase activity, these include ckit, the receptor for SLF, and *c-fins*, the receptor for M-CSF (25). Other tyrosine kinases are cytoplasmic proteins, which may become translocated to the cell membrane to receive and pass on signals. More recently, kinases have been located in the cell nucleus. *C-abl*, a protein with tyrosine kinase activity, was shown to bind to specific DNA sequences. Its DNA binding ability is altered depending on its phosphorylation state, which is regulated in

a cell-cycle specific manner (124). The structure, activity, and cellular location of *c-abl* are altered in chronic myelogenous leukemia (CML), which has important implications for cellular dysregulation in this disorder. Studies using inhibitors of kinases and of phosphatases have demonstrated that both kinase and phosphatase activity are required in order for hematopoietic cells to respond to cytokines (125). In addition, certain signal transduction pathways appear to be common to different cells, whereas some pathways are present in some cells but not in others. For example, transfection of *c-fms* was shown to enable cells to respond to its ligand CSF-1, and to rescue them from dependence on SLF (126), an indication that signals transduced by *c-fms* and *c-kit*, the receptor for SLF, use common pathways. In another study, transfection of *c-fms* enabled cells of the myeloid lineage, but not T cells, to respond to CSF-1 (127), suggesting that the signal transduction pathway used by *c-fms* is absent or non-functional in T cells. Both kinases and phosphatases specifically active in hematopoietic cells have recently been identified (128, 129, 130), as have some substrates of phosphorylation (131, 132).

One of the first signal transduction pathways to be elucidated is that of protein kinase C (PKC). In this pathway, binding of ligand to receptor results in the activation of phospholipase C. This enzyme cleaves inositol triphosphate (IP₃) to phosphatidylinositol phosphate (PIP₂) and diacylglycerol (DAG). PIP₂ causes the release of Ca²⁺ from intracellular stores in the endoplasmic reticulum and mitochondria, resulting in the activation of protein kinase A (PKA, cyclic AMP-dependent protein kinase). DAG binds to cytoplasmic PKC, resulting in its translocation to the cell membrane and activation of its kinase activity (133). Some of the substrates of PKC have been identified. Although its function is not understood, one of the substrates phosphorylated by PKC is CD34 (134).

Since CD34 is a marker of primitive myelopoietic cells, the phosphorylation of CD34 by protein kinase C may prove to be an important event in hematopoiesis.

Also involved in signal transduction from the cell surface are the receptor-associated G proteins and the related p21^{ras}. Binding of ligand to the appropriate receptor brings about activation of its associated G protein, and release of the α subunit from the alphabeta-gamma G protein complex. Release of the α subunit results in alteration of the activity of adenylate cyclase. If the α subunit is a G_s, or stimulatory subunit, adenylate cyclase becomes activated, and intracellular levels of cyclic adenosine monophosphate (cAMP) rise, activating cAMP dependent kinases, or protein kinase A. If the α subunit is a G_i, an inhibitory subunit, the reverse will occur. Recently, a G protein α subunit specifically expressed in hematopoietic cells, G- α -16, was identified (135).

G proteins depend on the binding of guanosine triphosphate (GTP) for their activity. They possess intrinsic GTPase activity, but hydrolysis of GTP is slow. GTPase activity is potentiated by GTPase-activating proteins, or GAPs, thus hastening the inactivation of the G protein. It is thought that GAPs may also be regulated by G proteins as a downstream step in signal transduction (136). A recent finding likely to have considerable impact in the understanding of chronic myelogenous leukemia is that *bcr*, a gene product of previously unknown function which is involved in the characteristic translocation of CML between chromosomes 9 and 22 that results in a *bcr-abl* fusion protein, encodes a GTPase activating protein (137, see below).

Common and diverging downstream pathways of signal transduction activated by the interaction of ligand with cytokine receptors are being elucidated. For example, using antisense oligodeoxynucleotides to N-ras, it was demonstrated that N-ras is required for colony formation supported by IL-3, GM-CSF, and M-CSF, but not G-CSF (138). Moreover, ras GAP has been shown to immunoprecipitate with the P210 bcr-abl protein present in cells of chronic myelogenous leukemia, and to be phosphorylated by the kinase activity of P210 bcr-abl, a finding which implies that mitogenic signals mediated by p21 ras could be altered in *bcr-abl* positive cells (139). In addition, it has been shown that the M-CSF receptor c-fms, the SLF receptor c-kit, and the platelet-derived growth factor (PDGF) receptor, all of which have intrinsic tyrosine kinase activity, and are activated by autophosphorylation on ligand binding, all form complexes with phosphatidylinositol 3'kinase (PI3-kinase), an indication that they may initiate signalling along a common pathway. In contrast, the PDGF receptor and *c-kit*, but not *c-fms*, form complexes with phospholipase C-gamma 1, and only the PDGF receptor forms a complex with ras GAP (114). Hence, different receptors appear to initiate similar signalling pathways, but in different combinations. Moreover, the PDGF receptor was shown to bind GAP and PI3kinase through different sites, thus activating different signal transduction pathways (140). PI3-kinase binds p21 ras in addition to ras GAP, an illustration of the complexity of interactions between signalling proteins known to influence each other's activity (141).

Several nuclear protein factors important in the regulation of transcription have been identified. These proteins bind each other in a specific manner, which influences their capacity to bind DNA. Specific DNA recognition sequences have been identified for various transcription factors, and located upstream of families of genes, an indication that regulation of the expression of certain genes has some common features. The nuclear factors interact with each other via leucine zippers, alpha-helical structures with leucine residues at intervals of every seventh amino acid. Leucines on the helices of adjacent factors interact in a staggered conformation and "zip" the proteins together (142). Transcription factors interact with DNA through structures such as zinc fingers, zinc coils and zinc twists. These were originally identified as structures in which four cysteines or two cysteines and two histidines, which could be quite separate by primary structure, become apposed by tertiary structure and incorporate a zinc atom, thus influencing their DNA binding (143). This has more recently become a somewhat generic term for similar structures which may incorporate other metals such as copper or iron (123).

Examples of known transcription factors are AP-1, which is a complex of *c-fos* and *c-jun*, NF κ B, which binds to sequences upstream of immunoglobulin genes as well as others, and *c-myc* and *c-myb*, which are important for progression through the cell cycle. As an example of the complexity of transcriptional control, the transcription of *c-fos* has been shown to be downregulated by the underphosphorylated, DNA-binding form of the product of the Rb gene locus, a ubiquitously expressed tumour suppressor gene which is inactivated in retinoblastoma (144). Phosphorylation of the nuclear Rb is associated with progression through the cell cycle, and is prevented by TGF- β (145), as well as by interferons and interleukin 6 (146). Transcription of TGF- β and IL-6 are in turn suppressed by Rb (145), and by Rb and the nuclear phosphoprotein p53 (147), respectively. The DNA binding capability of many other nuclear factors is altered by their phosphorylation state as well. Thus, the activation or repression of specific genes or sets of genes, both in hematopoietic and in non-hematopoietic tissues, is regulated by a series of

complex interactions between numerous gene products, the details of which are currently being elucidated.

Many translocations involved in several types of leukemias, particularly the acute leukemias, involve the dysregulation of nuclear transcription factors (148). For example, the t(15;17) translocation of acute promyelocytic leukemia disrupts the retinoic acid receptor- α locus. Retinoic acid, in turn, is a negative regulator of AP-1 responsive genes (149), which mediates transcription induced by phorbol ester tumour promoters (123) and cytokines (150), as well as DNA replication (151, 152). Hence, this translocation disrupts the ability of the cell to downregulate a specific set of genes involved in growth control. Other types of nuclear factors known to be involved in hematopoiesis include proteins containing helix-loop-helix (HLH) domains, such as Id, which inhibits myeloid differentiation by sequestering other HLH proteins (153), and homeodomain containing proteins, the products of homeobox (HOX) genes, which are differentially expressed during terminal differentiation along different hematopoietic lineages (154).

The function of cyclins, which are involved in passage through the cell cycle, is also being clarified. Cyclin levels are known to increase during the cell cycle just prior to the first growth phase/DNA synthesis (G1/S) and second growth phase/mitosis (G2/M) transitions, by inhibition of specific proteolysis. Injection of cyclin messages into *Xenopus* oocytes induces entry into M phase (155). Cell division cycle (cdc2) kinase which is known to phosphorylate histone H1 and other nuclear proteins such as the tumor suppressor genes p53 and Rb, and may also phosphorylate RNA polymerase. Cdc2 kinase is in turn activated by removal of a phosphate group by cdc25 phosphatase (156). Active cdc2 kinase and a cyclin then associate to form M phase promoting factor, which promotes progression through the cell cycle. Thus, phosphorylation and dephosphorylation are important not only in the activation of mature cell functions, but in the passage of cells through the cell cycle in addition. The growth inhibitory effect of TGF- β is reported to be in part mediated by a decrease in phosphorylation and kinase activity of cdc2 at the G1/S transition (157).

In summary, activation of positive or negative regulatory signals influencing cell function or cycling results in a complex set of interactions between signalling molecules, which act in interrelated cascades at the cell surface, in the cytoplasm, and in the nucleus. These signals ultimately result in alteration of the expression of different sets of genes, which in turn alter cellular functions. Changes in the structure or expression of these signalling molecules can lead to the dysregulation of cellular growth, and to oncogenesis.

1.2: Oncogenesis and leukemogenesis;

The clinical features and etiology of myeloid leukemias, with a focus on chronic myelogenous leukemia (CML), are described in this section. A general discussion of oncogenesis is included, with a description of the involvement of cellular oncogenes, tumour suppressor genes, and transcription factors. Also included is a description of current evidence for leukemogenesis by disruption of the balance between self-renewal and

differentiation, with a focus on CML. Evidence that CML is a pluripotent stem cell disorder is described. Current understanding of the translocation between chromosomes 9 and 22, which results in the Philadelphia chromosome and P210 *bcr-abl* fusion product, and the role of *bcr-abl* in the development of CML, is summarized. Factors involved in the alteration of hematopoiesis and potentially in leukemogenesis are also described. Included are factors which downregulate normal hematopoiesis, and factors which promote the outgrowth of myeloid leukemia cells.

Oncogenesis results from an uncoupling of the processes that regulate proliferation, terminal differentiation, and cell death. Multiple independent mechanisms are thought to regulate growth and differentiation, and several separate events are needed to subvert these controls and to induce other aspects of transformation (122). In order for a cell to become transformed, changes in cell regulation must become fixed or heritable, and passed on to daughter cells. Multiple genetic changes are thought to be required for a cell to become fully transformed. For example, human T lymphotropic virus-I (HTLV-1), which results in adult T cell leukemia, has a latent period in some cases of decades, suggesting that sufficient time is required to acquire further mutations in order to achieve cell transformation (158). Moreover, progression of tumours to more aggressive forms, which are in some cases refractory to previously effective therapy, has long been recognized to be associated with increasingly bizarre mitotic figures and with karyotypic changes (159). These include loss or duplication of chromosomes, and inversions, translocations, and deletions of various chromosomal segments (160). Solid tumours are thought to become invasive and metastasize as a result of the positive selection of one or more aggressive

subclones, in which a further genetic change has presumably occurred (161). Changes which result in increased proliferation can occur in two ways; activation of oncogenes, and inactivation of tumour suppressor genes. Examples of these changes have been found in many tumour types, in all probability both activation of oncogenes and inactivation of tumour suppressor genes are involved in any particular tumour (159). Moreover, oncogenes have been noted to cooperate in the production of malignant phenotypes (162). This cooperation occurs in many cases between a nuclear oncogene and a cytoplasmic oncogene (159, 163). Alteration of a nuclear oncoprotein results in immortalization, whereas alteration of a cytoplasmic oncoprotein reduces growth factor requirements, induces cell shape changes, and leads to anchorage-independent growth (122).

Cellular proto-oncogenes can become activated by several mechanisms; point mutation, truncation or disruption of coding or of regulatory sequences, or by the introduction of a strong promoter or enhancer. For example, point mutations of *ras* proteins have been shown to be involved in various human malignancies (122). Juxtaposition of unaltered *c-myc* coding sequences near strong immunoglobulin promoters for lambda, kappa, or heavy chains by chromosomal translocation is a hallmark of Burkitt's lymphoma (159). Balanced translocations involving nuclear oncogenes occur in several leukemias (159). Truncation of *erb-b*, the epidermal growth factor receptor, results in constitutive activation of receptor kinase activity in the absence of ligand binding, and in cell transformation. Similarly, tumour suppressor genes can be inactivated by several mechanisms. Point mutations, deletions, and rearrangements of p53, a sequence-specific DNA binding phosphoprotein, are involved in several solid tumours, such as cancers of the colon, lung, breast, ovary, and bladder (164), and are present in many myeloid leukemia

cell lines (165), but in few myeloid leukemia primary cells (166). Unproductive complexes are formed between altered p53 and other regulatory proteins in the nucleus, including wild-type p53, thus inactivating them in a dominant manner (144, 167). P53 is constitutively expressed in normal marrow blast populations (168), and is destabilized and localized to the nucleus during growth arrest (169), implying that its presence is important to the function and cycling of these cells. P53 is the most frequently altered gene in human tumours (144), and transgenic mice homozygous for a p53 null mutation showed increased susceptibility to spontaneous tumour formation (170). It was recently found that p53 forms a stable covalent linkage with RNA, suggesting that it may be involved in RNA metabolism (171). Similar to p53, Rb is inactivated in retinoblastoma (144), and is thought to be inactivated in megakaryoblastic crisis of CML (172). The observation of consistent karyotypic involvement in certain malignancies has led to the identification of other cell cycle regulatory genes; an example is the recently cloned DCC (Deleted in Colon Carcinoma, reference 159). DCC was identified by consistent loss of heterozygousity in colon cancers. It has the predicted structure of an integral membrane receptor; or cellular adhesion protein (144); its loss presumably confers a growth advantage on the affected cells. Progression of colon cancer following loss of DCC is correlated with subsequent inactivation of p53.

1.2.1: Leukemia; classification, etiology:

Leukemias are classified according to their aggressiveness, and further subdivided by the cell lineage involved. Common to leukemias is a clonal expansion that occurs in

cells with high potential for self-renewal, likely stem or progenitor cells. The precise site of expansion varies in different cases, accounting for the diverse phenotypes of the leukemias (173). The chronic leukemias include chronic myelogenous leukemia (CML) and chronic lymphocytic leukemia (CLL). CLL is further classified as B-CLL or T-CLL. The chronic leukemias are relatively indolent disorders, which in many cases are asymptomatic, and may be diagnosed on routine blood testing. In other cases, recurrent infections may occur due to defective functioning of cells of the leukemic clone; this results in symptoms such as fatigue and fever. Characteristic of chronic myelogenous leukemia is a partial uncoupling of proliferation and differentiation (174). There is an expansion of myeloid progenitors in the bone marrow and peripheral blood. These differentiate, however, into relatively normal neutrophilic granulocytes, which may be defective in some functions. For example, a defective respiratory burst is found in the neutrophils of CML patients in some cases. This can lead to impaired killing of phagocytosed bacteria, and thus to recurrent infections (175). Additional symptoms may be seen in CML, these include splenomegaly and bleeding or bruising, due to the suppression of normal myelopoiesis. which leads to decreased platelet counts.

The chronic phase of CML lasts for an average of about 3 years; after this time it enters what is referred to as an accelerated phase, followed by blast crisis and conversion to acute leukemia. The blastic transformation of CML involves increasing resistance to treatment, increasing proliferation, and maturation arrest. About two-thirds of cases of CML convert to AML (acute myelogenous leukemia, or acute non-lymphocytic leukemia, ANLL), whereas one-third convert to ALL (acute lymphocytic leukemia) or acute undifferentiated leukemia (176). Further karyotypic changes accompany the conversion of CML to a more aggressive leukemia, a further indication that leukemogenesis is a multistep process involving several genetic changes (177). These changes frequently include a duplication of the Philadelphia chromosome (25 to 30%), trisomy 8 (25 to 30%) and isochromosome 17 (20%) (175). Non-random acquired chromosome abnormalities are observed in other leukemias as well, frequent examples being loss of chromosomes 7 or 5 or their long arms (-7 or -7q or -5 or -5q) in AML after treatment with chemotherapy or radiation (178). An accumulation of immature blast cells occurs in the acute leukemias; the impediment to differentiation thus appears to be more complete. The acute leukemias are rapidly fatal. Average survival without treatment is three to four months (179).

The incidence of leukemia is 9 to 10 persons per 100,000 per year in the United States. About half are acute leukemias, and half are chronic. ALL and AML occur at approximately equal frequency. CLL occurs at four times the frequency of CML. The maximum incidence of ALL is in childhood, between ages 2 and 6. AML comprises about 20% of childhood leukemias, but is more common in older persons, and the incidence increases with age. CLL is rarely seen before mid-life, and the incidence increases with advancing age. CML occurs at any age, with the peak incidence in mid-life (1, 176).

Other leukemia-related disorders include the myeloproliferative syndromes, in which clonal growth expansion occurs (1, 176). These disorders include polycythemia rubra vera and essential thrombocythemia. These disorders often progress to myelofibrosis, in which the marrow is infiltrated by an overabundance of megakaryocytes which stimulate the replacement of hematopoietic tissue by fibroblasts and collagen fibers. Myelofibrosis, in turn, often terminates as acute leukemia. The myelodysplastic

syndromes are characterized by abnormal differentiation, and an increased risk of transformation to AML (1, 176).

Causative environmental or predisposing factors have in general not been clearly identified, aside from the involvement of HTLV-1 in ATL. Exposure to ionizing radiation is thought to result in an increased incidence of CML; a higher incidence of CML AND AML was reported in Hiroshima and Nagasaki following the atomic bomb explosions (180). Ionizing radiation results in the generation of free radicals which can introduce double strand DNA breaks and induce reciprocal translocations in cells in the quiescent (G_0) or first growth (G_1) phases of the cell cycle, as well as chromatid exchanges in the second growth phase (G₂) and in the phase of DNA synthesis (S, reference 181), in addition to disrupting the functions of proteins and other nucleic acids (182). Ionizing radiation results in a higher incidence of myeloid than of lymphoid leukemias (183). Generally, in the case of lower level exposure to radiation, such as near power stations, or a nuclear reactor accident such as occurred at Chernobyl, the overall incidence of leukemia is too low and/or records are not accurate enough for appropriate comparisons to be made and accurate conclusions to be reached (180). However, patients who previously received radiation therapy for ankylosing spondylitis, a condition of chronic inflammation in the spine, have a higher incidence of leukemia. Hereditary disorders which are associated with chromosomal abnormalities, genetic instability, or defects in DNA repair, such as Bloom's syndrome, Down's syndrome, and Fanconi's anemia, result in a higher incidence of acute leukemia (176). Neurofibromatosis, a hereditary autosomal dominant disorder, is associated with a higher rate of hematological malignancies. It has been suggested that the G-CSF gene may be involved in leukemic development, as the neurofibromatosis gene

maps to the same chromosomal band as does the G-CSF gene (184). However, the finding that the NF-1 gene has *ras* GAP homology, and is thus highly similar to *bcr*, is likely to prove to be of importance (122, see below). Exposure to alkylating agents, for example in the treatment of previous malignancies, results in a higher incidence of leukemia and lymphoma (1). Chronic benzene exposure is also associated with an increased risk of developing a hematological malignancy (185).

CML is one of the more thoroughly studied human malignancies. This is not only because tissue is relatively readily accessible, but also because it was the first human malignancy for which a consistent chromosomal aberration was described. The Philadelphia chromosome was described by Nowell and Hungerford in 1960 (186). It results from a reciprocal translocation between the long arms of chromosomes 9 and 22 [t(9;22)(q34;q11)] and is detectable in the blood and bone marrow cells of more than 90% of CML patients. The result of this translocation is the in frame juxtaposition of coding sequences for the 5' portion of bcr with 3' sequences of c-abl (187), resulting in the expression of a *bcr-abl* fusion protein, P210, the function of which is dysregulated in several ways (see below). Progression of CML in a majority of cases is associated with the acquisition of further karyotypic changes. Molecular studies have revealed that 30-50% of Philadelphia chromosome negative (Ph-) CML have variant translocations resulting in the *bcr-abl* fusion product. The remainder are considered 'atypical', and are a heterogeneous group with different cytological features including a lower incidence of basophilia and higher degrees of thrombocytopenia (low platelet counts) and anemia, and higher monocyte counts. Atypical CML responds poorly to chemotherapy, progresses

more quickly to blast transformation, and may constitute a separate clinical entity (175, 187, 188, 189).

Although CML presents clinically as an outgrowth of neutrophils and their progenitors, it is considered to be a stem cell disorder. This has been demonstrated in several ways; by analysis of the ratio of glucose-6-phosphate dehydrogenase (G6PD) isoenzymes in various hematopoietic cell lineages as compared to normal tissue, and by observation of the Philadelphia chromosome, *bcr-abl* transcripts, and fusion protein in cells of all hematopoietic lineages (175, 190, 191). This conclusion is supported by the observation that CML derived Ph⁺ cell lines are capable of differentiating along several cell lineages (192). Thus, the genetic lesion occurs in a pluripotent stem cell, but the transformed phenotype does not appear until the stage at which an expansion of the neutrophil progenitor pool is manifest.

In contrast, the genetic defects in many of the acute leukemias are thought to occur in more committed cells, although this is variable. For example, the French-American-British (FAB) classification subdivides AML into seven types, M1 through M7, on the basis of cytological and clinical criteria, and the predominant pathway of differentiation and degree of maturation (1, 185). M1, for example is an undifferentiated leukemia, in which the malignant phenotype is manifest in a relatively early cell. M6 and M7, in contrast, show clear characteristics of erythroid and megakaryocyte differentiation, respectively. About 10 to 15% of acute leukemias show characteristics of both myeloid and lymphoid lineage to varying degrees, such as cell surface markers and rearrangements of immunoglobulin or T cell receptor genes. Previously, this was thought to be an indication of "lineage infidelity", or aberrent expression of markers of one lineage in cells partially differentiated for the other lineage. However, more recently, it has been suggested that this phenomenon is an indication that the leukemic lesion occurs in a more primitive cell than previously thought, one that is earlier than the myeloid-lymphoid branch point (162, 193, 194). In keeping with this idea, it has been shown that cross-lineage rearrangements were found more commonly in acute leukemias following CML blastic transformation as compared to *de novo* cases (195). This is not surprising, since the leukemic defect in CML is known to occur in a very early cell. As cell surface markers and genetic lesions are becoming more well defined, they are being found to correlate fairly well with the cytological working classification, for example, of the FAB system in AML. Large studies have been undertaken in order to define cell phenotypes more clearly in these leukemias in order to facilitate diagnosis, biological understanding, and rational and effective therapy (185).

1.2.2: The 9;22 translocation in CML; the Philadelphia chromosome and P210 bcr-abl:

The balanced reciprocal translocation between chromosomes 9 and 22 [t(9;22)(q34;q11)] which results in the expression of P210 *bcr-abl* is the hallmark of CML. More than 90% of CML patients show evidence of the Philadelphia chromosome (Ph⁺). Of patients that are Ph⁻, at least another 5% express a rearranged *bcr-abl* transcript which is detectable by Northern blot analysis or by reverse transcriptase polymerase chain reaction (PCR). Thus, over 95% of CML patients are *bcr-abl*⁺, and, as discussed, the remainder may form a separate clinical entity. The t(9;22) translocation in CML results in

juxtaposition of the N-terminal sequences of bcr with downstream sequences of c-abl (196). The breakpoint in the bcr gene occurs within a 5.8 kb region, the major breakpoint cluster region, or M-bcr. The resulting fusion protein, P210, incorporates sequences from the first two to three exons of bcr, depending on the exact location of the break, excluding the fourth exon, and in some cases the third, and resulting in a b2a2 or b3a2 fusion. Expression of the b2a2 versus b3a2 product has been suggested to correlate with differing clinical features, such as differing cytogenetic abnormalities during the development of blast crisis (197) and platelet count (198). The breakpoint in *c-abl* occurs within a 100 kb region. The regulatory SH3 (src homology 3) domain near the N terminus of *c-abl* is deleted in the resulting P210 fusion protein, resulting in upregulation of abl tyrosine kinase activity, which in *c-abl* is low. A second breakpoint, the minor breakpoint cluster region, or m-bcr is involved in ALL arising de novo. In these leukemias, the first exon only of the bcr gene is incorporated into the fusion protein, P190, which also exhibits upregulated tyrosine kinase activity, but to a greater extent than does P210 (199). ALLs arising from pre-established CML express the P210 bcr-abl gene product. Balanced translocations can result by double strand DNA breaks in G_0 or G_1 induced by exposure to ionizing radiation, but more commonly occur by chromatid exchanges induced by radiation or chemicals in G_2 or S phase. Of interest is the presence of alu sequences near the breakpoints on chromosomes 9 and 22, suggesting that these sequences may be involved in the recombination (181). Although CML is rare in children, the bcr-abl rearrangements in juvenile CML have been shown to be similar to those in adult CML (200).

1.2.2.a: Signal transduction by c-abl, bcr, and P210 bcr-abl;

C-abl is highly conserved, is ubiquitously expressed in mammalian tissues, and has considerable homology with the src family of tyrosine kinases (196). It has been shown to be a nuclear protein, the first protein with kinase activity to be demonstrated to locate to the nucleus (201). Phosphorylation of c-abl is cell-cycle dependent; it is phosphorylated by the cell cycle associated cdc2 kinase on two sites in interphase cells, and on seven in mitotic cells (202), and the hyperphosphorylated form is translocated to the cytoplasm (201). Recently, it was shown that *c-abl* has sequence-specific DNA binding capability (124). Using antisense to *c*-abl, *c*-abl expression was shown to be essential for the formation of CFU-GM and CFU-G by hematopoietic cells, whereas CFU-E and BFU-E were unaffected (203). In addition transgenic mice lacking *abl* activity are severely affected, with high perinatal mortality, runting, and abnormal development of several organs (204). These observations suggest that *c-abl* is important in the development and function of many cell types. In addition, TNF- α and IL-1 increase *c-ab1* expression in marrow stromal cells (205). Expression of both abl and bcr-abl were found to decrease on differentiation of a CML cell line (206). Moreover, *c-abl* is thought to be regulated by a cellular inhibitor; this interaction could be disrupted by the *bcr-abl* translocation (207). These results strongly suggest that dysregulation of abl in CML is important in the selective expansion of cells of the neutrophilic lineage.

Until very recently, little was known about the normal cellular function of *bcr*. It was originally thought to be a passive player in the *bcr-abl* translocation, with upregulation of *abl* tyrosine kinase activity occurring by replacement of the *c-abl* SH3 regulatory

domain. More recently, first exon *bcr* sequences were shown to specifically activate the *bcr-abl* tyrosine kinase activity (208). *Bcr* is now known to have serine/threonine kinase activity encoded within its first exon. This is a novel coding structure for a kinase; sequences are usually spread over five to seven exons (209). In addition, bcr has been shown to encode a GTPase-activating protein (GAP) for p21rac(137). Little is known about the function of the rac proteins, but they are ras-related, so they presumably are involved in signal transduction, and they are known to be more highly expressed in myeloid cells (137). Phagocyte oxygen radical production has been reported to be regulated by rac 2 at the level of NADPH oxidase activity, so alteration of racGAP activity by the bcr-abl translocation may be related to the observed defects in the respiratory burst observed in CML cells (210). Moreover, it was shown that the rodent homologue of rac encodes a serine-threonine kinase which contains an SH2 domain, and is carried in a retrovirus (211). Intriguingly, one of the known GAPs for $p21^{ras}$ is the neurofibromatosis gene product NF-1 (144, recall that there is a higher than normal incidence of hematological malignancies in neurofibromatosis). In addition, the 85 K subunit of phophatidylinositol 3-kinase has been shown to have homology to *bcr*. Thus, evidence is accumulating that bcr plays an as yet undefined, but likely important, role in signal transduction.

It was demonstrated that *bcr* binds to the SH2 domain of *c-abl* (212). SH2 domains are important in interactions between proteins involved in signalling via tyrosine kinase pathways. The presence of two putative signal transducing activities, GAP and serine/threonine kinase, in addition to its demonstrated binding to the *abl* SH2 domain, has led to the suggestion that *bcr* may be involved at the intersection of cellular signal transduction pathways (209). *C-abl* is known to be expressed from the unrearranged allele

in CML cells (213), however, others report that it is undetectable by immunoblotting, implying that it may be expressed at lower levels than in normal cells (214). It is unclear whether normal *bcr* is expressed in CML cells. The inactivation or constitutive activation of the putative signal transducing functions of *bcr* could be expected to have significant effects on the biological functions of these cells, as could the alterations in *abl* activity. Long range mapping of the *bcr* gene demonstrated the presence of three genes with homology to 3' sequences in *bcr*. The significance of these genes is unknown (215).

1.2.2.b: Transforming capabilities of bcr-abl;

The form of *c-abl* expressed in Abelson murine leukemia virus, *v-abl*, which is a *gag-abl* fusion protein, is known to be transforming in hematopoietic cells (216). Both P190 and P210 *bcr-abl* have been demonstrated to have transforming capabilities as well. Introduction of P210 *bcr-abl* into hematopoietic cell lines caused them to become growth factor independent and tumorigenic (217). Reconstitution of mice with bone marrow cells infected with a retroviral vector carrying P210 *bcr-abl* resulted in various hematological malignancies, including a CML-like syndrome, macrophage tumours, and lymphoid malignancies (218, 219). One group found these tumours to be rarely transplantable, and concluded that *bcr-abl* confers a proliferative advantage and that complete transformation involves additional genetic changes (218). Other investigators found that transfection of bone marrow enriched for multipotent progenitor cells with P210 *bcr-abl* resulted in the formation of *in vitro* colonies which were responsive to growth factor regulation but subsequently became growth factor independent. The growth factor independent cells,

however, were not leukemogenic in mice with severe combined immunodeficiency (220). These studies support the concept of leukemogenesis as a multi-step process; acquisition of P210 bcr-abl appears to confer on a clone the ability to expand in a relatively benign manner, and further genetic lesions appear to be involved in the acquisition of an aggressive phenotype. Another group found that passage of bone marrow cells from mice with the CML-like syndrome to secondary recipients resulted in induction of one AML, several T-ALLs, and one CML, a situation the authors compare to blast crisis (221). Mice reconstituted with P190 or P210 bcr-abl displayed a similar spectrum of hematological abnormalities, which were more aggressive in those receiving P190 (222). This result is an indication that the target cells affected by the two fusion proteins are similar. Conversely, mice receiving v-abl transfected cells were reported to display hematological abnormalities which differed from those receiving P210 in some respects, such as the involvement of other organs, for example the lymph nodes and spleen (223). Interestingly, transfection of P210 bcr-abl into an IL-3 dependent myeloid cell line was found to result in the phosphorylation of similar sets of proteins as are involved in signal transduction by IL-3, indicating that growth factor independence may be facilitated by bcr-abl (224), and potentially implicating bcr and/or abl in the transduction of cellular signals stimulated by growth factors under normal conditions. In this regard, it is thought that src related kinases are involved in the transduction of signals from cytokine receptors lacking intrinsic kinase activity, such as the interaction of lck with CD4 or CD8, or the interaction of fyn with the T cell receptor. Recall that the absence of a kinase domain is characteristic of most of the CSF receptors, and that abl is a src related kinase; c-abl could be involved in the transduction of signals from these receptors. This is supported by the observation,

discussed above, that treatment of hematopoietic progenitor cells with antisense deoxynucleotides to *c-abl* abrogates the formation of CFU-GM and CFU-G (224).

1.2.2.c: *P210 bcr-abl in the suppression of apoptosis;*

In normal tissues, self-renewal is balanced by terminal differentiation and programmed cell death or apoptosis. Apoptosis is an active process that requires RNA transcription and protein synthesis (225). It is induced in hormone-dependent tissues on withdrawal of hormone (226), in HL60 cells during retinoic acid-induced differentiation (227), and in aging normal neutrophils during inflammation (228, 229). The colony-stimulating factors erythropoietin, IL-3, GM-CSF and G-CSF have all been shown to suppress apoptosis in cells that depend on them for survival (225, 226, 230). Messenger RNAs associated with apoptosis in immature thymocytes have been identified; one encodes a zinc finger protein, suggesting that it is involved in DNA regulation, and the other encodes an integral membrane protein (231). IL-1 has been found to be processed and released during apoptosis, which is presumably a cellular response to cell stress and injury (232).

Clues as to how the balance between self-renewal and apoptosis may become dysregulated can be found in the functioning of *bcl*-2 in B cell follicular lymphoma. *Bcl*-2 is a protein normally associated with the inner mitochondrial membrane, and is expressed in tissues in which apoptosis accounts for cell turnover, such as germinal centres in lymph nodes and at the base of intestinal crypts (233). Survival of IL3-dependent cell lines was

supported by the expression of bcl - 2 (225, 226, 230). Moreover, stimulation of lymphocytes with mitogens correlates with bcl - 2 expression (234). The t(14;18) translocation in follicular B cell lymphoma juxtaposes intact bcl - 2 coding sequences with the immunoglobulin heavy chain region, upregulating the expression of bcl - 2. Enforced bcl - 2 expression in B-lymphoid cells in transgenic mice resulted in prolonged antibody responses and a high incidence of autoimmune disease, suggesting that expression of bcl - 2promotes prolonged cell survival (235). Moreover, the latent membrane protein 1 (LMP 1) of Epstein Barr Virus (EBV) was shown to up-regulate bcl - 2 expression and to protect cells from apoptotic cell death (236). Taken together, these results suggest that bcl - 2functions in prolonging the life of cells in which it is expressed.

Follicular lymphoma characteristically has a relatively indolent course (1), however, a leukemic phase may arise after some time. The leukemic cells often harbour the t(14;18) translocation, suggesting that the more aggressive malignancy arose in the clone in which cell survival was prolonged by *bcl*-2 (225, 226). Many of the tissues that express *bcl*-2, such as skin, colon, and breast, have a high incidence of cancer (233). 70% of AML cells, irrespective of FAB classification, were found to express *bcl*-2, whereas most CML cells were *bcl*-2 negative (237). These findings imply that a similar function may be performed by P210 *bcr-abl* in CML as is performed by *bcl*-2 in follicular lymphoma. Prolongation of the lifetime of a clone of cells by P210 *bcr-abl* could provide a long lived pool of cells in which further genetic changes could accumulate, eventually resulting in a more aggressive malignancy. Indeed, a recent report demonstrates that apoptosis is in fact suppressed in a cell line rendered growth factor independent by transfection of P210 *bcr-abl*; inhibition by tyrphostins inhibitors of *bcr-abl* kinase activity resulted in apoptosis (238). Prolongation

of the lifetime of the cells in the expanded clone supported by P210 *bcr-abl* can be viewed as correlating with chronic phase CML. Further genetic changes would then result in accelerated phase, blast crisis, and the acute leukemias.

1.2.2.d: Other characteristics of bcr-abl;

C-abl was recently demonstrated to have microfilament binding capability, which is upregulated in P210 bcr-abl. Recently, it was reported that activation of abl actin binding activity requires bcr N-terminal sequences (239). The actin binding domain is located in the C-terminus (240). As the Drosophila abl homologue is involved in cell adhesion (241), the authors suggest that mammalian abl may be part of a signal transduction mechanism regulating cell adhesion, and that the bcr-abl translocation may be involved in the defective adhesion observed in CML. This might occur via phosphorylation of cytoskeletal proteins involved in the interaction between hematopoietic and stromal cells. However, using antibodies to cross-link cell adhesion molecules, one group found that cell adhesion results in the phosphorylation of cellular proteins and presumably in the activation of signal transduction pathways (110). In addition, GPI-anchored cell surface molecules are known to be complexed to protein tyrosine kinases (242). Furthermore, a defect in a GPI-anchored adhesion molecule is known to be involved in the reduced adhesion of CML cells to stroma (243). Thus, rather than altered adhesion being a result of altered signal transduction, altered signal transduction could be a result of altered adhesion. Also of potential importance is the observation that pp60 *c*-src is translocated to the cytoskeleton

during platelet aggregation and in cell transformation (244), reminiscent of the cytoskeletal binding capacity of *c-abl*, a protein with considerable *src* homology.

Using a set of *bcr-abl* constructs, it was shown that upregulation of *c-abl* kinase activity is not due to the removal of SH3 sequences alone, implying an active role for bcr in the increased kinase activity of bcr-abl. Analysis of the bcr promoter demonstrated structural similarity to the *c*-abl promoter, suggesting that structural alteration is likely to be more important that transcriptional dysregulation in conferring transforming ability on bcr-abl (213). In addition, it was recently found that bcr sequences necessary for transformation by bcr-abl bind to the abl SH2 regulatory domain (212). P210 bcr-abl was shown to coimmunoprecipitate with ras GAP, which is tyrosine phosphorylated in Ph⁺ cell lines (139). This finding is significant, since p21^{ras} is known to be activated by hematopoietic growth factors (115). In addition, P210 bcr-abl was found to form a complex with wild-type P160 bcr in K562, a CML-derived cell line. P210 was also shown to form a complex with the tumour suppressor gene product p53 in the same study (245). This observation is intriguing, since it has been reported that p53 levels increase with maturation of hematopoietic cells (246). Moreover, introduction of wild type p53 into a murine myeloid leukemia cell line, M1, induced apoptosis (247). Furthermore, transforming viruses such as SV40, adenovirus, and papillomavirus are known to exert their effects by the sequestration of the tumour suppressor genes Rb and p53 into ineffective complexes (144). Hence, bcr-abl may delay differentiation or suppress apoptosis by sequestering p53.

1.2.3: Adhesion defects in CML;

Defects in the adhesive interactions of hematopoietic cells have been described in CML. These adhesion defects account for the fact that no differences in the proportion of CD34⁺ cells can be detected between the blood and bone marrow in CML patient material, whereas in normal material, subpopulations of cells expressing CD34 in the peripheral blood are undetectable. In contrast, between 1 to 5% of normal bone marrow cells express CD34 (248). These results are an indication that immature cells in CML are inappropriately released into the circulation. CML cells are known to be reduced in their capacity to bind to normal stromal layers in LTMC (249). Conversely, stromal layers derived from CML cells are unable to support hematopoiesis by normal cells (250). The numbers and/or binding capacity of bl-CFCs to stromal layers was also reduced in cultures derived from AML cells (251).

As mentioned, a phosphatidyl-inositol (PI)-linked cellular adhesion molecule (CAM) has been found to be missing from CML cells (243). This result is interesting since an *abl*-related kinase in *Drosophila* is known to interact with a PI-anchored cellular adhesion molecule (CAM, reference 241). The authors suggest that the CAM in CML may be a target of the altered *bcr-abl* kinase. Also, treatment of normal progenitor cells with medium containing hematopoietic growth factors resulted in decreased adhesion (252). Taken together, these results suggest that growth factors may stimulate the *c-abl* pathway, resulting in altered adhesion. Upregulated *abl* kinase activity in the P210 *bcr-abl* fusion protein in CML cells may lead to a prolonged signal, resulting in poor adhesion.

Adherence of normal bone marrow cells to stromal layers can be competitively inhibited by exogenously added heparan sulfate proteoglycan (18). In other cell systems, heparan sulphate has been found to have anti-proliferative activity, which is associated with the transfer of heparan sulphate from the cell surface to the nucleus (253). This could account for the relatively quiescent state of adherent hematopoietic progenitor cells. HL60 cells, derived from a promyelocytic leukemia, have been shown to synthesize and express on their surface excess amounts of the proteoglycan chondroitin sulphate (254). Moreover, neutrophil adherence in inflammation correlates with shedding of chondroitin sulphate and synthesis of heparan sulphate (161), and platelet aggregation is inhibited by the expression of chondroitin sulphate (255). Platelet heparitinase has been shown to be activated by factors derived from metastatic tumor cells (256). Hence, changes in adherence in CML could correlate with changes in proteoglycan expression. Alterations in the adherence of CML cells could also explain the finding that Ph⁻ hematopoiesis overtakes Ph⁺ hematopoiesis in long term marrow culture in some cases (257), since additional factors which may promote the survival of leukemic cells in the absence of normal adherent interactions in vivo may not be completely reproduced in this culture system.

1.2.4: Alterations of cytokine expression in leukemogenesis;

Alterations in the expression of cytokines and their receptors have been described in AML. GM-CSF is secreted by AML blasts (258, 259), and it has been suggested that autocrine stimulation may contribute to the growth of these cells. In contrast, circulating leukocytes from patients with CML in chronic phase, accelerated phase, blast crisis, and
relapse were found to produce lower colony stimulating activity than cells from CML in remission or normal cells, as evaluated by a two-layered colony assay (260). However, the gene for IL-1 was shown to be expressed in blast cells in almost all cases of CML in myeloid blast crisis, which could initiate a paracrine mechanism of blast cell growth by the induction of cytokine expression in accessory cells (261). In a separate study, IL-1, IL-6 and LIF were found to be expressed in long term marrow culture adherent layers derived from a majority of patients in myeloid blast crisis, but were undetectable in those in lymphoid blast crisis, suggesting that a paracrine mechanism is indeed operative in these cases. The authors also suggest that the involvement of stromal cells in growth stimulation in blast crisis may be related to the poor results of marrow transplantation in this phase of CML (262). In contrast, stromal layers from normals and CML in chronic phase were found to express G-CSF, and evidence for abnormal autocrine or paracrine mechanisms of stimulation could not be found (263). Stromal layer expression of G-CSF was commonly lost during blast crisis (264). Involvement of the microenvironment in a paracrine fashion in the development of CML is also suggested by reports of CML developing in donor cells in patients who had undergone allogeneic BMT (175, 265). Thus, a picture emerges of the operation of relatively normal cytokine control mechanisms in chronic phase followed by paracrine stimulation in blast crisis, and progression to autocrine stimulation and full growth factor independence in acute phase; this dysregulation could be involved in stimulating the growth of the leukemic clone, at least in some cases.

There are additional examples of the possible involvement of cytokines in leukemogenesis. The t(1;14) translocation in acute pre-B cell leukemia juxtaposes the immunoglobulin heavy chain locus with IL-3 sequences. Transgenic mice expressing cytokines (GM-CSF) or mice reconstituted with cytokine-expressing retroviruses showed changes of a myeloproliferative nature, but did not develop leukemia (54). Introduction of the *bcr-abl* gene into many hematopoietic cell lines rendered them growth factor independent and tumorigenic (162, 217). It is postulated that the full leukemic phenotype requires alterations of genes regulating both growth, resulting in myeloproliferation, and differentiation, resulting in full transformation.

Progression of CML was recently described to be marked by changes in the methylation pattern of the calcitonin gene (266). Abnormal methylation patterns were found in few patients in chronic phase, but in a majority of cases of accelerated phase or blast crisis, in 90% of cases of acute leukemia (267), as well as in lung cancers, lymphomas, and colonic neoplasms (268, 269). The calcitonin gene is located on the short arm of chromosome 11 (11p), a region known to contain tumour suppressor genes such as that associated with Wilms' tumour. Loci adjacent to the calcitonin gene show abnormal methylation patterns as well. Moreover, the authors report abnormally high expression of the DNA methyltransferase gene. In addition, different patterns of methylation in the myeloperoxidase gene were reported in AML (270). Undoubtedly more details of the methylation story will emerge in coming months.

1.2.5: Other abnormalities in CML;

Other abnormalities in CML usually apply to only a minority of cases. An exception is increased erythropoetin receptor expression on CD34⁺ cells in CML, which

has been reported to occur in 5 of 5 cases examined (271). A novel receptor tyrosine kinase, *axl*, was isolated from CML cells and found to be transforming when overexpressed. The gene for this kinase was localized to chromosome 19; alterations in chromosome 19, particularly trisomy, are associated with progression to blast crisis in 18% of CML cases (272). Loss of p53 function was demonstrated in 25% of patients in clinical transition (144, 273). Activating *ras* mutations in CML have also been described, but in a minority of cases, and they usually occur late (274). Finally, the mRNA and precursor protein of HP-1, a short peptide 'defensin' involved in the formation of voltage-dependent ion permeability channels in target cell membranes was found to be overexpressed in CML (275). This observation may be related to the defective killing functions of CML neutrophils observed in some cases (175), and also may provide a convenient marker for CML cells.

1.2.6: Negative regulators of hematopoiesis:

It has been recognized for some time that the outgrowth of the leukemic clone in myeloid leukemias, as in many malignancies, is not due to an inherently faster progression through the cell cycle on the part of the malignant cells (1, 185, 276, 277, 278). In fact, a significant proportion of the leukemic cells in a population may be quiescent, a characteristic that makes these cells refractory to treatment with cell cycle-specific agents (185). Myeloid leukemias in some way produce conditions that inhibit both the development and the function of normal hematopoietic cells. In this way, the leukemic clone gradually gains dominance over normal cell lineages. How this is achieved is not

well understood, but inhibitors of normal hematopoiesis have been described, as well as factors which support the growth of leukemic cells.

Many negative regulators of hematopoiesis are being investigated in the context of protecting normal stem cells from cycle specific chemotherapy. These include AcSDKP, a tetrapeptide originally isolated from fetal bovine marrow and shown to be a potent inhibitor of normal myelopoiesis (279). The effect of AcSDKP is mediated by prevention of the entry of stem cells in G_0 or G_1 into S phase. AcSDKP has no effect on leukemic myelopoiesis (280). It has recently been isolated from human placenta (281). AcSDKP was shown to increase the survival of mice treated with cytosine arabinoside (Ara-C), a cycle specific chemotherapeutic agent used in the treatment of AML, and is entering clinical trials for this application (282).

The pentapeptide pEEDCK, also known as HP5b or SP1, is derived from neutrophilic granulocytes. As a monomer, pEEDCK is suppressive to hematopoiesis and protects CFU-S from Ara-C induced cytotoxicity (283). Oxidation of sulfhydryl groups results in dimerization of the pentapeptide through the formation of a disulfide bond. As a dimer, (pEEDCK)2 is stimulatory to hematopoiesis via stimulation of the production of a synergistic activity by stromal cells (284). A synthetic version of the dimer, SK&F 107647 (Smith, Kline and French), has been synthesized, in which the disulfide bond is replaced by a dimethylene carbon bridge (285). Interestingly, the sequence EEDCK is part of the effector domain of G_i alpha proteins, leading to the suggestion that pEEDCK may interfere with signal transduction mediated by G_i alpha proteins (286).

Physiological inhibitors of normal hematopoiesis include transforming growth factor- β (TGF- β), tumour necrosis factor- α (TNF- α), the interferons, and IL-10. TGF- β , like most physiological modulators of hematopoiesis, has pleiotropic effects on progenitor cells depending on their cell lineage, stage of differentiation, immediate microenvironment, and other cytokines present (26). TGF- β is synthesized by most normal and neoplastic cells, but a major source is the blood platelets. In general, TGF- β is inhibitory to primitive normal progenitor cells while stimulating committed progenitors (69, 287). TGF- β is reported to inhibit leukemic cell growth in addition (287). TNF- α is a 17 kDa cytokine produced mainly by monocytes. It is released from AML cells (288), and is suppressive to the proliferation of normal CFU-GM. The effects of TNF- α on colony formation, like those of TGF- β , are pleiotropic. In general TNF- α increases the responsiveness of primitive cells to other cytokines, while decreasing colony formation in cells of more restricted lineage (289, 290).

Interferon-gamma induces terminal differentiation in human leukemic cells which are blocked in differentiation by overexpression of *c-myc*, thus inhibiting leukemic cell proliferation (71). Interferons generally have a suppressive effect on cell growth, which is mediated by the selective inhibition of expression of several mitochondrial gene products (70). The inhibitory effect of interferons, however, seems to be greater on leukemic cells than on normal cells, and their use in the treatment of CML is being investigated (291). The suppressive effects both of the interferons and of TNF- α on colony formation are reported to be mediated through direct actions on the progenitor cells. In contrast, these factors can stimulate myelopoiesis indirectly by enhancing the production and release of stimulatory cytokines by accessory cells (292). Moreover, TNF- α has been reported to induce the expression of receptors for IL-3 and GM-CSF on AML cells, which, in addition to its suppressive effect on normal CFU-GM, could confer a growth advantage on leukemic cells (288). TGF- β , in contrast, has been reported to downmodulate receptors for some growth factors on normal cells, which could account for its suppressive activity at least in part (292).

IL-10 is a cytokine produced by monocytes, which is inhibitory to hematopoiesis through inhibition of the synthesis of stimulatory cytokines by monocytes, including IL-1, IL-6, IL-8, TNF- α , GM-CSF, and G-CSF (46). Interestingly, IL-10 has been reported to have homology to the Epstein-Barr virus (EBV) gene BCRFI, suggesting that EBV may have captured this cellular gene and uses it to inhibit the immune response (27).

Inhibin and activin are members of a family of proteins which include TGF- β . Inhibin and activin are most well known for their activities on the release of folliclestimulating hormone (FSH) from pituitary cells, which they inhibit and stimulate, respectively. Inhibin and activin also inhibit and stimulate, respectively, the production of CFU-GM, BFU-E, and CFU-GEMM through indirect effects which are mediated by T lymphocytes and/or monocytes (292).

Macrophage inflammatory protein-1 alpha (MIP-1 α or stem cell inhibitor SCI), is a macrophage-derived member of a family of heparin-binding proteins. MIP-1 α is suppressive to colony formation by primitive cells stimulated by optimal concentrations of colony-stimulating factors, but enhances colony formation when CSF concentrations are

suboptimal (293, 294). MIP-1 α is stimulatory to colony formation by more committed progenitor cells, as are the related proteins MIP-1 β and MIP-2. The inhibitory activity of MIP-1 α on primitive progenitor cells is direct, and can be blocked by MIP-1 β (292). Primitive hemopoietic colonies were inhibited by an extract from normal marrow (normal bone marrow extract, NBME), which may be identical to MIP-1 α , or SCI (295). MIP-1 α , as well as TGF- β and TNF- α are reported to be constitutively produced in bone marrow stromal cells, which may constitute a physiological mechanism by which the proliferation of hematopoietic stem cells is downregulated (263, 296).

Lactoferrin is an iron-binding glycoprotein derived from neutrophilic granulocytes which inhibits hematopoiesis indirectly by decreasing IL-1 secretion by monocytes (297). This effect can be overcome by the addition of IL-1, IL-6, or bacterial lipopolysaccaride (LPS). The blocking effect of LPS on lactoferrin-mediated suppression of myelopoiesis is mediated through formation of a complex between LPS and lactoferrin. This complex binds preferentially to the LPS receptor versus the lactoferrin.receptor (292). Defects in the quantity and activity of lactoferrin as well as decreased sensitivity to its suppressive effects on colony formation have been reported in patients with CML (292).

H-ferritin, the heavy chain subunit of the acidic isoferritins, is suppressive to colony formation by immature progenitor cells through a direct action on these cells. The actions of H-ferritin are mediated by its ferroxidase activity. Cycling cells require iron for proliferation, which is carried bound to transferrin in the blood in the ferric (Fe³⁺) form. Release from transferrin requires conversion to the ferrous (Fe²⁺) form, which is opposed by the ferroxidase activity of H-ferritin (292). Acidic isoferritin has been reported to

stimulate differentiation of normal neutrophilic granulocyte progenitors (298). Leukemia inhibitory activity (LIA) is identical to acidic isoferritin (299).

Prostaglandin E_2 (PGE₂) has a growth inhibitory effect on normal CFU-GM (300), while myeloid leukemia cells are insensitive to this effect (301). This effect of PGE₂ is indirect and is mediated through abrogation of the secretion of GM-CSF, M-CSF and G-CSF by macrophages (27). In addition, both PGE₁ and PGE₂ are directly inhibitory to CFU-M. In contrast, through indirect actions on CD8⁺ T lymphocytes, both PGE₁ and PGE₂ can enhance the proliferation of BFU-E (292).

Finally, a less well characterized inhibitor of myelopoiesis produced by normal cells is a granulopoietic inhibitory activity (GIA) of >100 kDa, which is produced by unstimulated lymphocytes (302).

It has been suggested that the suppressive effects of these inhibitors may be mediated through the induction of a common secondary suppressor molecule. Bone marrow and spleen cells from mice treated with lactoferrin, H-ferritin, or PGE all release an 8 kDa molecule which suppresses colony formation in vitro in a manner similar to MIP-1 α , but which is distinct from MIP-1 α . This molecule is being characterized (292).

It has been known for some years that myeloid leukemia cells secrete factors that are inhibitory to normal colony formation (303). In contrast, factors from lymphoid leukemias have been reported to enhance normal colony formation (304). Several leukemia-derived factors inhibitory to myelopoiesis deserve mention. As discussed, TNF- α is secreted by some AML cells, which may confer a growth advantage on the leukemic cells, as TNF- α is inhibitory to normal myelopoiesis in some circumstances. Leukemia associated inhibitor (LAI) is a 125 kDa polypeptide which causes reversible suppression of normal CFU-GM formation by blocking the cell cycle in S phase. LAI has no effect on colony formation by leukemic cells (299). A factor isolated from the culture medium of HL60 cells suppresses activation of normal lymphocytes but does not affect colony formation by GM-CFC (305). This observation may have implications for the disruption of immune surveillance in leukemia. A factor in the sera of AML patients was found to suppress both natural killer cell activity and lectin-induced cellular cytotoxicity (306). T lymphocytes of AML in remission have been described to inhibit granulopoiesis (307). Finally, one report describes disruption in the ability of normal CFU-S to bind stroma after treatment with sera from CML patients (308).

Inhibition of normal myelopoiesis is one mechanism by which the leukemic clone can gain dominance over normal cells; another is stimulation of leukemic myelopoiesis itself. In addition to the paracrine and autocrine mechanisms described in the previous section, which could promote outgrowth of the leukemic cells, one factor which stimulates leukemic myelopoiesis deserves mention. Myeloblastin is a recently cloned serine protease that was isolated from the azurophilic granules of human neutrophils. It has been shown to be identical to proteinase 3, the autoantigen in Wegener's granulomatosis (309). This is a disorder in which neutralizing antibodies are produced against myeloblastin, resulting in granulomatous lesions. Downregulation of myeloblastin has been shown to result in the growth arrest and differentiation of HL60 (310). In contrast to these inhibitory effects on hematopoiesis, leukemia inhibitory factor (LIF, or human interleukin for DA cells, HILDA) alters hematopoiesis in the opposite direction. LIF induces the monocytic differentiation of M1, a murine leukemic cell line. In addition, LIF suppresses the differentiation and maintains the proliferation of embryonic stem cells. The use of LIF in promoting the survival of retrovirally transfected hematopoietic stem cells for gene therapy is under investigation (84). LIF has been reported to be constitutively expressed by stromal layers in long term culture (262), and to stimulate the development of multipotential progenitor cells (311). Oncostatin M and IL-6, related proteins, have similar actions on M1 cells (85).

In addition to these factors, it must be recognized that many cytokines, singly or in combination, exert a variety of effects on cells which could influence outgrowth of the leukemic clone. However, other than the involvement of P210 *bcr-abl* in CML, and the t(15;17) translocation in acute promyelocytic leukemia, few consistent changes have been described which clearly influence leukemogenesis; this is in part due to the great complexity involved in the normal regulation of hematopoiesis, which is far from completely understood. Hence, much work in this area deals with phenomena rather than precise information. Deviations from normal, then, have been difficult to characterize.

1.3: Therapy;

Current therapies for myeloid leukemias are briefly described in this section. Included is a discussion of traditional chemotherapy, as well as investigational therapies such as bone marrow transplantation, the use of recombinant cytokines, immunologically based therapies, and molecular techniques. The success rate and limitations of allogeneic and autologous bone marrow transplantation are described in addition to techniques and agents under investigation for purging residual leukemic cells from autografts. This section is intended to give the reader a sense of the inadequacies of current treatments for myeloid leukemias and the importance of gaining a clearer understanding of how these leukemias develop in order to treat them more effectively.

1.3.1: Chemotherapy;

The treatment of leukemia with traditional chemotherapy provides little more than symptomatic control (179). Intensive chemotherapy in acute phase can induce remission, or re-establish chronic phase, but relapse is inevitible in most cases, with the exception of childhood ALL, in which permanent remissions are achieved. Long-term survival in childhood ALL is achieved in approximately 50% of cases using chemotherapy (312). Chemotherapy for AML, however, results in long-term survival in only 15 to 35% of cases (313). Toxicities of chemotherapeutic agents are unpleasant, and in many cases are related to cell cycle specific effects. These include disruption of the mucosa of the gastrointestinal tract, and marrow ablation with its accompanying neutropenia and increased risk of infection, as well as thrombocytopenia (decreased platelet counts) with increased risk of bleeding. Difficulties with efficacy such as multi-drug resistance are also encountered (314), hence combinations of drugs are often used. In many cases, toxicity is dose limiting (175), and new approaches are being evaluated for improving the delivery of chemotherapeutic agents. These techniques include delivery via liposomes, which reduces toxicity (315), and delivery of toxins linked to monoclonal antibodies (316). It is unclear, however, whether increasing chemotherapeutic doses in this way will result in increased cures (317). Many chemotherapeutic agents, for instance, are cell cycle specific, however, leukemic cells do necessarily proliferate more rapidly than normal cells, and substantial proportions of the leukemia cell population may be out of cycle at any given time (278). Cytokines are being used clinically in an attempt to increase the fraction of leukemic cells in cycle and render them more sensitive to chemotherapeutic agents is under evaluation; normal cells might be expected to be forced into cycle and rendered susceptible to cytotoxic effects as well.

Chemotherapeutic agents commonly used in the treatment of CML include busulfan, a bifunctional alkylating agent, and hydroxyurea, an inhibitor of DNA synthesis (175). Toxicities of these treatments, especially busulfan, include prolonged marrow aplasia and pulmonary damage.

One problem with chemotherapy involves the development of resistance. Multidrug resistance (mdr) is mediated by the P-glycoprotein or p170, an energy-dependent membrane pump which pumps out several structurally unrelated naturally derived cytotoxic

agents such as doxorubicin and etoposide, drugs used in the treatment of acute leukemias (314). The promoter of the mdr-1 gene has been shown to be a target for the *c-Ha-ras-1* oncogene and the p53 tumour suppressor genes, both associated with tumour progression (318). It has been suggested that targetting P170 with immunotoxins may be useful in purging resistant cells from the marrow prior to autologous BMT (319). P170 may be involved in the secretion of peptides or cellular proteins during normal cell metabolism (320).

Most patients achieving complete remission using chemotherapy for myeloid leukemias eventually suffer a relapse. As it has been estimated that patients in remission harbour a burden of 10⁸ to 10⁹ leukemic cells, this is not surprising (185). Long-term survival following chemotherapy for AML is only 15 to 35% (313). In CML, chemotherapy does not result in cures (321). For the majority of adult leukemias, the possibility of cure is currently thought to be limited mainly to allogeneic bone marrow transplantation (188).

1.3.2: Allogeneic bone marrow transplantation;

Of leukemia patients, only about 25% are eligible for allogeneic bone marrow transplantation (322). Half of patients are considered to be too old; allogeneic BMT is currently limited to patients under the age of 50 (323). Best results are achieved with patients under 30 years of age as the risk of complications, particularly graft versus host disease (GVHD) increases with age beyond this point. Five year survival rates following

allogeneic BMT are 50 to 60% for patients under 20 years of age, and only 20 to 30% for those over 40 (312, 322). Of the half of patients young enough for BMT, an appropriate HLA matched donor will be found for 50%; a related donor will be found for one third of these, or 15% of patients overall, and an unrelated donor for one fifth, or 10% overall (179, 324). Patients are treated with high dose myeloablative chemotherapy and often total body irradiation, subsequently their hematopoietic and immune system is reconstituted mostly with transplanted donor cells, thus, they become permanent chimeras. In addition to killing many of the leukemic cells, the conditioning regimen has been reported to aid engraftment by disrupting the bone marrow endothelium, which the engrafting cells must traverse to reach the marrow (325). Well-known toxicities of total body irradiation (TBI) include gastroenteritis, mucositis, myelosuppression, and alopecia (313).

Complications of allogeneic BMT include failure to engraft, and graft versus host disease (GVHD). GVHD is an unpleasant complication which manifests in two forms, acute and chronic. Acute GVHD involves inflammatory destruction of epithelial cells in the skin, gastrointestinal tract, and liver, and is mediated by CD8⁺ T lymphocytes. It develops within the first few weeks post-BMT. Chronic GVHD is characterized by increased collagen deposition resulting in fibrosis. It is mediated by CD4⁺ T lymphocytes, and develops later, usually after 6 months. It may be preceded by, and be continuous with, acute GVHD (326). Complications of chronic GVHD include epidermal atrophy and contractures. In an attempt to avoid GVHD, some allogeneic grafts were depleted of T cells. It was found, however, that although T cell depletion decreased the incidence of GVHD (20% vs. 40% in non-depleted grafts), the incidence of relapse increased (60% vs. 20%) and survival rates decreased (20% vs. 55%) (179). This led to the recognition that a

significant graft versus leukemia (GVL) effect is present in non T cell depleted grafts. Moreover, T cell depletion led to an increased incidence of graft failure or rejection (326). In addition, it is known that there is a higher incidence of relapse in patients receiving transplanted cells from identical twin donors (188, 239). These observations illustrate the importance of an anti-leukemia immune effect in successful BMT for CML. GVHD is currently treated using immunosuppressive therapy, including steroids, methotrexate, and cyclosporine (312, 327, 328). Recently, the well known mutagen thalidomide has been used to treat chronic GVHD resistant to conventional therapies (329, 330). Other complications following BMT include cytomegalovirus (CMV) infection, either from CMV reactivation or from viral infusion with donor cells, and hepatic toxicity (313).

Leukemic relapses occur in 20 to 25% of patients receiving allogeneic BMT overall, and is higher in patients receiving T cell depleted grafts, as discussed (175, 185). Engraftment and the incidence of relapse may be related to the number of donor cells transplanted. It was recently shown that both the quality and the quantity of stem cells in donor marrow are important for engraftment; significant numbers of host cells reconstituted long term hematopoiesis in lethally irradiated mice transplanted with low numbers of syngeneic marrow cells. The syngeneic cells presumably supported survival in the short term until the host hematopoietic system could recover (10, 13). It may be possible to exploit this observation for the treatment of leukemias arising in committed progenitor cells. Low numbers of allogeneic cells could be transplanted in order to reconstitute immunity in the short term, until clones arising from primitive host cells are able to recover. In this way, it may be possible to avoid long term GVHD (10). This kind of therapy, however, would be unrealistic in CML, since the leukemic defect occurs in a very primitive stem cell.

An additional complexity to consider in allogeneic BMT is the finding that there are strain-specific differences in the ability of hematopoietic cells to contribute to short term and long term reconstitution (331, 332). Moreover, colony formation and DNA synthesis in bone marrow cells were demonstrated to be circadian stage dependent and to show seasonal variation (333). In addition, successful engraftment in experimental animals was blocked by the administration of anti-class II antibodies (334). Thus, many factors need to be considered to maximize the possibility of successful reconstitution, including the number of cells transplanted, the HLA type of the patient and donor, and, apparently, the time of day and year at which donor marrow is harvested.

Survival rates following allogeneic BMT for the treatment of CML appear to correlate to the clinical phase in which the patient underwent transplantation. Two to 3 year leukemia-free survival rates for patients transplanted in chronic phase are in the range of 40 to 70% (175, 188). In contrast, three year survival in patients transplanted in accelerated phase in one study was 36%, and 12% in acute transformation (188). Moreover, patients undergoing BMT in accelerated or blastic phase have a very high incidence of leukemia relapse; 30 to 60% for accelerated phase, and 40 to 90% for blastic phase, as compared to 10 to 20% in chronic phase (188, 239). Mortality is strongly correlated with the patient's age.

Leukemia-free survival at 5 years following allogeneic BMT for the treatment of CML is 50 to 60% for patients receiving marrow from an HLA-identical sibling. Patients receiving marrow from an HLA-matched unrelated donor have a 35% leukemia free survival at 3 years, which correlates to the patients' age and the degree of matching between donor and recipient (239). The three year clinical relapse rate in CML following allogeneic BMT is 20% overall, while the cytogenetic relapse is up to 35% (175). Cytogenetic studies revealed that recurrence of CML was in the original clones, indicating that most failures are due to ineffective eradication of residual leukemic cells.

Sources of stem cells other than bone marrow for transplantation are under evaluation. These include peripheral blood; several collections by means of apheresis are thought to be sufficient for transplantation, and the pool of progenitor cells can be expanded by exposure to recombinant cytokines (335, 336). A series of peripheral blood donations can be supported by the administration of erythropoietin (337). In addition, the use of umbilical cord blood, which is highly enriched for progenitor cells is being investigated (338). It is thought that enough stem cells are present in the blood from one umbilical cord to engraft an adult, particularly when the stem cell pool is expanded using cytokines (339).

Although the toxicities and morbidity of allogeneic BMT are significant, this therapy at least provides a possibility of cure. For example, BMT performed in first remission in AML has a 10 year disease free survival rate of 50%. While remissions are induced with chemotherapy in a majority of cases of AML, the long-term survival rate after consolidation treatment, depending on the study, ranges from a mere 10 to 15% (323) to 15

to 35% (313). Unfortunately, allogeneic BMT, as discussed, is limited to a minority of patients due to age or lack of a suitable donor, or in other countries, due to its cost.

1.3.3: Autologous bone marrow transplantation (ABMT), purging;

Patients ineligible for allogeneic BMT may be considered for autologous BMT (ABMT). In ABMT, the advantages of purging leukemic cells from the graft are obvious. For the first 6 months to one year following ABMT many patients harboured cells in which expression of the *bcr-abl* message could be detected, as evaluated by the polymerase chain reaction (PCR), and significant numbers of patients remained Ph⁺ by cytogenetic analysis . After this time many progressed to PCR or Ph negativity (265, 340, 341). This is an indication that some leukemic cells can be tolerated and overcome by the body, however, it is not known what levels are dangerous. Limited success in ABMT of unpurged cells has been achieved, due to the apparent greater sensitivity of leukemic cells to cryopreservation, however, the majority of patients transplanted in this way suffer leukemic relapse (342).

The high rates of leukemic relapse in ABMT for CML may be related to the possibility that the graft versus leukemia effect is lower with autografted cells than using allogeneic grafts, which points to the importance of immune effects in controlling the leukemic cells. Although the incidence of GVHD is much lower in ABMT, it does occur in a minority of patients, and this is correlated with an improved prognosis (239). Leukemia free survival at two years following ABMT for CML is currently a mere 10% (239).

Various agents are being evaluated for their efficacy in purging leukemic cells while sparing normal cells. CML is a convenient model system for studying the efficacy of these agents, as residual cells can be detected by PCR analysis for the presence of the *bcr-abl* transcript. However, CML treated by autologous BMT has a poor prognosis compared to other leukemias, due to the primitive nature of the cell from which it is derived, and the difficulty in eradicating this cell while sparing enough normal stem cells to reconstitute hematopoiesis. Some specificity has been shown for cells of other leukemias using agents such as 4-hydroperxycyclophosphamide (4-HC), the cyclophosphamide derivative mafosfamide (ASTA-Z, reference 343), and Merocyanine 540 (MC-540), a photoactivatible drug, in combination with light. In addition, hyperthermia is reported to affect AML CFU-GM to a greater extent than normals (344).

4-HC has been used to purge autografts in ANLL. Primitive stem cells, however, are resistant to 4-HC, limiting its use in CML (345). Although it is toxic to normal cells in addition to malignant cells, a difference in kill of 2 logs was achieved using 4-HC for the treatment of lymphoma. In one study, the probability of relapse at two years following ABMT for AML in first complete remission was 35% using marrow purged with the 4-HC related drug mafosfamide, as compared to 47% using unpurged marrow (346). Using the photoactivatible drug MC-540 plus light, it was possible to reduce the clonogenicity of HL60 cells by 4 logs, while one third of normal colony forming cells were spared (347). More recently, promising work has been done using the photoactivatible agent benzoporphyrin derivative (BPD). BPD has been shown to be taken up preferentially by leukemic cells, and treatment of cells with BPD plus light results in a greater than 4 log reduction in leukemic cells. In contrast to Merocyanine 540, BPD shows no toxic effects

toward colony formation by normal cells at doses which are therapeutic in a murine model, and at some levels appears to be stimulatory to normal colony formation (348). Improvement in survival in BPD-purged grafts has also been demonstrated in a murine model using L1210 leukemia cells (349).

A variety of novel techniques are being evaluated in order to improve purging for ABMT. Cytokines are being tested in combination with various purging and chemotherapeutic regimens in an attempt to force leukemic cells into cycle and improve cell kill. Induction of proliferation in AML cells by GM-CSF or IL-3 has been reported to enhance the cytotoxicity of the cell cycle specific drug cytosine arabinoside (Ara-C, reference 350). Immunomagnetic beads coated with anti-CD10 antibodies have been used to purge CD10 (CALLA) positive common acute lymphoblastic leukemia cells (351). Similar strategies have been used to remove metastatic breast cancer (352) and neuroblastoma cells (313) from autografts. Finally, AML and CML cells were shown to be differentially sensitive to the inhibitory effects of c - myb antisense oligodeoxynucleotides, and *bcr-abl* expressing cells were completely eradicated at levels that spared normal progenitor cells (353). Antisense oligodeoxynucleotides may prove to be useful in purging marrow *ex viva*. Difficulties in the evaluation of purging protocols include the inability to detect minimum numbers of residual malignant cells due to the limitations of the detection procedures (313, see below).

There have been reports of the establishment of Ph⁻ hematopoiesis in long-term marrow cultures derived from CML marrow, and suggestions that patient's cells can be purged for autografting in this way (257, 342). However, other groups have reported that

this occurs in a minority of cases, and is dependent on the preconditioning regimen (354). Busulfan has long-term effects on progenitor cells compared to hydroxyurea, which is an obstacle to long-term culture techniques with CML cells (312). In other studies, CFU-GM from CML LTMC initiated both from CD34⁺CD33⁻ and CD34⁺CD33⁺ cells were found to be predominantly clonally derived (355). In addition, the number of CD34⁺CD33⁻ cells, the cells important for long term reconstitution, were found to decline rapidly in LTMC (356). Combination of this method with other methods of purging, however, may be a promising therapeutic alternative in selected cases. Normal cells have also been reported to grow selectively in cultures derived from ALL and AML (355, 357).

1.3.4: Cytokines in leukemia therapy;

The use of cytokines for the treatment of leukemias is being examined in several contexts. These include speeding the recovery of hematopoiesis following myeloablative therapy, increasing the fraction of leukemic cells in S phase in order to increase cell kill by chemotherapeutic agents, forcing the differentiation of leukemic cells in an attempt to extinguish the leukemic clone, and enhancing the anti-leukemia immune response (317).

Both chemotherapy and BMT are followed by marrow and immune suppression. This includes neutropenia, with an increased risk of infection, as well as thrombocytopenia, or decreased megakaryocytes and hence platelet counts, with increased risk of bleeding. Patients must be kept isolated in sterile rooms until their neutrophil counts are restored to acceptable levels. Half of deaths following BMT are due to infective

complications during severe myelosuppression (358). The use of various recombinant cytokines in speeding restoration of hematopoietic function is under investigation. GM-CSF and G-CSF are currently widely used in conjunction with BMT and chemotherapy, and result in fewer neutropenic days post-BMT and a reduction in complications (27, 185, 358, 359, 360). GM-CSF has been used to promote recovery after graft rejection following allogeneic BMT, which occurs in 2% of cases (358). In addition to speeding recovery of neutrophil counts, G-CSF has also been reported to improve platelet recovery after chemotherapy (361). Both GM-CSF and G-CSF also activate the effector functions of mature neutrophils. Side effects occurring with GM-CSF include fever, myalgias, anorexia, bone pain, fluid retention, pericarditis, and pleural effusions. These effects, however, are generally found to be tolerable. GM-CSF also supports the development of eosinophils and basophils, which could lead to allergic complications. The systemic side effects seen with GM-CSF may be related to its induction of TNF- α and IL-1 (30). Pentoxifylline, a TNF antagonist, when administered in conjunction with GM-CSF, was reported to decrease the GM-CSF induced pulmonary sequestration of neutrophils and thus preserve neutrophil migration to sites of infection (362). G-CSF is well tolerated, the major side effect being bone pain, and occasional reactions at the site of injection (53). G-CSF and GM-CSF were both recently approved for use in the United States in conjunction with myelosuppressive therapy for non-myeloid malignancies (59).

Combination with other cytokines such as IL-1, IL-3, and Steel factor (SLF) may potentiate the actions of GM-CSF and G-CSF. IL-3 was shown to improve the recovery of platelets and reticulocytes, as well as neutrophils. IL-3 administered sequentially with GM-CSF acted synergistically to stimulate myelopoiesis (185). IL-1 caused a significant

improvement in platelet recovery. It was found to be well tolerated at low doses, although organ toxicities occurred at higher doses (363). SLF has been shown to stimulate hematopoiesis in vivo in primates (364), and in mice in combination with G-CSF, where it increased the number of cells of all lineages (365). Due to its documented actions on mast cells (100), it seems possible that SLF could increase the risk of allergic reactions and anaphylaxis, however, this has not been reported. Finally, activation of the anti-tumour effects of macrophages using M-CSF is being investigated (366).

Cytokines are being used in conjunction with chemotherapeutic agents in an attempt to increase the cycling of leukemic cells and thus their susceptibility to cycle specific chemotherapy. GM-CSF administered in conjunction with Ara-C increased the S phase fraction of AML myeloblasts (367). Similarly, exposure to Ara-C in the presence of IL-3, GM-CSF, and G-CSF resulted in preferential kill of leukemic versus normal clonogenic cells (368). In contrast, other groups report that GM-CSF and IL-3 protected AML blasts from Ara-C toxicity (369). It has been suggested that the susceptibility of leukemic cells to cytotoxic agents may depend on the fraction of cells undergoing self-renewal vs. differentiation rather than corresponding to the fraction of cells in S phase (370). Leukapheresis was formerly used to decrease the burden of leukemic cells, and is occasionally still used to prevent vaso-occlusive complications. Like cytokines, leukapheresis is now being used to induce cell cycle changes in order to increase the efficacy of cycle-specific chemotherapy (371).

It remains to be determined whether the use of cytokines will increase the risk of leukemic relapse by preferentially stimulating the proliferation of residual leukemic cells

(27). Although overexpression of cytokine genes when transfected into cells experimentally is generally not found to be transforming (54, 59), it is possible that cytokines may aid in leukemic outgrowth. For example, GM-CSF and IL-3 are known to stimulate the proliferation of AML cells *in vitro* in at least some cases (55, 185). AML cells were also found to proliferate in response to SLF in combination with other cytokines (372), and in response to IL-3, GM-CSF, and G-CSF alone or in combination (373). Both of these studies, however, found considerable variation in the response of cells from different patients to growth stimulation by cytokines. Also, the regrowth of AML cells stimulated by GM-CSF treatment was found to be reversible on withdrawal of GM-CSF (374). In still other studies, GM-CSF and G-CSF were shown to decrease the growth of AML cells by increasing differentiation (375). It therefore seems difficult to predict what effect cytokine therapy will have in a particular patient or in a particular leukemia subtype without an increased understanding of the biology and biochemistry of the leukemic cells.

Cytokines inhibitory to leukemic cells are also being tested. One group reports long term inhibition of tumour growth by tumour necrosis factor (TNF) in the absence of cachexia, indicating a therapeutic window for possible clinical exploitation (376). In addition, cytokines inhibitory to normal, but not leukemic cells, such as transforming growth factor- β (TGF- β), are being used in an attempt to protect the normal cells from the effects of myeloablative therapy (185). Finally, it was shown that the grafting efficiency of donor cells in BMT could be improved by treatment of the donor cells with IL-3 and GM-CSF (377).

1.3.5: Alterations of adhesive interactions - interferon- α in CML;

Interferon- α (ifn- α) shows selective growth inhibition toward CML CFU-GM as compared to normal cells (378), and induces complete hematological remissions in a majority of patients with previously untreated CML in chronic phase, often with partial or complete suppression of the Ph⁺ clone (379). The mechanism of action of this effect is under investigation, and involves at least in part alterations of the adhesive interactions between hematopoietic and stromal cells. Greater numbers of CML CFU-GM were found to locate in the adherent layer of ifn- α - treated LTMC, suggesting a reduction in the number of CFU-GM in active cycle (291). Of possible relevance is the finding that ifn- α reduces phosphorylation of P210 *bcr-abl* during differentiation of a CML cell line, an indication that the activity of *bcr-abl* may be affected (380). Class II MHC antigen expression, which is reduced on CML cells, is enhanced by either ifn- α or ifn- γ , which also enhance the diminished natural killer (NK) cell activity observed in CML (175). Significant suppression of the malignant clone, however, occurs in only a minority of patients using interferon alpha or gamma singly or in combination (381). The use of interferons in combination with chemotherapeutic agents is being investigated.

1.3.6: Lymphokine activated killer cells;

Following the pioneering work of Rosenberg, who used IL-2 and lymphokine activated killer cells (LAK) cells to treat solid tumours (382), LAK are being generated toward CML cells (383). NK cell activity in CML is generally reduced, however,

significant numbers of LAK cells could be generated ex vivo which were cytotoxic to K562, a CML-derived Ph⁺ cell line. The LAK cells generated in this way were not derived from the leukemic clone despite considerable contamination of the starting culture with leukemic cells. Selective recognition of AML cells by LAK appears to be mediated by the differential expression of adhesion molecules by normal and leukemic cells (384). Experience with solid tumours, however, showed that it was necessary to administer IL-2 to patients in addition to the LAK cells in order to obtain a significant anti-tumour effect. Side effects were sometimes severe, and treatment in many cases had to be discontinued (382). However, this approach may prove to be a useful adjunct to other therapies, for example in ridding the body of residual leukemic cells following BMT. This approach was used successfully by one group in managing a variety of leukemias, and the amount of target cell lysis was improved by exposing the cells to IL-2 and TNF- α (385). IL-2 administered intravenously has been found to induce the expression of a variety of positive regulators of hematopoiesis, while failing to induce the expression of negative regulators, hence a net stimulatory effect was obtained (386). Interestingly, however, one of the side effects of IL-2/LAK therapy was suppression of hemopoiesis; LAK were found to have cytotoxic activity against normal CFU-GM. This effect was shown to be mediated by soluble factors, possibly interferon- γ or TNF- α , and was abolished by the inhibition of LAK DNA synthesis using irradiation or hydroxyurea, without affecting cytotoxicity toward tumour targets (387).

1.3.7: Induction of differentiation - retinoic acid in acute promyelocytic leukemia;

Another promising avenue of therapy is the induction of differentiation. A consistent abnormality in AML M3, or acute promyelocytic leukemia (APL) is the t(15;17) translocation, which occurs in 70-90% of cases (388, 389). This translocation was recently shown to juxtapose the *myl* locus within the retinoic acid receptor- α (RAR- α) coding sequence (388), resulting in a fusion transcript and protein. This finding sheds light on the good clinical response of patients with APL to treatment with all-trans-retinoic acid; 37 out of 46 patients were reported to achieve complete hematological remissions using this therapy (185). It is thought that high levels of retinoic acid restore relatively normal function to the abnormal fusion protein (148). Unfortunately, remissions induced using retinoic acid are only temporary, however, this is an intellectually gratifying example of a correlation between clinical efficacy and biological understanding (163).

It has been found that some established treatments in fact act by inducing the differentiation of leukemic cells and cell lines; examples are low doses of methylprednisone (390), Ara-C, hydroxyurea, interferons (175), and tumour necrosis factor (391). Others, rather than having directly cytocidal activity, induce programmed cell death (apoptosis) in the malignant cells (225). GM-CSF and G-CSF have also been reported to induce the differentiation of AML cells, although in some cases maturation is incomplete and subpopulations of leukemic cells appear to escape the differentiating effect (392, 393). Other compounds that have been reported to induce the differentiation of the Ph⁺ cell line K562 are the well known mutagens thalidomide and its metabolites (394) and ethidium bromide (395). Retinoic acid may be useful in the treatment of CML in

promyelocytic blast crisis (396). 1,25-dihydroxyvitamin D3 is known to cause the differentiation of HL60, a cell line derived from an acute promyelocytic leukemia (397). This effect is accompanied by the dephosphorylation of specific proteins (398), and is thought to involve cAMP-dependent protein kinase (399). Finally, it should be noted that part of the therapeutic effect obtained in unpurged transplants of autologous marrow may be derived from the forced differentiation of leukemic cells by dimethylsulfoxide (DMSO), in which the cells are cryopreserved (313).

1.3.8: Immunotoxins;

One group has reported the use of an anti-CD33 monoclonal antibody linked to the toxin ricin for purging in AML. This approach is possible since clonogenic AML cells express CD33 in 80% of cases. The ricin B-chain was blocked, in order to prevent non-specific binding to galactose residues of cell surface molecules. Using this Ab, a greater than 4 log selective kill of clonogenic CD33 AML cells mixed with an excess of normal marrow cells was achieved (316). When used for purging autografts, sustained engraftment occurred in all cases, but neutropenia was prolonged due to the removal of CD33⁺ lineage-committed progenitors (316). It is possible to shorten this period to tolerable levels using cytokine therapy (400).

1.3.9: Techniques for targetting bcr-abl;

Transforming abl proteins, including P210 *bcr-abl*, P190 *bcr-abl*, and P160 *gag-abl* were found to have a higher affinity for ATP and synthetic tyrosine containing substrates than did *c-abl*, and for tyrosine kinase blockers of the tyrphostin family. These observations raise the possibility that specific *abl* kinase inhibitors could be designed that affect only transforming *abl* (401). Similarly, it has been possible to induce both the formation of antibodies and T cell immunity directed toward the joining region of P210 *bcr-abl* (402, 403). The significance of these observations is unclear, since *bcr-abl* is an intracellular protein, and thus presumably inaccessible to immune reactions.

1.3.10: Molecular biological techniques;

Polymerase chain reaction (PCR) amplification of transcripts is used in diagnosis and in the detection of minimum residual disease following therapy (404, 405, 406, 407, 408, 409, 410). One in 10^5 to 10^6 tumour cells can be detected using this method whereas cytogenetic techniques detect the Ph chromosome at levels of 1 in 10 to 1 in 100 (265, 405). Interestingly, a group of patients that were Ph⁺ post-BMT had a higher incidence of severe chronic GVHD than did Ph⁻ patients, presumably an indication of an active GVL effect (340). It should be kept in mind that of 10^{12} nucleated bone marrow and blood cells, 10^6 to 10^7 leukemic cells could be present below the limits of detection of PCR (411). Similarly, a burden of 10^{10} to 10^{11} leukemic cells would be just at the threshold of detection of cytogenetic analysis (265). It has been suggested that in some cases cytogenetic techniques may be more sensitive than PCR for detecting the presence of residual CML cells, since the *bcr-abl* fusion gene may not be continually transcribed (239). Useful PCR-based techniques are being developed for facilitating diagnosis in neoplasia, examples are fluorescence in situ hybridization (FISH), and the detection of mutations in p53 in tumour cells identified on the basis of immunohistochemistry; the cells are subsequently removed from the slide for molecular analysis (412).

Approaches to inhibiting leukemic cell growth based on molecular biological techniques are under investigation. As discussed, antisense oligodeoxynucleotides to *c*-*myb*, a phosphorylation dependent DNA binding factor, and the related *B-myb*, were shown to inhibit the proliferation of myeloid leukemia cell lines (413) and CML colony formation (413, 414). Analysis of residual colonies by PCR for *bcr-abl* message showed greatly reduced signal, and replating of these colonies resulted in the formation of normal colonies only (415). Interestingly, the promoter region of the *bcr-abl* gene has at least one putative *myb* binding site (213). Similarly, *bcr-abl* antisense oligodeoxynucleotides selectively inhibited CML CFU-GM leaving residual normal colonies (416). These observations are likely to find application in purging for autologous BMT.

It may be possible to apply some of these observations to gene therapy. For example, the autograft could be transfected with a retroviral construct expressing antisense *bcr-abl* message, which should selectively target the leukemic clone in CML. To date, gene therapy has been successfully used in the treatment of adenosine deaminase deficiency, a genetic disorder, in a small number of cases (417). In addition, experimental animals have been reconstituted with tumour infiltrating lymphocytes transfected with a

TNF construct. Although these had significant anti-tumour effects, they also showed significant systemic toxicity, such as cachexia (113). Genes lost in tumour progression such as p53 or Rb could be replaced by gene therapy (418). Methods by which to increase the efficiency of gene transfer are being investigated; these include supporting the survival of primitive hematopoietic stem cells using leukemia inhibitory factor (LIF, references 83, 84), and stromal feeder layers (419, 420), and increasing the efficacy of gene transfer using liposomes (315). Retroviral transfection of transplanted cells has also been used to track the source of relapse after ABMT for AML and to evaluate the efficacy of purging (421).

1.3.11: Positive selection of stem cells;

Recently, interest has focussed on separating primitive stem cells from the total leucocyte population on the basis of the expression of CD34. These cells, which comprise 1 to 3% of the normal bone marrow cell population, can then be used to reconstitute hematopoiesis following BMT. Purification of CD34⁺ cells proved to be more difficult than anticipated, due to difficulties in recovering cells without destroying the CD34 antigen or the cells themselves. Initial protocols involved the use of anti-CD34 antibodies coupled to immunomagnetic beads. This required incubation to remove beads from the cells, which resulted in capping and antigen turnover. Similarly, releasing the cells with proteolytic enzymes resulted in removal of CD34, and homing antigens, from the cell surface. Panning was also used, but resulted in low yields, with significant contamination by CD34-cells. Fluorescence activated cell sorting of leucocytes provided pure populations, but was

too slow to isolate the numbers of cells required for transplantation. One group succeeded in enriching for CD34⁺ cells using avidin-biotin affinity column chromatography, with 25 to 85% yields and 35 to 92% purity (422). Another group enriched for CD34⁺ cells using immunomagnetic beads, from which the cells were released by cleavage with a glycoprotease from *Pasteurella haemolytica*, which recognizes O-sialoglycan structures. Yield and purity were reported to be 90-95% and 94-98%, respectively. It is reported that neither the functional competence of the cells nor the levels or distribution of surface CD34 were affected (423). Stem cells enriched in this way can be rid of leukemic cells and used for transplantation. Transplantation of these cells results in long term engraftment due to their primitive nature. Several patients have undergone transplantation for breast cancer metastatic to the bone marrow using this method with successful engraftment in 100% of cases (424).

Although it is thought that some leukemic cells can be tolerated by the body without resulting in clinical relapse, the CD34⁺ population is an important population in which to ensure removal of leukemic cells, since this is the population which supports long-term hematopoietic reconstitution. This is especially true of CML, since the genetic lesion in CML is thought to arise in an earlier stem cell than in most other leukemias. Although some patients with CML showed Ph⁺ hematopoiesis following BMT, which became Ph⁻ over time (265), it is unknown what levels of leukemic cells can be tolerated and overcome by the body, and the presence of stem cells harbouring the leukemic phenotype clearly increases the likelihood of relapse. Positive selection of stem cells may be coupled with one of the purging techniques outlined previously. Since it is a fraction of the total leucocyte population, fewer leukemic cells would need to be removed from the CD34+

population, and presumably the chances of successful purging of residual cells should be increased. Promising results from one laboratory indicate that the majority of CD34⁺HLA-DR⁺ cells give rise to Ph⁺ colonies, whereas CD34⁺HLA-DR⁻ cells give rise to Ph⁻ colonies (425). Positive selection for CD34 followed by negative selection for HLA-DR may thus prove to be a useful protocol for ridding autografts of leukemic cells in autologous BMT for CML. Unlike protocols which rid the graft of CD33⁺ cells, the period of neutropenia using CD34⁺ selected cells is not extended, since CFU-GM are included in the CD34⁺ population and not depleted from the autograft (422).

In summary, several new therapeutic approaches based on improved understanding of leukemia biology are under development and evaluation. However, many of these approaches are complex, invasive, associated with significant toxicity, and, in the case of cytokine therapy, may increase the risk of leukemic relapse. Moreover, there is some uncertainty as to the optimal use of established therapies; which dose, timing and combinations are most effective. The mechanism of action of many existing therapies is currently being elucidated, and is contributing to an increased understanding of the biology of leukemic cells (163). The development of new therapies based on increased understanding and targetting of the specific mechanisms involved in maintenance and progression of the leukemic clone is warranted.

1.4: Biological activities of proteases;

Proteases, and serine proteases in particular, have recently been shown to exert a variety of intriguing, and sometimes surprising, biological effects. In addition to their traditional role in releasing signal peptides, causing the maturation of other proteins, and their well-defined roles in the blood clotting cascade and complement system, proteases have been shown to be associated with a variety of tumours, including leukemias, to be important in development, and to be involved in the development and functions of hematopoietic cells.

1.4.1: Proteases in the functions of mature hematopoietic cells;

Cytolytic T cells produce a series of granule-associated serine proteases, granzymes, which are involved in antigen-specific cytolysis. (426). Although the specific roles of these proteases are not clear, cytolysis is partially inhibited by inhibitors of serine protease activity (427, 428). Granzyme A has been shown to localize to the nucleus of the target cell, where it cleaves the protein nucleolin, a nucleolar protein involved in the synthesis and assembly of ribosomes; this results in loss of chromatin structure, activation of an endogenous endonuclease, and programmed cell death (429). In addition, a serine protease which is cytostatic to a variety of cell lines has been purified from a natural killer cell line (430). Mast cell subtypes can be distinguished by the specific serine proteases

present in their granules. The transcription of these proteases is influenced by hematopoietic signalling cytokines (431, 432).

Neutrophil proteases are involved in a variety of functions, including the degradation of connective tissue, which facilitates leucocyte migration to sites of infection and inflammation. Human leucocyte elastase and cathepsin G cleave connective tissue proteins such as elastin, collagen, and proteoglycans (433). These proteases are able to specifically cleave their polypeptide inhibitors, which are present in plasma and tissues. Cleavage of the inhibitors inactivates the ability to inhibit the protease, prolonging its half-life, and converting the inhibitor to a chemotactic signal, which results in the influx of additional inflammatory cells (434, 435). Neutrophil elastase was shown to inactivate tissue factor pathway inhibitor, regenerating tissue factor activity, thus favouring local coagulation. Elastase and other enzymes are also known to inactivate several other inhibitors of coagulation, including antithrombin II, heparin cofactor II, C1 inactivator, and alpha 2-antiplasmin (436). In addition, the neutrophil serine proteases have bactericidal activity which is independent of their proteolytic capabilities (437, 438). Hence, they are multifunctional proteins which exert a variety of biological effects through more than one mechanism.

1.4.2: Surprising functions of proteases;

Recently, some surprising functions have been described in which the inhibition of proteolytic activity appears to be involved. Apolipoprotein a, a component of low density

lipoproteins (LDL), has 80% amino acid identity with plasminogen. It is thought that apolipoprotein a may compete with plasminogen for access to fibrin and to plasminogen activators; this is consistent with the observation that persons with high LDL levels are susceptible to atherosclerosis (439). Intriguingly, the amyloid-b protein of extracellular plaques in Alzheimer's brains was found to contain domains that exhibit sequence identity with protease inhibitors (440), implicating the involvement of aberrant proteolysis in the development of Alzheimer's disease.

1.4.3: *Proteases in development;*

Pattern formation in the *Drosophila melanogaster* embryo in part involves the gene products of *snake* (441) and *easter* (442); both are serine proteases.

1.4.4: *Proteases in cell signalling;*

More importantly, proteolytic activity has been shown to be associated with signalling in hematopoietic and non-hematopoietic cells. For example, an epidermal growth factor binding protein (EGF-BP) has serine protease activity which processes EGF to its active form. In addition, EGF-BP, free of contaminating EGF, potentiates the proliferative response of fibroblasts to EGF (443). The gamma unit of nerve growth factor (NGF), which has serine protease activity, proteolytically processes the beta unit to its active form (443). Fibronectin-degrading serine protease activity was found in the heparin-
binding domain of human plasma fibronectin (444), suggesting that proteolysis may be involved in modulating cell adhesion to the extracellular matrix. Sequence similarity to a family of serine protease inhibitors was found in a heat shock protein in the endoplasmic reticulum of myoblasts (445), suggesting that the inhibition of proteolysis may be involved in cellular stress responses. Modification of two isozymes of protein kinase C with a resultant loss of kinase activity in human neutrophils is mediated by serine protease activity (446). In addition, the labile nuclear oncoproteins *c-myc*, *c-fos*, p53 and E1A, are rapidly degraded by the ubiquitin system *in vitro* (447, 448).

In hematopoietic cells, the phorbol ester induced down-modulation of the CSF-1 receptor (449) as well as release of active CSF-1 from a membrane bound form (107) are both the results of proteolytic processing. The transmembrane form of Steel factor, which has structural homology to CSF-1 (450), has a putative proteolytic cleavage site in its extracellular domain (95). The membrane-bound form of CSF-1 was shown to be biologically active; it was able to stimulate cells expressing CSF-1 receptor (451), and may be involved in its downregulation following IL-3 binding. A serine protease inhibitor suppresses the secretion of tumour necrosis factor by peripheral blood mononuclear cells (112). In addition, TNF has been found to exist as an integral membrane protein, suggesting that it may by involved in cell-cell adhesion or signalling by interacting with its receptor on an adjacent cell. The TNF receptor exists *in vivo* in a soluble as well as a membrane-bound form. The soluble form may act to neutralize soluble TNF, but also may act as a ligand for membrane-bound TNF (112). Downregulation of G-CSF cell-binding capacity can be blocked by inhibitors of protease activity (290). FGF,

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IL-1 and TGF- β have all been shown to increase plasminogen activator activity. Both IL-1 and TGF- β are converted to their soluble forms by plasmin. Hence, this protease appears to be important in influencing hematopoiesis through its actions on cytokines (452). Sequence analysis of the hematopoietic adhesion molecule CD44 indicated that trypsin-like proteases might cleave within sequences included in the isoforms CD44R1 and CD44R2, generating soluble forms that could mediate adhesive interactions (20). Finally, proteolytic activity has been shown to be involved in the initiation of signal transduction pathways resulting in cell activation at the cell surface, as follows. The serine protease thrombin causes activation of platelets, resulting in their aggregation. Platelet activation is mediated by the thrombin receptor, one of the family of receptors with seven transmembrane domains. Activation of the thrombin receptor requires the serine protease activity of thrombin, and can be blocked using specific thrombin inhibitors. Receptor activation was shown to be mediated by the release of a short peptide from the N terminus of the receptor. The newly created N terminus of the receptor then binds to another site on the receptor, resulting in platelet activation and aggregation. Thus, protease activity from the extracellular milieu to the nucleus has been demonstrated to influence cell behaviour (453).

1.4.5: Proteases in malignancies, including leukemias;

The involvement of proteolysis in the physiology of several malignancies has been documented. A serine protease and its associated inhibitor have been found in elevated levels in the urine of patients with gynecological cancers (454). In addition, protease inhibitors were found to inhibit the growth of transformed murine fibroblast cell lines (455), and to decrease the frequency of radiation transformation in a murine cell line (456). Proteases have for some time been known to be essential for the metastasis of solid tumours (161). In some cases, this effect may be mediated by familiar cytokines; TGF- β was found to induce protease production and invasion by transformed fibrosarcoma cells, while it suppressed protease transcription in non-transformed fibroblasts (457).

More important is the demonstrated involvement of proteolytic activity in several leukemias. The alpha 2-macroglobulin receptor, which binds serine proteases, is expressed on the cells of a high proportion of monocytic leukemias. This receptor also is known to bind cytokines such as interleukins 1, 2, and 6, TGF-\beta and FGF, leading the investigators to speculate that its interactions with both proteases and growth factors may affect the turnover of the malignant cells (458). The presence of serum plasminogen activator inhibitor-2 (PAI-2) was found to be a marker of active leukemias with monocytic components (459). Recently, serine protease activity was shown to be associated with the estrogen receptor when bound to its ligand (460). Intriguingly, in a separate report, estrogen receptors were demonstrated to be expressed in a case of AML M4, as well as in the cell line HL60. This observation followed reports of spontaneous remission of AML in pregnancy following parturition, and of the presence of estrogen receptors in multiple cases of CLL, some or which responded to the estrogen receptor blocking drug tamoxifen (461). CALLA (Common Acute Lymphoblastic Leukemia Antigen, also known as CD10, enkephalinase, and membrane metalloendopeptidase) is a cell surface neutral endopeptidase (NEP) known to be associated with lymphoid leukemias (462). Finally, the downregulation of a recently cloned serine protease, myeloblastin, was found to cause the growth arrest and differentiation of the promyelocytic leukemia cell line HL60 (310).

These examples clearly set precedents for the involvement of proteases in the development, functioning, and oncogenesis of hematopoietic cells.

1.5: Background on 'CAMAL';

Background studies which led to the current investigations are summarized in this section. The original isolation of 'CAMAL' (Common Antigen in Myelogenous Acute Leukemia) from acute non-lymphocytic leukemia (ANLL) leucocytes, and the preparation of anti-CAMAL antibodies is described. Immunoperoxidase studies using anti-CAMAL antibodies in which it was demonstrated that the detection of the CAMAL antigen is diagnostic of myeloid leukemias, and is of prognostic value in these leukemias, are summarized. Also described are *in vitro* 'CAMAL' addition studies using CAMAL-1 enriched preparations of myeloid leukemia cell lysates cultured with normal and CML progenitor cells. These studies demonstrated that exposure of progenitor cells to CAMAL-1 enriched material resulted in alterations of myelopoiesis which could impart a growth advantage on the leukemic clone. The purpose of the current study was to further characterize and define these effects in order to gain greater insight into the possible role of CAMAL-1 enriched material in the events of leukemogenesis, and to facilitate the design of new strategies to block or reverse the leukemic progression.

The original isolation of 'CAMAL' from ANLL leucocytes involved subtractive precipitation of normal cellular components from ANLL leucocyte lysates by the sequential addition of increasing amounts of rabbit antiserum raised against preparations of pooled normal human leucocytes. Material that remained once precipitation was complete was used to prepare rabbit anti-CAMAL antisera (463), and to screen monoclonal hybridoma supernatants during the preparation of the CAMAL-1 antibody (464).

The anti-CAMAL antibodies were used for immunophenotyping studies of myeloid leukemias. It was shown by enzyme-linked immunosorbent assay (ELISA), fluorescence activated cell sorting analysis (FACS), and an immunoperoxidase slide test that antibodies raised against 'CAMAL' preparations reacted to a far greater extent with the mononuclear cells of persons with myelogenous leukemias, both ANLL and CML, than with normal cells or cells of persons with various lymphoproliferative disorders (463, 464, 465). For example, using FACS analysis, it was found that the rabbit antibodies reacted with the cells, both peripheral blood leucocytes (PBL) and bone marrow (BM), of 44 out of 45 patients with active AML, with 19 out of 19 patients with CML, and with 13 out of 13 patients with AML in remission. However, they did not react with any of 14 normals nor with 40 out of 42 patients with a variety of lymphoproliferative disorders (466, 467). Similarly, in an immunoperoxidase slide test using CAMAL-1, it was found that of cells from patients with ANLL in primary presentation, 35/36 and 21/23 were positive ($\geq 1\%$ staining by cell number, BM and PBL, respectively). In addition, 7/7 PBL from CML were positive. In contrast, 0/13 and 0/30 BM and PBL from normals reacted with this antibody. 1/5 PBL from ALL in primary presentation showed reactivity, as did 1/12 PBL from CLL. Average percentages of positive cells were 15.8 ± 2.8 and 8.9 ± 3.9 for BM

and PBL of ANLL in primary presentation, and 19.3 ± 2.6 for CML. In contrast, values were 0.3 ± 0.1 and 0 for BM and PBL of normals, 0.4 ± 0.2 for PBL of ALL, and 0.2 ± 0.1 for CLL PBL (465). Thus, detection of 'CAMAL' on the bone marrow or peripheral blood cells, as indicated by the reactivity of these cells with the antibody CAMAL-1, appeared to be indicative of myeloid leukemia. Few other markers common to both chronic and acute myeloid leukemias have been reported. One is oncofetal protein (OFP), a cytoplasmic protein of 50 - 55 kDa which locates to the nuclear pores and may be involved in the transport of mRNA. OFP was detected in HL60 cells using a mAb raised against hepatoma cells, and is common to all tumour cell types examined (468).

The same immunoperoxidase studies indicated that the cells of many patients with ANLL in clinical remission continued to express the CAMAL marker (24/25, 11.8 \pm 1.7 for BM; 16/24, 4.9 \pm 1.4 for PBL). In order to determine whether levels of cells reactive with the antibody CAMAL-1 remained consistent over time, a double blind study was undertaken in which specimens from patients with a variety of hematological malignancies were evaluated over a course of three years. It was found that of patients undergoing bone marrow transplantation for ANLL, 10 of 12 who remained in remission during the study period had 'CAMAL' bone marrow values of less than 1.0%, as did 10 of 10 patients who died in remission of causes other than leukemia. In contrast, 6 of 6 patient with ANLL who relapsed, two of whom died in relapse, had greatly elevated 'CAMAL' values, ranging from 5 to 100%. In these patients, 'CAMAL' values were found to become elevated up to three months prior to relapse (469). In further studies of patients with ANLL undergoing chemotherapy, it was found that patients whose 'CAMAL' bone marrow values fell significantly on induction of remission had significantly longer survival

times than those whose 'CAMAL' values rose or remained the same (6.8 months and 19.2 months, respectively, reference 470). These results were taken as an indication that increases in detection of the CAMAL antigen on bone marrow cells preceded the recurrence of clinical symptoms, that levels of the CAMAL antigen were of prognostic value in predicting survival time, and that the CAMAL antigen might be indicative of a molecular entity involved in the outgrowth of the leukemic clone.

The possibility that the entity detected by the antibody CAMAL-1 was involved in the outgrowth of myeloid leukemia cells was investigated by the addition of protein preparations enriched from lysates of myeloid leukemia cells using CAMAL-1 immunoaffinity chromatography to in vitro colony assays of progenitor cells from normal healthy donors or from patients with CML. These studies showed that colony formation by normal progenitor cells was inhibited by the addition of CAMAL-1 eluted material, often profoundly. Colony formation by CML progenitor cells, however, was not inhibited by the addition of the same preparations of CAMAL-1 enriched material, and in some cases was stimulated (471). Outgrowth of the leukemic clone, then, could occur in two ways; the suppression of normal myelopoiesis could impart a selective growth advantage on leukemic cells by reducing competition for space and growth requirements in the hematopoietic microenvironment, and the enhancement of leukemic myelopoiesis could give the leukemic cells a more direct growth advantage. The purpose of the current study was to further characterize and define these effects in order to gain greater insight into the role of CAMAL-1 eluted material in the early events of leukemogenesis, and to facilitate the design of new strategies to block or reverse the leukemic progression. As CAMAL-1 eluted material consisted of several protein species by silver-stained SDS-PAGE analysis,

the first step taken was to separate these proteins from each other and determine which component in these preparations mediated the alterations of myelopoiesis (472, Chapter 2).

Studies were undertaken in order to address the biochemical characterization of the marker and in order to further define the activities on colony formation. The biochemical characterization of the marker was the subject of a separate research project, and was the Ph.D. research of another graduate student in this laboratory (473). The biological characterization of the activities on colony formation was the subject of research by the author. When these studies were undertaken, it was thought to be probable that the CAMAL marker detected on myeloid leukemia cells by CAMAL-1 in the immunoperoxidase assay and the material which mediated the effects on myelopoiesis by normal and leukemic progenitor cells were equivalent. As work progressed, however, it became clear that the marker and the activity were not in fact equivalent entities. In this paper, the marker on leukemic cells recognized by CAMAL-1 is referred to as 'the CAMAL antigen', 'the CAMAL marker', or 'CAMAL'. The material shown to mediate the effects on *in vitro* myelopoiesis is designated 'P30-35 CAMAL', unless otherwise specified.

It was recently shown by Western blot analysis that the antibody CAMAL-1 is not reactive with the P30-35 CAMAL material shown in this study to mediate the effects on *in vitro* myelopoiesis by normal and CML progenitor cells (474). Thus it is likely that CAMAL-1 does not bind directly to P30-35 CAMAL. However, CAMAL-1 is known to enrich for P30-35 CAMAL. This was shown by passing material from lysates of myeloid leukemia cells which fell through a CAMAL-1 immunoaffinity column over an immunoaffinity column prepared with α -P30/35, a monoclonal antibody prepared against

highly enriched P30-35 material. No further P30-35 CAMAL material eluted from the a-P30/35 column (474), an indication that the vast majority of P30-35 CAMAL was removed from the leukemic cell lysates by the antibody CAMAL-1. CAMAL-1 thus enriches for P30-35 CAMAL, which explains its usefulness during P30-35 CAMAL enrichment protocols.

The enrichment of P30-35 CAMAL by the antibody CAMAL-1 in the apparent absence of direct binding is somewhat of a mystery. It is known, however, that CAMAL-1 is reactive with human serum albumin (HSA, reference 474). HSA is known to carry a number of biologically active molecules, so it is possible that P30-35 CAMAL could itself be carried by HSA (reviewed in 475). The monoclonal antibody against the active P30-35 CAMAL material, α -P30/35, which was screened for lack of reactivity with HSA, was raised in order to better address these questions, as well as to facilitate purification of P30-35 CAMAL (Chapter 2). α -P30/35 is known to react by Western blot analysis and by ELISA with P30-35 CAMAL, and thus appears to bind directly to P30-35 CAMAL (474, Chapter 2). The similarity in patterns of staining of CML vs. normal nucleated cells by immunoperoxidase using CAMAL-1 as compared to α -P30/35 (Appendix 1) suggest an association between the CAMAL-1 reactive CAMAL marker and the P30-35 material recognized by α -P30/35. However, no definitive answers are available at this point, and this matter is the subject of ongoing research (476).

For the majority of the studies described in this thesis, P30-35 CAMAL was enriched in a two-step protocol using immunoadsorbent columns prepared with CAMAL-1 and α - P30/35, since this protocol was shown to result in optimal removal of materials

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done using a one-step immunoaffinity protocol with α -P30/35 (474). Recent work has shown that active material can be obtained using either procedure (Chapter 2, 474, 477). It was shown that P30-35 CAMAL was highly enriched for inhibitory activity on *in vitro* colony formation by normal progenitor cells (Chapter 2), and that stimulatory activity on colony formation by CML progenitor cells resides in the same P30-35 CAMAL fraction (Chapter 4). Recent information suggests that the inhibitory activity on normal colony formation might in fact reside in less than ten percent of the P30-35 CAMAL fraction (477). Experiments presented in Chapter 5 suggest the involvement of serine protease activity in the effects on normal and CML myelopoiesis, activity which might be unique (Appendix 2). Further biochemical definition of the entity (or entities) to which the activities on normal and CML myelopoiesis can be attributed are the subject of ongoing research (476).

CHAPTER 2

CHARACTERIZATION OF THE INHIBITORY EFFECTS OF P30-35 CAMAL ON NORMAL MYELOPOIESIS

2.1: INTRODUCTION

Chronic myelogenous leukemia (CML) is a myeloproliferative hematopoietic stem cell disorder in which an elevation of neutrophilic granulocytes and their progenitors are observed in the bone marrow and peripheral blood. How the leukemic clone gains dominance over normal hematopoietic cells is not well understood, but inhibitors of normal hematopoiesis have been described (Chapter 1). The CAMAL antigen was originally isolated by elution of ANLL or CML cell lysates from a CAMAL-1 immunoaffinity column and shown to inhibit colony formation by normal progenitor cells *in vitro*. This observation was important, as suppression of normal hematopoiesis could provide an environment in which the leukemic clone could gain dominance. As CAMAL-1 eluted material used for these original experiments consisted of several protein components, the proteins were further separated in order to identify the component to which the inhibitory activity on normal colony formation is mediated by the component in CAMAL-1 eluted material which migrates at 30-35 kilodaltons (kDa) by sodium dodecyl sulphate polyacrylamide gel electropheresis (SDS-PAGE) analysis is described; this material is referred to as P30-35

CAMAL. In cultures of cells from normal healthy donors, P30-35 CAMAL at low concentrations was demonstrated to be profoundly inhibitory to colonies of neutrophilic granulocytes (CFU-G) and to all colony types at higher levels. In addition, P30-35 CAMAL was inhibitory to normal myelopoiesis in long term marrow culture, a condition which more closely approximates the hematopoietic microenvironment.

2.2: MATERIALS AND METHODS

2.2.1: Purification of P30-35 CAMAL:

The major focus of this study was the characterization of the biological activity of CAMAL-eluted material, with a minor focus on the reactivity of anti-CAMAL and anti-P30/35 antibodies at a cellular level. Separation of the components in CAMAL-1 eluted material, optimization of protein purification, characterization of the protein, and activities and characteristics of CAMAL at a subcellular level, and characterization of the reactivities of the anti-CAMAL antibodies at a subcellular level were and are the subject of ongoing research by another graduate student in this laboratory, as part of a separate project (472, 473). Characterization of the biological activity of CAMAL-1 eluted material involved, as an initial step, the identification of the component in CAMAL-1 eluted preparations inhibitory to colony formation by progenitor cells from normal healthy donors. This involved a collaborative effort. CAMAL-1 eluted material was separated for the experiments in this initial phase of the study, including experiments described in Figures 1

through 8, Figure 9b, and Table II, by Joan Shellard, using FPLC gel filtration or preparative non-reducing SDS-PAGE to separate the components in CAMAL-1 eluted material (472). The active P30-35 CAMAL material for all subsequent experiments was enriched by the author using sequential elution from CAMAL-1 and α -P30/35 immunoadsorbent columns.

2.2.1.a: Antibodies;

Antibodies used in these studies are all monoclonals. CAMAL-1 was raised against original preparations of the CAMAL antigen, which were in turn prepared by subtractive methods from lysates of cells from patients with myeloid leukemias, as described in Chapter 1. The original immunoperoxidase studies which demonstrated that recognition of CAMAL was diagnostic of myelogenous leukemias were performed using this antibody (465). CAMAL-1 has demonstrated reactivity with proteins other than P30-35 CAMAL. It was shown by Coomassie Blue-stained SDS-PAGE, and by immunoprecipitation, that the major species recognized by CAMAL-1 is a protein which migrates in the range of 64 - 68 kDa by SDS-PAGE (475, 478). Similarly, CAMAL-1 was shown to be highly reactive with human serum albumin (HSA) using the enzyme-linked immunosorbent assay (ELISA) technique (474). However, silver-stained SDS-PAGE analysis showed that protein species other than the 64 - 68 kDa material were present in CAMAL-1 eluted material (472, Figure 1a), leading to the experiments in which these components were separated and the inhibitory activity identified.

A new monoclonal antibody was prepared against the 30-35 kDa material in CAMAL-1 eluted preparations. This was done in order to facilitate purification of P30-35 CAMAL, and in order to facilitate investigations into whether the antigen recognized by CAMAL-1 in the immunoperoxidase test and the inhibitory material might be the same entity (Appendix 1). This was investigated since rabbit polyclonal antibodies raised against original preparations of the CAMAL antigen were shown both to recognize the marker on myeloid leukemia cells (465) and to react with P30-35 CAMAL by Western blot analysis (474). More recently, it was shown by Western blot analysis that the monoclonal antibody CAMAL-1, used for the majority of the immunoperoxidase studies, does not react with P30-35 CAMAL (474), however, similarities in cell staining using CAMAL-1 or α -P30/35 suggest an association between the CAMAL antigen, and the inhibitory material, P30-35 CAMAL (Appendix 1). The possibility of an association between the CAMAL antigen and P30-35 CAMAL is supported by the observation that CAMAL-1 does enrich for the P30-35 CAMAL active material (474).

 α -P30/35 was raised against highly enriched material which migrated between 30 and 35 kDa (< 2% contaminants by silver stained SDS-PAGE analysis). This material was purified by subjecting CML or AML cell lysates which had been eluted from a CAMAL-1 immunoaffinity column to fast protein liquid chromatography (FPLC) gel filtration or to preparative non-reducing SDS-PAGE as described in the following section. Since the antibody CAMAL-1 is known to react with HSA, hybridoma supernatants were screeend for lack of reactivity with HSA, and α -P30/35 does not react with HSA by ELISA (data not included). Recently performed reverse phase high performance liquid chromatography (HPLC) separations of P30-35 CAMAL preparations, which were performed after the

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completion of this study, have shown that other protein components are present in preparations purified using α -P30/35, components with which α -P30/35 appears to be reactive by Western blot analysis (474); these include elastase, azurocidin, and cathepsin G, but not myeloblastin. There is, however, a protein peak in preparations of P30-35 CAMAL which is distinct from the other proteins present, which have been characterized (473, 476, 479). In addition, experiments described in Chapter 5 and in Appendix 2 demonstrate clearly that there is an activity in preparations of P30-35 CAMAL which appears to be distinct from the activities of these other components. This is discussed in greater detail in sections 2.4, 5.4, 6.1, and in Appendix 2.

 α -BLV was raised against the major coat protein of bovine leukosis virus at the facilities of Quadra Logic Technologies (QLT), and was obtained from QLT. α -BLV was used as a control antibody for the evaluation of purification protocols and for diagnostic studies.

2.2.1.b: *Class identification;*

CAMAL-1, α -P30/35, and α -BLV were all determined to be of the immunoglobulin subclass IgG₁ by the Ouchterlony technique. 10 microliter (µl) volumes of CAMAL-1, α -P30/35 or α -BLV ascites were loaded into wells cut into 1% agar gels prepared on 7.5 X 5 cm strips of gel-bond (FMC Bio-Products) fixed onto glass plates. These were prepared by heating agar (Difco) to boiling in the microwave, pipetting 7.5 ml

onto each plate, and allowing it to solidify at room temperature. Antisera (Meloy) to various Ig classes and subclasses, including IgG_1 , IgG_{2a} , IgG_{2b} , IgG_3 , and IgM were loaded into adjacent wells (10 µl volumes), and gels incubated overnight in a humid atmosphere at 4 degrees centigrade (°C) in order to allow diffusion of antisera to occur. Gels were rinsed with 0.15 M NaCl to remove soluble proteins, rinsed in distilled water (dH2O), and stained with 0.5% amido black in 5% acetic acid for 1 minute (min). Gels were destained with a solution of 45% MeOH and 10% acetic acid.

2.2.1.c: Preparation and screening of α -P30/35;

The fraction in CAMAL-1 eluted material which migrated at between 30 and 35 kDa by SDS-PAGE analysis was determined to be the component in these preparations which was inhibitory to colony formation by normal progenitor cells *in vitro*. Hence, a monoclonal antibody was raised against this material in order to facilitate purification and diagnostic studies. Material further separated from CAMAL-1 immunoaffinity preparations by FPLC gel filtration or elution from preparative non-reducing SDS polyacrylamide gels, and which corresponded to material that migrated at between 30 to 35 kDa by analytical SDS-PAGE, was used to immunize Balb/C mice by standard methods. The material used for immunization was prepared by another graduate student in this laboratory, and the procedure is described elsewhere (472). Immune spleen cells were fused with an NS-1 fusion partner by the method of Oi and Herzenberg (480). Cell fusion and cloning were performed at the facilities of Quadra Logic Technologies by Herma Neyndorff.

Hybridoma supernatants were screened for reactivity with P30-35 CAMAL material by enzyme-linked immunosorbent assay (ELISA). P30-35 CAMAL used for screening hybridoma supernatants was material from lysates of leucocytes from patients with chronic myelogenous leukemia (CML) or acute myelogenous leukemia (AML) which eluted from a CAMAL-1 immunoadsorbent column, and which was further fractionated by FPLC gel filtration or preparative non-reducing SDS-PAGE. As the major contaminating protein with which CAMAL-1 reacts was shown by ELISA to be human serum albumin (HSA, reference 474), hybridoma supernatants were also screened for lack of reactivity with HSA (Sigma). Cells from wells showing reactivity with P30-35 CAMAL, and lacking reactivity with HSA were cloned, and supernatants from the clones rescreened.

The ELISA procedure is as follows. 96 well ELISA plates (Immunlon 2) were coated with 1 μ g/ml of P30-35 CAMAL or HSA in 50 mM carbonate coating buffer, pH 9.6, in 100 μ l/well volumes. Plates were incubated overnight at 4 °C. Buffer and excess protein were removed by shaking and vigorous washing with PBS-Tween (phosphate buffered saline containing 0.05% Tween-20). Hybridoma supernatants were added in 100 μ l of volumes, and plates were incubated for 60 min at 37°C. Plates were washed with PBS-Tween and alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulins (Jackson) diluted 1:3000 in PBS-Tween was added in 100 μ l volumes. Plates were again incubated at 37°C for 60 min, washed, and substrate added (*p*-nitrophenyl disodium, Sigma, 1 mg/ml in 10% diethanolamine, DEAE, buffer, pH 9.8). Plates were incubated for 30 min to 4 hours at 37°C until visible colour formation occurred. Reactivity was determined by absorbance at 405 nanometers (A₄₀₅) using a Titertek ELISA reader. Absorbance was compared to controls containing culture media but no cell supernatant.

Three clones were obtained that were reactive with P30-35 CAMAL, and non-reactive with HSA. These were evaluated for their ability to purify P30-35 CAMAL by immunoaffinity as described below.

2.2.1.d: Preparation of ascites;

Hybridomas were cultured in Dulbecco's modified Eagle's Medium (DME), supplemented with 10% fetal calf serum (FCS). Cells were cryopreserved in liquid nitrogen at a cell density of 3 X 10% milliliter (ml) in 40% media and 50% FCS by volume with a final concentration of 10% dimethylsulfoxide (DMSO; Sigma, tissue culture grade). DMSO was added dropwise, with gentle shaking, to cells in media containing FCS. 1 to 1.5 ml fractions were then frozen in cryovials (Nunc). Initially, cryovials were insulated in styrofoam containers to slow freezing overnight in a at -70°C, after which time they were transferred to liquid nitrogen (IN_2). Later cryopreservation procedures involved freezing of cells were in liquid nitrogen vapour overnight, following which they were submerged in IN_2 .

Female Balb/C mice between 6 weeks and one year old were injected intraperitoneally (i.p.) with 0.5 ml pristane (2,6,10,14-tetramethylpentadecane, Aldrich). Ascites was prepared by i.p. injection of hybridoma cells 5 to 14 days after injection of pristane. Between 2 to 5 X 10^6 cells were injected per mouse. Between 5 to 10 mice were used per group for each ascites preparation. Hybridoma cells from culture, or cryopreserved cells which had been thawed, were used to prepare ascites. Cryopreserved

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cells were thawed in a 37°C water bath with gentle shaking, and injected immediately after thawing. Ascites was collected from mice 7 to 21 days after injection of hybridoma cells by intraperitoneal placement of a 16 gauge needle (tapped). Mice were tapped one to three times before euthanization. Ascites from each hybridoma group was pooled for purification of immunoglobulins and centrifuged for 10 min at 5000 rotations per minute (rpm) to remove fibrin clots.

2.2.1.e: Purification of immunoglobulins from ascites;

Immunoglobulins were initially purified from ascites using hydroxyapatite column chromatography. 75 ml columns were prepared in degassed 0.01 M sodium phosphate buffer, pH 6.8. 15 ml volumes of ascites were diluted 1:2 in the same buffer, and pumped over the column at a flow rate of 3.5 ml/min. The column was washed with starting buffer to an A₂₈₀ of less than 0.05, then proteins were eluted with 0.5 M sodium phosphate buffer, pH 6.8. Protein containing fractions as determined by A₂₈₀ from column fallthrough, and eluted protein, were pooled separately and concentrated by centrifugation at 5000 X gravity (g, 8000 rpm on a Sorvall centrifuge using an SS35 rotor) in minicentricons (Amicon) with a molecular weight cutoff of 30 kDa. Protein concentration was determined by the Lowry assay, and protein content analyzed by silver stained SDS-PAGE. Fall-through material consisted mainly of a 66 to 68 kDa protein species, in all likelihood albumin, and eluted material was enriched for immunoglobulins. Between 5 to 25% of eluted proteins appeared to be immunoglobulin heavy and light chain proteins by SDS-PAGE analysis. This corresponded to an approximately eight fold enrichment. Subsequent purification of antibodies was performed using a goat α -mouse Ig column (Hyclone). 15 ml of ascites was diluted 1:2 in PBS and pumped onto the goat α -mouse Ig column at a flow rate of 0.8 ml/min. The column was washed with PBS to A₂₈₀ < 0.05, then eluted with 0.1 normal (N) hydrochloric acid (HCl). The eluated material was immediately neutralized by the addition of 1.5 M Tris-HCl (Tris hydroxymethyl aminomethane HCl, Sigma), pH 7.5. Protein containing fractions as determined by A₂₈₀ were pooled. The column eluate was dialyzed against PBS, and concentrated and analyzed as above. The column was immediately neutralized by this method appeared to be immunoglobulin heavy and light chain proteins by SDS-PAGE analysis. This corresponded to an approximately fifty fold enrichment.

2.2.1.f: Immunoadsorbent column preparation;

Purified monoclonal antibodies were dialyzed against 0.01 M Hepes (N-[2hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid], Sigma) buffer, pH 8.0 (prepared with degassed deionized distilled water, ddH₂O) for coupling to agarose column beads (Bio-Rad Affi-Gel-10). 20 mg/ml of hydroxyapatite-purified antibodies or 5 mg/ml of antimouse Ig purified antibodies were used per ml of beads. All steps were performed at 4°C. Beads were washed in ddH₂O, ddH₂O was removed, and Ig in coupling buffer added. An equal volume of Ig solution to bead volume was used. Mixtures were allowed to react for 4 hours with gentle shaking, at which time the reaction was stopped by the addition of 0.1 ml 1 M glycine ethyl ester, pH 8.0 per ml of gel beads. Beads were packed into Bio-Rad columns (10 cm X 1.5 cm), washed extensively with PBS, and stored at 4°C in PBS containing 0.02% NaN₂.

Antibodies were evaluated for their ability to enrich for P30-35 CAMAL. One CML cell lysate was divided into fractions and these were purified by immunoaffinity using various columns and combinations of columns, including preabsorption against a column prepared with the control mAb, α -BLV. Optimal enrichment was obtained using CAMAL-1 eluted material further fractionated over a column prepared from one of the three hybridoma antibodies, which was called α -P30/35, and this protocol was used for subsequent preparations. The other hybridoma antibodies were not used subsequently.

2.2.1.g: Protein analysis;

Protein concentrations were determined using the Micro-Lowry assay (481).

Purified mAbs and preparations of P30-35 CAMAL were analyzed by SDS-PAGE according to the method of Laemmli (482). 10% polyacrylamide gels were run using the Bio-Rad mini-gel system. Protein was diluted in sample buffer containing 2% SDS, 10% glycerol, 0.15 M Tris-HCl pH 6.8 and bromphenol blue with 20 mM dithiothreitol (DTT, Bio-Rad), heated at 70°C for 10 min, cooled and loaded onto 10% polyacrylamide mini-gels (7.5 X 10 cm). Gels were run at a constant current of 25 milliamps (mA). Gels were silver stained as follows. Briefly, gels were fixed in a solution of 50% methanol and 10%

acetic acid, then rehydrated in a solution of 10% methanol and 10% acetic acid, with heating. Gels were washed in dH₂O, heated in dH₂O containing 30 μ M DTT, and stained in a solution of 0.1% silver nitrate in dH₂O for 15 minutes at room temperature. They were rinsed with dH₂O and developed in a solution of 3% sodium bicarbonate (Na₂CO₃) with 0.037% formaldehyde (Fisher) in dH₂O. Development was stopped by the addition of 2.3 M citric acid or acetic acid.

2.2.2: Preparations of P30-35 CAMAL:

2.2.2.a: Sources of P30-35 CAMAL;

Preparations of P30-35 CAMAL were derived from three sources. One preparation was purified from ANLL leucocytes from a patient who had undergone apheresis to reduce the white blood cell burden. These cells were obtained from the Vancouver General Hospital Division of Hematology. One preparation was purified from leucocytes obtained by apheresis from a patient with CML. These cells were obtained from the Toronto General Hospital by Dr. Armand Keating. The remaining preparations were purified from CML remission bone marrow buffy coat cells, which are from the fraction enriched for leucocytes, and which includes plasma. These cells were originally intended for autologous transplantation, but the patients had since died. These cells were obtained from the Terry Fox Laboratory and the Canadian Red Cross Society. They had originally been stored in liquid nitrogen, and were subsequently stored at -70°C.

2.2.2.b: Cell lysis;

Cells were thawed and mixed with an equal volume of lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM sodium chloride [NaCl], 1.0 mM ethylenediamine tetraacetic acid [EDTA], 1.0% Triton X-100, 0.5% sodium deoxycholate [NaDOC]), containing 2000 units/ml of pancreatic deoxyribonuclease (DNase, Sigma). This mixture was allowed to stir at room temperature for 2 to 3 hours and then at 4°C for an additional 12 hours. Subsequent steps were all performed at 4°C. The lysate was centrifuge at 15000 rpm for 30 min using a Sorvall SS35 rotor, in order to remove insoluble debris.

2.2.2.c: *Protein purification;*

Soluble components of cell lysates were affinity purified over CAMAL-1 immunoadsorbent columns prepared as described above. The eluted material was then further purified in one of three ways; by preparative non-reducing SDS-PAGE, by FPLC gel filtration using a Superose-12 column, or by affinity purification using an α -P30/35 immunoadsorbent column. Protein which was not bound by the α -P30/35 column, and which fell through, was collected, concentrated and used as control protein in biological assays.

Preparative gels and gel filtration were performed by another student in this laboratory, and are described elsewhere (472). Briefly, material eluted from a CAMAL-1

immunoaffinity column was subjected to electropheresis through a 16 cm 12% SDS polyacrylamide gel. The separating gel was cut horizontally into 25 mm slices, which were eluted for analysis by reducing SDS-PAGE in order to determine the molecular weight of the protein contained in each fraction. Fractions to be tested in the progenitor cell assay were dialyzed against PBS and protein content determined by the Micro Lowry assay. Alternatively, CAMAL-1 eluted material was dialyzed against gel filtration buffer (0.1 M ammonium [NH₄] acetate, pH 5.0) and run over a Superose-12 gel filtration column at a flow rate of 0.35 ml/min using FPLC. Individual peak fractions, as monitored by A₂₈₀ were pooled, concentrated and analyzed for protein content by silver-stained reducing SDS-PAGE and the Micro Lowry assay. P30-35 containing fractions were dialyzed against PBS for evaluation of biological activity in the colony assay.

2.2.2.d: Control protein;

Protein which fell through the α -P30/35 column was used as control protein in biological assays. In addition, human serum albumin (HSA,Sigma) was used as a further control protein in colony assays using normal progenitor cells. N-terminal amino acid sequence suggested that a protein with significant sequence identity to human neutrophil elastase was present in preparations of P30-35 CAMAL (see Discussion), hence the effect of elastase (Calbiochem) on normal and CML progenitors was evaluated in colony assays.

2.2.3: Hematopoietic progenitor cell assays:

2.2.3.a: Cells;

Normal leucocytes for colony assays were obtained from the following sources; all leucocytes were obtained with informed consent. Normal peripheral blood leucocytes (PBL) were obtained from normal healthy volunteers. Normal bone marrow was obtained from donors for allogeneic bone marrow transplantation through the Cancer Control Agency of British Columbia by Dr. Gordon Phillips. Normal marrow was also obtained from persons undergoing thoracic surgery at St. Paul's Hospital by Drs. Hilton Ling and William MacDonald. CML bone marrow was obtained from the Vancouver General Hospital (VGH) Division of Hematology, and CML peripheral blood from the VGH Division of Hematology and the Munroe Clinic at VGH. Both bone marrow and peripheral blood were collected into tubes containing enough sodium heparin (Fisher) for a minimum of 50U heparin/ml of bone marrow or blood.

Mononuclear cells were separated by centrifugation over Ficoll-Hypaque or Percoll (Pharmacia). This procedure separates the mononuclear cell fraction, including neutrophils, from erythrocytes and mature neutrophils. Blood or marrow was diluted 1:2 to 1:4 in fresh Iscove's medium (Gibco) which had been warmed to 37° C. Between 7 to 10 ml of diluted blood or marrow was carefully layered over 3 ml Ficoll-Hypaque or Percoll (diluted to 1.077 g/ml in sterile PBS) in 15 ml polystyrene tubes and centrifuged at 400 X g (1500 rpm) for 12 minutes using a "Silencer" counter-top centrifuge (Fisher). Fat was removed from the top following centrifugation of bone marrow, using a sterile pasteur

pipette, and was discarded. The mononuclear cells at the interface of the Ficoll-Hypaque or Percoll and media/plasma were recovered by aspiration with a sterile pasteur pipette. Cells were washed twice and resuspended in fresh Iscove's medium. Cells were counted in white blood cell counting media, which causes the lysis of residual contaminating erythrocytes (5% acetic acid in PBS with a crystal of methylene blue, sterile filtered through a 0.2 μ m filter). Viability was determined by dye exclusion using 0.2% eosin Y (Fisher). Cells were plated in a colony assay only when cell viability exceeded 98%. Viability was rarely less than 100%.

Cells were often cultured fresh in the colony assay, however, at times, particularly in initial assays, it was necessary to cryopreserve cells for later use. This was done as described above for the hybridoma cells. It was found that greater recovery could be achieved when cells were cryopreserved using higher concentrations of FCS, hence 90% FCS (heat inactivated) was used, with 10% DMSO added dropwise. Cells were thawed in a water bath, as above, and fresh warm Iscoves's medium containing 20% FCS added dropwise with gentle shaking to 10 times the frozen volume. Cells were then centrifuged at 1000 rpm, washed once in fresh Iscove's medium with FCS and once in serum-free Iscove's medium, resuspended in Iscove's medium, and counted as described above.

2.2.3.b: *Preparation of conditioned medium and plasma for colony assays;*

Phytohemagglutinin-stimulated leucocyte conditioned medium (PHA-LCM) and human fasting plasma were obtained from normal healthy volunteers. 500 ml blood was drawn into a blood bag containing 50U sodium heparin (Fisher) per ml blood. Blood was transferred to 50 ml Falcon tubes, and allowed to settle for 45 to 90 min to form a buffy coat layer composed of white blood cells and plasma. The buffy coat layer was removed by aspiration with a pasteur pipette, cells pelleted by centrifugation for 10 min at 400 X g, and the plasma set aside. Cells were washed twice in Iscove's medium, resuspended at a density of $1X10^{6}$ /ml in Iscove's medium with 1% PHA by volume (Sigma) and cultured for 7 days at 37°C, 5% carbon dioxide (CO₂) in 200 ml flasks. Conditioned medium was collected into 50 ml Falcon tubes, cells removed by two rounds of centrifugation at 1500 rpm for 5 min, then PHA-LCM was divided into aliquots and frozen at -70°C. Plasma was collected from the buffy coat layer, and by centrifugation of the remaining fraction at 1500 rpm for 20 minutes. Plasma was centrifuged twice at 2500 rpm, filtered through 0.45 µm filters (Millex-HA, Millipore), divided into aliquots, and stored at -70°C. On thawing, plasma was again centrifuged at 2500 rpm for 10 min in order to remove fibrin clots before addition to colony assays.

2.2.3.c: Colony assays;

Colony assays were performed according to the method of Messner (483). Fresh or previously cryopreserved bone marrow or peripheral blood mononuclear cells were added to cultures containing Iscove's medium, 1% methylcellulose (Fluka AG), 30% human fasting plasma, and 10% PHA-LCM. These conditions supported the formation of a mixture of colony types, the most primitive of which were CFU-GM. Addition of 1 unit per milliliter (U/ml) erythropoietin (Epo-Conn, Connaught Laboratories), final concentration, supported the growth of CFU-GEMM in addition. 1 ml cultures were plated in duplicate or quadruplicate in 35 mm tissue culture dishes (Lux), and incubated at 37°C, 5% CO₂ for 14 days, at which time colonies comprised of \geq 20 cells were scored by visual inspection using an inverted microscope. Cultures were incubated under highly humid conditions by the inclusion of a tray in the incubator brimming with sterile dH₂O. In addition, duplicates in 35 mm plates were placed in larger 100 mm plates; a third 35 mm plate filled with sterile dH₂O was included with each pair of culture plates. Phase contrast photomicrographs were taken using a Nikon camera on a Zeiss Axiovert inverted microscope.

Colonies were plucked using a miniature pasteur pipette, placed on glass slides, and spread by gentle air pressure. They were fixed for 10 min in methanol, stained for 10 seconds (sec) with Wright's Giemsa (Camco Quick Stain, Bridge Chemical Products), rinsed for 20 sec in dH₂O, air dried, and examined using a light microscope. The plucked colonies examined in the experiments described in Figure 6 were evaluated by Dr. Patricia Logan at the facilities of Quadra Logic Technologies.

2.2.3.d: Long term marrow cultures;

Long term marrow cultures (LTMC) were performed as described by Keating and Toor (484), using fresh or cryopreserved normal bone marrow mononuclear cells. Cells were cultured at a density of 2 X 10⁶ cells/ml in 2 to 5 ml volumes in long term marrow culture media using Corning 24 or 6 well tissue culture plates or 10 ml flasks. LTMC

media consisted of McCoy's medium supplemented with 12.5% horse serum, 12.5% fetal calf serum, essential (2.5 X from concentrate) and non-essential amino acids vitamins (1 X serum)from concentrate) glutamine (2 mM), sodium pyruvate (1 mM), sodium bicarbonate (0.75% w/vol), penicillin (100 U/ml), streptomycin (100 U/ml), fungizone (all from Gibco), and $0.35 \,\mu$ g/ml hydrocortisone (Sigma). Medium was sterile-filtered using a $0.2 \,\mu$ filter and stored at -20°C. Once thawed, medium was stored at 4°C, and used within 2 weeks. Cultures were incubated for 7 days at 37°C, 5% CO₂, following which they were incubated for the remainder of the culture period at 33°C, 5% CO₂. Cultures were incubated under highly humid conditions. In addition to the inclusion of a tray in the incubator brimming with sterile dH_2O , unused wells on culture plates were filled with sterile dH₂O containing fungizone (0.04 U/ml). Cultures were fed weekly by removing half of the supernatant, including cells, and replenishing the culture with fresh medium. P30-35 CAMAL concentration was depleted weekly by feeding the cultures. Supernatant cells were washed, resuspended in Iscove's medium, counted, and plated in a methylcellulose colony assay as described above. Cells were plated at a constant cell density of 1 X 10⁵ cells per 1 ml culture, unless inadequate numbers were recovered from the cultures to achieve this cell density. In this case, cells from control or P30-35 CAMALtreated cultures were diluted to the cell density of whichever culture was lowest, so that cells from control and P30-35 CAMAL-treated cultures were cultured for colony formation at equivalent cell densities. At week 5, adherent layers were sacrificed by trypsinization using 0.25% trypsin-EDTA in Iscove's medium (Gibco-BRL) for 10 min at 37°C; adherent cells and supernatant cells were cultured separately for colony formation. Cultures were photographed using a Nikon camera and a Zeiss Axiovert inverted microscope, using differential interference contrast microscopy (DIC).

2.3: RESULTS

2.3.1: Identification of the component in CAMAL-1-enriched material inhibitory to normal colony formation;

Crude material enriched by CAMAL-1 immunoaffinity chromatography was previouslyshown to be inhibitory to colony formation by normal progenitor cells (471). Since material eluted from CAMAL-1 immunoadsorbent columns contained a variety of protein species, as determined by SDS-PAGE analysis, it was necessary to determine which component of CAMAL-1 eluted material exerted this inhibitory effect on colony formation. Components in CAMAL-1 eluted material were further separated using FPLC gel filtration or preparative non-reducing SDS-PAGE, for evaluation in the colony assay. The gel filtration and preparative SDS gel purification procedures have been described previously (472).

A silver stained SDS polyacrylamide gel showing the peak fractions resulting from a Superose-12 gel filtration column fractionation of CAMAL-1 eluated material is shown in Figure 1a (this gel was prepared by Joan Shellard). Figure 1b shows the mean number of day 14 colonies observed when gel filtration fractions were added to CFU-GM assays of normal progenitor cells at a protein concentration of 1 μ g/ml. Only fractions containing material that migrated at 30 to 35 kDa by SDS-PAGE significantly reduced colony formation; peak 2 (lane c), peak 3, (lane d), and peak 4 (lane e), but not peak 1 (lane b). Lane f is preparative SDS gel-purified P30 CAMAL material, which was used in the



FIGURE 1. Identification of the component in CAMAL-1 eluted material inhibitory to colony formation by progenitor cells from normal healthy donors. A CML cell lysate was prepared using CAMAL-1 immunoaffinity chromatography followed by FPLC gel filtration or preparative non-reducing SDS-PAGE. a. A silver stained analytical SDS polyacrylamide gel illustrates protein purification; unfractionated CAMAL-1 eluate, lane a; FPLC fractions corresponding to peaks 1 through 4, lanes b through e respectively; P30-35 CAMAL eluted from a preparative non-reducing SDS gel, lane f. Note that molecular weight standards differ between lane a and lanes b through f. b. The mean number of day 14 colonies observed when normal progenitor cells were cocultured with FPLC peak fractions at a protein concentration of $1 \mu g/ml$ in CFU-GM assays.

experiment labelled 'NPBL4/CML6a', Table II. A titration of FPLC fractions 2 and 3 (lanes c and d, Figure 1a) is illustrated in Figure 2. Inhibitory activity was maintained to lower protein concentrations in fraction 3, which consisted of essentially pure P30-35 CAMAL material by SDS-PAGE analysis, than in fraction 2, in which many protein components were present. Table II summarizes many of the experiments performed using material purified from CAMAL-1 eluates of CML or AML cell lysates by FPLC gel filtration or preparative SDS-PAGE. Colony formation was reduced by P30-35 CAMAL in these experiments both in number and in size. Inhibition at concentrations of P30-35 CAMAL above 1 µg/ml was profound; colony numbers were reduced by 90 to 100%. Inhibition of colony formation at concentrations of P30-35 CAMAL between 35 ng/ml and 1 μ g/ml was fairly constant at between 30 and 45%; at concentrations of P30-35 below 35 ng/ml, a titration of inhibitory activity occurred. Inhibition of normal colony formation by p30-35 CAMAL was a consistent effect; it was seen using six preparations of P30-35 CAMAL obtained from different donor sources of leukemic cells on six different sources of normal progenitor cells. The enrichment of inhibitory acitivity observed with increasing enrichment of P30-35 CAMAL is described in Table III. Enrichment of P30-35 CAMAL from CAMAL-1 eluated material was estimated to result in an approximately 200 fold enrichment of inhibitory activity.



FIGURE 2. Titration of inhibitory activity on colony formation by normal progenitor cells in fractions 2 and 3 (as described in Figure 1). Normal progenitor cells were cocultured with between 330 and 1 ng/ml of protein from fractions 2 or 3 in a CFU-GM assay; the inhibitory activities of these fractions was compared to sham treated (PBS) controls. Mean number of control colonies was 60.5 ± 1.5 .

TABLE II. Summary of P30-35 CAMAL inhibitory activity in colony assays using progenitor cells from five different normal healthy donors and six preparations of P30-35 CAMAL derived from different CML or AML patient leucocyte specimens.

				Percen	ut inhibiti	ion by P3	30-35 CA	AMAL co	ncentratio	u		Number of control	Number of
	P30-35			(ug/ml	(•						colonies per	mononuclear cells
Normal	CAMAL											milliliter	plated per milliliter
donor	source	15	5.0	1.7	1.0	0.6	0.2	0.06	0.035	0.01	0.004	culture ± SE	culture
NBM1	CML1	89						-				162.0±3.0	7.5 X 10 ⁴
NBM2	CML2		100	86		4	45	24				127.3 ± 5.5	1×10^{5}
NBM3	AML3	95										46.5 ± 6.5	3 X 10 ⁵
NPBL4	CML4				27		28					183.0 ± 5.0	3 X 10 ⁵
NPBL4	CML4 ^a						38	20b				75.0 ± 9.0	3 X 10 ⁵
NPBL4	CML5				100							129.5 ± 2.5	3 X 10 ⁵
NPBL4	CML6								35	26		60.5 ± 1.5	3 X 10 ⁵
NPBL4	CML6 ^a								17		10	163.5 ± 0.5	3 X 10 ⁵
NBM5	CML6 ^c							37	26	17		139.5 ± 7.0	2.5 X 10 ⁵
^a All P30-	35 CAMAL	prepara	ations we	re purifie	d using (CAMAL	-1 immu	noaffinity	chromat	ography	and FPLC	gel filtration except th	lose with the ^a
superscrip	t which wer	e purifi	ied using	preparativ	ve non-re	educing 5	SDS-PAG	GE.		•		-	

^cAll colonies were cultured under conditions that supported the growth of CFU-GM, except ^c, to which erythropoietin was added; these cultures ^bStudent's *t*-test analysis of results showed that all inhibitory levels were significant at p < 0.05 with the exception of ^b, in which p < 0.1. supported the growth of CFU-GEMM. TABLE III. Enrichment of P30-35 CAMAL and coenrichment of inhibitory activity on colony formation by progenitor cells from normal healthy donors^a.

Preparation	Concentration of protein effecting 50% inhibition	Units of activity per ug of protein ^b	Enrichment of biological activity			
CAMAL-1 eluate	20 ug/ml	0.05	-			
FPLC P30-35 enriched material	220 ng/ml	4.5	90 x			
Pure P30-35 (by PAGE analysis)	100 ng/ml	10.	200 x			
^a Shown are representative d	ata for the purification of Pa	30-35 CAMAL and its activit	ty in CFU-GM/CFU-GEMM			
assays.						
^b One unit is defined as the activity that effects 50% inhibition of CFU-GM/CFU-GEMM in a 1.0 milliliter						

2.3.2: Lack of inhibitory effect on CML colony formation by P30-35 CAMAL;

Since CAMAL-1 eluated material was observed to inhibit colony formation by normal progenitor cells, whereas no inhibition of colony formation by CML progenitor cells occurred (471), the effect of material enriched for P30-35 CAMAL on colony formation by CML cells was evaluated in order to verify that the component of interest had been isolated. An experiment in which the effect of P30-35 CAMAL eluted from preparative gels on colony formation by normal and by CML progenitor cells was compared is shown in Figure 3. An experiment in which the effect of P30-35 CAMAL purified by FPLC gel filtration on colony formation by CML cells was evaluated is shown in Figure 4. While it is recognized that these effects are not dramatic, they are not meant to stand on their own, but to be taken in the context of results from many assays using normal progenitor cells (Table II). It can be seen that, in both instances, whereas colony formation



FIGURE 3. Activity of preparative non-reducing SDS gel-purified P30-35 CAMAL on colony formation. Progenitor cells were cocultured with P30-35 CAMAL eluted from preparative gels in CFU-GM assays and the mean number of day 14 colonies compared to SDS controls. a. 250 ng/ml P30-35 CAMAL material in cultures of cells from normal healthy donors. b. 37 ng/ml P30-35 CAMAL from a second preparation in cultures of normal cells. c. 37 ng/ml P30-35 CAMAL from the same preparation as in 'b' in cultures of cells from a patient with CML. Material used in 'a' is illustrated in Figure 1a, lane f. In these experiments 'CAMAL' refers to P30-35 CAMAL.


FIGURE 4. Activity of P30-35 CAMAL purified using FPLC gel filtration on CML-CFU. A CML-derived preparation of P30-35 CAMAL was cultured with normal or CML progenitor cell assays and the mean number of day 14 colonies compared to PBS controls. a. Cultures of normal cells. b. Cultures of cells from a patient with CML. In these experiments, 'CAMAL' refers to P30-35 CAMAL.

by normal progenitor cells tested concurrently was inhibited by incubation with P30-35 CAMAL, colony formation by CML progenitor cells was not.

In addition to a reduction in number, some of the remaining colonies within P30-35 CAMAL treated cultures were reduced in size compared to colonies in control cultures in most experiments. This effect is illustrated in Figure 5.

2.3.3: Preferential targetting of CFU-G by P30-35 CAMAL;

The observation that inhibition of colony formation was maintained at similar levels (30 to 45%) over a wide range of protein concentration (35 ng/ml to 1 μ g/ml, Table 1) suggested that some progenitor cell types might be more sensitive than others to inhibition by P30-35 CAMAL. Two experiments in which colonies plucked from control and P30-35 CAMAL treated cultures were examined are illustrated in Figure 6. At the levels of P30-35 CAMAL used in these experiments (100 ng/ml and 50 ng/ml for 6a and 6b respectively, levels at which inhibition of colony formation was around 30%) CFU-G were dramatically reduced by treatment with P30-35 CAMAL. Interestingly, eosinophil colony numbers were increased under these conditions.









FIGURE 6. Preferential inhibition by P30-35 CAMAL of neutrophilic granulocyte colonies (CFU-G) cultured from normal progenitor cells: Normal progenitor cells were cultured with levels of P30-35 CAMAL known to inhibit colony formation by between 30 to 45%. Colonies were plucked and evaluated for morphology. a. An experiment in which 37% inhibition resulted at a P30-35 CAMAL concentration of 100 ng/ml. Mean number of control colonies was 139.5 \pm 6.9; mean in P30-35 CAMAL-treated cultures was 88.0 \pm 15.0 (p < 0.025 by Student's *t*-test analysis). One plate was chosen from each category, and 50 colonies plucked from each. b. An experiment in which 31% inhibition resulted at a P30-35 CAMAL concentration of 50 ng/ml. Mean number of control colonies was 76.8 \pm 3.0; mean in P30-35 CAMAL-treated cultures was 53.0 \pm 2.0 (p < 0.005 by Student's *t*-test analysis). One plate was chosen from each category and the entire plate plucked; 64 colonies from the control culture and 50 colonies from the P30-35 CAMAL-treated culture.

2.3.4: Experiments using α -P30/35 enriched P30-35 CAMAL;

Subsequent experiments were performed with P30-35 CAMAL which was enriched from CAMAL-1 eluted CML cell lysates by fractionation using an α -P30/35 immunoaffinity column. Figure 7 is an SDS-polyacrylamide gel of one such preparation. CAMAL-1 eluted material is shown in lanes a and b, material which fell through the α -P30/35 column and was used as control protein in subsequent experiments in lanes c and d, and material which eluted from the α -P30/35 column in lanes e and f. It can be seen that the α -P30/35 eluted material was highly enriched for P30-35, with little contaminating material.

In order to ensure that no inhibitory activity on normal colony formation was lost due to sequential acid elutions, the activity of equivalent levels of P30-35 in material eluted from a CAMAL-1 column and material sequentially eluted from a CAMAL-1 column followed by fractionation using an α -P30/35 column were compared. Figure 8a shows that inhibitory activity on colony formation by normal cells was equivalent in both cases. In neither case was colony formation by CML cells affected (Figure 8b).



FIGURE 7. P30-35 CAMAL prepared using sequential CAMAL-1 and α -P30/35 immunoaffinity chromatography. The silver-stained SDS polyacrylamide gels show a CML lysate eluted from a CAMAL-1 column, lanes a and b. CAMAL-1 eluted material was further fractionated using an α -P30/35 immunoadsorbent column. α -P30/35 column fall-thru protein, lanes c and d, α -P30/35 column eluted material, lanes e and f. 1 µg of protein was loaded in each of lanes a, c, and e, and 4 µg was loaded in each of lanes b, d, and f.



FIGURE 8. Comparison of inhibitory activity on normal colony formation by CAMAL-1 eluted material ('CAMAL-1') and material prepared using sequential CAMAL-1 and α -P30/35 immunaffinity column chromatography ('C1:P30/35'). Protein was added to the cultures to an estimated final P30-35 concentration of 300 ng/ml. a. Cultures of cells from a normal healthy donor. b. Cultures of cells from a patient with CML.

2.3.5: Lack of inhibitory activity on normal colony formation by other proteins in CAMAL-1 eluted material;

Additional control experiments were performed in order to determine whether other protein components in CAMAL-1 eluated material had an effect on colony formation. CAMAL-1 eluted material which fell through an α -P30/35 column, and which migrated at around 66 kDa by SDS-PAGE analysis (Figure 7, lanes c and d) was cultured in a colony assay using normal progenitor cells. This material did not inhibit colony formation, nor did it potentiate the inhibition mediated by P30-35 when added to the α -P30/35 eluted material. This result is illustrated in Figure 9a. Since the material which migrated at around 66 kDa was shown to consist predominantly of human serum albumin (HSA, reference 474), the effect of purified HSA obtained from Sigma was evaluated in a colony assay. Figure 9b shows that HSA had no inhibitory effect on colony formation by normal cells.

2.3.6: Evidence that the effects of P30-35 CAMAL on colony formation are not mediated by elastase;

N-terminal amino acid sequence suggested the presence of a serine protease with some sequence identity to human neutrophil elastase in preparations of P30-35 CAMAL (see Discussion). Figure 10 shows that the effect of elastase on colony formation is different from that exerted by P30-35 CAMAL. In contrast to P30-35 CAMAL, which inhibited colony formation by normal cells but did not inhibit colony formation by CML



FIGURE 9. Comparison of P30-35 CAMAL and control protein activity on colony formation by normal progenitor cells. 300 ng/ml of P30-35 CAMAL material and 15 μ g/ml 'P66' control protein were used where indicated. a. Inhibitory activity of P30-35 CAMAL is compared to 'P66' control protein which fell through an α -P30/35 column. b. Inhibitory activity of P30-35 CAMAL is compared to HSA. 15 μ g/ml of P30-35 CAMAL material or HSA were added to these cultures.



FIGURE 10. Inhibition of colony formation by both progenitor cells from normal healthy donors and from patients with CML mediated by elastase. Mean number of control colonies was 413.0 ± 14.5 for NBM, 225.0 ± 7.0 for CML 1, which was PBL, and 66.0 \pm 3.7 for CML 2, which was BM.

cells, elastase inhibited colony formation by both normal and CML progenitor cells. In order to rule out the possibility that preparations of P30-35 CAMAL were contaminated by elastase, all subsequent preparations were screened for lack of elastase activity, i.e. lack of inhibition of colony formation by CML cells.

2.3.7: Experiments using fresh progenitor cells;

The experiments described above were all performed using cryopreserved progenitor cells. CFU-G, however, are known to be more sensitive to cryopreservation than are other progenitor cell types (485), Since CFU-G were shown to be more sensitive to the inhibitory effect of P30-35 CAMAL material than were other progenitor cell types, the effect of P30-35 CAMAL on colony formation by fresh progenitor cells was evaluated in order to determine whether the inhibitory effect seen using cryopreserved progenitor cells was optimal. Table IV summarizes results observed using fresh normal progenitor cells treated with P30-35 CAMAL. At a P30-35 CAMAL concentration of 100 ng/ml there was a larger variation in levels of inhibition of colonies grown from fresh progenitors; inhibition ranged from 27% to 71%, as compared to between 30 and 45% inhibition at the same protein levels using cryopreserved progenitor cells. Two titrations of P30-35 CAMAL using fresh progenitor cells are illustrated in Figure 11 a and b; inhibitory activity titrated over the same range of protein concentration as was seen using cryopreserved progenitor cells in all four cases tested.

TABLE IV. Summary of inhibitory activity mediated by P30-35 CAMAL on colony formation in assays using six different fresh progenitor cells from six different normal healthy donors.							
Normal bone marrow donor	Percent inhibition at 100 ng/ml P30-35 CAMAL	Number of control colonies per milliliter culture ± SE	Number of mononuclear cells plated per milliliter culture				
1	71	84.0 ± 1.8	1 X 10 ⁵				
2	43	30.75 ± 1.3	3 X 10 ⁴				
3	27	108.75 ± 4.2	7 X 10 ⁴				
4	54	189.75 ± 6.5	9 X 10 ⁴				
5	42	200.0 ± 8.1	1 X 10 ⁵				
6	36	154.75 ± 7.0	2×10^4				



FIGURE 11. Two experiments in which P30-35 CAMAL inhibitory activity on normal colony formation was titrated using fresh progenitor cells. a. Mononuclear cells were preincubated with between 100 and 0.4 ng/ml P30-35 CAMAL. Mean number of control colonies was 84.0 ± 1.8 . b. Mononuclear cells were preincubated with between 100 and 4 ng/ml P30-35 CAMAL. Mean number of control colonies was 154.8 ± 7.0 .

2.3.8: Apparent reduction in release of cells from the adherent layer in P30-35 CAMALtreated long term marrow cultures;

Tables V and VI summarize data from experiments testing the effect of P30-35 CAMAL material, which had been characterized in colony assays using normal and CML progenitor cells, on normal bone marrow cells in long-term marrow culture. The number of supernatant cells in these cultures was reduced by treatment with P30-35 CAMAL. This reduction was maintained to the fifth week of culture, at which time the cultures were sacrificed. Since the supernatant cells from control and P30-35 CAMAL-treated cultures were plated for colony formation, and since equivalent numbers of colonies resulted (Table VI), this reduction in supernatant cell numbers is equivalent to a reduction in the number of colony forming cells. The number of cells in the adherent layer in P30-35 CAMAL treated cultures, however, was increased. This is evident from cell counts of trypsinized adherent layers (Table VI), and by visual inspection of P30-35 CAMAL treated and control cultures (Figure 12). Numerous cobblestone areas, areas of active granulopoiesis (2) are evident in the cultures treated with P30-35 CAMAL (Figure 12 a and b, panel ii in both cases). These areas of granulopoies are absent from control cultures (Figure 12 a and b, panel i in both Whether colony formation was affected in the experiment presented in Table V cases). cannot be evaluated since the culture size was too small and cell numbers too low to support appropriate colony formation. However, colony formation in the experiment described in Table VI was quite

equivalent when equivalent numbers of cells from P30-35 CAMAL-treated and control cultures were plated, an indication that the reduction in supernatant cell numbers

TABLE V. Inhibition by P30-35 CAMAL of cell numbers in the non-adherent supernatant compartment of a long-term marrow culture using cells from a normal healthy donor ²								
[P30-35 CAMAL] (ng/ml) ^b	110	44	26	16	8			
CELL COUNT	week 1	week 2	week 3	week 4	week 5 non-adherent	adherent		
+ CAMAL ^C	2.2 X 10 ⁶	2.4 X 10 ⁵	2.5 X 10 ⁵	6.7 X 10 ³	1.9 X 10 ⁴	3.4 X 10 ⁴		
NO CAMAL ^C	2.1 X 10 ⁶	4.1 X 10 ⁵	5.4 X 10 ⁵	5.3 X 10 ³	3.9 X 10 ⁴	4.2 X 10 ⁴		
% inhibition cf control	-5	41	56	-26	52	19		
NUMBER OF COLONIES ^d								
+ CAMAL ^C	0	5,10	12,10	1,0	0	0		
NO CAMAL ^C	0	3,6	4,2	0	0	0		

^aTwo milliliter cultures were used in this experiment.

^bP30-35 CAMAL was added to culture at the beginning of the culture period and subsequently progressively depleted with removal of culture supernatant for purposes of feeding and recovery of supernatant cells to plate for colony formation.

^c'CAMAL' is used here to refer to P30-35 CAMAL.

^dequivalent numbers of cells recovered from control and P30-35 CAMAL-treated LTMC were plated for colony formation.

corresponds to a reduction in the numbers of colony-forming cells. Similarly, cell numbers recovered from the adherent layer in the experiment described in Table V were too low to draw conclusions, as were the colony numbers derived from the adherent layer cells in the experiment described in Table VI. The increase in cell numbers recovered from the adherent layer in the experiment described in Table VI, however, as well as the increase in cell numbers in the adherent layers of both experiments, which is obvious by visual inspection, supports the idea that cell numbers in the adherent layers in these experiments are increased in conjunction with a decrease in the numbers of cells found in the non-

adherent compartment. This is consistent with the idea that cells are held in the adherent layers of P30-35 CAMAL-treated LTMC of normal bone marrow cells, and that their release into the non-adherent compartment is prevented or delayed.

TABLE VI. Inhibition by P30-35 CAMAL of cell numbers in the non-adherent supernatant compartment of a lon-term marrow culture and of the formation of colonies cultured from non-adherent cells, using cells from a normal healthy donor^a.

100	50	25	13	6	
week 1	week 2	week 3	week 4	week 5	
				non-adherent	adherent
5.35 X 10 ⁶ 1.05 X 10 ⁷	1.85 X 10 ⁶ 2.7 X 10 ⁶	1.17 X 10 ⁶ 1.95 X 10 ⁶	6.5 X 10 ⁵ 1.0 X 10 ⁶	7.4 X 10 ⁵ 1.65 X 10 ⁶	9.6 X 10 ⁶ 3.5 X 10 ⁶
49	31	40	37	55	
$284 \ 0 \pm 6.4$ 265.8 ± 17.2	47.7 ± 3.8 46.3 ± 2.5	49.5 ± 1.5 56 0 ± 2.0	26.8 ± 2.3 18.8 ± 1.1	23.5 ± 8.5 17.8 ± 2.2	6.0 ± 1.1 14.3 ± 0.9
	100 week 1 5.35×10^{6} 1.05×10^{7} 49 $284 \ 0 \pm 6.4$ 265.8 ± 17.2	100 50 week 1 week 2 5.35×10^6 1.85×10^6 1.05×10^7 2.7×10^6 49 31 284 0 ± 6.4 47.7 ± 3.8 265.8 ± 17.2 46.3 ± 2.5	100 50 25 week 1 week 2 week 3 5.35×10^6 1.85×10^6 1.17×10^6 1.05×10^7 2.7×10^6 1.95×10^6 49 31 40 284 0 ± 6.4 47.7 ± 3.8 49.5 ± 1.5 265.8 ± 17.2 46.3 ± 2.5 $56 \text{ 0} \pm 2.0$	100 50 25 13 week 1 week 2 week 3 week 4 5.35×10^6 1.85×10^6 1.17×10^6 6.5×10^5 1.05×10^7 2.7×10^6 1.95×10^6 1.0×10^6 49 31 40 37 284 0 ± 6.4 47.7 ± 3.8 49.5 ± 1.5 26.8 ± 2.3 265.8 ± 17.2 46.3 ± 2.5 $56 \ 0 \pm 2.0$ 18.8 ± 1.1	100 50 25 13 6 week 1 week 2 week 3 week 4 week 5 5.35×10^6 1.85×10^6 1.17×10^6 6.5×10^5 7.4×10^5 1.05×10^7 2.7×10^6 1.95×10^6 1.0×10^6 1.65×10^6 49 31 40 37 55 284 0 ± 6.4 47.7 ± 3.8 49.5 ± 1.5 26.8 ± 2.3 23.5 ± 8.5 25.8 ± 17.2 46.3 ± 2.5 $56 \text{ 0} \pm 2.0$ 18.8 ± 1.1 17.8 ± 2.2

^aFive milliliter cultures were used in this experiment.

^bP30-35 CAMAL was added to culture at the beginning of the culture period and subsequently progressively depleted with removal of culture supernatant for purposes of feeding and recovery of supernatant cells to plate for colony formation.

^C'CAMAL' is used here to refer to P30-35 CAMAL.

 d Equivalent numbers of cells recovered from control and P30-35 CAMAL-treated LTMC were plated for colony formation.



i.

a



FIGURE 12. Increase in cobblestone areas in the adherent layers of P30-35 CAMALtreated long-term marrow cultures using bone marrow cells from normal healthy donors. All photographs are 460 X magnification. a. 2 ml cultures grown from cryopreserved progenitor cells at week 4 of culture. i. Control culture. ii. P30-35 CAMAL-treated culture. b. 5 ml cultures grown from cryopreserved progenitor cells at week 5 of culture. i. Control culture. ii. P30-35 CAMAL-treated culture. b

i.



ii.

2.4: DISCUSSION

The inhibitory activity of CAMAL-1 eluted material on colony formation by progenitor cells from normal healthy donors was shown to be mediated by the component of 30 to 35 kDa enriched from this CAMAL-1 eluted material (Figures 1 and 2 and Table II). In contrast, neither P30-35 CAMAL eluted from preparative SDS gels nor that purified by FPLC gel filtration inhibited colony formation by CML progenitor cells (Figures 3 and 4). The inhibition of colony formation by normal progenitor cells mediated by P30-35 CAMAL was directed to colony size as well as colony number (Figure 5). This effect on colony number and on colony size is an indication that, in addition to reduction of the clonogenicity of progenitor cells in P30-35 CAMAL-treated cultures, the proliferative capacity of progenitor cells giving rise to remaining colonies in P30-35 CAMAL-treated cultures appears to be reduced.

While it is recognized that the inhibitory effect of P30-35 CAMAL on colony numbers is in many cases not dramatic (Figure 3b, Figure 4), these results are not meant to stand alone, but to be taken in the context of the results of many experiments (Table II, and subsequent experiments in Chapter 2, Chapter 3, and reference 477). Several factors contributed to the sometimes small inhibitory effects on colony formation. The material used in the experiments summarized in Figure 3 was eluted from preparative SDS gels. Controls including SDS-containing fractions were used in these experiments, which was detrimental to colony growth. Although massive titrations were undertaken, only a narrow window occurred at which the inhibitory effect of P30-35 CAMAL could be discerned

above and beyond the inhibitory effect of SDS. In addition, P30-35 CAMAL was likely partially denatured by the SDS, and the fact that any inhibition at all above control was discerned is by itself impressive. Moreover, the protein concentrations used in these experiments fell in the range in which a dramatic inhibition of CFU-G was observed (Figure 6, see below). Hence, an inhibition of 30% (Figure 4a) mediated by P30-35 CAMAL at levels of 100 ng/ml can be attributed to the fact that around 30% of total control colonies are likely CFU-G. This was evident by visual inspection of control and P30-35 CAMAL-treated cultures, and confirmed in experiments in which colonies were plucked and examined for morphology (Figure 6, see below). Moreover, these experiments were performed using cryopreserved progenitor cells, for technical reasons. CFU-G, however, are known to be more sensitive to the effects of cryopreservation than are other colony types, hence the inhibition seen in these experiments was likely not optimal. Experiments in which the effects of the same levels of P30-35 CAMAL was evaluated on fresh progenitor cells support these arguments; inhibition in many cases was greater than resulted in the experiments described so far, depending on the specimen (see below). The differential inhibition of CFU-G at these levels of P30-35 CAMAL is supported by the results of experiments using murine colonies cultured with recombinant G-CSF; these colonies were profoundly inhibited (Chapter 3). All these data indicate that the effect on CFU-G at the levels of P30-35 CAMAL evaluated in these experiments is greater than is apparent from some of the assays described to this point. The validity of the results is further supported by the fact that the inhibitory effect on the formation of murine colonies in assays using conditioned medium have subsequent to these studies been observed in experiments performed independently by a separate investigator, in both single and double

blind experiments. In some of these double blind studies, colony counts were verified, again blindly, by two additional investigators (477).

Inhibitory activity mediated by CAMAL-1 eluted material was enriched by approximately 200 fold with separation of P30-35 CAMAL from CAMAL-1 eluates of CML or AML cell lysates. P30-35 CAMAL had a significant inhibitory effect on normal colony formation to levels of 4 ng/ml or 10⁻¹¹ M. This is in a range compatible with the concentrations at which many mediators of biological effects are active in plasma. For example, colony stimulating factors are known to exert half maximal effects on colony formation in the 1 to 10 picomolar range (486).

P30-35 CAMAL profoundly inhibited colony formation at levels greater than 1 μ g/ml (Table II). This is an indication that all progenitor cell types were affected at this protein concentration. CFU-G were demonstrated to be sensitive to inhibition by P30-35 CAMAL at concentrations of 100 ng/ml and 50 ng/ml (Figure 6). At these levels, CFU-G were dramatically inhibited whereas other colony types were not. The plateau in inhibitory activity observed at levels between 1 μ g/ml and 35 ng/ml is in all probability due to this differential sensitivity of CFU-G to inhibition by P30-35 CAMAL.

Inhibition of normal colony formation mediated by P30-35 CAMAL was a consistent effect; it was observed using P30-35 CAMAL enriched from six different sources by CAMAL-1 immunoaffinity chromatography followed by gel filtration or by preparative SDS-PAGE, and using six different sources of normal progenitor cells (Table II). Subsequent to these experiments, inhibition of normal colony formation was observed

using three additional sources of P30-35 CAMAL enriched by immunoaffinity chromatography; in these preparations, fractionation using a CAMAL-1 immunoadsorbent column was followed by further separation using an α -P30/35 immunoadsorbent column (Figures 8, 9, 10 and Table IV). In addition, inhibition of *in vitro* myelopoiesis by P30-35 CAMAL was observed using two culture systems; the colony assay and long term marrow culture of normal bone marrow cells (Tables V and VI, Figure 12).

In contrast to the consistent inhibition of colony formation by normal progenitor cells mediated by P30-35 CAMAL material, other protein components in CAMAL-1 eluted material were not inhibitory to colony formation by normal cells, nor did they potentiate the inhibition mediated by P30-35 CAMAL (Figure 9a). In addition, these protein components had no effect on colony formation by CML progenitor cells (Figure 9b).

In contrast to the differential effect of preparations of P30-35 CAMAL on colony formation by normal and CML progenitor cells, human neutrophil elastase was inhibitory to colony formation by both normal and CML progenitor cells (Figure 10).

Colony formation in cultures of fresh cells from normal donors was inhibited by 30 to 70% at P30-35 CAMAL levels of 100 ng/ml, depending on the progenitor cell specimen, as compared to the inhibition of 30 to 45% observed using cryopreserved progenitor cells. CFU-G are known to be more sensitive to cryopreservation than are other progenitor cell types (485); the higher levels of inhibition observed with colonies cultured from fresh cells in some experiments is likely due to this effect. Proportionally fewer neutrophil colonies would be expected in cultures grown from cryopreserved cells. Comparison by visual

inspection of colonies from cultures grown from fresh and cryopreserved progenitor cells supports this conclusion; proportionally more CFU-G were evident in control cultures of normal specimens showing a higher degree of inhibition on treatment with P30-35 CAMAL. The range of inhibitory activity seen using fresh progenitor cells is likely due to variations in the proportions of CFU-G between bone marrow specimens.

In contrast to the observed inhibition of normal colony formation, CML colony formation was not inhibited using the same preparations of P30-35 CAMAL at the same concentrations (Figures 3 and 4). Since CML is a myeloproliferative disorder characterized by a sometimes dramatic overabundance of neutrophilic granulocytes and their progenitors, the observation that P30-35 CAMAL inhibits normal granulopoiesis but has no inhibitory effect on *in vitro* myelopoiesis by CML progenitor cells suggests that inhibition of normal cells might be a mechanism by which leukemic progenitor cells gain a growth advantage over their normal counterparts. The dramatic inhibition of all colony types at higher concentrations of P30-35 CAMAL could result in a high proportion of leukemic cells in comparison to normals.

The observation that eosinophil colonies were increased in P30-35 CAMAL-treated cultures is interesting and possibly significant. Eosinophils are known to be involved in allergic responses and responses to parasitic infections, where they kill by antibody dependent cellular cytotoxicity. Eosinophils are also thought to be active in graft rejection and in the rejection of tumour cells. In lung cancer, carcinoma of the colon, and gastric carcinoma, an infiltration of eosinophils is known to be associated with a good prognosis. In addition, a good prognosis in Hodgkin's lymphoma was found to be indicated by blood

eosinophilia (487). The increase in eosinophil colonies, then, seen on the addition of a protein purified from leukemia cells which is seemingly important in the outgrowth of the leukemic clone, might be related to an anti-tumour response.

Long-term marrow cultures are considered to be a more physiological condition than are colony assays for the assessment of *in vitro* myelopoiesis. An adherent layer of stromal cells forms in long-term cultures which is thought to regulate proliferation and differentiation of the hematopoietic cells (Chapter 1). Primitive stem and progenitor cells are held in tight association with the stroma. As the cells differentiate, they are released into the supernatant. Equivalent numbers of cells were plated for colony formation from control and P30-35 CAMAL-treated cultures, and equivalent numbers of colonies resulted. The observation that the number of colony forming cells released into the supernatant in P30-35 CAMAL-treated cultures is reduced, and that a disproportionate number of cells in P30-35 CAMAL treated cultures appear to be held in the adherent stromal layer, suggests that these cells might be blocked in differentiation. In addition, these observations provide evidence that the inhibition of normal myelopoiesis by P30-35 CAMAL is maintained under more physiological conditions than those of the colony assay.

The observation that P30-35 CAMAL is inhibitory to normal myelopoiesis in colony assays and in long-term marrow culture is important, since this downregulation of normal myelopoiesis could provide a growth advantage for the leukemic clone. Immunoperoxidase studies suggested that P30-35 CAMAL might be overproduced in leucocytes derived from myeloid leukemias as compared to normal cells (465, 470); the apparent ability of P30-35 CAMAL to feed back on the formation of normal neutrophilic

granulocytes could provide an environment in which leukemic neutrophils and their progenitors gain a growth advantage and become predominant. The profound inhibition of all colony types observed using higher levels of P30-35 CAMAL could be involved in the progression of CML to its more aggressive stages.

Questions arise as to the mechanism of inhibition at the level of the single colonyforming cell. The recruitment of colony-forming cells into S phase could be reduced or delayed, or the ability of daughter cells to undergo subsequent divisions could be decreased (279). In order to address the question of whether the recruitment of cells into S phase is reduced, colonies numbers are evaluated early, i.e. at day 4 of culture. It was not possible to evaluate colony formation at day 4 during the course of these studies, since under conditions in this laboratory, even brief removal of the cultures from the incubator prior to day 10 of culture was found to jeopardize colony formation, in both control and P30-35 CAMAL-treated cultures. The recruitment of cells into S phase did not appear to be delayed, since colony counts were steady from day 10 of culture up to day 30 (data not included). Although not evaluated quantitatively, the reduction in size of some colonies within P30-35 CAMAL-treated cultures, which was seen consistently in these experiments (Figure 5), suggests that the ability of daughter cells to undergo subsequent divisions might be decreased. The reduction in colony size is not likely to be due to a reduction in 'feeder effects' (the effect of cytokines released by the cells of adjacent colonies), since similar levels of inhibition were observed in experiments in which the numbers of colonies per plate varied greatly (from a low of 30 to a high of 200, Table IV). Generally, the effect on the ability of daughter cells to undergo subsequent divisions is indicated by the presence of an equal number of clones of reduced size. Although not evaluated directly, the reduction

in the number of colonies in P30-35 CAMAL-treated cultures suggests that the entry of colony-forming cells into S phase might have been decreased.

Preparative SDS gel-purified P30-35 CAMAL material was analyzed for N-terminal amino acid sequence. The sequence was determined at Genentech, and is as follows: Nile-val-gly-gly-arg-lys-ala-arg-pro-arg-gln-phe-pro-phe-leu-ala-ser-ile-gln-asn-gln-gly. The protein sequence data bank showed that the closest sequence identity of this peptide was with elastase of human neutrophils, a serine protease, and was 59% over 22 amino acids. Other related proteins were human complement factor D, 45%, human cathepsin G, 42%, a 28 kDa protein from adipocyte precursors, 41%, murine granzyme A, 27%, murine granzyme C, 27%, and a 28 kDa serine protease from human T cells, 32% (474). All of these proteins are serine proteases, which suggested that the material in preparations of P30-35 CAMAL might also be a serine protease, and lead to the set of experiments described in Chapter 5. The close identity with human neutrophil elastase led to colony assays using elastase which determined that the activity of preparations of P30-35 on colony formation in was in fact distinct from that of elastase (Figure 10). This apparently close amino acid identity also lead to the screening of all subsequent preparations of P30-35 CAMAL for lack of elastase activity, i.e. lack of inhibition on colony formation by CML progenitor cells. Further information on this amino acid sequence, which became available after the experiments described in this paper to the end of Appendix 1 were completed, showed that the sequence obtained from preparations of P30-35 CAMAL was in fact identical to that of azurocidin. Azurocidin is a protein with serine protease homology, but no serine protease activity, which was isolated from the azurophilic granules of normal

neutrophils (438). At the time that the N-terminal sequence was obtained from preparations of P30-35 CAMAL, the azurocidin sequence was not entered into the protein sequence data bank. Experiments described in Chapter 5 and Appendix 2 demonstrate that the activity of P30-35 CAMAL is in fact distinct from any that could be ascribed to azurocidin. Experiments described in Chapter 5 demonstrate that the activity of P30-35 CAMAL on colony formation requires serine protease activity, both in the case of inhibition of colony formation by progenitor cells from normal healthy donors, and in the case of enhancement of colony formation by progenitor cells from donors with CML. Azurocidin, in contrast, has amino acid substitutions in two of the three residues of the serine protease catalytic triad, and hence lacks protease activity (437). Reverse-phase high performance liquid chromatography (HPLC) separations of P30-35 CAMAL preparations performed since this study was completed, demonstrated that several components are present in preparations of P30-35 CAMAL, which correspond to characterized neutrophil proteins and serine proteases, but that a component unique from these characterized proteins is present in addition (474, 479). Experiments described in Appendix 2, in which the possible effects of azurocidin and characterized neutrophil proteases were tested on colony formation by normal progenitor cells, showed that the inhibitory activity on normal colony formation is not mediated by any of these characterized neutrophil proteins. More recent data indicates that protein in the fraction unique to P30-35 CAMAL preparations is inhibitory to colony formation by murine progenitor cells (477). In addition, enzyme assays using chromogenic peptide substrates and peptide blockers of protease activity showed that there is a serine protease activity in preparations of P30-35 CAMAL which appears to be distinct from the activities of characterized neutrophil serine proteases (Chapter 5, Appendix 2). Hence, there is a component present in P30-35 CAMAL preparations which is unique to

these preparations and distinct from characterized neutrophil proteins, and there is a serine protease activity in P30-35 CAMAL preparations which appears to be distinct from the activities of the characterized neutrophil proteases. These results are discussed in greater detail in sections 5.4, 6.1, and in Appendix 2.

In summary, experiments described in this chapter demonstrate that the inhibitory activity on colony formation by normal progenitor cells described using crude CAMAL-1 eluted material (471) co-enriched with P30-35 material enriched from such preparations. This inhibitory activity on normal colony formation was a consistent effect, and colony size was affected as well as colony numbers. Furthermore, the inhibitory effect was also observed in long term cultures of normal bone marrow cells. Inhibition was preferentially directed toward CFU-G at low concentrations of P30-35 CAMAL. In contrast, colony formation by CML progenitor cells was not inhibited by P30-35 CAMAL, suggesting that inhibition of normal myelopoiesis by leukemia-derived P30-35 CAMAL might be a mechanism by which the progressive outgrowth of the leukemic clone seen in CML could occur.

CHAPTER 3

THE INHIBITORY EFFECT OF P30-35 CAMAL ON NORMAL MYELOPOIESIS CROSSES SPECIES BARRIERS; MURINE ASSAYS WITH P30-35 CAMAL

3.1: INTRODUCTION

In this chapter, the effects of P30-35 CAMAL on murine myelopoiesis are described. These were tested *in vitro* and in an *ex vivo* spleen colony assay. Like myelopoiesis by human cells *in vitro*, myelopoiesis by murine progenitors was inhibited by P30-35 CAMAL, and this effect was directed toward neutrophilic granulocyte progenitors. In addition, spleen colonies were reduced in number by treatment with P30-35 CAMAL in an *ex vivo* assay. The inhibitory effect of P30-35 CAMAL on myelopoiesis, then, crosses species barriers, and is maintained under the conditions of the *ex vivo* spleen colony assay. The inhibitory effect of P30-35 CAMAL on myelopoiesis therefore appears to be conserved, and the physiological ligands or substrates recognized by P30-35 CAMAL are likely to be similar on murine and human cells.

Traditional therapies for CML, such as combination chemotherapy, provide little more than symptomatic relief (179). Investigative therapies for CML show promise, but are associated with significant toxicity and morbidity, hence there is a need for novel approaches toward managing this disorder. The observation that normal human myelopoiesis is suppressed by P30-35 CAMAL *in vitro* is important since this suppression

could impart a selective growth advantage to the leukemic clone. The observation that murine myelopoiesis is inhibited by P30-35 CAMAL to an extent equivalent to that seen in human hematopoietic cells provides a convenient model for studying the mechanisms by which the regulatory effect mediated by P30-35 CAMAL is exerted, and for evaluating agents which might block this inhibitory activity on normal myelopoiesis.

3.2: MATERIALS AND METHODS

3.2.1: *Cells;*

Bone marrow was harvested from the femurs of male DBA/2J mice over 12 weeks of age into Iscove's medium, washed once and resuspended in fresh medium.

3.2.2: Colony assays;

Murine *in vitro* colony assays were performed according to the method of Metcalf (488), with modifications. Bone marrow cells from DBA/2J mice (5 X 10⁴ per 1.1 ml culture) were added to cultures containing Iscove's medium, 20% fetal calf serum, 10% bovine serum albumin (Boehringer-Mannheim, tissue culture grade), 2 to 5% pokeweed mitogen-stimulated spleen cell conditioned medium (PWM-SCCM, Terry Fox Laboratories

Media Services), and 0.3% Bacto-Agar (Difco). These conditions supported the growth of a mixture of colony types, the most primitive of which were CFU-GEMM. Cultures were plated in duplicate or quadruplicate in 35 mm culture dishes and incubated at 37°C, 5% CO_2 for 10 days, at which time colonies comprised of ≥ 20 cells were scored and photographed using a Zeiss Axiovert inverted microscope. In preliminary experiments, cells were co-cultured with P30-35 CAMAL. In later experiments, cells were preincubated with P30-35 CAMAL for 60 minutes at 37°C in Iscove's medium under serum-free conditions. Cells were then centrifuged at 1500 rpm for 5 min using a countertop centrifuge to remove the P30-35 CAMAL material, washed once in fresh media, resuspended, and cultured for colony formation.

CFU-G assays were conducted as described above, but colony formation was stimulated using recombinant G-CSF (Amgen, 1 ng/ml final concentration) rather than PWM-SCCM. 5 X 10⁴ to 1 X 10⁵ cells were plated per 1.1 ml culture.

Colony assays with human cells were performed in methylcellulose cultures as described in Chapter 2.

These experiments were performed using a preparation which had been enriched for P30-35 CAMAL using CAMAL-1 followed by α -P30/35 immunoadsorbent columns as described in Chapter 2. The preparation was shown to be inhibitory to colony formation by normal human progenitor cells, and not to be inhibitory to colony formation by progenitor cells from patients with CML.

3.2.3: Spleen colony assay:

Female DBA/2J mice 12 to 14 weeks old were lethally irradiated with 9.5 Grays using a ⁶⁰Co gamma source. They were reconstituted within 3 hours with marrow cells of syngeneic mice. The marrow cells had been incubated for 45 to 60 minutes with P30-35 CAMAL, equivalent amounts of control protein, or alone, then 1 X 10⁵ cells were injected intravenously into a tail vein in a volume of 0.2 ml. Four to six mice were used per group. The control protein used was protein that fell through the α -P30/35 column. This consisted mainly of material that migrated at about 66 kDa by SDS-PAGE (see Figure 7, Chapter 2).

Mice were maintained after treatment four to six per cage in the experimental groups. Each cage was isolated with a nylon filter for sterility. Mice were given unlimited access to food (breeding mix, which is softer and easier for the mice to eat after irradiation, which is damaging to the oral mucosa) and acid water.

Mice were euthanized on day 10. Spleens were removed, immersed in Bouin's fixative for 10 minutes, and surface colonies counted using a dissecting microscope. Day 10 colonies were evaluated in order to see both primitive (day 12) and more restricted (day 8) colonies (10).

3.3: **RESULTS**

3.3.1: Inhibition of in vitro colony formation by P30-35 CAMAL

Initial murine cultures were carried out using PWM-SCCM in order to support a mixture of colony types. Figure 13 shows results from an experiment in which cells were cocultured with P30-35 CAMAL. Colony formation was inhibited consistently in these experiments by 35 to 40% at a P30-35 CAMAL concentration of 100 ng/ml. This was a comparable effect to that seen with cultures using normal human progenitor cells, as can be seen in Figure 13, in which the effect on human and murine colony formation using identical material is directly compared.

In subsequent experiments, cells were incubated with P30-35 CAMAL, washed, and then cultured for colony formation. Results from such an experiment are shown in Figure 14; the inhibitory effect on colony formation was essentially the same as that seen in experiments in which cells were cocultured with P30-35 CAMAL. In addition to a reduction in the number of colonies, there was frequently a qualitative difference in the size of some colonies; colonies which formed in P30-35 CAMAL-treated cultures were usually significantly smaller than those in control cultures (Figure 15). This effect of P30-35 CAMAL on colony size was also frequently seen in cultures using human cells, and when P30-35 CAMAL was added directly to colony assays.

In order to determine whether, as in human cultures, murine CFU-G were preferentially targetted by P30-35 CAMAL, colony assays were performed using



FIGURE 13. Comparison of the inhibitory activity of P30-35 CAMAL on colony formation by normal human and murine progenitor cells cultured *in vitro* under conditions supporting the growth of a mixture of colony types; using SCCM to support the growth of murine colonies and LCM to support the growth of human colonies. Cells were cocultured with P30-35 CAMAL in these experiments. Comparison of the inhibitory activity of P30-35 CAMAL on colony formation by murine and by human progenitor cells. Mean number of control colonies was 68.25 ± 3.25 for the murine cultures and 60.5 ± 1.5 for the human cultures.



FIGURE 14. Comparison of the inhibitory activity of P30-35 CAMAL on colony formation by murine progenitor cells under conditions of coculture vs. preincubation with P30-35 CAMAL. Cells were cultured for colony formation *in vitro* under conditions supporting the growth of a mixture of colony types. Mean number of control colonies was 68.25 ± 3.25 and 136.25 ± 7.4 for coculture and preincubated cultures respectively.


FIGURE 15. Inhibitory activity of P30-35 CAMAL on the size of colonies cultured from murine progenitor cells. Colonies from the experiment described in Figure 13, using conditions of preincubation. Colonies from control cultures are shown in a and b. Some of the remaining colonies in P30-35 CAMAL-treated cultures (100 ng/ml), c and d, were usually reduced in size in comparison. All photographs are 115 X magnification.



recombinant G-CSF rather than PWM-SCCM. Figure 16 directly compares inhibition of colony formation in the two culture systems. Colony formation was profoundly inhibited in the CFU-G cultures; by 80% at a P30-35 CAMAL concentration of 100 ng/ml. This result directly supported the observation made with P30-35 CAMAL using human progenitor cells that neutrophilic granulocytic progenitor cells are preferentially targetted for inhibition by P30-35 CAMAL.

3.3.2: Inhibition of spleen colony formation by P30-35 CAMAL

Two experiments in which the effect of P30-35 CAMAL on spleen colony formation was evaluated are summarized in Figure 17. Significantly fewer spleen colonies formed in mice receiving cells treated with P30-35 CAMAL at 100 ng/ml (Figure 17a). Spleen colony formation in those mice receiving cells treated with 100 ng/ml of 'P66' control protein from a preparation of P30-35 CAMAL, however, did not differ significantly from controls which received cells alone. A titration of the inhibitory effect on spleen colony formation is shown in Figure 17b. Inhibition remained highly significant (p <0.0005 by Student's *t*-test analysis) to P30-35 CAMAL levels of 4 ng/ml.



FIGURE 16. Inhibitory activity of P30-35 CAMAL on murine CFU-G; direct comparison of the inhibitory effect on cultures stimulated with G-CSF to that on cultures stimulated with SCCM. Mean number of control colonies was 49.25 ± 3.1 for the G-CSF cultures and 136.25 ± 7.4 for the SCCM cultures.



FIGURE 17. Inhibitory activity of P30-35 CAMAL on spleen colony formation. a. Results of an experiment in which lethally irradiated mice were reconstituted with bone marrow cells treated with 100 ng/ml P30-35 CAMAL (32.5% inhibition, p < 0.0005 by Student's *t*-test analysis) or 100 ng/ml control protein (p > 0.4). b. Titration of the inhibitory effect of P30-35 CAMAL on spleen colony formation. p < 0.0005 in groups receiving cells treated with 33, 11, and 37 ng/ml P30-35 CAMAL, 0.05 in the group receiving cells treated with 1.2 ng/ml. Six mice per group were used in these experiments.

3.4: DISCUSSION

Myelopoiesis by murine cells was inhibited by P30-35 CAMAL in vitro and in an ex vivo spleen colony assay. The inhibition by P30-35 CAMAL of murine colony formation *in vitro* was similar to the inhibitory effect of P30-35 CAMAL on colony formation by normal human progenitor cells; the extent of inhibition of murine and human colonies of mixed types, supported by conditioned medium, at similar levels of P30-35 CAMAL is virtually superimposable (Figure 13). The observation that inhibition of colony formation by murine bone marrow cells occurred to a comparable extent whether cells were cocultured or preincubated with P30-35 CAMAL (Figure 14) suggested that this inhibitory effect is immediate. Like human colonies, in addition to a reduction in the number of colonies observed, some colonies in P30-35 CAMAL-treated cultures were reduced in size compared to those from control cultures (Figure 15). In addition, as in the human system, the inhibitory effect was preferentially directed toward murine neutrophilic granulocyte progenitors at levels tested in these studies (between 100 ng/ml and 1.2 ng/ml, Figure 6, Chapter 2, and Figure 16). These were levels at which a saturation of inhibitory activity on human colony formation was observed in cultures of mixed colony types; at levels of P30-35 CAMAL between 50 ng/ml and 1 μ g/ml, inhibition of between 30 to 40% of colonies was seen; most of the inhibited colonies were CFU-G (Table 2 and Figure 6, Chapter 2).

The observation that the inhibitory effect of P30-35 CAMAL was directed toward CFU-G in both human and murine assays is important for the following reasons. It suggests that the physiological ligand(s) and/or substrate(s) for P30-35 CAMAL on human and murine neutrophil progenitors are likely to be similar, and that down regulation of granulopoiesis by P30-35 CAMAL might be a highly conserved and important regulatory mechanism in myelopoiesis. In addition, murine colony assays thus appear to be a convenient and reproducible system for further studies with P30-35 CAMAL.

While the observed reduction in spleen colony numbers (Figure 17) using the assay conditions described here does not exclude the possibility that homing of progenitor cells to the spleen might have been affected by treatment with P30-35 CAMAL, the observations that *in vitro* colony formation using both human and murine cells was inhibited by P30-35 CAMAL in addition to this inhibition of spleen colony formation support the idea that the hematopoietic capacity of the CFU-S may have been reduced by P30-35 CAMAL. The observed inhibition of spleen colony formation thus provides evidence that the effects of P30-35 CAMAL are maintained under the physiological conditions of this *ex vivo* assay. The inhibitory effect of P30-35 CAMAL on spleen colony formation appears to be specific, since control protein from preparations of P30-35 CAMAL did not affect spleen colony formation at comparable levels. The effect of P30-35 CAMAL on murine hematopoietic progenitor cells appears to be immediate, since inhibition of colony formation in cultures preincubated or cocultured with P30-35 CAMAL was found to be comparable.

As in cultures of human cells, both the size and the number of colonies in P30-35 CAMAL-treated cultures was reduced as compared to control cultures. Colonies were not

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evaluated prior to day 7 of culture since removal of cultures from the incubator for even brief periods was found to jeopardize colony formation under conditions used in this laboratory. However, colony numbers were found to be steady from day 7 of culture to day 30 (data not included), excluding a mechanism of action by which the entry of colonyforming cells into S phase is delayed. Although neither of the following effects were documented quantitatively, the reduction in colony size suggests a reduction in the ability of daughters of affected colony-forming cells to undergo subsequent division, whereas the concomitant reduction in colony numbers suggests that the ability of cells to enter into S phase might have been reduced.

The observation that the inhibitory effect of P30-35 CAMAL on myelopoiesis crosses species barriers is significant since it suggests that the mechanism by which the effects of P30-35 CAMAL on progenitor cells are mediated might be of fundamental regulatory importance in myelopoiesis. In addition, this observation is important since these assays provide convenient experimental systems for further studies with P30-35 CAMAL. In particular, the spleen colony assay provides a model system for the evaluation of potential agents which block the activity of P30-35 CAMAL. Since the inhibition of myelopoiesis by P30-35 CAMAL provides an environment in which the leukemic clone might gain a growth advantage, agents which target this activity and block this growth advantage could prove to be useful in the treatment of CML. A physiological model system is essential for the evaluation of such agents; the spleen colony assay provides such a system.

In summary, the experiments described in this chapter provide evidence that the inhibitory effect of P30-35 CAMAL on normal myelopoiesis cross species barriers and may be an important regulatory mechanism in hematopoiesis. The observation that the inhibitory effect was equivalent when P30-35 CAMAL was removed from the progenitor cells before culturing them for colony formation suggested that the effect of P30-35 CAMAL was immediate, consistent with the activity of a protease. Finally, the murine assays developed herein provide a convenient model system for further studies with P30-35 CAMAL.

CHAPTER 4

CHARACTERIZATION OF THE STIMULATORY EFFECT OF P30-35 CAMAL ON COLONY FORMATION BY CML PROGENITOR CELLS AND MYELOID LEUKEMIA-DERIVED CELL LINES

4.1: INTRODUCTION

CML is a myeloproliferative hematopoietic stem cell disorder in which an elevation of neutrophilic granulocytes and their progenitors are seen in the bone marrow and circulating in the peripheral blood. How the leukemic clone gains dominance over normal hematopoietic cells is not well understood, but both inhibitors of normal hematopoiesis and factors which appear to promote leukemic myelopoiesis have been described (Chapter 1). The described inhibition of normal granulopoiesis mediated by P30-35 CAMAL provides one mechanism by which the leukemic clone could gain dominance over normal cells. In addition, a stimulatory effect on leukemic myelopoiesis has been defined. In this chapter, the enhancing effect of P30-35 CAMAL on colony formation by progenitor cells from patient donors with CML is described. Enhancement of colony formation by CML progenitor cells was originally observed in studies using CAMAL-1 eluted material, but was seen infrequently and at inconsistent protein concentrations. In order to further characterize this effect, the effect of material highly enriched for P30-35 CAMAL on mononuclear cells from CML clinical specimens was evaluated. Enhancement of colony formation by CML progenitor cells, occurred consistently using P30-35 CAMAL. Colony formation was enhanced both in number and in size, and in a biphasic fashion; colony formation was enhanced at low (1 to 10 ng/ml) and high (100 to 200 ng/ml) concentrations of P30-35 CAMAL but enhancement was reduced or absent at an intermediate concentration of P30-35 CAMAL (20 to 70 ng/ml), and was not observed at levels of P30-35 CAMAL above 200 ng/ml. The colony types in cultures of CML clinical specimens targetted for enhancement by P30-35 CAMAL were identified. At low concentrations of P30-35 CAMAL, primitive colonies were increased, whereas at high concentrations of P30-35 CAMAL, an increase in all colony types occurred. In addition, two cell lines derived from myeloid leukemias, EM2 and EM3, which respond to treatment with P30-35 CAMAL in a similar manner as do CML clinical specimens, were identified.

4.2: MATERIALS AND METHODS

4.2.1: Colony assays;

Colony assays were performed as described in Chapter 2, using cryopreserved or freshly separated mononuclear cells from CML clinical specimens. All assays described in this chapter were supplemented with erythropoietin. In initial experiments, cells were cocultured with P30-35 CAMAL. In later experiments, cells were incubated with P30-35 CAMAL at 37° C in 100 µl volumes in Iscove's medium under serum-free conditions for 60 minutes. Cells were then centrifuged at 1500 rpm (400 X g) to remove the P30-35

CAMAL material using an Eppendorf counter-top centrifuge (Brinkman), washed in fresh medium, resuspended, and plated for colony formation.

4.2.2: Cell lines;

The cell lines EM2 and EM3 were both derived from the bone marrow cells of the same CML patient, and were obtained from Dr. Armand Keating of the Toronto General Hospital Division of Hematology. EM2 was derived from cells taken immediately following treatment for CML by bone marrow transplantation, whereas EM3 was derived from cells taken at a later time, when the CML was in a more aggressive phase (489, 490). Both EM2 and EM3 are positive for the Philadelphia chromosome and the P210 *bcr-abl* transcript. HL60 was derived from an acute promyelocytic leukemia. HEL (human erythrocytic leukemia) was derived from an erythrocytic leukemia, and was obtained from Mr. Paul Cyopik of the Toronto General Hospital.

Cell lines were maintained in culture at 37° C, 5% CO₂, and split one in five to one in forty every three to four days. EM2 and EM3 were maintained in RPMI (Roswell Park Memorial Institute) medium supplemented with 20% FCS, and split one in five to one in ten. HL60 and HEL were maintained in DME (Dulbecco's Modified Eagle's) medium supplemented with 10% FCS and split one in ten to one in forty. For plating in a colony assay, cells were centrifuged at 400 X g, and washed twice with fresh Iscove's medium. Cells were resuspended in Iscove's medium and viability counts performed by dye exclusion using eosin Y. A minimum viability of 95% was considered acceptable for performing the clonogenicity assay. Viability was usually greater than 98%. Identical culture conditions were used for clonogenicity assays with cell lines as for colony assays with clinical specimens (Chapter 2).

Colonies were scored, photographed, plucked, stained, and evaluated as described in Chapter 2.

4.3: RESULTS

4.3.1: Definition of the enhancing effect of P30-35 CAMAL on colony formation by CML progenitor cells;

Colony formation by progenitor cells from patients with CML was enhanced by treatment with P30-35 CAMAL. Enhancement of CML colony formation was biphasic in nature (Figure 18); it was observed at a high concentration of P30-35 CAMAL (100 to 200 ng/ml) and at a low concentration of P30-35 CAMAL (2.5 to 10 ng/ml) but enhancement was reduced or absent at intermediate concentrations of P30-35 CAMAL (20 to 70 ng/ml). Figure 19 shows a comparative titration using P30-35 CAMAL from the same preparation over the same range of protein concentration on normal progenitor cells; in contrast to the enhancement of colony formation by CML progenitor cells seen over this concentration range, normal colony formation was inhibited by P30-35 CAMAL at these concentrations.



FIGURE 18. Stimulatory activity of P30-35 CAMAL on colony formation by progenitor cells from patients with CML. a. Colonies were cultured from cryopreserved CML peripheral blood mononuclear cells, which were cocultured with P30-35 CAMAL. b. Colonies were cultured from freshly drawn and separated CML peripheral blood mononuclear cells, which were preincubated with P30-35 CAMAL.



FIGURE 19. Inhibitory activity of P30-35 CAMAL on colony formation by progenitor cells from a normal healthy donor over the same concentration range of P30-35 CAMAL at which enhancement of CML colony formation was observed. Mean number of control colonies was 40.75 ± 3.1 .

The stimulatory effect of P30-35 CAMAL on colony formation by cells from patients with CML was consistent. Titrations were performed using P30-35 CAMAL from three different preparations and using either cryopreserved or freshly separated mononuclear cells from several CML clinical specimens, both peripheral blood and bone marrow. Enhancement of colony formation on treatment with P30-35 CAMAL was seen in all experiments without exception, and this enhancement occurred in a similar biphasic fashion. Enhancement of colony formation occurred at P30-35 CAMAL concentrations of 100 and 200 ng/ml and at concentrations of 1 to 10 ng/ml, but in all cases enhancement was either reduced or was not above control levels using P30-35 CAMAL concentrations of 30 or 70 ng/ml. These results are summarized in Table VII and Figure 18a. No enhancement of CML colony formation resulted at levels of P30-35 CAMAL of 0.4 ng/ml (Table VII) or less (data not included) or at levels above 200 ng/ml (up to 1 µg/ml, Table VII).

In contrast, control protein from preparations of P30-35 CAMAL did not enhance colony formation by CML progenitor cells. This result is illustrated in Figure 20.

In addition to an increase in colony number, a sometimes dramatic increase in the size of some colonies in P30-35 CAMAL-treated cultures in many experiments. Photomicrographs of CML colonies from control and P30-35 CAMAL treated cultures illustrate this effect (Figure 21). In these experiments, treatment with a low concentration of P30-35 CAMAL (7.4 ng/ml or 10 ng/ml) resulted in the appearance of an abundance of mixed colonies, some of which were very large. Treatment with a high concentration of P30-35 CAMAL (200 ng/ml) also resulted in an increase in colony size and an increase in

TABLE VII. Summary of P30-35 CAMAL simulatory activity in colony assays using progenitor cells from eight different patients with CML and three different preparations of P30-35 CAMAL.

M. mosenitor	P30-35 Camal	Percen	t enhance	ment per	P30-35 (CAMAL	concentr	ation (ng	(Im)	Number of control colonies	Number of mononuclear cells plated per milliliter
cell source	source	1000	300	100	30	10	4	1	0.4	culture ± SE	culture
fresh 1 PBL	"			100*	-23	133*	67	33		9.0 + 2.0	1 ~ 104
2 PBL		•	ı	*62	42*	100*	26*	19	11	112.5 ± 6.6	201 X 105
2 PBL	3	1	•	64*	31*	71*	32*	18*	•	122.8 ± 3.1	1×10^{5}
3 PBL	3	·	ı	66 *	20	± 04	*96	ı	ı	13.3 ± 2.5	2 X 10 ⁴
1 PBL	3	•	•	49*	10	63*	4	. I	ı	138.0 ± 5.0	1×10^{5}
1 PBL			·	*69	0	65*	23	9	ı	115.5 ± 9.7	7 X 10 ⁴
4 PBL	3	•	ı	*86	-15	111^{*}	10	ŝ	•	53.0 ± 5.1	7 X 10 ⁴
cryopreserved											
5 BM	1	L-	æ	71*	•	•	•	. •	•	82.5 ± 0.5	2.5 X 10 ⁴
6 PBL	2		7	31^{*}	7	82*	S.	-5	, I	31.5 ± 2.1	1.5 X 10 ⁴
7 PBL	5	١.	ı	40*	6	-2	32*	7	ı	13.3 ± 0.9	6 X 10 ⁴
8 BM	5	•	ì	27*	10	10	40*	10	•.	41.3 ± 2.2	4.4 X 10 ⁴
* All '*' were sign All fresh specimen	ifficant to $p < 0$ s were preincul	0.05 by Stud	ent's <i>t</i> -tes 30-35 CAI	st analysis MAL. All	. '-' mear	is not don erved spec	ie. cimens we	ere cocult	ured with	P30-35 CAMAL.	

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FIGURE 20. Lack of stimulatory activity by control protein from preparations of P30-35 CAMAL on colony formation by progenitor cells from a patient with CML.



Figure 21. Stimulatory activity of P30-35 CAMAL on the size of colonies cultured from progenitor cells from patients with CML in two experiments. Phase contrast photomicrographs of selected colonies. Mean number of control colonies was 130.5 ± 3.0 for 'a' and 13.3 ± 2.5 for 'b'. i. Colonies from control cultures. ii. Colonies from cultures treated with a low concentration of P30-35 CAMAL (7.4 ng/ml in 'a', enhancement in colony numbers was 47%, p < 0.0005 by Student's *t*-test analysis, 10 ng/ml in 'b', enhancement was 70%, p < 0.0005). iii. Colonies from cultures treated with a high concentration of P30-35 CAMAL (200 ng/ml in 'a', enhancement was 45%, p < 0.0005, 100 ng/ml in 'b', enhancement was 66%, p < 0.01).





the number of cells per colony. The effect on colony size at high concentrations of P30-35 CAMAL (200 ng/ml or 100 ng/ml), however, was less dramatic than the effect seen at low concentrations of P30-35 CAMAL.

4.3.2: Identification of colony types stimulated by P30-35 CAMAL;

In order to determine which progenitor cell populations were affected by P30-35 CAMAL treatment in these assays using CML cells, colonies were plucked from control and P30-35 CAMAL-treated cultures, stained, and examined for cell morphology. In cultures treated with low concentrations of P30-35 CAMAL, more primitive colony types (CFU-GEMM) were increased (Figure 22). At these concentrations of P30-35 CAMAL, a decrease in more differentiated colony types was seen. These include the single lineage colonies CFU-G in both cases, as well as CFU-M, and possibly CFU-eos (Figure 22b). At high concentrations of P30-35 CAMAL, all colony types were increased; this is evident since the proportion of no progenitor type was increased as compared to colonies plucked from control cultures, yet the overall number of colonies in P30-35 CAMAL-treated cultures were increased as compared to controls.



FIGURE 22. Identification of colony types in cultures of cells from patients with CML enhanced by P30-35 CAMAL in two experiments. a. 72 colonies were plucked from a control culture, 90 from a culture treated with a P30-35 CAMAL concentration of 4 ng/ml, and 84 from a culture treated with a P30-35 CAMAL concentration of 100 ng/ml. Mean number of control colonies was 130.0 ± 4.0 . Colony formation was enhanced by 45% at a P30-35 CAMAL concentration of 4 ng/ml (p < 0.0005 by Student's *t*-test analysis) and by 37% at a P30-35 CAMAL concentration of 100 ng/ml (p < 0.005). b. 54 colonies were plucked from each group. Mean number of control colonies was 31.0 ± 2.1 . Colony formation was enhanced by 82% at a P30-35 CAMAL concentration of 10 ng/ml (0.005), and by 31% at a P30-35 CAMAL concentration of 100 ng/ml (<math>0.025). In this figure, 'CAMAL' refers to P30-35 CAMAL.

4.3.3: Stimulatory effect of P30-35 CAMAL on colony formation by myeloid leukemiaderived cell lines;

Several cell lines derived from myeloid leukemias were treated with P30-35 CAMAL and their clonogenicity evaluated. Results from these experiments are summarized in Table VIII. Colony formation by EM2, derived from a CML patient following BMT, responded to treatment with P30-35 CAMAL in a manner similar to the CML clinical specimens; colony formation was enhanced at 100 and at 10 ng/ml, but at 30 ng/ml was not increased above control levels. Colony formation by EM3, derived from the same CML patient in blast crisis, was enhanced more dramatically, and over a broader range of P30-35 CAMAL concentrations, but also in a biphasic fashion. Colony formation by HL60, a promyelocytic leukemia which has both monocytic and neutrophilic characteristics, was enhanced over a broad and low range of P30-35 CAMAL concentrations, between 40 pg/ml and 30 ng/ml, with the peak effect seen at 1 ng/ml. Colony formation by HEL, or human erythrocytic leukemia, was not affected by P30-35 CAMAL. Individual titrations of P30-35 CAMAL on EM3 and HEL are illustrated in Figure 23.

TABLE VIII. Summary of P30-35 CAMAL activity in colony assays using cell lines derived from myeloid leukemias.

Number of cells plated per milliliter	culture	$1 \ge 10^3$	3 X 10 ³	5 X 10 ²	$1 \ge 10^3$	3 X 10 ⁴	$3 X_{-10}^{4}$	$2 X 10^2$	2×10^3	
Number of control colonies per milliliter	culture ± SE	23.0 ± 3.0	19.3 ± 4.8	10.5 ± 1.3	19.0 ± 0.7	115.0 ± 5.1	162.5 ± 8.5	85.5 ± 3.6	71.8 ± 3.1	
(ng/ml)	0.04		ı	ı	ı	ŀ	14*	ı	t	
AL concentration	0.1	,	ı	ŀ	!	ı	26*	ı	ı	.0
	0.4	ı	ı	62	13	•	44*	,	-7	not don
5CAM	1	ı	51*	109*	29*	ı	48*	0	'n	'-' means
er P30-3	4	17	53*	329*	39*	38*	ŀ	S.	ę	nalysis.
cement p	10	28*	105*	310*	58*	20*	ı	0	Ņ	t -test a
nt enhan	30	20	35	71*	13*	17*	!	1	7	Student's
Percer	100	48*	*69	423*	50*	-12	ı	'n	L-	r less by
P30-35 CAMAL	source	2	2	3	e,	7	7	7	e	t to p < 0.05 o
	treatment	cocultured	cocultured	preincubated	preincubated	cocultured	cocultured	cocultured	preincubated	*' were significan
cell	line	EM2	EM3	EM3	EM3	HL60	HL60	HEL	HEL	* All *

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FIGURE 23. Activity of P30-35 CAMAL on colony formation by two cell lines derived from myeloid leukemias. a. Enhancement of colony formation by EM3 on treatment with P30-35 CAMAL. b. Colony formation by HEL was not affected by P30-35 CAMAL.

4.4: DISCUSSION

Colony formation by specimens from CML patients was enhanced by treatment with P30-35 CAMAL. This effect consisted of an increase in colony number, as well as a frequent, and sometimes dramatic, increase in colony size (Figures 18 and 21, and Table VII). These effects of P30-35 CAMAL are diametrically opposed to those exerted by P30-35 CAMAL on normal human progenitor cells, which are reduced both in number and in size over the same concentration range (Figure 19). Hence it appears that P30-35 CAMAL upregulates myelopoiesis by CML cells at the same levels at which it negatively influences myelopoiesis by normal cells.

The stimulatory activity of P30-35 CAMAL on the formation of colonies by CML progenitor cells was consistent; enhancement of colony formation was similar in eleven of eleven experiments using progenitor cells from eight different CML clinical specimens and using three different preparations of P30-35 CAMAL (Figure 18 and Table VII). Enhancement of CML colony formation in P30-35 CAMAL -treated cultures was observed at a low concentration of P30-35 CAMAL (1 to 10 ng/ml) and at a relatively higher concentration (100 to 200 ng/ml), but enhancement was either reduced or absent at an intermediate concentration of P30-35 CAMAL (20 to 70 ng/ml. Figure 18 and Table VII). In contrast, control protein from preparations of P30-35 CAMAL had no enhancing activity on colony formation by CML progenitor cells (Figure 20). It was not possible to evaluate the effect of P30-35 CAMAL on bone marrow as compared to peripheral blood from the same patient, since these specimens were not available. However, Table VII shows that

the stimulatory effect of P30-35 CAMAL on CML colony formation is similar whether bone marrow or peripheral blood was used.

Initial cultures of CML cells were performed using cryopreserved mononuclear cells from CML clinical specimens. Leukemic cells, however, are known to be more sensitive to a variety of manipulations and agents, including cryopreservation, than are normal cells; this is the basis of chemotherapy. In addition, not all progenitor cells in CML are derived from the leukemic clone, at least in the initial chronic phase of this leukemia. Normal (Philadelphia chromosome negative) progenitor cells are present, but their capacity to contribute to overall hematopoiesis is reduced. Since it seemed possible that a disproportionate number of leukemic vs. normal progenitor cells might be lost by freezing and thawing the CML specimens (491), the effect of P30-35 CAMAL on freshly obtained CML specimens was evaluated. The enhancing effect of P30-35 CAMAL on colony formation observed using fresh CML specimens was indeed greater than that seen using cryopreserved cells (Table VII). It should be kept in mind that the degree of enhancement in colony numbers overall in CML cultures is the result of a balance between the enhancement, presumably of leukemic progenitor cells in the cultures, and the inhibition of normal colony formation. Hence, the effect on leukemic progenitors is in all probability larger than is indicated by this culture system. Similarly, since colony formation by normal cells is inhibited by P30-35 CAMAL, the colonies which are enhanced are in all probability derived from the leukemic clone.

The increase in colony size observed in P30-35 CAMAL-treated CML cultures is illustrated in Figure 21. This effect suggests that P30-35 CAMAL may increase the

capacity for self-renewal of the progenitor types affected (60). In contrast, the size of colonies derived from normal progenitor cells was often decreased by treatment with P30-35 CAMAL. This suggests that the opposite is the case for normal progenitors; a block in the capacity for self-renewal and/or differentiation. Since discordant maturation, or the partial uncoupling of self-renewal and differentiation, is considered to be an important feature of CML (174), the apparent ability of P30-35 CAMAL to shift the balance in these progenitor cells away from differentiation or toward self-renewal might be an important step in the development of CML.

The observation that two peaks of enhancement of colony formation by CML progenitor cells occurred suggested that two progenitor cell populations were the targets of the activity of P30-35 CAMAL. These populations were identified by examining colonies plucked from control and P30-35 CAMAL-treated cultures. At low concentrations of P30-35 CAMAL (7.4 or 10 ng/ml), colonies derived from more primitive progenitor cells were increased in number, whereas at high concentrations of P30-35 CAMAL (200 or 100 ng/ml), an increase was seen in the numbers of all colony types. This result was evident by visual inspection of colonies in P30-35 CAMAL-treated cultures using an inverted microscope; at low concentrations large and numerous CFU-GEMM were often obvious. These colonies clearly arose from primitive progenitors. This is evident by virtue of the fact that they are multilineage colonies, and hence arose from an early cell capable of giving rise to several cell types, but also by virtue of their size; they obviously arose from progenitors with a high capacity for self-renewal. At a high concentration of P30-35 CAMAL, however, although colonies were often increased in size, no increase in one colony type over the others was obvious (Figure 21). Since overall colony numbers were

increased in these cultures, this was an indication that all colony types were increased in number. These observations were confirmed in the described experiments in which colonies were plucked and examined for cellular morphology (Figure 22).

In cultures using normal progenitor cells, CFU-G were preferentially inhibited by P30-35 CAMAL at the same concentrations evaluated here using CML progenitor cells; at higher concentrations, a more profound inhibition of colony formation resulted (up to 100%). This is an indication that the clonogenicity of all progenitor cell types was suppressed in normal cultures at high concentrations of P30-35 CAMAL (Chapter 2). Since CML is a disorder in which levels of neutrophilic granulocytes and their progenitors are elevated, the inhibition of normal CFU-G, which was significant at concentrations above 30 ng/ml, could give the leukemic CFU-G a growth advantage. Similarly, the outgrowth of primitive leukemic progenitors at a low concentration of P30-35 CAMAL could result in an initial expansion of the primitive leukemic cell population and might be an important early event in the development of this leukemia. It was previously found by immunoperoxidase staining of cells from leukemic patients that the number of cells from patients in clinical remission reactive with the antibody CAMAL-1 often rose dramatically up to several weeks prior to clinical relapse (470). In addition, the cells found to express the CAMAL antigen in specimens from CML patients in stable phase did not appear to be restricted in lineage; many cell types were found to be CAMAL antigen-positive. Assuming that the antibody CAMAL-1 recognizes an epitope with which the P30-35 CAMAL material forms an association (Appendix 1), this result is consistent with the observation that all colony types cultured from CML clinical specimens are increased at high concentrations of P30-35 CAMAL, since material with which P30-35 CAMAL

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interacts may be present on the progenitors which give rise to these cells, and retained on the maturing cells. In addition, it is well known that the Philadelphia chromosome, a characteristic marker of CML, is present in all marrow elements, including cells of the lymphoid lineage. The finding that P30-35 CAMAL interacts with and increases the number of all CML-derived colony types assayed, then, is interesting, since, judging by the differential effects of P30-35 CAMAL on colony formation by normal vs. CML progenitor cell, the CML-derived colonies stimulated by P30-35 CAMAL are presumably derived from the leukemic clone.

It was not possible to evaluate whether the enhancement of colony formation in CML cultures treated with P30-35 CAMAL started early, since it was found that removal of cultures from the incubator for even brief periods prior to day 10 of culture jeopardized colony formation under conditions in this laboratory. However, colony counts were found to be steady from day 10 of culture to day 30, an indication that the rate of recruitment of CML colony-forming cells is not likely increased as compared to controls, since the control cultures never did catch up. Although the increase in colony size was not evaluated quantitatively (Figure 21), it was a consistent effect, and could be the result of an effect of P30-35 CAMAL on the ability of CML colony-forming cells to undergo daughter cell divisions. This effect on colony size is not likely due to feeder effects (effects of cytokines released by the cells of adjacent colonies, since enhancement of colony formation was seen in cultures with control counts ranging from 10 colonies per culture to 140 (Table VII). The concomitant increase in colony numbers could be a result of an increased recruitment of these colony-forming cells into S phase.

The effect of P30-35 CAMAL on cell lines derived from myeloid leukemias was evaluated for two reasons; in order to compare the effects of P30-35 CAMAL on cell populations derived from different myeloid leukemias, and in order to facilitate further investigations. Cells in cell line populations, although clonally derived, are not equal in their progenitor-like characteristics and clonogenicity. Many cell lines have characteristics of more than one cell type, for example HL60 cells can be forced to differentiate to neutrophils, monocytes, eosinophils, or basophils, depending on which differentiating agent and conditions are used. In addition, cells within the cell line population are not equal with respect to their ability to form colonies; the cloning efficiency of cell lines tested in this study ranged from 1 in 2 to 1 in 200 (Table 8). This is an indication that the cells within each cell line population responded unequally in culture to positive and negative regulatory signals. Resulting colonies in the same culture, also, differ somewhat in their size and morphology, indicating the presence of progenitor-like cells in the cell line population with differences in self-renewal and differentiative capacity. For these reasons cell lines were tested using colony assays, rather than other culture conditions, under identical conditions to those used for CML clinical specimens.

Colony formation by EM2, a Ph⁺ cell line derived from a patient with CML following bone marrow transplantation, was enhanced in a manner identical to CML clinical specimens; enhancement occurred at P30-35 CAMAL concentrations of 100 ng/ml and 10 ng/ml, but was not increased above control levels at an intermediate concentration of P30-35 CAMAL. Colony formation by EM3, a Ph⁺ cell line derived from the same patient in clinical relapse, was enhanced following treatment with P30-35 CAMAL in a manner similar to that seen with EM2; two peaks of enhancement occurred. Stimulation of the

formation of EM3 colonies by P30-35 CAMAL, however, was more dramatic in degree than that seen with EM2. This result suggests that the extent of response of clonogenic cells to treatment with P30-35 CAMAL may correlate with the phase or degree of aggressiveness of the leukemia.

Colony formation by HL60, derived from an acute promyelocytic leukemia, responded to P30-35 CAMAL over a broad range of concentrations (40 pg/ml to 30 ng/ml), with a peak of enhancement at a lower concentration (1 ng/ml) than that seen with EM2 and EM3. HEL, an erythrocytic leukemia, did not respond to P30-35 CAMAL over the range of concentrations tested. These results are summarized in Table VIII and Figure 23. Thus, not all cell lines derived from myeloid leukemias responded to P30-35 CAMAL in a manner similar to clinical specimens, but two were identified; EM2 and EM3.

It is interesting that the degree of sensitivity of those myeloid leukemia cell lines that responded by enhancement of colony formation to treatment with P30-35 CAMAL appeared to correlate with the aggressiveness of the leukemia. EM3 was derived from the same patient with CML as was EM2, but was derived when the patient was in clinical relapse. The degree of enhancement of EM3 colony formation was substantially more than that of EM2. Colony formation by HL60, derived from an acute leukemia, was enhanced by treatment with P30-35 CAMAL at significantly lower concentrations than were colony formation by EM2 and EM3 (1 ng/ml peak vs. 10 and 100 ng/ml) Thus, levels of P30-35 CAMAL and/or the sensitivity of the particular leukemia cell type involved might be an important factor in determining the degree of modulation in the self-renewal or

differentiative capacity of leukemic cells in response to P30-35 CAMAL, and the resulting degree of clonal expansion.

It is interesting and possibly significant that the same cell lines that responded to P30-35 CAMAL in these studies are those that stain positive by immunoperoxidase using α -P30/35 and thus might produce P30-35 CAMAL and/or express a ligand or substrate with which it interactis (Appendix 1). It is possible that P30-35 CAMAL might function as an autocrine regulatory mechanism gone awry in myeloid leukemias.

In summary, experiments described in this chapter demonstrate that colony formation by CML progenitor cells is enhanced by treatment with P30-35 CAMAL. Enhancement is both in number of colonies and in the size of many colonies in P30-35 CAMAL-treated cultures, is directed toward primitive colonies at a low concentration of P30-35 CAMAL, and toward all colony types at a high concentration of P30-35 CAMAL. These effects are diametrically opposed to those seen using cultures of normal progenitor cells, in which colony formation is inhibited by treatment with P30-35 CAMAL. Since colony formation by normal progenitor cells is inhibited by P30-35 CAMAL over the same concentration range at which an enhancement of colony formation by CML progenitor cells was observed, the colonies stimulated by P30-35 CAMAL are likely derived from the leukemic clone. In addition, colony formation by several cell lines derived from myeloid leukemias was enhanced by P30-35 CAMAL, and the degree of this enhancement appeared to correlate with the aggressiveness of the leukemia from which the cell lines were derived. Thus, two mechanisms by which the leukemic clone can gain dominance in CML appear to

be mediated by P30-35 CAMAL; downregulation of normal myelopoiesis, and concomitant upregulation of leukemic myelopoiesis.

CHAPTER 5

THE MODULATING EFFECTS OF P30-35 CAMAL ON MYELOPOIESIS REQUIRE SERINE PROTEASE ACTIVITY, WHICH CAN BE BLOCKED BY THE PEPTIDE ALA-PRO-PHE-CMK

5.1: INTRODUCTION

Traditional therapies for CML, including combination chemotherapy, provide little more than alleviation of symptoms (179). Investigational therapies, although promising, are associated with significant toxicity and morbidity (Chapter 1). Hence, novel approaches to managing CML are warranted. The involvement of a variety of proteases and serine proteases in development, in the development and functioning of hematopoietic and other cells, and in tumourigenesis have recently been described (Chapter 1). Moreover, serine protease activity has been specifically blocked in other systems as a way of treating clinical disorders. N-terminal protein sequence indicated the presence of a protein in preparations of P30-35 CAMAL with serine protease homology, most closely related to human neutrophil elastase (59% sequence identity). In this chapter, evidence that serine protease activity is required for the influences of P30-35 CAMAL on myelopoiesis by both normal and CML progenitor cells is described. Protease activity was investigated for two reasons; as mentioned, N-terminal amino acid sequence derived from a preparation of P30-35 CAMAL had homology to known serine proteases. This sequence was
subsequently shown to be that of azurocidin, a protein with serine protease homology but no protease activity, however, this result influenced the course of the studies described here (Chapter 2). In addition, preparations of P30-35 CAMAL in which inhibitors of protease activity had been included had been found to be inactive in colony assays. The involvement of protease activity in the alterations of myelopoiesis mediated by preparations of P30-35 CAMAL was demonstrated as follows. The inhibition of colony formation by normal progenitor cells and the enhancement of colony formation by progenitor cells from patients with CML mediated by preparations of P30-35 CAMAL were shown to be immediate and irreversible, and both activities were demonstrated to be retained in the supernatant of treated cells; both observations are compatible with the activity of a protease. The effects of P30-35 CAMAL on colony formation by both normal progenitor cells and by CML progenitor cells were blocked using PMSF, an inhibitor of serine protease activity. In separate experiments, both of these activities of P30-35 CAMAL on colony formation were also blocked using a peptide substrate recognized by P30-35 CAMAL, which was linked to a chloromethyl ketone group, a group which blocks serine protease blocking activity. Hence, serine protease activity is required for the alterations of normal and leukemic myelopoiesis mediated by P30-35 CAMAL.

5.2: MATERIALS AND METHODS

5.2.1: Experiments in which colony-forming cells were preincubated with P30-35 CAMAL;

Cells were incubated with P30-35 CAMAL in Iscove's medium under serum free conditions in a total volume of 100 μ l for 45 to 60 minutes at 37°C, 5% CO₂, washed with fresh medium, resuspended, and plated for colony formation. These experiments were performed using cultures of normal bone marrow (Chapter 2), cultures of murine bone marrow stimulated with PWM-SCCM, or with G-CSF, in addition to the spleen colony assays (Chapter 3), cultures of cells from CML clinical specimens (Chapter 4), and cultures using CML cell lines (Chapter 4).

5.2.2: Retention of activity on colony formation in the supernatant of P30-35 CAMAL treated cells;

Experiments were performed to determine whether activity was retained in or absorbed from the supernatant of P30-35 CAMAL-treated cells. Cells were incubated with P30-35 CAMAL for 60 minutes, then centrifuged at 400 X g for 5 min. The supernatant was carefully removed by pipetting without disturbing the cell pellet, and transferred to a second set of cells which had been incubated for 60 minutes in parallel with the first set, but in Iscove's medium alone, and from which the medium had been removed in order to avoid dilution of the transferred supernatant. The first set of cells was then washed, resuspended in fresh Iscove's medium, and plated for colony formation. The second set of cells was resuspended in the supernatant recovered from the first (P30-35 CAMAL-treated) set of cells, incubated for 60 minutes, washed, resuspended in fresh medium, and plated for colony formation. These experiments were performed using normal human bone marrow cells, murine bone marrow cells stimulated with recombinant G-CSF, cells from CML clinical specimens, and the cell line EM3.

5.2.3: Colony assays with PMSF-treated P30-35 CAMAL;

In order to determine whether serine protease activity was required for the effects of P30-35 CAMAL on colony formation, P30-35 CAMAL was incubated with PMSF at a 10:1 PMSF to P30-35 CAMAL molar ratio (0.3 μ M final concentration; PMSF was resuspended in absolute ethanol, and diluted in Iscove's medium). Incubations were in a 10 μ l volume in Iscove's medium at 37°C for 60 minutes. Following this incubation, 90 μ l of cells in Iscove's medium were added to bring the total volume to 100 μ l. This mixture was incubated for a further 60 minutes, at which time cells were washed and plated for colony formation. These experiments were performed using normal human bone marrow cells and cells from CML clinical specimens.

5.2.4: Enzyme assays using chromogenic peptide substrates and CMK-linked peptides;

P30-35 CAMAL has been shown to recognize the chromogenic peptide substrate ala-ala-pro-phe-NA (Bachem, NA=nitroanilide). This observation was originally made as part of the research project of another graduate student in this laboratory, and will be described in detail elsewhere (474). This substrate was not cleaved by the serine protease elastase of human neutrophils. Elastase was used for comparison in these experiments due to the close sequence identity between elastase and the N-terminal amino acid sequence obtained from preparations of P30-35 CAMAL. Both P30-35 CAMAL and elastase cleave the substrate, ala-ala-pro-val-NA. Versions of both these peptides linked to a chloromethyl ketone (CMK) group were obtained (Bachem). CMK groups block the activity of serine proteases by forming a covalent bond in their active site (492). Specificity of fit into the active site and therefore specificity of activity blockage is mediated by the peptide sequence to which the CMK group is attached.

Enzyme (elastase or a preparation of P30-35 CAMAL) was incubated with the appropriate substrate in 30 mM Tris buffer, pH 8.0, containing 500 μ g/ml bovine serum albumin (BSA, Sigma). Total reaction volumes of 125 μ l were used in 96 well ELISA plates, as follows. Enzyme was added in 100 μ l volumes (300 ng/ml P30-35 CAMAL, 100 ng/ml elastase). To this was added 25 μ l of the appropriate chromogenic substrate (ala-ala-pro-phe-NA for P30-35 CAMAL, ala-ala-pro-val-NA for elastase) diluted from a stock solution of 100 mM in DMSO to 4 mM in Tris-BSA buffer. Reactivity was quantified by A₄₀₅ using a Titertek ELISA reader once visible colour formation had occurred (4 to 12 hours).

P30-35 CAMAL was preincubated with ala-pro-phe-CMK at various peptide-CMK:P30-35 CAMAL molar ratios in Tris-BSA buffer for 60 minutes at 37°C, following which enzyme activity was determined using the chromogenic peptide substrate ala-ala-prophe-NA. As controls, P30-35 CAMAL was preincubated with ala-ala-pro-val-CMK following which its reactivity with ala-ala-pro-phe-NA was determined, and elastase was preincubated with ala-ala-pro-val-CMK or ala-ala-pro-phe-CMK following which its reactivity with ala-ala-pro-val-NA was determined.

5.2.5: Colony assays with CMK peptide-treated P30-35 CAMAL;

P30-35 CAMAL was incubated with ala-pro-phe-CMK at a 10:1 CMK to P30-35 CAMAL molar ratio (0.3 μ M final concentration of CMK-peptide; these were resuspended in DMSO, dilutions were made in Iscove's medium) in 10 μ l volumes in Iscove's medium under serum-free conditions for 60 minutes at 37°C. Following this incubation, 90 μ l of cells in Iscove's medium were added to bring the total volume to 100 μ l. This mixture was incubated for a further 60 minutes, at which time cells were washed and plated for colony formation. These experiments were performed using normal human marrow and CML clinical specimens. Experiments were performed incubating P30-35 CAMAL with three other CMK peptides under identical conditions using normal bone marrow. These peptides were ala-ala-pro-val-CMK, phe-pro-arg-CMK, and gly-gly-phe-CMK.

5.3: RESULTS

5.3.1: Alterations of colony formation under conditions of preincubation with P30-35 CAMAL were similar to effects in coculture experiments;

The inhibitory effect of P30-35 CAMAL on colony formation by murine progenitor cells was directly compared in experiments in which colony formation was stimulated using PWM-SCCM. Under these conditions, inhibition by P30-35 CAMAL on the formation of murine colonies of a mixture of types was virtually identical in extent whether progenitor cells were cocultured or preincubated with P30-35 CAMAL. This result indicated that the effect of P30-35 CAMAL on the progenitor cells was immediate, and was maintained after the P30-35 CAMAL material was removed. (Figure 14, Chapter 3). In addition, P30-35 CAMAL inhibited the formation of normal human colonies and murine CFU-G under conditions of preincubation at levels comparable to those described for cells which were cocultured with P30-35 CAMAL (Table IV, Chapter 2). This effect was observed in six of six experiments using bone marrow from normal healthy donors, and in four of four experiments using murine bone marrow cells.

The stimulatory effect of P30-35 CAMAL on colony formation by progenitor cells from CML clinical specimens (Table VII, and Figure 18, Chapter 4) and on colony formation by myeloid leukemia-derived cell lines (Table VIII, Chapter 4) also occurred consistently under conditions of preincubation, at levels comparable to those observed when cells which were cocultured with P30-35 CAMAL. This effect was observed in seven of seven experiments using CML clinical specimens from five different donors, and using two different preparations of P30-35 CAMAL under conditions of preincubation. These results are comparable to those observed in four experiments under conditions of coculture with P30-35 CAMAL, using progenitor cells from three additional donors, and P30-35 CAMAL from two preparations, one of which was a different preparation than those used in the preincubation experiments (i.e. a third preparation, Table VIII and Figure 18a, Chapter 4). Examples of experiments in which cells were preincubated with P30-35 CAMAL are illustrated in Figure 24.



FIGURE 24. Effects of P30-35 CAMAL on colony formation under conditions of preincubation with P30-35 CAMAL in selected experiments. a. Inhibition of colony formation in cells from a normal healthy donor. Mean number of control colonies was 189.75 ± 6.5 . b. Inhibition of murine CFU-G. Mean number of control colonies was 49.25 ± 3.1 . c. Enhancement of colony formation in cultures of cells from a patient with CML. d. Enhancement of colony formation in cultures of EM3 cells. Number of experiments showing similar results: six of six for 'a', four of four for 'b', seven of seven for 'c', three of three for 'd'.

5.3.2: Retention of activity in the supernatant of P30-35 CAMAL-treated cells;

P30-35 CAMAL activity on colony formation was retained in the supernatant of treated cells and was fully transferrable to previously untreated cells. This was true both of inhibitory activity on normal human colonies and murine CFU-G, and of the stimulatory activity on colonies from CML clinical specimens and the cell line EM3. Results from representative experiments are illustrated in Figure 25.

5.3.3: Colony assays with PMSF-treated P30-35 CAMAL;

Both the P30-35 CAMAL-mediated inhibition of colony formation by normal cells and the P30-35 CAMAL-mediated enhancement of CML colony formation were fully blocked by incubation of P30-35 CAMAL with PMSF, an inhibitor of serine proteases. Results from representative experiments using PMSF are illustrated in Figure 26.

5.3.4: Enzyme assays with CMK-peptide treated P30-35 CAMAL;

Figure 27a shows that *in vitro* incubation of P30-35 CAMAL with ala-pro-phe-CMK blocked cleavage of the chromogenic substrate recognized by preparations of P30-35 CAMAL, ala-ala-pro-phe-NA, almost completely at a CMK-peptide:P30-35 CAMAL molar ratio of 1:1; colour formation as determined by A_{405} was reduced by 86%. At higher



FIGURE 25. Adsorption studies with P30-35 CAMAL; retention of activity on colony formation in the supernatant of P30-35 CAMAL-treated cells. Effect on colony formation of treatment with P30-35 CAMAL, set 1, or with supernatants from P30-35 CAMAL-treated cells, set 2. Following a 60 minute incubation, supernatants of cells from set 1 were directly transferred to set 2 cells, which had been centrifuged, and from which medium had been removed. Set 1 cells were then plated for colony formation, whereas set 2 cells were incubated for a further 60 minutes, washed, and plated for colony formation. a. Inhibition of normal colony formation. Mean number of control colonies was 189.75 ± 6.5 and 212.25 ± 9.9 for sets 1 and 2 respectively. b. Inhibition of murine CFU-G. Mean number of control colonies was 100.25 ± 1.7 and 92.5 ± 4.4 for sets 1 and 2 respectively. c. Enhancement of CML colony formation. Mean number of control colonies was 260.5 ± 13.4 and 243.5 ± 13.4 29.8 for sets 1 and 2 respectively. d. Enhancement of EM3 colony formation. Mean number of control colonies was 260.5 ± 13.4 and 243.5 ± 29.8 for sets 1 and 2 respectively. Number of experiments showing similar results: two of two for each of 'a', 'b', and 'c', one of one for 'd'.



FIGURE 26. Blocking effect of PMSF on P30-35 CAMAL-mediated alterations of colony formation in selected experiments. a. Blocking effect of PMSF on P30-35 CAMAL-mediated inhibition of normal colony formation. b. Blocking effect of PMSF on P30-35 CAMAL-mediated enhancement of CML colony formation. PMSF was preincubated with P30-35 CAMAL at a constant PMSF:P30-35 CAMAL molar ratio. Numbers refer to P30-35 CAMAL concentration in ng/ml in P30-35 CAMAL treated cultures. Cells were incubated with PMSF only in the "PMSF" group. Number of control colonies was 53.0 ± 5.1 . In these experiments, 'CAM' or 'CAMAL' refer to P30-35 CAMAL. Number of experiments showing similar results: two of two for each of 'a' and 'b'.



FIGURE 27. Assays for enzyme activity with P30-35 CAMAL and elastase using CMKlinked peptides as putative blockers of enzyme activity. P30-35 CAMAL or elastase were incubated with a CMK-linked peptide, following which enzyme activity was determined on the appropriate substrate; P30-35 CAMAL activity on ala-ala-pro-phe-NA and elastase activity on ala-ala-pro-val-NA. a. Blocking effect of P30-35 CAMAL enzyme activity but not of elastase enzyme activity using ala-pro-phe-CMK. b. Blocking effect of elastase and P30-35 CAMAL enzyme activities at different levels using ala-ala-pro-val-CMK. CMK:P30-35 CAMAL molar ratios (10:1), colour formation was completely blocked. In contrast, ala-pro-phe-CMK did not block the activity of elastase on the elastase substrate ala-ala-pro-val-NA at up to an 100 fold molar excess. In Figure 27b, it can be seen that elastase activity was blocked by ala-ala-pro-val-CMK, by 50% at a 1:1 molar ratio, and completely at higher (10:1) molar ratios. P30-35 CAMAL activity was also blocked by ala-ala-pro-val-CMK, however, higher levels of this CMK peptide were required to block the activity of P30-35 CAMAL than were required to block the activity of elastase; P30-35 CAMAL activity was not decreased by ala-ala-pro-val-CMK at a 1:1 molar ratio, but was blocked at 10:1.

5.3.5: Colony assays with CMK-peptide treated P30-35 CAMAL;

Incubation of P30-35 CAMAL with ala-pro-phe-CMK fully blocked the P30-35 CAMAL-mediated inhibition of normal colony formation. In addition, the P30-35 CAMAL-mediated enhancement of CML colony formation was fully blocked by incubation of P30-35 CAMAL with this CMK peptide. Results from colony assays using ala-pro-phe-CMK are shown in Figure 28. In contrast, three other CMK-linked peptides did not block P30-35 CAMAL-mediated inhibition of normal colony formation. These results are illustrated in Figure 29.



FIGURE 28. Blocking of P30-35 CAMAL-mediated alterations of colony formation by ala-pro-phe-CMK. a. Blocking effect on P30-35 CAMAL-mediated inhibition of normal colony formation. b. Blocking effect on P30-35 CAMAL-mediated enhancement of CML colony formation. The peptide ala-pro-phe-CMK was incubated with P30-35 CAMAL at a constant peptide:P30-35 CAMAL molar ratio. Numbers refer to concentration of P30-35 CAMAL in ng/ml in P30-35 CAMAL-treated cultures. Cells were incubated with ala-pro-phe-CMK only in the "CMK" group. In these experiments 'CAMAL' or 'CAM' refer to P30-35 CAMAL. Number of control colonies was 122.75 ± 3.1 . Number of experiments showing similar results: two of two for each of 'a' and 'b'.



FIGURE 29. Lack of blocking effect of three CMK-linked peptides on P30-35 CAMALmediated inhibition of normal colony formation. a. Using ala-ala-pro-val-CMK ('AAPV'). b. Using phe-pro-arg-CMK ('FPR'). c. Using gly-gly-phe-CMK ('GGF'). In these experiments 'CAMAL' or 'CAM' refer to P30-35 CAMAL.

5.4: DISCUSSION

P30-35 CAMAL-mediated alterations of myelopoiesis by normal and CML progenitor cells were demonstrated to require serine protease activity. The presence of a serine protease in preparations of P30-35 CAMAL was originally suggested by N-terminal amino acid sequence. This sequence proved in retrospect to be azurocidin, a protein with serine protease homology, but no protease activity (Chapter 2), however, taken together with the following results, the N-terminal sequence obtained from P30-35 CAMAL preparations influenced the course of experiments described in this paper. The importance of protease activity in the P30-35 CAMAL-mediated alterations of *in vitro* myelopoiesis was suggested by the observation that P30-35 CAMAL prepared using protease inhibitors during cell lysis was inactive. Protease activity was further suggested by the observation that the effects of P30-35 CAMAL on progenitor cells were immediate and were maintained even after the P30-35 CAMAL material was removed.

Experiments in which progenitor cells were incubated with P30-35 CAMAL, following which the P30-35 CAMAL material was removed, then cells cultured for colony formation, demonstrated that the activity of P30-35 CAMAL on progenitor cells was immediate. Inhibition of normal colony formation and murine CFU-G occurred at levels comparable to those described for cells which were cocultured with P30-35 CAMAL, as did enhancement of colony formation by CML clinical specimens and the CML-derived cell line EM3 (Figure 24; also Table IV, chapter 2; Tables VII and VIII, chapter 5). In addition, inhibition of the formation of murine colonies of mixed type (stimulated with

spleen cell conditioned medium) in experiments where the progenitor cells were preincubated with P30-35 CAMAL was virtually identical in extent to the inhibition observed when cells were cocultured with P30-35 CAMAL (Figure 14, Chapter 3). These results are an indication that the activities of P30-35 CAMAL on the progenitor cells were immediate and were maintained even after the P30-35 CAMAL material was removed. The possibility that protease activity might be involved in the P30-35 CAMAL-mediated alterations of colony formation was further supported by the finding that P30-35 CAMAL activity was retained in the supernatant of treated cells, and not adsorbed by the cells. Experiments in which inhibitory activity on normal myelopoiesis and enhancing activity on leukemic myelopoiesis were demonstrated to be retained in the supernatant of treated cells and transferrable to a second set of cells (Figure 25) indicate that P30-35 CAMAL in all probability is active at the cell surface. These observations are compatible with the activity of a protease.

Serine protease activity was more directly demonstrated in experiments using phenyl methyl sulfonyl fluoride (PMSF), an agent which irreversibly blocks the activity of serine proteases by forming a covalent bond in the protease active site. Both the P30-35 CAMAL-mediated inhibition of normal colony formation and the enhancement of leukemic colony formation were fully blocked by PMSF (Figure 26). These observations confirm that the effects of P30-35 CAMAL on both normal and leukemic myelopoiesis require serine protease activity, either directly or indirectly.

Protease activity was evaluated in an enzyme assay using chromogenic peptide substrates. Cleavage of a chromogenic substrate recognized by preparations of P30-35

CAMAL but not by elastase in this system was blocked using the peptide ala-pro-phe-CMK. Elastase activity on an elastase substrate, however, was not blocked by this peptide (Figure 27a).

Both the inhibition of normal colony formation and the enhancement of leukemic colony formation mediated by P30-35 CAMAL were fully blocked by the peptide ala-prophe-CMK (Figure 28). The P30-35 CAMAL-mediated inhibition of normal colony formation, however, was not blocked by three other CMK peptides (Figure 29). Thus, the alterations of normal and leukemic *in vitro* myelopoiesis mediated by P30-35 CAMAL have been blocked. This finding may have implications in the management of CML. Barring toxicity, the peptide ala-pro-phe-CMK or other agents which block the activity of P30-35 CAMAL could potentially be useful in a clinical setting in the treatment of CML singly or in conjunction with other therapies.

Elastase was used for comparison in these experiments due to the similarity between elastase and the amino acid sequence obtained from preparations of P30-35 CAMAL in order to evaluate whether the activity of P30-35 CAMAL was distinct from that of elastase. Although elastase and P30-35 CAMAL were shown to have some similarities in sequence preference (both recognize the substrate ala-ala-pro-val-NA, reference 474), they do have activities which are distinct. Elastase does not recognize the substrate ala-ala-pro-phe-NA which is recognized by preparations of P30-35 CAMAL, and there were differences in the ability of two CMK-linked peptides to block the activity of elastase as compared to the activity of P30-35 CAMAL preparations (Figure 27). In order to ensure minimal contamination of P30-35 CAMAL preparations by elastase, preparations of P30-

35 CAMAL were screened for lack of elastase-mediated inhibitory activity on colony formation by CML progenitor cells (Chapter 2).

Subsequent to the completion of these studies, it was recognized that the N-terminal amino acid sequence obtained from a preparation of P30-35 CAMAL was that of azurocidin, and reverse phase high performance liquid chromatography (HPLC) separation of P30-35 CAMAL preparations was undertaken (474). These demonstrated the presence of several proteases in P30-35 preparations; the relative proportion of each protease varied between preparations. How the activity on colony formation correlates with the presence and relative proportions of these proteases is still in the early stages of evaluation, however, preliminary experiments indicate that the inhibitory activity on murine colony formation can be assigned to a major protein peak which is distinct from those of characterized neutrophil proteins, which constitute the other protein peaks in these preparations (474, 477). Although it cannot be conclusively excluded that some of the effects of P30-35 might be indirect and require the activity of a characterized protease, and that P30-35 CAMAL itself might not be a unique protease, from what is known about the activity of these characterized proteins on chromogenic peptide substrates, the pattern of blocking of protease activity by CMK-linked peptides, and the activity of the proteins on colony formation, it appears that there is a serine protease activity present in preparations of P30-35 CAMAL which is distinct from those of characterized proteases. This is discussed in more detail in Appendix 2.

In summary, experiments demonstrating that P30-35 CAMAL-mediated alterations of colony formation require serine protease activity, either directly or indirectly, are described. The effects of P30-35 CAMAL on colony formation by both normal and CML progenitor cells were shown to be immediate, and maintained after P30-35 CAMAL was removed from the cells. The activities on colony formation were retained in the supernatant of treated cells, and not adsorbed by the cells. Moreover, the activities on colony formation by normal and CML progenitor cells were fully blocked by the serine protease inhibitor PMSF, and by the peptide ala-pro-phe-CMK. These activities are distinct from those of the characterized protease elastase, and might be unique.

CHAPTER 6

DISCUSSION

6.1: Overview of results reported in this study;

This study was undertaken in order to define the effects of CAMAL-1 eluted material on myelopoiesis by normal and leukemic hematopoietic progenitor cells. Previous studies in this laboratory had demonstrated that material enriched from lysates of myeloid leukemia peripheral blood or bone marrow cells, using the antibody CAMAL-1, was inhibitory to colony formation by progenitor cells from normal healthy donors. In contrast, the same CAMAL-1 eluted material lacked inhibitory activity on colony formation by progenitor cells from patients with CML or AML, and in some cases colony formation by these cells was enhanced by the CAMAL-1 eluted material (471). These observations are important since the observed effects on normal and leukemic myelopoiesis provide a mechanism by which the leukemic cells could gain a growth advantage over normal cells and become predominant. The mechanism by which leukemic cells become predominant in the development of myeloid leukemias is not well understood. Inhibitors of normal myelopoiesis have been described, but in most cases these are not well defined, and in many cases these same substances are inhibitory to leukemic myelopoiesis in addition (Chapter 1). An increased understanding of the mechanisms by which normal regulatory mechanisms in myelopoiesis are subverted in leukemia is desirable since this might shed

light into normal hematopoietic regulatory function. In addition, increased understanding of how the myeloid leukemias develop is desirable from a clinical standpoint, since specific aberrent mechanisms could be targetted in the treatment of these leukemias, thus avoiding the significant toxicities and morbidity of established and investigative therapies, and possibly resulting in increased efficacy. These are important issues particularly in CML, which is refractory to cures due to the primitive nature of the stem cell affected (Chapter 1).

Since preparations used in previous studies, which were isolated using an immunoadsorbent column prepared with the monoclonal antibody CAMAL-1, were crude and contained several protein components, the first objective of this study was to identify the protein component inhibitory to colony formation by normal progenitor cells. Using myeloid leukemia leucocyte lysates eluted from CAMAL-1 immunoadsorbent columns, which were further fractionated using FPLC gel filtration or preparative non-reducing SDS-PAGE, it was demonstrated that the inhibitory activity on colony formation by progenitor cells from the peripheral blood or bone marrow of normal healthy donors was mediated by the material which migrated at 30 to 35 kDa by SDS-PAGE analysis (Chapter 2). The observed inhibition was both in the number of colonies and in the size of many colonies in P30-35 CAMAL-treated cultures. P30-35 CAMAL-mediated inhibition of normal colony formation was a consistent effect and was shown to be preferentially directed toward neutrophilic granulocyte progenitors (CFU-G) at low concentrations of P30-35 CAMAL (50 to 100 ng/ml), whereas profound inhibition of the formation of all colony types occurred at higher concentrations of P30-35 CAMAL (over 1 µg/ml). In addition, release of hematopoietic colony-forming cells from the primitive cell compartment in the adherent layer of long term cultures of normal bone marrow cells, an assay considered to support

more physiological conditions than the colony assay, was also inhibited by treatment with P30-35 CAMAL, suggesting that the affected cells might be blocked in differentiation. A differentiation block might also account for the reduction in colony number and size in P30-35 CAMAL treated cultures observed using colony assays, since a signal to reduce differentiation might also reduce proliferation. Hence the inhibitory effect of P30-35 CAMAL on normal myelopoiesis was observed in two culture systems using progenitor cells from normal healthy human donors; the colony assay, and the LTMC.

The formation of colonies cultured from murine bone marrow cells was inhibited by P30-35 CAMAL to a similar degree as was observed in cultures of normal human cells, using equivalent concentrations of P30-35 CAMAL. As in the assays using normal human progenitor cells, both the number of colonies and the size of many of the remaining colonies in P30-35 CAMAL-treated cultures were reduced, and this inhibitory effect was preferentially directed toward murine CFU-G. These results are interesting as they are an indication that the inhibitory effect of P30-35 CAMAL on normal myelopoiesis is conserved across species barriers, and suggest that the physiological ligand for and/or substrate recognized by P30-35 CAMAL on murine and human cells must be similar. In addition, the murine colony assay provides a convenient experimental model for future studies. Similarly, spleen colony formation in lethally irradiated mice reconstituted with syngeneic bone marrow cells was also inhibited by treatment of the reconstituting cells with P30-35 CAMAL. This result demonstrates that the inhibitory effect of P30-35 CAMAL on myelopoiesis is maintained under the more physiological conditions of the ex vivo spleen colony assay. It also provides a straightforward model system for the evaluation of potentially therapeutic agents which might block or reverse the activity of P30-35 CAMAL.

Since CML is a proliferative disorder in which the levels of neutrophilic granulocytes and their progenitors are elevated, the observed inhibition of normal CFU-G at low concentrations of P30-35 CAMAL (50 to 100 ng/ml) could give leukemic CFU-G a growth advantage. Similarly, the apparent shutdown of normal myelopoiesis by all progenitor cell types observed at higher concentrations of P30-35 CAMAL (greater than 1 μ g/ml) could permit a dramatic elevation in the leukemic cell population, which does occur in CML; in some cases, more than 99% of circulating blood cells are leukemic blasts (175). An interesting observation was the increase in eosinophil colonies in cultures of normal cells in cultures treated with low concentrations of P30-35 CAMAL (50 to 100 ng/ml). Eosinophils are documented to have anti-tumour cytotoxicity, and eosinophilia with several tumour types is associated with a good prognosis (487). Hence, the increase in normal eosinophil colonies observed in P30-35 CAMAL-treated cultures might be related to an anti-tumour response.

In contrast to the P30-35 CAMAL-mediated inhibition of colony formation observed using normal cells, P30-35 CAMAL material exerted a stimulatory effect on *in vitro* colony formation by progenitor cells from patients with CML. Like the inhibition observed using normal cells, in which both the number of the and the size of colonies were reduced in P30-35 CAMAL-treated cultures, the observed enhancement of colonies cultured from CML progenitor cells was both in the number of colonies and in the size of many colonies within the P30-35 CAMAL-treated cultures. The effects of P30-35 CAMAL on colony formation by normal and CML progenitor cells, then, while similar in quality, were opposite in direction. P30-35 CAMAL-mediated enhancement of colony formation

cultured from CML progenitor cells was consistent; it was observed with every CML specimen tested, and occurred at consistent concentrations of P30-35 CAMAL. The stimulatory activity on colony formation by CML progenitor cells was shown to be directed toward two progenitor cell populations in cultures of CML cells. Expansion of colonies arising from primitive progenitor cells (CFU-GEMM) occurred at low concentrations of P30-35 CAMAL (7.4 and 10 ng/ml), whereas expansion of all colony types, including primitive colonies, occurred at higher concentrations of P30-35 CAMAL (100 and 200 ng/ml). At an intermediate concentration of P30-35 CAMAL, the observed enhancement of colony formation was not as great, and was often not above control levels. Similarly, the enhancement of colony formation was not significant at concentrations of P30-35 CAMAL treatment resulted in an increase in CML colony numbers at concentrations at which an inhibition of colony formation by normal cells resulted, it is probable that the colonies stimulated in CML cultures are derived from the leukemic clone.

Precedents have been set in the literature for factors which have more than one, seemingly opposing, effect on hematopoietic cells, as well as on normal vs. transformed cells. A familiar example is tumour necrosis factor- α (TNF- α), which stimulates the growth of immature colony forming cells, but inhibits the growth of more mature progenitor cells (289). Moreover, in juvenile chronic myelogenous leukemia, a leukemia which is considered to be a separate clinical entity than Ph⁺ CML, both growth-inhibitory effects on normal cells and growth-promoting effects on leukemic cells were shown to be mediated by TNF- α (493). In addition, TNF- α *in vivo* induces cachexia at high levels, whereas at low levels it is thought to be involved in normal tissue development and turnover (113). In contrast to the effects of TNF- α *in vitro*, TGF- β inhibits the colonystimulating factor driven growth of immature hematopoietic progenitor cells, but stimulates the growth of more mature progenitor cells (69). In addition, TGF- β suppresses the transcription of proteases in normal fibroblasts. Transcription of proteases in transformed fibroblasts, however, is augmented by TGF- β (457, 494). In light of these observations, the seemingly opposing effects of P30-35 CAMAL on myelopoiesis by normal and CML progenitor cells are neither unprecedented nor unusual.

Stimulation of colony formation by primitive CML progenitor cells at low concentrations of P30-35 CAMAL could result in an expansion of the primitive leukemic cell population and might be an important early event in the development of CML. Indeed, it was previously found by immunoperoxidase staining using the monoclonal antibody CAMAL-1 that the number of cells expressing the CAMAL antigen in mononuclear cells from patients with myeloid leukemias in clinical remission often rose dramatically up to several weeks prior to clinical relapse. These results suggest that a clonal expansion of leukemic cells is temporally, and possibly causally, linked (470).

The increase in colony size observed in P30-35 CAMAL-treated cultures of CML cells suggests that the capacity for self-renewal of the progenitor cells affected is increased. Since discordant maturation, or the partial uncoupling of self-renewal and differentiation, is considered to be an important feature of CML (174), the apparent activity of P30-35 CAMAL in shifting the balance in these colony-forming cells toward self renewal, or away from differentiation, is a potentially important step in the development of CML. This apparent differentiation block is consistent with the observation that normal cells in P30-35

CAMAL-treated long-term marrow cultures appeared to be blocked in differentiation as well. A decrease in the number of cells in the non-adherent compartment of these cultures, and an apparent increase in the number of colony-forming cells in the adherent layer indicated that release of cells from the adherent stromal layer, in which more primitive cells are held, into the supernatant, which consists of more mature cells, appeared to be impaired. In the case of normal cells, a block in differentiation might not result in increased proliferation, since, unlike in CML, maturation is not discordant. The observation that the numbers and size of colonies in P30-35 CAMAL-treated cultures of normal cells was decreased suggests that a block in differentiation might be coupled to a block in selfrenewal in these cells. Alternatively, this effect might be a function of the assay system used. While the number of colony forming cells in the non-adherent fraction of long-term cultures of normal cells was decreased, the number of cells in the adherent layers appeared to be increased, suggesting that the self-renewal capacity of the cells affected might not be decreased, merely their ability to differentiate and be released from the adherent layer. Conditions in long-term culture might provide additional signals required for the survival of these cells which are absent in colony assays.

The effects on colony number and size in P30-35 CAMAL-treated cultures of normal and CML cells indicate that the recruitment of colony forming cells into S phase and the subsequent ability of daughter cells to undergo cell divisions might be affected. However, in order to draw conclusions about these possible mechanisms of action of P30-35 CAMAL on colony-forming cells, more studies quantitating the changes in colony size, and colony counts early in the culture period must be performed.

It was previously found that cells which were reactive with the monoclonal antibody CAMAL-1 in an immunoperoxidase slide test using specimens from patients with CML in chronic phase did not appear to be restricted in type; many cell types were found to be CAMAL antigen-positive, including cells of the lymphoid lineage (465). These immunoperoxidase studies suggest that the CAMAL antigen recognized by the antibody CAMAL-1 interacts with cells of all lineages. Assuming that the antigen recognized by CAMAL-1 and the P30-35 CAMAL shown to mediated alterations in colony formation interact in some way (Appendix 1), the observation that the CAMAL antigen interacts with cells of all lineages is consistent with data from the colony assays. In cultures of normal cells, the myelopoietic development of cells of all lineages assayed was decreased by treatment with P30-35 CAMAL at high concentrations. Conversely, in cultures of CML cells, the myelopoietic accumulation of cells of all lineages assayed is increased. These cells are presumably derived from the leukemic clone, since colony formation by normal cells is inhibited by treatment with P30-35 CAMAL at the same concentrations. These observations provide a mechanism by which normal leucocytes could become progressively displaced by leukemic leucocytes during the development of CML. If it is assumed that the bulk of P30-35 CAMAL is produced by the leukemic cells, and that the concentration of P30-35 CAMAL in the hematopoietic microenvironments *in vivo* increases with expansion of the leukemic clone, the following situation can be envisioned. At low concentrations of P30-35 CAMAL (1 to 10 ng/ml), a stimulation of primitive CML GEMM-CFC occurs. No effect on normal myelopoiesis occurs at this level. As the CML GEMM-CFC expand, their progeny produce P30-35 CAMAL, causing the concentration of P30-35 CAMAL to rise to the level (100 ng/ml) where normal G-CFC are inhibited, resulting in clinical infections. In addition, a more general stimulation of CML-CFC of all

lineages occurs at this level. As the leukemic population further expands, the concentration of P30-35 CAMAL rises further, to levels (> 1 μ g/ml) where a more complete shutdown of normal myelopoiesis is seen, and complications such as bleeding and fatigue occur due to decreased platelet production, and due to anemia from decreased red blood cell production, respectively.

Colony formation by several cell lines derived from myeloid leukemias was stimulated by treatment with P30-35 CAMAL. These included EM2 and EM3, derived from the leucocytes of the same patient with CML at different stages of leukemic progression. Colony formation by both of these cell lines was stimulated in a manner similar to observed using CML clinical specimens. Colony formation by HL60, derived from the leucocytes of a patient with acute promyelocytic leukemia, was also stimulated by P30-35 CAMAL. In contrast to EM2 and EM3, a single peak of enhancement occurred over a broad concentration range of P30-35 CAMAL with HL60, and peak enhancement occurred at a lower concentration than with the CML-derived cell lines. Colony formation by the cell line HEL, derived from an erythrocytic leukemia, was not affected by treatment with P30-35 CAMAL. It is interesting and possibly significant that the degree of responsiveness of those cell lines which responded to treatment with P30-35 CAMAL appeared to correlate with the aggressiveness of the leukemia from which the cell lines were derived. Colony formation by EM3, derived when the patient's CML was in a more aggressive phase, was enhanced more dramatically than was colony formation by EM2. HL60, derived from an AML, was stimulated at a lower concentration than were either of the CML-derived lines. Thus, different leukemic cell populations appear to respond to treatment with P30-35 CAMAL in non-identical manners, and responsiveness of cells to

the effects of P30-35 CAMAL might be as important as the levels of P30-35 CAMAL *in vitro* or *in vivo* in determining the outcome of exposure of these cells to P30-35 CAMAL. The similarity in responsiveness to P30-35 CAMAL between the cell lines EM2 and EM3 and CML clinical specimens provides a convenient experimental system for future studies. The responsiveness of HL60 to P30-35 CAMAL provides a potentially useful model for the study of the activity of P30-35 CAMAL on AML cells.

Several lines of evidence suggested that protease activity was involved in the effects of P30-35 CAMAL on colony-forming cells. First, preparations of P30-35 CAMAL in which inhibitors of protease activity had been included were found to be inactive in colony assays. In addition, N-terminal amino acid sequence analysis of preparative gel-purified material indicated that a protein with amino acid identity to known serine proteases was present (Chapter 2, Appendix 2). Finally, it was observed that the effects of P30-35 CAMAL on colony formation were maintained even after P30-35 CAMAL was washed from the cells, suggesting that these effects were immediate and maintained even after the P30-35 CAMAL material was removed. For these reasons, the possibility of the involvement of protease activity in the alterations of *in vitro* myelopoiesis mediated by P30-35 CAMAL was investigated.

Colony formation by normal human cells and by murine progenitor cells was inhibited to a similar extent whether the cells were cocultured or preincubated with P30-35 CAMAL. In addition, colony formation by CML progenitor cells and myeloid leukemiaderived cell lines was enhanced in a similar manner whether cells were cocultured or

preincubated with P30-35 CAMAL. These results indicate that the effects of P30-35 CAMAL on hematopoietic cells and cell lines are immediate and may well take place at the cell surface. This finding was of significance also from a technical standpoint; preincubation of cells with P30-35 CAMAL allowed treatment in small volumes, requiring far less P30-35 CAMAL material than did coculture experiments.

Experiments in which previously untreated cells were incubated with supernatant retrieved from P30-35 CAMAL-treated cells demonstrated that the activities of P30-35 CAMAL on colony formation were fully retained in the cell supernatant, and not absorbed by the cells. This was demonstrated using normal human cells and murine cells in assays for CFU-G; in both cases inhibitory activity on colony formation was fully retained in the supernatant. Similarly, enhancing activity on colony formation by both CML cells and the cell line EM3 was also fully retained in the supernatant. These results suggest that the effects of P30-35 CAMAL on myelopoiesis are exerted at the cell surface, and that the active component is not taken up by target cells.

The observations indicating that the effects of P30-35 CAMAL on colony formation are immediate and likely occur at the cell surface are both consistent with the activity of a protease. Hence, more direct evidence of protease involvement was sought. The effect of phenyl methyl sulfonyl fluoride (PMSF), an inhibitor of serine protease activity, on P30-35 CAMAL-mediated alterations of myelopoiesis was tested. It was found that both the inhibition of normal colony formation and the enhancement of CML colony formation mediated by P30-35 CAMAL were fully blocked by treatment of P30-35 CAMAL with PMSF.

Some progress had been made in this laboratory toward defining the substrate preference of preparations of P30-35 CAMAL using a panel of chromogenic peptide substrates. It was found that P30-35 CAMAL preparations recognize the substrate ala-ala-pro-phe-NA, whereas human neutrophil elastase, a serine protease related to the sequence obtained from a preparation of P30-35 CAMAL, did not. Both P30-35 CAMAL and elastase recognize the substrate ala-ala-pro-val-NA (474).

A peptide highly similar to the putative P30-35 CAMAL substrate, ala-pro-phe, which was linked to a chloro-methyl ketone (CMK) group, was obtained. Chloromethyl ketones are inhibitors of serine protease activity which form a covalent bond in the active site of the enzyme. Specificity of blockage using these peptides is mediated by the amino acid sequence to which the CMK group is linked. In an enzyme assay using chromogenic peptide substrates, it was demonstrated that the activity of P30-35 CAMAL was blocked by ala-pro-phe-CMK, whereas the activity of elastase was not blocked by this peptide. Furthermore, both the inhibition of normal colony formation and the enhancement of CML colony formation mediated by P30-35 CAMAL were fully blocked on treatment of P30-35 CAMAL with ala-pro-phe-CMK. In contrast, the activity of P30-35 CAMAL on normal colony formation was not blocked by three other CMK-linked peptides, including the peptide which blocks elastase activity, ala-ala-pro-val-CMK. These results provide a second line of evidence that serine protease activity is required for P30-35 CAMALmediated alterations of myelopoiesis. In addition, they demonstrate that the activity of P30-35 CAMAL can be blocked independent of effects on other protease activities, and raise the possibility that specific blocking of the activity of P30-35 CAMAL using the peptide alapro-phe-CMK or other substances might be clinically useful in aiding the restoration of normal myelopoietic balance in the treatment of myeloid leukemias.

A monoclonal antibody raised against preparations of P30-35 CAMAL, α -P30/35, was shown to react with cytospin preparations of nucleated cells from patients with CML to a far greater extent than with nucleated cells from normal healthy donors. This is a similar pattern of positive staining to that previously observed using CAMAL-1, a monoclonal antibody raised against original preparations of the CAMAL antigen obtained by subtractive methods from the lysates of cells from patients with myeloid leukemias; CAMAL-1 has demonstrated reactivity with proteins other than P30-35 CAMAL. It has been shown, however, by ELISA, and more recently by Western blot analysis, that CAMAL-1 does not react with P30-35 CAMAL (473). Hence the antigen recognized as diagnostic of CML in studies using CAMAL-1 and the P30-35 CAMAL material that mediates the described inhibition of normal myelopoiesis and the enhancement of leukemic myelopoiesis do not appear to be identical. However, the similarities in the pattern of staining obtained using these two monoclonal antibodies, in addition to the observation that CAMAL-1 enriches for P30-35 CAMAL from lysates of myeloid leukemia cells, suggest that the entities recognized by CAMAL-1 and α -P30/35 might form an association.

Several cell lines derived from myeloid leukemias were found to react extensively with α -P30/35 in the immunoperoxidase test. It is interesting and possibly significant that the cell lines shown to react with α -P30/35 by immunoperoxidase, and which thus might produce P30-35 CAMAL, also showed enhanced colony formation on treatment with P30-35 CAMAL. The only cell line tested which did not demonstrate enhancement in the

colony assay, HEL, also did not react with α -P30/35 in the immunoperoxidase test. These results raise the possibility that P30-35 CAMAL might be involved in regulating proliferation and differentiation in an autocrine fashion in these cell lines.

Subsequent to the completion of the studies described above, it was recognized that the N-terminal amino acid sequence obtained from a preparation of P30-35 CAMAL was identical to that of azurocidin (Chapter 2, Appendix 2). The azurocidin sequence was not in the protein data bank at the time that the N-terminal sequence from the P30-35 CAMAL preparation was obtained, but its homology to known serine proteases taken together with the observations described above influenced the course of the experiments addressing serine protease activity (Chapter 5). Azurocidin was cloned by a group investigating the antibacterial properties of proteases isolated from normal neutrophils. By purification of proteins from azurophilic granules, this group isolated four proteins which were serine proteases or had serine protease homology. Two were well-known proteases, elastase and cathepsin G (479). One was shown by amino acid sequence to be identical to myeloblastin (309, 495, 496). The fourth, azurocidin, has two important changes; the serine and histidine of the catalytic triad are both substituted. Consequently, azurocidin has no protease activity on any chromogenic peptide substrates tested, including several of those with which P30-35 CAMAL preparations were shown to react (438, 474).

P30-35 CAMAL preparations were fractionated using reverse-phase HPLC as part of the project of another graduate student in this laboratory dealing characterization of P30-35 CAMAL at a subcellular level (474). In these preparations, peaks corresponding to

elastase, cathepsin G, and azurocidin could be identified, in addition to a major peak which was unique to preparations of P30-35 CAMAL (474), as compared to preparations derived from normal neutrophils (479). Additional studies were undertaken in order to determine whether the observed alterations in myelopoiesis could be attributed to the characterized proteins in these preparations.

The results presented in Chapter 5 show clearly that P30-35 CAMAL-mediated alterations of myelopoiesis require serine protease activity, so the possibility that azurocidin in preparations of P30-35 CAMAL could mediate the observed effects on colony formation seemed unlikely. Elastase is known not to mediate P30-35 CAMAL activity on colony formation, since, in contrast to P30-35 CAMAL, elastase was shown to be inhibitory to colony formation by CML progenitor cells, and since preparations of P30-35 CAMAL were screened for lack of this elastase activity. In addition, it was shown that the activity of elastase was not blocked by the peptide ala-pro-phe-CMK at even an 100:1 molar excess, whereas ala-pro-phe-CMK did fully block P30-35 CAMAL-mediated alterations of colony formation by normal progenitor cells and by CML progenitor cells at a 10:1 molar excess. Since downregulation of myeloblastin was found to cause the growth arrest and differentiation of promyelocytic leukemia cells (310), it appeared that the effects of P30-35 CAMAL and myeloblastin might be similar. However, myeloblastin, also known as proteinase 3, was found to be essentially absent from P30-35 CAMAL preparations. In addition, the chromogenic peptide subtrates recognized by myeloblastin are substantially different from those recognized by P30-35 CAMAL (438, 474). The effects of azurocidin, myeloblastin, and cathepsin G were evaluated in a colony assay using normal human bone marrow cells. None of these proteins inhibited colony formation in this assay (Appendix

2). In contrast, recent experiments have demonstrated that protein from the unique peak in preparations of P30-35 CAMAL was inhibitory to colony formation in cultures of murine cells (477). In addition, protein from this unique peak had activity on the chromogenic peptide substrate recognized by preparations of P30-35 CAMAL (474). Protein from this peak is being prepared for N-terminal amino acid analysis.

The same peptide substrate recognized by preparations of P30-35 CAMAL is also recognized by the serine protease cathepsin G. Although it was shown that colony formation by normal progenitor cells was not altered by cathepsin G, it remains a possibility that the alterations of colony formation by CML progenitor cells might be mediated by cathepsin G, or that cathepsin G activity might be required to render treated cells susceptible to alterations in myelopoiesis mediated by other substances in P30-35 CAMAL preparations. However, enzyme assays using CMK-linked peptides to block the activity of P30-35 CAMAL preparations or cathepsin G on the substrate which is recognized by both, ala-ala-pro-phe-NA, clearly demonstrated the presence of a protease activity in preparations of P30-35 CAMAL which is distinct from that of cathepsin G (Appendix 2). Specifically, the peptide ala-ala-pro-val-CMK fully blocked P30-35 CAMAL activity at a CMK:P30-35 CAMAL molar ratio of 10:1 or less, whereas cathepsin G activity was not blocked by this same peptide at a CMK:cathepsin G molar ratio of even 100:1 (Figure 34a). This difference in activity cannot be attributed to a block of elastase activity, since elastase does not recognize the tchromogenic peptide substrate used to evaluate activity in this assay (474). Similarly, the peptide ala-pro-phe-CMK blocked P30-35 CAMAL activity substantially at a molar ratio of 1:1, whereas cathepsin G activity was essentially unaffected at this level, and significant activity was retained even at a ratio of
10:1 (Figure 34b). More recently, the unique peak in reverse phase HPLC-separated preparations of P30-35 CAMAL has been shown to have activity on the P30-35 CAMAL/cathepsin G substrate (474). It remains a possibility, then, that the alterations of myelopoiesis described in this study are mediated by a serine protease which is distinct from characterized proteases. Whether the enhancement of colony formation by CML cells is mediated by cathepsin G or by the unique peak in preparations of P30-35 CAMAL is the subject of ongoing studies (476).

In many systems involving proteolysis, a cascade of zymogen activations occurs, with an altered biological effect as the end result. Proteases activated in a cascade include the proteases of the blood clotting system (497, 498), the complement system, (499, 500), and apparently the proteases involved in pattern formation in the *Drosophila melanogaster* embryo (501). It is possible that the presence of more than one protease in P30-35 CAMAL preparations is required for the final alterations of *in vitro* myelopoiesis to occur.

Other substances which are inhibitory to normal myelopoiesis have been described; these include leukemia inhibitory activity (LIA), leukemia associated inhibitor (LAI), lactoferrin, acidic isoferritins, macrophage inflammatory protein-1 α , inhibin, TGF- β , TNF- α , the interferons, interleukin 10, the tetrapeptide Ac-ser-asp-lys-pro, and the pentapeptide pEEDCK (Chapter 1). Substances which alter the growth of leukemic cells, such as the related proteins leukemia inhibitory factor (LIF) and oncostatin M, also have been investigated. In addition, there is an enormous volume of literature documenting the actions of various cytokines and combinations of cytokines on normal and malignant

hematopoietic cells. The effects of these cytokines vary depending on several factors, for example on which other factors are present, on the immediate microenvironment, such as whether cytokine-producing accessory cells are present which are able to influence target cells in a paracrine fashion, on whether target cells are undergoing particular adhesive interactions, and on the state of dependence of target cells to the cytokine in question, for example some leukemic cells are thought to be growth factor independent, and may respond to factors in an autocrine fashion. Proteases, and serine proteases in particular have been described which function in or alter the function of hematopoietic cells. In addition to the functions of proteases in cytotoxic T lymphocytes, neutrophils, and mast cells, these include, among other examples, protease involvement in the release of TNF- α from a membrane-bound form, protease involvement in the release of biologically active M-CSF from a membrane-bound form, proteolytic downregulation of the M-CSF and G-CSF receptors, modulation of protein kinase C activity in hematopoietic cells, myeloblastin activity in promyelocytic leukemia, and CALLA in CLL (Chapter 1). Thus, many events in differentiation and function in the hematopoietic system involve proteolytic activity. The involvement of proteolysis in alterations of hematopoiesis has become increasingly well documented in recent years, and appears to be coming under increasing scientific scrutiny. P30-35 CAMAL appears to be one further example of a proteolytic activity which functions in the alteration of hematopoiesis.

6.2: Speculation on possible mechanisms of action of P30-35 CAMAL;

The following ideas about mechanisms by which alterations in myelopoiesis could be mediated by P30-35 CAMAL have not been experimentally tested, and are speculative.

Although normal and CML progenitor cells responded to treatment with P30-35 CAMAL at different concentrations, possible common themes were noted. In the cases of both normal and of CML colony formation, one progenitor cell type responded to a relatively lower concentration of P30-35 CAMAL; CML CFU-GEMM were consistently affected at between 4 and 10 ng/ml, and normal CFU-G were affected at between 50 ng/ml and 1 μ g/ml. Similarly, at relatively higher concentrations of P30-35 CAMAL, multiple progenitor cell types were affected in both cases; all CML colony types were increased at 100 to 200 ng/ml, whereas all normal colony types were decreased at levels greater than 1 μ g/ml. The effects on one progenitor cell type as compared to multiple progenitor cell types occurred at a concentration difference of 10 fold or greater in both the CML and the normal assays. These observations imply that the signal initiated by P30-35 CAMAL in the signal initiated in the multiple progenitor cell types in both cases.

These observations raise questions about the substrate(s) or ligand(s) recognized by P30-35 CAMAL on the affected progenitor cells. Several possibilities exist as to the pattern of ligands recognized by P30-35 CAMAL under the various treatment conditions. For example, the ligand recognized on a single class of progenitor cells might be similar on

both CML and normal progenitor cells, whereas the ligand recognized on multiple progenitor cell types might also be similar on both CML and normal progenitor cells but different from that affected on the single class of progenitor cells in each case. The difference in P30-35 CAMAL concentration required between CML and normal progenitor cells to effect changes in colony formation in either the case of a single progenitor cell class or multiple progenitor cell classes might be accounted for by a change in regulatory mechanisms in CML cells which increase or stabilize signalling mechanisms initiated by P30-35 CAMAL.

It seems possible that two ligands are affected by P30-35 CAMAL, at least in the case of CML progenitor cells, since enhancement of colony formation at an intermediate concentration of P30-35 CAMAL was reduced or absent. If the same cell surface molecule were involved in P30-35 CAMAL-mediated enhancement of colony formation in the two progenitor cell populations affected, enhancement of primitive colony types at intermediate concentrations of P30-35 CAMAL would be expected. It is possible, however, that P30-35 CAMAL might interact with the same cell surface molecule in the two cell populations, but activate different signal transduction pathways, resulting in the enhancement of different colony populations.

A stabilizing change to a putative signal transducing portion of a P30-35 CAMAL ligand, or a signal transducing molecule downstream of a P30-35 CAMAL ligand, could result in a continuous signal favouring growth over differentiation in CML progenitor cells. An example from the literature which may be illustrative is that of signal transduction involving IL-3. It is thought that proteolytic cleavage of the IL-3 receptor might be

involved in signal transduction mediated by IL-3. Stimulation of IL-3 responsive cells by IL-3 results in the disappearance of a 140 kDa IL-3 binding protein, and the appearance of two 68 kDa proteins, each of which can be phosphorylated, one on serine, and the other on tyrosine (451). These phosphoproteins are thought to have signal transducing capabilities, and might be rapidly degraded under normal circumstances as a downregulatory mechanism. It is possible that P30-35 CAMAL might affect a similar cell signalling mechanism; interaction with a cell surface ligand such as a colony-stimulating factor (CSF) receptor could result in intracellular release of a signalling molecule into the cytoplasm, which in normal cells might be rapidly degraded. Under these circumstances, proteolytic cleavage of the cell surface ligand would result in a net loss of the signalling molecule from the cell surface. In normal cells, intracellular degradation of the signalling molecule would result in downregulation of the signal transduced. Removal of the ligand from the cell surface would lead to a loss of responsiveness to stimulatory signals resulting in decreased colony formation. If a stabilizing change to the signalling portion of the putative ligand were to occur in CML cells, however, stimulation of these cells would result in continued intracellular signalling, due to lack of downregulation, in the absence of further stimulatory signals, and might be a mechanism involved in the progression of CML from chronic phase to accelerated phase and blast crisis (Figure 30a). This line of reasoning is consistent with the observation that one of the hallmarks of full cellular transformation is a loss of dependence on growth factors (25, 122, 161, 502). This or other models require expression of a ligand or substrate for P30-35 CAMAL on CML progenitor cells of all types since the growth of all colony types is enhanced by P30-35 CAMAL. A ligand or substrate for P30-35 CAMAL must be present on all normal progenitor cell types also since

at high concentrations of P30-35 CAMAL colony formation by all types of progenitor cells was inhibited.

An alternative explanation for the changes observed in colony formation on treatment of progenitor cells with P30-35 CAMAL involves differential expression of ligands or substrates for P30-35 CAMAL, resulting in differential sensitivity on the part of progenitor cells to the effects of P30-35 CAMAL on myelopoiesis. If aberrant expression of a P30-35 CAMAL-reactive molecule in CML were the sole cause of enhancement, however, CML CFU-G, like normal CFU-G, would be expected to be downregulated by P30-35 CAMAL. Plucked colony data, however, suggest that CML CFU-G, in addition to other colony types, are increased by concentrations of P30-35 CAMAL of 100 to 200 ng/ml. Alternatively, CML cells might produce more P30-35 CAMAL than do normal cells, resulting in higher intrinsic culture or serum levels and an apparent increased sensitivity to exogenously added protein. However, the effect of P30-35 CAMAL on cultures of CML cells was consistent at consistent concentrations of exogenously added P30-35 CAMAL, despite the fact that the number of presumably P30-35 CAMAL, producing colonies varied greatly between experiments. Consistent intrinsic culture levels of P30-35 CAMAL under these conditions seem unlikely.

Insight into how the activation of a signal transduction pathway might be initiated at the cell surface may be gained through the example of platelet activation by thrombin. Platelet activation and aggregation is blocked by agents which inhibit the serine protease activity of thrombin. The activation signal is transmitted via the thrombin receptor, an integral membrane protein with seven transmembrane domains. Thrombin has been shown

to release a short peptide from the N terminus of the receptor, allowing the newly created N terminus to act as a "tethered ligand"; the new N terminus binds to another site on the receptor protein and results in platelet activation, as measured by calcium flux, and aggregation (453). It is possible that the protease activity required for the effects of P30-35 CAMAL on myelopoiesis might act to initiate signal transduction via a similar mechanism.

It is possible that a signal transduction pathway involving bcr and/or abl, or P210 *bcr-abl*, in the case of normal and CML cells respectively, are activated by P30-35 CAMAL. In this model, P30-35 CAMAL might activate the same pathway in both normal and CML cells upstream of bcr and/or abl or P210 bcr-abl, resulting in different effects downstream. The altered kinase activity and cellular location of P210 bcr-abl could result in a quantitatively greater or prolonged signal, resulting in a differentiation block, for example. Since differentiation and proliferation are partially uncoupled in CML, by unknown mechanisms which could conceivably involve bcr-abl, a prolonged stimulatory signal might shift the balance toward cell proliferation, or away from differentiation, resulting in increased clonogenicity and larger colonies. In contrast, in normal cells, in which differentiation and proliferation are coupled, activation of the same pathway might result in interaction with unaltered bcr and/or unaltered abl. The net result in this case would be a signal of normal duration, and a decrease in differentiation/proliferation, leading to a decrease in colony formation and in colony size. These speculations are consistent with the observation that higher levels of P30-35 CAMAL are required for observable alterations in myelopoiesis in normal colony-forming cells than in CML cells. A higher concentration of P30-35 CAMAL would be expected to be required for a significant effect to result in cells in which normal regulatory mechanisms are operative, and signalling is

efficiently downregulated. Conversely, the alterations in kinase activity and cellular location of signal transducing proteins such as *bcr-abl* in CML would be expected to abnormally stabilize and prolong signal transduction, hence lower concentrations and/or a shorter duration of an activating signal would be required in order to obtain an observable effect.

Using antisense oligodeoxynucleotides to *ras*, it was shown that *ras* is required for colony formation supported by IL-3, GM-CSF, and M-CSF, but not by G-CSF (138). Recall that it has been shown that *bcr* encodes a GTPase activating protein (GAP) for *rac*. If it is assumed that G-CSF induced colony formation is mediated by the *ras* -related protein *rac*, partial inactivation of *rac*GAP activity due to the *bcr-abl* translocation would explain the increase in cells of the granulocytic lineage seen in CML, since the ability of *bcr/rac*GAP to downregulate *rac* activity would be impaired. P30-35 CAMAL fits into this picture as follows.

In normal cells, at low concentrations of P30-35 CAMAL, CFU-G would be expected to be targetted if P30-35 CAMAL were to somehow interfere with *rac* activity, possibly by activating *bcr/rac* GAP activity. This would result in downregulation of *rac* activity, thus disrupting the ability of *rac* to support the formation of CFU-G. (It is possible, for example, that P30-35 CAMAL induces the ability of *bcr* to bind to *c-abl*; recall that it has been documented that *bcr* binds *bcr-abl*, see below). In normal cells at high concentrations of P30-35 CAMAL, this interference might extend to the *ras* pathway by virtue of the fact that *rac* and *ras* are similar; i.e. *ras* GAP activity would be upregulated. This would result in downregulation of *ras* activity, which is required for

colony formation supported by IL-3, GM-CSF and M-CSF. Hence, all colony types formed by normal cells would be reduced at these concentrations. In contrast, in CML cells, the ability of P30-35 CAMAL to activate bcr/racGAP would be disrupted by virtue of the fact that bcr is altered by the bcr-abl translocation. Signals stimulating rac activity would thus be prolonged (Figure 30b). At low concentrations of P30-35 CAMAL, P30-35 CAMAL treatment of CML cells causes increases in primitive colony types. It would have to be postulated that *rac* is involved in signal transduction in more primitive progenitor cells than CFU-G in order for this to occur, which is not unreasonable since bcr-abl is known to be present in pluripotent stem cells. The inability to activate bcr/racGAP might be expected to result in no net effect on CML colony formation. However, the observed increase in colony formation might be mediated by the altered cellular location of *bcr-abl* or its upregulated tyrosine kinase activity. Signalling mechanisms initiated by P30-35 CAMAL might result in a change, such as phosphorylation of bcr-abl, which alters this kinase activity. Or, an increase in the ability of bcr-abl to bind and sequester normal bcr/racGAP could result, thus further decreasing the ability of the cell to downregulate stimulatory signals mediated through *rac*, and resulting in increased colony formation. Primitive colony formation might be predominant because the dysregulated bcr-abl activity in primitive cells might be preferentially expressed in these cells, resulting in an expansion in the clone from which leukemic CFU-G arise. At the same time, colony formation in normal clones would be inhibited by virtue of the normal racGAP activity in these cells. In CML cells at high concentrations of P30-35 CAMAL, P30-35 CAMAL treatment might initiate the binding of ras GAP by bcr-abl, resulting in the decreased downregulation of ras activity, and prolonged stimulatory signals promoting colony formation supported by IL-3, GM-CSF, and M-CSF (ras), and by G-CSF (rac). In this regard, ras GAP has been



ii. CML cells

FIGURE 30. Possible mechanisms by which P30-35 CAMAL treatment could affect signal transduction, resulting in the observed alterations in myelopoiesis. a. Interaction of P30-35 CAMAL with a substrate (ς) at the cell surface results in activation (*) of an intracellular signal transduction pathway. i. In normal cells, the positive signal is rapidly downregulated by a cellular inhibitor (]). Release of the P30-35 CAMAL substrate from the cell surface makes the cells refractory to stimulatory signals, resulting in an overall suppressive effect. ii. In leukemic cells, a stabilizing change to the signalling molecule (+) results in inefficient downregulation ([]) of signals, and in an overall stimulatory effect (!). Removal of the P30-35 CAMAL substrate from the cell surface in these cells is inconsequential. b. Cellular activation by P30-35 CAMAL of a pathway involving *rac*. i. In normal cells, stimulatory signals transmitted via *rac* are rapidly downregulated by the *rac* GAP activity of *bcr* ([). ii. In CML cells, *bcr-abl*, shown bound to the cytoskeleton, sequesters *bcr*, allowing prolonged activation of *rac* (*rac) and a prolonged stimulatory signal (!). The *ras* pathway could be affected in a similar manner, as *bcr-abl* has been shown to bind *ras* GAP. In these schemes, 'CAMAL' refers to P30-35 CAMAL.



i. Normal cells



ii. CML cells

shown to immunoprecipitate with P210 *bcr-abl* and to be phosphorylated by it, a finding which implies that mitogenic signals mediated by p21 *ras* could be altered by *bcr-abl* (139). Recall that differences in which colony types were affected by P30-35 CAMAL occurred at approximately 10 fold differences in concentration in the case of both normal and CML cells. A 10 fold increase in P30-35 CAMAL concentration and signals initiated in this way might be what is required to affect *ras* activity.

The technology to evaluate whether P30-35 CAMAL initiates signal transduction pathways upstream of *bcr-abl* is currently available. Treating CML cells with antisense oligodeoxynucleotides to *bcr-abl* should abrogate the P30-35 CAMAL mediated stimulation of CML colony formation, if *bcr-abl* pathways are involved. A block in expression of the *bcr-abl* fusion gene might even result in a decrease in colony formation on treatment with P30-35 CAMAL, if the lack of *bcr-abl* expression restores a 'normal' phenotype to these cells. The expression of *bcr-abl* can be evaluated by the polymerase chain reaction.

If P30-35 CAMAL is indeed found to be an upstream signal which acts extracellularly and alters signalling by *bcr-abl*, this has important therapeutic implications in CML. Specific therapies for CML are still in the very experimental stages. Specific targetting of *bcr-abl* by antisense oligodeoxynucleotides, for example, requires uptake into the cells, which must be incubated *ex vivo*. This is possible so far only in conjunction with bone marrow transplantation, an invasive procedure associated with significant toxicity, mortality, and a significant incidence of leukemic relapse. If the activity of an upstream extracellular signal affecting *bcr-abl* could be targetted and blocked, it might be possible to

carry this out in a far less invasive way such as by intravenous injection of a blocking agent.

To summarize, the ligands or substrates recognized by P30-35 CAMAL at the surface of normal and CML progenitor cells are unknown. However, interaction of P30-35 CAMAL with these ligands or substrates might result in activation of cell signalling by a mechanism similar to that described for the thrombin receptor, in which proteolytic cleavage unmasks a 'tethered ligand' which causes activation of signalling pathways. Alternatively, it might activate signalling in a manner similar to that described for the IL-3 receptor, in which a signalling protein is released from the receptor intracellularly by proteolytic cleavage. Stabilizing changes to regulatory proteins in signalling pathways could result in the enhancement of colony formation in CML cells mediated by P30-35 CAMAL in contrast to the inhibition of colony formation by normal cells. P30-35 CAMAL might initiate signalling upstream of *bcr/rac* GAP and *ras* GAP in normal cells, and upstream of *bcr-abl* and *ras* GAP in CML cells. These ideas, while attractive, and while supported in part by observations described in this study as well as by data published by other groups, are speculative, and must be tested experimentally before conclusions can be drawn.

6.3: *Future directions*;

6.3.1: Basic science;

Purification procedures are being optimized as part of the research project of another graduate student in this laboratory in order to make the purification of P30-35 CAMAL less labour intensive yet still reproducible. The apparent similarity in amino acid sequence between that obtained from a preparation of P30-35 CAMAL and elastase, for instance, made it necessary to ensure that preparations of P30-35 CAMAL were free of contaminating elastase activity. This was an issue since elastase has a similar molecular weight to the P30-35 CAMAL entity shown to mediate the described alterations of *in vitro* myelopoiesis. Most preparations of P30-35 CAMAL were shown to be free of elastase activity by virtue of the fact that elastase is inhibitory to colony formation by CML progenitor cells (Chapter 2; all preparations of P30-35 CAMAL used in these studies were free of elastase activity), however screening P30-35 CAMAL preparations for lack of elastase activity was a time and labour-intensive enterprise.

Analysis of reverse phase HPLC separations of P30-35 CAMAL preparations indicates the presence of a protein which might be unique, and which appears to mediate inhibitory activity on murine colony formation (474, 477). Whether this same material mediates the enhancement of colony formation by CML progenitor cells remains to be determined. This material which is inhibitory to colonies cultures from murine cells has been sent for N-terminal amino acid analysis. Assuming that P30-35 CAMAL proves to be

a unique protein, cloning of the gene from cell lines derived from myeloid leukemias and/or CML clinical specimens would be an obvious asset for further studies. Recombinant protein might be a convenient source of P30-35 CAMAL for future biological assays. For example, the effects of P30-35 CAMAL on myelopoiesis could be examined in greater detail using long-term cultures of normal or CML progenitor cells, in one step cultures, or in two step cultures in order to determine the effects of P30-35 CAMAL on myelopoiesis by cells exposed to a preformed adherent layer, and the possible interactive effects of P30-35 CAMAL in conjunction with other cytokines or regulatory factors could be examined. Moreover, the effects of P30-35 CAMAL on the gene expression of other regulatory factors could be examined in this system. In addition, it could be determined whether the effects of P30-35 CAMAL on myelopoiesis are mediated by a direct action on the affected progenitor cells, or require the presence of accessory cells. This analysis would require support of colony formation by recombinant colony-stimulating factors in cell populations depleted of accessory cells.

Analysis of the effects of P30-35 CAMAL on the clonogenicity and proliferation of AML cells was not possible during the course of these studies due to rare access to these clinical specimens, and due to the fact that a minority of specimens that were obtained formed colonies in the culture conditions used; they tended to form large numbers of small cell clusters which were difficult to evaluate. The effect of P30-35 CAMAL on AML myelopoiesis could be examined using long term cultures, and access to recombinant protein would facilitate this. Alternatively, liquid suspension cultures using AML cells have been described (503), and, although the cloning efficiency is reported to be lower than that obtained in methylcellulose cultures (504), these could be pursued. In addition, the

effect of P30-35 CAMAL on cell lines derived from AML leucocytes, such as HL60, could be examined in greater detail.

Recombinant protein would also facilitate definition of the reactivities of α -P30/35 at a cellular level. For example, it would be interesting to determine whether the recognition of CML cells by α -P30/35 could be competitively inhibited by the protein of interest. In addition, it would be interesting to determine whether incubation of normal cells with this protein increases reactivity with this antibody. This would indicate the presence of a ligand or substrate for P30-35 CAMAL on the surface of these cells.

Molecular probes would allow identification of the cell type(s) which produce P30-35 CAMAL and might shed light on its apparent regulatory role in myelopoiesis. It has been assumed that the major cell population producing P30-35 CAMAL is the leukemic neutrophils and/or their progenitors. However, P30-35 CAMAL could equally well be produced by other cell types and act in a paracrine fashion on the leukemic cells. For example, it is possible that P30-35 CAMAL might be produced by a minority of cells from normal bone marrow, and could be a normal regulatory molecule in hematopoiesis which becomes dysregulated in CML.

One intriguing study would be the examination of CML cell populations using a molecular probe for P30-35 CAMAL, and a probe for the *bcr-abl* fusion gene product. These probes could be fluorescently labelled to determine whether the P30-35 CAMAL-producing cell population is indeed the leukemic population (505). Similarly, obviously enlarged CML colonies could be tested by polymerase chain reaction (PCR) for *bcr-abl*

expression to determine whether, as would be expected on the basis of these studies, the responsive progenitor cell population is indeed derived from the Philadelphia chromosome positive clone. Alternatively, leukemic and normal cells could be separated in specimens of leucocytes obtained from CML patients and the effects of P30-35 CAMAL tested on these sorted cell populations. In this regard, it has recently been shown that the cell population with the surface characteristics CD34⁺DR^{-lin⁻} (DR = class II HLA-DR) contain almost exclusively progenitor cells that give rise to colonies which are *bcr-abl* negative (425). Cells which are CD34⁺DR⁺, conversely, give rise almost exclusively to *bcr-abl* positive colonies. Finally, cloning of the putative gene encoding P30-35 CAMAL would enable determination of its chromosomal location, and its possible involvement in genetic events in myeloid leukemias could be evaluated.

It might be possible to identify the physiological ligand(s) and/or substrate(s) recognized by P30-35 CAMAL by analysis of cells which are susceptible to the effects of P30-35 CAMAL. For example, it might be possible to analyze two dimensional gels of cell lysates of P30-35 CAMAL treated cells using antibodies specific for likely substrate candidates. For example, the group investigating myeloblastin believe its substrate to be the retinoic acid receptor (310). Western blots could be done using anti-retinoic acid receptor Abs, or Abs directed to cell surface molecules known to be involved in growth control and differentiation and which are possible candidates as ligands or substrates for P30-35 CAMAL; examples include the IL-3 receptor, *c-kit* and its ligand Steel factor, CD34, and adhesion molecules. One cell surface molecule which is a possible P30-35 CAMAL substrate is the G-CSF receptor. It was recently shown that downregulation of the G-CSF receptor can be blocked by inhibitors of serine protease activity (290). This is

an intriguing observation in light of the inhibitory activity of P30-35 CAMAL on normal CFU-G, and one that deserves to be pursued.

6.3.2: Development of a clinically useful agent;

Agents which inhibit the activity of specific proteases have been used in a clinically relevent manner. For example, angiotensin converting enzyme (ACE) inhibitors are commonly used in the treatment of hypertension. Inhibitors are being designed by protein engineering which inhibit proteases specifically without the untoward effects of other agents. For example, alpha-1 antithrombin has been modified by site-directed mutagenesis to have thrombin blocking activity, making it potentially useful as an anticoagulant, and making it possible to avoid the risk of hemorrhage associated with the use of heparin (506). Other groups are designing protease inhibitors by computer modelling of the three dimensional structure of a particular enzyme based on its primary sequence (507). Hence, blocking the protease activity involved in the alterations of myelopoiesis mediated by preparations of P30-35 CAMAL might prove to be a realistic goal.

Studies demonstrating that P30-35 CAMAL-mediated alterations of myelopoiesis could be blocked using the peptide ala-pro-phe-CMK, that this effect could be separated from effects on the serine protease elastase, and that the activity of P30-35 CAMAL on myelopoiesis was not blocked by several other CMK-linked peptides raise the possibility that ala-pro-phe-CMK or a similar agent could be clinically useful in the treatment of CML.

A first step in the development of a clinically useful blocker of the activity of P30-35 CAMAL is the determination of the optimal substrate for P30-35 CAMAL, and blocking the activity of P30-35 CAMAL must be shown not to significantly affect other important protease activities. As a preliminary evaluation, the potential toxicity of putative blocking agents on primitive progenitor cells should be tested in long-term marrow cultures using normal bone marrow cells. If an agent which blocks the activity of P30-35 CAMAL is to be clinically useful, it must be shown to spare primitive normal stem and progenitor cells. If, however, such an agent proves to be toxic to normal stem cells, it might be possible to reduce this toxicity by coupling the agent to a carrier protein. Alternatively carriers which improve delivery, and reduce toxicity, such as liposomes, could be used (315). Alternatively, physiological substrates such as inhibitors of proteases found in the plasma could be altered by protein engineering to a form which is bound but not cleaved by P30-35 CAMAL. Such a strategy has been used to block proteases in the treatment of hypertension (506).

If putative agents which block the activity of P30-35 CAMAL prove to be non-toxic to normal stem cells, long-term marrow cultures should be done using CML cells in order to determine whether the blocking agent is inhibitory to the growth of the CML clone. The effect on the CML clone can be determined by screening for the presence of the P210 *bcr-abl* transcript by polymerase chain reaction (PCR). This system is already in place in this laboratory and has been used to evaluate the selective toxicity of benzoporphyrin derivative (BPD) toward Ph⁺ progenitors (348). If a P30-35 CAMAL-blocking agent were to be found to differentially inhibit the growth of the Ph⁺ clone, it could be used in conjunction with purging agents to treat remission marrow intended for autologous bone marrow

transplantation. Or, it is possible that such an agent could be used to control this malignancy in a less invasive way; by intravenous injection, for example, singly, or in conjunction with chemotherapeutic agents.

Should long term marrow culture studies prove promising, toxicity and clearance studies with agents which block the activity of P30-35 CAMAL can be conducted in mice. Efficacy studies in mice can be conducted using the *ex vivo* spleen colony assay described in Chapter 3.

SUMMARY AND CONCLUSIONS;

Studies reported in this paper document the effects of P30-35 CAMAL, enriched from lysates of leucocytes obtained from patients with myeloid leukemias, on in vitro myelopoiesis by normal and CML progenitor cells. The inhibition of colony formation by normal progenitor cells originally noted using crude preparations eluted from immunoaffinity columns prepared with the monoclonal antibody CAMAL-1, which was in turn prepared against protein isolated by substractive methods from lysates of myeloid leukmia cells, was shown to be mediated by the P30-35 material in these preparations. Normal colonies were inhibited in number and in size by this P30-35 CAMAL material, and the effect was preferentially directed toward CFU-G at low concentrations of P30-35 CAMAL. At high concentrations of P30-35 CAMAL, colony formation by all progenitor cell types was inhibited. Cell numbers in the non-adherent fraction of long term cultures using normal bone marrow were reduced, but were increased within the adherent layers, indicating that these cells might be blocked in differentiation. The inhibitory effect on colony formation was also observed in assays using murine progenitor cells. Like in assays of human cells, colonies were inhibited in number and in size. Colony formation was inhibited to a similar extent in assays of human and murine cells, and the effect titrated over a similar range of P30-35 CAMAL concentration. As was observed in cultures of human cells, the inhibitory effect was directed toward murine CFU-G. In addition, spleen colony formation in an ex vivo assay was reduced. These results are an indication that the

inhibition of normal granulopoiesis mediated by P30-35 CAMAL is conserved across species barriers, and might be an important regulatory mechanism in myelopoiesis.

In contrast, the same P30-35 CAMAL material that was inhibitory to colony formation by normal progenitor cells enhanced colony formation by CML progenitor cells. This enhancement of colony formation was both in number of colonies and in the size of colonies within the P30-35 CAMAL-treated cultures. These effects were diametrically opposed to the effects observed in cultures of normal progenitor cells. The enhancement of colony formation by CML progenitor cells was consistent, and was directed toward two progenitor cell populations. At a low concentrations of P30-35 CAMAL, primitive colony types were enhanced, whereas at a higher concentration, increases were seen in colonies of all types. Colony formation by several cell lines derived from the leucocytes of patients with myeloid leukemias was enhanced in addition.

The differential alterations in colony formation by normal and CML progenitor cells mediated by P30-35 CAMAL are important, since they provide a mechanism by which leukemic cells might gain a growth advantage over normal cells and become predominant. Suppression of normal myelopoiesis and an often large increase in levels of circulating leukemic neutrophils and their progenitors are well known features of CML.

The observed alterations in colony formation by normal and CML progenitor cells were demonstrated to require serine protease activity. Experiments in which the P30-35 CAMAL-mediated activities on colony formation were maintained after the P30-35 CAMAL material was removed from the cells, and were shown to be retained in the

supernatants of treated cells demonstrated that the effects on *in vitro* myelopoiesis were immediate, were not adsorbed by the cells, and likely occurred at the cell surface. P30-35 CAMAL-mediated activities on colony formation were blocked by treatment of the P30-35 CAMAL material with PMSF, an inhibitor of serine protease activity, and using a peptide for which material in preparations of P30-35 CAMAL had some selectivity, which was linked to a chloromethyl ketone group, an inhibitor of serine protease activity. It might be possible to block this P30-35 CAMAL activity in the treatment of CML using a highly specific CMK-linked peptide or other agents which are found to block the activity of P30-35 CAMAL.

A monoclonal antibody was raised against highly enriched P30-35 CAMAL material, and was used to enrich P30-35 CAMAL from myeloid leukemia cell lysates, and to examine cells in an immunoperoxidase slide test. The immunoperoxidase analysis showed that nucleated cells derived from patients with CML showed far greater reactivity with this antibody than did normal cells. This was a similar pattern of reactivity as was observed using CAMAL-1, an antibody with known reactivity with other proteins, and was an indication that the antigen recognized in the studies using CAMAL-1 as diagnostic of myeloid leukemias might be associated with the P30-35 CAMAL material that was shown to alter colony formation by normal and CML progenitor cells. In addition, several cell lines derived from the leucocytes of patients with myeloid leukemias were highly positive using this antibody raised against P30-35.

Since the completion of these studies, the P30-35 CAMAL material was further separated into several constituents using reverse phase HPLC. The inhibitory activity on

normal colony formation was shown not to be mediated by known proteins in these preparations, and it was shown using cultures of murine cells that inhibition was mediated by the only protein peak in these preparations which was unique. One of the contaminating proteins, the serine protease cathepsin G, has known reactivity with the peptide substrate with which preparations of P30-35 CAMAL are reactive, however, protein in the unique peak showed activity on this substrate in addition, and it was shown using CMK-linked peptides that a protease activity distinct from that of cathepsin G was present in preparations of P30-35 CAMAL. While it remains a possiblity that the described activities on colony formation could require cathepsin G activity in addition to that of other substances in preparations of P30-35 CAMAL, it is also possible that the observed alterations of *in vitro* myelopoiesis might be mediated by this apparently unique protease activity.

In conclusion, the P30-35 CAMAL, prepared from lysates of leucocytes obtained from patients with myeloid leukemias, was shown to mediate an inhibitory effect on colony formation by normal progenitor cells, which was directed toward CFU-G at low concentrations of P30-35 CAMAL, and toward all progenitor cell types at higher concentrations. A reduction in the number of cells in the non-adherent compartment of long term marrow cultures also occurred, whereas cell numbers were increased in the adherent compartment, suggesting that the cells were held in the stromal layer and blocked in differentiation. The inhibitory effect on colony formation was maintained in cultures of murine bone marrow cells. Similar to the human assays, this effect was directed preferentially toward CFU-G. CFU-S were also reduced. These results suggest that

inhibition of normal colony formation by P30-35 CAMAL is conserved and might be an important regulatory mechanism in myelopoiesis. In contrast, the same preparations of P30-35 CAMAL mediated a stimulatory effect on colony formation by CML progenitor cells, which was directed toward CFU-GEMM at low concentrations of P30-35 CAMAL, and toward all progenitor cell types at higher concentrations of P30-35 CAMAL. The reduction of colony formation by normal progenitor cells and enhancement of colony formation by CML progenitor cells provides a mechanism by which the leukemic clone of cells could gain a growth advantage over normal cells. These effects were demonstrated to require serine protease activity in the P30-35 CAMAL material, which appears to be distinct from that of characterized proteins and proteases from normal neutrophils. Leucocytes from CML patients and the cells of several cell lines derived from the leucocytes of patients with myeloid leukemias showed greatly increased reactivity by immunoperoxidase with a monoclonal antibody raised against P30-35 CAMAL, suggesting that the antigen recognized in previous studies as diagnostic of myeloid leukemias using an antibody directed toward crude preparations of the CAMAL antigen might be associated with the P30-35 CAMAL material as was shown to alter in vitro myelopoiesis by normal and CML progenitor cells.

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APPENDIX 1

DIFFERENTIAL IMMUNOPEROXIDASE STAINING OF CML AND NORMAL CYTOSPIN PREPARATIONS USING α -P30/35

9.1: INTRODUCTION

Immunoperoxidase staining of cytospin preparations using the monoclonal antibody CAMAL-1 was previously shown to be diagnostic of myeloid leukemias (465). In addition, the number of positive cells in CML specimens was found to decrease on induction of clinical remission and an increase in the number of positive cells frequently preceded clinical relapse by up to several weeks (470). Patients with lower bone marrow values of the CAMAL antigen as determined by CAMAL-1 immunoperoxidase staining had significantly longer disease free survival than did patients with higher values. Thus, the detection of the CAMAL antigen was shown to be of prognostic value in these leukemias. The purpose of the studies described in this section was to determine whether the antigen recognized in the studies using CAMAL-1 was the P30-35 component in material enriched from lysates of myeloid leukemia cells using CAMAL-1 immunoaffinity chromatography. Since this P30-35 CAMAL material was demonstrated to be inhibitory to normal myelopoiesis, and stimulatory to leukemic myelopoiesis, these studies were to search for evidence regarding whether or not the CAMAL antigen and the P30-35 CAMAL activity were the same entity. Leucocytes from CML clinical specimens and normal healthy donors enriched for mononuclear cells were stained in an immunoperoxidase slide test using an α -P30/35 monoclonal antibody raised against highly enriched P30-35 CAMAL material. The staining of cell lines derived from myeloid leukemias were also evaluated using this antibody. Conclusions that can be drawn from these studies, are limited by the recent finding that preparations of P30-35 CAMAL are not homogeneous (Appendix 2), however, interesting patterns of reactivity with α -P30/35 did result.

9.2: MATERIALS AND METHODS

9.2.1: Antibodies;

Monoclonal antibodies used in this study, their preparation, and purification, were described in Chapter 2. Briefly; CAMAL-1 was raised against original preparations of the CAMAL antigen, which was isolated by subtractive methods from lysates of myeloid leukemia cells as described in Chapter 1, α -P30/35 was raised and screened against P30-35 material further purified from CAMAL-1 immunoaffinity preparations of CML and or ANLL cell lysates by preparative SDS-PAGE or gel filtration, and α -BLV, used as a control Ab, was raised against P24, the major coat glycoprotein of the bovine leukosis virus. α -P30/35 hybridomas were screened by ELISA for positive reactivity with P30-35 and negative reactivity with HSA, with which CAMAL-1 is known to react. All antibodies were shown to be of the IgG1 subclass by the Ouchterlony technique. Ascites was

prepared from which antibodies were purified by immunoaffinity chromatography using a goat α - mouse Ig immunoadsorbent column, as described in Chapter 2.

9.2.2: Cell lines;

EM2 and EM3 were derived from a patient with CML, HL60 was derived from an acute promyelocytic leukemia, and HEL was derived from an erythrocytic leukemia. K562 was derived from a CML and is Ph⁺. KG1 was derived from an AML. Cell lines were maintained at 37° C, 5% CO₂, and split one in five to one in forty every three to four days. EM2 and EM3 were grown in RPMI medium supplemented with 20% FCS. These cell lines were split one in five to one in ten. HL60, HEL, K562 and KG1 were grown in DME supplemented with 10% FCS. These cell lines were split one in ten to one in forty.

9.2.3: *Preparation of cytospins*;

Cytospins of freshly separated leucocytes enriched for mononuclear cells, from normal or CML peripheral blood, or of cells freshly removed from cell line cultures were prepared. Separation of leucocytes enriched for mononuclear cells from whole blood was by Percoll density centrifugation and is described in Chapter 2. Contaminating red blood cells were removed, when necessary, by lysis with 0.017 M Tris-ammonium chloride (Tris NH₄Cl), pH 7.2. Cell pellets were resuspended in 1 ml Tris NH₄Cl, incubated for 5 min at 37°C, and washed once with fresh Iscove's medium. Cells from cell line cultures were washed twice in Iscove's medium in order to remove FCS. Cytospins were prepared from cell lines with a minimum viability of 98% as determined by dye exclusion counts performed using eosin Y. Cells were resuspended at a density of 1 X 10⁶ cells/ml and spun onto glass slides at 5000 rpm (approximately 100 μ l volume, two drops from a pasteur pipette per slide) using a cytospin apparatus (Shandon).

9.2.4: Serum;

Normal human serum was prepared by drawing blood from a healthy volunteer into a vacutainer tube containing no anticoagulant. Blood was allowed to clot for 30 min, then the clot was removed by centrifugation at 400 X g. The serum was divided into aliquots and stored at -70°C.

9.2.5: Cell staining;

Cytospins were stained by immunoperoxidase within fourteen days of preparation. Cells were fixed by immersion in methanol with 2% H_2O_2 (Fisher) for 30 minutes. Slides were transferred to a staining rack and washed three times with fresh phosphate buffered saline (PBS). Slides were incubated for 30 min at room temperature with the appropriate primary antibody (30 µg/ml in PBS). Slides were again washed three times with PBS, following which they were incubated for 30 minutes with the secondary antibody solution. This consisted of rabbit α -mouse Igs (DAKO) at a 1:100 dilution in PBS containing 4%
normal human serum. Slides were washed three times in PBS, and immersed in a solution of 0.0013% DAB (diaminobenzidine, 3,4,3',4'-tetra-aminobiphenyl hydrochloride, BDH) with 0.06% H_2O_2 for 10 minutes. Slides were then immersed in fresh PBS and counterstained for 90 seconds with hematoxylin (0.5%, Banco, Harris formula, Oxford Scientific). Slides were rinsed with gently running tap water for 5 minutes, then allowed to air dry. Dry slides were mounted with Permount (Fisher) and coverslipped.

Staining was evaluated using an Olympus microscope. Positive reactivity resulted in a brown precipitate. The percentage of cells staining positive in each preparation was estimated by counting positive and negative cells. Photomicrographs were taken of representative preparations.

9.3: RESULTS

Cytospin preparations of mononuclear cell-enriched leucocytes from CML clinical specimens showed greater reactivity with α -P30/35 than did cytospins of mononuclear cell-enriched leucocytes from normal healthy donors. This is a similar pattern of reactivity to that previously described using the CAMAL-1 monoclonal antibody and reproduced here as a positive control. These results are summarized in Table IX. Photomicrographs of representative specimens are shown in Figure 31. In individual CML clinical specimens, positive staining was usually found to be more extensive using α -P30/35 than using CAMAL-1 (Figure 31a and b). In all cases, background reactivity as determined using a

non-specific monoclonal antibody of the same subclass, α -BLV, was low (Figure 31c). Although the CML cells appear to stain non-specifically to a greater extent than do normal cells (Figure 31c), the level of staining obtained using either a-P30/35 of CAMAL-1 was clearly greater than that obtained using the control antibody (Figures 31 a anf b). In each CML specimen, the amount of staining with control antibody was defined as 'background', and the number of cells staining to a clearly greater extent than this level was evaluated. The difference in staining between CML and normal cells was greater using α -P30/35 than using CAMAL-1 (Figure 31a, b, and d, Table IX).

Several human cell lines derived from myeloid leukemias showed reactivity with α -P30/35. These results are summarized in Table X, and photomicrographs shown in Figure 32. The cell lines EM2, EM3, HL60, K562, and KG1 all stained extensively with α -P30/35 (Figure 32a, d, and f, and Table X). In all cases, background reactivity asdetermined using a non-specific monoclonal antibody of the same subclass, α -BLV, was low (Figure 32c). In addition, the cell line HEL was negative with all antibodies.(Figure

normal healthy donors.				
PBL specimen		% positive cells		
· · · · · · · · · · · · · · · · · · ·	CAMAL-1	α-P30/35	α-BLV	
CML			· · ·	
1	<2	>5	<2	
2	>5	>25	<2	
3	>25	>50	<2	
4	<2	>25	<2	
5	>10	>10	<2	
6	>10	>50	<2	
normal				
1	>5	<2	<2	
2	>5	<2	<2	
3	>5	<2	<2	
4	>5	<2	<2	
5	<2	<2	<2	
6	<2	>5	<2	

TABLE IX. Immunoperoxidase staining of peripheral blood mononuclear cells from CML patients and normal healthy donors.

32e). Staining of positive cell lines was far less extensive using CAMAL-1 than using α -P30/35, but was above background (Figure 32a and b), with the exception of HL60, which reacted to a similar extent using either CAMAL-1 or α -P30/35 (Figure 32f and g).



a

FIGURE 31. Preferential staining by immunoperoxidase of mononuclear cells obtained from the peripheral blood of patients with CML as compared to normal healthy donors using α -P30/35. Cells in panels a through c were derived from the same CML donor. a. CML peripheral blood cells stained using α -P30/35. b. CML peripheral blood cells stained using CAMAL-1. c. CML peripheral blood cells stained using the control antibody α -BLV. d. Normal peripheral blood cells stained using α -P30/35. e. Peripheral blood cells from a second CML donor stained using α -P30/35.





e



TABLE X. Immunoperoxidase staining of cell lines derived from human myeloid leukemias.					
CELL LINE	% positive cells				
	CAMAL-1	α- P 30/35	α-BLV		
EM2	> 5	> 75	< 2		
EM3	> 5	> 75	< 2		
HL60	> 80	> 80	< 5		
K562	. > 5	> 80	< 2		
KG1	> 10	> 80	< 2		
HEL	< 2	< 2	< 2		



FIGURE 32. Immunoperoxidase staining of myeloid leukemia-derived cell lines. a. EM3 stained using α -P30/35. b. EM3 stained using CAMAL-1. c. EM3 stained using α -BLV. d. EM2 stained using α -P30/35. e. HEL stained using α -P30/35. f. HL60 stained using α -P30/35. g. HL60 stained using CAMAL-1.

d









9.4: DISCUSSION

It was of interest to determine whether the antigen recognized in immunoperoxidase studies using the CAMAL-1 antibody as diagnostic of myeloid leukemias was the same P30-35 CAMAL material enriched using CAMAL-1 immunoaffinity chromatography, and shown mediate an inhibitory effect on colony formation by normal progenitor cells and a stimulatory effect on colony formation by CML progenitor cells. That the CAMAL antigen and the P30-35 CAMAL material active on myelopoiesis might be the same entity was originally suggested by the finding that a rabbit polyclonal antibody raised against original preparations of the CAMAL antigen, and which was found to preferentially stain cells from patients with myeloid leukemias (465) was reactive with P30-35 CAMAL by Western blot analysis (474).

A similar pattern of staining was observed using the monoclonal antibodies α -P30/35, raised against preparations highly enriched for P30-35 CAMAL, or CAMAL-1, raised against original preparations of the CAMAL antigen; mononuclear cell-enriched leucocytes from CML clinical specimens generally stained to a greater extent using either antibody than did cells from normal healthy donors. This result initially suggested that the two antibodies might recognize the same component (P30-35 CAMAL) on and within these cells. However, more recent data indicate that the CAMAL-1 antibody is not reactive with P30-35 CAMAL by Western blot analysis (474, 476). P30-35 CAMAL and the entity recognized by CAMAL-1 in the immunoperoxidase studies thus do not appear to be the same entity. The similarities in patterns of cell staining obtained using these antibodies,

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however, suggest that the CAMAL antigen and P30-35 CAMAL might form a complex, the different components of which might be recognized by the two antibodies.

 α -P30/35 was found to stain a greater number of cells in CML clinical specimens and cell lines derived from myeloid leukemias than did CAMAL-1 in many cases. This result suggests that α -P30/35 has greater specificity for myeloid leukemia-derived cells than does CAMAL-1. Recent further separation of preparations of P30-35 CAMAL has shown that other protein species such as elastase, cathepsin G and azurocidin are present within these preparations (Appendix 2). It must be recognized, then, that this antibody might recognize these proteins within the myeloid leukemia cells. In this regard, recent evidence shows that α -P30-35 is reactive with elastase, azurocidin, and cathepsin G, but not with myeloblastin, by Western blot analysis (474, 476).

Staining by α -P30/35 and CAMAL-1 appeared in non-identical cell compartments. For example, CAMAL-1 was found to stain membranes (465, Figure 31b) to a greater extent than did α -P30/35, which localized mainly to the cytoplasm and perinuclear space (Figure 31a and e). This result supports the idea that the two antibodies might recognize different proteins. The similarities in patterns of staining, however, suggest that these proteins might form an association. For example, CAMAL-1 might preferentially recognize a P30-35 CAMAL substrate or carrier molecule, such as HSA, which is known to carry many molecules, on the cell surface, whereas α -P30/35 might recognize free P30-35 CAMAL in the cytoplasm or P30-35 CAMAL in the process of being synthesized or packaged.

Positive staining of cell lines using CAMAL-1 and α -P30/35 suggests that they might produce P30-35 CAMAL. This is consistent with the previously reported observation that a rabbit antiserum raised against highly purified P30-35 recognized a protein in the range of 30 to 35 kDa by Western blot analysis in lysates from the myeloid leukemia-derived cell lines HL60 and K562 (472). It is interesting that the cell lines that stained positive using α -P30/35 are the same cell lines that responded to P30-35 CAMALmediated enhancement in colony assays. Colony formation by EM2, EM3, and HL60 was stimulated by P30-35 CAMAL (Chapter 4); these cell lines all stained extensively using α -P30/35. In addition, preliminary results showed that colony formation by K562 and KG1 was also stimulated by P30-35 CAMAL (data not included). These cell lines also stained extensively using α -P30/35. In contrast, colony formation by the cell line HEL was not affected by treatment with P30-35 CAMAL (chapter 4); this was the only cell line tested that did not stain using α -P30/35. Hence, the cell populations that appear to produce P30-35 CAMAL are the same cells that are affected by upregulation of their clonogenicity. This might be an indication that P30-35 CAMAL could function as an autocrine regulatory mechanism gone awry in CML.

The cell types showing positive reactivity with these antibodies was not evaluated. This was due to the recognition, after the slides were prepared, but before they were evaluated, that the material against which α -P30/35 was raised consists of a mixture of proteins (Appendix 2). Subsequent work has shown that α -P30/35 does indeed react with several of the proteins known to be present in this mixture by Western blot analysis (474).

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It is the opinion of the author that the similarities in pattern of staining of CML and normal cells using the antibodies α -P30/35 and CAMAL-1 suggest an association between the CAMAL antigen and the P30-35 CAMAL material which mediates the alterations of myelopoiesis described in this paper. Definitive evaluation of cell types which produce or react with P30-35 CAMAL, however, must await conclusive definition of the entity involved in these effects at a biochemical level. The cell types in question can then be defined using antibodies raised against this material, or using molecular probes.

In summary, mononuclear cell-enriched leucocytes from CML patients and several myeloid leukemia-derived cell lines stained to a greater extent than did mononuclear cells from normal healthy donors in an immunoperoxidase slide test using a monoclonal antibody raised against P30-35 CAMAL. This is a similar result to that previously reported using CAMAL-1, a monoclonal antibody raised against original crude preparations of the CAMAL antigen, and suggests that the antigen recognized as diagnostic of myeloid leukemias in the studies using CAMAL-1 might be associated with P30-35 CAMAL, the material shown to alter myelopoiesis by normal and CML progenitor cells. Cell lines which stained using α -P30/35 were the same cell lines that responded by enhancement of colony formation to treatment with P30-35 CAMAL, suggesting that they might produce P30-35 CAMAL and respond to it in an autocrine manner. Conclusions that can be drawn from these studies, however, are limited by the recent finding that preparations of P30-35 CAMAL, which α -P30/35 was raised against, are not homogeneous, and that this antibody likely reacts with several components in these preparations (Appendix 2).

APPENDIX 2

FURTHER EVIDENCE THAT P30-35 CAMAL ACTIVITY IS MEDIATED BY A COMPONENT IN PREPARATIONS OF P30-35 CAMAL WHICH IS DISTINCT FROM CHARACTERIZED NEUTROPHIL PROTEINS

The investigations reported up to this point were completed by August, 1991. There was a hiatus of nine months between completion and documentation of the described studies. During this time, progress was made with the definition of P30-35 CAMAL at a subcellular level, the project of another graduate student in this laboratory. P30-35 CAMAL prepared by enrichment from CML cell lysates using an α -P30/35 immunoadsorbent column was separated by reverse phase HPLC and shown to consist of several protein components (476). Three of the peaks obtained in these separations corresponded to known neutrophil proteins, including cathepsin G, elastase, and azurocidin. A fourth peak, which was the major species in many of the reverse phase HLPC preparations, was unique to preparations of P30-35 CAMAL as compared to previously published information on separations of neutrophil proteins (477). Additional studies were undertaken in order to determine whether the effects of P30-35 CAMAL on myelopoiesis by normal and CML progenitor cells might be mediated by characterized proteins present in these preparations. It should be noted that studies of the biological activities of P30-35 CAMAL were largely performed using material enriched from CML cell lysates by sequential elution from CAMAL-1 and α -P30/35 immunadsorbent columns,

whereas reverse-phase HPLC separations were performed using material enriched from CML cell lysates using an α -P30/35 column only. The relative proportions of neutrophil proteins in material enriched from CML lysates using sequential elution from CAMAL-1 and α -P30/35 columns is currently under investigation (496).

Elastase was previously shown to be inhibitory to colony formation by progenitor cells from normal healthy donors. In contrast to P30-35 CAMAL, however, elastase is also inhibitory to colony formation by progenitor cells from patients with CML (Figure 10, Chapter 2). Since preparations of P30-35 CAMAL were screened to ensure that no inhibitory activity against CML colony formation was present, elastase is in all probability a minor contaminant of the preparations that were used to characterize the activities of P30-35 CAMAL reported in this study. Studies using CMK-linked peptides in an attempt to block the activity of P30-35 CAMAL on colony formation support this; the elastase blocker alaala-pro-val-CMK did not block the inhibitory activity of P30-35 CAMAL preparations on colony formation by normal progenitor cells (Figure 27, Chapter 4). This is an indication that the inhibition of colony formation by normal progenitor cells on treatment with P30-35 CAMAL is not mediated by elastase. Similarly, the enhancement of colony formation by CML progenitor cells on treatment with P30-35 CAMAL cannot be mediated by elastase since elastase was shown to be inhibitory to colony formation by CML progenitor cells.

Myeloblastin, or proteinase 3, was reported to be present in preparations purified from the azurophilic granules of normal neutrophils (438). The activity of P30-35 CAMAL is not likely to be mediated by myeloblastin for several reasons. The activity of myeloblastin was evaluated in a colony assay using normal human cells; it was not inhibitory to colony formation, and, in fact, a slight enhancement of colony formation was seen (Figure 33a). Enhancement of colony formation by CML progenitor cells is not likely to be due to myeloblastin since myeloblastin was found to be a very minor component of reverse phase HPLC-separated preparations of P30-35 CAMAL (<1%). In addition, myeloblastin is known to preferentially recognize chromogenic peptide substrates that are substantially different than those which are recognized by preparations of P30-35 CAMAL (438).

It was considered to be unlikely that the observed alterations in colony formation by normal and by CML progenitor cells were due to azurocidin, since azurocidin has substitutions in two of the three amino acid residues of the serine protease active site, and has no protease activity on a panel of chromogenic peptide substrates (438). In contrast, protease activity was shown to be required for the effects on colony formation by both normal and CML progenitor cells (Chapter 5). However, the activity of azurocidin was evaluated in a colony assay using normal human cells nonetheless. Azurocidin was not inhibitory to colony formation by normal progenitor cells, and in fact a slight enhancement of colony formation was observed (Figure 33a). Enhancement of colony formation by CML progenitor cells is not likely to be attributable to azurocidin since protease activity was shown to be required for this effect as well.

Similarly, cathepsin G was not inhibitory to colony formation by normal progenitor cells, at a protein concentration of up to 15 μ g/ml (Figure 33 a & b). The possibility, however, that the enhancement of colony formation by CML progenitor cells could be mediated by cathepsin G, cannot yet be ruled out, since cathepsin G is known to recognize

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FIGURE 33. Lack of inhibitory activity of myeloblastin, azurocidin, and cathepsin G on colony formation by progenitor cells from a normal healthy donor. Methylcellulose cultures were used, as described in Chapter 2. a. Conditions of preincubation, as described in Chapters 3 and 4, using myeloblastin, azurocidin, and cathepsin G, as compared to the activity of a P30-35 CAMAL preparation. Cells were treated with 100 ng/ml of protein in each case. In this experiment, 'CAMAL' refers to P30-35 CAMAL. b. Conditions of coculture, as described in Chapter 2, using cathepsin G.

the substrate ala-ala-pro-phe-NA, which is the same substrate with which preparations of P30-35 CAMAL were found to react. This possibility is currently under investigation (496). It is also possible that the alterations in colony formation by CML progenitor cells might be mediated by the action of a non-protease substance in preparations of P30-35 CAMAL in conjunction with cathepsin G. For example, cathepsin G might alter structures on the cell surface in such a way that they are rendered susceptible to the activities of a second substance. Enzyme assays using cathepsin G and preparations of P30-35 CAMAL, however, indicate the presence of a protease activity in preparations of P30-35 CAMAL which appears to be unique. A preparation of P30-35 CAMAL which was fully characterized as to its effects on myelopoiesis in all systems described in this paper (inhibition of normal human colony formation, effect on long-term culture of human marrow, inhibition of murine CFU-G, stimulation of CML colony formation, and stimulation of colony formation by the CML-derived cell line EM3) was used for this comparison with cathepsin G. The activity of P30-35 CAMAL on the substrate ala-ala-prophe-NA was fully blocked by the ala-ala-pro-val-CMK peptide in an enzyme assay at a CMK:P30-35 CAMAL molar ratio of 10:1, whereas the activity of cathepsin G on the same substrate was not affected by ala-ala-pro-val-CMK at a CMK:cathepsin G molar ratio of 100:1 (Figure 34a). This difference in activity cannot be attributed to a blockage of elastase activity, since elastase does not react with the chromogenic peptide substrate used to evaluate activity in this experiment (476). Similarly, the activity of P30-35 CAMAL was blocked by the peptide ala-pro-phe-CMK almost fully at a molar ratio of 1:1, whereas the activity of cathepsin G using this same CMK-linked peptide as a putative blocker of activity was only minimally affected at a molar ratio of 1:1, and was not fully blocked even at a level of 10:1 (Figure 34b). Thus, there appears to be a protease activity in preparations of





P30-35 CAMAL which is unique, and the possibility remains that the enhancement of colony formation by CML progenitor cells mediated by P30-35 CAMAL preparations might be due to this activity. Other than elastase, cathepsin G, and the unique peak, no other major peaks were present in reverse phase HPLC separations of P30-35 CAMAL preparations (476, 486).

The unique peak in preparations of P30-35 CAMAL, which is distinct from the peak known to contain cathepsin G, has been shown to cleave the chromogenic peptide substrate ala-ala-pro-phe-NA, with which preparations of P30-35 CAMAL are reactive (476). Moreover, it was recently shown that this fraction unique to preparations of P30-35 CAMAL is inhibitory to colony formation by murine progenitor cells (486). The inhibitory activity of P30-35 CAMAL on normal myelopoiesis, then, appears to be mediated by protein in this peak. Reverse phase HPLC-purified material is undergoing amino acid sequence analysis, and further material is currently being purified for evaluation of its activity on colony formation by CML progenitor cells (496).

In summary, P30-35 CAMAL was separated by reverse-phase HPLC as part of a separate ongoing study, and was shown to consist of more than one protein species. Many of these proteins have been characterized; these include elastase, myeloblastin, azurocidin, and cathepsin G. A major peak which was unique to preparations of P30-35 CAMAL and not found in preparations derived from normal neutrophils was also present in reverse-phase HPLC separated P30-35 CAMAL (477). The activities of P30-35 CAMAL preparations on colony formation by normal and CML progenitor cells could not be

attributed to elastase, myeloblastin, or azurocidin. Similarly, the inhibitory activity of P30-35 CAMAL on colony formation by normal progenitor cells could not be attributed to cathepsin G, and was shown to be mediated by the unique peak in preparations of P30-35 CAMAL. The possibility remains that the stimulatory activity on colony formation by CML progenitor cells might be mediated by cathepsin G. However, an enzyme assay which compared the activity of a preparation of P30-35 CAMAL to that of cathepsin G demonstrated the presence of a protease activity in the P30-35 CAMAL preparation which appears to be unique, and it remains a possibility that the activity of P30-35 CAMAL on colony formation by CML progenitor cells is mediated by this fraction.