

The molecular characterization of a common  
human myelogenous leukemia-associated  
antigen (CAMAL)

by

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## Abstract

Previous studies had demonstrated the presence of the p70 (CAMAL) molecule in human myeloid leukemia cells and the promyelocytic leukemia cell line HL60, but not in equivalent preparations of normal cells (Malcolm et al., 1982, 1984; Shipman et al., 1983; Logan et al., 1984). Subsequent studies demonstrated that the p70 (CAMAL) protein was detectable and expressed in human myeloid leukemia cells and the leukemic cell lines HL60, KG1, K562 and U937. The association of p70 (CAMAL) expression with human myeloid leukemia cells prompted its consideration as a candidate leukemia-associated antigen.

The demonstration, following CAMAL purification and peptide sequencing, that two tryptic peptides (tp27, tp31) displayed significant homology to sequences present in human serum albumin (HSA) and human alpha-1-fetoprotein (AFP), while one tryptic peptide (tp20) displayed unique peptide sequence, suggested that CAMAL might represent a protein that was structurally and functionally related to the albumins. Consequently, a comparative biochemical analysis of CAMAL and HSA was initiated.

The results of the comparative studies demonstrated that although CAMAL and HSA shared conformational antigenic determinants, both proteins were also shown to be distinct molecules by a number of other criteria. The possibility that the CAMAL preparation, used for protein sequencing and comparative studies, was contaminated with HSA was thought likely, in view of the HSA/AFP-related peptide sequences from the CAMAL tryptic peptide sequence analysis.

However, other results, particularly the antibody reactivity and ligand binding studies, showed that the CAMAL preparation was not contaminated with HSA. The unique CAMAL tryptic peptide (tp20) sequence supported further the contention that CAMAL was a distinct protein with regions homologous to HSA and AFP.

Further analysis of the CAMAL molecule, through extensive protein sequencing, will be, in all likelihood, the only means by which to establish the degree of relatedness between CAMAL, HSA and AFP.

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## Chapter 1. Introduction

### 1.1. The human hematopoietic system.

Circulating red and white blood cells have finite lifespans and must therefore be replaced constantly throughout the life of the individual. This process of differentiation and functional maturation of specific lineages of red and white blood cells, termed hematopoiesis, is both expansive, in terms of the number of cells regulated within the bone marrow and in the blood, and intricate, in the sense that all the progenitor cells and functional subsets of all the hematopoietic cell lineages coexist, at varying levels of maturity, in the bone marrow. The liberation of committed progenitor cells or 'mature' hematopoietic cells from the bone marrow, in response to the demand for functionally active blood cells, is also a dynamic and complex process. The existence of a small population of multipotential stem cells in the mammalian bone marrow and their ability to maintain the status of the hematopoietic system throughout life, as well as retaining an extensive capacity for self-generation, represents one of the most novel and complex cellular differentiation programs known.

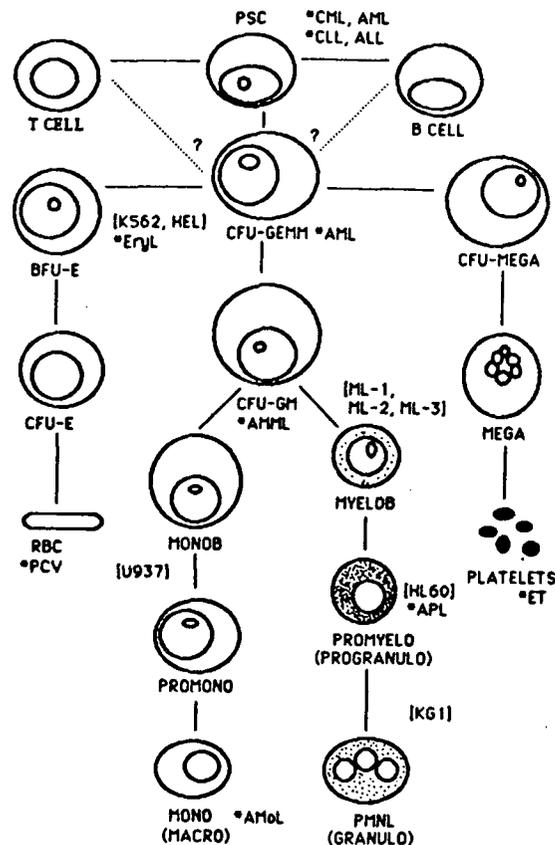
The capacity of these stem cells to give rise to progenitor cells committed to differentiation into all cells of the myeloid, lymphoid and erythroid lineages is well documented (Metcalf et al., 1984). The ability of each individual hematopoietic stem cell to regenerate itself, by giving rise to new pluripotent stem cells ['self-renewal'], or to produce committed progenitor cells is one of the central postulates in

Figure 1. Hematopoietic stem cell differentiation.

PSC : pluripotent stem cell, CFU-GEMM : colony forming unit-granuloid/erythroid/myeloid/megakaryocytoid, BFU-E : burst forming unit-erythroid, CFU-E : colony forming unit-erythroid, CFU-MEGA : colony forming unit-megakaryocytoid, CFU-GM : colony forming unit-granuloid/myeloid, RBC : red blood cell/erythrocyte, MEGA : megakaryocyte, MONOB : monoblast, MYELOB : myeloblast, PROMONO : promonocyte, PROMYELO/GRANULO : promyelocyte/granulocyte, MONO/MACRO : monocyte/macrophage, PMNL/GRANULO : polymorphonuclear leukocyte/granulocyte, AML/CML : acute/chronic myeloblastic leukemia, ALL/CLL : acute/chronic lymphoblastic leukemia, AMML : acute myelomonoblastic leukemia, AMoL : acute monoblastic leukemia, APL : acute promyelocytic leukemia, PCV : polycythemia vera, EryL : erythroleukemia, ET : essential thrombocytosis.

\* denotes disease states associated with specific progenitor/precursor cells.

[ ] denotes leukemic cell lines derived from specific leukemic cells.



hematopoiesis and one of the least understood. It is assumed that homeostasis of the hematopoietic system is achieved through the interaction of an invariant population of pluripotential bone marrow stem cells with both serum or cellular factors as well as factors liberated by stromal (non-hematopoietic, fibroblastic, dendritic, adipocytic) cells within the bone marrow microenvironment (Chan et al., 1972; Dexter et al., 1977; Onada et al., 1980; Lanotte et al., 1982). As such, these interactions must mobilize the bone marrow stem cell population in such a way that the capacity to produce committed, lineage-specific progenitor cells, in response to the demand for fully mature, functionally active blood cells, does not affect the capacity of these same cells to regenerate, and thus keep constant, the bone marrow population of pluripotential stem cells. Abnormalities in the interaction between stromal cells and hematopoietic progenitor cells are therefore likely to be important in the pathogenesis and clinical expression of hematopoietic malignancies in humans (Greenberg et al., 1981; Lanotte et al., 1982). The process by which the pluripotential stem cell is able to secure progenitor cell fidelity, such that specific blood cell lineages are generated, and furthermore, how the transition from stem cell to progenitor cell to mature blood cell is regulated, is still unclear. The control of stem cell regeneration, proliferation or differentiation is presumed to involve an interactive matrix of diffusible cellular growth factors and direct cell contact interactions. However, the precise mode(s) of interaction and transmission of the growth-modulatory signals have yet to be elucidated.

## 1.2. The control of hematopoiesis.

Mechanisms governing hematopoiesis, at the level of the bone marrow stem cell, must involve, in part, microenvironmental regulation by stromal cells through either direct cell contact or the production of specific cellular factors at the sites of blood cell formation in the bone marrow (Chan et al., 1972; Lanotte et al., 1982). It is presumed that microenvironmental cell interactions and the liberation of short-range cellular factors are not the only means by which blood cell formation is regulated. *In vitro* systems have been established which implicate a family of serum/cellular glycoprotein factors in the regulation of normal bone marrow cell proliferation, differentiation and functional activation (Metcalf et al., 1984).

The identification and characterization of these growth regulatory factors, from tissue or cell conditioned medium, was due, in large part, to the development of specific *in vitro* methods for the propagation of hematopoietic stem/progenitor cells in semisolid culture medium. Using bone marrow cells and factor-containing supernatants, or purified factor, this technique permitted the formation of colonies of mature hematopoietic progeny cells, clonally expanded from individual progenitor cells present in the initial bone marrow sample. That the stimulation of hematopoietic stem cell differentiation is due to specific growth factor(s), present in the conditioned medium, and that subsequent maturation is also dependent on the continued presence of this factor(s) is an established fact. Although the original culture technique only permitted the generation of granulocytic or monocyte-macrophage colonies, modifications of the initial method supported the

clonal proliferation of stem cells, from both human and animal sources, into 'mature' colonies representative of all the known hematopoietic cell lineages (Metcalf et al.,1984). It is assumed, from the analysis of *in vitro* differentiation experiments, that the process by which 'mature' progeny cells are generated from stem cell progenitors is both functionally and mechanistically analogous to the process of hematopoietic stem cell differentiation *in vivo* .

The observation that hematopoietic stem cells have an absolute requirement for factors, in order to permit cell division and the progression through the cell cycle, and that these factors are capable of maintaining growth, proliferation and functional differentiation of mammalian bone marrow cells *in vitro* into colonies of maturing progeny cells, prompted their designation as 'colony stimulating factors' [CSFs]. The best characterized CSFs are the granulocyte-macrophage colony stimulating factors [GM-CSFs]. These CSFs have been characterized extensively in the murine hematopoietic system (Metcalf et al.,1984) and are currently the focus of critical examination in the human hematopoietic system (Metcalf et al.,1985b).

### 1.3. The colony-stimulating factors (CSFs).

Four major GM-CSFs have been characterized, in the murine system, that are thought to interact to an extent that control of both the formation and functional activation of granulocytes and monocyte-macrophages is achieved (Metcalf et al.,1985a; Table 1). The purification, to homogeneity, of three of the four known human counterparts of these murine CSFs has been accomplished but the extent

to which these human CSFs interact is still largely unknown (Table 2). However, two distinct human factors, CSF  $\alpha$  and CSF  $\beta$ , have been identified which exhibit some functional overlap with murine GM-CSF and G-CSF, respectively (Metcalf et al., 1985b).

Each of the four murine CSFs was purified to homogeneity from medium conditioned by cells or tissue. All four CSFs are extensively glycosylated proteins, 40-60% carbohydrate, and it has been suggested from both deglycosylation experiments and the analysis of CSFs from tunicamycin-treated cells, that the carbohydrate moiety is not required for biological activity *in vitro*. Proteolytic cleavage of purified GM-CSFs abrogates activity *in vitro* and, as yet, no active proteolytic cleavage fragments of the GM-CSFs have been detected. Treatment of GM-CSFs with reducing agents also destroys activity *in vitro*, suggesting that disulfide bonds and the tertiary structure specified by these intramolecular bonds is obligatory for biological activity.

Although a wide variety of normal tissues and cells have been shown to produce one or more of the GM-CSFs, the minute quantities produced (eg. 2-10  $\mu$ g per 1000 mouse lungs) have restricted the structural analysis of the individual purified GM-CSFs (Burgess et al., 1977; Sparrow et al., 1985). As a consequence, molecular cloning of the genes encoding the GM-CSFs was undertaken. cDNA has been cloned for both murine multi-CSF (Yokota et al., 1984; Fung et al., 1984) and GM-CSF (Gough et al., 1984) as well as human GM-CSF (CSF  $\alpha$ ; Wong et al., 1985; Nagata et al., 1986; Nomura et al., 1986) and urinary CSF (Kawasaki et al., 1985). Expression of these cloned cDNAs in mammalian or bacterial expression systems has demonstrated that the CSFs produced in this manner exhibit biological activity indistinguishable

Table 1. The murine granulocyte-macrophage colony stimulating factors.<sup>1,2</sup>

Factor	M <sub>r</sub> <sup>*</sup>	Source	Amino acid sequence	Major progeny cells	Receptor M <sub>r</sub>	Receptor number per cell
GM-CSF <sup>a</sup>	23000 monomer	Mouse lung	Full	Granulocytes and macrophages	51000	70-350
G-CSF <sup>b</sup>	25000 monomer	Mouse lung	N-term.	Granulocytes	150000	50-700
Multi-CSF <sup>c</sup>	23000-28000 monomer	WEHI-3B cell line	Full	Granulocytes, macrophages, erythroid cells, megakaryocytes, mast and stem cells	55000, 75000	50-5000
M-CSF <sup>d</sup> (CSF-1)	45000-76000 dimer 28000-35000 monomer 14000-16500 glycosidase-treated monomer	Mouse L cells	N-term	Macrophages	165000	3000-50000

\* Relative molecular weight; ND: not determined.

<sup>1</sup> Burgess et al., Growth Factors and Stem Cells,(Sydney, Academic Press), 1983.  
Burgess et al., Biochem.J. 185:301-314, 1980.

<sup>2</sup> Metcalf et al., The Hemopoietic Colony Stimulating Factors,(New York, Elsevier), 1984.

<sup>a</sup> GM-CSF: granulocyte-macrophage colony stimulating factor  
Burgess et al., JBC 252:1998-2003, 1977; Gough et al., Nature 309:763-767, 1984;  
Sparrow et al., PNAS 82:292-296, 1985; Walker et al., EMBO J. 4:933-939, 1985;  
Metcalf et al., Blood 55:138-145, 1980.

<sup>b</sup> G-CSF: granulocyte colony stimulating factor  
Nicola et al., JBC 258:9017-9023, 1983; Nicola et al., PNAS 81:3765-3769, 1984;  
Tsuchiya et al., PNAS 83:7633-7637, 1986; Nicola et al., J.Cell.Physiol. 124:313-321,  
1985; Lopez et al., J.Immunol. 131:2983-2988, 1984; Metcalf et al., J.Cell.Physiol.  
116: 198-206, 1983; Nicola et al., JBC 258:9017-9021, 1983.

<sup>c</sup> Multi-CSF: multipotential colony stimulating factor Yokota et al., PNAS 81:1070-1074, 1984. Also described as Interleukin 3 (IL-3; Fung et al., Nature 307:233-237, 1984; Palaszynski et al., J.Immunol. 132:1872-1878,1984; Inle et al., J.Immunol. 129:2431-2436, 1982), haemopoietic cell growth factor (HCGF; Bazill et al., Biochem.J. 210:747-759, 1983), mast cell growth factor (MCSF; Yung et al., J.Immunol. 127:794-799, 1981) or P cell-stimulating factor (PSF; Clark-Lewis et al., J.Immunol. 127:1941-1947, 1981).

<sup>d</sup> M-CSF (CSF-1): macrophage colony stimulating factor (colony stimulating factor 1)  
Stanley et al., JBC 252:4305-4312, 1977; Tushinski et al., Cell 28:71-81, 1982;  
Morgan et al., BBRC 119:35-41, 1984.

Table 2. The human granulocyte-macrophage colony stimulating factors.

Factor	M <sub>r</sub> *	Source	Amino acid sequence	Major progeny cells	Receptor M <sub>r</sub>	Receptor number per cell
GM-CSF <sup>a</sup> (CSF $\alpha$ )	22000 monomer	Mo T-lymphoblast cell line	Full	Granulocytes, macrophages	ND	ND
G-CSF <sup>b</sup> (CSF $\beta$ )	30000 monomer	Human placental-conditioned medium	ND	Granulocytes	ND	300-480
U-CSF <sup>c</sup> (CSF-1) (M-CSF)	45000-60000 dimer 28000 monomer	Urine	Full	Macrophages	ND	ND
Pluripoietin <sup>d</sup>	18000 monomer	Human bladder carcinoma cell line 5637	ND	Granulocytes, macrophages, erythroid cells, mixed colonies	ND	ND

\* Relative molecular weight; ND: not determined.

- <sup>a</sup> GM-CSF (CSF $\alpha$ ): granulocyte-macrophage colony stimulating factor (colony stimulating factor). Murine and human GM-CSF share sequence homology but are not species cross-reactive (Nicola et al. *Nature* 314:625-628, 1985). Human GM-CSF contains erythroid burst-promoting activity (BPA; Linch et al., *Nature* 312:775-777, 1984) but not erythroidpotentiating activity (EPA; Westbrook et al., *JBC* 259:9992-9996, 1984; Gasson et al., *Nature* 315:768-771, 1985). Gasson et al., *Science* 226:1339-1342, 1984; Sieff et al., *Science* 230:1171-1173, 1985; Wong et al., *Science* 228:810-814, 1985.
- <sup>b</sup> G-CSF (CSF  $\beta$ ): granulocyte colony stimulatig factor (colony stimulating factor  $\beta$ ). Murine G-CSF and human CSF  $\beta$  are fully species cross-reactive and may be homologous (Nicola et al., *Nature* 314:625-628, 1985). Nicola et al., *Blood* 54:614-627, 1979.
- <sup>c</sup> U-CSF (CSF-1, M-CSF): urinary colony stimulating factor (colony stimulating factor; macrophage colony stimulating factor). Murine M-CSF and human U-CSF share sequence homology and both are species cross-reactive (Kawasaki et al., *Science* 230:291-296, 1985). Human U-CSF also shows structure-activity similarities to human  $\beta$  nerve growth factor ( $\beta$ -NGF; Server et al., *Adv.Prot.Chem.* 31:339-409, 1977). Stanley et al., *Fed.Proc.* 34:2272-2278, 1975; Das et al., *Blood* 58:630-641, 1981; Das et al., *JBC* 257:13679-13684, 1982.
- <sup>d</sup> Pluripoietin: pluripotential hematopoietic colony-stimulating factor. May be homologous to human GM-CSF (Wong et al., *Science* 228:810-814, 1985). Welte et al., *PNAS* 82:1526-1530, 1985.

from that of the purified CSF. Amino acid sequence data indicate that none of the four murine CSFs share any significant homology with each other even though the CSFs of the murine hematopoietic system, and presumably the human system, demonstrate extensive functional homology and individual progenitor cells seem to be able to respond to more than one CSF.

#### 1.4. Function of the CSFs.

The CSFs were originally characterized on the basis of their absolute requirement in the stimulation of hematopoietic cell colony formation. Although this function is of primary importance to hematopoiesis, CSFs also possess several other important properties: (i) the promotion and maintenance of stem/progenitor cell and colony survival, (ii) the ability to secure commitment of progenitor cells to lineage-specific differentiation and (iii) the functional activation of 'mature' hematopoietic cells. Hematopoietic stem cells and/or 'mature' blood cells in active cell cycle, *in vitro* and presumably *in vivo*, can not complete the cycle in the absence of the appropriate CSF. This abrupt cessation of growth *in vitro* is attributable to the complete abrogation of nucleic acid and protein synthesis and eventually results in cell death, the 'lag-time' being dependent on the type of cell affected and its level of 'maturity' (Metcalf et al.,1982a; Whetton et al.,1983; Metcalf et al.,1985c). CSFs are also required to maintain cell proliferation *in vitro*. The ability of these cells to proliferate in a normal fashion can only be restored by the addition of exogenous CSF(s). The proliferation of hematopoietic cells and the magnitude of the concomitant

proliferation of these cells is determined in a dose-dependent manner by the extracellular concentration of CSF(s) and by the distribution of specific CSF receptors on the responsive sub-population of hematopoietic cells (Whetton et al.,1983). The precise biochemical processes by which CSFs induce cells to traverse the cell cycle, divide and differentiate are unclear. However, autophosphorylation of specific CSF receptors, changes in the synthesis rates of cytoplasmic and nuclear proteins, phosphorylation of intracellular proteins, most notably p21<sup>ras</sup> and p53, have all been observed in CSF-stimulated hematopoietic cells (Metcalf et al.,1985a; Stanley et al.,1983).

All four murine CSFs differ in their ability to stimulate the proliferation of hematopoietic cells of other cell lineages. Multi-CSF is able to stimulate the proliferation of multipotential, erythroid, megakaryocytic and mast cells whilst M-CSF can only affect the proliferation of macrophage progenitors. Studies to date suggest that this spectrum of lineage-specificity of the CSFs is not due to CSF promiscuity but, more likely, due to the variation in the distribution of specific CSF receptors on the membranes of hematopoietic stem/progenitor cell subpopulations (Metcalf et al.,1980,1983,1984). Since CSFs act directly on both multipotent stem cells and bipotent progenitor cells, to elicit the formation of all the hematopoietic cell lineages, it is evident that the CSFs are not merely mediators of cellular proliferation. The ability of the CSFs to irreversibly commit progenitor cells to the production of hematopoietic cells of a defined cell lineage is extensively documented (Metcalf et al.,1984). Although the CSFs are able to restrict the differentiation of progenitor cells, both multipotent stem cells and bipotent progenitor cells exhibit considerable variation in

the degree of responsiveness to a given CSF, assessed mainly by the 'burst size' [the number of progeny cells generated] and the CSF concentration needed to stimulate and maintain progeny cell division (Metcalf et al.,1984).

The CSFs are also responsible for inducing a variety of functional activities in a number of 'mature' hematopoietic blood cells. These include phagocytosis,  $F_c$ -receptor expression, antibody-dependent cytotoxicity, prostaglandin E production, activation of nonspecific esterases ( $\alpha$ -naphthyl acetate and  $\alpha$ -naphthyl butyrate esterases; Yourno et al., 1984), lactoferrin and isoferritin production, plasminogen-activator synthesis and the elaboration of other inhibitory (MIF) or stimulatory (MAF, CSFs) factors by stromal cells or cells of myeloid, lymphoid and erythroid origin (Metcalf et al.,1985a). Consequently, the CSFs can be separated into two 'functionally' distinct groups. The first contains multi-CSF and GM-CSF, which deliver primarily a mitogenic stimulus to progenitor cells but can induce concentration-dependent differentiation indirectly, through CSF receptor modulation, and the second, containing G-CSF and M-CSF, which affect proliferation and differentiation directly through interaction with their specific receptors on responsive cells. These effects of the CSFs on hematopoietic cells presumably involve membrane receptor-mediated processes and, most likely, elicit the production of specific intracellular mediators of proliferation, differentiation or functional activation in these cells.

### 1.5. The CSF receptors.

Specific, high-affinity membrane receptors for the CSFs are coexpressed on all lineages of granulocytes and macrophages regardless of their stage of differentiation or maturation. Since each CSF receptor appears to bind only its cognate CSF and most hematopoietic cells are responsive to more than one CSF, these cells must necessarily express more than one type of CSF receptor (Tables 1 and 2). It is not known whether all hematopoietic cells express the full complement of CSF receptors or if some cells express only a single species or if all cells express the same numbers of each CSF receptor. The CSF receptors in the murine system differ in both their molecular weights and the number of receptors found on normal responsive cells (Morgan et al., 1984; Palaszynski et al., 1984; Nicola et al., 1985a; Walker et al., 1985). In spite of the variation in the distribution of CSF receptors and the relatively low receptor numbers per cell, CSFs can induce proliferation with only 5-10% receptor occupancy, a property directly attributable to the high affinity of the CSFs (eg.  $10^{-11}$  to  $10^{-13}$  M) for their cognate receptors (Metcalf et al., 1984).

CSFs appear to act synergistically in promoting the proliferation of granulocyte-macrophage progenitors, presumably through their specific membrane receptors, but the underlying basis for this synergism is unclear and likely to be fairly complex. It has been observed that CSF:CSF receptor interaction can lead to the down-modulation of other CSF receptors *in vitro*, for example, multi-CSF down-regulates GM-CSF, G-CSF and M-CSF receptors and GM-CSF down-regulates G-CSF and M-CSF receptors (Walker et al., 1985). This hierarchical receptor down-

modulation has been shown to be as effective in stimulating progenitor cell proliferation and differentiation as the direct binding of G-CSF and M-CSF to their respective receptors. However, it has yet to be established that this observed down-regulation of CSF receptors and the effects of the CSF: CSF receptor interaction *in vitro* are tenable analogs for the same processes *in vivo*. Recently, the murine M-CSF (CSF-1) receptor was shown to be related to the protein product of the *c-fms* proto-oncogene, an oncogene known to be expressed in human bone marrow and in blood mononuclear cells and also involved in the 5q- chromosomal deletion syndrome in human acute myelogenous leukemia (Sherr et al.,1985; Nienhuis et al.,1985). This growth factor receptor-oncogene association bears a strong resemblance to the mammalian erythroblast system describing the production of a protein encoded by the *v-erb-B* oncogene and its identification as a truncated form of the mammalian EGF receptor (Downward et al.,1984). Recently, several other growth factor/growth factor receptor-oncogene associations have been identified : human oestrogen receptor/*v-erb-A* (Green et al., 1986); human glucocorticoid receptor/*v-erb-A* (Weinberger et al., 1985; Hollenberg et al., 1985); avian progesterone receptor/*v-erb-A* (Jeltsch et al., 1986; Conneely et al., 1986); human thyroid hormone receptor/*c-erb-A* (Sap et al., 1986; Weinberger et al., 1986); human transferrin, ovotransferrin, lactoferrin/ Blym-1 (Goubin et al., 1983); and human insulin receptor, EGF receptor, transmembrane domain of human MHC class II antigen/*c-fur* (*c-fes/fps* upstream region) (Roebroek et al., 1986). Since the CSFs, via their specific receptors, are the only identified regulators of granulocyte-macrophage proliferation, autocrine production of the CSFs or aberrant expression of normal or truncated

forms of the CSFs or CSF receptors may be involved in the genesis or maintenance of myeloid leukemia.

#### 1.6. The CSFs, CSF receptors and human myeloid leukemia.

The myeloid leukemias, in mammals, are clonal neoplasms of the granulocyte-macrophage progenitor cells found in the bone marrow. The aberration in normal hematopoiesis observed in these leukemias is presumed to be due to the dysfunction of normal cell growth regulation elicited by the intervention of a wide variety of *in vivo* and *ex vivo* factors, amongst which the viral or cellular oncogenes/oncogene products play a paramount role (Weinberg et al., 1985; Duesberg et al., 1985). That these viral or cellular oncogenes encode 'normal' or 'aberrant' growth factors or growth factor receptors and that the attendant leukemic cell proliferation is due to these oncogene products is consistent with a number of autocrine stimulation theories of leukemogenesis (Deuel et al., 1984; Kris et al., 1985; Sporn et al., 1985). Since the CSFs are the only known proliferative factors for granulocyte and macrophage progenitor cells, it is of interest to determine whether the evolution of myeloid leukemia is ascribable to autocrine stimulation of 'normal' or 'aberrant' CSF or CSF receptor synthesis. Evidence for the autocrine production of CSFs in myeloid leukemia cells is unequivocal in its assertion that, like normal hematopoietic cells, leukemic cells have an absolute requirement for the addition of exogenous CSF in order to proliferate *in vitro*. It has also been demonstrated that leukemic cells produce the same CSFs, to the same level, as normal cells (Moore et al., 1973; Golde et al., 1974; Goldman et al., 1976). On the other hand, it is

also apparent that since leukemic cells are absolutely dependent on CSF for growth, the emergence and maintenance of the leukemic clone would also be dependent on CSF. This observation establishes the CSFs as mandatory cofactors in the induction of leukemia. These observations suggest that the generation of leukemia by autocrine growth factor production is unlikely. It may be better to consider that myeloid leukemia cells, although able to synthesize CSFs to normal levels, may have a defect unique to leukemic cells which promotes a bias toward CSF-stimulated regeneration of undifferentiated cells rather than the induction of lineage-specific differentiation.

That this defect may be ascribable to the intervention of oncogenes is still within the realm of possibility. The induction of leukemia in non-leukemic cells has been correlated with the ability to synthesize CSF constitutively (Hapel et al.,1981; Schrader et al.,1983), which has been further established by inducing leukemia in non-leukemic cell lines by transfecting these cells with constructs containing GM-CSF cDNA under the control of viral promoters (Lang et al.,1985). The expression of the *c-fms* oncogene (the M-CSF receptor analog) in normal hematopoietic cells, and its deletion in human AML, may represent an example of such a process in the induction or maintenance of myeloid leukemia (Nienhuis et al.,1985). Since a wide variety of chemical and biological agents are able to induce the terminal differentiation of both human and murine myeloid cells, it was thought that the CSFs might be employed as therapeutic agents to induce differentiation in myeloid leukemia cells. It has been shown that murine G-CSF is able to suppress murine myeloid leukemia, presumably by suppressing self-generation and stimulating terminal differentiation

(Metcalf et al.,1982b), which raises the possibility that human G-CSF (CSF ) might be used in an analogous manner to treat human myeloid leukemia (Nicola et al.,1985b).

### 1.7. The myeloid leukemias.

Chronic myelogenous leukemia (CML) and acute myelogenous leukemia (AML) are two representatives of a group of hematopoietic stem cell malignancies referred to as myeloproliferative disorders. Other members of this group include polycythemia vera (PCV) and essential thrombocytosis (ET). These malignancies are characterized by the expansion of committed myeloid progenitor cells, ultimately resulting in the accumulation of mature/immature granulocytes (CML/AML), red blood cells (PRV) or platelets (ET) in both the bone marrow and peripheral blood (Fialkow et al., 1977, 1978; Abkowitz et al., 1985). Although most research indicates that the myeloproliferative disorders arise from the neoplastic transformation of a pluripotent myeloid stem cell, the involvement of mature lymphoid cells in these disorders suggests that a more primitive stem cell, common to both myeloid and lymphoid lineages, may be involved (Raskind et al., 1985). These findings do not, however, preclude the influence of transformational events, secondary to myeloid stem cell transformation, acting on the pluripotent lymphoid stem cell(s).

Both CML and AML are well documented examples of clonal neoplasms, resulting from the neoplastic transformation of a pluripotent hematopoietic stem cell (Fialkow et al., 1981a, 1983). The demonstration that all myeloid cells in CML and AML display the same

chromosomal anomalies and G6PD isoenzyme patterns further supports this contention (Moore et al., 1973; Douer et al., 1981). The chronic stage of this disease (CML, CGL) normally persists for 3-5 years and is characterized by the clonal proliferation and differentiation of committed myeloid progenitor cells, leading to the accumulation of 'mature-leukemic' granulocytes in the bone marrow and peripheral blood. These 'leukemic' granulocytes appear to mature normally and the clonal proliferation of CML bone marrow cells *in vitro* does not differ significantly from normal bone marrow progenitors, except that the gross levels of bone marrow progenitor cells are elevated in CML (Moore et al., 1973; Hara et al., 1981). The distinction to be made at this point is that, in CML, all the myeloid cells arising from the malignant clone retain the capacity to develop into mature, functional end cells, in contrast to AML. CML ultimately evolves into a fatal acute leukemia (AML), preceded by a phase called "blast crisis", whose hallmark is the accumulation of immature, non-differentiating myeloid blast cells (Abkowitz et al., 1985). This process presumably involves the evolution and proliferation of a leukemic stem cell clone from within the CML stem cell population. In contrast to CML, the abnormal stem cell population in AML does not respond to differentiation signals and consequently cannot undergo normal myelopoiesis. The accumulation of immature myeloblasts is thus the major impetus behind the failure of normal hemopoiesis in AML (Saunders et al., 1969; Fialkow et al., 1981b).

The occurrence of unique chromosomal abnormalities in the myeloid leukemias has been established (Rowley et al., 1973; First and Second International Workshops on Chromosomes in Leukemia, 1978

and 1980; Yunis et al., 1981; Abkowitz et al., 1985; Bartram et al., 1985). The acquisition of these chromosomal changes suggests that the processes responsible for the appearance of these abnormalities are involved in the evolution of myeloid leukemia. The processes creating these heritable chromosomal abnormalities are probably not the primary causative events in leukemia but rather represent one of a number of interrelated processes contributing to leukemogenesis. In CML, greater than 90% of leukemic patients display a truncated chromosome 22, the Philadelphia (Ph1) chromosome, which arises from a reciprocal translocation between chromosomes 9 and 22 (Wiggans et al., 1978; Rowley et al., 1982). Although some AML patients display the Ph1 chromosome, the majority display other consistent chromosomal anomalies, most notably, trisomy 6 and 8, monosomy 5, 7 and 10, deletions 5q<sup>-</sup> and 7q<sup>-</sup>, inversion 16 and multiple translocations involving chromosomes 1, 5, 7, 8, 11, 17 and 21 (Rowley et al., 1977; First and Second International Workshops on Chromosomes in Leukemia, 1978 and 1980; Berger et al., 1980; Sandberg et al., 1980; Yunis et al., 1981; Le Beau et al., 1983,1985a; Rowley et al., 1983; Holmes et al., 1985). Concomitant with the occurrence of these chromosomal abnormalities is the activation or expression of cellular proto-oncogenes resident on these chromosomes (Klein et al., 1981; Westin et al., 1982; Rowley et al., 1983; Table 3).

#### 1.8. The cellular proto-oncogenes (c-oncs) and human myeloid leukemia.

i) c-abl, c-sis (Table 3).

The best characterized chromosomal abnormality involving cellular proto-oncogenes is the Ph1 chromosome found in CML patients (Rowley et al., 1973). The event generating the Ph1 chromosome involves a reciprocal translocation between chr.9 and chr.22 in which the c-abl oncogene of chr.9 (9q34) is fused to the breakpoint cluster region (bcr; 22q11) of chr.22 and the c-sis oncogene of chr.22 (22q12.3-q13.1) is fused to the distal portion of chr.9 (9q+) or chr.11 (11q-). The segregation of c-sis to chr.9q+ or chr.11q- in Ph1+ CML and the non-segregation of c-sis from chr.22 in Ph1- CML has not been correlated with the appearance of c-sis-related transcripts. These results support the view that translocation of c-abl rather than c-sis is one of the determinative steps in the development of Ph1+ CML (Bartram et al., 1984).

Fusion of the c-abl sequence to bcr in Ph1+ CML generates a hybrid bcr-abl gene (Collins et al., 1984; Shtivelman et al., 1985; Grosveld et al., 1986; Hariharan et al., 1987). The chimeric bcr-abl protein (p210) derives its amino-terminal portion from bcr and contains most of the c-abl coding sequence, including the tyrosine-kinase domain, as its carboxy-terminus. Unlike the normal c-abl (p145) and bcr (p143; Hariharan et al., 1987) gene products, the bcr-abl fusion gene product (p210) has demonstrable *in vitro* tyrosine-kinase activity, autophosphorylates and is phosphorylated on tyrosine residues *in vivo* (Konopka et al., 1984; Kloetzer et al., 1985). The gag-abl transforming fusion protein (p160) from Abelson murine leukemia virus possesses kinase activity *in vitro* and *in vivo* analogous to that observed with

Table 3. Proto-oncogenes expressed in hematopoietic cells and diseases.

<u>c-onc</u>	<u>chromosomal location</u> <sup>1</sup>	<u>chromosomal abnormality</u> <sup>2</sup>	<u>mRNA in</u>	<u>induced by</u>	<u>protein (location)</u>	<u>disease</u>
c-abl	9	t(9:22)* t(9:11)# *with c-sis and bcr #with c-ras <sup>H</sup>	HL60, K562, granulos		p145 p210 (bcr-abl fusion) (cytoplasm)	CML AML AMML
c-sis (PDGF-2/B)	22	t(9:22)* t(8:22)# *with c-abl #with c-myc	HL60, U937, monos, macros	TPA	p30-32 (secreted)	CML
c-myc	8	t(8:22)* t(8:21) *with c-abl #to IgH locus +8	HL60, monos, macros, granulos	PDGF, EGF, IL-3, FGF	p58 (nuclear)	AML CML ANLL
c-myb	6	t(6:14)* t(6:9)# *to IgH locus 6q+ #with c-abl +6	HL60, monos, macros, granulos	PDGF, EGF, IL-3, FGF	p80 (nuclear)	MPD
c-fos	14	t(6:14)* *with c-myb	HL60, U937, monos, macros	TPA, CSF-1, PDGF, IL-3	p56-72 (nuclear)	CML
c-fes/fps	15	t(15:17)* *with c-erb A	HL60		p92/98 (cytoplasm)	APML
c-fms (CSF-1-receptor)	5	5q-	HL60, monos, macros	TPA	gp165 (cytoplasmic, plasma memb.)	AML CML
c-ras <sup>H</sup>	11	11p-	HL60		p21	AML
c-ras <sup>K</sup>	12	+12	HL60		p21	AMoL

c-ras <sup>N</sup>	1		HL60	p21 (plasma memb.)	APML
c-erb A (ThyR, EstR, GluR)	17	t(15:17)* *with c-fes/fps	erythros	p46-52 (cytoplasm)	APML
c-erb B (EGF-receptor)	7	7q-	erythros	p68-74 (cytoplasmic, plasma memb.)	AMML AMoL AML

<sup>1</sup> Rowley et al., Nature 301: 290, 1983; Bartram et al., Blut 51: 63-71, 1985.

<sup>2</sup> First International Workshop on Chromosomes in Leukemia, Br. J. Hematol. 39: 311-316, 1978.

Second International Workshop on Chromosomes in Leukemia, Cancer Genet. Cytogenet. 2: 108-133, 1980.

<sup>3</sup> Hunter et al., Scientific American 251: 70-79, 1984.

AML	Acute Myelogenous Leukemia (FAB M1 and M2)
AMML	Acute Myelomonocytic Leukemia (FAB M3)
AMoL	Acute Monocytic Leukemia (FAB M4)
ANLL	Acute Nonlymphocytic Leukemia
APML	Acute (hypergranular) Promyelocytic Leukemia (FAB M5)
CML	Chronic Myelogenous Leukemia
MPD	Myeloproliferative Disease
CSF-1	Colony Stimulating Factor-1 (human mononuclear phagocyte colony stimulating factor; human U-CSF or M-CSF)
EGF	Epidermal Growth Factor
EstR	Human Estrogen receptor
FGF	Fibroblast Growth Factor
GluR	Human Glucocorticoid receptor
IL-3	Interleukin-3
PDGF	Platelet-Derived Growth Factor
ThyR	Human Thyroid Hormone receptor
TPA	12-O-Tetradecanoyl-Phorbol-13-Acetate

erythros	erythroblasts
granulos	granulocytes
macros	macrophages
monos	monocytes
HL60	human promyelocytic leukemia cell line

K562 human erythroleukemic cell line  
U937 human monocytic leukemia cell line

p210. This raises the possibility that replacement of the amino-terminal portion of c-abl by bcr- or gag-encoded sequences alters both the activity and specificity of the c-abl gene product.

Since c-abl is expressed in a variety of hematopoietic cells and bcr is expressed in fibroblast, epithelial, lymphoid and myeloid cells, it is reasonable to assume that the bcr-abl gene could also be expressed in the same variety of cells. The observation that the specific translocation of bcr and c-abl occurs preferentially in CML cells, suggests that the factors governing p210 expression are restricted to these cell lineages (Westin et al., 1982; Gale et al., 1984; Heisterkamp et al., 1985). It is possible that the action of p210 on specific substrates, within a subset of myeloid stem cells, mediates a pivotal step in leukemic transformation.

ii) c-myb, -myc, -fes/fps (fur), -fms, -fos, -ras, -sis (Table 3).

The activation and expression of two or more proto-oncogenes in mammalian cells is widely accepted as a mechanism for inducing transformation and the acquisition of a neoplastic phenotype (Bishop et al., 1983, 1985; Blick et al., 1984; Duesberg et al., 1985; Weinberg et al., 1985). In the myeloid leukemias a number of cellular proto-oncogenes have been implicated, via their amplification and inducible expression, in the differentiation of hematopoietic cells and possibly in the induction, maintenance and proliferation of the leukemic phenotype (Collins et al., 1982; Gonda et al., 1984; Kruijer et al., 1984; Muller et al., 1984, 1985; Slamon et al., 1984a; Sariban et al., 1985; Mavilio et al., 1987). That these cellular oncogenes represent normal cellular

components of growth regulatory or mitogenic pathways, rather than singular determinants of malignant transformation, has yet to be determined.

The cellular oncogenes *myb*, *myc*, *fes/fps*, *fms*, *fos*, *ras* and *sis* are all expressed in leukemic cells and cell lines (HL60, K562, KG1, U937), the level of expression being determined by the differentiation state of the cell (Blick et al., 1984; Slamon et al., 1984b). Most of these oncogenes are inducible by growth factors (CSF-1, EGF, FGF, IL-3, PDGF) and chemical morphogens (TPA, DMSO, *trans*-retinoic acid) in cells and cell lines. These oncogenes are also super-inducible by the combination of growth factor/morphogen and cycloheximide, indicating that longevity of the oncogene-specific mRNA is regulated by an intracellular protein, presumably a labile nuclease or repressor protein (Kelly et al., 1983; Cochran et al., 1984; Muller et al., 1984; Conscience et al., 1986; Pantazis et al., 1986).

*c-myc* is known to be amplified in uninduced HL60 cells and the primary acute promyelocytic leukemia cells from which HL60 was derived (Dalla-Favera et al., 1982). *c-ras<sup>H</sup>*, *c-ras<sup>K</sup>* and *c-ras<sup>N</sup>* are also known to be expressed in leukemic cells and cell lines and, at least in the case of *c-ras<sup>N</sup>*, known to co-exist in HL60 cells with an altered *c-myc* gene (Eva et al., 1983; Murray et al., 1983; Gambke et al., 1984). Recent findings suggest that the *c-myb*, *-myc* and *-fos* genes represent 'competence factors' in normal cell growth and are responsible for determining the transition of cells from  $G_0$ - $G_1$  to S-phase in the cell cycle (Gonda et al., 1984; Einat et al., 1985). Due to the nuclear localization and DNA-binding properties of the cognate oncogene products, it is possible that the control over cell growth and

proliferation is manifest through transcriptional regulation. The differentiation of myelo-monocytic cells has been correlated with a decrease in the level of c-myb and c-myc expression whereas c-fos expression increases steadily during the later stages of myelo-monocytic differentiation. This suggests that c-fos expression is linked to the terminal differentiation and functional activation of monocytes and macrophages in normal and leukemic hematopoiesis (Gonda et al., 1984; Kruijer et al., 1984; Muller et al., 1985; Klempnauer et al., 1986; Mitchell et al., 1986; Mavilio et al., 1987).

c-fms has also been implicated in normal and leukemic monocytic differentiation. Induction of monocytic differentiation in HL60 cells with TPA is associated with the induction of c-fms expression, preceded by the expression of c-myc and c-fos (Gonda et al., 1984; Sariban et al., 1985). Recently the c-fms oncogene was identified as the cognate cellular gene for mononuclear phagocyte growth factor, CSF-1 (Sherr et al., 1985). This correlates well with the detection of c-fms transcripts in monocytes but not granulocytes or lymphocytes, suggesting a specific association between c-fms and monocyte-macrophage differentiation (Sariban et al., 1985). Since several cellular oncogenes (myc, fos, fms) are co-regulated and expressed temporally during monocytic differentiation, it is tempting to ascribe a regulatory role to c-fms in the expression of c-myc and c-fos. Aberrations in the expression of c-fms, such as its deletion in the 5q<sup>-</sup> syndrome in AML, could lead to abnormal c-myc and c-fos expression and be manifest as defects in both monocytic cell differentiation and proliferation (Nienhuis et al., 1985).

The regulated expression of c-fes/fps transcripts in hematopoietic cells has also been associated with functional maturation. The existence

of a putative regulatory region 5' to *c-fes/fps* has been demonstrated in hematopoietic cells. The 'fur' (*fes/fps* upstream region) gene displays significant homology to the cysteine-rich regions of human insulin receptor and epidermal growth factor receptor (Roebroek et al., 1986). The capacity of this region to regulate the expression of *c-fes/fps* in hematopoietic cells has yet to be demonstrated.

The *c-sis* cellular oncogene is known to encode the platelet-derived growth factor 2/ $\beta$  chain (PDGF-2) of the heterodimeric human platelet-derived growth factor (PDGF) (Chiu et al., 1984; Johnsson et al., 1984; Josephs et al., 1984). *c-sis* transcripts have been demonstrated in malignant cells of mesenchymal origin, cells whose normal counterparts are responsive to PDGF. *c-sis* activation, by PDGF, in these cells induces the synthesis and secretion of a PDGF-like mitogen (Eva et al., 1982; Graves et al., 1984; Pantazis et al., 1985; Shimokado et al., 1985; Johnsson et al., 1986; Martinet et al., 1986). Recently, the induction of *c-sis* expression and production of PDGF-2-related proteins was shown to occur in TPA-stimulated monocytic differentiation of HL60 and U937 cells (Pantazis et al., 1986). Untreated cells do not contain detectable amounts of *c-sis*/PDGF-2 mRNA. Treatment of HL60 cells with DMSO, thereby inducing granulocytic differentiation, does not induce the expression of *c-sis* which correlates with the absence of *c-sis* transcripts in normal human granulocytes or leukemic cell lines K562 and KG1 (Sariban et al., 1985). Like *c-myb*, *c-myc* and *c-fos*, *c-sis* expression is superinducible by the combination of TPA and cycloheximide in the monocytic differentiation of HL60 and U937 cells (Gonda et al., 1984; Pantazis et al., 1986). Concomitant with the expression of *c-sis* is the the synthesis and secretion of PDGF-2-derived proteins. These 30 kd

and 32 kd proteins, homodimers of disulphide bonded 14 kd and 15 kd monomers, are secreted following TPA-induced differentiation of HL60 and U937 and display biological activity similar to PDGF (Pantazis et al., 1986). Activation of c-sis gene expression in myeloid leukemia is therefore associated with the concerted expression of other cellular oncogenes (c-myb, c-myc, c-fos and c-fms) in the TPA-induced differentiation of HL60 and U937 leukemic cells. The observation that bone marrow-derived stromal cells, from both normal and leukemic sources, express receptors for and are stimulated by PDGF and EGF further implicates c-sis/PDGF-2 in the regulation of the hematopoietic microenvironment and, indirectly, in the regulation of normal and leukemic hematopoiesis (Rosenfeld et al., 1985; Grossman et al., 1986).

The temporal regulation of cellular oncogene expression during myelo-monocytic cell differentiation suggests that the cellular oncogene products play a central role in normal cell growth, proliferation and responsiveness to mitogenic stimuli. In fact, the accumulated evidence to date suggests that anomalous expression or over-expression of a cellular oncogene/oncogene product confers a growth advantage to the malignant phenotype and that the attendant activities of these oncogene products mediate leukemic transformation.

### 1.9. Myeloid leukemia-associated proteins and oncofetal proteins.

The expression of cellular proto-oncogenes and the ability of these genes/gene products to affect a cell's capacity to differentiate and proliferate is considered to be one of several pivotal steps in the multi-step process of oncogenesis. The "clonality" of a wide variety of cancers

is indicative of the involvement of processes causing heritable genetic defects in genes intimate to normal cellular growth regulation. Since these genetic defects are manifest as "neoplastic phenotypes", only cells that are endowed with a capacity to proliferate and differentiate, whilst harbouring the genetic defect(s), will give rise to the neoplastic phenotype. The involvement of stem cells, immature progenitor cells capable of proliferation and differentiation, as the "target(s)" of neoplastic transformation is well documented (Yunis et al., 1983; Griffin et al., 1985). The observation that 90% of adult human cancers arise from tissues that undergo continual renewal throughout life, eg. external/glandular epithelium and bone marrow, further supports this suggestion (Cairns et al., 1981).

Another feature common to the neoplastic phenotype is the expression of embryonic or fetal proteins. The renewed synthesis of these fetal proteins in association with neoplasia has prompted their definition as "oncofetal proteins/antigens". Alpha-1-fetoprotein (AFP; teratocarcinomas, hepatocellular carcinoma, liver injury or necrosis, hepatitis: Abelev et al., 1971; Sell et al., 1978; Adinolfi et al., 1985), the common lymphocytic leukemia-associated antigen (CALLA; acute lymphocytic leukemia, some chronic myeloid leukemias: Ritz et al., 1980; Rosenthal et al., 1983), carbohydrate moieties of cellular glycoproteins and glycolipids (carcinomas, melanomas, lymphomas, neuroendodermal cancers, leukemias, anemias: Hakomori et al., 1984; Feizi et al., 1985a, 1985b) and the murine  $Q_a/Tl_a$ -like cellular oncogene family (increased expression in all murine tumours: Bricknell et al., 1983; Murphy et al., 1983) are all developmentally regulated proteins in the transition from embryo to fetus to the adult and as such are

functionally defined as "onco-developmental/oncofetal proteins". At present, the functional role that these proteins play in stem cell proliferation, commitment, differentiation or the oncogenic process remains unclear. Many of these fetal proteins continue to be synthesized in normal adults, albeit in much smaller quantities, and as such the designation of these proteins as "onco-developmental or oncofetal proteins" is somewhat misleading since it is suggestive of a direct involvement of these proteins in oncogenesis. Though the renewed expression of these proteins is often directly correlated with neoplasia, no evidence has been found to suggest that there is a causal relationship between expression of these oncofetal proteins and neoplasia. However, the disparity in the synthesis of these proteins between the normal and neoplastic state has been exploited in the development of monoclonal antibodies directed to individual "oncofetal proteins". The utility of these monoclonal antibodies (MoAbs) as diagnostic reagents is unparalleled, but the application of these MoAbs as cytotoxic reagents for killing metastatic cells, "purging" leukemic bone marrow of leukemic stem cells or as adjuncts to radio-/chemotherapy is of more immediate importance.

The isolation and characterization of the unique antigens detected by these MoAbs has proven to be more complex than initially anticipated. This complexity is largely due to the characteristics of the antigenic structures to which many of these "tumour-specific" MoAbs are directed. A large proportion of the MoAbs, thought to be specific for certain carcinomas, lymphomas or leukemias, are actually directed to carbohydrate structures of glycoproteins and glycolipids expressed on the surface of most tumour cells and certain normal tissues. These

carbohydrate moieties have subsequently been shown to be developmentally regulated and differentiation-dependent in a number of tumor cell systems and in normal tissue as well. As a result many of the antigenic carbohydrate structures recognized by these "tumour-specific" MoAbs have been designated as onco-developmental antigenic structures (Hakomori et al., 1984; Feizi et al., 1985a, 1985b).

Cell surface antigen analysis of myeloid leukemic cells with MoAbs, directed to leukemic or normal myeloid cells, has led to the development of several myeloid stem cell differentiation schemes (Knapp et al., 1981; Ball et al., 1983; Griffin et al., 1983; Todd III et al., 1985). Phenotyping myeloid cells on the basis of their reactivity with various MoAbs specific for the cell stages subsequent to the myeloid progenitor cell (Figure 1) and correlation with the expression of myeloperoxidase and alpha-naphthyl-acetate/butyrate esterase activity is an accepted method for establishing cellular differentiation pathways and the maturation stage of isolated myeloid cells. Unfortunately, this method does not define the nature of the antigenic determinants recognized by the MoAbs and does not distinguish between synthesis, cytoplasmic accumulation and expression at the cell surface. Since most of these MoAbs were prepared by immunization with whole cells it is not surprising to find that most of them demonstrate a wide latitude of reactivity with leukemic cell extracts. The reactivity of these MoAbs with a variety of proteins in leukemic cell extracts has precluded most attempts at detailed antigenic analysis of leukemic cells. However, they do seem to define a restricted set of cell surface antigens which are expressed on differentiating myeloid cells.

Although many MoAbs were screened simply for reactivity with myeloid cells, a proportion were also analysed for the ability to specifically immunoprecipitate proteins from ectopically-labelled or *in vitro* -labelled myeloid leukemia cells and cell lines. These myeloid leukemia-associated proteins are shown in Table 4.

A number of these proteins were shown to be cell stage-dependent such that morphogen-induced differentiation or maturation resulted in their disappearance, and in some cases, the appearance of a novel protein (p180;gp160/gp130; p88/p86, p83-84). The monomeric p180 was detected on immature leukemic cells and was also shown to be completely lost upon DMSO, retinoic acid or TPA-induced maturation of the leukemic cell lines HL60 and KG1. p180 was not detectable on normal peripheral blood leukocytes and has not been investigated for its expression on normal bone marrow progenitor cells. On the basis of available data, p180 can be tentatively defined as a differentiation-associated/blast cell antigen in myeloid leukemia (Askew et al., 1985).

gp160 and gp130 were detected as maturation-associated proteins in the DMSO-induced maturation of the leukemic cell line HL60. gp160 is detectable on uninduced HL60 cells (promyelocytes) and rapidly disappears when HL60 is induced to mature (monocytes). Concomitant with the loss of gp160 expression is the synthesis of a "granulocyte-specific" antigen, gp130. gp130 expression has been correlated with the acquisition of phagocytic and chemotactic activities and granulocytic cell morphology in both normal granulopoiesis and chemically-induced granulocytic maturation of leukemic cell lines. The detection of gp130 as the major cell surface glycoprotein in normal peripheral blood granulocytes suggests that gp130 is a marker for the

Table 4. Myeloid leukemia cell-associated proteins defined by antibody.

<u>protein</u> $M_r$ (kd)	<u>detected in</u>	<u>function</u>	<u>cited by</u>
gp350-400	myeloblasts	?	Taub,1978
gp200 (monomer)	T-cell leukemia	T200 antigen, leuk. dev/diff Ag	Omary,1980
gp210, 120	AMLs	?	Andersson,1979b
gp120, 100-110	CMLs	?	"
gp130	AMLs, CMLs, granulocytes	?	"
gp190	AMLs, CMLs, K562	fibronectin- receptor	Virtanen,1987
gp190 (dimer) gp90 (monomer)	myeloid leuk. cells, K562, HL60, U937	transferrin- receptor	Sutherland,1981 Trowbridge,1981
p180 (monomer)	HL60, KG1, K562, HEL, AMLs	leuk.blast cell dev/diff Ag	Askew,1985
gp177( $\alpha$ ), 95( $\beta$ ) (heterodimer)	granulocytes	LFA-1, leuk. dev/diff Ag	Springer,1984
gp177( $\alpha$ ), 95( $\beta$ ) gp165( $\alpha$ ), 95( $\beta$ ) gp150( $\alpha$ ), 95( $\beta$ ) (heterodimer)	lymphocytes, granulocytes, monocytes, U937	LFA-1 glyco- protein family, leuk. diff Ags	Sanchez-Madrid, 1983
gp160 gp130	granulocytes, HL60 granulocytes	myeloid diff Ag, functional diff Ag	Gahmberg,1979b "
p145 (pI 4.5) p105 (pI 3.5-6.3)	granulocytes, HL60, K562, U937, ML1, ML2, ML3	?	Skubitz,1983
gp100 (CALLA)	ALLs, AMLs granulocytes, monocytes, lymphocytes	? (oncofetal antigen)	Ritz,1980

gp100 (monomer)	myeloid progenitor cells in adult bone marrow and fetal liver, KG1, Ph1 <sup>+</sup> myelob	?	Katz,1985
p95	monos, macros granulos	? (diff Ag for phagocytic cells)	Vartio,1985
p94 (monomer)	monos, macros, all myeloperoxidase <sup>+</sup> U937	? (not related to 94kd $\beta$ subunit of LFA-1 family)	Todd III,1985
p88 p86 p83-84 p68-70 p53	HL60, CMLs, granulos HL60, ML1, CMLs KG1, monos HL60, ML1, CMLs ML1, CMLs	? (myeloid leuk. dev/diff Ag)	Mulder,1981
gp75-80 (pI 7.6-7.9)	AMMLs, AMLs	?	Baker,1982
p68 (monomer) (pI 6.2-6.3) p205(trimer) (pI 5.8-6.1)	HL60, ML1, AMLs, monos	monocyte non- specific esterase	Yourno,1986
p68	monos, myelob promyelos, AMLs CMLs, erythrobs	? (diff Ag for myeloid progenitor cells)	Griffin,1984
p70 (CAMAL) (pI 7.0-7.2) (monomer)	HL60, K562, KG1, U937, AMLs, CMLs	inhibitory to normal bone marrow cell growth <i>in vitro</i>	Shipman,1983 Logan,1987
p65	AMLs, CMLs, all T-cells	T65 antigen, T-cell antigen	Roystan,1980

p55	monos, granulos promyelos, myelos metamyelos, AMLs	?	Griffin,1982 (diff Ag for myeloid progenitor cells)
p37	K562, erythros, all hemato. cells	?	Allen,1985 (diff Ag in erythroid cell lineage)
gp110 (pI 5.5-6.0)	AMLs, myelobs	?	de Jong,1984
gp100 (pI 7.5-8.0)	AMLs	?	"
gp90 (pI 5.0-5.5)	AMLs, myelobs	? (trfn-r)	"
gp67 (pI 4.5-5.0)	AMLs, myelobs	? (HSA)	"
gp40 (pI 7.5-8.0)	AMLs, myelobs	? (actin)	"
p38 (pI 5.0)	AMLs	?	"
p32 (pI 4.5)	AMLs, myelobs		

AMLs	Acute Myelogenous Leukemia cells
AMMLs	Acute Monomyelocytic Leukemia cells
CMLs	Chronic Myelogenous Leukemia cells
erythrobs	erythroblasts/immature erythrocytes
granulos	granulocytes
hemato	hematopoietic
lymphos	lymphocytes
macros	macrophages
metamyelos	metamyelocytes
monos	monocytes
myelos	myelocytes
myelobs	myeloblasts
promyelos	promyelocytes

HEL	human erythroleukemic cell line (AML)
HL60	human acute promyelocytic leukemia cell line (AML)
K562	human erythroleukemia cell line (CML)
KG1	human myelocytic leukemia cell line (AML)
ML1/2/3	human myeloid leukemia cell lines (AML)
U937	human monocytic leukemia cell line (histiocytic lymphoma)

CALLA	Common Acute Lymphocytic Leukemia-Associated Antigen
CAMAL	Common Myelogenous Leukemia-Associated Antigen
diff Ag(s)	differentiation antigen(s)
LFA-1	Leukocyte Function-Associated Antigen -1

Mac-1	Macrophage Adhesion Protein -1 (C3b <sub>i</sub> -complement receptor)
leuk. dev/ diff Ag	leukocyte developmental/differentiation antigen
trfn-r	transferrin receptor
fnc-r	fibronectin receptor
HSA	human serum albumin

functional maturation of granulocytes. Neither of these proteins have been investigated for their modulation of expression in normal myeloid cells, other leukemic cell lines or in HL60 cells induced to mature with other chemical morphogens (TPA, Na-butyrate). As such it is not clear whether gp160/gp130 expression is a general consequence of myeloid cell maturation or a DMSO specific event in HL60 cells (Gahmberg et al., 1979b).

The expression of p88/p86 on normal granulocytes and untreated HL60 cells and the concomitant loss of p88/p86 expression upon DMSO/TPA-induced maturation of HL60 cells is suggestive of aberrant expression of mature granulocytic markers on immature myeloid precursor cells. The appearance of p83-84 upon TPA-induced differentiation of HL60 cells which is detectable on mature monocytes is indicative of differentiation/maturation state-dependent expression of p83-84. The analyses performed on p88/p86 and p83-84 suggest that all are markers of particular stages in myeloid differentiation and that the inability to detect these proteins in cell lines U937, K562 and KG1 is due to the more "mature" character of these cell lines or that genetic defects resulting in the loss of p88/p86/p83-84 has occurred in these cell lines (Mulder et al., 1981).

Other proteins were detected on both normal and leukemic cells, albeit at different levels, and were also shown to be modulated by the maturation state of the cell (T200/T65; gp190; gp180-200; LFA-1 glycoprotein family; gp105; gp100 [CALLA]; gp100; p95; p94; p68; p55; p37). The T200 and T65 antigens are known to be leukocyte-specific cell surface antigens whose developmentally regulated expression is restricted to hematopoietic cells. The T200 antigen is the T-cell

equivalent of the B-cell-specific T220 antigen expressed on normal B cells (Omary et al., 1980). The T65 antigen is expressed on T-cells and has been implicated as a differentiation-associated antigen due to the modulation of its expression during T-cell development (Roystan et al., 1980).

The LFA-1 glycoprotein family, including the leukocyte function-associated antigen (LFA-1), the C3b<sub>i</sub> complement receptor (Mac-1/OKM1) and the gp150,95 molecule, specifies a number of leukocyte-specific functions, such as complement-mediated phagocytosis and adherence, and demonstrates cell-specific expression, Mac-1 is expressed only in myeloid cells, and morphogen-induced modulation of expression, TPA induction of leukemic monoblast cell line U937 causes an increase in Mac-1 and gp150,95 mRNA and a substantial decrease in LFA-1 mRNA (Sanchez-Madrid et al., 1983). The LFA-1 glycoprotein family is expressed in both normal and leukemic myeloid cell populations and has also been implicated as the inherited deficiency in patients with recurring bacterial infections. The granulocytes of these patients have been shown to be completely deficient in the expression of the LFA-1 glycoprotein family. The heritable defect seems to reside in the gene encoding the 95kd  $\beta$ -subunit since normal levels of the  $\alpha$ -subunit precursors are detected in the cytoplasm. Presumably, the formation of  $\alpha\beta$  complexes are required prior to processing and transport to the cell surface to constitute functional  $\alpha\beta$  glycoprotein complexes (Springer et al., 1984).

The gp190 and gp180-200 proteins encode the fibronectin receptor (fnc-r) and transferrin receptor (trfn-r) respectively. Both of these proteins are expressed on normal adherent (fnc-r) and dividing

(trfn-r) cells but are expressed to a much greater extent in leukemic cells and cell lines (Sutherland et al., 1981; Trowbridge et al., 1981; Virtanen et al., 1987).

gp105 has been shown to express a fetal type(i) carbohydrate antigen in the erythroleukemic cell line K562. The distribution of this type(i) antigen on glycoproteins in mature erythrocytes differs completely with its distribution in K562 cells and specifically with its association with gp105, which is barely detectable in mature erythrocytes. The fetal type(i) antigen distribution on K562 was essentially the same as fetal cord erythrocytes indicating that although K562 shares some features with mature erythrocytes, eg. glycophorin and band 3, K562 is essentially a proerythroblastic/fetal erythroblast cell line. The presence of "unique" glycoproteins and associated fetal carbohydrates on the K562 cell surface is both suggestive of both "leukemia-specific" cell surface antigen expression and oncodevelopmental antigen expression (Gahmberg et al., 1979a; Fukuda et al., 1980).

The gp100 (CALLA) antigen has long been recognized as a "specific" marker for acute lymphocytic leukemia. Recently, it has been shown that the CALLA antigen is detectable on normal lymphocytes, granulocytes, monocytes and acute myelocytic leukemia cells. The level of CALLA expression does differ substantially between normal and leukemic cells such that the designation of CALLA as an "oncodevelopmental antigen" is still valid (Ritz et al., 1980).

gp100, p95 and p94 all demonstrate a restricted pattern of expression in relation to the cellular maturation state. gp100 is specifically associated with hematopoietic progenitor cells in human

bone marrow and fetal liver but is also detectable on Ph1<sup>+</sup> myeloblasts and cells from the leukemia cell line KG1. Modulation of gp100 expression and the level of expression in normal or leukemic cell lines has not been investigated (Katz et al., 1985). p95 was identified as a gelatin-binding protein in differentiated human macrophages and granulocytes. Adherent human macrophages produce p95 and it has been shown to be distinct from other fibronectin binding proteins and the M<sub>r</sub> 70000 polypeptide (the gelatin-binding protein produced by mesenchymal and epithelial cell types). Mature monocytes, macrophages and granulocytes express p95 whereas their corresponding progenitor cells, the monocytic leukemia cell line U937, non-myeloid leukemias and other hematopoietic stem cell disorders do not. However, maturation-induced U937 cells and leukemias presenting with mature granulocytes express p95, lending support to the suggestion that p95 is a differentiation marker for phagocytic cells and may be involved in phagocyte-related functions (Vartio et al., 1985). p94 is expressed on all myeloperoxidase<sup>+</sup> bone marrow myeloid precursors (promyelocytes, myelocytes, metamyelocytes) and mature monocytes and macrophages. p94 expression can be increased in the leukemic cell line U937 by treatment with the Ca<sup>2+</sup>-ionophore A23187, TPA, complement factor C5a or FMLP (formyl-met.leu.phe). No induction of p94 expression is seen in the cell lines HL60 or KG1 upon treatment with TPA even though morphological transformation has occurred. Peripheral blood and bone marrow B- and T-lymphocytes do not express detectable levels of p94 either. Both peripheral blood and bone marrow macrophages and monocytes express p94 which seems to suggest that p94 is a normal myelomonocytic plasma membrane glycoprotein rather

than a developmentally regulated oncofetal antigen (Todd III et al., 1985).

p68, p55 and p37 have been shown to be associated with hematopoietic cells of either the myeloid (p68, p55) or erythroid (p37) lineages. p68 and p55 have been detected on all myeloid progenitor cells but are not expressed by mature erythrocytes, lymphocytes, platelets, granulocytes or mature bone marrow myeloid cells. Information regarding their levels of expression during myelopoiesis or modulation in response to morphogen-induced maturation is not available. However, due to their expression on myeloid precursor cells and on AML/CML blast cells, p68 and p55 may represent differentiation antigens for myeloid progenitor cells and immature, committed myeloid cells (Griffin et al., 1982; 1984). p37 is expressed in all hematopoietic cells but its expression in the erythroid lineage is developmentally regulated. p37 is expressed on the outer surface of the plasma membrane in all hematopoietic cells except mature erythrocytes, where it is found on the inner surface of the plasma membrane. The reason for or the function of this variable membrane organization of p37 in erythroid cells is unknown. It is possible that p37 in erythroid cells functions in a manner analogous to the avian p34-39 inner plasma membrane protein, which serves as a target for pp60<sup>src</sup>-mediated membrane phosphorylation.

The remaining proteins have been isolated as leukemia-associated antigens shed from the surface of leukemic cells (gp350-400, gp75-80) or specifically detected using MoAbs directed to specific cells or cell components. The antigens shed from leukemic cells have not been characterized further but their expression at the cell surface is

consistent with a number of reports in the literature which ascribe an increased degree of glycoprotein and glycolipid synthesis to the neoplastic phenotype (Hakomori et al., 1984). Consequently, the distribution of these antigens between normal and leukemic cells and possible differences in maturation specified expression could be significant but to date, have not been demonstrated (Taub et al., 1978; Baker et al., 1982).

The gp210 series (Andersson et al., 1979b) and the gp110 series (de Jong et al., 1984) were identified by screening ectopically-labelled leukemic cell samples using MoAbs and 2D-PAGE. Beyond the demonstration that the same protein arrays seem to be associated with leukemic cell samples, no further attempts were made to identify or characterize these proteins or determine whether or not they were represented in equivalent preparations of normal cells.

The p145/p105 proteins were identified as granulocyte-specific antigens by using a series of MoAbs directed to normal granulocytes. These proteins were expressed on all the myeloid cells and leukemic cell lines tested and were not expressed on a variety of non-myeloid cell lines (RPMI 6410, RPMI 8226, Raji, Molt 4, Daudi). Maturation induction experiments were not performed to assess the possible modulation of p145 or p105 expression (Skubitz et al., 1983). Apart from the designation of some of the aforementioned proteins as "onco-developmental antigens", no attempt has been made to ascribe any function to these proteins in the normal or leukemic cell.

The p68 (monomer)/p205 (trimer) complex was purified from myeloid leukemia cell lines HL60 and ML-1. This complex was subsequently identified as one of the monocyte nonspecific esterases.

The activation of these monocyte esterases in mature monocytes or macrophages and their inactivity in immature myeloid cells and cell lines indicates that esterase activity is a marker for terminal/functional differentiation of myeloid cells. The activation of monocyte esterases in the cell line HL60 by TPA-induced maturation to monocytes / macrophages further supports this assertion (Yourno et al., 1984, 1986).

The p70 (CAMAL) protein was detected in all non-lymphoblastic leukemic cell samples tested and was also shown to be unaffected by the maturation state of the leukemic cells examined. p70 (CAMAL) was detected on a small percentage of normal bone marrow cells but it has not been determined what the nature of these bone marrow cells are. Of particular interest is the finding that p70 (CAMAL) is inhibitory to the growth of normal, but not leukemic, bone marrow cells *in vitro*. It has been demonstrated previously that leukemic cells liberate growth suppressive moieties into culture supernatants (lactoferrin, prostaglandins E<sub>1</sub> and E<sub>2</sub>, "LIA", "LAI") and that these substances have specific inhibitory effects on normal, but not leukemic, granulocyte-macrophage progenitor cells *in vitro* (Broxmeyer et al., 1982; Olofsson et al., 1984). The specific synthesis of p70 (CAMAL) and subsequent secretion of this protein may endow the leukemic cell with a growth advantage over its normal counterparts and thus allow expansion of the leukemic clone in the bone marrow. Continued synthesis and secretion of p70 (CAMAL) by leukemic cells may also contribute to the maintenance of the leukemic phenotype in patients with myeloid leukemia.

The aims of the studies described in this thesis were first, to determine whether p70 (CAMAL) was expressed in leukemic cell lines as well as leukemic patient cell samples, second, to determine whether p70 (CAMAL) expression was related to cell cycle or cellular differentiation and, third, to determine, by protein/peptide sequencing, if purified p70 (CAMAL) represented a novel leukemia-associated antigen or an aberrant form of a normal myeloid developmental antigen and whether p70 (CAMAL) exhibited any relatedness to proteins regulating the growth and differentiation of normal myeloid cells.

## Chapter 2. Materials and Methods.

### 2.1. Cell culture and induction.

Cell lines HL-60 (acute promyelocytic leukemia; Collins et al., 1977), K562 (chronic myelogenous leukemia/erythroleukemia; Lozzio et al., 1975; Andersson et al., 1979; Rutherford et al., 1981), KG-1 (acute myelogenous leukemia/myelomonocytic leukemia; Koeffler et al., 1978), U937 (histiocytic leukemia/lymphoma; Sundstrom et al., 1976) and NS-1 (mouse myeloma; Kohler et al., 1976) have been described previously. All cell lines were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 0.12% (w/v) NaHCO<sub>3</sub>, 12 mM pyruvic acid, penicillin (50 units/ml) and streptomycin (50 ug/ml). Cultures were maintained at 10% CO<sub>2</sub>/37°C and passaged at 2 x10<sup>5</sup> cells/ml every 3 days. For morphological induction experiments with either TPA (12-O-tetradecanoyl-phorbol-13-acetate), DMSO (dimethyl sulphoxide) or retinoic acid (all trans -retinoic acid), cells were washed and then resuspended in fresh RPMI-1640 containing TPA (20 ng/ml), DMSO (1.25% v/v) (Collins et al., 1978; Westin et al., 1982a, 1982b; Craig et al., 1984; Gonda et al., 1984) or retinoic acid (0.5 ng/ml) (Breitman et al., 1980). Cells were allowed to differentiate over a 3 day period at which point cell suspensions were assessed for altered cell morphology by histochemical staining with Wright's stain.

### 2.2. Leukemia-associated antigen (CAMAL) isolation and purification.

Cells from patients with AML or CGL were obtained from the Cell Separator Unit, Vancouver General Hospital. Buffy coats were collected from heparanized leukemic cell samples by sedimentation at 1x g using Plasmagel (Laboratoire Roger Bellon, Neuilly, France). Cells were washed in PBS pH 7.3/5% FCS, and the cell pellet was resuspended in 17 mM Tris pH 7.2/140 mM ammonium chloride to lyse contaminating erythrocytes, pelleted and washed in PBS pH 7.3.

Leukemic cells or cell lines ( $1 \times 10^8$ - $10^9$  total) were washed three times in PBS pH 7.3 and resuspended in sterile water to approximately  $1 \times 10^8$ /ml prior to processing. An equal volume of a 2X stock solution of lysis buffer (300 mM NaCl/40 mM Tris pH 7.5/2% Triton X-100 or NP-40/1% NaDOC/0.2%SDS/10 mM PMSF) was added to the cell sample and the resulting cell suspension was allowed to lyse for 60 minutes, on ice, with continuous stirring. Unlysed cells and cell debris were removed by centrifugation at 17000 rpm for 20 minutes in a Sorvall SS34 rotor. The clarified supernatant (cell lysate) was collected and dialysed against 50 volumes of 0.01M  $\text{NH}_4\text{HCO}_3$  pH 9.0 and then 50 volumes of distilled water prior to concentration by lyophilization. The protein content of the cell lysate, prior to lyophilization, was determined by the Lowry assay (Lowry et al., 1951).

The lyophilized cell lysate was resuspended in BBS (300 mM NaCl) pH 8.5, to approximately 5 mg/ml, and clarified by centrifugation prior to application to the equilibrated CAMAL-1 immunoabsorbent column. The cell lysate was passed over the immunoabsorbent column 5 times and the final column pass collected. Prior to elution the column was washed free of unbound material by applying at least 25 column volumes of BBS (300 mM NaCl) pH 8.5. When the absorbance of the

column pass was less than 0.01 OD<sub>280 nm</sub>, the column was eluted in 1 column volume of 0.1N HCl followed by BBS (300 mM NaCl) pH 8.5 to re-equilibrate the column bed to pH 8.5. 1 ml fractions were collected, monitored for absorbance at 280 nm and the peak fractions were pooled, dialyzed against 0.01M NH<sub>4</sub>HCO<sub>3</sub> and distilled water and then lyophilized. Purified CAMAL pools were resuspended in 1-2 ml distilled water, protein content determined, ELISA reactivity with specific antiserum assessed and sample purity examined using SDS-PAGE. Immunoprecipitation of iodinated CAMAL pools, followed by either SDS-PAGE or 2D-PAGE and autoradiography, were also employed to assess purity.

### 2.3. Immunological reagents.

#### (i) Preparation of polyclonal anti-CAMAL antibody.

Fractions from preparative non-reducing polyacrylamide gels containing CAMAL were used to immunize young female albino rabbits. The eluted material was mixed 1:1 with complete Freund's adjuvant to a total volume of 1.0 ml, and was injected intramuscularly into four distal sites. Rabbits were given another immunization four weeks later and were bled seven days after the second immunization. The specificity of this antibody was assessed by the ELISA procedure (Malcolm et al., 1982).

Polyclonal antibody was also produced to immunoaffinity column-purified CAMAL in an analogous manner. The reactivity of this

antibody was also assessed by the ELISA procedure and a variety of other immunoassays.

(ii) Preparation of monoclonal anti-CAMAL antibody.

Female Balb/c mice (Jackson Laboratory, Bar Harbor, Maine) were immunized intraperitoneally with 20 ug of gel-purified CAMAL in 50% Freund's complete adjuvant. A second immunization was given four weeks later, in which 20 ug CAMAL in PBS pH 7.3 was administered on three consecutive days intravenously. Mice were sacrificed one day later, and their spleens used for fusion with the NS-1 myeloma cell line. Positive clones were identified using the ELISA procedure, cloned by limiting dilution three times, then expanded and grown as ascitic fluid in pristane-treated Balb/c mice (Malcolm et al., 1984).

(iii) Control antiserum.

Pre-immune rabbit serum was obtained from young female albino rabbits prior to immunization with either gel-purified or immunoaffinity column-purified CAMAL and used as a specificity control for the polyclonal anti-CAMAL antibody in the ELISA and other immunoassays.

Polyclonal anti-human serum albumin and anti-human alpha-1-fetoprotein antibodies were obtained from Cooper Biomedical Inc. [Cappel, Worthington Biochemicals] (Malvern, PA 19355) and Calbiochem [Behring Diagnostics] (La Jolla, CA 92037), respectively.

Both antisera were used as controls in the ELISA, immunoprecipitation assays and Western immunoblots.

Normal mouse serum was obtained from Cedarlane Laboratories Ltd. (Hornby, Ont. L0P 1E0), purified for immunoglobulin by DEAE-Sephacel gel chromatography and used as a specificity control for the monoclonal anti-CAMAL antibody in the ELISA and other immunoassays.

Monoclonal anti-human HLA-A,-B,-C antibody was obtained from Dr. R McMaster (Dept. Medical Genetics, University of British Columbia) and used as both a specificity control in the ELISA and as positive control for the immunoprecipitation of phosphorylated HLA-A,-B heavy chain in the  $^{32}\text{P}_i$ -labelling studies.

Monoclonal anti-Ferredoxin antibody (Weaver et al., 1982) was produced in our laboratory and was shown to be of the same immunoglobulin isotype as the monoclonal anti-CAMAL antibody. Consequently, the monoclonal anti-Ferredoxin antibody was used as a negative control antibody in both the ELISA and immunoprecipitation assays.

(iv) Control proteins.

Human serum albumin (HSA) was obtained from the Sigma Chemical Co. (St. Louis, MO 63178) as a crystalline protein, essentially fatty acid free (<0.005%) and essentially pure albumin (96-99%). HSA was used as a specificity control for the reactivity of the anti-CAMAL antisera with purified CAMAL in the ELISA and other immunoassays.

HSA was also used as a control for protein and peptide studies involving purified CAMAL.

Human alpha-1-fetoprotein (AFP) [fetal albumin] was obtained as iodination grade (98.2% pure) AFP from Calbiochem [Behring Diagnostics] (La Jolla, CA 92037). AFP was also used as a specificity control in the ELISA and a control for protein and peptide studies.

#### 2.4. Isotopic labelling of CAMAL *in vitro*.

##### (i) Labelling of cell lysates and purified protein with $^{125}\text{I}$ iodine.

Purified CAMAL (5 ug) or leukemic/normal cell lysates (1-5 mg) were labelled with 100 uCi or 1 mCi respectively of  $^{125}\text{I}$  (New England Nuclear) in PBS pH 7.3 using IODO-BEADS (Markwell et al. 1982). Protein-associated radioactivity, following dialysis of the iodination mixture against PBS pH 7.3 for 18 hr at 4°C, was determined by TCA-precipitation. A 10 ul aliquot of the iodinated protein sample was spotted onto a 2.5 cm glass fiber filter (Whatman GFA) and air-dried. The filter was then washed with 2 X 1 ml ice-cold 25% trichloroacetic acid, 5 ml absolute methanol and air-dried. Following drying the filter was counted in a gamma radiation counter.

##### (ii) Labelling of cells with $^{35}\text{S}$ -methionine and $^{35}\text{S}$ -cysteine.

Leukemic cell lines (HL60, U937, KG1 and K562) were harvested in mid-log phase (day 3), washed three times in RPMI 1640 medium, resuspended in 10 ml PBS pH 7.3 and an aliquot counted using 0.2%

Eosin Y to assess viability. A cell sample corresponding to  $1 \times 10^7$  cells was then transferred to a sterile tube, pelleted, and resuspended in 1 ml of RPMI 1640 medium (-)met and (-)cys. The cell sample was then incubated for 2 hr at  $37^\circ\text{C}/10\%$   $\text{CO}_2$ . Subsequent to this "starvation" period the cells were pelleted once more and then resuspended in 1 ml RPMI 1640 medium (-)met and (-)cys containing 250 uCi each of  $^{35}\text{S}$ -met/-cys (Amersham). The cell sample was then returned to the incubator for a period of 4 hr following which both cells and supernatant from the labelling mixture were collected. The cells were washed twice in RPMI 1640 medium, twice in PBS pH 7.3 and resuspended in 1 ml distilled water. An equal volume of 2X stock lysis buffer (300 mM NaCl/40 mM Tris pH 7.5/2% Triton X-100/1% NaDOC/0.2% SDS/10 mM PMSF) was added and the cell suspension allowed to lyse for 30 minutes, on ice, with vortexing at 10 minute intervals. Following lysis, cell debris was removed by centrifugation of the cell lysate in a microfuge for 15 minutes at  $4^\circ\text{C}$ . The supernatant was collected and protein-associated radioactivity assessed by TCA-precipitation as described above. Filters were air-dried after the methanol wash, immersed in 4 ml 0.4% PPO/BIS MSB premix (98:2 PPO:Bis-MSB, Syndel Laboratories Ltd.) in toluene and counted in a United Technologies-Packard Tri-Carb 4550 scintillation counter.

(iii) Labelling of cells with  $^3\text{H}$ -leucine.

Leukemic cell lines were harvested, washed, counted and assessed for viability as described above. Cells were "starved" in RPMI 1640

medium (-)leu and labelled with 1 mCi of  $^3\text{H}$ -leu (Amersham) in fresh RPMI 1640 (-)leu medium as described above.

(iv) Labelling of cells with  $^{32}\text{P}_i$  (Poher et al., 1978).

Leukemic cells ( $1 \times 10^7$ ) were washed free of medium in phosphate-free buffer A (150 mM NaCl/5 mM  $\text{MgCl}_2$ /5 mM KCl/2 mM glutamine/1.8 mM glucose/10 mM Tris-acetate pH 7.4) and incubated in buffer A for 1 hr at  $37^\circ\text{C}/10\% \text{CO}_2$ . The cells were then resuspended in 10 ml of buffer A containing 5mCi of  $^{32}\text{P}_i$  (Amersham) and incubated for an additional 3 hr at  $37^\circ\text{C}/10\% \text{CO}_2$ . The cells were then brought to ice temperature, pelleted and washed three times with phosphate-containing buffer B (137 mM NaCl/2.6 mM KCl/1.4 mM  $\text{K}_2\text{HPO}_4$  and 8.6 mM  $\text{Na}_2\text{HPO}_4$  pH 7.2). The final pellet was lysed on ice in 2% NP40 in buffer C (25 mM NaCl/5 mM  $\text{MgCl}_2$ /25 mM Tris-HCl pH 7.4/1 mM PMSF). The cell sample was allowed to lyse for 30 minutes on ice and clarified by centrifugation at 17000 rpm for 20 minutes in a Sorvall SS34 rotor. The labelled cell lysate was then subjected to immunoprecipitation assays with either the polyclonal or monoclonal anti-CAMAL antibody and control antibodies, followed by SDS-PAGE and autoradiography.

## 2.5. Immunoprecipitation of CAMAL from cell lysates.

(i) Immunoprecipitation with CAMAL-specific antibodies.

Labelled cell lysate was usually pre-cleared by the addition of 100 ul IgG-sorb/500 ul of lysate for 45-60 minutes on ice. Non-specifically adsorbing material in the lysate was removed by pelleting the IgG-sorb for 2 minutes in a cooled microfuge. The lysate was then transferred to a clean microfuge tube and 10 ul (5 ug) of specific antibody (polyclonal or monoclonal) added to the lysate. Control immunoprecipitations were performed in parallel using either non-immune serum or irrelevant polyclonal/monoclonal antibody. This mixture was incubated on ice for 60-90 minutes followed by the addition of 25 ul IgG-sorb and further incubation on ice for 45-60 minutes. The immune complexes were collected by centrifugation in a cooled microfuge for 2-3 minutes. The supernatant was discarded and the pellet washed once in ice-cold wash buffer (150 mM NaCl/20 mM Tris-HCl pH 7.5/1% Triton X-100/0.1% SDS) and twice in ice-cold PBS pH 7.3. The final pellet was resuspended in reduction mix (1% SDS/10 mM DTT/10% glycerol pH 7.0), incubated at 37°C for 15 minutes to dissociate the immune complexes and pelleted in a cooled microfuge to remove IgG-sorb. The supernatant was then transferred to a clean microfuge tube prior to heating at 100°C for 5 minutes and application to SDS-PAGE, vertical IEF slab gels or 2D-PAGE.

(ii) Immunoprecipitation using the CAMAL-1 immunoabsorbent.

Monoclonal antibody specific for CAMAL (CAMAL-1; Malcolm et al., 1984) coupled to cyanogen bromide (CNBr)-activated Sepharose CL4B beads (Cuatrecasas et al., 1970) was prepared for use by equilibration with lysis buffer (150 mM NaCl/20 mM Tris-HCl pH

7.5/1% Triton X-100/0.5% NaDOC/0.1% SDS/5 mM PMSF). Typically, 100-200 ul of immunoadsorbent was used per 1 ml of labelled lysate and the resulting suspension was incubated at room temperature for 1-2 hr, with intermittent mixing to keep the beads in suspension. Non-specific adsorption was assessed using beads to which irrelevant monoclonal antibody had been coupled. Following adsorption, the suspension was pelleted at 2000 rpm in a Sorvall GSC centrifuge. The supernatant was removed and the remaining "pellet" was washed twice with 1 ml of cold wash buffer (150 mM NaCl/20 mM Tris-HCl pH 7.5/1% Triton X-100/0.1% SDS) and three times with 1 ml of cold PBS pH 7.3. Elution of bound material was effected by incubation of the beads with 2 volumes of 0.1N HCl. The resulting "acid" supernatant was concentrated, using a Savant Speed-Vac centrifuge, prior to SDS-PAGE.

## 2.6. Electrophoretic techniques.

### (i) SDS-PAGE (Sodium dodecyl sulphate-polyacrylamide gel electrophoresis).

Electrophoresis was performed on concentrated protein samples by resuspending the sample in 25 ul of distilled water, if necessary, and adding an equal volume of 2X reduction mix (20 mM dithiothreitol/2% SDS/20% glycerol pH 7.0). Samples were heated at 100°C for 5 minutes and applied to 0.75 mm or 1.5 mm thick SDS-polyacrylamide gels. 10% SDS-PAGE gels were run, in a water-cooled apparatus, at constant voltage (pre-run at 70 volts for 20 minutes, samples added, stacked at 70 volts, run at 150 volts); stained using Coomassie Blue ([0.03% Serva

Coomassie Blue/50% methanol/10% acetic acid]; Fairbanks et al., 1971) or fixed in 50% methanol (2-18 hr) followed by alkaline silver nitrate staining (Wray et al., 1981) or 2% periodic acid cleavage of carbohydrate moieties followed by alkaline silver nitrate staining (Dubray et al., 1982).

(ii) Vertical slab IEF (Isoelectric focussing).

Isoelectric focussing of immunoprecipitates of purified protein(s) was performed essentially according to established procedures (O'Farrell et al., 1975), except that IEF gels were polymerised vertically in a standard SDS-PAGE apparatus using 0.75 mm or 1.5 mm thick spacers. 7% acrylamide /0.2% bis-acrylamide/8M urea/2% ampholine (LKB-Bromma ampholines; pH 4-6/6-8/7-9/3.5-10 at a ratio of 2:1:1:1) IEF gels were allowed to polymerise at room temperature for at least 2 hr, following which the comb was removed, cathode ([-]/20 mM NaOH) and anode ([+]/10 mM H<sub>3</sub>PO<sub>4</sub>) buffers were added and the IEF gel pre-focussed for 15 minutes at 50 volts, 15 minutes at 200 volts, 15 minutes at 300 volts and 30 minutes at 400 volts in a water-cooled SDS-PAGE apparatus. IEF samples were prepared by resuspending the concentrated protein sample in 25 ul of distilled water, if necessary, and adding an equal volume of 2X reduction mix (20 mM dithiothreitol/2% SDS/20% glycerol pH 7.0), heating at 100°C for 5 minutes, applying the reduced sample to the IEF gel and focussing the protein sample in a pH gradient for 10000 volt-hr. Establishment of the pH gradient could be observed after approximately 3 hr since the SDS in the reduction mix focusses to the extreme acidic end of the IEF gel and forms a yellow-

white precipitate. pH gradients were determined by sectioning the IEF gel, equilibrating sections in 1 ml distilled water and measuring the pH or by using pI marker proteins or by using carbamylation trains of carbonic anhydrase (Anderson et al., 1979). Following electrophoresis, the IEF gels were immersed in a 4% 5-sulphosalicylic acid/12% trichloroacetic acid solution for 1-2 hr to remove most of the ampholines and irreversibly precipitate the proteins. Prior to staining, the gels were equilibrated in destain (7.5% methanol/5% acetic acid) for 1 hr, to remove the remainder of the ampholines and adjust the pH of the gel to the pH of the staining solution, and then stained with either Coomassie Blue (Fairbanks et al., 1971) or alkaline silver nitrate (Wray et al., 1981).

(iii) 2D-PAGE (Two-dimensional polyacrylamide gel electrophoresis).

Two-dimensional electrophoresis of purified protein(s), immunoprecipitates or cell lysates was performed according to the procedure of O'Farrell (1975), as modified by Anderson et al. (1977). Isoelectric focussing of reduced protein samples was performed in either a vertical IEF slab gel apparatus or a Bio-Rad IEF tube gel apparatus. Lanes from the IEF slab gels were cut out of the gel and equilibrated in 5 ml of SDS-PAGE reduction mix (10 mM dithiothreitol /1% SDS/10% glycerol pH 7.0) for 30 minutes at 37°C. IEF tube gels were extruded from the glass tubes and treated as above. Both IEF slab gel lanes and IEF tube gels were applied to 10% SDS-polyacrylamide gels (5% polyacrylamide stacking gel) and electrophoresed as in Section 6(i). The final pH gradient of IEF slab gel lanes or IEF tube gels was

determined by sectioning the gel as in Section 6(ii). The SDS-polyacrylamide gels were stained using either Coomassie Blue or alkaline silver nitrate, as described in Section 6(i).

## 2.7. ELISA (Enzyme-linked immunosorbent assay) and related techniques.

### (i) Titration of antisera using the ELISA.

The use of the ELISA for the titration of antiserum and assessment of antiserum specificity has been described previously in detail (Kelly et al. 1979; Al-Rammahy et al. 1980). Both polyclonal, monoclonal and control antibodies were titrated in the ELISA using purified CAMAL (100 ng/well) to determine the "limiting" dilution for inhibition assays.

### (ii) Inhibition ELISA.

Inhibition assays were performed using the ELISA by incubating a constant dilution of antiserum with increasing concentrations of inhibitor, usually cell lysate. A parallel inhibition assay was always performed using purified CAMAL as the inhibitor to allow quantitative determinations of the amount of CAMAL present in the cell lysate. Polyclonal/monoclonal antiserum was diluted in PBS pH 7.3 and aliquots of the diluted antiserum were mixed with dilutions of cell lysate or known amounts of purified CAMAL (3.0, 1.0, 0.3, 0.1, 0.03, 0.01, 0.003,

0.0 ug) and incubated at 4°C for 18-24 hr. Following incubation the antiserum-inhibitor mixture was transferred to an ELISA plate coated with purified CAMAL at 100 ng/well. The ELISA plate was incubated at 37°C for 2 hr after which standard ELISA protocol was followed.

Following colour development, the amount of CAMAL present in the cell lysate was determined by comparing the dilution of cell lysate that gave the same degree of inhibition as a known amount of purified CAMAL.

## 2.8. Protein (Western) blotting technique.

### (i) Electrophoretic methods.

SDS-PAGE was performed using the discontinuous buffer system of Laemmli (1970). 100-300ug of cell lysate was concentrated in a Savant Speed-Vac, resuspended in 50 ul of reduction mix, heated at 100°C for 5 minutes and loaded into a single lane on an SDS-PAGE gel. 1-5ug of purified CAMAL, HSA, AFP or BSA were routinely included as positive and negative control proteins. Proteins were transferred to nitrocellulose membranes (S&S BA85, Schleicher and Schull, Inc), in a BioRad Trans-Blot apparatus, by the methods of Towbin et al. (1979), with a transfer time of 90 minutes at 0.5 A or 18 hr at 25 mA.

### (ii) Immunological reagents.

Purified CAMAL and antibodies specific for CAMAL have been described previously (Malcolm et al., 1984). Antibodies specific for a number of human plasma proteins were obtained from Cappel

Laboratories, Inc. Purified plasma proteins were obtained from the Sigma Chemical Company. Alkaline-phosphatase conjugated rabbit anti-mouse and sheep or goat anti-rabbit IgG were either purchased from Tago, Inc. or prepared according to the methods of Avrameas et al. (1978).

(iii) Development of blot (Blake et al., 1984).

Following transfer, the nitrocellulose sheet(s) was immersed in PBS pH 7.3 containing either 3% BSA, 3% fat-free casein or 3% Pacific milk powder for 1 hr at 37°C with constant, gentle agitation. After incubation in this "blocking" solution, antibody (1:1000 in "blocking" solution) was added to the nitrocellulose sheet and incubation was continued for an additional 90 minutes at 37°C. The blot was washed with warm PBS-Tween pH 7.3 three times for 10 minutes each. The alkaline-phosphatase conjugate (1:2000 in "blocking" solution) was added to the nitrocellulose sheet and incubated for 90 minutes at 37°C. The blot was washed three times in warm PBS-Tween pH 7.3 for 10 minutes each, and once in 150 mM Tris-acetate pH 9.6 for at least 5 minutes. Each nitrocellulose sheet was then incubated in 10 ml of 150 mM Tris-acetate pH 9.6 containing 20 ul 2M MgCl<sub>2</sub>, 100 ul 5-bromo-4-chloro-3-indolyl phosphate (Sigma, 5 mg/ml in 5,5' dimethyl formamide) and 1 mg Nitroblue tetrazolium (Sigma). Blots were developed at room temperature for about 15 minutes following which the reaction was terminated by replacement of the developing solution with distilled water.

## 2.9. Reduction and carboxymethylation (RCM) of proteins.

HSA, BSA and AFP were used as standards for immunological assays and protein chemistry studies. The purity of these proteins was assessed by the manufacturers and stated as >98%. CAMAL was purified from both leukemic cell (AML/CGL) and cell line (HL60, KG1, K562, U937) lysates by immunoaffinity chromatography on the CAMAL-1 immunoabsorbent. CAMAL purified in this manner was subjected to further purification by gel-size exclusion and anion-exchange high pressure liquid chromatography (HPLC). CAMAL purified by this protocol was shown to be >90% by both HPLC analysis and one or two-dimensional PAGE. Purified protein(s) was reduced and carboxymethylated (RCM) prior to amino acid analysis (Crestfield et al., 1963; Gurd et al., 1967). 1 nmole of lyophilized protein was resuspended in reduction mix (500 mM Tris-HCl pH 8.5/50 mM dithiothreitol/5 mM EDTA/6M guanidine-HCl) and incubated, under argon or nitrogen, at 37°C for 90 minutes in the dark. Following the reduction step, the sample was carboxymethylated by the addition of iodoacetic acid to a final concentration of 100 mM (stock solution; 1M iodoacetic acid in reduction mix without dithiothreitol) and incubated, under argon or nitrogen, at 37°C for 30 minutes in the dark. Following carboxymethylation, the "free" iodoacetic acid was "scavenged" by the addition of 2-mercaptoethanol to a final concentration of 1% v/v. The RCM-protein sample was then exhaustively dialyzed against 0.01M  $\text{NH}_4\text{HCO}_3$  pH 9.0 and finally distilled water after which it was concentrated by lyophilization using a Savant Speed-Vac centrifuge. Prior to amino acid analysis, the RCM-

protein was resuspended in a minimal volume of 6N HCl/0.1% thioglycolic acid/0.1% phenol and heated at 110°C *in vacuo* for 24 hours. Following the acid hydrolysis, the protein sample was washed free of acid by repeated lyophilization from water, resuspended in 0.2M Na-citrate buffer pH 3.2 and applied to a Beckman 6300 automated amino acid analyser. Amino acid analysis data is presented as the mean value of at least three separate analyses.

## 2.10. Trypsin cleavage of RCM-protein(s).

For analytical reverse-phase HPLC runs, 1 nmole of RCM-protein was used. When purified tryptic peptides were required for sequence analysis, 12 nmole of RCM-protein was used.

RCM-protein samples were citraconylated (Atassi et al., 1967) to block lysine (lys) residues prior to enzymatic cleavage with TPCK-trypsin (Worthington Enzymes). RCM-protein was resuspended in 1 ml HPLC water and adjusted to pH 9.0 with NaOH. Citraconic anhydride (CTA) was added in four equal aliquots at 1 hr intervals such that the final concentration of CTA was 100 times that of the lys residues present (as indicated by amino acid analysis). The mixture was kept stirring for an additional 2 hr, at room temperature, following the last CTA addition. The CTA-protein mixture was then dialysed against HPLC water for 24 hr at 4°C after which it was concentrated in a Savant Speed-Vac centrifuge. The CTA-RCM-protein sample was resuspended in a minimal volume of 0.1M  $\text{NH}_4\text{HCO}_3$  pH 9.0 containing TPCK-trypsin (2 mg/ml in 0.1M  $\text{NH}_4\text{HCO}_3$  pH 9.0) at an enzyme:substrate ratio of 1:200. Two consecutive 3 hr digestions were performed at 37°C,

following which the digestion was terminated by the addition of acetic acid to 10% v/v. The protein/peptide mixture was stirred overnight at room temperature to deblock the citraconylated lys residues and then dried down once from 0.01M  $\text{NH}_4\text{HCO}_3$  pH 9.0 and twice from HPLC water.

## 2.11. High pressure liquid chromatography (HPLC) analysis of peptides.

### (i) Size-exclusion and anion-exchange chromatography of CAMAL, CAMAL cyanogen bromide (CNBr) and TPCCK-trypsin peptides.

Immunoaffinity column-purified CAMAL was reduced and carboxymethylated, as described in Section 9, and subjected to HPLC size-exclusion chromatography to remove low molecular weight contaminants prior to chemical or enzymatic protein cleavage procedures. Following dialysis and lyophilization, RCM-CAMAL was resuspended in a minimal volume of a 1:1 (v/v) mixture of 0.05% trifluoroacetic acid (TFA) in HPLC water and HPLC grade acetonitrile (ACN). Isocratic elution of RCM-CAMAL was accomplished using a Waters Associates Inc. HPLC system consisting of one Waters M45 pump, a Waters Model 441 fixed wavelength absorbance detector (254 nm) and a Waters I-125 gel size-exclusion column (1.2cm X 35cm) at a flowrate of 1 ml/minute. Peak fractions from size-exclusion HPLC were collected manually and lyophilized directly from TFA/ACN using a Savant Speed-Vac centrifuge prior to further analysis.

Linear gradient elution of CAMAL or CAMAL CNBr-peptides from an anion-exchange column was accomplished using a Waters Associates Inc. HPLC system consisting of two Waters M45 pumps, a Waters Model 660 solvent programmer (gradient curve 6), a Waters Model 441 fixed wavelength absorbance detector (254 nm for CAMAL, 215 nm for peptides), and either a Waters Protein-Pak DEAE-5PW (7.5mm X 7.5cm) or a Pharmacia Mono Q (HR 5/5, 5mm X 50mm) column. Bound protein or peptides were eluted in a linear gradient from 100% buffer A (20mM Tris-Cl, pH 8.5) to 100% buffer B (20mM Tris-Cl, pH 8.5/0.5M NaCl) over 25 minutes at a flowrate of 1 ml/minute. Peak fractions for anion-exchange HPLC were collected manually, dialyzed exhaustively against water and lyophilized prior to further analysis.

(ii) Reverse-phase chromatography of CAMAL tryptic peptides.

Linear gradient elution of peptides from a reverse-phase column was accomplished using a Waters Associates Inc. HPLC system consisting of two Waters M45 pumps, a Waters Model 660 solvent programmer (gradient curve 6), a Waters Model 441 fixed wavelength absorbance detector (215 nm) and an RPC3 (Beckman [Altex] Ultrapore C3, 4.6 mm x 75 mm) column. Digested protein was resuspended in buffer A (0.1% trifluoroacetic acid [TFA] in HPLC water) and aliquots were applied, using a U6K universal injector, to the RPC3 column. Bound peptides were eluted in a linear gradient of 10-40% buffer B (0.1% TFA in isopropanol) over 40 minutes at a flowrate of 1 ml/minute. Peak fractions from repetitive RPC3 runs were collected manually, pooled, dried and subjected to both amino acid analysis (section 9) and

peptide sequencing. The automated N-terminal sequence analysis of purified CAMAL peptides was performed by Dr. Don McKay at the University of Calgary Protein Sequencing Facility, using the resident sequencing program in an Applied Biosystems Model 470A gas-phase protein sequencer.

## 2.12. Two-dimensional thin layer chromatography (2D-TLC) of derivatized peptides.

Peptides were dansylated (Gray et al., 1967; Gerday et al., 1968), iodinated with diiodo-Bolton-Hunter reagent (Bolton et al., 1973), iodinated with  $^{125}\text{I}$  using Iodo-beads (Markwell et al., 1982) or reacted with fluorescamine (Stephens et al., 1978), prior to 2D-TLC analysis or thin-layer electrophoresis (TLE)/TLC.

### (i) Dansyl-N-terminal amino acid analysis (Hartley et al., 1970).

End-group analysis for accessible N-terminal amino acid(s) of derivatized RCM-protein(s) was determined as follows; dansylated, intact RCM-protein was hydrolysed in 6.7N constant boiling HCl (Pierce Chemical Co.)/0.1% thioglycolic acid/0.1% phenol in vacuo for 24 hr at 110°C. The resulting hydrolysate was dried and resuspended in a minimal volume of acetone:acetic acid 1:1 v/v. This solution was then applied 2 cm in and 2 cm up from the bottom left-hand corner of a 25  $\mu\text{m}$  X 15 cm X 15 cm micropolyamide TLC foil (Schleicher & Schull). The TLC foil was subjected to chromatography in an equilibrated TLC chamber in water:formic acid 50:1.5 v/v, dried, rotated 90°, subjected to

chromatography in toluene:acetic acid 9:1 v/v, dried, visualised and photographed under short-wave ultraviolet light. "Free" or accessible N-terminal amino acids appeared as blue-green spots under ultraviolet light. The secondary dansyl chloride derivatives of tyrosine (tyr), O-dansyl-tyr, and lysine (lys),  $\epsilon$ -dansyl-lys, also appeared consistently in all analyses and were used to approximate the yield of the N-terminal dansyl amino acid by quantitating the observed fluorescence based on 100% yield of either O-dansyl-tyr or  $\epsilon$ -dansyl-lys. Two other non-dansyl amino acid spots appeared consistently on the TLC foils as well. These spots corresponded to the aqueous decomposition products of dansyl chloride, namely, dansyl sulphonic acid (DNS-OH) and dansyl sulphonamide (DNS-NH<sub>2</sub>).

(ii) TLE/TLC of derivatized peptides

RCM-protein cleaved with CNBr (Gross et al., 1967) or TPCK-trypsin was derivatized with <sup>125</sup>I. TLE/TLC was performed on 100  $\mu$ m X 20 cm X 20 cm cellulose acetate sheets (Art. 5577, E. Merck). Electrophoresis in the first dimension was accomplished in a Desaga thin-layer electrophoresis chamber at pH 2.1 (2% formic acid, 8% acetic acid, 90% water), using Whatman 1MM wicks, at 1000 volts for 60 minutes. Crystal violet, spotted at the top left-hand corner, was used as a tracking dye and usually migrated approximately 5 cm by the end of the run. The peptide sample was resuspended and spotted as in section 6(i). Following electrophoresis the TLC sheet was allowed to dry in a fume hood overnight before being subjected to chromatography in the second dimension. The dry TLC sheet was subjected to chromatography

in an equilibrated TLC chamber in butanol:acetic acid:pyridine:water (BAWP) 15:3:12:10. The TLC sheet was allowed to dry in a fume hood and peptide spots were localised by autoradiography.

### 2.13. Comparison of ligand binding to human serum albumin (HSA) and CAMAL

The binding of fluorescent probes to both HSA and CAMAL was examined using fluorescent methods previously described by Sudlow et al. (1973,1975 and 1976) and Chen (1967). Within the limits of sensitivity, excitation wavelengths were chosen to minimize interference from incident light. The optical density change of the sample solution, at the chosen excitation wavelength, did not exceed 0.3 during DNS-probe or ligand titrations. Emission and excitation wavelengths for individual fluorescent probes were chosen to yield maximum fluorescence when the DNS-probe was bound to HSA, while yielding minimal fluorescence in the unbound state in sample buffer (Sudlow et al., 1975). Titration of a limiting amount of fluorescent probe with HSA was used to quantitate the maximum fluorescence yield when all the available DNS-probe was bound to HSA. This value was then used to calculate the concentrations of "free" and "bound" DNS-probe in a titration of a fixed amount of HSA or CAMAL with increasing amounts of fluorescent probe (Sudlow et al., 1973). Results were plotted by the method of Scatchard or by using the double-reciprocal method of Hughes and Klotz, as described by Sudlow et al. (1975), and binding parameters calculated by linear regression.

Solutions of crystalline human serum albumin (Sigma) and lyophilized CAMAL were made up in 100 mM  $\text{NaH}_2\text{PO}_4$  pH 7.4/0.9% NaCl buffer to a concentration of 1 mg/ml and the concentration verified using the published extinction coefficient for HSA of  $E_{1\text{cm}}^{1\%} = 5.3 \text{ OD}_{280 \text{ nm}}$  (Chignell et al., 1969). An additional solution of HSA at 10 mg/ml was made in the same buffer and was used for probe saturation studies. Solutions of dansyl chloride (1-dimethylaminonaphthalene-5-sulphonyl chloride [DNS-]) derivatives of selected amino acids (Sigma; DNS-glycine, DNS-sarcosine [methyl-glycine], DNS-proline) were made by dissolving 5 mg of the selected dansyl-amino acid in 1 ml of a 50% ethanol/50% 100 mM  $\text{NaH}_2\text{PO}_4$  pH 7.4/0.9% NaCl solution. DNS-amino acid solutions were further diluted in 100 mM  $\text{NaH}_2\text{PO}_4$  pH 7.4/0.9% NaCl buffer to a concentration of 0.5 mg/ml. Fluorescence measurements were made in a Perkin-Elmer 650-10S Fluorescence spectrophotometer using a Perkin-Elmer Model 150 Xenon power supply. Relative fluorescence yields were quantitated using a Beckman Industrial Model 310 digital multimeter (set to 200 DC mV range) connected, in series, to both the fluorescence spectrophotometer and a 10 mV "strip-chart" recorder. All fluorescence measurements were determined using excitation/emission slit widths of 10 nm, the appropriate excitation/emission wavelengths (Sudlow et al., 1975) and protein/DNS-probe solutions made and used at room temperature.

(i) Titration to measure maximum fluorescence (limiting probe)

Titration of the dansyl chloride derivatives of the chosen amino acids with HSA were used to quantitate the limiting or maximum

fluorescence when all of the particular DNS-amino acid was bound. Solutions of DNS-gly, -sarc and -pro were made up in 100 mM  $\text{NaH}_2\text{PO}_4$  pH 7.4/0.9% NaCl buffer to a concentration of 5ug/ml. 0.2ul (1ng) of the DNS-amino acid solution was then added to 1ml of 100 mM  $\text{NaH}_2\text{PO}_4$  pH 7.4/0.9% NaCl buffer and mixed in a 1cm pathlength, low UV, quartz cuvette before placing the cuvette into the spectrofluorometer.

Background fluorescence of this solution was usually 0.3-0.4 RFU (relative fluorescence units) and, typically, did not increase unless protein was added. Buffer blanks containing equivalent concentrations of added protein, but not DNS-probe, did exhibit a gradual increase in absorbance, due primarily to the increase in the optical density of the solution upon addition of protein, throughout the titration and this value was subtracted from the observed RFU value in samples containing both protein and fluorescent probe.

HSA, at 10 mg/ml in 100 mM  $\text{NaH}_2\text{PO}_4$  pH 7.4/0.9% NaCl buffer, was added in 10ul (100ug) aliquots to one cuvette containing the DNS-probe, while an additional 10ul aliquot of HSA was added to another cuvette containing only buffer. Both cuvettes were read sequentially and relative fluorescence recorded on the "strip-chart" recorder and quantitated using the digital multimeter. As mentioned previously, background fluorescence (buffer cuvette) was subtracted from the observed fluorescence (DNS-probe cuvette) to give the overall increase in fluorescence of the DNS-probe upon addition of protein. After each addition of HSA, the fluorescence was measured using excitation/emission wavelengths of 370 nm/475 nm for DNS-gly, 370 nm/475 nm for DNS-sarc and 375 nm/460 nm for DNS-pro. This titration, in protein excess, was continued until no further increase in

DNS-probe fluorescence was observed. At this point, saturation of the fluorescent probe with protein had been achieved and this maximal fluorescence was used to calculate the amount of "protein-bound" DNS-probe by applying the derived maximum RFU/[ng DNS-probe bound] value to the observed fluorescence values when using limiting amounts of protein and increasing amounts of DNS-probe.

DNS-probe saturation studies were performed for all three DNS-amino acid derivatives over a sequential range of HSA concentrations from 14.3  $\mu$ M to 142.9  $\mu$ M. Initial DNS-probe concentrations were 3.24 nM for DNS-gly, 2.45 nM for DNS-sarc and 2.87 nM for DNS-pro.

(ii) Titration of HSA/CAMAL using fluorescent probes (limiting protein)

The maximum RFU/[ng DNS-probe bound] value was then used to determine the amount of DNS-probe "bound" and "free" at each point on a titration of HSA or CAMAL with DNS-probe. Solutions of HSA and CAMAL at 1mg/ml and DNS-probe at 0.5 mg/ml in 100 mM  $\text{NaH}_2\text{PO}_4$  pH 7.4/0.9% NaCl buffer were employed in this titration. 2.5  $\mu$ l (2.5  $\mu$ g) of HSA, or CAMAL, was added to 1ml of 100 mM  $\text{NaH}_2\text{PO}_4$  pH 7.4/0.9% NaCl and mixed in a 1cm pathlength, low UV, quartz cuvette before placing the cuvette into the spectrofluorometer. Background fluorescence was determined as in Section 13(i), except that buffer blanks contained DNS-probe but no protein. DNS-probe was added to the cuvettes, one containing either HSA or CAMAL and the other containing only buffer, mixed and the increase in fluorescence recorded on the "strip-chart" recorder and the digital multimeter simultaneously. After each addition of DNS-probe the fluorescence was recorded at the

appropriate excitation and emission wavelengths and quantitated as described in Section 13(i). This titration, in DNS-probe excess, was continued until no further increase in fluorescence, upon further addition of DNS-probe, was observed. DNS-probe binding parameters were then determined by calculating the amount of "bound" versus "free" DNS-probe at each point in the titration and plotting the values according to the method of Scatchard or by using the double-reciprocal method of Hughes and Klotz, as described by Sudlow et al. (1975).

Protein saturation studies were performed for both HSA and CAMAL using both proteins at a limiting initial concentration of 357 nM (2.5 ug/ml). All three DNS-probes were used to saturate each protein by using a sequential range of DNS-probe concentrations from 4.1uM to 162.1uM for DNS-gly, 3.07 uM to 122.7 uM for DNS-sarc and 3.59 uM to 143.5 uM for DNS-pro. In each case, this corresponded to a sequential increase in DNS-probe concentration from 1.25 ug/ml to 50 ug/ml.

## Chapter 3. Expression and detection of CAMAL in leukemic cell populations.

### 3.1. Introduction

The detection and isolation of antigens specifically associated with particular leukemias has long been sought as a means for developing reagents which could be employed in the diagnosis and therapy of acute leukemias. With the advent of hybridoma technology and the attendant production of monoclonal antibodies (MoAbs), the desire for leukemia-specific diagnostic reagents has, at least, been partially realized. Unfortunately, many of these MoAbs were raised to whole leukemic cells or cell extracts and consequently exhibit substantial reactivity with normal hematopoietic cells. While this reactivity with normal tissue may not affect differential diagnosis significantly, it most certainly limits the use of these MoAbs in the "purging" of autologous bone marrow prior to reinfusion. The use of proteins purified from leukemic cells or cell extracts, not detectable in equivalent preparations of normal cells, as primary immunogens allows the production of MoAbs with greater specificity for leukemic cells and a decreased level of reactivity with normal cells. Using such MoAbs it then becomes possible to delineate the expression of the antigen(s) recognised by the MoAb in both leukemic and normal cell populations. As well as providing a basis for judging the specificity of antigen expression in particular cell lineages within a leukemic or normal cell population, the reactivity of the MoAb with leukemic cells versus normal cells allows the evaluation

of the MoAb and its cognate antigen as a diagnostic reagent for and a marker of particular leukemias.

The use of immunoprecipitation, with MoAbs and polyclonal antibodies, and labelled leukemic and normal cells has permitted both the detection and demonstration of differential antigen expression in leukemic cells versus normal cells (Al-Rammahy et al., 1980; Malcolm et al., 1982; Malcolm et al., 1983; Malcolm et al., 1984).

Immunohistochemical staining of leukemic or normal cell preparations has detected MoAb-defined antigens associated with specific leukemias and has also shown these antigens to be undetectable or present at significantly reduced levels in normal cells (Logan et al., 1984; Levy et al., 1985). The association of antigen expression with particular cell lineages, cellular proliferation or differentiation can be established using both leukemic cell samples and cell lines. The detection of such leukemia-associated antigens is significant in that associated expression of these antigens with leukemia may reflect an integral role for these antigens in the genesis or maintenance of the leukemic phenotype.

In our laboratory, we have identified a common antigen (CAMAL) that is associated with cells of patients with acute non-lymphocytic leukemia (ANLL) and chronic granulocytic leukemia (CGL). CAMAL has been detected on or in the cells of patients with myeloproliferative disorders (AML, CGL, ANLL) whereas, preparations of normal peripheral blood leukocytes (PBLs) or bone marrow cells did not demonstrate the presence of CAMAL in more than 1% of the cells tested (Logan et al., 1984). Cells from patients with other hematologic malignancies or lymphoproliferative disorders did not usually demonstrate elevated levels of CAMAL either. This putative leukemia antigen was also shown

to be expressed in the promyelocytic cell line HL60. Preliminary characterization of this antigen showed CAMAL to be a protein of  $M_r$  70000 with a pI of 7.2. Both polyclonal and monoclonal antisera have also been raised to CAMAL (Malcolm et al., 1982; Shipman et al., 1983; Malcolm et al., 1984).

Polyclonal anti-CAMAL antiserum was produced in rabbits by immunization with native gel-purified CAMAL. This antiserum exhibited marked specificity for human AML and CGL cells or cell extracts whereas, equivalent cellular preparations from normal individuals or patients with other non-myelocytic lymphoproliferative disorders (ALL, CLL, lymphomas, myelomas) demonstrated no significant reactivity when tested in the ELISA (Al-Rammahy et al., 1980; Malcolm et al., 1982).

Polyclonal antiserum produced in rabbits to immunoaffinity column-purified CAMAL also exhibited specificity for AML and CGL cell preparations in a variety of immunoassays and was indistinguishable, in its specificity, from the polyclonal antibody raised to gel-purified CAMAL. As expected, this antiserum did not exhibit any significant reactivity with normal PBLs or bone marrow cells or cells from patients with lymphoproliferative disorders.

Monoclonal anti-CAMAL antiserum (CAMAL-1) was produced to gel-purified CAMAL and used, subsequently, to purify CAMAL by passing leukemic cell or cell line extracts over a Sepharose CL4B:CAMAL-1 immunoaffinity column. CAMAL-1 had been shown to react specifically with AML and CGL PBLs or bone marrow cells or cell extracts but exhibited no specific reactivity with PBLs or bone marrow cells from normal individuals or patients with lymphocytic leukemia,

lymphoma or myeloma, as assessed by flow cytometry (Malcolm et al., 1984).

CAMAL-1 was also shown to react specifically with AML/CGL PBLs and bone marrow cells in an indirect immunoperoxidase staining procedure of single cell slide preparations (Logan et al., 1984). A small percentage (<1%) of bone marrow cells, but not PBLs, from normal individuals and patients with lymphoproliferative disorders was detected by CAMAL-1 in the immunoperoxidase staining technique. The significance of this finding has yet to be established, however, the occurrence of elevated levels of CAMAL in both leukemic cells and leukemic "remission" cells, as determined by immunoperoxidase staining, suggests that CAMAL expression might represent an underlying pathology in the maintenance of the leukemic phenotype.

The detection of p70 (CAMAL) expression in leukemic cells and cell lines is the subject of this chapter.

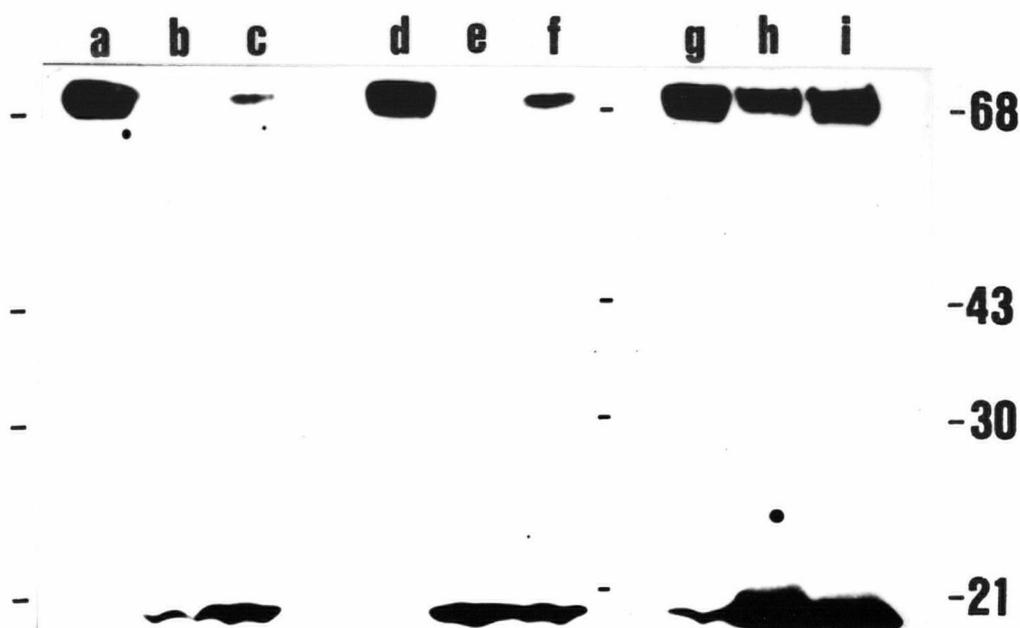
### 3.2. Results

To establish the presence and specific expression of p70 (CAMAL) in association with leukemic cells or cell lines, a survey study was initiated involving immunoprecipitation, with either polyclonal or monoclonal (CAMAL-1) anti-CAMAL antibody, of a variety of labelled leukemic clinical samples and cell line lysates. Figure 2 illustrates the typical pattern observed with immunoprecipitation of p70 (CAMAL) from iodinated cell lysates. p70 (CAMAL) was detected by both the polyclonal and monoclonal antibodies in all the samples tested. Broad bands of  $M_r$  68-70000 are evident in Figure 2 (lanes a, d, g, h, i). The presence of such broad bands is most likely due to overloading of the SDS-gel with  $^{125}\text{I}$ -p70 (CAMAL) immunoprecipitates, rather than indicating the presence of two proteins with  $M_r$ s of 68000 and 70000. The relative amount of p70 (CAMAL) detected by the polyclonal antibody did not seem to differ significantly between clinical material (AML, CGL) and leukemic cell lines (HL60, K562) which indicated, at least for p70 (CAMAL) "expression", that the leukemic cell lines used in this study were equivalent to leukemic cells from clinical sources. The 5-10 fold difference seen in the amounts of  $^{125}\text{I}$ -p70 (CAMAL) immunoprecipitable by the polyclonal antibody as compared to the monoclonal antibody (Figure 2, lanes a & c, d & f) is most likely due to the destruction or modification of the antigenic determinant recognised by the monoclonal on the p70 (CAMAL) molecule by the oxidative iodination procedure. The observed differences between the monoclonal and polyclonal antibodies in immunoprecipitation might also be due to a decreased affinity of the monoclonal antibody for iodinated

Figure 2. SDS-PAGE of p70 (CAMAL) immunoprecipitates from  $^{125}\text{I}$ -labelled leukemic cells and cell lines.

Lanes a-f : AML cell lysates. Lane g : HL60 cell line lysate.  
 Lane h : CGL cell lysate. Lane i : K562 cell line lysate.  
 Lanes a, d : precipitation with polyclonal anti-CAMAL antibody.  
 Lanes b, e : precipitation with normal rabbit (b) or mouse (e) serum.  
 Lanes c, f : precipitation with monoclonal anti-CAMAL antibody  
 Lanes g, h, i : precipitation with polyclonal anti-CAMAL antibody.

$1 \times 10^5$  TCA-precipitable cpm of iodinated cell lysate was used in each immunoprecipitation. The dried gel was exposed to Kodak X-Omat AR film for 12 hours at  $-70^\circ\text{C}$ . Relative molecular weights (kilodaltons) of protein standards are as indicated.



CAMAL. In either case, both the polyclonal and monoclonal antibodies demonstrated the presence and specific association of p70 (CAMAL) with the leukemic cells and cell lines. Since the leukemic cell lines appeared to be equivalent to clinical material in all aspects relevant to p70 (CAMAL) detection, the cell lines were used as the primary sources for the examination of p70 (CAMAL) expression. To establish whether p70 (CAMAL) could be detected in all the leukemic cell lines, immunoprecipitation of iodinated cell lysates was performed for each cell line (Figures 3 and 4, lanes a & b). The monoclonal antibody failed to immunoprecipitate a protein of  $M_r$  70000 in any of the cell lines tested, even though these same cell lines showed specific reactivity with the monoclonal in the ELISA and on Western blots (data not shown). These data suggested that the lack of reactivity with iodinated cell lysates was most likely attributable to iodination-induced changes in the antigenic determinant on p70 (CAMAL) recognised by CAMAL-1. Immunoprecipitation from these iodinated cell lysates with the polyclonal antibody detected a protein of  $M_r$  72000. The apparent discrepancy in relative molecular weight of the antigen recognised by the polyclonal antibody in Figures 2, 3 and 4 may, in part, be due to changes in antigenic determinants on CAMAL induced by the iodination procedure or to differences in processing of the CAMAL molecule in these cell lines. The relative amounts of p70/72 (CAMAL) detected by the polyclonal antibody in these cell lines was approximately the same for K562 (erythroleukemia), KG1 (myeloid leukemia) and HL60 (promyelocytic leukemia). However, approximately 10-fold less CAMAL was detected in the U937 (monocytic leukemia) cell

Figure 3. SDS-PAGE of p70 (CAMAL) immunoprecipitates from  $^{125}\text{I}$ -labelled HL60 and U937 leukemic cell lines.

Lane i : initial unprecipitated cell lysate.

Lane a : normal mouse serum.

Lane c : normal rabbit serum.

Lane b : CAMAL-1 antibody.

Lane d : polyclonal anti-CAMAL antibody.

$1 \times 10^5$  TCA-precipitable cpm of iodinated cell lysate was used in each immunoprecipitation. The dried gel was exposed to Kodak X-Omat AR film for 12 hours at  $-70^\circ\text{C}$ . Relative molecular weights (kilodaltons) of protein standards are as indicated.

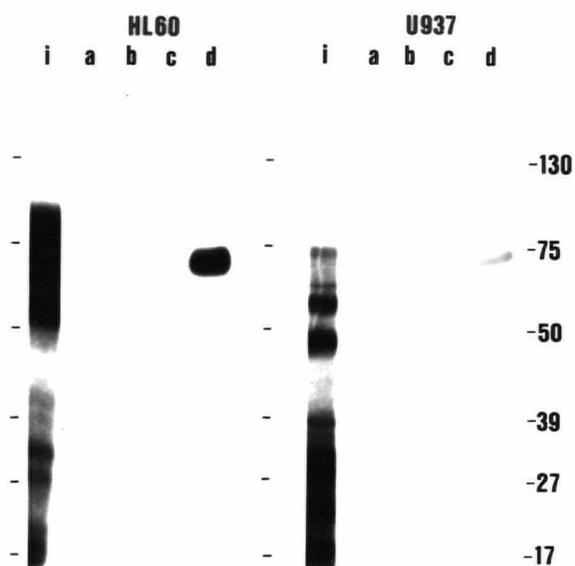


Figure 4. SDS-PAGE of p70 (CAMAL) immunoprecipitates from  $^{125}\text{I}$ -labelled KG1 and K562 leukemic cell lines.

Lane i : initial unprecipitated cell lysate.

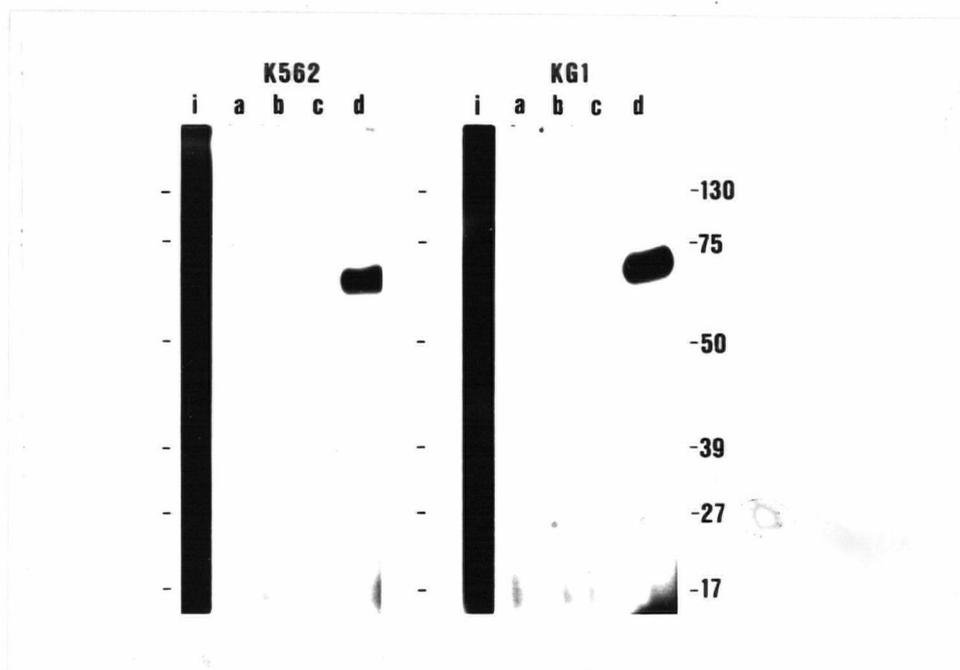
Lane a : normal mouse serum.

Lane c : normal rabbit serum.

Lane b : CAMAL-1 antibody.

Lane d : polyclonal anti-CAMAL antibody.

$1 \times 10^5$  TCA-precipitable cpm of iodinated cell lysate was used in each immunoprecipitation. The dried gel was exposed to Kodak X-Omat AR for 12 hours at  $-70^\circ\text{C}$ . Relative molecular weights (kilodaltons) of protein standards are as indicated.



line. In order to determine whether the observed differences in the amount of CAMAL detectable by immunoprecipitation reflected cell lineage-regulated expression of CAMAL, immunoprecipitations were performed on lysates of metabolically labelled cell lines.

The immunoprecipitation profiles, using the polyclonal antibody, of  $^{35}\text{S}$ -methionine- or  $^3\text{H}$ -leucine-labelled cell extracts or cell-free supernatants are shown in Figures 5 and 6 for all the leukemic cell lines. The most obvious feature in Figure 5 was the precipitation of two proteins of  $M_r$  72000 and 58000 from the cell-free supernatant fraction of the labelled cell culture. The implication of this finding was that both the p72 and p58 proteins are actively secreted by these cell lines, a suggestion which was corroborated by the lack of precipitation of these proteins from the cell-associated fraction of the labelled cell culture. The co-precipitation of p72 and p58 was evident in all the cell lines indicating that the expression of both of these proteins was not cell lineage-dependent and was approximately the same for all four cell lines. p58 might represent a degradation product of p72, an unprocessed form of p72, a unique protein which is co-expressed with p72 or a unique protein that forms a stable complex with p72. It is also possible that p58 may interact specifically or non-specifically with the polyclonal antibody or immune complex. The suggestion that the interaction was non-specific was supported by the finding that a small amount of p58 was immunoprecipitated by the control antibody. Whether this association was functional or coincidental is still unresolved but proteins of  $M_r$  53-55000 have been detected in equivalent leukemic cell samples and cell lines using other independently derived antibodies (Mulder et al., 1981; Griffin et al.,

Figure 5. SDS-PAGE of p70 (CAMAL) immunoprecipitates from  $^{35}\text{S}$ -methionine labelled leukemic cell line supernatants and cell lysates.

Lanes 1 & 3 : non-immune rabbit serum.

Lanes 2 & 4 : polyclonal anti-CAMAL antibody.

Lanes 1 & 2 : precipitation from cell line supernatants (1 ml).

Lanes 3 & 4 : precipitation from cell line lysates ( $1 \times 10^7$  cells).

$2.5 \times 10^5$  TCA-precipitable cpm of labelled cell supernatant or cell lysate was used in each immunoprecipitation. The dried photofluor-impregnated gel was exposed to Kodak X-Omat AR film for 18 hours at  $-70^\circ\text{C}$ . Relative molecular weights (kilodaltons) of protein standards are as indicated.

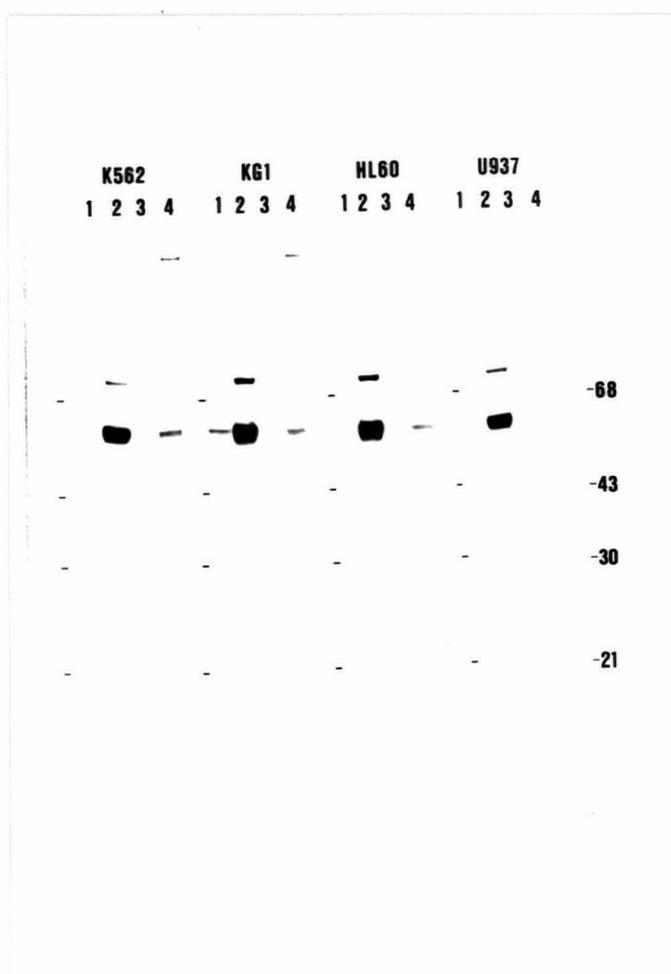


Figure 6. SDS-PAGE of p70 (CAMAL) immunoprecipitates from  $^3\text{H}$ -leucine labelled leukemic cell line supernatants and cell lysates.

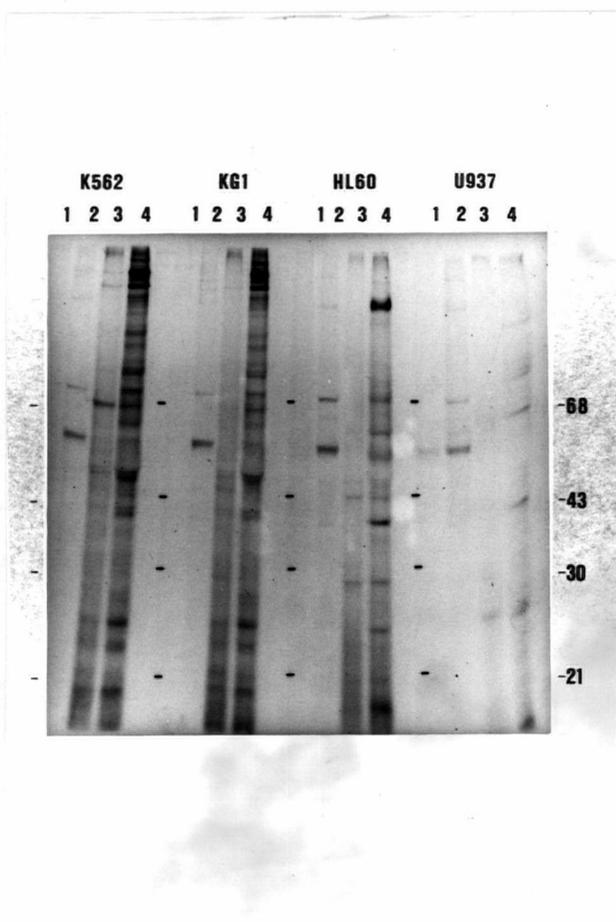
Lanes 1 & 3 : non-immune rabbit serum.

Lanes 2 & 4 : polyclonal rabbit anti-CAMAL antibody.

Lanes 1 & 2 : precipitation from cell line supernatants (1 ml).

Lanes 3 & 4 : precipitation from cell line lysates ( $1 \times 10^7$  cells).

$2.5 \times 10^5$  TCA-precipitable cpm of labelled cell supernatant or cell lysate was used in each immunoprecipitation. The dried photofluor-impregnated gel was exposed to Kodak X-Omat AR film for 32 days at  $-70^\circ\text{C}$ . Relative molecular weights (kilodaltons) of protein standards are as indicated.



1982). The monoclonal antibody (CAMAL-1) did not specifically immunoprecipitate any proteins from metabolically-labelled cell line lysates, while it reacted specifically with these same lysates in the ELISA or on Western blots (data not shown). The discrepancy in reactivity of CAMAL-1 in solution versus solid-phase assays suggested a decreased affinity of CAMAL-1 for p70 (CAMAL), as compared to the polyclonal antibody, or indicated that some degree of antigenic determinant modification on nascent CAMAL molecules occurred during metabolic labelling of the leukemic cell lines. The lack of CAMAL-1 reactivity might also reflect differences in affinity for labelled versus non-labelled CAMAL. In order to assess the affinity of the monoclonal antibody for p70 (CAMAL) directly, an immunoadsorbent column was made by coupling CAMAL-1 to cyanogen bromide (CNBr)-activated Sepharose CL4B beads and assessed for its ability to purify p70/72 (CAMAL) from a metabolically-labelled leukemic cell line lysate. The immunoadsorbent column was pre-eluted with 0.1N HCl, re-equilibrated with BBS (300 mM NaCl) pH 8.5 and then used as described in section 2.5 (ii).

Cell lysates from both  $^{35}\text{S}$ -met-/ $^{35}\text{S}$ -cys-labelled NS1 (mouse myeloma) and U937 (human monocytic leukemia) cell lines were passed over a Sepharose-CL4B:mouse immunoglobulin (mouse-Ig) column prior to application to the CAMAL-1 immunoadsorbent to remove material in the cell lysate that might non-specifically adsorb to the column matrix or mouse immunoglobulin. The column pass from the mouse-Ig column was applied directly onto the CAMAL-1 immunoadsorbent. The autoradiogram for the CAMAL-1 column-bound material from both cell lines is shown in Figure 7. The NS1 cell lysate did not exhibit any

Figure 7. SDS-PAGE of p70 (CAMAL) immunoprecipitated from  $^{35}\text{S}$ -methionine and  $^{35}\text{S}$ -cysteine labelled U937 leukemic cell line using the CAMAL-1 immunoabsorbent column.

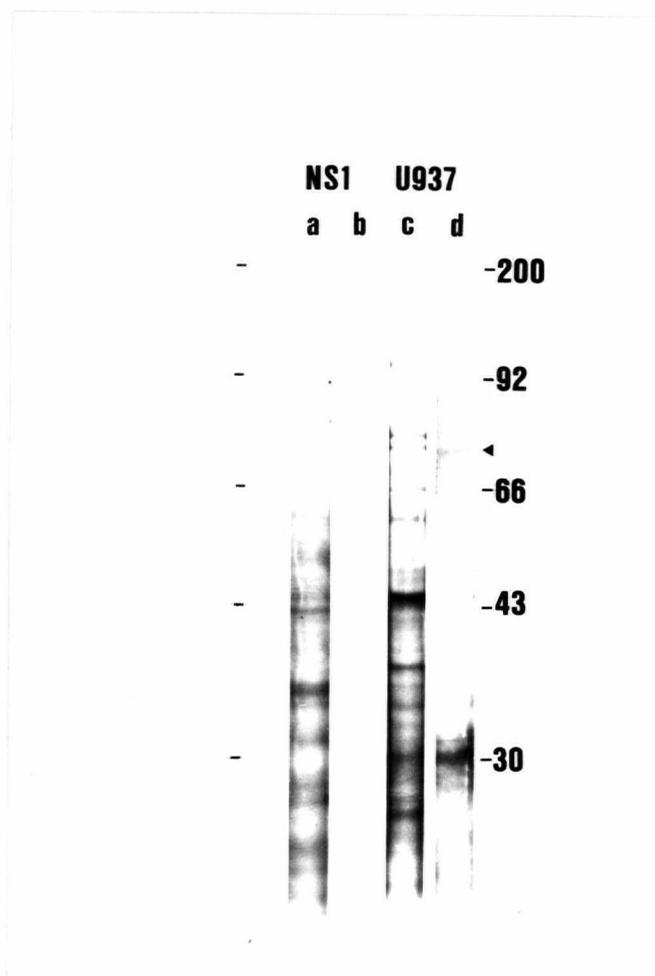
Lane a : initial unprecipitated NS1 cell lysate.

Lane b : NS1 cell lysate material eluted from the CAMAL-1 column.

Lane c : initial unprecipitated U937 cell lysate.

Lane d : U937 cell lysate material eluted from the CAMAL-1 column.

$1 \times 10^8$  TCA-precipitable cpm of labelled cell lysate was applied to the immunoaffinity column and processed as described in Materials and Methods, Section 2.5 (ii). The dried photofluor-impregnated gel was exposed to Kodak X-Omat AR film for 5 days at  $-70^\circ\text{C}$ . Relative molecular weights (kilodaltons) of protein standards are as indicated.



proteins that were retained by the CAMAL-1 immunoadsorbent column while the U937 cell lysate displayed the specific retention of proteins of  $M_r$ s 71000 and 30000. Leukemic cells and cell lines have been shown to express a higher level of cellular protein phosphorylation than normal cells, this increased level of cellular phosphorylation being associated with constitutive expression of genes/proteins involved in cellular proliferation and growth. The phosphorylation of p70 (CAMAL) in the leukemic cell lines was examined by immunoprecipitation, with CAMAL-specific antibodies, from  $^{32}P_i$ -labelled cell lysates. The human HLA-A,-B molecule has been shown to be phosphorylated in the carboxy-terminal region of its heavy chain in both human lymphoid and myeloid cells (p44; Pober et al., 1978) and consequently, immunoprecipitation of this protein, with monoclonal anti-human HLA antibody, from  $^{32}P_i$ -labelled cell lysates served both as a control for the phosphorylation efficiency of cellular proteins in the leukemic cell line and as a specificity control for the anti-CAMAL antibodies. Figure 8 shows the results for immunoprecipitation of  $^{32}P_i$ -labelled HL60 cells. As is apparent from the autoradiogram, only the p44 HLA molecule was phosphorylated (lane c) while the lanes containing labelled cell lysate immunoprecipitated with either polyclonal or monoclonal anti-CAMAL antibodies, did not reveal the presence of a phosphorylated p70 (CAMAL) molecule (lanes b & e). Morphogen-induced differentiation of the cell lines did not induce phosphorylation of the p70 (CAMAL) molecule, as assessed by immunoprecipitation, and did not affect the phosphorylation of the HLA-A, -B heavy chain (p44) molecule either (data not shown). Although changes in cellular morphology, consistent with cellular differentiation, of the treated cell lines were apparent, no

Figure 8. SDS-PAGE of p70 (CAMAL) immunoprecipitated from  $^{32}\text{P}_i$ -labelled HL60 leukemic cell line lysates.

Lane i : initial unprecipitated cell lysate.

Lane a : normal mouse serum. Lane b : CAMAL-1 antibody. Lane c : anti-human HLA monoclonal antibody (W6/32). Lane d : non-immune rabbit serum. Lane e : polyclonal anti-CAMAL antibody.

$1 \times 10^5$  TCA-precipitable cpm of  $^{32}\text{P}_i$ -labelled cell lysate was used for each immunoprecipitation. The dried gel was exposed to Kodak X-Omat AR film for 5 days at  $-70^\circ\text{C}$ . Relative molecular weights (kilodaltons) of protein standards are as indicated.

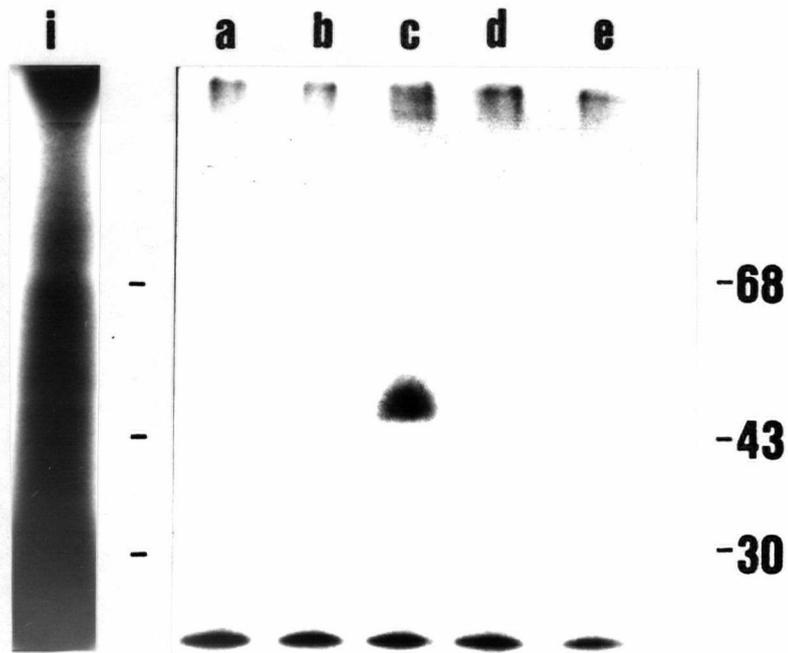


Figure 9. SDS-PAGE profile of column fractions from leukemic (AML/CGL) cell lysates after application and elution from the CAMAL-1 immunoadsorbent column.

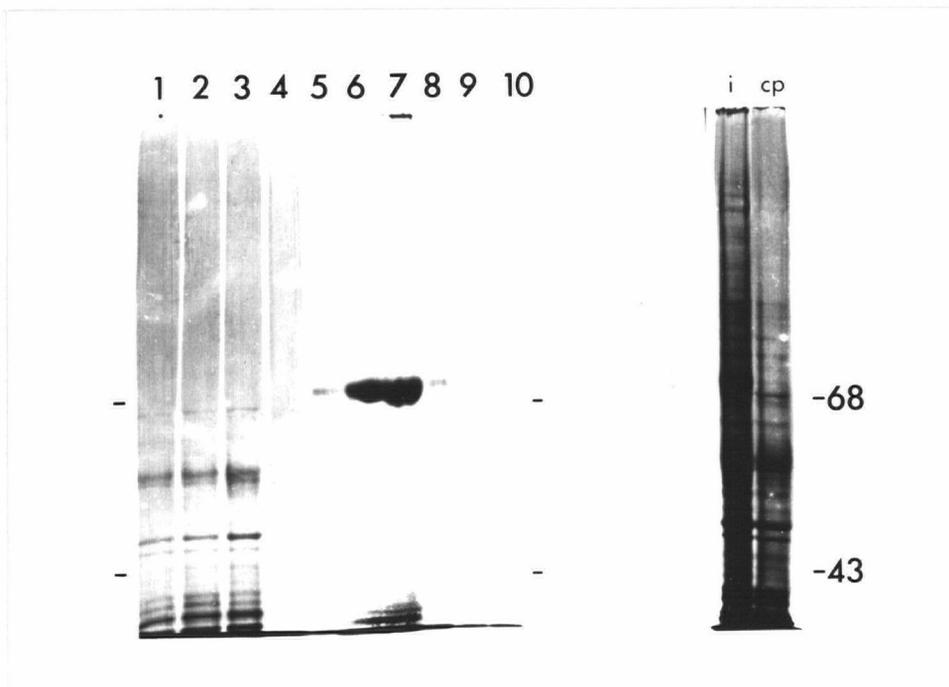
Lane i : initial cell lysate before passage over CAMAL-1 column.

Lane cp : CAMAL-1 column pass of applied cell lysate.

Lanes 1-4 : material eluted from CAMAL-1 column with BBS (300mM NaCl) pH 8.5.

Lanes 5-10 : material eluted from CAMAL-1 column with 0.1N HCl.

Treatment of cell lysate and application to the immunoaffinity column was as described in the Materials and Methods, Section 2.2. Relative molecular weights (kilodaltons) of protein standards are as indicated.



detectable level of phosphorylated p70 (CAMAL) was immunoprecipitated from these cell lines . The amount of phosphorylated HLA-A, -B heavy chain immunoprecipitable from these cell lines did not vary appreciably with cellular differentiation either (data not shown).

Purification of p70 (CAMAL) was performed using the CAMAL-1 immunoabsorbent, a representative elution profile being shown in Figure 9. The leukemic cell lysate (AML/CGL leukemic cell lysate, 5-10 mg total protein in 5 mls BBS [300 mM NaCl] pH 8.5) was applied to the column and allowed to pass through the immunoabsorbent. This sample was re-applied to the immunoabsorbent column 4 additional times, after which the column bed was washed thoroughly with BBS (300 mM NaCl) pH 8.5. Elution of column-bound material was accomplished using one column volume of 0.1N HCl. Lanes 1-4 show the material washed off the column in high salt buffer, while lanes 5-10 show the acid-eluted material. Specific elution of p70 (CAMAL) was achieved using this protocol although minor contaminants were observed. CAMAL purified in this manner exhibited an  $M_r$  of 70-72000 and an isoelectric point of 7.1-7.2, as determined by both isoelectric focussing and two-dimensional electrophoresis (Figure 10). Trace amounts of protein(s) of  $M_r$  55000 most likely represents immunoglobulin heavy chain from the polyclonal anti-CAMAL antibody which has become iodinated due to the degradation of iodinated CAMAL, and subsequent release of reactive  $^{125}I^-$ , during the immunoprecipitation procedure.

Affinity-purified CAMAL and the polyclonal anti-CAMAL antibody were tested against a panel of antisera directed to and antigens purified from non-human primate and mammalian retroviruses. As demonstrated by the results shown in Table 5, CAMAL was not

Figure 10. Two-dimensional polyacrylamide gel electrophoresis profile of  $^{125}\text{I}$ -p70 (CAMAL) immunoprecipitated with the polyclonal anti-CAMAL antibody.

$5 \times 10^4$  TCA-precipitable cpm of iodinated, purified p70 (CAMAL) was immunoprecipitated with the polyclonal antibody prior to application of the sample to the IEF gel. The dried gel was exposed to Kodak X-Omat AR film for 12 hours at  $-70^\circ\text{C}$ . Relative molecular weights (kilodaltons) of protein standards are as indicated.

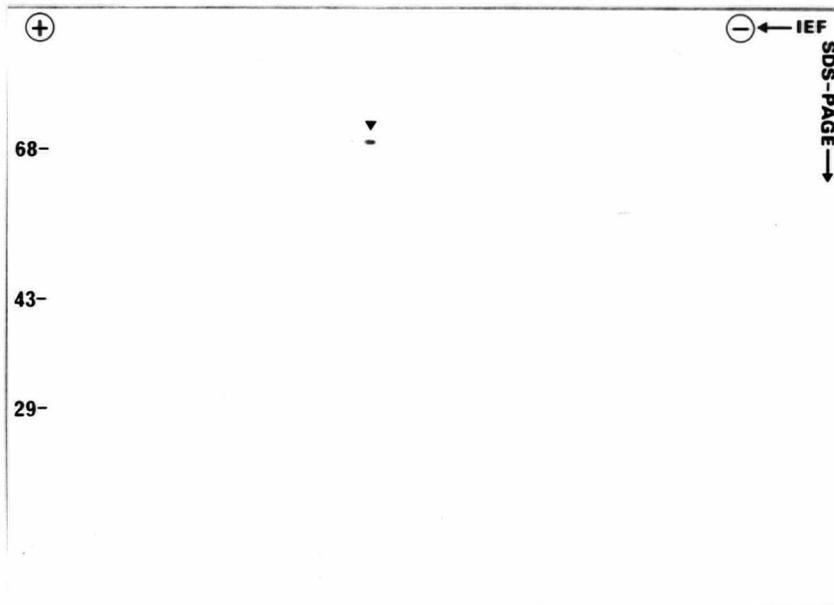


Table 5. Maximum immunoprecipitation of  $^{125}\text{I}$ -CAMAL by antisera directed against mammalian retroviral antigens.<sup>1,2</sup>

Source of antiserum	Specificity	Maximum % precipitation of $^{125}\text{I}$ -CAMAL
rabbit	non-immune	7
rabbit	CAMAL	70
rabbit	p30 SSAV-strain K42. (simian sarcoma-associated virus)	8
rabbit	p30 FLV-strain K13. (feline lymphoma virus)	10
goat	non-immune	10
goat	p30 SSV-strain 2s-696. (simian sarcoma virus)	6
goat	whole virions SSV-strain 3s-172	7
	goat whole virions GALV-strain 3s-321. (gibbon-ape leukemia virus)	4
goat	whole virions BEV:NC37-strain 75s-295 (baboon endogenous virus)	4
goat	whole virions BEV:BCKT-strain 77s-200	9
goat	p28 BEV:NC37-strain 75s-260.	3
goat	whole virions RD 114-strain 73s-73. (feline rhabdomyosarcoma virus)	7
goat	p28 RD 114-strain 72s-781.	4
goat	whole virions MPMV-strain 2s-752.	<1
goat	p27 MPMV-strain 75s-88.	<1
goat	whole virions SMRV-strain 78s-188. (squirrel monkey retrovirus)	8
goat	whole virions FeLV-strain 0s-264. (feline leukemia virus)	5
goat	gp70 FeLV-strain 76s-455.	15
goat	p27 FeLV-strain 76s-520.	7
goat	whole virions BoLV (bovine leukemia/leukosis virus)	12
cow	non-immune	10
cow	whole virions BoLV	11

<sup>1</sup> All antisera (50 ul) were preabsorbed on mixed cell pellets (50 ul) consisting of human embryo fibroblasts and peripheral blood leukocytes resuspended 1:1 in PBS pH 7.3 / fetal calf serum.

<sup>2</sup> All assays consisted of 100000 TCA-precipitable cpm  $^{125}\text{I}$ -CAMAL input antigen.

antigenically related to any of the retroviral antigens present in the survey and consequently was not likely to be a viral gene product.

### 3.3. Summary

The results presented in this chapter demonstrated the existence of an  $M_r$  70000 protein in all ANLL cell samples and leukemic cell lines tested. Minor variations in the predicted relative molecular weight of CAMAL,  $M_r$  70-72000, are attributable to either conformational changes in the CAMAL molecule caused by the iodination procedure or to anomalous migration of pre-stained protein standards as compared to underivatized protein standards in SDS-PAGE. Variations in the relative molecular weight of p70 (CAMAL) might also be attributable to post-translational modifications of the CAMAL protein in different leukemic cells or cell lines, but no direct evidence for this suggestion has been found to date. It would seem more plausible that the observed differences in mobility in SDS-PAGE are due to the anomalous migration of pre-stained molecular weight standard proteins, however, alterations of p70 (CAMAL) conformation such that less SDS is bound to the protein may also contribute to changes in mobility in SDS-PAGE.

p70 (CAMAL) was not detectable in normal peripheral blood leukocytes by immunoprecipitation and was detectable in less than 1% of normal bone marrow cells using immunoperoxidase staining of fixed cells (Logan et al., 1984), suggesting that the increased expression of p70 (CAMAL) may be specific for leukemic cells. Although CAMAL does not exhibit antigenic relatedness to any non-human primate or mammalian retroviral proteins tested, these results do not preclude the involvement of virally-induced modulation of CAMAL expression in the leukemic cell lines examined. CAMAL was present in or on leukemic cells and cell lines, was detected as an  $M_r$  70000 protein monomer with

a pI of 7.1-7.2, was not phosphorylated, appeared to be actively secreted and was not affected by induced differentiation of the leukemic cell lines. The detection of p70 (CAMAL) in the leukemic cell lines HL60 (promyelocytic), KG1 (myelocytic), K562 (erythroleukemic) and U937 (monocytic) suggested that the expression of p70 (CAMAL) may be a general consequence of leukemic transformation. The implication of constitutive p70 (CAMAL) expression in all these leukemic cell lines is that CAMAL, produced by leukemic cells in the bone marrow, may preferentially affect the growth of normal myeloid progenitor cells, allowing the outgrowth of the leukemic cells and their eventual domination of the bone marrow. This function of p70 (CAMAL) in the "leukemic" bone marrow is consistent with the pathology of the disease in which immature myeloid cells dominate the bone marrow. The implication of constitutive p70 (CAMAL) expression in leukemic cells is that p70 (CAMAL) might be responsible for the "maturation block" or bias toward "self-renewal", rather than proliferation, exhibited by "leukemic" bone marrow cells. The detection of p70 (CAMAL) in association with all the leukemic cell samples and cell lines examined suggested that p70 (CAMAL) might be considered as a putative leukemia-associated antigen.

Since p70 (CAMAL) was expressed in both leukemic cells and cell lines, purification of p70 (CAMAL) from leukemic cells or leukemic cell line lysates was performed. Using the CAMAL-1 immunoaffinity column, a sufficient quantity of p70 (CAMAL) was purified from both patient material and leukemic cell line lysates to permit the characterization of the p70 (CAMAL) molecule.

## Chapter 4. Molecular characterization of CAMAL.

### 4.1. Introduction

Amino acid sequence data has permitted comparisons between proteins to define the relationship between functional or structural homology and amino acid sequence homology. Protein sequence analysis has also facilitated the delineation of regulatory sites and functional domains within proteins, the organization of coding and non-coding regions within cloned eukaryotic genes, the location of potential glycosylation or phosphorylation sites within proteins, the establishment of reading-frame and, in conjunction with X-ray crystallographic analysis, the prediction of protein structure. Since sufficient quantities of purified CAMAL were available and functional studies had suggested that CAMAL exerted an inhibitory effect on normal bone marrow progenitor cell growth *in vitro*, protein sequencing studies were initiated with purified CAMAL to establish whether this protein exhibited homology to any known growth regulatory proteins.

CAMAL was purified from both leukemic cell (AML/CGL) and leukemic cell line (HL60, K562, KG1, U937) lysates by immunoaffinity chromatography on the CAMAL-1 immunoabsorbent column. Protein sequence analysis of peptides derived from purified CAMAL revealed that two of the three tryptic peptides (tp27, tp31) sequenced exhibited substantial homology to human serum albumin (HSA) and a lesser degree of homology to human alpha-1-fetoprotein (AFP). The third

tryptic peptide (tp20) sequence exhibited no homology to HSA, BSA, AFP, trypsin or any sequences present in the NBRF database. On the basis of these results the tp20 peptide sequence was considered to be unique to CAMAL. The examination and significance of the homology demonstrated between CAMAL, HSA and AFP are the subjects of this chapter.

## 4.2. Results

CAMAL purified from either AML/CGL cell lysates or leukemic cell line lysates, by immunoaffinity chromatography, was shown to be identical in both relative molecular mass ( $M_r$  70000) and isoelectric point (pI 7.2), suggesting that the processes involved in CAMAL synthesis were equivalent in both clinical leukemic cell samples and leukemic cell lines propagated *in vitro*. Since no demonstrable differences in p70 (CAMAL) synthesis or processing were evident between p70 (CAMAL) from leukemic patient cell lysates or leukemic cell line lysates, purified CAMAL from either source was considered to be equivalent. Further purification of these CAMAL preparations by size-exclusion (Waters Associates I-125 protein column) and anion-exchange (Waters Associates DEAE-5PW or Pharmacia Mono Q column) high performance liquid chromatography (HPLC) (Figure 11), to remove low molecular weight protein and non-protein contaminants, demonstrated that these preparations of CAMAL were approximately 90%  $M_r$  70000 (CAMAL). The major peak fraction (1) in figure 11A was collected and subjected to SDS-PAGE. The "shoulder" fraction of peak 1 was not collected separately however, this proved to be of no significance since the only protein present in the stained SDS-PAGE gel was p70 (CAMAL) (data not shown). The two peak fractions (1,2) in figure 11B were collected separately and subjected to SDS-PAGE. Peak 2 was shown to contain only p70 (CAMAL), whereas peak 1 did not demonstrate any material that was detectable by either Coomassie blue or silver staining (data not shown).

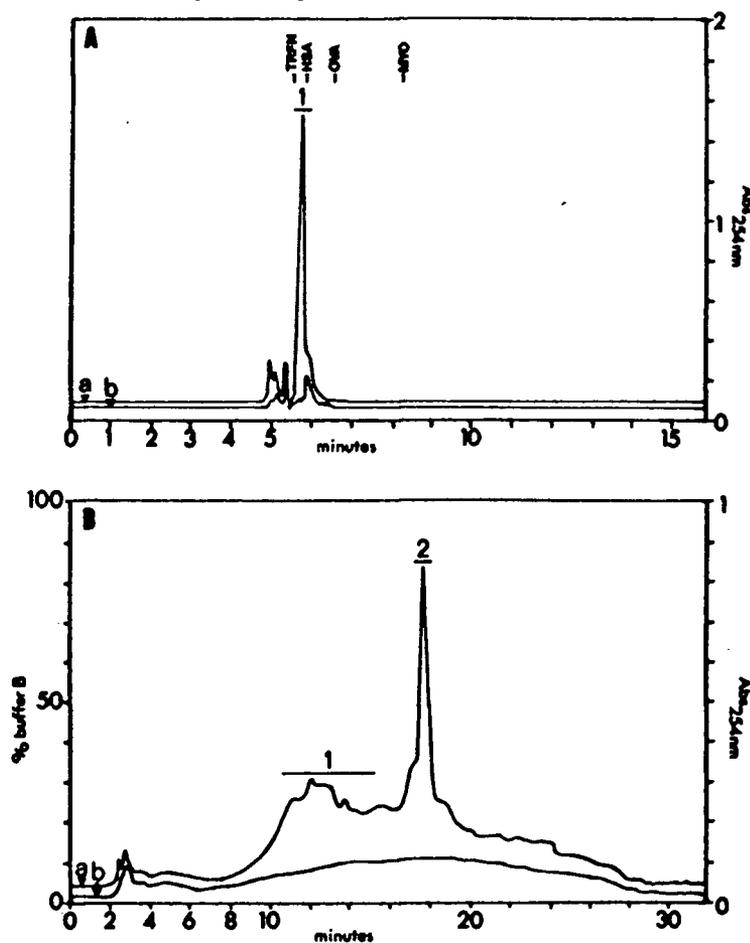
Figure 11. Analysis of immunoaffinity-purified p70 (CAMAL) by HPLC.

Panel A. Size-exclusion HPLC of p70 (CAMAL).

5 $\mu$ g (5 $\mu$ l) of purified p70 (CAMAL), in 0.05% TFA:ACN (1:1 v/v), was applied to a Waters I-125 protein column, pre-equilibrated in 0.05% TFA:ACN (1:1 v/v), and analysed as described in Section 2.11(i). Tracing 'a' represents a single 5 $\mu$ g injection of purified p70 (CAMAL). Tracing 'b' represents a single 5 $\mu$ l blank/baseline injection. Molecular weight (kilodaltons) of protein standards are TRFN (transferrin; 80000), HSA (human serum albumin; 68000), OVA (ovalbumin; 43000) and MYO (myoglobin; 18000). Peak 1 was analysed by SDS-PAGE.

Panel B. Anion-exchange HPLC of p70 (CAMAL).

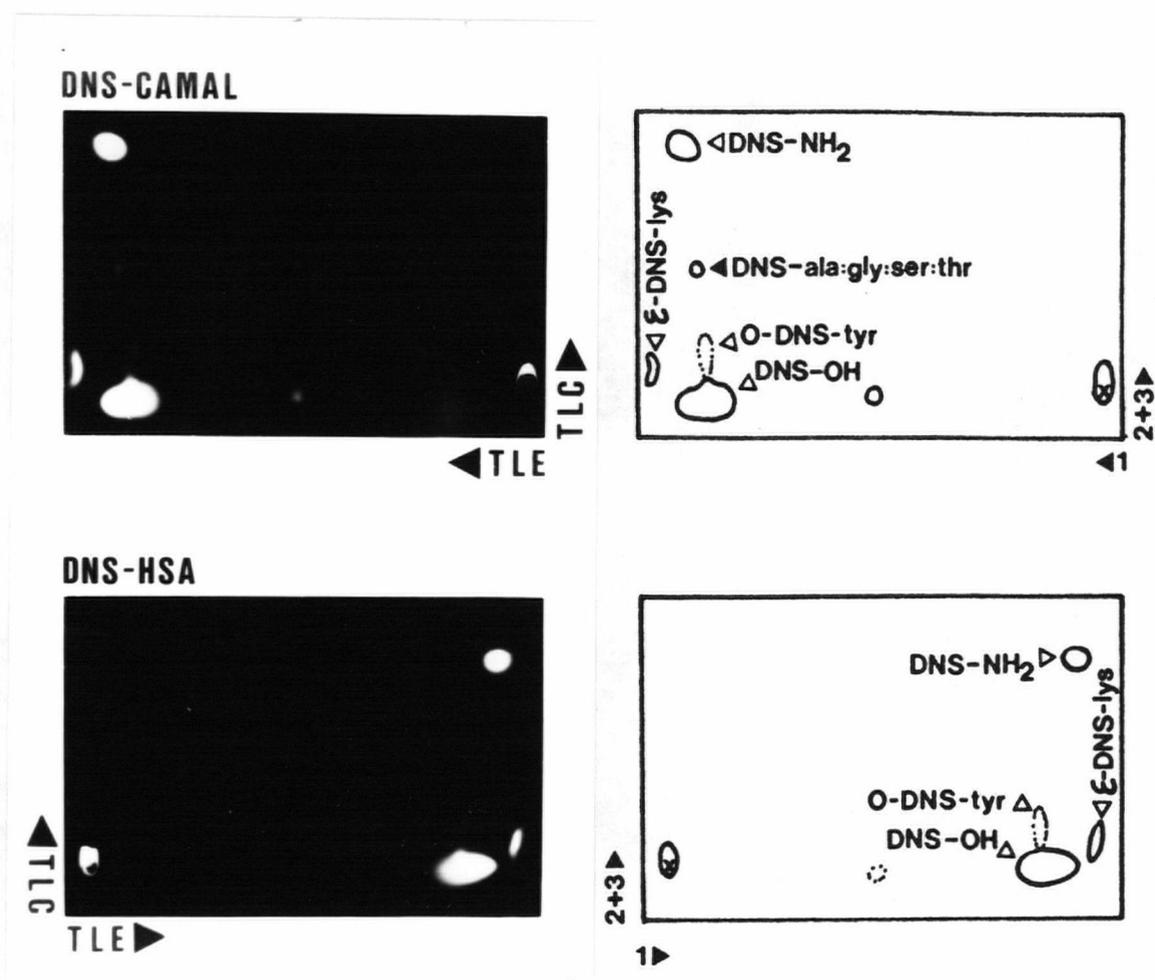
5 $\mu$ g (5 $\mu$ l) of purified p70 (CAMAL), in 20mM Tris pH 8.5, was applied to a Pharmacia MonoQ HR5/5 column, pre-equilibrated in 20mM Tris pH 8.5, and eluted in a linear gradient from 0-100% buffer B (0.5M NaCl in 20mM Tris pH 8.5) and analysed as described in Section 2.11(i). Tracing 'a' represents a single 5 $\mu$ g injection of purified p70 (CAMAL). Tracing 'b' represents a single 5 $\mu$ l blank/baseline injection. Peaks 1 and 2 were analysed by SDS-PAGE.



Protein sequencing of native CAMAL or reduced and carboxymethylated (RCM) CAMAL failed to produce any sequence data, suggesting that the N-terminus of CAMAL was modified in a manner that precluded N-terminal sequencing. N-terminal amino acid analysis of either native or RCM-CAMAL (section 2.12[i]) revealed that both protein preparations possessed an accessible N-terminus (Figure 12). The liberated N-terminal amino acid migrated with the same relative mobility as dansyl (DNS) -alanine, DNS-serine, DNS-threonine or DNS-glycine, these four dansyl amino acid derivatives having approximately the same mobility in most TLC solvent systems. O-DNS-tyr and  $\epsilon$ -DNS-lys were also detected and based on the expected yield of these derivatives and their relative fluorescence, the amount of liberated N-terminal amino acid was estimated to be between 0.5 and 1 pmole. Possible contamination of the CAMAL preparations with glycinate from either polyacrylamide gels, residual electrophoresis buffer or the immunoaffinity column, which might result in the appearance of a dansylated species migrating analogously to DNS-gly (Vandekerckhove et al., 1985), was minimized by using HPLC-purified CAMAL. Since N-terminal sequencing did not reveal an accessible N-terminus, both native and RCM-CAMAL must undergo some degree of N-terminal modification, such as acetylation or the formation of a cyclic pyroglutamyl residue, during sample preparation which precluded N-terminal sequencing. Aggregation of the protein prior to application to the gas-phase sequencer or aggregation of the protein on the "frit" in the reaction chamber of the gas-phase sequencer might also prevent the sequencing of the protein by permitting the N-terminus to "bury" itself within the protein aggregate.

Figure 12. Dansyl-N-terminal amino acid analysis of p70 (CAMAL) and HSA.

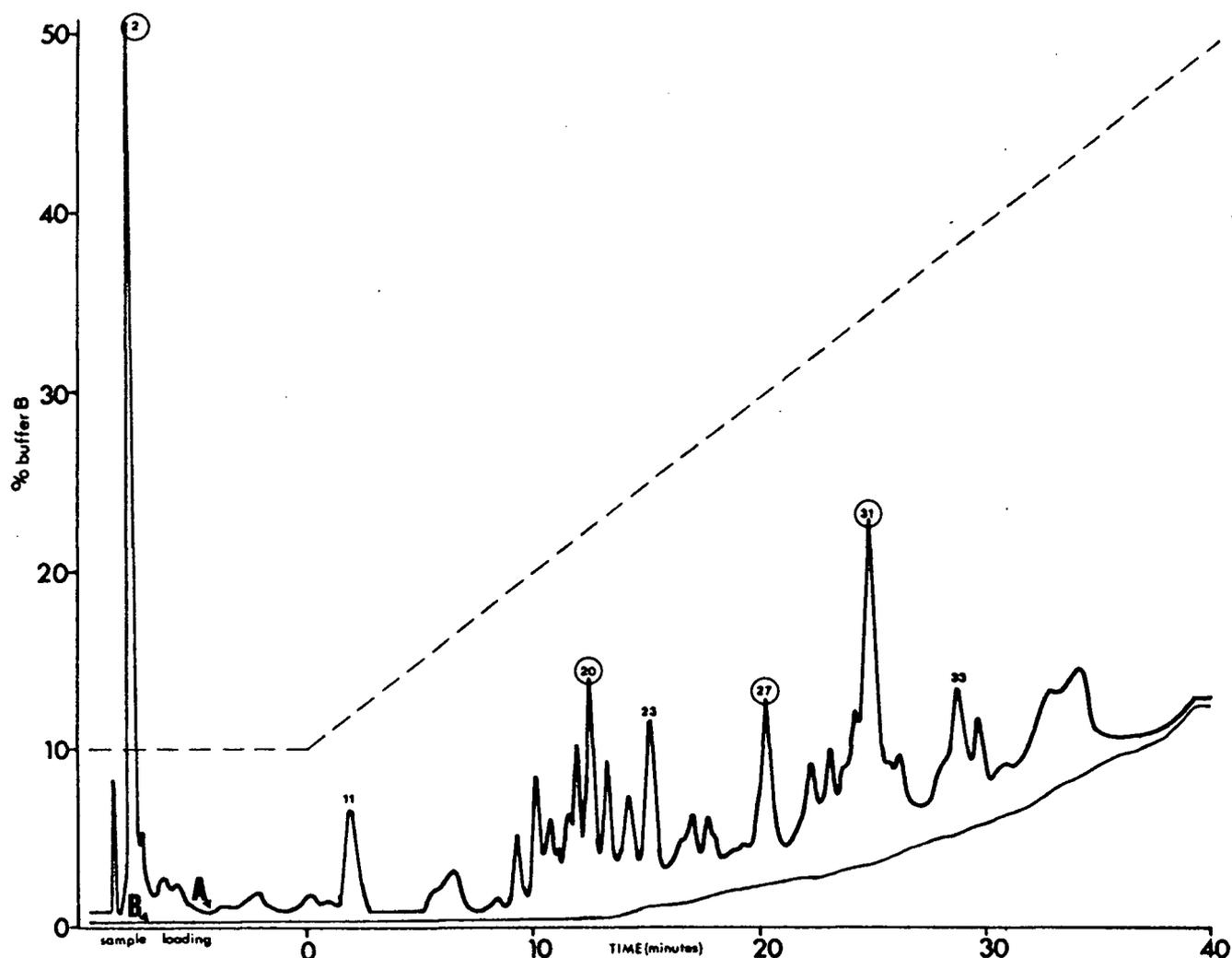
5 $\mu$ g each of RCM-CAMAL and RCM-HSA was dansylated, hydrolysed and subjected to 2D-TLC, as described in Section 2.12(i). The directions of the TLC dimensions are indicated by arrows. The polyamide TLC foils were photographed under UV light using Polaroid 4 X 5 Land film. The illustrations accompanying the DNS-CAMAL and DNS-HSA panels indicate the positions of the relevant DNS-amino acid standards and decomposition products of dansyl chloride.



Purified CAMAL was subsequently subjected to fractionation procedures designed to liberate peptides which could be purified and sequenced. Separation and purification of CAMAL CNBr peptides, by RP-HPLC or anion-exchange HPLC, proved difficult since all the peptides exhibited the same degree of hydrophobicity or net charge at pH 7.3 and 8.5. Consequently, TPCK-trypsin digestion of purified CAMAL was used to generate CAMAL tryptic peptides. 12 nmoles (850 ug) of purified CAMAL was reduced, carboxymethylated, citraconylated and digested at 37°C for 6 hours with two consecutive additions of TPCK-trypsin, at 3 hour intervals, at an enzyme to substrate ratio of 1 to 200. Since application of the CAMAL tryptic-peptide mixture to an HPLC RPC18 column resulted in irreversible absorption of the peptide mixture to the column matrix, a less hydrophobic column matrix was chosen. The CAMAL tryptic-peptide mixture was fractionated on a C3-reverse-phase (RPC3) HPLC column and peptides were isolated as single peaks in a 10-40% linear gradient with 0.1% trifluoroacetic acid:isopropanol as the mobile phase (Figure 13). Peak fractions were pooled from repetitive RPC3-HPLC runs, concentrated and sequenced in an automated Applied Biosystems Model 470A gas-phase protein sequencer. Peptide 2, which eluted prior to initiation of the gradient, contained a significant proportion of the protein applied to the RPC3 column and contained several peptides, as revealed by SDS-PAGE and multiple amino acids at each cycle in the sequencing procedure. As a result, no usable sequence data was derived from this peptide pool. Peptide 2 was subsequently separated into several discrete peaks by a combination of size-exclusion and anion-exchange HPLC. Protein sequencing was not attempted with these peak fractions since there

Figure 13. Reverse-phase (RPC3) HPLC profile of purified p70 (CAMAL) tryptic peptides.

12 nmole of RCM-CTA-p70 (CAMAL) was digested with TPCK-trypsin (Enzyme:substrate, 1:200) for 6 hours at 37°C. The dried sample was then resuspended in 500ul Buffer A (0.1% TFA:H<sub>2</sub>O) and 100ul sample volumes applied to the RPC3 column. Bound peptides were eluted from the RPC3 column in a linear gradient from 10-50% Buffer B (0.1% TFA:Isopropanol). Running conditions were as described in Section 2.11(ii). Circled, numbered peaks represent peptide pools that were used for protein sequencing. Tracing 'A' represents a single 100ul (2.5nmole) injection of trypsin-digested p70 (CAMAL). Tracing 'B' represents a single 100ul blank/baseline injection.



were still indications that these fractions contained more than one peptide. Peptides 20, 27 and 31, when sequenced, gave readable amino acid sequences of 9, 10 and 11 amino acids, respectively. Comparison of these derived amino acid sequences to protein sequences in the NBRF database revealed that peptide 27 was homologous to sequences present in human serum albumin (HSA, 9/10 match) and human alpha-1-fetoprotein (AFP, 7/10 match), peptide 31 was homologous to HSA sequences (8/11 match), whereas peptide 20 displayed a unique sequence. Tryptic peptide 20 (tp20) had a predicted length of 30 amino acids and displayed no homology to amino acid sequences present in HSA, AFP, bovine serum albumin (BSA), bovine or porcine trypsin (Table 6). Tryptic peptide 27 (tp27) had a predicted length of 32 amino acids. Comparison of the amino acid composition and sequence between tp27 and the corresponding tryptic peptide from HSA revealed one non-conservative change in sequence and several differences as determined by amino acid composition, most notably, in cys and gly content (Table 7, 8). Comparison of tp27 and the comparable tryptic peptide from AFP revealed three non-conservative changes in the peptide sequence and several differences based on amino acid analysis data (Table 7, 8). Tryptic peptide 31 (tp31) had a predicted length of 51 amino acids and exhibited two changes in amidation state, asn to asp and gln to glu, as well as one non-conservative change, val to glu, when compared to the corresponding peptide sequence from HSA. Comparison of the amino acid compositions of tp31 and HSA also revealed several differences in cys, his and met content (Table 9, 10). Taken as a whole, the CAMAL tryptic peptides, tp27 and tp31, displayed significant sequence homology to the corresponding sequences in HSA and AFP, within the

Table 6. Amino acid and sequence analysis of CAMAL tryptic peptide20.

	CAMAL tp20			CAMAL tp20 (3 nmoles by amino acid analysis) sequence analysis*		
	aa ratio <sup>a</sup>	aa comp <sup>a</sup>	MPN <sup>b</sup>	cycle#	1° aa (nmol) <sup>c</sup>	2° aa (nmol)
Asx	3.543	3.528	4	1	K (2)	L (1), V(1)
Thr	1.888	1.880	2	2	V (2)	E (.5), Q(.2)
Ser	1.135	1.130	1	3	N (.5)	D (.3)
Glx	4.373	4.354	4	4	A (1)	E (.7)
Pro	1.004	0.999	1	5	V (1)	L (.5)
Gly	1.495	1.489	2	6	H (.3)	-
Ala	2.001	1.993	2	7	E (.5)	-
Cys	0.588	0.586	1	8	F (.5)	-
Val	2.640	2.628	3	9	A (.3)	-
Met	0.149	0.149	1?			
Ile	0.441	0.440	1?			
Leu	3.175	3.161	3			
Tyr	0.607	0.605	1?			
Phe	1.228	1.222	1			
His	0.763	0.760	1?			
Lys	1.825	1.817	2			
Arg	1.000	0.996	1			
Total			33			

\* tp20 sequence not found in HSA, AFP, BSA, porcine or bovine trypsin.

<sup>a</sup> amino acid ratios and compositions are based on the % recovery of 5 nmoles norleucine injected with the hydrolysate prior to analysis and assuming 1 mole arg per mole peptide.

<sup>b</sup> MPN : mean probable number of amino acid(s) from amino acid composition.

<sup>c</sup> yield of primary and secondary PTH-amino acids from each cleavage cycle in an Applied Biosystems gas-phase protein sequencer given as nmoles amino acid based on a 1 nmole internal standard of PTH-norleucine.

Table 7. Comparison of the amino acid compositions for CAMAL tryptic peptide 27 and the comparable peptides from human serum albumin and alpha-1-fetoprotein.

	CAMAL tp27		HSA <sup>c</sup>	AFP <sup>d</sup>
	aa ratio <sup>a</sup>	aa comp <sup>a</sup>	MPN <sup>b</sup>	MPN
Asx	2.280+0.712	2.077+0.792	3	4
Thr	1.132+0.275	1.057+0.401	2	1
Ser	2.216+0.592	2.073+0.823	3	3
Glx	3.443+0.495	3.109+0.505	4	3
Pro	2.104+0.0	2.116+0.0	2	3
Gly	3.413+1.105	3.001+0.476	4	1
Ala	1.645+0.406	1.542+0.594	2	3
Cys	0	0	0	2
Val	1.631+0.466	1.320+0.560	2	4
Met	0.308+0.0	0.224+0.0	0	0
Ile	0.646+0.160	0.529+0.159	1	1
Leu	1.772+0.474	1.602+0.434	2	3
Tyr	0.891+0.075	0.774+0.167	1	1
Phe	1.322+0.526	0.929+0.279	1	4
His	0.539+0.159	0.499+0.186	1	1
Lys	1.545+0.266	1.424+0.466	2	2
Arg	1.000+0.0	1.000+0.0	1	1
Total			32	37

<sup>a</sup> amino acid ratios and compositions are based on the % recovery of 5nmoles norleucine injected with hydrolysate prior to analysis and assuming 1mole arg per mole peptide. Values are presented as the mean+standard deviation.

<sup>b</sup> MPN : mean probable number of amino acid(s) from amino acid compositions.

<sup>c</sup> MPN for HSA from published sequence; Dugaicznyk et al., 1982.

<sup>d</sup> MPN for AFP from published sequence; Morinaga et al., 1983.

Table 8. Comparison of the deduced amino acid sequence for CAMAL tryptic peptide 27 and the comparable sequences from human serum albumin and alpha-1-fetoprotein.

CAMAL tp27	R P N F S A L E V D
	*
Human serum albumin (domain 3B) <sup>a</sup>	510 R P C F S A L E V D E T Y V P K E F N A E T F T F H A D I C T L S E K E R <sub>546</sub>
CAMAL tp27	R P N F S A L E V D
	* * *
Human alpha-1-fetoprotein (domain 3B) <sup>b</sup>	490 R P C F S S L V V D E T Y V P P A F S D D K F I F H K D L C Q A Q G V A L <sub>527</sub>

CAMAL tp27 (4 nmoles by amino acid analysis) sequence analysis.

cycle#	1° aa (nmol) <sup>c</sup>	2° aa (nmol) <sup>c</sup>
1	R (.2)	P (.2), M/V (.1), K/F (.1)
2	P (1)	-
3	N (<.1)	D (.1)
4	K/F (.4)	-
5	S (<.1)	-
6	A (<.1)	-
7	L (<.1)	-
8	E (<.1)	-
9	M/V (.1)	-
10	D (<.1)	-

<sup>a</sup> from published sequence; Dugaiczky et al., 1982.

<sup>b</sup> from published sequence; Morinaga et al., 1983.

<sup>c</sup> yield of primary and secondary PTH-amino acid(s) from each cleavage cycle in an Applied Biosystems gas-phase protein sequencer given as nmoles PTH-amino acid based on a 500 pmole internal standard of PTH-norleucine.

\* denotes positional mismatches in amino acid sequence.

Table 9. Comparison of the amino acid compositions for CAMAL tryptic peptide 31 and the comparable peptides from human serum albumin and alpha-1-fetoprotein.

	CAMAL tp31		MPN <sup>b</sup>	HSA <sup>c</sup>	AFP <sup>d</sup>
	aa ratio <sup>a</sup>	aa comp <sup>a</sup>		MPN	MPN
Asx	4.011+2.459	3.478+2.723	6	5	1
Thr	1.697+0.511	1.692+0.523	2	3	3
Ser	2.986+0.951	2.412+0.422	3	2	4
Glx	5.398+2.511	4.602+2.817	7	12	12
Pro	2.598+1.368	2.596+1.381	4	4	1
Gly	5.156+2.406	4.512+2.900	8	2	3
Ala	1.979+0.854	1.667+0.921	3	1	8
Cys	0	0	0	1	1
Val	1.589+1.173	1.413+1.292	3	7	1
Met	0.266+0.112	0.172+0.033	0	0	2
Ile	1.050+0.429	0.865+0.396	2	1	2
Leu	2.922+1.383	2.487+1.537	4	8	8
Tyr	0.739+0.212	0.493+0.006	1	2	4
Phe	2.184+1.604	1.922+1.750	3	4	2
His	0.810+0.436	0.696+0.488	1	0	0
Lys	2.139+1.444	1.880+1.600	3	6	6
Arg	1.000+0.0	1.000+0.0	1	2	2
Total			51	60	60

<sup>a</sup> amino acid ratios and compositions are based on the % recovery of 5nmoles norleucine injected with the hydrolysate prior to analysis and assuming 1nmole arg per mole peptide. Values are presented as the mean+standard deviation.

<sup>b</sup> MPN : mean probable number of amino acid(s) from amino acid compositions.

<sup>c</sup> MPN for HSA from published sequence; Dugaicznyk et al., 1982.

<sup>d</sup> MPN for AFP from published sequence; Morinaga et al., 1983.



limits of the available sequence. However, the amino acid compositions for tp27, tp31 and the corresponding peptides from HSA suggested that the CAMAL tryptic peptides might be related to rather than identical to the corresponding HSA "tryptic" peptides. Within the limits of the available sequence data, it was difficult to determine whether the CAMAL tryptic peptide sequences were indicative of genuine homology to HSA or merely the result of contamination of CAMAL purified from clinical cell samples with HSA.

A closer examination of the PTH-amino acid yields from the sequence analysis of tp20, tp27 and tp31 reveals that tp27 and tp31 do not give the expected yields of PTH-amino acids based on the amount of peptide applied to the gas-phase sequencer (Table 8 and 10). The primary PTH-amino acid from tp27 would be expected to give a signal of 2-3 nmoles instead of 0.2 nmoles. The yields of all the subsequent PTH-amino acids from tp27 are also much lower than would be expected. This situation also holds true for tp31 which would be expected to give primary PTH-amino acid yields in the range of 2-3 nmoles. The yields of PTH-amino acids from the sequence analysis of tp31 were between 0.1 and 0.5 nmoles. The large discrepancy between the expected and actual yields of the primary PTH-amino acids from tp27 (5% of expected yield) and tp31 (16% of expected yield) as well as the low yields of subsequent amino acids from the tp27 and tp 31 sequence analysis suggests that these sequences might be derived from a contaminating peptide or that tp27 and tp 31 have become modified in a manner that prevents sequencing. This latter suggestion is supported by the result that acid hydrolysis of protein bound to the filter support from the gas-phase sequencer and subsequent amino acid

analysis of the hydrolysate reveals that all the protein added to the filter was bound. This result precludes the low yields of PTH-amino acids being attributable to lower than expected amounts of protein binding to the filter in the gas-phase sequencer. The suggestion that the obtained sequences for tp27 and tp31 might be due to contamination with the equivalent peptides from HSA is unlikely. Although contamination of the CAMAL preparation with low levels of HSA is possible, it is unlikely that these contaminating peptides would have been detected as major peptide peaks in the HPLC purification of CAMAL tryptic peptides (Figure 13). The CAMAL preparation used for peptide sequencing has been shown to be >90% p70 (CAMAL) by a number of criteria, such that any sequences similar to HSA or AFP present in CAMAL most likely represent genuine homology to HSA or AFP rather than these sequences being a consequence of contamination of the CAMAL or the CAMAL peptide preparations with HSA or AFP. Analysis of the tp20 sequencing results reveals that the yields of the primary PTH-amino acid and subsequent amino acids were within the expected range based on the initial amount of peptide added to the gas-phase sequencer. The fact that the tp20 sequence is a novel sequence and in view of the relatedness of the tp27 and tp31 sequences to HSA and AFP sequences, CAMAL might represent an "albumin-like" molecule that is more related to HSA and AFP at the level of protein conformation rather than the primary amino acid sequence level.

This type of relationship has been demonstrated between HSA and AFP, which are 39% homologous at the amino acid sequence level. It has been demonstrated that both HSA and AFP share immunological cross-reactivity between both the native proteins and peptides and

Table 11. Comparison of the amino acid compositions for CAMAL, human serum albumin (HSA), human alpha-1-fetoprotein (AFP) and bovine serum albumin (BSA).

	CAMAL		HSA		BSA	
	aa ratio <sup>a</sup>	aa comp <sup>a</sup>	aa ratio	aa comp	aa ratio	aa comp
Asx	10.8+0.3	64.6+1.3	10.8+0.4	64.7+1.4	16.3+0.3	64.2+0.4
Thr	5.2+0.1	30.9+1.0	5.0+0.1	30.2+0.9	9.1+0.2	36.1+1.3
Ser	5.9+0.1	36.1+1.7	4.3+0.4	25.7+1.7	7.2+0.6	28.3+2.0
Glx	13.6+0.1	79.9+2.6	15.3+0.4	91.7+1.1	22.3+0.6	87.8+0.6
Pro	5.8+0.4	34.3+2.0	5.0+0.2	30.0+0.5	8.5+0.4	33.7+0.7
Gly	9.2+0.3	54.6+1.3	2.7+0.1	15.9+0.5	4.8+0.2	18.9+0.4
Ala	7.6+0.2	45.0+1.6	11.1+0.2	66.4+2.1	12.7+0.0	50.0+1.0
Cys	1.8+0.3	10.7+1.6	3.6+0.1	21.3+0.7	5.4+0.4	21.4+1.2
Val	7.4+0.5	43.9+3.1	7.5+0.4	45.2+3.2	10.6+0.5	41.8+2.6
Met	1.0+0.0	6.0+0.04	1.0+0.0	6.0+0.1	1.0+0.0	5.95+0.0
Ile	3.8+0.1	22.6+0.3	1.1+0.0	6.6+0.1	3.0+0.1	12.0+0.1
Leu	9.3+0.1	55.2+0.2	11.0+0.2	65.5+0.3	16.9+0.2	66.7+0.4
Tyr	3.3+0.1	19.7+0.5	3.1+0.1	18.7+0.1	5.3+0.1	20.8+0.2
Phe	4.3+0.1	25.7+0.3	5.4+0.1	32.6+0.1	7.3+0.1	28.8+0.3
His	2.8+0.0	16.8+0.2	2.6+0.1	15.8+0.2	4.5+0.1	17.6+0.2
Lys	8.5+0.2	50.4+0.7	9.6+0.2	57.2+0.5	14.9+0.7	58.9+1.7
Arg	5.8+0.1	34.5+0.3	4.2+0.1	24.9+0.1	6.2+0.1	24.6+0.2
Total	631.9+0.0		618.5+0.9		615.8+0.2	

<sup>a</sup> amino acid ratios and compositions are based on the % recovery of 5 nmoles norleucine injected prior to analysis of the hydrolysate. Values are presented as the mean+standard deviation. The calculated values were derived from amino acid analysis data of three separate, consecutive analyses of 1 nmole reduced and carboxymethylated CAMAL, HSA and BSA.

Table 12. Comparison of the amino acid compositions for CAMAL, human serum albumin (HSA), human alpha-fetoprotein (AFP) and bovine serum albumin (BSA).

	A-CAMAL <sup>a</sup>	HSA <sup>a</sup>	HSA <sup>b</sup>	HSA <sup>c</sup>	HSA <sup>d</sup>	AFPe	BSA <sup>a</sup>	BSA <sup>f</sup>
Ala	45	67	62	63	62	50	50	46
Arg	35	25	24	22	24	21	25	23
Asx	65	65	53	54	53	42	64	53
Cys	10	22	35	35	35	32	21	35
Glx	81	92	81	83	82	97	88	78
Gly	55	16	13	12	12	26	19	15
His	17	16	16	16	16	16	18	17
Ile	25	7	8	8	8	32	12	14
Leu	55	65	61	61	62	57	66	61
Lys	50	58	59	59	59	41	59	59
Met	6	6	6	6	6	8	6	6
Phe	26	33	31	30	30	29	29	26
Pro	35	30	24	25	24	21	34	28
Ser	36	26	24	23	24	36	28	28
Thr	31	30	28	29	28	35	36	36
Trp	-	-	1	1	1	1	-	2
Tyr	20	19	18	18	18	17	21	19
Val	44	45	41	39	40	29	43	36
Total	636	622	585	584	584	590	619	582

<sup>a</sup> values represent the mean probable number of amino acids calculated from amino acid analysis of 1 nmole reduced and carboxymethylated protein.

<sup>b</sup> from sequence; Dugaicznyk et al., 1982.

<sup>c</sup> from sequence; Behrens et al., 1975.

<sup>d</sup> from sequence; Meloun et al., 1975.

<sup>e</sup> from sequence; Moringa et al., 1983.

<sup>f</sup> from sequence; Brown et al., 1976.

share equivalent binding specificities for various biological compounds (Hirano et al., 1984a, 1984b). This degree of immunological cross-reactivity between HSA and AFP, and its relationship to the shared binding abilities of both proteins, has shown that the similar binding abilities of both HSA and AFP are due mainly to their comparable conformational character rather than to their primary amino acid sequences (Hirano et al., 1984b). In order to establish whether the observed peptide sequence homology between CAMAL and HSA was manifest at the level of the whole molecule, comparative studies were initiated to determine the differences between CAMAL and HSA at both the structural and immunological level.

Comparison of the amino acid compositions for CAMAL, HSA (human serum albumin) and BSA (bovine serum albumin) are shown in Tables 11 and 12. Three consecutive amino acid compositions were performed on 1 nmole samples of RCM-CAMAL, HSA and BSA using the methods described in Section 2.9. All analyses were performed sequentially and under identical conditions. As far as amino acid composition was concerned CAMAL displayed some similarity to HSA although minor differences in amino acid content were apparent for most amino acids between CAMAL and HSA. Major differences were evident in the gly, ile and cys content and the predicted amino acid length of the two molecules. The contributions of the increased gly and ile content of CAMAL to molecular conformation are difficult to predict, however, it was likely that the decreased cys content of CAMAL might permit CAMAL to assume a less constrained conformation than HSA. The differences seen in gly content between HSA, BSA and CAMAL might be explained by "carry-over" of glycinate from SDS-PAGE gels or

glycine from buffers during the purification of CAMAL. This is possible, however the HPLC purification step, following immunoaffinity chromatography, should have removed any residual material that might have contributed to elevated yields of gly in the amino acid analysis. The differences seen in the cys content between CAMAL, HSA and AFP are less certain in view of the fact that CAMAL was not performic acid oxidized prior to amino acid analysis, to protect cys and met residues, and consequently some degree of cys and met degradation may have occurred. Whether differential degradation of CAMAL cys residues, as compared to HSA and BSA cys residues, could result in the 2-fold difference seen in cys yields between CAMAL, HSA and BSA is uncertain. The yields of cys residues in both HSA and BSA are lower by amino acid analysis than the value predicted by sequence analysis which suggests that some degree of degradation has occurred (Table 11). Again, though, the suggestion that CAMAL cys residues undergo a greater rate of degradation than the equivalent residues in HSA and BSA is speculative. Assuming that the amino acid analysis values for gly, ile and cys are valid for CAMAL, HSA and BSA, differences in protein conformation between CAMAL and HSA might be discernable as differences in protein cleavage patterns and reactivity with specific antibody.

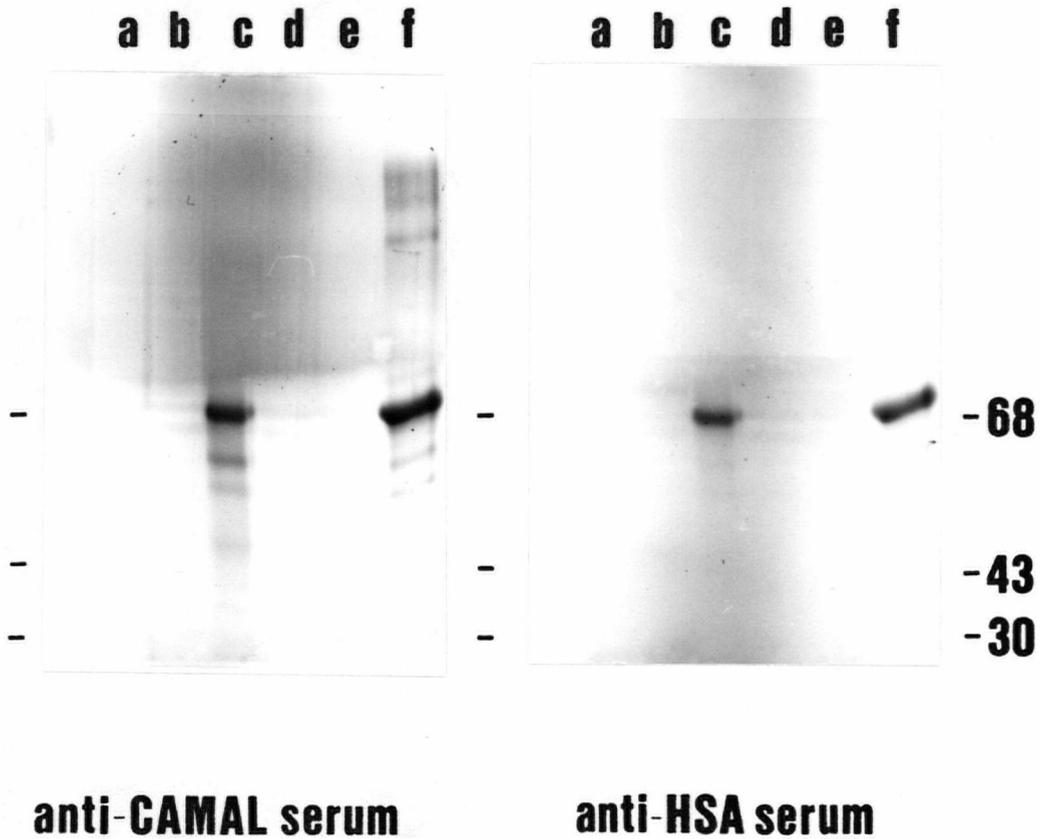
As shown in Figure 14, when equivalent amounts of CAMAL and HSA were subjected to SDS-PAGE, blotted electrophoretically onto nitrocellulose sheets and then incubated with either polyclonal anti-CAMAL or anti-HSA antibody, both CAMAL and HSA exhibited the same degree of reactivity. Equivalent protein blots incubated with pre-immune or normal rabbit serum failed to react with any proteins on the

Figure 14. Protein immuno-blots of purified p70 (CAMAL), HSA and leukemic cell line lysates.

Cell lysate and protein samples were subjected to SDS-PAGE, electro-blotted onto nitrocellulose sheets and reacted with the indicated polyclonal antibodies. Processing of the immuno-blots is described in Section 2.8.

Lane a : 100ug HL60 cell lysate  
 Lane b : 100ug U937 cell lysate  
 Lane c : 5ug purified p70 (CAMAL)

Lane d : 100ug KG1 cell lysate  
 Lane e : 100ug K562 cell lysate  
 Lane f : 5ug HSA



blots (data not shown). This data suggested that either both CAMAL and HSA were related antigenically or that both polyclonal antisera contained two specificities, one for CAMAL determinants and one for HSA determinants. In order to assess further the degree of antigenic relatedness between CAMAL and HSA, inhibition assays, with the CAMAL-1 MoAb, in the ELISA were employed. The results presented in Figure 15 indicated that CAMAL and HSA were completely cross-reactive at the level of the antigenic determinant recognised by the CAMAL-1 MoAb. The possibility that the antigenic determinant(s) shared by CAMAL and HSA, recognised by both the polyclonal and monoclonal antibodies, represented a shared conformational determinant rather than a shared linear sequence determinant was investigated by comparing the reactivity of the various antibodies to native versus reduced and carboxymethylated protein. In order to assess the degree of immunological cross-reactivity between CAMAL, HSA and AFP, ELISA plates were coated with either CAMAL, HSA or AFP and reacted with antibodies specific for CAMAL, HSA and AFP. The results shown in Figure 16 further demonstrated that CAMAL and HSA shared common antigenic determinants, in their native protein conformations, that were not shared by AFP, a protein known to be homologous to HSA in both conformation, sequence and ligand binding kinetics (Hirano et al., 1984a, 1984b). In order to determine whether the anti-CAMAL and anti-HSA antibodies were recognizing a common conformational determinant on CAMAL and HSA, both proteins were reduced and carboxymethylated and then examined in the ELISA for their reactivity with specific antibodies. As shown in Figure 17, reduction and carboxymethylation of the CAMAL molecule

Figure 15. Inhibition of the monoclonal anti-CAMAL (CAMAL-1) antibody by CAMAL and HSA in the ELISA.

The inhibition ELISA was performed according to the protocol described in Section 2.7(ii). A constant dilution of CAMAL-1 was incubated with various amounts of protein inhibitor for 18 hr at 4°C. Protein inhibitor concentrations were 0.003, 0.01, 0.03, 0.1, 0.3, 1.0 and 3.0ug. The solution was centrifuged at 12000 rpm for 10 min, the supernatant collected and titrated on ELISA plates coated with purified CAMAL or HSA at 300 ng/ml.

- (●) CAMAL-1 inhibited with purified CAMAL and tested on CAMAL.
- (○) CAMAL-1 inhibited with purified CAMAL and tested on HSA.
- (■) CAMAL-1 inhibited with HSA and tested on HSA.
- (□) CAMAL-1 inhibited with HSA and tested on CAMAL.

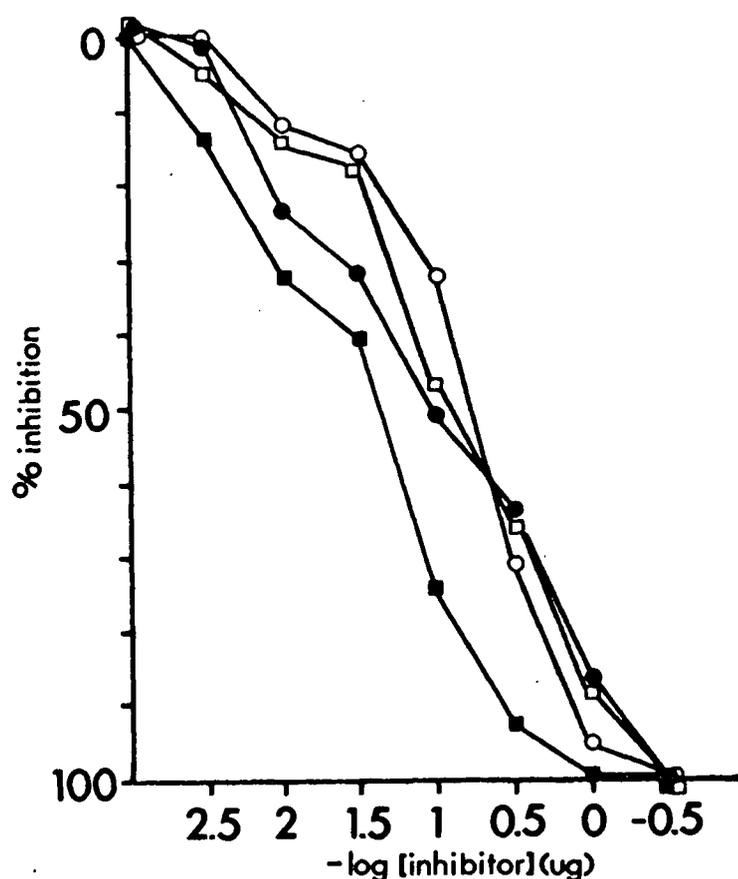


Figure 16. Reactivity of polyclonal anti-CAMAL, anti-HSA and anti-AFP antibodies with CAMAL, HSA and AFP in the ELISA.

The ELISA was performed as described in Section 2.7(i). ELISA plates coated with HSA, AFP or CAMAL at 300 ng/ml were reacted with NRS, polyclonal anti-HSA, anti-AFP and anti-CAMAL antibodies. The plates were developed and the reactivity of the various antibodies on the various proteins quantitated in a Flow Laboratories Multiscan ELISA plate reader. Results are presented as the mean absorbance<sub>405nm</sub> + standard deviation of three separate trials. Proteins used to coat ELISA plates are indicated on the x-axis, the antibodies used are indicated within the boxed areas and the degree of reactivity of each antibody in the ELISA represented by the bar graph.

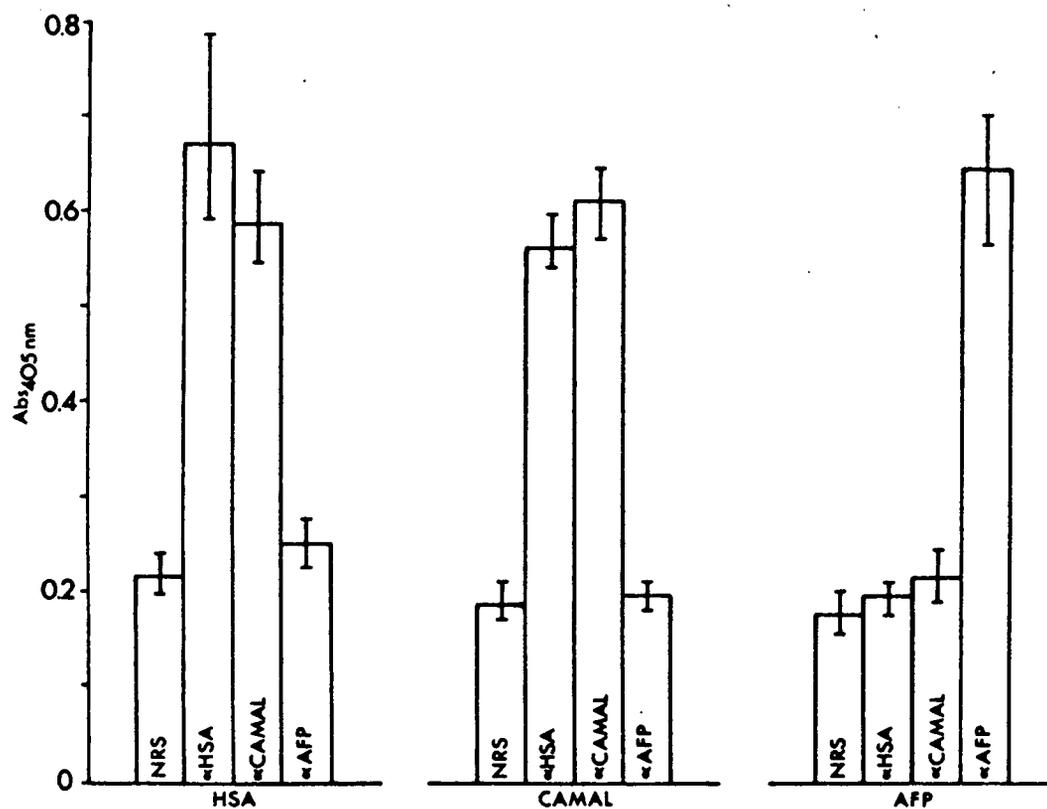
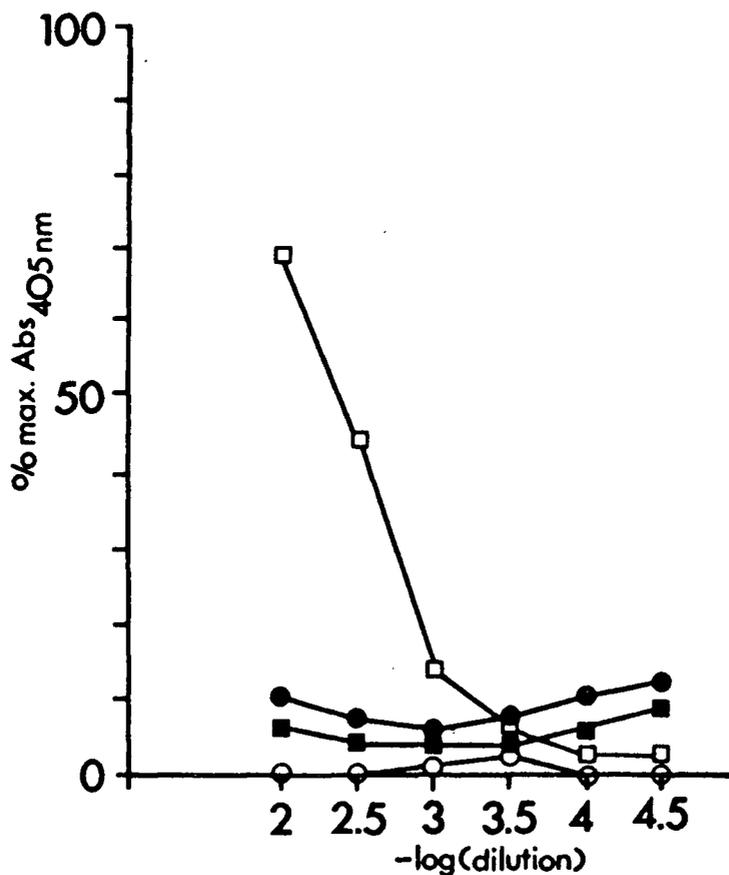


Figure 17. Reactivity of anti-CAMAL and anti-HSA antibodies on native and RCM-CAMAL and -HSA.

The ELISA was performed on both native and RCM-proteins according to the procedure described in Section 2.7(i). ELISA plates coated with either CAMAL, HSA, RCM-CAMAL or RCM-HSA at 300 ng/ml were reacted with NRS, polyclonal anti-CAMAL and anti-HSA antibodies. The plates were developed and the reactivity of the various antibodies on the various protein samples quantitated in a Flow Laboratories Multiscan ELISA plate reader. Results are presented as the percent of the maximum absorbance<sub>405nm</sub> when comparing the degree of reactivity of the antibody on the native protein versus the RCM-protein. Antibody and protein combinations are as indicated.

- (●) reactivity of anti-CAMAL with CAMAL versus RCM-CAMAL.
- (○) reactivity of anti-CAMAL with HSA versus RCM-HSA.
- (■) reactivity of anti-HSA with CAMAL versus RCM-CAMAL.
- (□) reactivity of anti-HSA with HSA versus RCM-HSA.



essentially ablated the reactivity of both the anti-CAMAL and anti-HSA antibodies. However, reduction and carboxymethylation of HSA did not affect both antibodies equivalently. The reactivity of the anti-CAMAL antibody was ablated by the reduction and carboxymethylation of HSA, but the reactivity of the anti-HSA antibody was not as affected by reduction and carboxy-methylation of HSA. Since both antibodies had been shown to titrate equivalently in the ELISA on both CAMAL and HSA, the observed difference in reactivity with native protein versus RCM-protein was not due to differences in the respective antibody titres. The results presented in Figure 17 demonstrated that the determinant(s) shared by CAMAL and HSA were sensitive to reduction and carboxymethylation and were most likely conformational, rather than primary sequence-specified, antigenic determinants. A more significant conclusion to be drawn from the data shown in Figure 17 was that the preparation of CAMAL used for these studies, and the sequence analysis, was not substantially contaminated with HSA. Consequently, peptides derived from contaminating HSA would not be expected to represent significant peaks in the HPLC analysis of CAMAL-derived peptides.

An additional demonstration that CAMAL and HSA represented related but distinct molecules was accomplished by using a combination of mixed immunoprecipitation and 2D-PAGE. Comparable amounts of  $^{125}\text{I}$ -labelled CAMAL and HSA were immunoprecipitated separately with antibody specific for each molecule. The immune complexes were collected, washed and either loaded separately onto 2D gels or mixed together prior to 2D-PAGE. The results of both individual and mixed immunoprecipitations followed by 2D-PAGE are shown in Figure 18.

Figure 18. Mixed immunoprecipitation and 2D-PAGE of  $^{125}\text{I}$ -CAMAL and  $^{125}\text{I}$ -HSA.

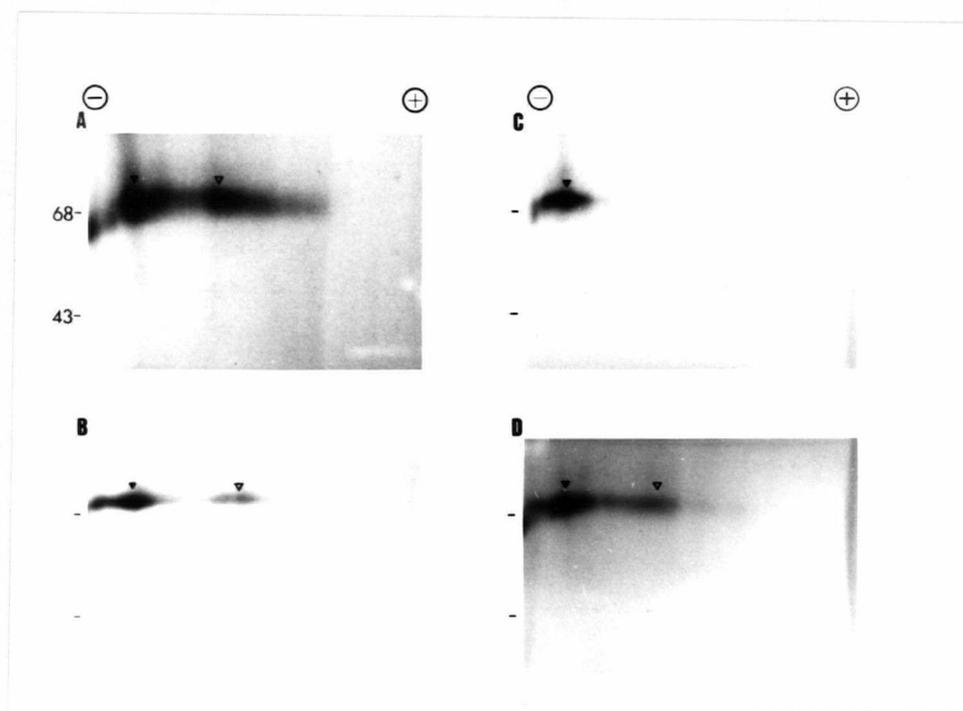
Immunoprecipitation of iodinated CAMAL with anti-CAMAL antibody and iodinated HSA with anti-HSA antibody was performed. The washed immunoprecipitates were subjected to 2D-PAGE individually or in combination, as described in Section 2.6(iii). The dried gel was exposed to Kodak X-Omat AR film for 18 hours at  $-70^{\circ}\text{C}$ . The direction of isoelectric focussing and SDS-PAGE are as indicated. Relative molecular weights (kilodaltons) of protein standards are as indicated.

Panel A. 2D-PAGE of  $^{125}\text{I}$ -HSA immunoprecipitated with anti-HSA antibody.

Panel B. 2D-PAGE of  $^{125}\text{I}$ -HSA immunoprecipitated with anti-HSA antibody plus  $^{125}\text{I}$ -CAMAL immunoprecipitated with monoclonal anti-CAMAL antibody.

Panel C. 2D-PAGE of  $^{125}\text{I}$ -CAMAL immunoprecipitated with polyclonal anti-CAMAL antibody.

Panel D. 2D-PAGE of  $^{125}\text{I}$ -CAMAL immunoprecipitated with polyclonal anti-CAMAL antibody plus  $^{125}\text{I}$ -HSA immunoprecipitated with anti-HSA antibody.



As amino acid composition analysis would predict, both CAMAL and HSA behaved as distinct molecules, at least as far as pI is concerned, even though both molecules had been shown to be antigenically and structurally related. The observation that the anti-HSA polyclonal antibody precipitates a protein (Figure 18A) with an  $M_r$  and pI similar to CAMAL suggests a number of possibilities. If the right-hand "spot" in figure 18A is HSA, then the precipitation of CAMAL from HSA can be explained by the antigenic cross-reactivity demonstrated between HSA and CAMAL and suggests further that CAMAL is a "normal" protein constituent of serum that co-purifies with HSA. An additional interpretation of this result is that the left-hand "spot" is HSA and the remaining "spot" represents a glycosylated or modified form of HSA. This interpretation suggests that both CAMAL and HSA are identical molecules. On the basis of this gel alone it would not be possible to refute this suggestion, however on the basis of the other data presented in this thesis and the fact that myeloid leukemia cells have not been shown to synthesize HSA, this is not likely to be the case. The overall conclusion to be drawn from the results of figure 18 is that CAMAL represents a molecule that exhibits antigenic homology to HSA but is itself a distinct entity from HSA.

Since immunochemical techniques indicated significant antigenic relatedness between CAMAL and HSA, protein cleavage methods were used to establish whether this antigenic relatedness was reflected as a similarity in protein structure between CAMAL and HSA, as assessed by two-dimensional thin layer chromatography analysis of the protein cleavage products. Equivalent amounts of  $^{125}\text{I}$ -RCM-CAMAL/HSA were cleaved in the presence of a 100-fold molar excess of cyanogen bromide

(CNBr), over the met residues present. The resulting peptide mixture was dried, resuspended in a minimal volume of thin-layer electrophoresis (TLE) buffer pH 2.1 and spotted onto a 20cm X 20cm cellulose acetate thin layer chromatography (TLC) plate. The plate was wetted with TLE buffer pH 2.1, loaded into a TLE apparatus and electrophoretic separation of the CNBr-peptides carried out for 1 hour at 1000 volts (until crystal violet tracking dye moved 6cm). The plates were dried overnight in a fume hood and then placed in an equilibrated thin layer chromatography (TLC) chamber. Chromatographic separation of the CNBr-peptides was performed at room temperature for approximately 6 hours (until solvent front was 2cm from the top edge of the TLC plate). The plate was dried and subjected to autoradiography. The composite  $^{125}\text{I}$ -CNBr-peptide maps for CAMAL and HSA are shown in Figures 19, 20 and 21. As can be seen from the autoradiograms and composite tracings, both CAMAL and HSA exhibited coincident and unique peptides, although some of the peptides identified as "HSA unique" in Figure 21 may not be unique since possible coincident peptides are "hidden" in Figure 19. It is important to emphasize, at this point, that coincident peptides in 2D-TLC are not necessarily indicative of primary amino acid sequence homology between coincident peptides, since coincident migration in 2D-TLC may merely be a reflection of shared charge, size and hydrophobic character between the coincident peptides.

To further delineate structural similarities between CAMAL and HSA, tryptic 2D-TLC peptide maps of  $^{125}\text{I}$ -RCM-CAMAL/-HSA were generated. Both CAMAL and HSA were digested with TPCK-trypsin, as described previously, and the resulting peptide mixture processed in

Figure 19. CNBr peptide map of  $^{125}\text{I}$ -RCM-CAMAL.

5 $\mu\text{g}$  RCM-CAMAL was iodinated with 100 $\mu\text{Ci}$   $^{125}\text{I}$ , acetone precipitated, dried, digested with CNBr and subjected to TLE at pH 2.1 and TLC in BAWP (15:3:10:12) on cellulose acetate TLC sheets, as described in Section 2.12(ii). The dried TLC sheet was exposed to Kodak X-Omat AR film for 4, 8, 12, 24, 48 and 72 hours at  $-70^\circ\text{C}$ . A composite peptide map is represented by the illustration accompanying the photograph. The directions of the TLE and TLC dimensions are indicated by the arrows.

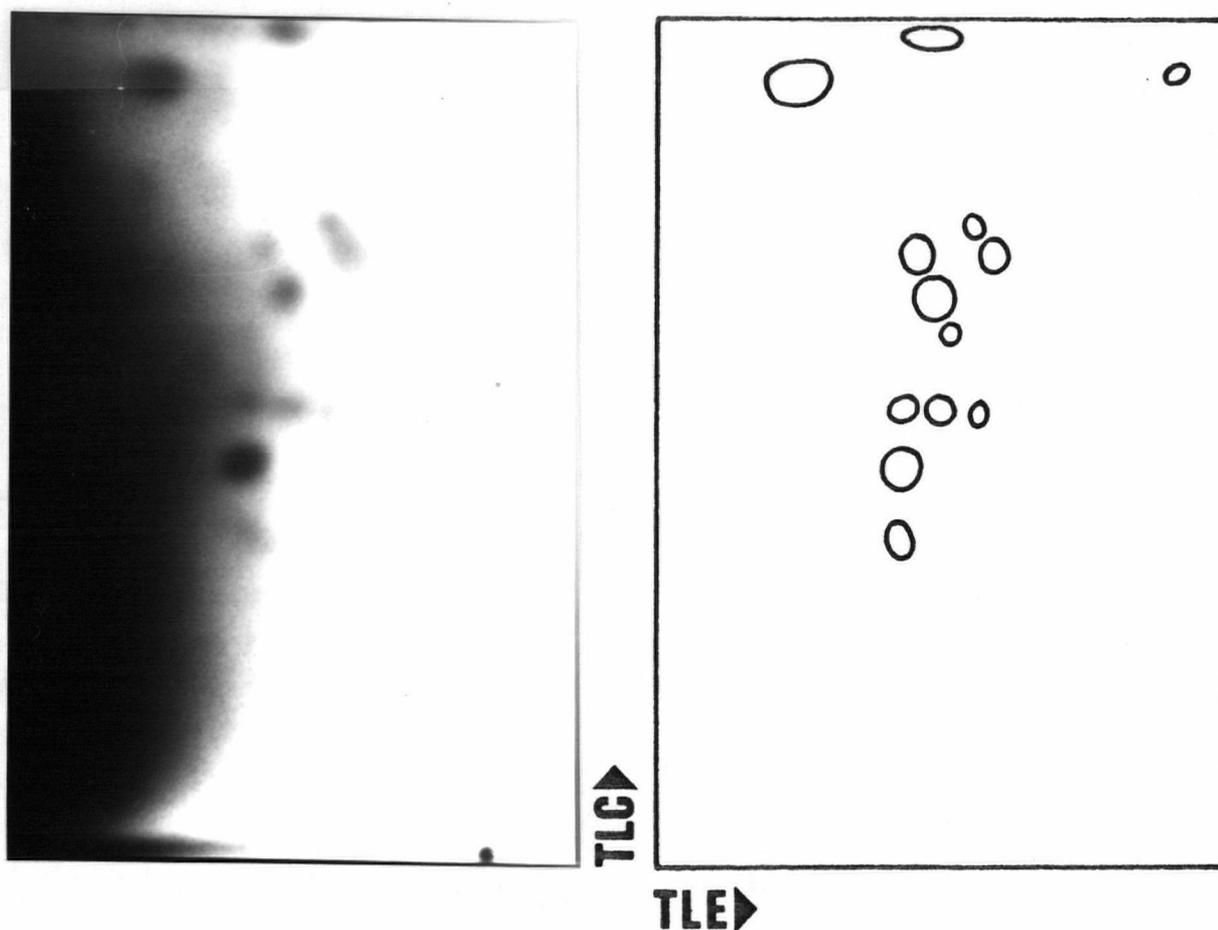


Figure 20. CNBr peptide map of  $^{125}\text{I}$ -RCM-HSA.

5 $\mu\text{g}$  RCM-HSA was iodinated with 100 $\mu\text{Ci}$   $^{125}\text{I}$ , acetone precipitated, dried, digested with CNBr and subjected to TLE at pH 2.1 and TLC in BAWP (15:3:10:12) on cellulose acetate TLC sheets, as described in Section 2.12(ii). The dried TLC sheet was exposed to Kodak X-Omat AR film for 4, 8, 12, 24, 48 and 72 hours at  $-70^\circ\text{C}$ . A composite peptide map is represented by the illustration accompanying the photograph. The directions of the TLE and TLC dimensions are indicated by the arrows.

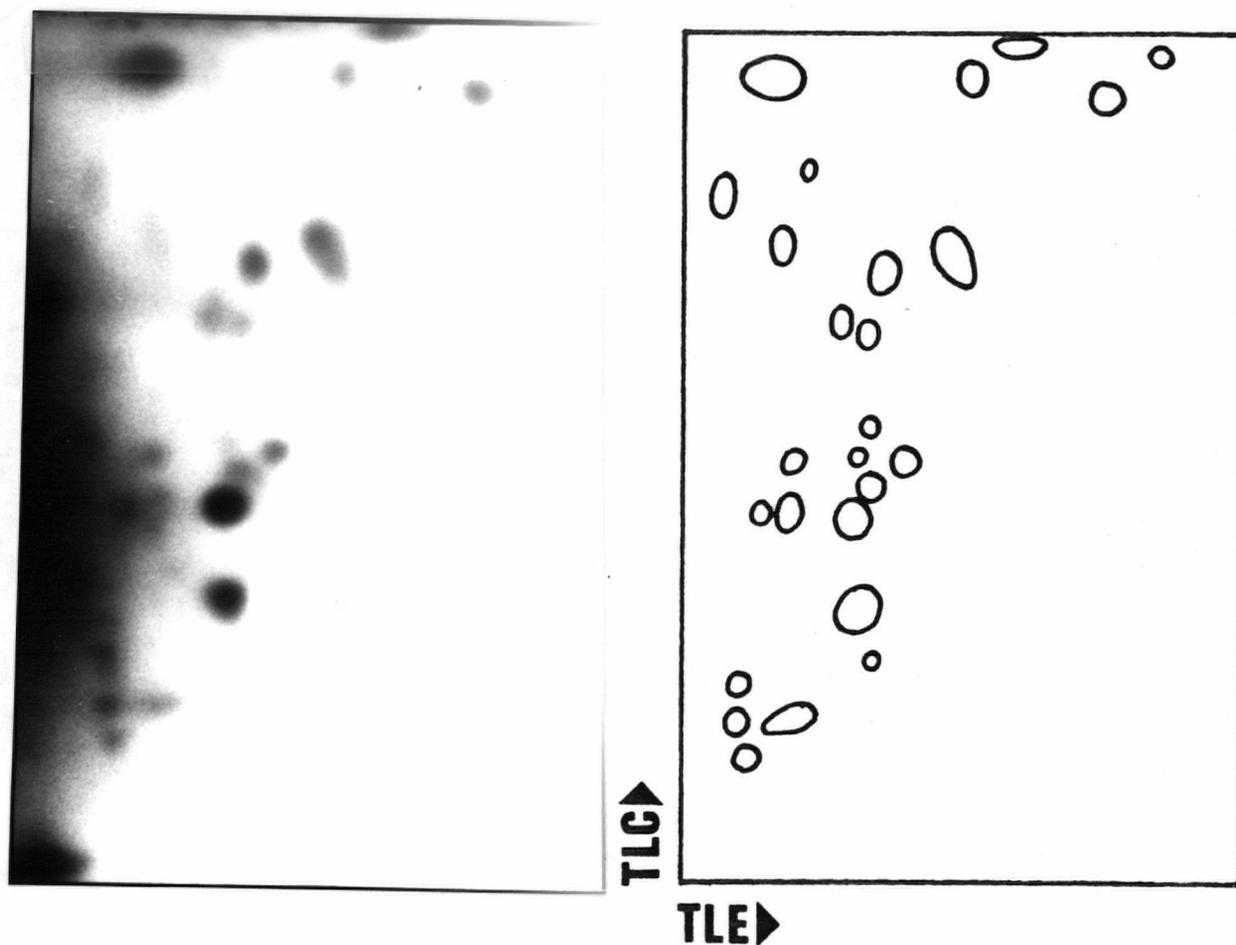
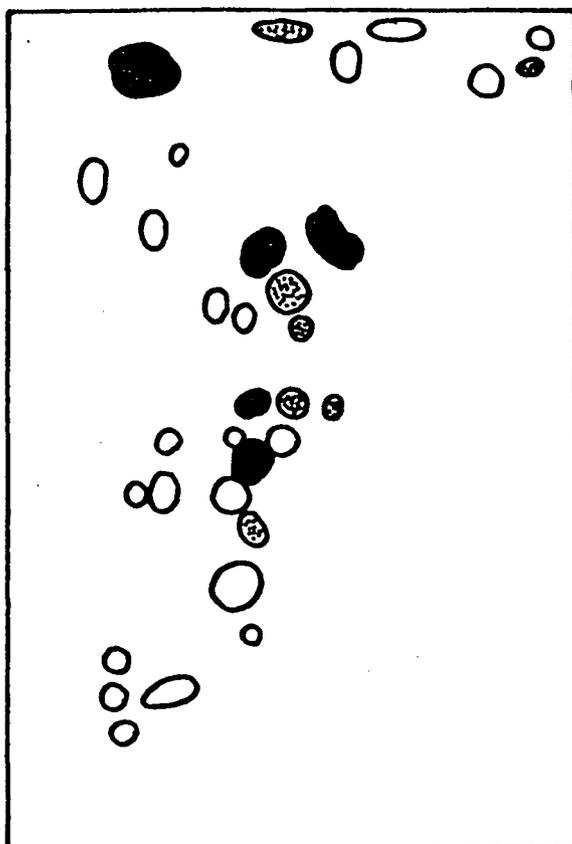


Figure 21. Composite CNBr peptide map of  $^{125}\text{I}$ -CAMAL and  $^{125}\text{I}$ -HSA.

Filled (●) peptide spots represent CNBr peptides common to CAMAL and HSA.

Stippled (◐) peptide spots represent CNBr peptides unique to CAMAL.

Open (○) peptide spots represent CNBr peptides unique to HSA.



the same manner as the CNBr-peptides. The autoradiograms and composite tracings for both CAMAL and HSA are shown in Figures 22, 23 and 24. The tryptic peptide maps reiterated the findings of the CNBr-peptide maps, in that, the tryptic peptide maps demonstrated coincident and unique peptides between CAMAL and HSA. The analysis of CAMAL/HSA CNBr-peptides and tryptic peptides demonstrated that, although both proteins possessed unique peptides, the nature of the "shared" peptides most likely contributed to homologous structural regions within the protein molecule, and thus to an overall conformational similarity between the two proteins in their "native" (non-reduced, non-carboxymethylated, in pH 7.3 or pH 8.5 buffer) states.

An indication that CAMAL protein conformation differed from HSA, in the "native" state, was the occurrence of a reproducible staining anomaly of CAMAL when comparing silver stained and Coomassie blue stained polyacrylamide gels containing both CAMAL and HSA. As shown in Figure 25, when equivalent amounts of CAMAL, HSA and BSA (protein concentration determined by Lowry protein assay and confirmed by amino acid analysis) were separated by SDS- PAGE and stained with Coomassie blue dye (Serva), CAMAL stained approximately 2-3 fold lower in intensity than either HSA or BSA whereas all three proteins stained to the same degree with silver stain (Figure 25; panels a & b). This staining anomaly was further demonstrated by comparing the sensitivity, in determining protein concentration, of the Lowry or Bradford (Coomassie blue reagent) protein microassays (Figure 25; panels c & d). As with protein staining, equivalent amounts of CAMAL, HSA and BSA bound varying amounts of the Bradford reagent, CAMAL

Figure 22. Tryptic peptide map of  $^{125}\text{I}$ -RCM-CAMAL.

5 $\mu\text{g}$  RCM-CAMAL was iodinated with 100 $\mu\text{Ci}$   $^{125}\text{I}$ , acetone precipitated, dried, digested with TPCK-trypsin and subjected to TLE at pH 2.1 and TLC in BAWP (15:3:10:12) on cellulose acetate TLC sheets, as described in Section 2.12(ii). The dried TLC sheet was exposed to Kodak X-Omat AR film for 4, 8, 12, 24, 48 and 72 hours at  $-70^\circ\text{C}$ . A composite peptide map is represented by the illustration accompanying the photograph. Peptides marked with a "t" represent trypsin autolytic peptides. The directions of the TLE and TLC dimensions are indicated by the arrows.

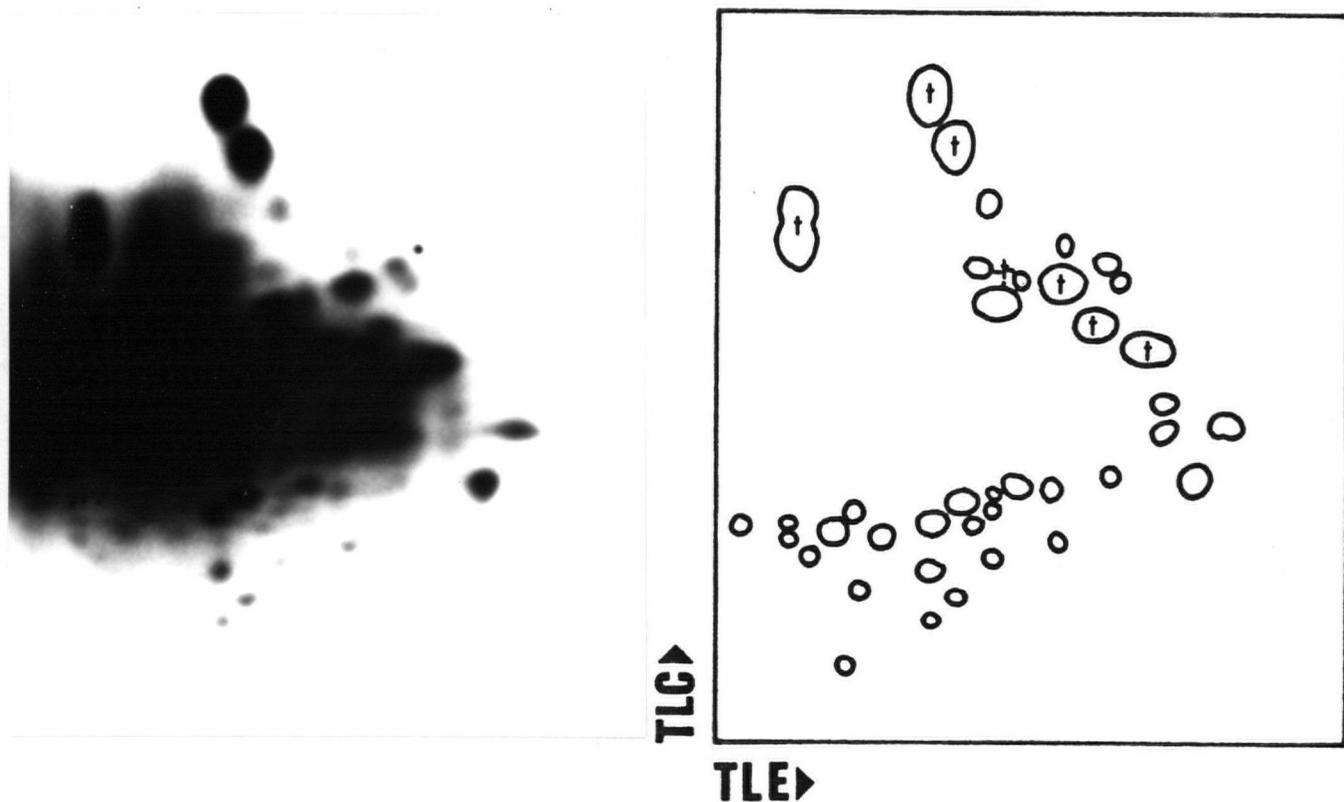


Figure 23. Tryptic peptide map of  $^{125}\text{I}$ -RCM-HSA.

5 $\mu\text{g}$  RCM-HSA was iodinated with 100 $\mu\text{Ci}$   $^{125}\text{I}$ , acetone precipitated, dried, digested with TPCK-trypsin and subjected to TLE at pH 2.1 and TLC in BAWP (15:3:10:12) on cellulose acetate TLC sheets, as described in Section 2.12(ii). The dried TLC sheet was exposed to Kodak X-Omat AR film for 4, 8, 12, 24, 48 and 72 hours at  $-70^\circ\text{C}$ . A composite peptide map is represented by the illustration accompanying the photograph. Peptides marked with a "t" represent trypsin autolytic peptides. The directions of the TLE and TLC dimensions are indicated by the arrows.

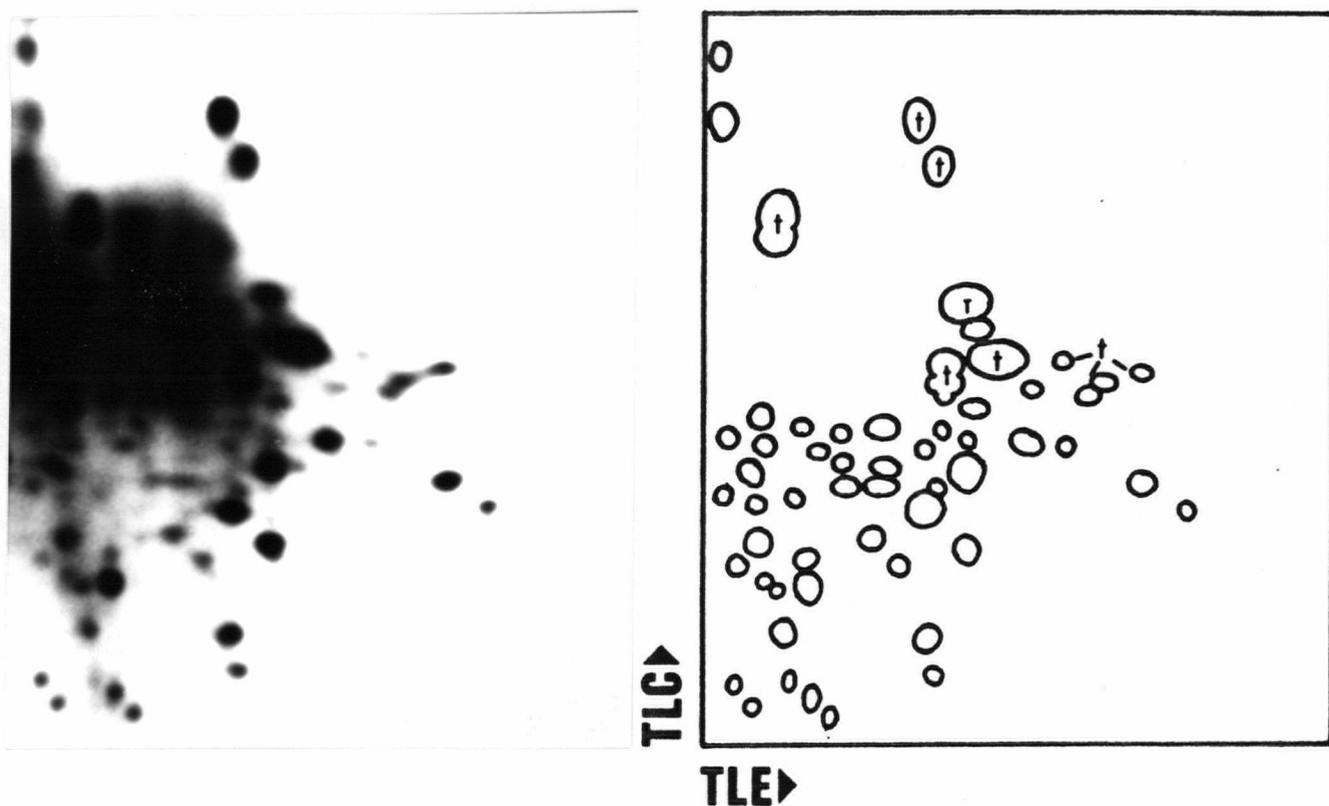


Figure 24. Composite tryptic peptide map of  $^{125}\text{I}$ -CAMAL and  $^{125}\text{I}$ -HSA.

Filled (●) peptide spots represent tryptic peptides common to CAMAL and HSA.

Stippled (◐) peptide spots represent tryptic peptides unique to CAMAL.

Open (○) peptide spots represent tryptic peptides unique to HSA.

Peptides representing trypsin autolytic peptides have been omitted for clarity.

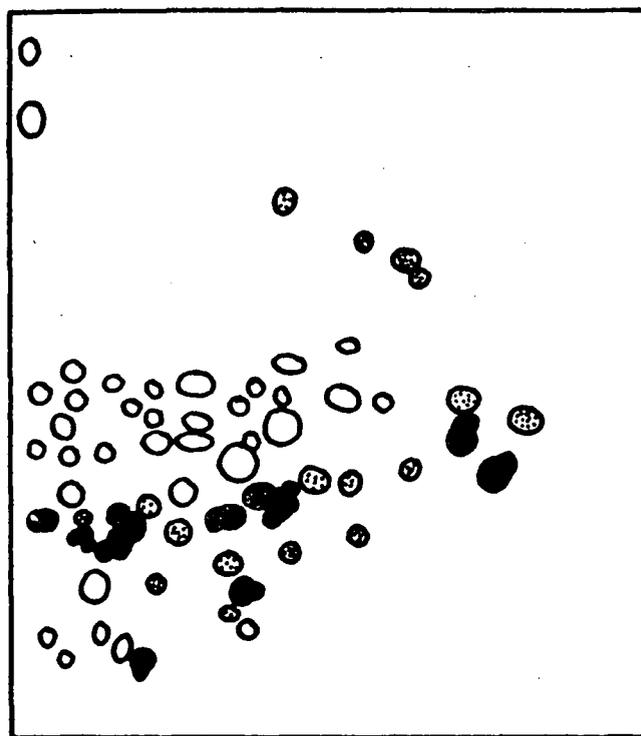


Figure 25. Comparison of protein stain dye binding and protein assay dye binding between BSA, HSA and CAMAL.

5 $\mu$ g BSA, HSA and CAMAL were subjected to SDS-PAGE and stained with either Coomassie Brilliant Blue or the alkaline silver nitrate staining reagent.

Panel A : BSA, HSA and CAMAL stained with Coomassie brilliant blue.

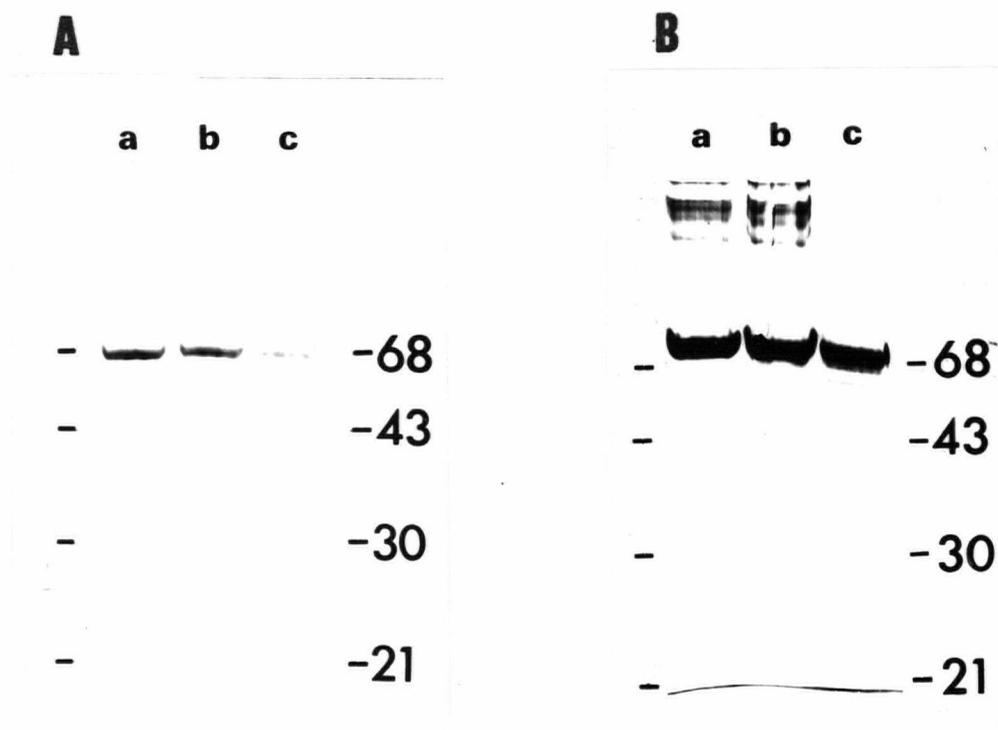
Panel B : BSA, HSA and CAMAL stained with silver nitrate.

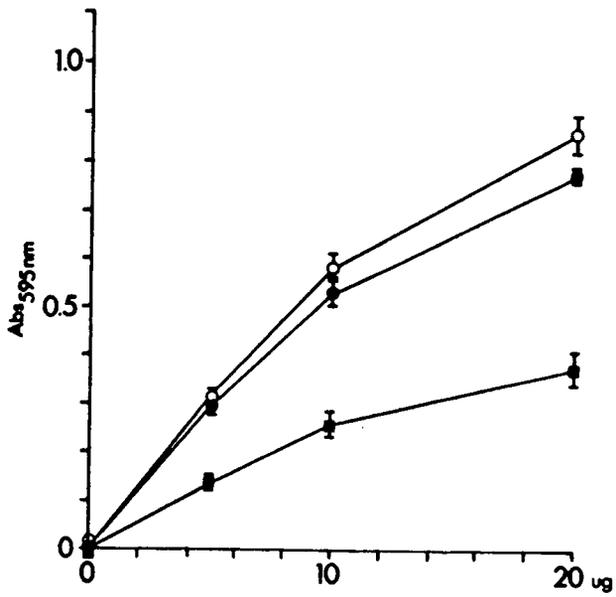
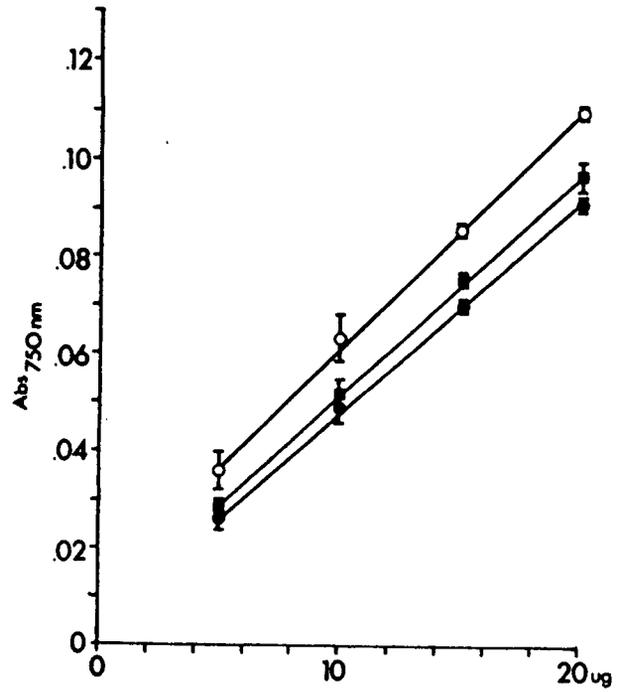
BSA, HSA and CAMAL were subjected to the Bradford (Coomassie blue) protein assay and the Lowry protein microassay.

Panel C : Bradford protein assay. (○) BSA, (●) HSA, (■) CAMAL.

Panel D : Lowry protein microassay. (○) BSA, (●) HSA, (■) CAMAL.

Protein concentrations of stock solutions were determined by Lowry protein assay and confirmed by amino acid analysis.



**C****D**

binding approximately 2-3 fold less reagent than HSA or BSA, whereas all three proteins reacted equally well with the Lowry reagent.

Many investigators have postulated that the interaction between proteins and heterocyclic aromatic compounds, such as dyes, is due largely to interactions at sites of increased local hydrophobicity. If this was the case for Coomassie blue dye interaction with CAMAL, HSA and BSA, there existed the possibility that further characterization of the kinetics and parameters of protein-dye binding could reveal additional differences between CAMAL and HSA.

HSA is known to serve as a "depot" protein and transport protein for many endogenous and exogenous substances present in human plasma (for review see U. Kragh-Hansen, 1981). Among the endogenous substances bound to HSA with high affinity are bilirubin, haemin, long-chain fatty acids (palmitate, linoleate), steroid hormones (progesterone, testosterone, aldosterone, estradiol, cortisol) and various inorganic ions ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Cl}^-$ ,  $\text{I}^-$ ,  $\text{SCN}^-$ ). Among the enormous variety of exogenous substances that exhibit "specific" interactions with HSA, are a wide spectrum of charged and neutral drugs. The specific interactions of these drugs, and their analogues, with HSA have been studied in great detail mainly because of the relevance of the kinetics of protein:drug interactions to the levels of drug(s) administered in the clinical setting to achieve an "effective dose". The interaction of these drugs with other plasma proteins is insignificant in comparison to HSA, which is most likely attributable to the overwhelming concentration of HSA in normal human plasma in relation to other plasma proteins. Included in this large group of drugs are the coumarins (Warfarin,

dicoumarol), benzodiazapines (Diazepam/Valium) and salicylates (ASA/Aspirin).

At least six separate binding sites have been identified on the HSA molecule, namely; binding region 1 (br1) = long-chain fatty acids; br2 = thyroxine,  $\text{Cl}^-$ ,  $\text{I}^-$ ; br3 = bilirubin, azo-dyes, benzodiazapines; br4 =  $\text{Cu}^{2+}$ , br5 = haemin and br6 = salicylate, indomethacin, Warfarin. Quite apart from the specific interactions of drugs with HSA is the observation that a number of dansyl amino acid (DNS-aa) analogues are able to function as specific antagonists/competitive "inhibitors" for the binding of certain drugs at specific binding sites on the HSA molecule. Three DNS-aa derivatives, DNS-gly, DNS-sarc and DNS-pro, have been shown to bind to br6 on the HSA molecule and are specifically displaceable by Warfarin. Since the binding of all of the exogenous ligands examined so far have been shown to be dependent on protein conformation (HSA binding site integrity), it seemed reasonable that this methodology could be used to distinguish the conformational relatedness of CAMAL to HSA on the basis of their ability to bind the various DNS-aa derivatives.

DNS-aa derivatives were chosen over other ligands as molecular probes primarily because quantitation of DNS-aa binding to HSA using spectrofluorometry was much more reproducible, simpler and faster than other non-fluorescent methods, such as equilibrium dialysis. In order to ensure that differences in DNS-aa probe binding to HSA, BSA and CAMAL was due to differences in the number of binding sites or binding site affinity and not to discrepancies in the amount of protein assayed, amino acid analysis was performed on standard solutions of HSA, BSA and CAMAL to confirm the concentrations derived by the

Lowry protein assay. The protein concentrations determined by the Lowry assay and amino acid analysis were shown to be equivalent. Protein concentrations used in the DNS-aa binding assays were therefore derived by dilution of the standard protein solutions. Figure 26 illustrates the binding curves and kinetic parameters for the binding of DNS-gly (panel a), DNS-sarc (panel b) and DNS-pro (panel c) to both HSA and CAMAL. Although all three DNS-aa derivatives exhibited approximately the same "affinity" ( $K_a$  = association constant) for CAMAL and HSA, the maximal amount of ligand bound per molecule at saturation differed markedly between the two proteins. Since the same amount of protein was used in each assay, the differences in binding capacity for the three DNS-aa can only be attributed to differences in the total number of binding sites or the accessibility of the DNS-aa to the binding sites, all of which suggested that CAMAL and HSA differ in both "native" conformation (binding site accessibility) and possibly sequence (binding site number).

Figure 26. Capacity of p70 (CAMAL) to bind ligands specific for the "Warfarin" binding site of human serum albumin.

Panel A : Binding kinetics for dansyl-glycine (DNS-gly).

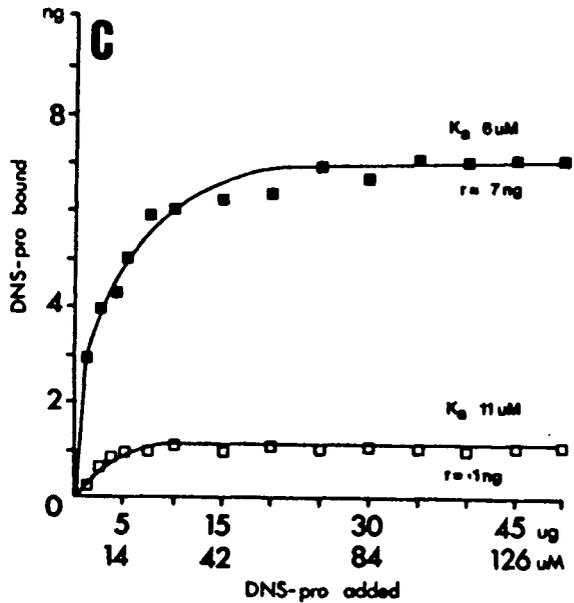
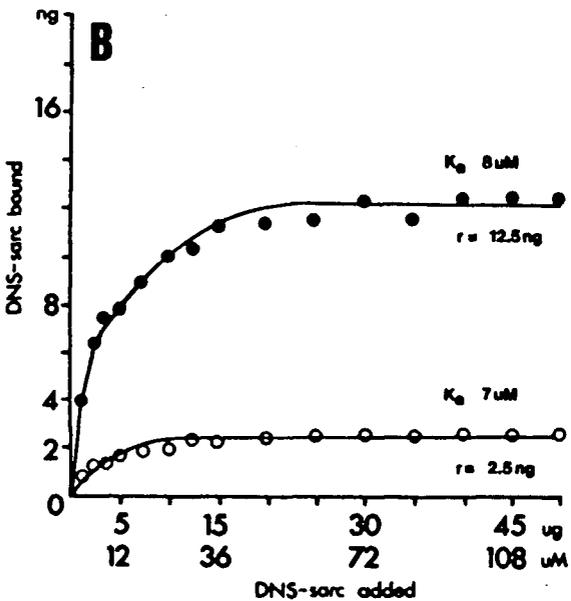
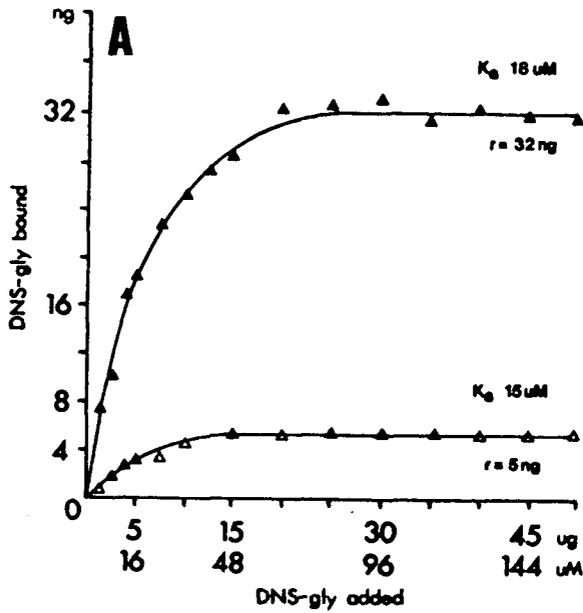
(▲) HSA, (△) CAMAL.

Panel B : Binding kinetics for dansyl-sarcosine (DNS-sarc).

(●) HSA, (○) CAMAL.

Panel C : Binding kinetics for dansyl-proline (DNS-pro).

(■) HSA, (□) CAMAL.



### 4.3. Summary

Analysis of the molecular characteristics of CAMAL and HSA (Table 13) demonstrated both similarities and differences between CAMAL and HSA and suggested that these molecules were related but not identical. The demonstration that CAMAL displayed peptide sequences homologous to HSA and AFP (tp 27, tp31) and unique sequences (tp20) led to difficulties in determining whether CAMAL was albumin, related to albumin or a distinct protein contaminated with albumin. The observation that CAMAL migrated as a protein distinct from HSA in 2D-PAGE, suggested that CAMAL was not substantially contaminated with HSA. However, this finding does not exclude the possibility that CAMAL represents an altered form of HSA, possibly an aberrantly glycosylated isoform of HSA. The relatedness of CAMAL to HSA through glycosylation has not been examined in detail but, neuraminidase or mixed glycosidase treatment of CAMAL did not affect its mobility in 2D-PAGE suggesting that CAMAL is not glycosylated to an extent that is detectable by 2D-PAGE and as a result does not appear to be a glycosylation variant of HSA (data not shown).

The results of ELISA titrations, inhibition assays and protein immunoblots using antibody specific for either CAMAL or HSA showed that an extensive degree of antigenic relatedness existed between CAMAL and HSA. Whether this antigenic relatedness was indicative of extensive primary sequence homology is still undetermined. The antigenic cross-reactivity exhibited between CAMAL and HSA was shown to be specified by a shared conformational determinant, since any disruption of the tertiary structure of either molecule led to a

Table 13. Comparison of the molecular characteristics of CAMAL and HSA.

	CAMAL	HSA
1. Molecular weight ( $M_r$ )	70-72000	68-70000
2. Isoelectric point (pI)	7.1-7.2	5.5-6.5
3. Amino acid composition		
glycine	55	16
isoleucine	23	7
cysteine	12	22
4. Dansyl-amino terminal amino acid(s)	DNS-gly, -ala -ser, -thr	"blocked" (Acetyl)-asp
5. Reactivity with antibody		
rabbit anti-CAMAL	+++	++
- (RCM-CAMAL)	-	- (RCM-HSA)
rabbit anti-HSA	++	+++
- (RCM-CAMAL)	-	++ (RCM-HSA)
rabbit anti-AFP	-	-/+
6. Lowry protein assay/ Alkaline silver staining	+++	+++
7. Bradford protein assay/ Coomassie blue staining	+	+++
8. Dansyl-amino acid binding		
DNS-glycine	$K_a=15\mu\text{M}$ , $r=5$ ng	$K_a=18\mu\text{M}$ , $r=33$ ng
DNS-sarcosine	$K_a=7\mu\text{M}$ , $r=3$ ng	$K_a=8\mu\text{M}$ , $r=13$ ng
DNS-proline	$K_a=11\mu\text{M}$ , $r<1$ ng	$K_a=6\mu\text{M}$ , $r=7$ ng
9. Peptide sequences		
tp20	unique sequence to CAMAL	
tp27	9/10 amino acids identical to HSA	
	7/10 amino acids identical to AFP	
tp31	8/11 amino acids identical to HSA	

significant loss in ELISA reactivity using either the monoclonal or polyclonal anti-CAMAL antibody, while the reactivity of the polyclonal anti-HSA antibody was maintained with both native and reduced and carboxymethylated HSA. Most importantly, this result demonstrates that the preparation of CAMAL used for sequence analysis and comparative studies was not substantially contaminated with HSA. This result, in light of the homologous peptide sequences, similar CNBr- and tryptic peptide map profiles and shared conformational antigenic determinant(s) between CAMAL and HSA, further strengthens the assertion that CAMAL is more "albumin-like" rather than "albumin".

The studies which provided data most supportive of the assertion that CAMAL represented a distinct, but related, protein from HSA were the ELISA assays, demonstrating a differential reactivity of anti-CAMAL and anti-HSA antibodies to native and RCM-CAMAL or HSA, the mixed immunoprecipitate /2D-PAGE analysis and the dye/DNS-aa binding studies. The existence and significance of a common conformational antigenic determinant on HSA and CAMAL has been discussed. The results of the mixed immunoprecipitate/2D-PAGE analysis demonstrated that CAMAL and HSA were electrophoretically distinct proteins and supported the contention that although CAMAL and HSA were related antigenically, it was likely that a significant amount of primary sequence unique to CAMAL also existed.

The dye binding studies using Coomassie blue, as a protein staining and protein assay reagent, demonstrated a decreased capacity of CAMAL to bind this dye when compared to HSA and BSA. These results coupled with the results of the DNS-aa binding studies, where CAMAL again exhibited a decreased capacity for ligand binding when

compared to HSA, indicated that the molecular topology of CAMAL and HSA differed significantly. Another interpretation of the DNS-aa binding studies is that the specific binding seen between CAMAL and the various DNS-aa probes may not be due to CAMAL but may represent low level contamination of CAMAL with HSA. The similar association constants and maximum amounts of ligand bound per mole protein would suggest that the CAMAL preparation used for these studies was approximately 20% contaminated with HSA, assuming that CAMAL does not bind any DNS-aa probe. This degree of HSA contamination of the CAMAL preparation is unlikely and incompatible with the results of the ELISA studies, peptide studies and 2D-PAGE analysis of CAMAL, since this level of contamination would have been detected. The results from both the Coomassie blue and DNS-aa binding studies, the CNBr- and tryptic peptide map profiles and the unique sequence of tp20 suggest more than conformational differences between CAMAL and HSA and are indeed indicative of primary sequence differences, at least as far as the sequences bordering or constituting binding sites on the CAMAL molecule are concerned. In the final analysis, only extensive protein/peptide sequencing will be able to establish whether CAMAL is related, and to what degree, to HSA.

## Chapter 5. General discussion.

The characterization of specific genes or gene products associated with normal hematopoietic cell growth and maturation has been one approach to developing a more detailed understanding of the differentiation process in normal cells and defining the abnormality in differentiation of leukemic cell populations. Morphologically distinct cell types can be identified during normal hematopoietic cell differentiation and the normal cellular precursors of these specific cell lineages isolated from both normal bone marrow and peripheral blood samples.

Isolated normal bone marrow and peripheral blood cells have been shown to differentiate and mature *in vitro* in a manner analogous to the situation *in vivo*. Leukemic cells are generally considered to be blocked in normal myeloid cell differentiation since the capacity of these leukemic cells to differentiate or mature *in vitro* is greatly reduced when compared to normal cells. Since the *in vitro* growth potential of both normal and leukemic cells is limited by the supply of exogenous growth factors, both cell types can be used as tools to analyse the steps involved in myeloid cell differentiation through the manipulation of the growth environment (Tables 1 and 2).

The regulation of cellular differentiation is proposed to reside at the cell surface and be controlled through the expression of growth factor receptors. The phenotyping of cells on the basis of their responsiveness to specific growth factors necessarily involves the analysis of the expression of the cognate growth factor receptors

(Metcalf et al., 1985a,1985b; Walker et al., 1985b). Consequently, immunological phenotyping of precursor versus mature cell would permit the isolation of cellular structures associated with proliferating stem cells and the mature phenotype. The identification of leukemia-associated antigens is therefore a reasonable approach to defining the entities associated with the stage of arrest in leukemic cell populations as well as permitting the development of leukemia-specific immunological reagents. The induction or loss of specific antigen expression upon induced maturation of leukemic cell lines would permit the isolation of differentiation-associated antigens through their modulated expression.

Biochemical characterization of a number of leukemia-associated antigens has demonstrated that a significant proportion of these antigens are expressed as developmental antigens in or on normal myeloid progenitor cells (Table 4). The expression of these normal myeloid differentiation-associated antigens in leukemic cells might merely reflect the nature of the normal cell lineage affected in leukemia; however, aberrant expression might also indicate a more primary involvement of these antigens in the leukemigenic process.

The  $M_r$  70000 (CAMAL) protein, characterized in this study, has been shown to be expressed in leukemic cells, although a small percentage (<1%) of normal bone marrow cells seem to express a low level of this protein (Logan et al., 1984). From the results of immunoperoxidase-staining of slide preparations of leukemic and normal cells, p70 (CAMAL) was shown to be expressed in a manner inconsistent with it being a blast cell antigen or a normal myeloid differentiation antigen. The expression of p70 (CAMAL) in leukemic

cells, and possibly CAMAL-positive normal cells, was shown to be constitutive, since p70 (CAMAL) expression in these cells was unaltered by induced differentiation or functional maturation. This data also supports the contention that p70 (CAMAL) is not a typical differentiation or maturation antigen.

Subsequent studies have demonstrated that purified CAMAL is inhibitory to the growth of normal bone marrow cells *in vitro*, whereas leukemic bone marrow cells were unaffected by CAMAL treatment. Since CAMAL expression and secretion by leukemic cells in the bone marrow could provide a mechanism for the outgrowth of the leukemic clone, at the expense of its normal counterparts, a detailed analysis of the protein was undertaken to establish the relatedness of CAMAL to known growth regulatory proteins.

The production of substances by leukemic cells and cell lines that are inhibitory to the growth of normal hematopoietic cells has been demonstrated by a number of investigators. The ability of these secreted products to specifically suppress the proliferation and cellular functions of normal myeloid and lymphoid cells *in vitro* has also been shown. The ability of these leukemia cell-associated inhibitors to suppress normal myelopoiesis and lymphopoiesis *in vitro* suggests that these same substances may be responsible for the observed imbalances in the growth and proliferation of myeloid and lymphoid cells associated with acute and chronic myeloid leukemia *in vivo* (Olofsson et al., 1980; Broxmeyer et al., 1982; Chiao et al., 1986).

Chiao and co-workers have reported the isolation of a leukemia-cell derived inhibitor that suppresses both lymphocyte activation and function (Chiao et al., 1986). Serum-free culture medium from the

human promyelocytic leukemia cell line HL60 contained an  $M_r$  58000 protein which was able to suppress the proliferative response of normal lymphocytes *in vitro*, in a dose-dependent manner. The observed suppression was specific for lymphocytes, since the proliferative potential of normal granulocytes or macrophages was not affected. The observation that suppression of lymphocyte proliferation was accompanied by a drastic reduction in lymphokine production, specifically interleukin-2 (IL-2), suggested that the mechanism of inhibition might result from alterations in IL-2 production or activity. The leukemia cell-associated inhibitor was also shown to be capable of blocking the activity of lymphokines that normally induce the terminal differentiation of myeloid leukemia cells (Leung and Chiao, 1985; Chiao et al., 1986).

Reports in the literature have also demonstrated that some leukemic cells and cell lines have the ability to produce autocrine growth factors which potentiate the production of the leukemia-associated inhibitor resulting in an increased predominance of leukemic cell growth (Brennan et al., 1981; Heil and Chiao, 1985). Induced differentiation of the leukemic cell line HL60 resulted in a rapid and irreversible loss of the capacity of these cells to produce the leukemia-associated inhibitor (Heil and Chiao, 1985). These findings suggest that myeloid leukemia cells, capable of producing an inhibitory factor that blocks the normal lymphocyte/ lymphokine-mediated regulation of leukemia cell differentiation, can proliferate in an unrestricted manner, leading to the outgrowth of the leukemic clone and eventual domination of the bone marrow by immature leukemic progenitor cells.

Leukemic cells are capable of liberating a variety of growth inhibitory factors including transferrin, acidic isoferritins, lactoferrin, prostaglandins E<sub>1</sub> and E<sub>2</sub>, with varying degrees of specificity for normal hematopoietic progenitor cells, which may account for the heterogenous cellular phenotypes seen in leukemic bone marrow cell samples (Broxmeyer et al., 1982, 1983). Leukemia cells and cell lines have also been shown to liberate inhibitors of normal granulopoiesis (Broxmeyer et al., 1982; Olofsson et al., 1980a & 1980b). In these studies, the granulopoiesis inhibitor was detected by the inhibition of granulocyte and monocyte/macrophage colony formation *in vitro*. Broxmeyer et al., 1982 have described a leukemia-associated inhibitory activity (LIA) that suppresses normal granulopoiesis *in vitro*, and have shown that the LIA is a glycoprotein with an apparent molecular weight of approximately 550,000 that is produced by leukemic bone marrow and peripheral blood cells and normal monocytes and macrophages. Further characterization has identified the LIA as acidic isoferritins, has shown the LIA to be a homopolymer of M<sub>r</sub> 21000 "heavy" subunits (as opposed to M<sub>r</sub> 19000 "light" subunits present in basic isoferritins) and has suggested that this activity might be better considered as a normal negative feedback regulator of granulopoiesis (Broxmeyer et al., 1982).

Olofsson et al., 1980 have demonstrated the suppression of normal, but not leukemic, granulocytic progenitor cells *in vitro* by using leukemia cell-conditioned medium (LCCM). The leukemia-associated inhibitor (LAI) was isolated from LCCM and shown to be an M<sub>r</sub> 300-500,000 glycoprotein with subunits of 150-170,000 (Olofsson et al., 1980b, 1984). LAI acts by limiting the progression of normal granulocyte progenitors to S-phase and seems to be produced by low

density, adherent, leukemic bone marrow and peripheral blood cells. LAI has also been shown to be produced by the leukemic cell line HL60. LAI has not, as yet, been characterized as an acidic isoferritin, even though it bears a strong resemblance to the LIA described by Broxmeyer et al., 1982.

Immunoprecipitation of labelled leukemic cell line lysates with the polyclonal anti-CAMAL antibody results in the precipitation of two proteins of  $M_r$ s 70000 and 58000. Since the  $M_r$  70000 protein has been shown to have growth regulatory effects on normal bone marrow cells (P. Logan, personal communication) and, more recently, peripheral blood lymphocytes (J. Shellard, personal communication), it would be interesting to determine whether the p70 and p58 proteins are related and if the p58 protein is indeed the leukemia cell-associated inhibitor described by Chiao et al. The detection of proteins similar to the LAI/LIA proteins have not been seen with either the polyclonal or monoclonal anti-CAMAL antibodies and the leukemic cell lines used in those studies. It is likely that the inhibitory activity associated with the p70 (CAMAL) protein is due to some intrinsic property of the protein itself or may be manifest through the interaction of the p70 (CAMAL) protein with an, as yet, unidentified protein or receptor on the surface of the target cell. It is not possible to totally exclude the involvement of some low molecular weight inhibitory compound that might be carried by p70 (CAMAL), since p70 (CAMAL) has been shown to be capable of binding exogenous ligands, but this interaction would have to occur subsequent to purification and prior to interaction of p70 (CAMAL) with the target cell. Candidates for this inhibitory activity would include the prostaglandins and steroid hormones, which are present in conditioned

medium, the target cell specificity being determined by the p70 (CAMAL) molecule (Fitzpatrick et al., 1983; Goodwin et al., 1983; Lewis, 1983; Tisdale, 1983; Chouaib et al., 1984; Moore et al., 1984).

Future studies involving a more detailed characterization of the p70 (CAMAL) protein at the molecular level are necessary in order to establish what p70 (CAMAL) is and how its structure is related to its *in vitro* growth effect on normal bone marrow cells. Although further protein sequencing of p70 (CAMAL) will be necessary to provide the required information, a potentially more useful approach would be the isolation of cDNA clones containing CAMAL sequences from a cDNA library of HL60, KG1, K562 or U937 cells. The detection of CAMAL-positive clones could be accomplished through the use of anti-CAMAL antibodies or oligonucleotide probes specific for CAMAL peptide sequences. DNA sequencing of CAMAL-positive clones would, at the very least, aid in establishing the entire primary sequence of p70 (CAMAL) and, more importantly, provide a more sensitive means of detecting and quantifying the degree of CAMAL expression in leukemic and normal cells. Although the molecular studies of p70 (CAMAL) are important, several aspects of p70 (CAMAL) biology deserve attention as well.

The modulation of p70 (CAMAL) expression in differentiation-induced leukemic cell lines has not been examined thoroughly enough to suggest that changes in p70 (CAMAL) expression are not associated with maturation. The possible regulation of p70 (CAMAL) expression between maturation-arrested leukemic cells and differentiation-induced leukemic cells could be important in establishing whether p70

(CAMAL) expression is responsible for or a consequence of the maturation-arrest in leukemic cells.

The idea that p70 (CAMAL) synthesis might be a cell-cycle dependent process has not been investigated in detail. Previous studies indicated that mRNA encoding the p70 (CAMAL) molecule was not demonstrable, by immunoprecipitation of labelled p70 (CAMAL) from *in vitro* <sup>35</sup>S-methionine translation assays, in leukemic cell lines, which were subsequently shown to produce p70 (CAMAL). These data suggested that p70 (CAMAL) mRNA was a rare mRNA, which would have to be highly translated during a specific point in the cell cycle in order to account for the observed amount of p70 (CAMAL) produced in these cell lines.

Another interesting and potentially important avenue of research would be the examination of the co-ordinate expression of p70 (CAMAL) with other cellular proteins known to be regulated by differentiation in leukemic cells and cell lines. These cellular proteins would include most of the proto-oncogene products shown in Table 3. Since both antibody and molecular probes exist for these proto-oncogenes, it would be interesting to determine whether compounds that affect proto-oncogene expression also affect p70 (CAMAL) expression.

The homology exhibited between p70 (CAMAL) and AFP, at the level of tp27 peptide sequence, is interesting since AFP has been shown to exert suppressive effects on lymphocyte growth and activation *in vitro* (Yachnin, 1976; Aver and Kress, 1977). p70 (CAMAL) also demonstrates suppressive effects *in vitro* on both myeloid and lymphoid cell growth and activation (P. Logan and J. Shellard, personal

communication). p70 (CAMAL), HSA and AFP have the ability to bind a variety of ligands (Nunez et al., 1974), some of which may be responsible for the suppressive effects associated with these proteins.

AFP expression has been shown to be elevated and constitutive in malignant tissue of endodermal origin. Elevated AFP expression has been observed in hepatitis, degenerative liver disease and necrosis, hepatomas, teratocarcinomas, hepatocellular carcinomas, endodermal sinus tumours and gastrointestinal or bronchial embryonic endodermal tissue tumours. Elevated expression of AFP has also been associated with some cases of leukemia, lymphoma and myeloma, but the elevation of AFP expression in non-embryonic endodermal tissue tumours has not been examined in detail (Abelev, 1971).

Chemical carcinogens, such as "azo-dyes", are capable of inducing "experimental hepatocarcinogenesis" (EH) in cells of endodermal origin, causing a rapid and persistent induction of AFP expression, even after the inducing agent is removed (Becker and Sell, 1974; Becker et al., 1975). Interestingly, many of the compounds that induce differentiation in leukemic cells and cell lines, such as retinoids, exert protective effects on the induction of EH by "azo-dyes" such as 3'-methyl-4-dimethylamino-azo- benzene and 2-acetyl- aminofluorene [N-2-fluorenyl- acetamide] (Daoud and Griffin, 1980). It would be useful to investigate whether compounds that induce EH have similar effects on normal lymphocytes and myeloid cells and, additionally, if any effects on p70 (CAMAL) expression are seen. The capacity for "azo-dyes" to induce differentiation in leukemic cell lines and modulate p70 (CAMAL) expression should also be examined.

Recently, the human p53 gene, a cellular phosphoprotein known to contribute directly to cellular transformation, was cloned and characterized (Matlashewski et al., 1984; Lamb and Crawford, 1986). Previous studies have shown that elevated levels of p53 are a common feature of a wide variety of murine and human transformed cells and cell lines. p53 has also been shown to form specific complexes with either large T antigen, in SV40-transformed cells (Lane and Crawford, 1979), and the M<sub>r</sub> 58000 E1B protein, in adenovirus-transformed cells (Sarnow et al., 1982), which are viral gene products known to be required for transformation. The p53 gene has also been shown to be located on human chromosome 17 and translocated in t(15:17) in acute promyelocytic leukemia (Benchimol et al., 1985; Le Beau et al., 1985b). The fact that transfected cloned p53 can also replace myc or E1A in transformation synergy experiments with an activated ras gene, and that cloned p53 can immortalize cells in a manner analogous to myc, suggested that p53 belongs to the nuclear oncogenes family (Ruley, 1983; Eliyahu et al., 1984; Parada et al., 1984; Harlow et al., 1986).

p53 has also been shown to form specific complexes with the major mammalian cellular heat shock proteins (Pinhasi-Kimhi et al., 1986). These cellular heat shock proteins (hsp), and heat shock-related or cognate genes/proteins (hsc), have been shown to be expressed in stressed and unstressed cells (Bensaude and Morange, 1983; Lowe and Moran, 1984; Ullrich et al., 1986). The hsp are members of a multigene family encoding proteins ranging from M<sub>r</sub> 24000 to M<sub>r</sub> 105,000. The normal cellular functions of the hsp, and possibly the hsc, have been associated with cellular proliferation, which is consistent with the cell-

cycle-dependent expression of the majority of hsp/hsc gene(s) (Pinhasi-Kimhi et al., 1986).

The possible interaction of viral elements with cellular proteins, like p53 and the hsp/hsc, might be responsible for the induction of p70 (CAMAL) expression. The formation of specific complexes between proteins in leukemic or transformed cells might represent a functional complex having some significance in maintaining or predisposing a cell to aberrant growth regulation. Although evidence suggesting the direct involvement of viral protein-cellular protein complexes in myeloid leukemias is rather sparse, the investigation of p70 (CAMAL) expression, as regards viral intervention, in myeloid leukemias deserves some consideration. Since antibodies to p53 and most of the hsp expressed in human and murine cells, particularly hsp70, exist, the examination of the co-ordinate expression and possible complex formation between CAMAL and these proteins is possible.

Overlying all of the above suggestions is the observation that some normal bone marrow cells seem to express p70 (CAMAL) as well. It is of some importance to establish whether these cells are indeed synthesizing p70 (CAMAL) or sequestering p70 (CAMAL) that is being produced by an unidentified subpopulation of normal bone marrow cells. The molecular characteristics of p70 (CAMAL) purified from normal cells, at all stages of maturation, should also be investigated in order to determine whether the protein isolated from normal tissue possesses the same activity on normal bone marrow progenitors that the leukemic protein displays.

Myeloid leukemia cells and cell lines can be induced by a variety of chemical compounds *in vitro* to form morphologically and

functionally mature granulocytes and monocytes-macrophages. The human leukemic cell lines HL60, KG1, K562, U937 and the murine leukemic cell lines M1, WEHI-3B, L1210 have been used as model systems for the examination of the regulation of myeloid cell differentiation and functional maturation. Both human and murine leukemic cell lines can be induced to differentiate by a number of chemical compounds. Representatives of this group are TPA [12-O-tetradecanoylphorbol 13-acetate], PDB [phorbol dibutyrate], DMSO [dimethylsulfoxide], DMF [dimethyl formamide], HMBA [hexamethylene bisacetamide], hypoxanthine, actinomycin D (Andersson et al., 1979; Rovera et al., 1979; Collins et al., 1980), butyric acid/sodium butyrate (Leder and Leder, 1975; Boyd and Metcalf, 1984), tunicamycin (Nakayasu et al., 1980), 1,25-dihydroxyvitamin D<sub>3</sub> (San Miguel et al., 1984; Rigby et al., 1984; Chaplinski and Bennet, 1987), retinoids (Breitman et al., 1980), interferons (Hemmi and Breitman, 1987; Michalevicz and Revel, 1987), anthracyclines (Schwartz and Sartorelli, 1982), cytosine/adenine arabinoside [Ara-C/-A], aphidicolin (Griffin et al., 1982; Munroe et al., 1984), 5-azacytidine (Bodner et al., 1981), arginase (Honma et al., 1980), 6-thio- guanine (Papac et al., 1980) and lymphotoxin (Hemmi et al., 1987). Since murine leukemic cell lines are also inducible by the same spectrum of compounds, it would be interesting to investigate whether human and murine leukemic cell lines exhibit coordinate expression of the same protein(s), specifically p70 (CAMAL), with respect to differentiation and maturation.

It has also been demonstrated that proteins analogous to the human hsp/hsc proteins are expressed in chemically-transformed murine cells (Croy and Pardee, 1983; Lowe and Moran, 1984). Since

expression of these proteins seems to be linked to cellular proliferation, a characteristic in common with p70 (CAMAL), it would be interesting to determine whether chemically-transformed or leukemic murine cells produce a protein(s) analogous to human p70 (CAMAL). If a murine p70 (CAMAL) protein does exist, the possibilities for investigating the effect of p70 (CAMAL) expression on murine hematopoiesis exists. Eventually, studies involving transgenic mice and p70 (CAMAL) expression may lead to an understanding of the *in vivo* consequences of p70 (CAMAL) expression in normal murine and human hematopoiesis.

The relationship established between CAMAL, HSA and AFP in this thesis, is provocative from both a functional and developmental standpoint. Both HSA and AFP have been shown to share similar binding specificities and affinities for a number of biologically relevant ligands such as steroid hormones, fatty acids, vitamins and divalent cations (Kragh-Hansen et al., 1981; ). The function of AFP as a "carrier" protein is consistent with its similar structure to HSA and in fact these "carrier" functions of AFP may be due more to its conformational similarity to HSA rather than to sequence homology (Hirano et al., 1984a, 1984b). That CAMAL displays both primary sequence and conformational homology to HSA suggests that CAMAL might also function as a "carrier" protein. The observation that CAMAL causes a dose-dependent inhibition in the growth of normal bone marrow cells *in vitro* supports this view and suggests that CAMAL or a "factor" that CAMAL "carries" may be responsible for the observed effects on normal bone marrow cell development (P. Logan and J. Shellard, unpublished observations). An analysis of the cell population(s) affected by CAMAL treatment in normal bone marrow may also shed light on how CAMAL

exerts its growth inhibitory effect, for example, does CAMAL bind to normal bone marrow cells through a specific cell-surface receptor?

The relatedness of CAMAL, HSA and AFP is also interesting from a developmental standpoint. The implication of CAMAL homology to HSA and AFP and CAMAL expression in myeloid leukemic cells is that the mechanism(s) inducing leukemia in normal myeloid progenitor cells may coincidentally or coordinately induce the expression of albumin or albumin-like proteins. The expression of albumin (HSA) or albumin-like (CAMAL, AFP) proteins in myeloid leukemia cells is significant in light of the fact that albumins are normally expressed in hepatic (embryonic endoderm) and not hematopoietic tissue (embryonic mesoderm). The expression of albumins in hematopoietic cell malignancies would be significant since only malignancies involving cells of endodermal origin (liver) have been shown to express abnormally high levels of albumin (Abelev, 1971). The significance of the proposed association between CAMAL, HSA and AFP and their expression in myeloid leukemia cells becomes more apparent when the relationship between hepatic and hematopoietic tissue during embryonic development is considered.

The primary function of the "bone marrow" in the embryo is to aid in the formation and development of bone, a function which it maintains throughout fetal and adult life. Initially the "bone marrow" consists of vascular sprouts, bone forming cells (osteoblasts, osteoclasts), other mesenchymal elements but little recognizable hematopoietic tissue. However, hematopoietic tissue is present early in embryonic development as "blood islands". These "blood islands" consist of tightly packed clusters of mesodermal cells linked to yolk sac blood vessels.

The cells in these "blood islands" become differentiated into two functionally disparate cell types. The cells on the periphery join to form a thin epithelial layer, the endothelium of future blood vessels, whereas the central cells become separated from one another and are differentiated as blood/hematopoietic cells (Balinsky, 1981; Tavassoli et al., 1983). Eventually, hematopoietic cells begin to migrate into the body mesenchyme of the embryo. Liver parenchyma also migrates into this site and begins to establish a network of hepatic cords amongst the undifferentiated hematopoietic "blast" cells. As such the liver initially becomes established as an hematopoietic organ in the embryo and maintains its hematopoietic association until close to birth. Eventually bone cavities form around the definitive hematopoietic tissue and hematopoiesis in the liver recedes. The bone marrow then becomes the primary source of myeloid and lymphoid progenitor cells in the adult (Weiss, 1972; Balinsky, 1981; Tavassoli et al., 1983).

The embryonic association of the hepatic and hematopoietic developmental programs suggests that one of the consequences of hepatic or hematopoietic tissue malignancy may be the expression of regulatory proteins common to both of these developmental programs. Thus the embryonic association of hepatic and hematopoietic tissue and the precedent for "fetal" protein expression in many tissue malignancies suggests that the expression of CAMAL in myeloid leukemia cells, and its homology to HSA and AFP, may be more significant, in a developmental sense, than initially believed. If CAMAL is a genuine structural and functional homologue of HSA and AFP, as the data presented in this thesis would suggest, then the event(s) leading to the expression of CAMAL in human myeloid leukemia results in the

expression of an albumin or "albumin-like" element in human myeloid leukemia cells. The examination of fetal tissue of both endodermal and mesodermal origin with the anti-CAMAL antibodies provides a means for determining whether CAMAL might be expressed in non-malignant tissues of endodermal origin (liver) and expressed in tissues of mesodermal origin (hematopoietic cells) as a consequence of malignancy.

Immunoperoxidase studies with the p70 (CAMAL)-specific monoclonal antibody demonstrated reactivity with both adult and fetal kidney tissue (embryonic mesoderm). Reactivity was observed with kidney stromal elements (epithelial cells, follicular dendritic reticulum cells) and mononuclear phagocytes present in the kidney stroma. The monoclonal antibody was also shown to react with proximal and distal tubules as well as interstitial and endothelial cells of both fetal and adult kidney (Horton et al., 1985). The observed reactivity of the monoclonal antibody demonstrates that kidney tissue expresses a product that is antigenically related to p70 (CAMAL). Whether this product is made by kidney tissue or is secreted by the mononuclear phagocytes present in the kidney is not known. It would be interesting to determine whether p70 (CAMAL) is expressed by kidney tissue from a developmental standpoint, since both kidney and hematopoietic tissue arise from embryonic mesodermal elements (Balinsky, 1981). The expression of p70 (CAMAL) in normal kidney and hematopoietic tissue may indicate a common regulatory role for this protein in the development of both tissue types.

Reactivity of the monoclonal anti-CAMAL antibody was also observed with various non-hematopoietic tissues, namely thymus, lung,

liver, tonsils and kidney. Although the embryonic origin of the epithelial part of these tissues is different, embryonic endoderm for thymus, lung, liver, tonsils and mesoderm for kidney, all of these tissues support secondary investments of mesodermal elements (tissue stromal elements). The cross-reactivity demonstrated between these different tissues, with the monoclonal anti-CAMAL antibody, may be due more to the existence of common mesodermal structural elements in all these tissue types rather than the expression of an element(s) common to both embryonic endoderm and mesoderm (Horton et al., 1985).

It is possible that CAMAL may be a variant of HSA, however, in light of the tp20 sequence, it is unlikely that CAMAL is related to the many electrophoretic mobility and "point" variants (single amino acid substitutions) of HSA documented in the literature (Schell et al., 1977; U. Kragh-Hansen, 1981). Consequently, the only definitive manner by which to establish identity or non-identity of CAMAL to HSA, as with the HSA "point" variants, is by deriving the entire primary sequence of the CAMAL molecule. The purification of CAMAL from leukemic cell or cell line lysates by immuno-affinity chromatography has been demonstrated in this study. Although not a guarantee, the application of immunoaffinity chromatography to purification of CAMAL from these sources does provide the means to purify sufficient quantities of CAMAL for the derivation of the complete primary sequence of the CAMAL molecule.

The determination of the complete primary sequence of the CAMAL molecule will allow the synthesis of CAMAL-specific oligonucleotide probes and should facilitate the isolation of the gene encoding the CAMAL molecule. Further analysis of CAMAL expression

in leukemic and normal cells, using these probes, will provide more definitive data on the degree of CAMAL expression in leukemic versus normal cells, permit the definition of the sites of CAMAL synthesis within leukemic/normal cells and cell populations, allow the analysis of the "CAMAL gene" organization in leukemic/normal cells and, in concert with the *in vitro* growth inhibition studies of CAMAL on normal myeloid progenitor cells, aid in establishing a role for CAMAL in the maintenance of the leukemic state.

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