

**The Expression of Stem Cell Inhibitor / Macrophage Inflammatory
Protein-1 α and Macrophage Inflammatory Protein-1 β mRNA in Murine
Macrophages.**

by

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Abstract

The proliferation of haemopoietic stem cells in the bone marrow is believed to be controlled by competing interactions between positive and negative regulators.

The identification and characterisation of specific negative regulators has been the subject of much investigation and one of these, an activity termed the Manchester Inhibitor, was shown to be effective in inhibiting the proliferation of CFU-S cells. The presence of this activity in murine bone marrow was also demonstrated to correlate inversely with CFU-S proliferative status.

Purification of the active component of the Manchester Inhibitor showed it to be functionally and antigenically indistinct from Stem Cell Inhibitor / Macrophage Inflammatory Protein-1 α (SCI/MIP-1 α), a member of the MIP-1 family of cytokines. Another, closely related, member of this family, Macrophage Inflammatory Protein-1 β (MIP-1 β), is ineffective as an inhibitor of haemopoietic stem cell proliferation but both SCI/MIP-1 α and MIP-1 β have been shown to be effective in a number of inflammatory assays and MIP-1 β may be a specific antagonist of the action of SCI/MIP-1 α .

Understanding the signals and mechanisms controlling the expression of the genes for both SCI/MIP-1 α and MIP-1 β is of fundamental importance for gaining a clear picture of the role played by these proteins in the physiology of stem cell proliferation regulation and in the inflammatory response.

Previous studies with the Manchester Inhibitor had shown that this activity was a product of bone marrow macrophages and that the ability to detect the activity correlated with stem cell quiescence; however, only limited studies had been carried out on the factors regulating the production of this activity.

In this project, an in vitro culture system was set up to study the regulation of the expression of the genes for SCI/MIP-1 α and MIP-1 β . Macrophages cultured in vitro

from normal murine bone marrow (bone marrow-derived macrophages, BMDM) were employed as a surrogate for the SCI/MIP-1 α and MIP-1 β producer cells in the bone marrow. This cultured population has characteristic proliferative properties and was demonstrated to express mRNA for SCI/MIP-1 α and MIP-1 β and secrete SCI/MIP-1 α protein. Previous, limited, studies had observed the expression of SCI/MIP-1 α and MIP-1 β mRNA in macrophage-like or monocyte-like cell lines. This study represents the first study of the expression of these genes in untransformed, primary macrophages.

The accumulation of SCI/MIP-1 α and MIP-1 β mRNA was observed to be greatly increased by treatment of BMDM with bacterial endotoxin and this was investigated in more detail. Both SCI/MIP-1 α and MIP-1 β mRNAs were observed to be induced in a rapid and transient fashion, which was dependent on the early stimulation of transcription, in a manner suggestive of this being a part of the early phase of the response of BMDM to stimulation by endotoxin. The accumulation of both mRNAs was also demonstrated to be independent of *de novo* protein synthesis, which places the expression of SCI/MIP-1 α and MIP-1 β within the "immediate early" group of genes. Furthermore, the glucocorticoid hormone hydrocortisone was seen to be able to downregulate the accumulation of both mRNAs and oppose the induction of accumulation by endotoxin. These characteristics of expression are typical of those observed for cytokine genes.

The accumulation of mRNA for SCI/MIP-1 α and MIP-1 β was induced by treatment of BMDM with conditioned medium from the cell line L929, a potent source of the monocyte/macrophage lineage-specific growth factor CSF-1. This induction was again observed to be rapid and transient. Attempts to demonstrate increased accumulation of either of the MIP-1 mRNAs using recombinant human CSF-1 were unsuccessful, possibly due to the poor activity of the preparations used. The macrophage priming/activating factor IFN γ , however, was able to induce the

accumulation of both of the MIP-1 mRNAs, again in a transient manner, demonstrating that accumulation of both of these mRNAs could be induced by a cytokine as well as endotoxin.

Studies in serum-free medium demonstrated the accumulation of both SCI/MIP-1 α and MIP-1 β mRNA on refeeding in the absence of donor horse serum. Both SCI/MIP-1 α and MIP-1 β mRNA was again observed to accumulate with a rapid and transient pattern. Donor horse serum was seen to downregulate, and fetal calf serum to upregulate, levels of both mRNAs.

The expression of many genes with the characteristics of expression observed for SCI/MIP-1 α and MIP-1 β in this study has been postulated to be linked to the induction of cellular proliferation. The proliferative characteristics of the BMDM population employed in this study allowed this question to be addressed. While several culture conditions were identified where induction of accumulation of SCI/MIP-1 α and MIP-1 β mRNA precedes the induction of proliferation of BMDM, the level of expression of these mRNAs can also be increased in the absence of proliferation. This suggests that, while there may be some overlap in the signals mediating the proliferation of BMDM and the expression of SCI/MIP-1 α and MIP-1 β genes, these signals are not identical and the two processes can be regulated independently.

In order to investigate the role played by the expression of the SCI/MIP-1 α gene in haemopoiesis *in vivo*, studies were carried out on the accumulation of SCI/MIP-1 α mRNA in total murine bone marrow. A low level of SCI/MIP-1 α mRNA was detected in normal, unfractionated murine bone marrow in agreement with ability of other investigators to detect Manchester Inhibitor activity by washing normal bone marrow and consistent with expression of the SCI/MIP-1 α gene playing a role in marrow haemopoiesis.

On treatment of mice with 5-fluorouracil, a drug with well documented effects on the proliferation of haemopoietic stem cells, levels of SCI/MIP-1 α mRNA were observed to change with patterns not inconsistent with changes in SCI/MIP-1 α gene expression playing a role in the control of myeloid stem cell proliferation.

Dedication

This thesis is dedicated to my parents.

Declaration

I state that all of the work in this thesis was performed personally unless otherwise acknowledged.

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Abbreviations.

³² Phosphorous	³² P
³⁵ Sulphur	³⁵ S
5-fluorouracil	5-FU
Actinomycin D	ActD
Adenosine Triphosphate	ATP
AF1-19T Conditioned Medium	AF1CM
Base Pairs	bp
Bone Marrow-Derived Macrophages	BMDM
Bovine Serum Albumin	BSA
Colony Forming Cell	CFC
Colony Forming Unit-Assay	CFU-A
Colony Forming Unit-Spleen	CFU-S
Colony Stimulating Factor	CSF
Complementary DNA	cDNA
Counts Per Minute	cpm
Cycloheximide	CHX
Cytidine Triphosphate	CTP
Deoxyadenosine Triphosphate	dATP
Deoxycytidine Triphosphate	dCTP
Deoxyguanosine Triphosphate	dGTP
Deoxyribonucleic acid	DNA
Deoxythymidine Triphosphate	dTTP

Donor Horse Serum	DHS
Fetal Calf Serum	FCS
Guanosine Triphosphate	GTP
High Proliferative Potential	HPP
Human Macrophage Inflammatory Protein	hMIP
Hydrocortisone	HC
Interferon- γ	IFN γ
Interleukin	IL
Kilobases	Kb
Kilodaltons	KDa
L929 Conditioned Medium	L929CM
Lipopolysaccharide	LPS
Macrophage Inflammatory Protein	MIP
Messenger Ribonucleic Acid	mRNA
Microgram	μ g
Microlitre	μ l
Millilitre	ml
Nanogram	ng
Phenylhydrazine	PHZ
Polyacrylamide Gel Electrophoresis	PAGE
Recombinant Human CSF-1	rhCSF-1
Recombinant Murine IFN γ	rmIFN γ
Ribosomal Ribonucleic Acid	rRNA
Serum-free	SF

Sodium Dodecylsulphate	SDS
Spleen-Derived Macrophages	SDM
Stem Cell Inhibitor	SCI
Transforming Growth Factor β	TGF β
Tritiated Deoxythymidine	³ HTdR
Tumour Necrosis Factor	TNF
Untranslated Region	UTR
Uridine Triphosphate	UTP
WEHI 3B Conditioned Medium	WEHICM

Chapter 1

Introduction.

1.1 Composition and Regulation of the Haemopoietic System.

In the adult mammal, blood cells are continually being manufactured in the bone marrow and destroyed in the periphery. This is achieved through processes of differentiation and cell division involving vast numbers of cells. Humans, for example, produce in the order of 10^9 platelets, erythrocytes and neutrophils per hour (Golde 1991). The fact that, in the normal situation, the numbers of blood and developing haemopoietic cells are maintained within fairly narrow ranges and the relative rarity of neoplastic transformation in these cells indicates that haemopoiesis is an extremely effectively controlled process.

The starting point for all blood cell production is the pluripotential haemopoietic stem cell. All of the mature haemopoietic cells are derived from this cell by a series of differentiation and amplifying divisions.

Conventionally, the haemopoietic system is thought of as consisting of three interlinked compartments: the stem cell, progenitor (transient amplifying) cell and maturing end cell compartments. These compartments are believed to comprise 0.1-0.4%, less than 10% and greater than 90% of the total marrow population respectively (Lord and Testa 1988). It should be stressed, however, that these categories are not absolute and there may be considerable overlap between the compartments.

1.1.1 Maturing End Cells.

The haemopoietic system gives rise to at least eight mature cell types. These cell-types have widely divergent functions in the blood and tissues such as carrying

oxygen and playing roles in host-defence and the response to injury. The production of the various cell lineages from stem cells via progenitor cell stages is illustrated in figure 1.1.

1.1.2 Progenitor (Transit Amplifying) Compartment.

It is generally believed that stem cells give rise to precursor cells which in turn give rise to unipotent progenitor cells through a series of ill-defined differentiation steps. The development of in vitro clonogenic assays for bone marrow lead to the identification and description of the lineage-committed progenitor cells. These cells differ from stem cells in that they are committed to one, or a few, lineages and have a much reduced proliferative capacity. A greater percentage of this compartment is proliferating at any one time than the stem cell compartment which reflects the fact that these cells are irreversibly committed to becoming post-mitotic end cells (Metcalf 1984).

The identification of the various progenitor cells is inextricably linked to the discovery and purification of the colony-stimulating factors (CSFs), glycoprotein hormones which are absolutely required for the survival, proliferation and differentiation of the progenitor cells, at least in vitro (for reviews see Metcalf 1989, Morstyn and Burgess 1988, Zipori 1991).

The type of progenitor under investigation is defined by the morphology of the resultant colonies which in turn are dependent on the CSF used. Thus, granulocyte-colony stimulating factor (G-CSF) will give rise to colonies derived from bone marrow consisting predominantly of granulocytic cells. The cell from which the colony derives, for which colony formation constitutes a retrospective assay, is termed granulocyte-colony forming cell (G-CFC). Colony assays for all of the identified blood cell lineages have now been developed (Metcalf 1984).

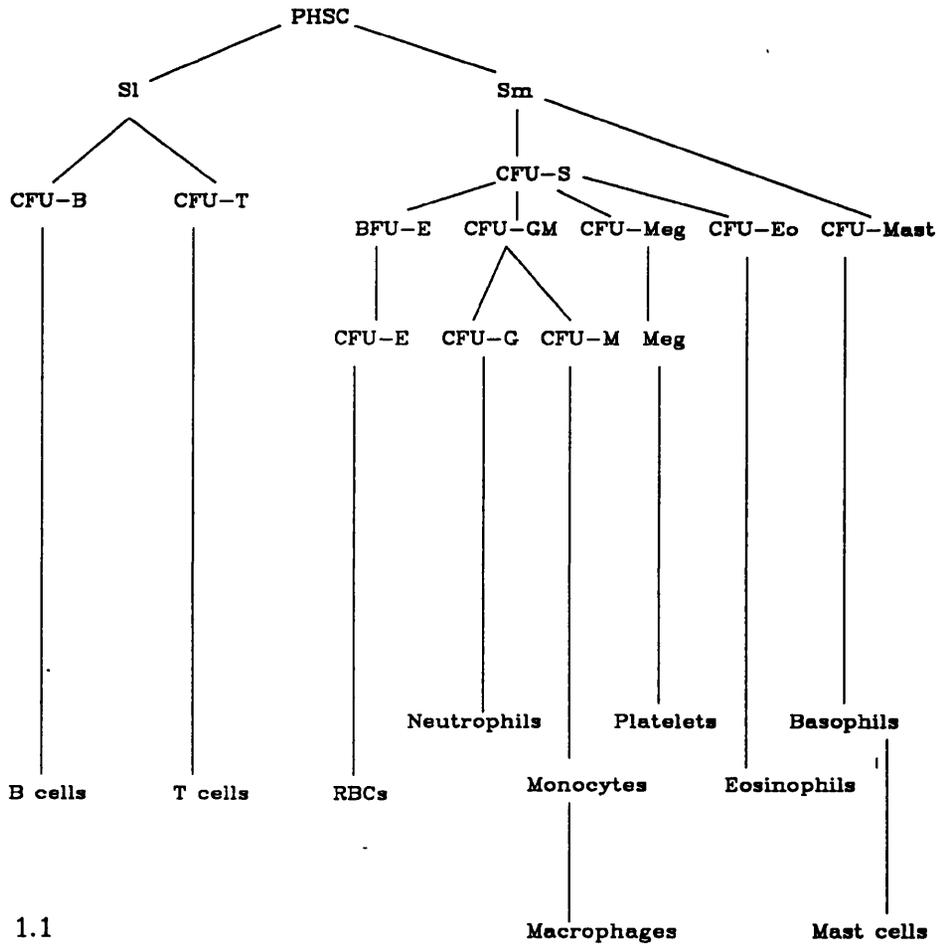


Figure 1.1

Figure 1.1 A model of the hierarchical organisation of the murine haemopoietic system.

The stem cells and progenitor cells reside predominantly in the marrow, whereas the morphologically recognisable cells (except megakaryocytes) are found in the peripheral blood. Macrophages and mast cells are found in tissues.

PHSC	pluripotential haemopoietic stem cell
SI	lymphoid-restricted stem cell
Sm	myeloid-restricted stem cell
BFU	burst forming unit
CFU	colony forming unit
Meg	megakaryocyte
RBC	red blood cell

That cell types approximating to the defined colony-forming cells exist in vivo, rather than the progeny of the in vitro colonies being derived purely by induction by the growth factor from an undifferentiated pool, was first demonstrated by the separation studies of Worton et al (1969). These studies have recently been extended in a series of publications by Weissman and colleagues (reviewed in Heimfeld et al 1992). Muller-Seiburg et al (1986) have demonstrated that most of the progenitor activity of the marrow is contained within a population which stains for the Thy-1 antigen with an intensity which is 10-fold lower than that on mature mouse T cells. This population can be further divided on the basis of lineage markers for all of the haemopoietic lineages. The lineage restricted capacity of these cells is demonstrated by in vivo reconstitution experiments (Muller-Seiburg et al 1986, Heimfeld et al 1990, 1991, Smith et al 1991). It should be noted, however, that some of these sorted subpopulations demonstrated a greater degree of developmental restriction in vitro than in vivo (Smith et al 1991), which suggests that there may be artificial limitations placed on the growth of haemopoietic progenitors in in vitro semi-solid media assays. This demonstrates a potential pitfall of all in vitro haemopoietic assays which should be borne in mind when interpreting data.

1.1.3 Stem Cell Compartment.

The distinguishing feature of haemopoietic stem cells is their ability to sustain a functional haemopoietic system in vivo over an extended period of time (Harrison et al 1988, Jordan and Lemischka 1990, Capel et al 1989). To achieve this the stem cells must be able to maintain a balance between two apparently antagonistic functions.

1) Firstly, the pluripotential stem cells must be able to undergo a process of differentiation which must be unique amongst the stem cells of the adult self-renewing tissues in that it ultimately gives rise to a large number of distinct cellular lineages of both the lymphoid and myeloid branches of the haemopoietic system.

Figure 1.1 shows the hierarchical organisation of the haemopoietic system, illustrating that the pluripotential haemopoietic stem cell (PHSC) precedes the major myeloid-lymphoid branch point and is therefore capable of repopulating both the lymphoid and myeloid branches of the haemopoietic system.

The existence of the PHSC has now been well established by several functional studies (Abramson 1977, Harrison 1980, Lemischka et al 1986, Lerner and Harrison 1990, Jordan and Lemischka 1990, Szilvassy et al 1990).

2) Secondly, a quota of potentially active stem cells sufficient to enable the constant repopulation of all of the haemopoietic lineages must be maintained. This could be achieved in two basic ways.

A) Self-renewal, by which stem cells divide to replicate themselves, has been widely postulated to account for the maintenance of the stem cell compartment of the marrow (Siminovitch et al 1964, Sprangude et al 1988, Keller 1992, Harrison 1992) but has never been formally proven to exist. However, self-renewal of haemopoietic stem cells can be inferred from the ability of individual retrovirally marked stem cell clones to give rise to mature end cells over an extended period (Lemischka et al 1986, Jordan and Lemischka 1990).

B) Alternatively, it is possible to envisage that haemopoiesis might be maintained by the successive activation of a reserve of primitive cells which are sufficient in number to maintain haemopoiesis for several life-spans, in a way directly analogous to the female reproductive system (Kay 1965). Mintz et al (1984) have reported sequential activation of uniquely retrovirally marked stem cell clones which would support such a model; however, these data may be representative of repopulating marrow rather than steady-state haemopoiesis.

Regardless of which mechanism is responsible for the maintenance of a functional stem cell compartment, it is generally accepted that proliferation in the stem cell compartment is minimal relative to the rest of the haemopoietic system, as assessed

by the ability of cells of the stem cell compartment to resist the lethal effects of cycle-specific agents both in vivo (Lerner and Harrison 1990) and in vitro (Shibagaki et al 1986). This could be achieved in two ways. First, as is conventionally considered to be the case, only a small fraction of the stem cell compartment could be proliferating with the majority existing in a quiescent state, making no contribution to haemopoiesis. The demonstration by several investigators of pluripotential haemopoietic reconstitution by a small number of individually retrovirally marked stem cell clones (Keller et al 1985, Capel et al 1989, Jordan and Lemischka 1990) and the ability of the majority of assayed primitive myeloid progenitor cells from unstressed mice and humans to resist the lethal effects of cycle specific drugs in vitro (Shibagaki et al 1986, Cashman et al 1990) would support this idea.

On the other hand, Harrison et al (1988) have presented evidence, using mathematical analysis of a long-term repopulation assay (Harrison 1980) that large numbers of PHSCs are continually, albeit extremely slowly, proliferating; the low proliferation would presumably account for the resistance of most of the PHSCs to the action cycle-specific cytotoxic drugs. It is a corollary of this that multiple stem cell clones would be contributing to haemopoiesis in an individual.

It is probable that changes in the proliferative rate of haemopoietic stem cells makes little or no contribution to haemopoiesis in the unstressed animal, with most of the day to day regulation of blood-cell formation taking place at the progenitor cell level. However, due to the primary importance of the stem cell in blood cell development much attention has been focussed on understanding the basis for the control of stem cell proliferation.

It is clear from the studies of Lerner and Harrison (1990) and Harrison and Lerner (1991) that the proliferative rate of PHSCs can be reversibly increased during recovery from marrow ablation; however, investigation of biochemical, or otherwise, signals controlling the proliferation of stem cells is impossible using long-term

reconstitution assays. Therefore, studies aimed at understanding the control of stem cell proliferation and development have utilised assays which detect primitive, multi-lineage haemopoietic progenitors with a large proliferative capacity, the relationship of which to the PHSC is not always certain.

1.1.4 Actions of the Colony Stimulating Factors and Cytokines.

Committed haemopoietic progenitor cells, which have been immobilised *in vitro* in semi-solid growth medium can be stimulated to divide and multiply by specific growth factors, in which case they will form colonies consisting mainly of morphologically recognisable mature haemopoietic cells (Metcalf 1984). Growth factors (termed colony-stimulating factors, CSFs) able to promote the growth of colonies from bone marrow containing all of the major blood cell lineages have been identified using this technique.

The activities of the CSFs include promotion of cell survival, induction of proliferation coupled with differentiation and potentiation of the functions of mature haemopoietic cells (for reviews see Morstyn and Burgess 1988 and Metcalf 1989). Some of the CSFs are relatively restricted in their target cell populations, for example colony stimulating factor 1 (CSF-1) also known as macrophage-CSF (M-CSF), granulocyte-CSF (G-CSF) and erythropoietin (epo) are restricted to the production of colonies consisting of mature macrophages, granulocytes and erythroid cells, respectively (Metcalf 1989). Similarly, IL-5 is a specific CSF for the production of eosinophils (Sanderson et al 1985) and IL-2 specifically promotes the development of cells of the T and B lymphoid lineages (Smith 1988).

On the other hand, other CSFs appear to have a wider range of activities as judged by their ability to promote the formation of colonies containing cells from two or more lineages. For example, granulocyte-macrophage CSF (GM-CSF) and IL-3 (or multi-CSF) have a much broader target cell population, including cells of the myeloid and

megakaryocytic lineages, early erythroid cells and primitive myeloid progenitors (Morstyn and Burgess 1988). In some cases these growth factors are also able to exhibit strong synergy when acting together on a responding cell. These synergistic actions are likely to be important in vivo as most cells that produce cytokines secrete a mixture of these (Morstyn and Burgess 1988) and because most haemopoietic cells have receptors for more than one cytokine (Nicola 1989).

Receptors for several of the CSFs have now been described. The CSF-1 receptor is a member of the tyrosine kinase family of receptors. Receptors for the other CSFs described to date appear to be structurally related to each other and may mediate their effects through additional membrane proteins which bind the growth factor-receptor complex and also increase the apparent binding affinity of the growth factor (for review see Nicola and Metcalf 1991).

1.1.5 Stem cell assays.

Colony Forming Unit-Spleen (CFU-S).

Much of the work aimed at understanding the physiological control of stem cell behaviour has utilised the Colony Forming Unit-Spleen (CFU-S) assay of Till and McCulloch (1961). This is a clonal assay (Becker et al 1963) which measures the ability of a class of primitive bone marrow progenitor cells to seed in the spleen of a lethally irradiated mouse and form macroscopic colonies. The colonies appear on the spleen from around day seven after transplantation onwards and can contain erythroid cells, granulocytes and platelets as well as cells with the ability to give rise to colonies in secondary recipients (Becker 1963). The spleen colony founding cell (CFU-S) therefore has the ability to give rise to myeloid lineages and also to display self-renewal; this lead to the assumption that the CFU-S represented the pluripotent haemopoietic stem cell (Siminovitch et al 1963, Sprangude et al 1988).

Subsequent studies revealed considerable heterogeneity in the CFU-S population with regard to self-renewal ability, (Siminovitch et al 1963) multipotentiality (Curry and Trentin 1967) and proliferative status (Baines and Visser 1983, Shibagaki et al 1986). CFU-S displaying more and less primitive characteristics can be partially distinguished according to their time of appearance on the spleen of the recipient animal. Colonies appearing at day 12 after transplantation are generally more primitive than those appearing on day 8, although this distinction is not absolute as many day 8 colonies remain on the spleen through to day 12 (Wolf and Priestley 1986). The day 12 CFU-S are believed to be derived from a more primitive cell (pre-CFU-S) which resides in the marrow immediately after transplantation (Hodgson and Bradley 1979).

It is now also apparent that the cell responsible for the long-term engraftment of transplanted marrow (which is functionally analogous to the PHSC) is not contained within the CFU-S compartment. Jones et al (1990) and Sprangude and Johnson (1990) have demonstrated that the two activities can be physically separated. Given that no study has demonstrated repopulation of the lymphoid compartment by CFU-S (Abramson et al 1977) it is more likely that CFU-S represents a myeloid stem cell (or primitive myeloid progenitor if adhering to a strict definition of the stem cell) rather than the PHSC.

In Vitro Stem Cell Assays.

Attempts have also been made to assay for cells with the properties of stem cells by in vitro colony assay in semi-solid agar. For a haemopoietic progenitor cell assayed in vitro to be designated as having stem cell-like properties it must display at least some of the following properties: 1) Low frequency in unfractionated bone marrow 2) Large colony size, denoting a large proliferative potential. 3) The ability to give rise to multiple lineages. 4) Low proliferative activity. 5) Delayed onset of growth. 6) The

capacity of the primary colonies to give rise to secondary progeny. These properties are displayed to varying extents by the cells detected in the assays described below which illustrates the heterogeneous nature of the stem cell compartment.

High Proliferative Potential Colony Forming Cells (HPP-CFC).

HPP-CFC are amongst the most primitive haemopoietic cells identified in vitro. They have a fairly low frequency (approximately 250 per 10^5 normal bone marrow cells) and are extremely quiescent in normal marrow (Hodgson and Bradley 1984). In addition to this, they are multipotential and are able to give rise to transplantable and in vitro progenitors of various lineages (McNiece et al 1987).

The growth of HPP-CFC colonies is absolutely dependent on the presence of multiple cytokines acting in synergy, and the particular combinations used, in addition to differential sensitivity to the cytotoxic effects of 5-fluorouracil (5-FU), have allowed HPP-CFC to be divided into a least two groups according to their degree of primitiveness.

The 40% of HPP-CFC which survive 5-FU treatment can only be detected by the combined action of IL-1 α plus IL-3 plus CSF-1 (Bartlemez 1989). HPP-CFC with more restricted requirements for growth, displaying properties of less primitive cells reappear sequentially in the marrow in the regeneration period following 5-FU treatment (reviewed in Bertoncello 1992).

The resistance to 5-FU of HPP-CFC responsive to IL-1 α plus IL-3 plus CSF-1 indicates that these cells are probably more primitive than CFU-S whose numbers are ablated by about 98% within 24 hours by this treatment (Hodgson and Bradley 1979, Van Zant 1984, Lorimore et al 1990). In addition to this there is some evidence correlating 5-FU-resistant HPP-CFC and bone marrow populations capable of conferring long-term haemopoietic reconstitution in vivo, though this data is not conclusive (reviewed in Bertoncello 1992). Taken together, the data indicate that

HPP-CFC are probably the in vitro representatives of a fairly primitive component of the myeloid stem cell compartment.

Blast-Colony Forming Cells (Blast-CFC).

Nakahata and Ogawa (1982) have described a class of haemopoietic progenitor found in colonies of 40-1000 cells sixteen days after incubation of bone marrow in semi-solid medium in the presence of pokeweed mitogen spleen conditioned medium (PWMSCM), which is known to be a source of at least M-CSF, IL-3 and GM-CSF (Broxmeyer 1990). These cells display no signs of terminal differentiation (hence the designation blast-CFC). On replating, the colonies produced secondary multi-lineage colonies with some displaying 100% replating efficiency. Leary et al (1989) observed that blast-CFC may remain quiescent as single cells for more than two weeks of culture in the absence of exogenously added growth factors. The use of neutralising antibodies to IL-1 α , IL-3, IL-6 and G-CSF indicated that dormancy of blast-CFC is maintained independently of these factors. The frequency of blast-CFC (4 per 10⁵ normal marrow cells) and the properties described above suggest that these cells may be equivalent to a pre-CFU-S cell. This question may be resolved by in vivo repopulation studies.

Colony Forming Unit-Assay (CFU-A).

When normal unfractionated bone marrow is incubated in semi-solid medium at a low density (2.5.10³/ml) in the presence of medium conditioned by the cell lines L929 and AF1-19T (Franz et al 1985) a number of colony-types are found to be present after 11 days, including colonies of diameters of greater than 2mm, derived from a cell now designated CFU-A (Pragnell et al 1988, Eckmann et al 1989). On morphological examination, the CFU-A-derived colonies can contain cells from

several haemopoietic lineages, including macrophages, granulocytes and megakaryocytes (Eckmann et al 1989).

Several properties of the CFU-A cell, including its low proliferative status, incidence in bone marrow (150-200 per 10^5), buoyant density, recovery after 5-FU treatment in vivo, radial distribution in the femur and response to ionising radiation indicate overlap between CFU-A and CFU-S but not GM-CFC populations (Eckmann et al 1989, Lorimore et al 1990). In addition to this Pragnell et al (1988) report recovery of CFU-S cells from CFU-A-derived colonies.

The two conditioned media used in this assay are known to contain GM-CSF and M-CSF, however it is unlikely that stimulation of CFU-A growth can be achieved by these factors alone as the colonies stimulated by rGM-CSF and rM-CSF, although macroscopic (Pragnell et al 1988, McNiece et al 1988) do not display the same capacity for multipotentiality (I.Pragnell personal communication.); it is likely, therefore, that additional factors, probably contained in AF1-19T conditioned medium are contributing to the development of colonies from CFU-A. The AF1-19T line does not express mRNA for IL-3 (Pragnell et al 1988); however, Kriegler et al (1990) have reported evidence that these cells produce IL-1 α and an IL-6-like activity, so it is possible that these factors may be contributing to the assay. Pragnell et al (1988), on the other hand, found that a neutralising antibody against IL-1 α had no effect on CFU-A colony formation. Pragnell et al (personal communication) have recently demonstrated that addition of stem cell factor (SCF) in the CFU-A assay along with GM-CSF and M-CSF allows the detection of colonies with very similar appearance to those detected using conditioned media, so it is possible that SCF is an important regulator of CFU-A proliferation.

1.1.6 Positive Regulation of Haemopoietic Stem Cell Proliferation.

It is now a generally accepted hypothesis that the proliferation of haemopoietic cells is determined by a balance between positive and negative regulators produced by the stroma.

The importance of the stromal microenvironment in both the maintenance and regulation of stem cell proliferation is illustrated by the (Steel) Sl and W (white spotting) anaemic mice. These two mutants have similar phenotypic abnormalities, including deficiency of the activity of very primitive haemopoietic stem cells (Russell 1979). Transplantation and studies with long-term bone marrow cultures showed that the W defect is intrinsic to the stem cells themselves while the Sl deficiency is a function of the haemopoietic microenvironment (Russell et al 1979, Dexter and Moore 1977).

The gene products of the W and Sl loci have now been identified. The W locus product codes for the c-kit proto-oncogene, a tyrosine kinase receptor (Chabot et al 1988, Geissler et al 1988) which is structurally related to the receptors for CSF-1 and platelet-derived growth factor (PDGF) (Qui et al 1988).

The Sl locus encodes the ligand for the c-kit receptor. Several groups have isolated cDNAs for this growth factor (Williams et al 1990, Copeland et al 1990, Flanagan and Leder 1990, Zsebo et al 1990a, 1990b, Martin et al 1990, Huang et al 1990, Anderson et al 1990) called variously, mast cell growth factor (MGF), stem cell factor (SCF) or kit ligand (KL) which predicts a protein product with a signal peptide and intracellular, transmembrane and extracellular domains. Both the soluble and transmembrane forms of SCF are biologically active (Anderson et al 1990).

The biological effects of deficiency in SCF activity are a powerful demonstration of a role for this molecule in stem cell biology. Other factors which have been proposed to have a positive effect on stem cells have been identified by virtue of their effects in

the in vitro assays for primitive progenitors discussed above. This means that their effects on stem cells in vivo can only be inferred rather demonstrated.

One general feature which appears to be emerging from the in vitro studies is that colonies derived from very primitive stem cells are only observed upon stimulation with multiple cytokines as illustrated by the dependence of both CFU-A and the more primitive HPP-CFC on at least three different growth factors (Pragnell et al 1988, Bradley and Hodgson 1979, Bertoncello et al 1992) for colony formation.

Two types of activity appear to be necessary for the maximal colony formation by very primitive progenitors and these can be illustrated by considering the growth factor combinations necessary for HPP-CFC colony development.

SCF and IL-1 on their own have a minimal effect, if any, on the proliferation of purified or enriched primitive cells (De Vries et al 1991, Bartlemez and Stanley 1985). However, either of these factors, in conjunction with a later acting lineage-restricted cytokine (such as CSF-1 or G-CSF) can stimulate the proliferation of HPP-CFC colonies (Zsebo et al 1990a, McNiece et al 1989, Moore and Warren 1987), seemingly expanding the range of progenitor cells able to respond to the CSF in the primitive direction. Therefore, it can be seen that molecules conferring ability to respond to colony stimulating factors in addition to the CSFs themselves are essential for the proliferation of the most primitive cells of the haemopoietic system.

Bartlemez and Stanley (1985) have demonstrated the ability of IL-1 to increase the number of receptors for CSF-1 in cultures of bone marrow enriched for primitive cells. This is one possible mechanism by which SCF and IL-1 exert their effects on stem cell proliferation.

IL-3 and GM-CSF, as single agents, have been shown to promote the growth of a subpopulation of blast-CFC after a lag of several days (Koike et al 1988). This may be taken as evidence that single growth factors are able to induce the proliferation of stem cells; however, the primitive status of blast-CFC is a matter of controversy and

further studies await the resolution of this point. What is clear, however, is that a number of growth factors, which are ineffective on their own, including IL-1, IL-6, G-CSF (Ikebuchi et al 1987) IL-4 (Kishi et al 1989), IL-11 (Musashi et al 1990), LIF (Leary et al 1990) and SCF (Williams et al 1991) have been demonstrated to hasten the entry of quiescent blast-CFC cells into active cell cycle.

These data therefore, reinforce the idea that the proliferation of primitive haemopoietic progenitors with stem-like properties are regulated by combinations of growth factors.

1.1.7 The Role of Bone Marrow Stroma in Haemopoiesis.

In the marrow, the haemopoietic cells are associated with stromal tissue, a network of cells and extracellular matrix which not only physically supports the haemopoietic cells but is also believed to influence their differentiation through the creation of an inductive microenvironment. The bone marrow stroma contains several types of adherent cells including fibroblasts, reticular adventitial cells and macrophages (for reviews see Owen 1988, Dexter et al 1990, Greenberger 1991, Quesenberry 1992). Much of what is known about the interactions between stromal and haemopoietic cells is derived from studies involving the long-term bone marrow culture (LTBMC) system. If normal murine bone marrow is cultured *in vitro* under appropriate conditions, an adherent layer is formed on the surface of the culture dish which can subsequently support active haemopoiesis, in the absence of exogenously added cytokines, including the maintenance of CFU-S and the proliferation and development of progenitor cells able to produce erythrocytes, neutrophils, macrophages, eosinophils, megakaryocytes, mast cells for prolonged periods (Dexter et al 1977, Schofield and Dexter 1985).

The presence of the stromal layer is absolutely required for the maintenance of haemopoiesis over extended periods, from which it is inferred that the growth-

promoting, differentiation-inducing and regulatory activities necessary for haemopoiesis are produced by these cells. However, the mechanism(s) by which this is effected, especially the nature of the role played by cytokines, if any, is still a matter of some controversy.

The results of attempts to demonstrate the production of cytokines by the stromal cells of LT BMC have been highly variable.

Several groups investigating the expression of either bioactivity or mRNA expression of the CSFs by bone marrow stromal cells have only detected constitutive production of CSF-1 (M-CSF) (Fibbe et al 1988, Sieff et al 1988, Piersma et al 1984, Schaafsma et al 1989).

The expression of protein and/or mRNA for several cytokines, such as IL-6, GM-CSF, G-CSF and IL-7 by LT BMC stroma (Gualtieri et al 1984, Quesenberry et al 1990) and of mRNA for the positive regulators IL-7, IL-6, G-CSF and the negative regulators TGF β and TNF α in specific cloned stromal cell lines (Gimble et al 1989) is clearly demonstrable; however these factors are only readily detectable after stimulation.

Dexter et al (1990) and Zipori (1991) have argued that the available information does not support a role for the myeloid colony-stimulating factors in the stroma-dependent development of haemopoietic cells in LT BMC. However, it is possible that stromal cells which have not been exposed to strong inducing signals are capable of producing levels of cytokine mRNA and protein below the level of detection by conventional techniques.

Kittler et al (1992) have recently reported the detection of mRNA for IL-3 in LT BMC stroma by the highly sensitive polymerase chain reaction (PCR) technique. The ability of LT BMC stroma (depleted of haemopoietic cells by irradiation) to support the growth of factor-dependent haemopoietic cell lines and the abolition of this support by antibodies specifically directed against IL-3 or GM-CSF demonstrates that

these CSFs can be produced by LT BMC stroma and is strongly indicative of a role for these factors in the maintenance of haemopoiesis in this system.

Interestingly, Gualtieri et al (1984) demonstrated, using an *in situ* detection method which allows detection of very low levels of colony stimulating activity, an inverse correlation between colony-stimulating activity for granulocyte/macrophage colonies and haemopoietic activity in LT BMC. One interpretation of this data is that CSFs are being utilised or degraded by haemopoietic cells, which would account for the inability of many investigators to detect cytokine activity in haemopoietically active LT BMC.

An additional issue in this argument is whether the CSFs, even if they are produced by haemopoietic stroma are actually relevant to stroma-dependent haemopoiesis.

The role of SCF in the stromal control of stem cell proliferation has been discussed above (section 1.1.6).

Yoshida et al (1990) have demonstrated that the approximately 50% reduction in the numbers of macrophages and severe reduction in the number of osteoclasts in the *op/op* strain of mice is due to a failure to produce CSF-1. The fact that the *op/op* mutation can not be corrected by transfer of normal haemopoietic cells (Yoshida et al 1990) suggests that this is a stromal defect. It seems, therefore, that CSF-1 has a definite role to play in the maintenance of haemopoiesis.

The fact that some CSFs associated with stroma are found only in barely detectable amounts may mean that haemopoietic cells are only ever exposed to suboptimal concentrations of these factors; again throwing some doubt on the predominance of the CSFs as the determining factor in the differentiation and development of these cells. However, the context in which the CSF is presented to the growth factor may greatly influence the effective concentration of the CSF at the cell surface.

Gordon et al (1987) and Roberts et al (1988) have demonstrated that molecules of the extracellular matrix (in particular glycosaminoglycans) are capable of binding GM-

CSF and IL-3 in a biologically active form. Given that direct contact (Dexter et al 1977) or at least extremely close association (Verfaillie 1992) between haemopoietic cells and stroma is necessary for active haemopoiesis in LTBM, the concentration of even a small number of growth factor molecules to a discrete site on the stroma in this way might constitute an effective method way of directing haemopoietic development with a relatively small number of CSF molecules and of confining CSF-directed haemopoiesis to particular discrete sites on the stroma.

Interestingly, Rathjen (1990) have shown LIF can be secreted in two forms by embryonic fibroblasts, one which is soluble and another which is immobilised by being bound by molecules of the extracellular matrix by virtue of having a portion of the N-terminal end of the molecule encoded by an alternative first exon. It will be interesting to see whether this is a general feature of the expression of cytokines by stromal cells.

Basic fibroblast growth factor (bFGF), which has potent mitogenic activity in several cell types has been shown to bind extracellular matrix molecules, in particular the glycosaminoglycan heparin. Yayon et al (1991) have demonstrated that binding of bFGF to heparin is a prerequisite for binding to its specific high-affinity receptor. This represents yet another way of ensuring that the activities of growth factors is context-dependent and it is possible that such a mechanism could also be involved in controlling the action of cytokines in marrow haemopoiesis.

Another way in which cytokine activity can be localised to the surface is to have the cytokine expressed as an integral membrane protein rather than as a soluble molecule. Anderson et al (1990) have demonstrated that SCF exists in both soluble and membrane-bound forms in fibroblasts. Furthermore, Flanagan et al (1991) have demonstrated the defective haemopoiesis seen in the Dickie strain of steel (Sl^d) to be associated with the failure to express the membrane-bound form of SCF, indicating the potential importance of membrane-bound forms of cytokines.

It is also important to note that the maximal activity of many colony stimulating factors can be achieved by as little as 10% receptor occupancy (Nicola 1989) which is also consistent with extremely low levels of cytokines being biologically relevant.

1.1.8 Two Types of Haemopoiesis.

While the role of the CSFs and cytokines in normal steady-state haemopoiesis is still a matter of debate, the importance of these molecules in many processes involved in immune defence and tissue remodelling is now well established.

Cytokines are produced on activation of monocytes and macrophages, T and B lymphocytes and mast cells (Arai et al 1990, Gordon et al 1990). Many of these are induced as part of the early programme of these cells in response to stimulation by non-specific inflammatory signals (monocytes/macrophages) or as part of an antigen-specific activation programme (T and B cells and mast cells). The mechanisms underlying the expression of cytokine genes are discussed in section 1.4.

In addition to their effects on haemopoietic progenitor cells, cytokines are able to alter the behaviour of mature, functional haemopoietic cells. For example, GM-CSF has effects on both mature neutrophils and macrophages which include the maintenance of survival and induction of proliferation in vitro (as with haemopoietic progenitors) (Vairo and Hamilton 1991). In addition to this GM-CSF has also been demonstrated to enhance the capacity of neutrophils (Weisbart et al 1985, Vadas et al 1985) and macrophages (Hamilton et al 1988) to undergo oxidative burst. Adherence and chemotaxis of neutrophils and macrophages can be enhanced by the action of several cytokines. For example, it is now well established that IL-8 is a chemoattractant for neutrophils (for review see Schall 1991) and MCP-1 (monocyte chemoattractant protein-1) appears to be a specific chemoattractant for monocytes (for review see Rollins 1991). Both of these cytokines can be produced by a variety of cells such as stimulated endothelial cells and activated monocytes.

These latter effects are strongly indicative of a role for cytokines in the recruitment of leukocytes to sites of infection in injury. It is possible that the numbers of immune effector cells recruited to these sites could be augmented by the clonal expansion of blood-borne haemopoietic progenitors. Given that marrow haemopoiesis is exclusively associated with stromal cells, it is possible that the activities of cytokines uncovered by semi-solid clonal agar assays are more analogous to the action of soluble cytokines in the blood-stream. Indeed, Arai et al (1990) and Zipori (1990) have argued that the main role for the CSFs is as directors of "emergency" haemopoiesis associated with immune defence. Obviously, such a process would have to be well-regulated with mechanisms responsible for limiting the extent of the response both spatially and temporally.

1.2 Negative Regulation of Stem Cell Proliferation.

It has long been postulated that the size of a cellular population in a developing tissue could be controlled by regulating the production of mature cells from their progenitors. Specifically, it has been postulated that the production of cells in a biological system needs to be limited by a feedback message(s) which is proportional to the size of the resultant population (Lord 1988).

There are basically two ways in which such a negative feedback loop could be organised. Firstly, a negative regulator of producer cell activity could be produced by the resulting cells as illustrated in figure 1.2. In this way the level of negative regulator produced is proportional to the size of the mature population; hence, this is a self-regulating system, as fluctuations in mature cell numbers are directly compensated for by changes in the activity of the progenitor populations.

An example of such a system might be the regulation of megakaryopoiesis in the bone marrow by platelet factor 4 (PF4). PF4 is a multifunctional protein specifically

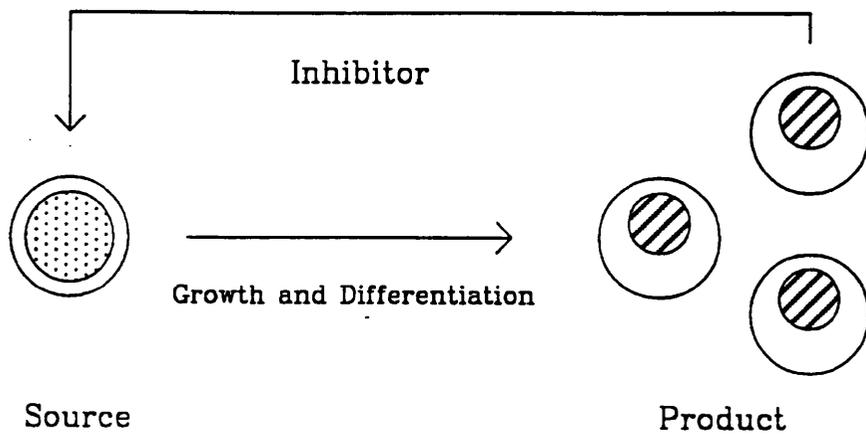


Figure 1.2

Figure 1.2 A model for the organisation of a simple feedback loop.

The source cells give rise, through a series of growth and differentiation events, to the product cells, which produce an inhibitor which feeds back to prevent the proliferation of the producer cell.

synthesised in megakaryocytes and stored and released by platelets which appears to play a role in the response to wounding. However, several investigators have demonstrated dose-dependent suppression of megakaryocyte progenitor maturation by platelet factor 4 in vivo and in vitro (Han et al 1991b). It is possible, therefore, that the production of megakaryocytes (and hence platelets) could be self-regulating via a system of simple negative feedback (for review see McDonald 1989).

Alternatively, it is possible to construct a model for negative feedback, whereby, the negative regulator is produced by an intermediary cell, the numbers of which do not fluctuate. The production of the negative regulator by the intermediary cell is dependent on signals received from the product cells. This system is more flexible than a simple negative feedback loop as it allows for the possibility of the integration of several types of signals, including stimulatory ones. It is likely in such situations that the production of the negative regulator would be controlled by signals from a number of sources.

It has been widely postulated that the proliferation of haemopoietic stem cells might be controlled, at least in part, by the modulation of production of negative feedback regulators.

Pluripotential haemopoietic stem cells (PHSC), as defined by the studies of Harrison and his co-workers appear to greatly increase their proliferative rate during the regeneration of marrow after administration of the cycle-specific drug 5-fluorouracil and return to quiescence within about five days (Harrison and Lerner 1991). It has been repeatedly demonstrated that the proliferation of CFU-S during marrow regeneration is induced and curtailed in a predictable time-frame (Siminovich et al 1963, Shibagaki et al 1986). These observations indicate that the control of proliferation of the haemopoietic stem cell compartment is under both positive and negative controls.

As indicated above, the level of a negative regulator should relate directly to the state of the haemopoietic tissue, but might also be regulated by other factors such as the relative level of positive regulators of stem cell proliferation. Understanding the signals and mechanisms which regulate the production of negative (and positive) regulators is essential for a full appreciation of the molecular basis of haemopoietic stem cell proliferation.

The stimulation of haemopoietic stem cell proliferation and differentiation was dealt with in section 1.1. This section will primarily deal with negative regulation.

Two seminal observations, in the 1970s, on the proliferation of murine CFU-S gave rise to the idea that the proliferation of these cells might be subject to control by specific, locally produced regulators. Gidali and Lajtha (1972) demonstrated that the rapid proliferation of marrow CFU-S induced by irradiation of mice could be prevented in a single limb protected from irradiation by a lead shield; indicating that control was exercised locally. Similarly, Rencricca et al (1970) observed a similar imbalance in the proliferation of CFU-S in the marrow and those which had migrated to the spleen after administration of the anaemia-inducing drug phenylhydrazine. It was widely postulated at the time that these effects might be due to differential production of CFU-S-specific proliferation inhibitors, whose production would in turn be directly related to the proliferative state of the CFU-S compartment.

Several specific regulators of stem cell proliferation have now been described.

1.2.1 The Manchester Inhibitor.

The first specific inhibitor of CFU-S proliferation was described by Lord et al (1976) and subsequently became known as the Manchester Inhibitor after the city in which the work was carried out. This inhibitor is prepared by washing fresh, normal bone marrow (in which CFU-S proliferation is minimal) and obtaining a conditioned medium. Inhibitory activity is measured by the ability of the conditioned medium to

block the entry of cycling CFU-S into the S-phase of the cell cycle as assayed by the ability to prevent the killing of CFU-S by cytotoxic drugs in vitro. The inhibitory material was not elaborated by regenerating bone marrow (in which CFU-S are proliferating rapidly); however a stimulatory, activity which could recruit quiescent CFU-S into S-phase, was demonstrated to be produced by this material (Lord et al 1977).

Molecular ultrafiltration of the conditioned media consigned the inhibitory and stimulatory activities to separate molecular weight fractions; the inhibitory activity to between 50 and 100kDa (fraction IV) and the stimulatory material to between 30 and 50kDa (fraction III).

The inhibitor and stimulator display specificities of action which centre on the CFU-S compartment and which are reversible and non-cytotoxic. The exact cellular populations affected by these two molecules overlap but are not identical.

Tejero et al (1984) demonstrated that the action of the inhibitor was confined solely to the CFU-S population as the proliferative behaviour of committed progenitor cells was unaffected. BFU-E, GM-CFC and CFC-mix were unaffected. The stimulator increased the proportion of both CFU-S and CFC-mix in DNA synthesis but did not affect BFU-E or GM-CFC.

In addition, Wright et al (1985) have demonstrated differential sensitivity of components of the CFU-S compartment to actions of the stimulator and inhibitor. In general, more primitive cells (represented by CFU-S day 12) are much more sensitive to inhibitor than the stimulator, while the opposite is true for the more mature CFU-S day 8 cells.

The actions of both the inhibitor and stimulator on CFU-S are reversible. This reversal, however, requires the presence of the opposing factor as opposed to merely the absence of the original factor (Lord et al 1977). This indicates that the movement

of CFU-S between quiescent and proliferative states is controlled by two separate mechanisms, rather than being dependent on one signal.

Very little is known about the mechanism of action of the inhibitor or stimulator; however, elegant studies by Lord et al (1979) suggest that the inhibitor acts by holding CFU-S in the G₀ phase of the cell cycle close to the G₁/S-phase transition point.

Knowledge of the cellular source of the inhibitor and stimulator is obviously important for a full picture of the biology of CFU-S proliferation and is essential if the mechanisms regulating the production of this molecule are to be investigated. A great deal of work to this end was carried out between 1979 and 1982 on fractionated cell populations of murine bone marrow and spleen by Wright and others.

Density separation of whole bone marrow on linear gradients of bovine serum albumin indicated that neither the inhibitor-producing nor stimulator-producing cells were likely to be the CFU-S cells themselves (Wright and Lord 1979, Wright et al 1980a, Wright et al 1982). This was an important finding as it is possible to envisage models where the stem cell compartment could be completely self-regulating.

The source of the inhibitory activity was shown to be confined to the adherent fraction of the bone marrow as removal of adherent cells of the bone marrow resulted in a loss of inhibitory activity. The inhibitory activity was also absent if bone marrow cells which had phagocytosed carbonyl iron were removed magnetically or if cells positive for the Fc receptor were removed by "panning" with immobilised immunoglobulin. Density separation showed that the inhibitor producing cells were confined to the fractions of density 1.052-1.062 g/ml (Wright et al 1980a, Lord and Wright 1982).

A similar series of experiments was carried out in an attempt to identify the cellular source of the stimulatory activity in regenerating bone marrow (Wright et al 1982).

The results obtained were exactly the same except that the stimulator producing cells were contained in a slightly heavier density fraction (1.064-1.072 g/ml).

From these studies it would seem that the inhibitory and stimulatory activities could be consigned to the same cell-type, which from the diagnostic tests seemed most likely to be the macrophage, but to different subpopulations of this cell-type.

Furthermore, Wright and Lord (1979) had shown using density fractionation that stimulatory material could be obtained on culturing cells of density 1.064-1.072 g/ml and that inhibitory material could be obtained on culturing cells of density 1.052-1.060 g/ml irrespective of the cycling status of the CFU-S in the source marrow.

However, there was no evidence for the simultaneous presence of both factors; indeed, fraction IV from regenerating marrow was found to contain no inhibitor and fraction III from normal bone marrow was found to contain no stimulator (Lord et al 1977; Lord et al 1976).

It would seem, therefore, that the two activities are produced by separable macrophage populations which are present in the marrow regardless of the kinetic status of the CFU-S population but whose ability to produce their respective activities is directly related to the aforementioned. These data, then, argue for a role for both of these activities in the control of CFU-S proliferation and also that the production of these can be modulated by appropriate physiological conditions.

Simmons and Lord (1985) attempted to further characterise the inhibitor-producing cell by separation of low-density marrow cells based on expression of the murine macrophage-specific antigen F4/80 using a fluorescence activated cell sorter (FACS).

The F4/80 positive population was found to have a 200-fold increased production of inhibitory material over unfractionated bone marrow. These cells were able to proliferate in the presence of conditioned medium from the cell lines L929 and WEHI3B which are sources of macrophage-colony stimulating factor (M-CSF) (Stanley and Guilbert 1981) and IL-3 plus M-CSF (Bazill et al 1983), respectively.

Proliferation was rapid in L929CM, but was more modest in WEHICM. The behaviour of these cells when cultured in the absence of conditioned media was not reported. Production of inhibitory material was enhanced a further 15 to 20-fold by culture of F4/80 positive cells in L929CM over several weeks. Morphologically, these cells resemble macrophages and have the ability to phagocytose latex particles. In addition to F4/80 they express the macrophage-specific antigen Mac-1 but not the neutrophil-specific 7/4 antigen. These cells are also negative for collagen types I and IV. In terms of enzyme histochemistry, these cells are positive for Acid-phosphatase, alpha-Naphthyl acetate esterase and Myeloperoxidase but negative for Alkaline phosphatase.

Which population of bone marrow macrophages is represented by these sorted cells is not clear. The F4/80 antigen has been demonstrated by Austyn and Gordon (1981) to be present on "resident" macrophages from several tissues including lung, spleen and thymus as well as staining resident macrophages of the marrow *in situ* (Crocker and Gordon 1985), suggesting that the fixed stromal bone macrophages of the marrow would have been sorted by Simmons and Lord (1985). However, the antibody is also reactive with murine blood monocytes (Austyn and Gordon 1981) which makes it possible that the most mature cells of the developing mononuclear phagocyte series in the marrow might also be part of the experimental population.

The fact that stimulator and inhibitor producing macrophages were demonstrated to be different macrophage sub-types suggested that the expression of both these activities might be very different in different macrophage sub-types. Accordingly, Pojda et al (1988) investigated the abilities of macrophages freshly isolated and cultured from different sources to produce inhibitory material.

Normal bone marrow, as expected, and cultured bone marrow macrophages were very strong producers while material obtained from both fresh and cultured peritoneal macrophages were only mildly inhibitory in comparison to the marrow-

derived macrophages. Fresh splenic macrophages produced a level of inhibitor comparable to that from peritoneal macrophages but cultured splenic macrophages produced a much higher level. Macrophages from mouse peripheral blood also produced a fairly high level of inhibitor. Wright and Lorimore (1987) also demonstrated the ability of bone marrow macrophages to elaborate inhibitory material.

Production of inhibitory material from species other than mouse has also been demonstrated. Wright et al (1980b) have reported that fraction IV from fresh human bone marrow and from human long-term marrow culture elaborated inhibitory material while fraction III from the same sources had no effect, either inhibitory or stimulatory. Pojda et al (1988) observed that the ability of cultured human blood macrophages to produce inhibitory material was variable. Cork et al (1981) also demonstrated that fraction IV from media conditioned by rat bone marrow was inhibitory to the proliferation of murine CFU-S while fraction III had no effect. The ultrafiltration characteristics of both the human and rat inhibitors were identical to that of the murine inhibitor. Material inhibitory to the proliferation of murine CFU-S has also been isolated from the bone marrow of pig and rabbit (Lord and Wright 1980).

It would appear, therefore, that the occurrence and activity of the inhibitor, at least, is not species specific. However, the vast majority of the investigations into the specific actions of both the stimulator and inhibitor have been carried out in the murine system.

Taken together, the data demonstrate that while both stimulator- and inhibitor-producing cells are present in the marrow at the one time, irrespective of the cycling status of the CFU-S, both stem-cell regulating activities can not be present in the marrow simultaneously. However, when inhibitor- or stimulator-producing cells are separated from each other they are able to resynthesise the appropriate factor

irrespective of the CFU-S cycling status of the marrow of origin (Lord and Wright 1982). Lord and Wright (1982) also demonstrated that, while the two factors are not mutually destructive, inhibitor could block the production of stimulator by cells of the appropriate density and vice versa. In addition, the active presence of the appropriate factor, not just the absence of the opposing factor, is necessary for changes in CFU-S proliferation state to take place (Lord and Wright 1982).

Clearly, any model for the regulation of CFU-S proliferation by the stimulator and inhibitor must take these factors into account. In addition to this, the predominance of either inhibitor or stimulator must be dependent on an appropriate physiological signal.

Lord and Wright (1982) and Lord (1988) have argued that this signal must come from the CFU-S compartment itself due to the heterogeneous nature of the maturing blood cells. A model for such an arrangement is illustrated in figure 1.3.

It can be seen that the proliferation of the CFU-S is dependent on the presence of either inhibitor (I) or stimulator (S). In the steady-state, where CFU-S are quiescent, the predominance of inhibitor is ensured by the production of a "feedback factor" from the CFU-S. The feedback factor acts to block the production of stimulator (S) from the appropriate macrophage subpopulation (m_s), ensuring the production of inhibitor (I) from the appropriate cells (m_i). Conversion to predominance of stimulator production is triggered by a decline in the production of the feedback factor from the CFU-S compartment, leading to the production of stimulator which then both induces proliferation of the CFU-S and blocks production of the inhibitor. The decline in inhibitor production could be caused simply by a reduction in CFU-S numbers or by a change in physiological state caused by, for example, the release of growth factors from the marrow microenvironment as a consequence of marrow ablation. Lending support to this model, Lord (1986) has reported the existence of an activity in medium conditioned by a purified population of CFU-S which is capable

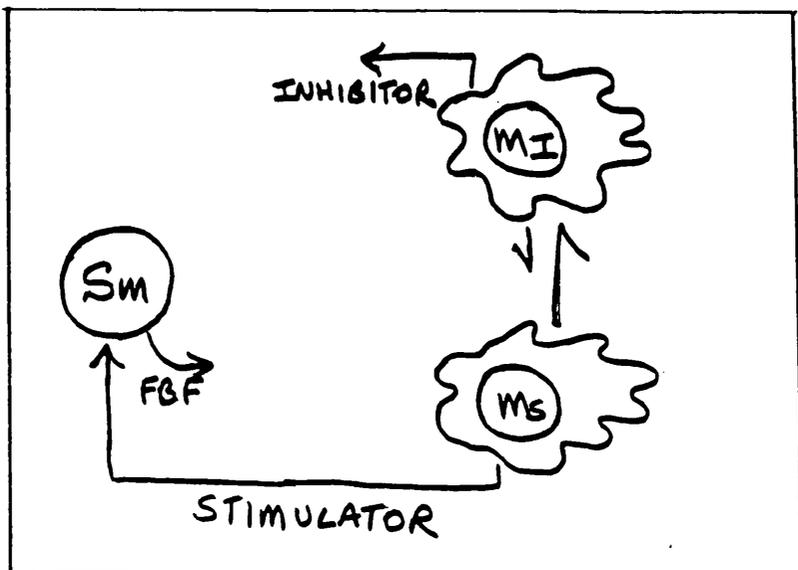
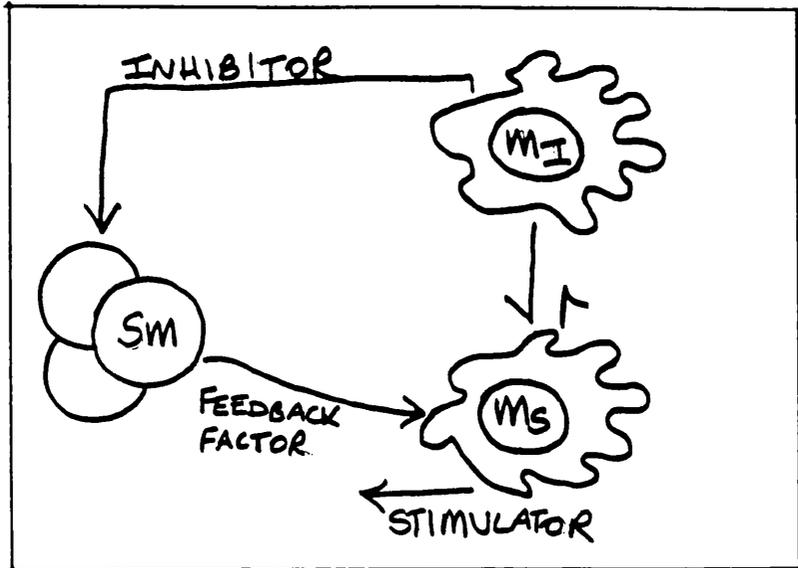


Figure 1.3 A model for the regulation of proliferation of myeloid stem cells by opposing inhibitory and stimulatory activities.

of blocking the production of stimulator, although it has not been characterised to any extent.

1.2.2 Other Negative Regulators of CFU-S

Other potentially physiologically important regulators of CFU-S proliferation have also been described and will be considered in this section.

1.2.2.1 The Terapeptide AcSDKP.

The tetrapeptide Acetyl-N-Ser-Asp-Lys-Pro (AcSDKP) was demonstrated by Lenfant et al (1989) to prevent the recruitment of marrow CFU-S into cycle by administration of the cell cycle-specific cytotoxic drug cytosine-arabioside *in vivo*. The action of AcSDKP appears to be specific to the CFU-S compartment as the formation of GM-CFC colonies is not affected by its presence (Monpezat and Frindel 1989).

Interestingly, studies on the mechanism of action of AcSDKP indicate that it is only capable of maintaining quiescent cells in the G₀ phase of the cell as it is unable to affect cells which are already actively cycling or indeed even cells at the G₁/S-phase boundary (Monpezat and Frindel 1989). This means that the tetrapeptide may exert its effects either early in G₁ or at the G₀/G₁ boundary.

Administration of specific anti-AcSDKP antiserum to mice significantly increases the percentage of CFU-S in cycle, supporting a role for AcSDKP in the maintenance of CFU-S quiescence in normal bone marrow. Interestingly, if this was the case it would mean that the the CFU-S compartment is maintained in the quiescent state by only one molecule; while this is certainly possible it seems unlikely given that redundancy appears to be a predominant feature of the action of cytokines on primitive haemopoietic cells.

The AcSDKP sequence has so far been found in only three proteins, thymosin β ₄, TNF α and rat liver phenylhydrazine hydroxylase (Lenfant et al 1989). It is possible,

therefore, that the tetrapeptide is produced by the proteolytic cleavage of one of these proteins; therefore the physiological activity of the tetrapeptide might be controlled by the secretion and/or activation of specific enzymes. Pradelles et al (1991, 1990) have demonstrated the presence of both AcSDKP and thymosin β 4 in extracts from a wide number of mouse tissues and in cultured human leukocytes. In addition, Wdzieczak-Bakala et al (1990) have demonstrated constitutive production of AcSDKP in long-term marrow culture, which again argues for a role for this molecule in haemopoietic regulation. Interestingly, Moscinski and Prystowsky (1990) have observed induction of expression of the thymosin β 4 gene in GM-CSF induced myeloid differentiation, which raises the possibility of this molecule acting, via proteolytic cleavage to produce the tetrapeptide, as a negative feedback factor.

1.2.2.2 The Hemoregulatory Peptide (HP5B)

The hemoregulatory peptide (HP5B) has the sequence pyroGlu-Glu-Asp-Cys-Lys (pEEDCK) (Laerum and Paukovits 1984a). This molecule is capable of inhibiting the formation of GM-CFC colonies in-vitro and CFU-S and GM-CFC in vivo, but has no effect on colony formation by erythroid progenitors (Laerum and Paukovits 1984a, Laerum and Paukovits 1984b, Laerum et al 1990). Paukovits et al (1987) have reported binding of ^3H -labelled pEEDCK to bone marrow but not spleen or thymus cells, suggesting that the action of the haemoregulatory peptide may be specific to the marrow.

The inhibition of GM-CFC colony-formation in vitro can be achieved with concentrations of pEEDCK as low as 10^{-13}M . The molecule can dimerise through the formation of intermolecular disulphide bonds; intriguingly, this form of the molecule, rather than being inhibitory, is stimulatory for the both murine and human GM-CFC in the concentration range 10^{-16} to 10^{-5}M .

Although the factor was initially identified in extracts of rodent bone marrow and human leukocytes, no further information has been reported on its cellular source in the marrow. Laerum et al (1990) have identified sequences similar to pEECDK in the G1 α subunit of G proteins. The significance of this fact is unclear, however, as these are intracellular proteins.

1.2.2.3 Transforming Growth Factor β (TGF β).

TGF β is a multifunctional regulator of cell behaviour and has a wide variety of effects on a number of cellular systems. It is a member of a large family of structurally-related multifunctional cytokines (for review see Massague 1990). It is ubiquitously expressed in a latent form which is activated to form an active 25kDa homodimer of two 12.5kDa chains linked by disulphide bonds. Activation of this form is required for biological activity (Sporn et al 1987). Mammals express three forms of TGF β (TGF β 1, 2 and 3) which are highly homologous and more than 90% conserved between species (Massague 1990).

Membrane receptor labelling studies have identified at least nine membrane-associated proteins which bind TGF β . However, the significance of most of these is at the moment uncertain (for review see Massague 1992). At least two of these proteins TGF β R type I and TGF β R type II have are associated with the transmission of antiproliferative signals in a lung epithelial cell line (Laiho et al 1991). Many of the antiproliferative effects of binding to the TGF β receptors are thought to be mediated ultimately at the level of tumour suppressor genes, such as the retinoblastoma gene product whose activity controls commitment to the cell cycle and by the inhibiting the expression of nuclear proto-oncogene c-myc (for review see Moses 1990). TGF β is also capable of down-regulating the expression of receptors for stimulatory growth factors as will be discussed below.

All three of the mammalian forms of TGF β are active in the haemopoietic system, with slightly different potencies (Keller et al 1992).

In situ hybridisation and immunohistochemical studies have demonstrated that TGF β is produced in areas of active haemopoiesis such as bone marrow and fetal liver (Seyedin et al 1985, Wilcox and Derynck 1988). In vitro studies have also demonstrated it to have a wide variety of effects on haemopoietic cells, including the modulation of the differentiated functions of mature cells. For example, it is an extremely potent chemotactic factor for monocytes (Wahl et al 1987) and is also able to deactivate macrophages (Tsunawaki et al 1988). In addition, TGF β can inhibit the production of cytokines from both T cells and monocytes (Wahl et al 1987).

The major biological effect of TGF β on the haemopoietic system, however, is the reversible inhibition of proliferation (for review see Keller et al 1992). In general, TGF β appears to act as a selective negative regulator of primitive haemopoietic cells, while more committed progenitors are unaffected. For example, Keller et al (1988) have shown that TGF β 1 is a potent inhibitor of the formation of mixed myeloid lineage colony formation induced by IL-3 plus erythropoietin, while colony formation by the single lineage progenitors is completely unaffected. In addition, the formation of colonies in the HPP-CFC in vitro stem cell assay is inhibited by TGF β 1 in a dose-dependent manner as is the formation of colonies in response to IL-3 of primitive human progenitor cells sorted directly from the marrow (Keller et al 1990), suggesting that the negative regulatory effects of TGF β extend to the stem cell compartment and that these effects are direct rather than mediated by the production of other factors by accessory cells.

Eaves et al (1991) and Cashman et al (1990) have studied the role of TGF β in the control of proliferation of primitive progenitor cells in human long-term bone marrow culture. Antibody versus TGF β could prolong the period of proliferation of previously cycling, and activate the proliferation of quiescent, primitive progenitors,

suggesting that TGF β has a role to play in the production and maintenance of the quiescent state in these cells.

The mechanism by which TGF β exerts its negative effects on the growth of primitive haemopoietic progenitors is unclear at present. However, Jacobsen et al (1991) have demonstrated that TGF β downregulates the numbers of the receptors for GM-CSF, IL-3 and G-CSF in factor-dependent and independent haemopoietic progenitor cell lines at concentrations similar to those which were inhibitory for proliferation; therefore it is possible that this mechanism could also operate on bone marrow progenitor cells.

It is now becoming apparent that TGF β can also display positive proliferative effects on haemopoietic progenitors in certain circumstances. For example, Keller et al (1991) have demonstrated that the number and size of bone marrow colonies induced by murine GM-CSF is increased 3 to 5-fold in the presence of TGF β . This ability to act as a bipotential regulator of haemopoiesis is also seen with the haemoregulatory peptide and with SCI/MIP-1 α (Broxmeyer et al 1989, 1990, 1991).

Given the ubiquity of expression of TGF β it is pertinent to ask how the activity of this molecule on the haemopoietic system is controlled.

Most cell-types examined so far have receptors for TGF β (Massague 1992) so specificity of action is unlikely to be mediated by differential receptor expression. One level of control is at the modulation of production from cell of origin. For example, human monocytes secrete 10-fold more TGF β activity after activation by LPS or ConA (Assoian et al 1987).

Yet another level of control could be the selective activation of TGF β from the latent form. Before use in the laboratory the molecule must be activated, usually by transient acidification (Lawrence et al 1985), however, it is not clear how the molecule might be activated *in vivo*. Plasmin has been shown to activate the molecule

(Lyons et al 1988), so it is possible that selective release of specific proteases at sites of action could be an important controlling factor.

In addition, TGF β has been shown to form an inactivating complex with α_2 -macroglobulin (Keller et al 1992) and it is possible that some of the differential effects seen TGF β 1 and TGF β 2 in inhibiting the growth of haemopoietic cells (Keller et al 1992) could be due to the different affinities of these molecules for α_2 -macroglobulin.

1.3. Macrophage Inflammatory Protein-1 α .

1.3.1 Structure and Genetics of SCI/MIP-1 α and MIP-1 β .

Biochemical characterisation of the Manchester Inhibitor was hindered for many years by the lack of a suitable assay. This situation was remedied by the development of the CFU-A assay by Pragnell et al (1988). This allowed Graham et al (1990) to purify the active protein component of the Manchester Inhibitor which they termed Stem Cell Inhibitor (SCI). When partially sequenced, SCI was found to be functionally and antigenically identical to the alpha chain of a previously described protein, Macrophage Inflammatory Protein-1 (Graham et al 1990; Wolpe et al 1988). MIP-1 is one of the two major heparin-binding proteins secreted on LPS activation of a murine macrophage cell line (RAW 264.7) that are found to elute with 0.7M sodium chloride; the other major protein being named MIP-2 (Wolpe et al 1988). MIP-1 purifies as a doublet, both components of which migrate on SDS-PAGE as proteins of approximately 8,000 daltons. The cDNA sequences of both components of MIP-1 have been obtained and termed SCI/MIP-1 α (Davatelis et al 1988, Brown et al 1989, Kwon and Weissman 1989) and MIP-1 β (Sherry et al 1988).

Purified recombinant MIP-1 α , as tested in the CFU-A assay, was found to be functionally and antigenically indistinguishable from the active component of the Manchester Inhibitor and purified natural SCI. Purified recombinant MIP-1 β was inactive in this

respect at the concentrations tested (Graham et al 1990). The active component, or components, of the Manchester Stimulator have not been identified.

Although MIP-1 α and MIP-1 β are very similar (see below) and co-purify, it is uncertain at the moment whether the two peptides would associate *in vivo*. MIP-1 appears to form multimers *in vitro* of α and β chains of varied molecular mass up to one million daltons; this is a non-covalent interaction which is most likely due to ionic interactions as high ionic buffers lessen the association (G.Graham personal communication).

The strong affinity of MIP-1 protein for heparin may have implications for the biological properties of both SCI/MIP-1 α and MIP-1 β .

The cDNA for SCI/MIP-1 α (Davatelis et al 1988) predicts a mature peptide of sixty-nine amino acids with a molecular weight of 7,889 daltons, from a ninety-two amino acid precursor molecule, which contains no apparent sites for N-glycosylation. The cDNA for MIP-1 β also predicts a mature peptide of sixty-nine amino acids from a ninety-two amino acid precursor, containing one potential site for N-glycosylation at position 53 (Asn-Pro-Ser).

Glycosylation of MIP-1 β might explain its slightly greater apparent molecular weight on resolution of the MIP-1 doublet on SDS-PAGE.

The two cDNAs share 57% identity at the nucleotide level. The predicted amino acid sequences are 60% identical over their entire lengths and 69% identical in the sequences of the mature peptides. Both sequences contain typical hydrophobic signal sequences (which are involved in the secretion of proteins and are subsequently cleaved to produce the mature peptide). The hydrophilicity plots of the two sequences are almost superimposable (Sherry et al 1988). Both sequences contain single open reading frames and have AT-rich sequences in their 3' untranslated regions which correspond to the consensus sequences defined by Caput et al (1986) and which are

found in the mRNA of many immunomodulatory and other transiently expressed genes and are believed to confer instability (Shaw and Kamen 1986).

SCI/MIP-1 α 3'UTR contains four interspersed, imperfect copies of the TATTTAT sequence defined by Caput et al (1986), while MIP-1 β contains one element with perfect correspondence to the consensus.

The cells which have been shown to express mRNA for SCI/MIP-1 α and MIP-1 β are monocytic cell lines (Davatelis et al 1988, Orlofsky et al 1991, Widmer et al 1991, Martin and Dorf 1991, T cell lines (Brown et al 1989, Kwon and Weissman 1989), mast cell lines (Burd et al 1989) and purified epidermal Langerhan's cells (G.Graham, K.Parkinson personal communication).

Human homologues of SCI/MIP-1 α and MIP-1 β have been cloned and characterised by several different groups. All of these cDNAs have been isolated as sequences induced on activation of haemopoietic cells. In addition, non-allelic variants of both of the human genes have been identified.

At least three different cDNAs for genes which appear to be human homologues of murine MIP-1 α (Obaru et al 1986, Zipfel et al 1989, Nakao et al 1990, Irving et al 1991, Siderovski et al 1990) have been isolated.

In addition, at least seven independent human cDNA sequences which display a high degree of identity to murine MIP-1 β have also been described (Lipes et al 1988, Zipfel et al 1989, Chang and Reinherz 1989, Miller et al 1989, Sporn et al 1990).

The genomic sequences of SCI/MIP-1 α (Grove et al 1990, Widmer et al 1991), human MIP-1 α (LD78) and human MIP-1 β (Act-2) have been obtained (Blum et al 1990; Irving et al 1990; Nakao et al 1990). This area has recently been reviewed by Schall (1991).

All of these genes exhibit a three exon, two intron structure. The first exon codes for the 5' untranslated sequence of the mRNA and the coding nucleotides for the leader sequence. The second exon codes for the amino-terminal end of the protein and the

carboxyl end of the protein and the 3' untranslated region of the mRNA are coded for by the third exon. This organisation is typical of that of members of the MIP-1 family (see below).

The organisation of the genomic and cDNA sequences of SCI/MIP-1 α is represented schematically in figure 1.4.

1.3.2 The MIP-1 Family.

The murine and human versions of SCI/MIP-1 α and MIP-1 β belong to a family of cytokine genes entitled variously the MIP-1, the SCY or the 17q family (Wolpe and Cerami 1989; Schall 1991; Stoeckle and Barker 1990). Membership of this family is conferred by sequence conservation, in particular that of four position-invariant cysteine residues, the first two of which are located adjacent to each other near the amino-terminal end of the protein (for review see Wolpe and Cerami 1989; Schall 1991).

The murine MIP-1 family has six members: MIP-1 α , MIP-1 β , JE (Cochran et al, 1983; for review see Rollins 1991), TCA3, (Burd et al 1987), p500 (Brown et al 1989) and C10 (Orlofsky et al 1991). TCA3 and p500 are alternatively spliced forms of the mRNA from one gene (Wilson et al 1990A). All of the genes for this family have been mapped to the same locus (q11-q21) on mouse chromosome 11 (Wilson et al 1990a) except for C10 which has not yet been assigned to a locus. The SCI/MIP-1 α gene, at least, appears to be present as a single copy (Kwon and Weissman (1989). The human MIP-1 family has five members, four of which are homologues of the murine members: LD78/464.1/GOS 19-1 (human MIP-1 α), Act-2/744.1 (human MIP-1 β), MCP-1 (Rollins et al 1989), I-309 (Miller et al 1989) and RANTES (Schall et al 1988). All of the known human MIP-1 genes have been mapped to chromosome

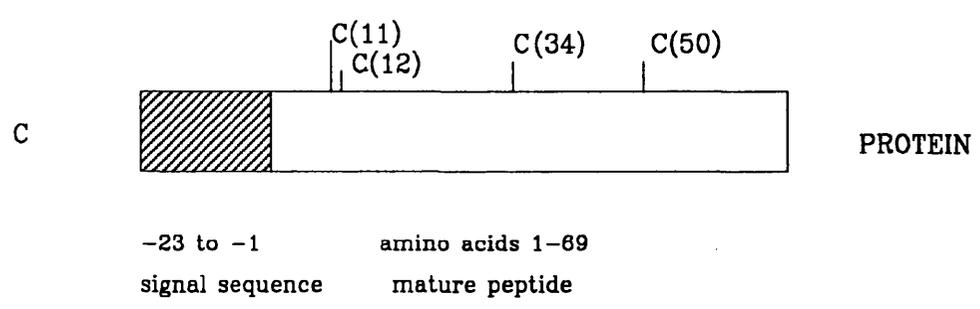
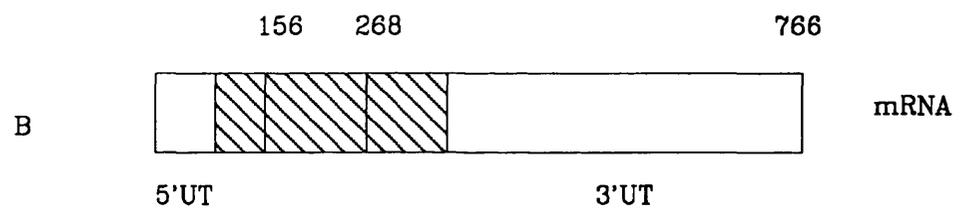
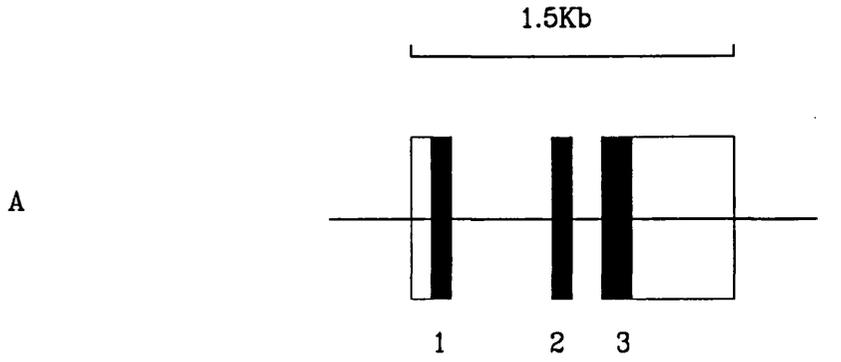
Figure 1.4 Organisation of the SCI/MIP-1 α gene, messenger RNA and protein.

A SCI/MIP-1 α genomic organisation: Boxes designate the intronic sequences; filled and open boxes signify the protein-coding and 5' and 3' untranslated sequences, respectively.

B SCI/MIP-1 α mRNA: Hatched sections are protein-coding. Numbers 156 and 268 refer to position of splice sites; number 766 refers to the polyadenylation signal.

C SCI/MIP-1 α protein: Hatched section designates leader sequence; open section, mature peptide. Numbers refer to the positions of the four conserved cysteine residues.

This figure is based on information contained in Davatellis et al (1988) and Widmer et al (1991). The organisation of MIP-1 β mRNA and protein is essentially identical (Sherry et al 1988).



17 (q11-q21) (Donlon et al 1990; Irving et al 1990; Miller et al 1990; Mehrabian et al 1991; Rollins et al 1991). The non-allelic variants of LD78/464.1/GOS 19-1 and Act-2/744.1 have also been shown to map to the same locus (Irving et al 1990).

Most of the cDNAs have been identified as sequences inducible on cellular stimulation which have been isolated by differential screening of haemopoietic cells (TCA3, LD78, Act-2, I-309) or fibroblasts (JE). RANTES and C10, neither of which as yet has an identified cross-species homologue, were also isolated from haemopoietic cells but have a slightly different pattern of expression in that their mRNA tends to be decreased on stimulation.

1.3.3 MIP-2 Family.

The MIP-1 family of cytokines is one of two branches of a cytokine superfamily, the platelet factor four (PF4) superfamily. Membership of the other branch, the archetype of which is PF4 is also conferred by sequence conservation. Members of the MIP-2 family also have four conserved cysteines. These are in similar positions to those of the MIP-1 family except that the first two cysteines are separated by a single amino acid rather than being adjacent (for reviews see Wolpe and Cerami 1989, Stoeckle and Barker 1990). The MIP-2 family contains both murine, human, rabbit and chicken members. The genes for the human proteins PF4, MGSA/gro and IP-10 have been mapped to the same locus (q13-q21) on human chromosome 4 (Stoeckle and Barker 1990).

1.3.4 Biology of the MIP-1 Family.

Understanding of the biology of the MIP-1 family cytokines is still at an early stage and some members of the murine branch (JE, p500, C10) have yet to be assigned any function. However, a body of evidence is beginning to emerge which is consistent with the members of the MIP-1 family acting as mediators responsible for attracting

immune effector cells to sites of inflammation and injury (for review see Schall 1991).

MIP-1 and TCA-3 have been shown to cause localised inflammation and swelling after injection into mouse footpads (Wolpe et al 1988, Wilson et al 1990b), suggesting that these proteins may play a role in mediating the inflammatory response. However, this may not be a specific effect of these proteins as very high protein concentrations and partially purified material, respectively were used by the investigators.

MCP-1 and I-309 have been shown to be monocyte-specific chemoattractants (Rollins 1991) and RANTES has been shown to be a specific attractant for monocytes and a specific subset of T lymphocytes (Schall et al 1990).

The ability of several members of the MIP-2 family to attract neutrophils (Stoeckle and Barker 1990) has led to suggestion that members of the MIP-2 family may be responsible for the early, acute inflammatory response associated with neutrophil infiltration while MIP-1 family members are responsible for the later, macrophage-borne chronic response. Further investigation of the biological properties of the MIP-1 family cytokines will enable the validity of this idea to be assessed.

The fact that mRNA and/or protein for MCP-1, RANTES, human MIP-1 α and human MIP-1 β has been found to be associated with atherosclerotic plaques, rheumatoid joints and some tumours (Wilcox et al 1990, Schall 1991, Graves et al 1989) has also led to suggestions that the proteins of the MIP-1 family could be involved in the pathology of these conditions; again these ideas await further experimental investigation.

Biology of MIP-1, SCI/MIP-1 α and MIP-1 β .

The macrophage inflammatory proteins appear to occupy a special position within the MIP-1 family in that they appear to have effects on both the function of mature blood

cells and also on the developmental process by which they are produced. In this section these functions will be described and discussed.

Effects on the Haemopoietic Progenitor Compartment

As has already been stated, SCI or the active component of the Manchester Inhibitor has been demonstrated to be antigenically identical to the alpha chain of MIP-1.

Graham et al (1990) demonstrated that highly purified MIP-1 α when added directly into the culture dish inhibited the formation of colonies by CFU-A. Purified MIP-1 α also reversibly reduced the proportion of CFU-A in DNA synthesis in regenerating bone marrow, while the proliferation of more mature progenitors such as granulocyte-macrophage colony forming cells (GM-CFC) were unaffected. These effects were seen at picomolar concentrations, which strongly indicates that the inhibition of primitive cell proliferation is a genuine property of SCI/MIP-1 α .

Studies with purified and recombinant material demonstrated that purified natural murine MIP-1 (nMIP-1) and recombinant murine MIP-1 α (rmMIP-1 α) but not rmMIP-1 β reversibly decreased the proportion of CFU-A in regenerating bone marrow in DNA synthesis, while neither rMIP-1 α nor rmMIP-1 β had any effect on GM-CFC colony formation (Graham et al 1990). These results then indicated that the haemopoietic effects of SCI/MIP-1 α were confined to the more primitive progenitor/stem cells in a way which is directly analogous to the CFU-S specificity displayed by the Manchester Inhibitor (Tejero et al 1984). Furthermore, recombinant SCI/MIP-1 α was shown in the same study to have similar effects on CFU-S as on CFU-A. A neutralizing MIP-1 antiserum blocked the effect of both rMIP-1 α and purified SCI (Manchester Inhibitor) on CFU-S DNA synthesis, demonstrating that MIP-1 α and SCI are functionally and antigenically indistinguishable; although it should be borne in mind that the formal possibility still exists that SCI/MIP-1 α and

the active protein component of the Manchester Inhibitor are distinct, although antigenically related, molecules.

Recently, Dunlop et al (1992) have demonstrated the ability both *in vitro* and *in vivo* of recombinant human MIP-1 α (LD78) to reduce the proportion of CFU-A and CFU-S in cycle in the regeneration period after treatment of mice with two drugs (5-fluorouracil and phenylhydrazine) which induce rapid proliferation of these cells. More primitive CFU-S are apparently more sensitive to the action of human MIP-1 α which is in agreement with previous results obtained by Wright et al (1985) with the Manchester Inhibitor.

Human MIP-1 α (LD78) has also been assessed for its ability to protect CFU-S from the cytotoxic effects of cycle-specific drugs. Dunlop et al (1992) and Lord et al (1992) have demonstrated protection of the CFU-S compartment from the effects of double injections of cytosine arabinoside and hydroxyurea, respectively, demonstrating that SCI/MIP-1 α has the capacity to act as a physiological mediator of CFU-S proliferation inhibition.

Investigations by other workers in the field have confirmed the ability of SCI/MIP-1 α to suppress the proliferation of primitive progenitors but have indicated that other haemopoietic compartments may also be affected.

Broxmeyer et al (1990) studied the effects of the murine MIP-1, SCI/MIP-1 α , MIP-1 β and MIP-2 proteins on primitive progenitor cells in mice and humans.

With murine bone marrow, they observed that natural murine (nm)MIP-1 and recombinant murine (rm)MIP-1 α but not rmMIP-1 β or rmMIP-2 inhibited murine CFU-GM colony formation stimulated by pokeweed mitogen spleen-conditioned medium (PWMSM) and suppressed colony formation of both BFU-E and colony forming unit Granulocyte Erythroid Megakaryocyte Macrophage (CFU-GEMM) stimulated by Epo plus PWMSM.

With human cells, none of the MIP proteins suppressed the formation of BFU-E stimulated by Epo alone but nmMIP-1 and rmMIP-1 α , but not rmMIP-1 β abrogated the enhanced formation of BFU-E stimulated by addition of rhIL-3 and rhGM-CSF to identical cultures. The formation of human CFU-GM colonies stimulated by rhGM-CSF plus rhIL-3 and of human CFU-GEMM by these growth factors plus erythropoietin was also affected in the same manner by the MIP proteins.

Bodine et al (1991) have also demonstrated that rmSCI/MIP-1 α is able to inhibit the increase in CFU-S numbers obtained on liquid culture of murine bone marrow with IL-3 and IL-6. This effect could be due either to inhibition of CFU-S self-renewal or by inhibition of the proliferation and differentiation of more primitive progenitors to produce CFU-S.

Taken together these data support the hypothesis that SCI/MIP-1 α , but not MIP-1 β , displays suppressive activity for primitive myeloid progenitors, although its suppression of both mouse and human CFU-GEMM may indicate that the primitive compartment affected by the protein is not as restricted as first envisaged.

In addition to this, there may seem to be a contradiction between the findings of Graham et al and Broxmeyer et al concerning the inhibition of colony formation by the lineage committed progenitor cell CFU-GM. However, the CFU-GM grown by Graham et al were stimulated by rGM-CSF whilst Broxmeyer et al observed inhibition of colonies stimulated by PWMSCM. As PWMSCM has been shown to contain GM-CSF, IL-3, M-CSF and other growth factors, it is likely that the CFU-GM of Broxmeyer et al are derived from a more primitive cell. Thus, this result may support rather than detract from the above hypothesis.

The MIP-1 family members JE, MCP-1, RANTES and TCA-3 have all been shown to be ineffective in inhibiting the proliferation of CFU-A cells, demonstrating the specificity of SCI/MIP-1 α in this activity. (G.Graham personal communication.).

Broxmeyer et al (1989, 1990) have also presented evidence that rmMIP-1 α , rmMIP-1 β and MIP-2 can affect the proliferation of lineage-committed myeloid progenitors, namely CFU-GM, by enhancing rather than suppressing colony formation in the presence of suboptimal concentrations nmM-CSF or rmGM-CSF. These proteins also enhanced the plating efficiency of a purified population of human progenitors (BFU-E and CFU-GM) demonstrating that the enhancing effects were caused directly. The ability to act as a bipotential regulator of progenitor cell proliferation has been seen with other inhibitors of haemopoiesis including transforming growth factor β (TGF β) (Keller et al 1992) and may have profound implications for the role played by SCI/MIP-1 α in the control of haemopoiesis. For example, it is possible that production of SCI/MIP-1 α in the haemopoietic microenvironment may lead to the proliferation of progenitors while at the same time affording protection, perhaps from extinction due to differentiation induced by growth factors, to stem cells. It is possible that a further layer of complexity exists in the action of SCI/MIP-1 α and MIP-1 β on the haemopoietic progenitor compartments. Broxmeyer et al (1991) have demonstrated the ability of rmMIP-1 β (but not MIP-2) to abrogate the suppressive effects of rmSCI/MIP-1 α on colony formation by BFU-E, CFU-GEMM and CFU-GM. A 4:1 molar ratio of MIP-1 β to SCI/MIP-1 α is required to block completely the myelosuppressive effects of SCI/MIP-1 α while a 1:1 ratio was ineffective in this respect. This fact presumably accounts for the myelosuppressive effects of the MIP-1 protein (Graham et al 1990, Broxmeyer et al 1990) which apparently consists of equimolar amounts of SCI/MIP-1 α and MIP-1 β (Wolpe et al 1988, Wolpe and Cerami 1989). This antagonistic effect of MIP-1 β is seen even when bone marrow cells are exposed to the protein for only one hour before washing and subsequent addition of SCI/MIP-1 α suggesting that the effect of MIP-1 β on primitive progenitor cells is achieved very rapidly and is not dependent on the continuous presence of the

factor. The antagonistic effect of MIP-1 β on the action of SCI/MIP-1 α appears to be specific as it does not affect the ability of another haemopoietic inhibitor, H-ferritin, to suppress colony formation by the same primitive progenitor populations (Broxmeyer et al 1991).

The fact that MIP-1 β has no effect on the more primitive haemopoietic cells itself and that the two MIP-1 proteins appear to be structurally closely related raises the possibility that MIP-1 β may exert its effects by acting as a direct antagonist of the receptor for SCI/MIP-1 α . Such an antagonist has recently been described for interleukin-1 (for review see Dinarello 1991).

It is also interesting to speculate that modulation of the relative levels of SCI/MIP-1 α and MIP-1 β synthesised by producer cells could be a mechanism for controlling the activity of SCI/MIP-1 α given that an excess of MIP-1 β can neutralise its inhibitory action.

Inflammatory Effects

MIP-1 protein was originally isolated as an inflammatory mediator (Wolpe et al 1988) and when tested in a number of in vivo and in vitro assays appeared to behave as such. Wolpe et al (1988) demonstrated that MIP-1 induced a localized inflammatory response, characterised by infiltration of polymorphonuclear neutrophils (PMNs) when injected into the footpads of (endotoxin-resistant) C3H/HeJ mice. MIP-1 also appeared to be mildly chemotactic for human PMNs in vitro and induces them to undergo an oxidative burst after a one to two hour lag (Wolpe et al 1988). It also induces these cells to degranulate. (Wolpe and Cerami, 1989).

Intracisternal injection of MIP-1 in rabbits causes a rapid influx of PMNs followed by monocyte infiltration (Wolpe and Cerami, 1989). The predominance of neutrophilic cells in the infiltrates attracted by MIP-1 as well as its apparent effect on human neutrophils led to the hypothesis that MIP-1 was a neutrophil-specific

chemoattractant. However, other investigators have found purified MIP-1 to be ineffective in affecting the behaviour of neutrophils (Schall 1991, G.Graham personal communication).

It is interesting to note that the maximal activity of MIP-1 in the studies of Wolpe et al (1988) and Wolpe and Cerami (1989) was observed at relatively high concentrations of protein (greater than 1µg/ml); these concentrations were thought to be necessary due to the propensity of the protein to aggregate. Schall (1991) has argued that the high concentrations used in these experiments make it possible that MIP-1 could be binding to a receptor on neutrophils for a related protein such as that for the MIP-2 family member Neutrophil Activating Peptide (IL-8); hence these activities might not reflect a true physiological role for MIP-1. As purified rather than recombinant material was used in these experiments it is also possible that neutrophil-activating contaminants could be giving misleading results. These issues could be resolved by using recombinant material or by the use of specific neutralising antibodies.

Schall (1991) have reported that human MIP-1α (LD78) protein is able to attract killer T cells and B lymphocytes in a dose dependent manner in vitro while recombinant human MIP-1β preferentially attracts naive helper T cells in vitro.

In addition to this, Fahey et al (1992) have recently studied the effects of MIP-1, MIP-1α and MIP-1β on its producer cell, the macrophage itself. Native MIP-1, at nanogram concentrations, had a modest, though significant proliferative effect on thyoglycollate broth-elicited peritoneal macrophages both alone and in combination with CSF-1. These macrophages also displayed enhanced antibody-independent cytotoxicity for tumour cells, which is a classic macrophage activation function (Adams and Hamilton 1984). On the other hand, MIP-1 was unable to trigger the oxidative burst, or to upregulate the expression of MHC antigens, which are normally

also associated with macrophage activation, suggesting that the macrophage response to MIP-1 may be complex.

In addition to these effects, Fahey et al (1992) observed that MIP-1 elicited the secretion of bioactive TNF α , IL-6 and immunoreactive IL-1 α from peritoneal macrophages and these effects were greatly enhanced by IFN γ . When the relative contributions of the two components of the MIP-1 doublet to these effects was assessed it was found that only SCI/MIP-1 α was active. Indeed, as with haemopoietic progenitors, an 8-fold excess of MIP-1 β abrogated the response of macrophages to SCI/MIP-1 α which further strengthens the idea that MIP-1 β may be a natural antagonist of SCI/MIP-1 α in some circumstances.

It seems possible, therefore, that the SCI/MIP-1 α and MIP-1 β proteins could play a role in the recruitment of immune defence cells to sites of infection which would correspond with the fact that they can be produced by activated macrophages and T cells, although the actual subset(s) of effector cells mobilised by these proteins remains unclear.

One interesting possibility is that the SCI/MIP-1 α secreted by macrophages may play a part in the autocrine loop which could both increase the number of effector macrophages at sites of inflammation and augment some of the effector functions of these cells, including the secretion of other cytokines.

Further evidence of a role for SCI/MIP-1 α and MIP-1 β in the inflammatory response and in tissue remodelling has been reported by Fahey et al (1990) who studied the induction of expression of several cytokine mRNAs in inflammatory cells invading sterile subcutaneous implants in mice. Using the polymerase chain reaction technique, they demonstrated the appearance of mRNA for SCI/MIP-1 α and MIP-1 β from day one to day seven after implantation. MIP-1 protein could also be detected by Western blotting. The fact that no SCI/MIP-1 α or MIP-1 β mRNA could be

detected in peripheral blood leukocytes indicates that these transcripts are specifically induced in response to injury.

In addition to its effects on inflammation in the periphery, SCI/MIP-1 α has been demonstrated to have inflammatory effects in the central nervous system.

Saukonen et al (1992) observed the efficacy of SCI/MIP-1 α in promoting the influx of inflammatory cells into the subarachnoid space, a site sequestered behind the specialised vascular endothelium comprising the blood-brain barrier. The leukocytosis was associated with blood brain barrier permeability and brain edema. Interestingly, MIP-1 β was found to be 100-fold less effective in generating these responses. These data suggest a possible role for SCI/MIP-1 α in the inflammatory response to and in the tissue damage associated with infections of the central nervous system such as bacterial meningitis.

Pyrogenic Activity of MIP-1.

MIP-1 appears to have a specific role to play in the systemic response to infection. Davatelis et al (1989) demonstrated that natural murine MIP-1, when injected into rabbits, induced a rapid monophasic fever which could not be suppressed by prostaglandin inhibitors. This febrile response was equal to those induced by the other known endogenous pyrogens, TNF α and IL-1, although the ability of MIP-1 to act independently of prostaglandin is unique among endogenous pyrogens.

Minano et al (1990) have demonstrated, using a direct injection technique on the rat that (murine) MIP-1 exerts its effects by acting directly on the hypothalamus. They observed that the potency of MIP-1 was in the femtomolar (10^{-15} M) range, making it almost certain that this is a specific activity of MIP-1. Interestingly, Davatelis et al (1989) injected a 300,000-fold higher dose systemically into rabbits to achieve a febrile response comparable to that observed in rats by direct injection. One reason for this may be that most of the heparin-binding MIP-1 protein is sequestered by

components of the vasculature when injected intravenously. It seems likely, therefore that MIP-1 acting on the hypothalamus would have to be released locally perhaps by blood-borne or brain-associated macrophages.

Given that the α and β moieties of MIP-1 have been shown to have differential effects on haemopoietic progenitors and peritoneal macrophages, it will be interesting to see whether this is the case for the action of MIP-1 on the hypothalamus.

1.4 The Expression of Cytokine Genes.

The cytokines and (colony stimulating factors) are extremely powerful biological regulators. As discussed in section 1.1 this means that activity of these proteins must be tightly regulated both physically and temporally. It has become apparent in the last few years that the expression of cytokine genes can be controlled at every possible level from mRNA transcription to translation and secretion of the final protein product. Cytokine genes can be expressed in a wide variety of cell types including leukocytes, fibroblasts, endothelial cells and smooth muscle cells (Taniguchi 1988, Arai et al 1990). It is now becoming apparent that mechanisms similar to those involved in the expression of cytokine genes are also involved in the regulation of expression of some nuclear proto-oncogenes, such as c-fos and c-myc. Therefore, some aspects of the regulation of expression of these genes will be considered where appropriate.

1.4.1 General Characteristics of Expression.

The two features of cytokine gene expression which are most striking are, firstly, the rapid and marked inducibility of expression and, secondly, the temporal control of that expression.

In general, in resting cells (which have not been exposed to a stimulus), messenger RNA (mRNA) is usually barely detectable, if at all. However, on cellular stimulation, levels of mRNA can rise rapidly (within minutes to a few hours) to as much as 20-100 times that in the resting cells (Taniguchi 1988, Davatellis et al 1988, Brown et al 1989). The accumulation of cytokine mRNA following cellular stimulation is usually independent of de novo protein synthesis (Taniguchi 1988). This, along with the rapidity of induction, places such genes in the "immediate-early" group (Rollins and Stiles 1989); therefore the expression of these genes is often induced as part of the initial response of a cell to stimulation.

It is also often the case that the accumulation of cytokine mRNA following stimulation is transient, with levels peaking after a few hours and then falling to either basal levels or to a much lower level than that seen at the peak of expression. The mechanisms by which these patterns are achieved have been the subject of considerable investigation over the last few years and will be considered in the following sections.

1.4.2 Transcriptional Control.

Transcription is the process whereby an RNA molecule complementary to the coding region of a gene is produced in the nucleus. This process has three main stages: 1) Initiation, where RNA polymerase II becomes attached to the promoter sequences 5' of the gene and then begins to synthesis the new RNA. 2) Elongation, in which the synthesis of the new RNA molecule takes place. 3) Termination, where the process is ended. (Lewin 1987). Initiation is normally the rate-limiting step for determining the level of transcription (Kornberg and Lorch 1991).

Transcription is directed by regulatory sequences lying closely 5' of (promoters) or at some distance from (enhancers) the gene (Lewin 1987, Ptashne 1988). RNA polymerase II does not directly bind to the gene on its own (Sowadogo and Sentenac

1990). Instead, usually several transcription factors, which recognise specific DNA sequences in the promoters and enhancers, must be present to facilitate the initiation of RNA synthesis. Other sequences, locus control regions (LCR), are responsible for the maintenance of an "open" chromatin structure which is important for the overall level of transcription (Kornberg and Lorch 1991).

Promoters, enhancers and LCRs appear to be constructed in a modular fashion in that they may consist of binding sites for several transcription factors. In this way regulation of transcription may be dependent on the simultaneous binding of several transcription factors (Ptashne 1988). Hence, changes in the availability of, or ability of, transcription factors to bind to specific motifs in the regulatory elements of genes is the primary mechanism for modulating expression.

Some transcription factors are expressed by a wide variety of cell types and probably serve a general role in transcription; for example the transcription factor TFIID, which binds an AT-rich sequence situated about 25 nucleotides from the transcription start-site of many RNA polymerase II promoters, has been shown to be essential for the binding of RNA polymerase II and subsequent transcription *in vitro*. Myers et al (1986) have also demonstrated that this sequence is one of the main determinants of fidelity of transcriptional initiation *in vivo* (for review see Sowadogo and Sentenac 1990).

Other transcription factors are restricted in their expression to one or a few cell types and are likely to be responsible for tissue-specific expression of genes, including those encoding cytokines. For example, a number of cytokine genes contain putative binding sites for the transcription factor PU.1, the expression of which in normal, untransformed cells is restricted to B cells and macrophages (Pongubala et al 1992). Still other transcription factors are only activated to bind promoters after the cell has been exposed to an extracellular stimulus. It is highly likely that the expression of

inducible genes could be specifically regulated by the control of activation of such transcription factors.

An example of such a transcription factor is NF- κ B. This transcription factor is found in a number of cell types and activates transcription through binding as a heterodimer of two proteins of 50kDa (p50) and 65kDa (p65) to a decameric consensus sequence 5'-GGGRNNYYCC-3'. This sequence is found in the 5' regulatory region of a number of genes including the cytokines IFN β , GM-CSF, G-CSF, IL-6, IL-2 and TNF α (Baeuerle 1991).

In resting cells, however, NF κ B is present in an inactive form complexed with a labile inhibitory molecule, I κ B. The NF κ B heterodimer is released from the inhibitory effects of I κ B, through a mechanism thought to involve changes in the phosphorylation state of I κ B (Ghosh and Baltimore 1990), only after cellular stimulation. Following this, the free NF κ B heterodimer can translocate to the nucleus where it is able to activate transcription of its target genes (for review see Baeurle 1991, Blank et al 1992).

Monocytes and B cells contain a certain amount of free, nuclear NF κ B which may be involved in the constitutive expression of some genes. Nevertheless, NF κ B activity can be greatly increased in these cells following stimulation which suggests that, for inducible gene expression, the same concepts apply to monocytes as to other cells (Blank et al 1992, Vincenti et al 1992).

Various agents are able to activate NF κ B including mitogens (phorbol esters, lectins), cytokines (IL-1 α , TNF α) and viruses (HIV-1, cytomegalovirus) and LPS (Bohnelein et al 1988, Sambucetti et al 1989, Collart et al 1990, Vincenti et al 1992), which makes activation of NF κ B an obvious candidate for the activation of cytokine genes. The fact that the activation of NF κ B can take place in the absence of new protein synthesis also makes this transcription factor a likely candidate for the activation of immediate early genes.

Collart et al (1986) have demonstrated the transcriptional induction of mRNA for TNF α in elicited peritoneal macrophages by LPS, the protein synthesis inhibitor cycloheximide and IFN γ . This induction appears to be mediated, in part, via NF κ B as treatment of the same cells with these agents stimulates its activation and translocation to the nucleus from the cytoplasm and specific binding of NF κ B to four NF κ B-binding sites in the promoter region of this gene is necessary for the stimulation of transcription. (Collart et al 1990). It is possible that cycloheximide stimulates TNF α transcription by blocking synthesis of the labile I κ B protein and hence leading to the production of free NF κ B.

Promoter-binding of NF- κ B has also been demonstrated to be involved in the transcriptional activation of the GM-CSF gene in T cells by PHA and phorbol ester (Schreck and Baeuerle 1990), the IL-6 gene by IL-1 α and TNF α (Shimizu et al 1990) and of the M-CSF gene in the human promyelocytic cell line HL-60 by TNF α (Yamada et al 1991).

One of the reasons for the transience of expression of mRNAs is the brevity of the enhancement of the rate transcription following cellular stimulation. For example LPS induces the transcription of the IL-1 β gene in human monocytes within fifteen minutes. This activity reaches a peak at 3-4 hours, is sustained for 6 to 8 hours and then decreases rapidly (Fenton et al 1987, 1988).

There is evidence to suggest that transcription of the c-fos proto-oncogene, which reaches a peak 15 minutes after stimulation and has returned to basal levels by 1 hour (Greenberg et al 1986), is downregulated by binding of the gene product to specific sites on the c-fos promoter (Sassone-Corsi et al 1988, Rivera et al 1990), thus ensuring that expression of c-fos is self-regulating.

While such an auto-regulatory mechanism might operate in the control of cytokine gene transcription it seems improbable, considering that cytokines are secreted molecules. Studies by Fenton et al (1987, 1988) suggest that LPS induces a repressor

of IL-1 β transcription in human monocytes which could be involved in the temporal control of IL-1 β transcription. Further studies will be required to determine whether this is a general mechanism for the control of cytokine gene transcription.

Inhibition of transcription is also a mechanism by which cytokine gene expression can be affected by downregulators. Various anti-inflammatory agents have been shown to be potent inhibitors of cytokine gene transcription. For example, glucocorticoids inhibit the accumulation of JE mRNA in fibroblasts in response to platelet-derived growth factor (PDGF) and of the IL-2 mRNA in T cells by phorbol ester and calcium ionophore by blocking the stimulation of gene transcription (Kawahara et al 1991, Vacca et al 1992).

Glucocorticoid transcriptional enhancement is known to occur through the interaction of the glucocorticoid receptor with specific DNA response elements (Yamamoto 1985). However, the mechanism by which glucocorticoids repress transcription are less well defined. Vacca et al (1992) have reported evidence that the glucocorticoid-mediated repression of IL-2 transcription is mediated by the activated glucocorticoid receptor interfering with the synergistic cooperation between proteins binding to consensus binding motifs for the two inducible transcription factors AP-1 and NF-AT. Northrop et al (1992) have reported evidence which supports a similar mechanism involving a previously uncharacterised transcription factor.

1.4.3 RNA Processing.

The production of translatable mRNA from the primary product of gene transcription, termed heterogeneous nuclear (hn)RNA, requires that internal sequences (introns) which are not part of the protein-coding sequence be removed by a nuclear protein and RNA complex, the spliceosome. In addition, the RNA molecules are also given a 3-methyl guanine cap at the 5' end, and a poly(A) tail at the 3' end of the molecule (Lewin 1987). These events take place before splicing.

In general, the precursor molecules of mRNA are not detectable. This is thought to be for two reasons: firstly, intron splicing is an extremely efficient process and is not thought to be a rate-limiting step and secondly, a large proportion of nuclear RNA is rapidly degraded. Thus RNA processing is not generally considered to be an important control point in gene expression.

Nevertheless, several cases do exist where nuclear precursors of cytoplasmic mRNA can be readily detected after cellular stimulation. Gudas et al (1988), for example, have demonstrated the accumulation of precursors of murine thymidine kinase mRNA in response to fetal calf serum in Balb/c 3T3 cells. This suggests slow maturation of the transcripts which could be a determining factor in the availability of mRNA for translation.

Similarly, Weil et al (1990) have observed the accumulation of precursors of tumour necrosis factor β (TNF β) mRNA in the nucleus of a murine T cell line CTLL-2 on stimulation by IL-2. The slow nuclear processing leads to a delay in the appearance of cytoplasmic TNF β mRNA, suggesting that RNA processing could be a rate-limiting step in the expression of the murine TNF β gene. Similar results were obtained with ConA-stimulated splenocytes, indicating that this phenomenon is not a consequence of cell immortalisation.

These data suggest that mRNA processing is a limiting step, and hence could be a control point, for the accumulation of some mRNAs, including those for cytokines, in response to cellular stimulation.

1.4.4 Cytokine mRNA Stability.

While the rate of production of mature mRNA molecules is an important control point for the regulation of expression of cytokine genes, posttranscriptional mechanisms also operate and can have profound effects on the overall level of expression.

Cytokine mRNAs are very unstable in comparison to constitutively expressed genes, being measured in minutes or a few hours; for example, the half-life of GM-CSF mRNA in PHA-treated T cells is 30 minutes, while that for β -globin mRNA is around 17 hours (Shaw and Kamen 1986). The instability of cytokine mRNAs is a major determinant, in addition to the brevity of transcription, of the transience of accumulation of these molecules.

Caput et al (1986) have observed that a large number of cytokine mRNAs have conserved UA-rich elements in their 3'-untranslated regions consisting of interspersed repeats of the consensus AUUUA. This sequence is believed to destabilise molecules which contain it, as Shaw and Kamen (1986) have demonstrated that transfer of a portion of the 3'-untranslated region of the GM-CSF mRNA containing the conserved AU-rich sequence confers instability on β -globin mRNA.

It is generally agreed that the first step in the degradation of mRNAs is the removal of the poly(A) tail which is then followed by the rapid endonuclease-mediated digestion of the mRNA in a general 3' direction (for review see Jackson and Standart 1990).

The description by Bernstein et al (1989) of a cytoplasmic 3' exoribonuclease in mammalian cells which *in vitro* readily degrades mRNA not protected by a 3' poly(A) tail lends credence to this idea.

Recent elegant work by Shyu and co-workers has led to a partial elucidation of the mechanisms underlying the rapid degradation of the mRNA for the nuclear proto-oncogene c-fos. Wilson and Treisman (1988) observed that removal of the poly(A) tail of c-fos mRNA occurred much more rapidly than that for β -globin, suggesting that this may be related to the short c-fos half-life. Shyu et al (1991) have extended this observation by demonstrating that removal of the poly(A) tail (deadenylation) is a prerequisite for subsequent mRNA degradation. Furthermore, these two processes appear to be mediated by two distinct sequence elements in the 3'UTR. Mutation of the AUUUA repeats demonstrated that deadenylation but not subsequent mRNA

degradation is independent of these elements. Deadenylation is still, however, dependent on the presence of 3' AU-rich sequence.

You et al (1992) have recently described four proteins which specifically bind a 20 nucleotide U-rich sequence in the 3'UTR of c-fos mRNA, the affinity of one of which is affected by agents shown to increase the stability of the c-fos mRNA. The binding of these factors is, however, independent of the presence of AUUUA pentanucleotides. This suggests that these four proteins may be involved in mediating the initial deadenylation of the c-fos mRNA.

Other investigators (Bohjanen 1991, Brewer, 1991, Vakalopoulou 1991) have described proteins which specifically bind the AUUUA pentanucleotides of several mRNAs. It is possible that these factors may be involved in regulating the mRNA degradation step.

Recent studies have indicated that the 3'AU-rich sequences are not the sole determinants of instability for labile mRNAs; other sequences within the coding region may be just as, if not more, important.

Han et al (1991a) have shown, using recombinant reporter constructs stably transfected into the macrophage cell line RAW 264.7 that decay of the mRNA for TNF α , after induction by LPS, is unaffected by the absence of a 3' UTR containing an AU-rich region. This suggests that other mechanisms for TNF α mRNA degradation, in addition to that mediated through the 3' AU-rich region, may operate. This "alternative" pathway is sensitive to the action of actinomycin D, whereas that mediated through the 3' AU-rich sequence is not which suggests that the two pathways operate through separate mechanisms.

Therefore, it appears that separate but superimposed mechanisms may determine the turnover of TNF α , and hence possibly other cytokine, mRNAs. Shyu et al (1989, 1991) have reported similar results for c-fos mRNA. In these studies, the "alternative" degradative pathway was located to the protein-coding region.

Han et al (1991a) have argued that the "alternative" pathway is actually the major determinant for TNF α mRNA degradation as it apparently operates constitutively, whereas the pathway which operates through the 3' UTR has to be induced by LPS. Evidence to support this has come from the studies of Peppel et al (1991) who demonstrated that degradation of IFN β mRNA in the L929 fibroblast cell line was independent of the presence or absence of an AU-rich element in the 3'UTR.

However, the AU-rich element was shown to be necessary for the increased turnover of IFN β mRNA induced by glucocorticoids.

Recent studies have indicated that the stability of several labile mRNA species can be increased on cellular stimulation, increasing the level of mRNA accumulated above that which would be achieved through transcription alone, for example the induction of GM-CSF and IL-3 mRNA in the mast cell line PB-3c by calcium and phorbol ester (Wodnar-Filipowicz and Moroni 1990). Indeed the induction of several cytokine mRNAs, such as that of IL-2, GM-CSF, TNF α and IFN γ in normal human T cells in response to mitogenic antibody and of GM-CSF (Thorens et al 1987) and JE (Koerner et al 1987) by LPS in murine peritoneal macrophages can take place in the absence of any change in transcriptional activity and hence are probably the result of increased mRNA stability.

Koeffler et al (1988) have demonstrated that the accumulation of GM-CSF mRNA in human fibroblasts by activators of protein kinase C, cycloheximide, IL-1 β and TNF α is achieved by a similar mechanism. The mRNA sequence-dependency of the stabilising effects of these agents has been investigated by Akashi et al (1991).

Removal of the 3'UTR AUUUA sequences completely abolished the ability of protein kinase activators and cycloheximide to confer increased stability, indicating that the AUUUA motifs may play a dynamic role in the regulation of mRNA stability. However, enhancement of GM-CSF mRNA instability by TNF α was independent of the presence or absence of the AUUUA motifs, demonstrating that

other mechanisms for stabilisation, presumably related to the utilisation of alternative degradative pathways may operate depending on the particular circumstance.

Modulation of mRNA stability appears to be a target for some agents which downregulate the expression of cytokine genes. For example, Poon et al (1991) have demonstrated that the inhibition of platelet-derived growth factor (PDGF)-induced expression of JE mRNA by the synthetic glucocorticoid dexamethasone, is mediated through destabilisation of the mRNA. Similarly, Lee et al (1988) have demonstrated that down-regulation of phorbol ester-induced expression of IL-1 β mRNA by the synthetic glucocorticoid dexamethasone is mediated through a reduction in the stability of cytoplasmic mRNA.

Further work on the molecular mechanisms underlying the modulation of labile mRNA stability is required to clarify the exact relationships between these competing effects.

1.4.5 Translational Control.

Another level at which the expression of cytokine genes can be regulated is that of the efficiency of translation into the protein product.

Various features of mature mRNA molecules are known to be necessary for the efficient translation of mRNAs in eukaryotes. For example, the presence of a cap structure, a favourable sequence surrounding an in-frame initiation codon at the 5' end of the molecule and the absence of stable secondary structures are all prerequisites for translation. In addition, the poly(A) tail at the 3' end of the mRNA is known to promote the initiation of translation. In yeast this effect is mediated through the poly(A)-binding protein whose gene is required for viability (reviewed in Jackson and Standart 1990).

Certain mRNAs, when injected into maturing and fully grown oocytes, such as that for tissue-type plasminogen activator (u-PA) in mouse oocytes (Huarte et al 1992)

can be stored in a translationally silent form by specific shortening (from greater than 150 to 15-90 nucleotides) of the poly(A) tail (de-adenylation) on entry to the cytoplasm. As the oocyte enters meiosis, re-adenylation takes place and the mRNA is translated (reviewed in Bacharova 1992).

Two sequence components of the mRNA 3' UTR have been shown to be necessary for readenylation to take place. First, the motif AAUAAA, which is also necessary for the polyadenylation of nascent RNA transcripts in the nucleus. Secondly, a signal known as the cytoplasmic polyadenylation element (CPE) or adenylation control element (ACE) which lies at a variable distance from the polyadenylation signal. The sequence element required for subsequent readenylation lies within the same ACE required for deadenylation to take place. The polyadenylation signal is not required for this process.

The consensus sequence for the ACE in the frog is UUUUUAU (Fox et al 1989, McGrew et al 1989). Huarte et al (1992) have demonstrated the sequence AUUUUAAU to be essential deadenylation and readenylation of both mouse and rat tissue-plasminogen activator mRNA. Therefore it seems likely that the AUUUUAAU sequence is the mouse and rat equivalent of the ACE sequence identified in the frog. This is strikingly similar to the (AUUUA)_n consensus sequence of the AU-rich elements in the 3'UTR shown to be at least partly responsible for conferring instability on cytokine and nuclear proto-oncogene mRNAs and discussed in the previous section (Caput et al 1986, Shaw and Kamen 1986, Brewer and Ross 1988, Wilson and Treisman 1988).

It is possible, therefore, that these sequences of cytokine mRNAs might also play a role in the control of production of cytokine proteins through modulation of the ability of the mRNAs to translated.

Messenger RNAs which are labile in murine and human cells are stable when injected into *Xenopus* oocytes. Kruys et al (1989) have used this system to

demonstrate that the translation of IFN β and GM-CSF mRNA is precluded by the presence of the 3'AU-rich element.

Han et al (1990) have used the transfection of appropriate chimaeric TNF α genes in the macrophage cell line RAW 264.7 to demonstrate that the 3'UTR of TNF α mRNA suppresses its translation, specifically through AUUUA motif. This process is separate from mRNA degradation as the recombinant mRNAs generated in these studies were completely stable over the course of the experiment.

This translational suppression can be overcome by LPS. The presence of the AUUUA sequence is also necessary for derepression to take place, although the presence of other regions of the 3'UTR greatly increases the magnitude of the derepression.

The mechanism by which the modulation of translational efficiency is carried out is not clear; obviously reversible deadenylation is a likely possibility.

1.4.6 Control of Cytokine Secretion.

It is axiomatic that cytokines must be secreted, or at least presented on the cell surface, by the producer cell before they can exert their effects on other cells.

Proteins to be secreted are synthesised in a precursor form which contains amino acid residues at the amino-terminal end additional to the mature form of the molecule.

These additional residues represent a signal sequence which is involved in the secretion of the protein (Alberts et al 1989). The protein coding sequences in the mRNA of most cytokines encodes putative signal sequences, indicating that these proteins are secreted via a conventional route.

While macrophages, T cells and B cells do not appear to store preformed cytokine proteins, mast cells have been demonstrated to contain extensive preformed stores of, at least, TNF α that are available for immediate release on appropriate stimulation of the cell (Plaut et al 1989, Gordon et al 1990). Therefore, it is possible that control of

secretion may play a part in the regulation of cytokine activity in certain circumstances.

IL-1 α and IL-1 β are unusual cytokines with respect to processing and secretion in that they are synthesised as precursor (31kDa) forms which are approximately twice the size of the mature form (17.5kDa). Only the mature form is secreted, by a route separate from other cytokines, and is biologically active (Dinarello 1991). The production of the mature form of IL-1 β in human cells has been shown to be dependent on the activity of a specific cytosolic protease, the IL-1 β converting enzyme (Black et al 1989, Kostura et al 1989). Ray et al (1992) have recently demonstrated that the decrease in IL-1 β activity seen on cowpox virus infection of human cells is mediated by a virally-encoded specific inhibitor of the IL-1 β converting enzyme. So, it seems that the control of cytokine protein processing and/or secretion, at least in disease states, may play a role in regulating the production of these molecules.

1.5 Expression of the SCI/MIP-1 α and MIP-1 β Genes

1.5.1 Expression of MIP-1 Family Genes.

In addition to the fact that the members of the MIP-1 family are structurally related, they also appear to display some similarities in their patterns of gene expression. Most of the members of the MIP-1 family were initially described as sequences which are rapidly and greatly inducible on cellular activation and this property is one of the most noticeable features of the expression of these genes. In fact, on induction, the mRNA for these genes can represent about 1% of the poly(A)+ RNA in the stimulated cell (Davatellis et al 1988, Brown et al 1989). Murine JE and hMCP-1 appear to be the most widely expressed in that mRNA and/or protein can be detected in a range of cell-types, including macrophage/monocytes, fibroblasts, and other

connective tissue cells and cells associated with epithelial tissues. JE was first identified as a sequence inducible on platelet-derived growth factor (PDGF) stimulation of fibroblasts (Cochran et al 1983) and is a classical "immediate-early" gene in that accumulation of its mRNA is induced within minutes of stimulation and is independent of de novo protein synthesis (for reviews see Rollins and Stiles 1989, Rollins 1991).

The expression of the other members of the family appears to be exclusively restricted to leukocytes (for review see Schall 1991).

It should be noted, however, that RANTES and C10 do not conform to the general pattern for MIP-1 gene expression. Schall et al (1988) demonstrated that mRNA for RANTES was induced on stimulation of peripheral blood leukocytes but only reached a peak after 5 to 7 days, while antigen stimulation of a cytotoxic T cell line resulted in a decrease in message (Schall et al 1988). The expression pattern of C10 is also context dependent (Orlofsky et al 1991).

1.5.2 Expression of MIP-1 α and MIP-1 β .

The murine SCI/MIP-1 α and MIP-1 β genes appear to be generally co-expressed and so far their expression appears to be confined to haemopoietic cells, more particularly macrophage and monocytic cell lines (Davatellis et al 1988, Orlofsky et al 1991, Widmer et al 1991, Martin and Dorf 1991) T cell lines (Brown et al 1989), mast cell lines (Burd et al 1989) and purified epidermal Langerhan's cells (G.Graham, K.Parkinson personal communication).

A similar pattern of expression appears to be emerging for the human equivalent genes. Both of these genes have been shown to be expressed in human monocytes, human monocytic cell lines, peripheral blood mononuclear cells (PBMCs), human peripheral blood T cells, human T cell lines, human B cells and freshly harvested

leukemic cells. There is one report of expression in cultured primary human fibroblasts and a human glioma cell line treated with PMA (Nakao et al 1990) but it is possible that this represents cross-hybridisation from a related member of the MIP-1 family which may possibly have not yet been described.

Expression in monocytes and macrophages.

A cDNA clone for SCI/MIP-1 α was first isolated by Davatelis et al (1988). Using this sequence as a probe for the expression of SCI/MIP-1 α mRNA they demonstrated that the message was barely detectable in unstimulated macrophage cell line RAW 264.7 but was rapidly induced (within one hour) after stimulation with LPS with the level of SCI/MIP-1 α mRNA reaching a peak at around 8 hours and staying elevated for at least 16 hours. The level of mRNA obtained after six hours was shown to be dependent on the dose of LPS. Orlofsky et al (1991) and Widmer et al (1991) reported similar kinetics in the same cell line. Widmer et al (1991) also reported that the level of MIP-1 β mRNA, while following similar kinetics to that of SCI/MIP-1 α , declined to a lower level after the early peak although it still remained above the basal level. No data other than the kinetics of mRNA accumulation was reported by these investigators.

The most extensive study of the expression of both SCI/MIP-1 α and MIP-1 β genes in murine macrophages so far has been carried out by Martin and Dorf (1991) in the macrophage cell line J774.1 and in the monocytic cell line P388D1. They demonstrated that LPS, used at a suboptimal 50ng/ml, increased the expression of mRNA for both SCI/MIP-1 α and MIP-1 β 4 to 7-fold above constitutive levels after six hours. The level of mRNA for JE was equally elevated by the same treatment. The kinetics of accumulation was not reported.

The macrophage activating factor interferon- γ (IFN γ) only slightly increased (1.5-fold) levels of mRNA for both SCI/MIP-1 α and MIP-1 β but increased the levels of

mRNA for JE approximately 7-fold in both cell lines. IFN γ also augmented the increase in JE mRNA induced by LPS but was ineffective in respect of SCI/MIP-1 α and MIP-1 β . Neither IL-1 α nor TNF α had any effect on the expression of MIP-1 α , MIP-1 β or JE in these cells nor did they enhance the increase in mRNA levels stimulated by LPS.

Martin and Dorf also investigated the effects of pharmacological agents on the accumulation of these mRNAs. Prostaglandin E1 (PGE1), which is produced by macrophages in response to various agents including LPS, IL-1 α and TNF α and has immunosuppressive properties (Phipps et al 1991) was able to reduce both constitutive and LPS-induced levels of SCI/MIP-1 α and MIP-1 β mRNA by 50-70% and JE mRNA levels by 43%. This suggests the possibility of PGE1 playing a feedback role in the controlling the accumulation of these mRNAs.

The effects of PGE1 could be almost exactly duplicated by dibutyl cyclic AMP (dcAMP), the soluble analogue of cyclic AMP (cAMP), save for the fact that dcAMP was ineffective in reducing constitutive levels. Increases in the levels of the three mRNAs induced by PMA were also inhibited by dcAMP. The effects obtained with PGE1 and dcAMP are consistent as the effects of prostaglandins of the E series are mediated through increases in intracellular cAMP (Phipps et al 1991).

Interestingly, in the same study, the expression of both mRNA and protein for IL-6 and TNF α was investigated. It was found that while LPS, PMA and IFN γ could increase the expression of mRNA for these genes, this was accompanied by an increase in protein secretion only with LPS. Such effects have also been reported for the expression of IL-1 α and IL-1 β (Dinarello 1991). It would obviously be of great interest to find out whether the expression of SCI/MIP-1 α , MIP-1 β and JE is similar in this respect.

The expression of hMIP-1 β (Act-2) mRNA has been shown to be rapidly inducible in isolated primary human monocytes, but not lymphocytes, by LPS, IL-7, calcium

ionophore and PMA (Lipes et al 1988, Ziegler et al 1991, Sporn et al 1990). Sporn et al (1990) also observed that mRNA for hMIP-1 β (MAD-5) was rapidly induced on adherence of human monocytes to plastic, type I collagen, immune complex and fibronectin. These data again demonstrate the ability of stimuli associated with the activation of macrophages to induce accumulation of one of the MIP-1 mRNAs. Ziegler et al (1991) demonstrated the ability of IL-4 to down-regulate both the LPS and the IL-7-induced rise in hMIP-1 β mRNA. Despite the fact that they identified three glucocorticoid response elements in the 5' regulatory region of this gene, they found that dexamethasone was unable to inhibit the induction of hMIP-1 β mRNA by LPS. However, in this study, the concentration of LPS used was 10 μ g/ml, which is almost certainly a vast excess when it is considered that murine MIP-1 β gene expression can be elevated by as little as 50ng/ml LPS; therefore it is possible that dexamethasone may successfully oppose the action of lower concentrations of LPS. Three investigators have studied the expression of either both human MIP-1 α and MIP-1 β (Zipfel et al 1989) or human MIP-1 α alone (Nakao et al 1990, Yamamura et al 1989) in human monocytic cell lines.

In the promyelocytic cell line HL60, Zipfel et al (1989) found that although expression of neither hMIP-1 α or hMIP-1 β could be detected in untreated cells, treatment with PMA, which induces a monocytic phenotype, induced expression of both transcripts when measured at both 4.5 and 72 hours. Treatment of HL60 with dimethyl sulphoxide, which induces a granulocytic phenotype, did not induce these mRNAs. It is possible, therefore, that accumulation of these transcripts following treatment with PMA is mediated indirectly through the differentiation of the cell rather than by a direct action on gene expression. However, as PMA has been shown to increase expression of (murine) SCI/MIP-1 α and MIP-1 β mRNA in J774.1 cells it is also possible that PMA is regulating both processes independently.

Both Yamamura et al (1989) and Nakao et al (1990) followed the kinetics of expression of human MIP-1 α (LD78) in the promonocytic cell line U937 after treatment with both PMA (to induce a mature monocytic phenotype) and PMA plus LPS. In both cases mRNA was detected after 2 hours with a peak of expression at 4-12 hours and then declined. A similar pattern was observed in the promyelocytic cell line HL60 by Nakao et al (1990) after treatment with PMA and the protein synthesis inhibitor cycloheximide. It should be noted, however, that Yamamura et al (1989) detected expression of hMIP-1 α mRNA in untreated HL60 cells. Expression was abolished completely by the transcriptional inhibitor actinomycin D which suggests that the mRNA is labile and that its presence is dependent on continuous transcription. Cycloheximide, however, induced a higher level of expression at one hour which then declined and had returned to resting levels by six hours. Nakao et al (1990) found that addition of cycloheximide did not inhibit the induction of hMIP-1 α (LD78) mRNA by PMA plus LPS which indicates that this induction is independent of de novo protein synthesis and indeed cycloheximide increased over four-fold the level of message obtained after treatment with PMA.

Expression in Lymphocytic Cells.

Brown et al (1989) have demonstrated that mRNA for both SCI/MIP-1 α (TY5) and MIP-1 β (H400) are induced on ConA-mediated activation of murine T helper cell lines. Similarly, Kwon and Weissman (1989) described SCI/MIP-1 α as a sequence induced on ConA stimulation of the T cell line L2.

Expression of the human genes in lymphocytic cells has been more extensively studied.

Zipfel et al (1989) demonstrated using isolated peripheral blood T cells that hMIP-1 α (pAT464) and hMIP-1 β (pAT744) were co-induced by four and a half hours by cycloheximide, PHA, PHA plus PMA, and PHA plus PMA plus cycloheximide in

ascending order of effectiveness. While PMA synergised with PHA it had no effect on the expression of these genes on its own. The immunosuppressant drug cyclosporin A (CsA) completely abolished the induction of the two messages by PHA plus PMA.

Chang and Reinherz (1989) observed that antibody versus the cell surface determinant CD2 induces a characteristic pattern of expression of hMIP-1 β mRNA in peripheral blood lymphocytes and in human peripheral blood T cells. Induction is rapid, peaks within two hours, and then decreases. A second peak of expression is seen at around twenty-two hours.

Taking these data together, it can be seen that T cell activation is necessary for the accumulation of hMIP-1 α and hMIP-1 β mRNA. On activation, accumulation is rapid, suggesting that expression of these genes, as in macrophages, is part of the early response to cellular stimulation.

Several groups have also investigated the expression of the two human genes in human peripheral blood mononuclear cells (PBMC) which consist mainly of lymphocytes with some monocytes (Lipes et al 1988, Chang and Reinherz 1989, Blum et al 1990, Siderovski et al 1990) and in human T cell lines (Lipes et al 1988, Chang and Reinherz 1989, Zipfel et al 1989). The characteristics of expression in these cells are, not surprisingly, extremely similar to that in isolated human T cells and serve to reinforce the idea of the expression of the two genes being induced early in T cell activation.

Yamamura et al (1989) have also demonstrated that both mRNA and protein for hMIP-1 α (LD78) is constitutively expressed in the human T-lymphotropic virus 1-infected T cell line MJ. The level of mRNA was not reduced on exposure to actinomycin D for upto six hours which suggests that the mRNA may be fairly stable in this cell line which was in contrast to the observation made in the promyelocytic HL60 cell line by the same authors.

Schall et al (1992) have demonstrated that both hMIP-1 α and hMIP-1 β are induced rapidly and transiently by the T cell line HA1.7 upon activation by either anti-CD3 antibody or anti-CD3 antibody plus IL-2. This rise in mRNA is mirrored by levels of secreted protein. However, when this cell line is made anergic (non-responsive to antigen) by exposure to the superantigen Staphylococcus Aureus enterotoxin B the levels of hMIP-1 α and hMIP-1 β induced are higher and these levels are sustained. Interestingly, the mRNA levels are not reflected in the level of MIP-1 protein secreted which suggests that there may be an uncoupling of mRNA expression and either translation or protein secretion in anergic cells.

Expression in Mast Cells.

There has so far been one report of expression of the MIP-1 mRNAs in mast cells. Burd et al (1989) demonstrated the expression of mRNA for both SCI/MIP-1 α and MIP-1 β in three out of four cloned murine mast cell lines in response to activation by specific antigen and Immunoglobulin E (IgE). This induction was blocked by the anti-inflammatory agent cyclosporin A (CsA). The expression of mRNA for TCA3 was identical to that of the MIP-1 genes. Levels of mRNA for JE, which is expressed constitutively in these cells, was unaffected by these treatments, indicating that the mechanisms underlying the expression of JE and the MIP-1 genes may be overlapping judging by the similarity of their expression patterns but they are not identical.

The characteristics of the accumulation of the MIP-1 mRNAs in mast cells again reinforces the idea of rapid accumulation of SCI/MIP-1 α and MIP-1 β mRNA being associated with cellular activation. The fact that the genes can be expressed in mast cells, adds to the number of processes in which the gene products could potentially be involved.

Expression in Leukemic Samples.

Yamamura et al (1989) found that mRNA for hMIP-1 α (LD78) was expressed in 8 from 8 fresh marrow sample from patients with Acute Non-Lymphoblastic Leukaemia (ANLL). ANLL samples with the most monocytic morphology expressed the highest levels. Two from three acute lymphocytic leukaemia (ALL) samples, eight from nine B cell-ALL samples and one of three T cell-ALL samples expressed detectable mRNA for hMIP-1 α . LD78 protein could be detected by Western blot in two out of four of the ATL cases but in no other samples.

Interestingly, the expression of a number of cytokine genes (Arai et al 1990, Chan et al 1986) including hMIP-1 α as discussed above (Yamamura et al 1989) have been shown to be upregulated in adult T cell leukemia (ATL) and derived cell lines. The etiological agent of this condition is human T lymphotropic virus 1 (HTLV1).

A nuclear protein encoded by the HTLV-1 genome, p40^{tax}, functions as a positive regulator of the promoters of a number of genes including those of IL-2 and GM-CSF (Maruyama et al 1987, Miyatake et al 1988), possibly through activation of NF κ B. It is possible that such a mechanism might also be responsible for the upregulation of hMIP-1 α expression.

Whether similar mechanisms might operate in other leukaemic cells is unclear; however it is highly likely that cytokine genes including hMIP-1 α and MIP-1 β are dysregulated in some way in transformed cells.

1.5.3 Summary

The characteristics of expression of both the murine and human MIP-1 α and MIP-1 β genes follows a pattern which now appears to be typical for most of the members of the MIP-1 family. Expression appears to be restricted to haemopoietic cells and the

accumulation of mRNA is either induced or greatly enhanced by the appropriate activating stimuli.

In all cases, the accumulation of mRNA appears to be extremely rapid following stimulation and, at least in primary cells, accumulation is transient. In several reports, the mRNA accumulation was unaffected by the presence of cycloheximide, indicating that this is independent of de novo protein synthesis. These characteristics are indicative of the expression of these genes being a part of the early response to the activation of either macrophages, T cells, B cells or mast cells.

It is interesting to note that mRNA accumulation can be downregulated by an immunosuppressant agent in macrophages, T cells and mast cells. Presumably this reflects the inflammatory role of the products of these genes. This may be a major physiological route for downregulating the expression of these genes.

It should also be noted that studying expression of the MIP-1 genes in cell lines may give very different results from using primary cells. For example, a high level of unstimulated expression of hMIP-1 α mRNA is seen in the T cell line MJ and in HL60 cells (in one report). The fact that some leukemic cells also express high levels of hMIP-1 α mRNA suggests that the expression of this gene might be dysregulated in transformed cells. For this reason, it is likely that primary cells would be most appropriate for the study of the regulation of expression of these genes.

1.6 Bone Marrow-Derived Macrophages.

The Resident Bone Marrow Macrophage (RBMM) population is believed to be quite distinct from the rapidly proliferating, immature, developing members of the monocyte/macrophage lineage of the haemopoietic system (monoblasts, promonocytes, monocytes) which outnumber the RBMM by five to one (Crocker et al

1988). In contrast to the developing haemopoietic cells RBMM appear to be non-replicating end cells (Crocker and Gordon 1985).

RBMM have been identified in situ by criteria such as morphology, ultrastructure, the presence of phagocytic inclusions and enzyme histochemistry and appear to be evenly distributed throughout the marrow (Hume et al 1984). It is thought that the stromal macrophages of the marrow are predominantly of this type.

In vitro, functional, studies have, unfortunately, been hampered by the inability of investigators to purify RBMM (Crocker et al 1988). As experimental surrogates, therefore, other types of recruited and cultured ex vivo populations of macrophages have been used to study macrophage function both as inflammatory macrophages and as surrogates for RBMM (Pojda et al 1989, Rich 1988).

1.6.1 Bone Marrow-Derived Macrophages and their Proliferation.

One cultured population which has been used to study macrophage gene expression and effector function and the biochemistry of the colony stimulating factors is the so-called bone marrow-derived macrophage (BMDM or BMM) population. This population is cultured directly from normal unfractionated bone marrow in vitro using a source of Colony Stimulating Factor 1 (CSF-1). The BMDM are derived directly from progenitors of the monocytic lineage in the marrow and more than ninety five percent of this population expresses the CSF-1 receptor (Tushinski et al 1982, Tushinski and Stanley 1985). Tushinski et al (1982) observed that the cells were dependent on a source of CSF-1 for survival and proliferation. Submitogenic concentrations of CSF-1 were still able to promote survival of BMDM. They also demonstrated, using iodinated CSF-1, that the molecule is selectively degraded by the BMDM over the range of concentrations which stimulate both cell survival and proliferation.

The proliferative response of BMDM to CSF-1 has been analyzed by Tushinski and Stanley (1985) who demonstrated, using tritiated thymidine labelling of nuclei, that 93-98% of the BMDM were in cycle when cultured in the presence of both CSF-1 and serum (15% fetal calf serum). Tushinski and Stanley (1985) reported that in the presence of serum, but in the absence of CSF-1, DNA synthesis was reduced by 98%. This was concomitant with a 35% reduction in total cellular protein synthesis, a 76% reduction in ribosomal protein synthesis and a 96% reduction in the synthesis of histones compared to exponentially growing cells. When cells deprived of CSF-1 (in 15% serum) for 10-12 hours were refed with CSF-1 they entered DNA synthesis after a lag period of approximately 12 hours in a concentration-dependent manner (Tushinski et al 1982; Tushinski and Stanley 1985).

Assessment of labelled nuclei after treatment of BMDM with tritiated thymidine indicated that these cells had a doubling time of 24-28 hours and an S-phase time of 8-9 hours. The continuous presence of CSF-1 up until the end of G1 phase is required for DNA synthesis to take place, indicating that the early programme of events induced by CSF-1 is insufficient on its own to induce proliferation. However, once cells have begun to synthesise DNA they can complete the cell cycle in the absence of CSF-1.

Matsushime et al (1991a) reported similar observations in the BMDM-like cell line BAC1.2F5, which is dependent on either CSF-1 or GM-CSF for survival, in response to CSF-1 using flow cytometric analysis of cellular DNA content.

In other systems in which cellular proliferation in response to growth factors has been studied, such as the Balb/c 3T3 fibroblast system, the sequential action of more than one growth factor is required to drive the cell from the resting quiescent (G0) state through early and late G1 and onto DNA synthesis (Rollins and Stiles 1989).

Tushinski and Stanley (1985) have cited the fact that the presence of CSF-1 is able to induce progression from the resting state to S-phase to argue that in the BMDM

system, this process can be driven by CSF-1 alone. However, two qualifications must be made to this statement. Firstly, BMDM, because of their dependence on CSF-1 for survival do not enter a G₀ quiescent state on the removal of growth factor but arrest in G₁ and then die (Sherr 1991). Therefore, CSF-1 may be driving cellular proliferation from a different point in the cell cycle. Secondly, it should be borne in mind that all of the studies of BMDM proliferation described above were carried out in the presence of serum (15% FCS) which means that a component(s) of serum could be contributing to the induction of proliferation. Indeed, two reports have demonstrated a need for the presence of serum for the optimal mitogenic response to CSF-1. Hume and Gordon (1984) reported that the proliferation of BMDM was dose-dependent on serum even with optimal CSF-1. In the absence of serum, but in the presence of optimal CSF-1, proliferation was negligible. Tushinski and Stanley (1985) also reported that CSF-1-induced proliferation was reduced by approximately 60% in the absence of serum. Tushinski and Stanley argued that this was due to non-specific trophic effects. However, this issue can only be resolved by restoration of a full proliferative response to CSF-1 by BMDM in a defined serum-free medium which has not yet been achieved. It would appear, therefore, that the proliferation of BMDM is absolutely dependent on the presence of CSF-1 but that other factors (as yet unidentified) may play an auxiliary role.

Interestingly, the initiation of BMDM cultures from normal bone marrow can be achieved using partially purified but not pure CSF-1 (even in the presence of serum) which suggests that additional factors to CSF-1 may be involved in the development of BMDM as well as in their proliferation.

Hamilton et al (1988) have also investigated the ability of other growth factors to stimulate the proliferation of BMDM, both alone and in combination with CSF-1. They observed that recombinant murine Granulocyte Macrophage-Colony Stimulating Factor (rmGM-CSF) could induce incorporation of tritiated thymidine in

BMDM with approximately one fifth of the efficiency of murine placental CSF-1. Recombinant murine interleukin-3 (rmIL-3) and the phorbol ester, and protein kinase C activator 12-O-tetradecanoyl-phorbol-13-acetate (TPA) were weak mitogens. Purified murine G-CSF had no detectable effect.

These cytokines also displayed synergy in their ability to induce proliferation in BMDM. In addition, TPA displayed dramatic synergy with each of CSF-1, GM-CSF and IL-3.

The macrophage activating factors LPS and ConA were not mitogenic for BMDM and indeed LPS almost completely inhibited the CSF-1 induced proliferation of BMDM, even at optimal CSF-1 concentrations.

Vairo et al (1991) have shown that the inhibition of CSF-1-stimulated BMDM proliferation by $\text{TNF}\alpha$, $\text{IFN}\gamma$ and LPS is not due to a general loss of responsiveness to CSF-1; the ability of all three of these agents to also inhibit proliferation stimulated by GM-CSF and IL-3 suggests that they act at a post-receptor level.

The synthetic glucocorticoid dexamethasone and also prostaglandin E2 (PGE2) have also been shown to inhibit CSF-1-induced DNA synthesis in BMDM; this effect can however be overcome at higher concentrations of CSF-1 (Hamilton et al 1988; Hume and Gordon 1984; Hamilton et al 1991). Agents which increase the intracellular levels of cyclic AMP (e.g. cholera toxin and PGE2) have been shown to inhibit the proliferation of BMDM late in G1 (Vairo et al 1990).

1.6.2 Macrophage Effector Functions and BMDM.

The ability of BMDM to carry out the effector functions of macrophages has also been investigated. Phillips and Hamilton (1989) and Hamilton et al (1989) have demonstrated that BMDM can be induced to produce the superoxide anion by TPA after they have been primed first by the growth factors GM-CSF and IL-3 (but not CSF-1) and macrophage activating factors such as LPS.

Knight et al (1992) have demonstrated the stimulation of fluid-phase pinocytosis by BMDM after stimulation by CSF-1, GM-CSF, IL-3 and LPS. Again, this function can be dissociated from the proliferation of these cells.

1.7 Gene Expression in Bone Marrow-Derived Macrophages.

Bone marrow-derived macrophages have proved a suitable system for the investigation of the inducible expression of several genes.

The genes expressed in BMDM in response to stimuli can be assigned to two groups; those coding for products involved in cell maintenance and the mitogenic response and those coding for products involved in the functional activities of macrophages. Gene expression in an SV40-transformed cell line BAC1.2F5, which has similar properties to BMDM, which have very similar growth properties to BMDM will also be considered where appropriate.

1.7.1 Genes involved in the mitogenic response.

c-fos, c-myc

The proto-oncogenes c-myc, c-fos and c-jun have been implicated in the proliferative response to growth factors in a number of cell types. All three gene products appear to be transcription factors; c-myc protein on its own and c-fos and c-jun as a dimer known as AP-1. These products appear to exert their effects on the cell cycle by affecting the expression of other genes, most probably those responsible for progression through the later stages of the cell cycle.

Hamilton et al (1989) demonstrated transient expression of mRNA for c-fos and c-myc in BMDM which had been starved of CSF-1 in response to a variety of mitogenic agents (CSF-1, GM-CSF, IL-3, TPA, exogenous phospholipase C).

Messenger RNA for c-fos was detectable after 20 minutes and then declined rapidly, while c-myc mRNA had its maximum level of expression at one hour and declined

thereafter. The level of mRNA for both genes is dependent on the dose of CSF-1. The observed kinetics place these expressions in the "immediate early" time frame which is in agreement with that seen in other cell types (Rollins and Stiles 1989).

Agents which "activate" macrophages by other criteria but were not mitogenic (ConA, Zymosan, FMLP and calcium ionophore) did not stimulate expression of c-fos or c-myc. These results are consistent with expression of c-fos and c-myc playing an essential role in the proliferation of BMDM.

Orlofsky and Stanley (1987) demonstrated a similar pattern of expression for c-fos and c-myc in response to CSF-1 or GM-CSF stimulation in BAC1.2F5 cells.

1.7.2 Genes encoding secreted molecules.

Macrophages play roles in the response to infection and in tissue remodelling (Adams and Hamilton 1984) as well as being an important component of the haemopoietic microenvironment (Rich 1988, Greenberger 1991). One of the ways in which macrophages exert an effect on other cell types is by the secretion of signalling molecules which can affect the behaviour of other cell types. One recent review estimated the number of secretory products produced by macrophages to be over three hundred (Nathan 1987). Not all of these products are secreted by a given macrophage at any one time which indicates that heterogeneity within the macrophage population may be an important consideration of the biology of any secreted product (Rich 1988).

JE and KC

Orlofsky and Stanley (1987) demonstrated that mRNA transcripts for the MIP-1 family member JE and the MIP-2 family member KC were inducible by the action of CSF-1 on BAC1.2F5 cells.

The protein products of JE and KC are secreted and can affect the behaviour of monocytes (Rollins 1991) and tumour cells (Schall 1991), respectively.

The kinetics of expression of these genes was similar to that observed in PDGF-stimulated 3T3 cells (Cochran et al 1983) in that accumulation of mRNA was seen within twenty minutes and reached a peak after 4 hours and was still elevated at eight hours. The levels of expression at times later than eight hours was not reported.

The expression of JE, but not KC, could be similarly induced by GM-CSF which suggests differences in the pathways for the signals leading to the induction of these genes. This result also demonstrates that expression of KC is not essential for proliferation of BAC1.2F5 cells as these cells proliferate in response to GM-CSF as well as CSF-1.

Although the expression of JE, KC and c-fos and c-myc (see above) is temporally associated with the early response to proliferation, Orlofsky and Stanley (1987) demonstrated that induction of these genes could be dissociated from the cellular response to a mitogen. All four of these genes could be induced by CSF-1 in a BAC1.2F5 variant (Aut1) which can proliferate independently of exogenous growth factor which suggests that the pathways mediating progression through the cell cycle and the early expression of genes are different. In addition to this, all four genes can be greatly elevated by CSF-1 in cells previously made asynchronous with respect to the cell cycle by prolonged culture in GM-CSF; this again demonstrates that the early gene programme can be induced independently of the induction of mitogenesis.

It should be noted, however, that these experiments do not disprove a role for c-fos or c-myc (or JE for that matter) in the early stages of cell cycle progression, although Rollins and co-workers have been unable to demonstrate any role for JE in the proliferative response (Rollins 1991).

IL-1 α , IL-1 β and IL-1ra

IL-1 α and β are pro-inflammatory cytokines which exert effects on practically every tissue of the body, including induction and suppression of gene expression, and cellular activation. The genes for IL-1 α and IL-1 β are expressed in a wide variety of cell-types. A natural inhibitor of the action of these molecules, IL-1 receptor antagonist (IL-1 ra) has been identified and the cDNA cloned in the mouse (Matsushime et al 1991b). This field has recently been reviewed by Dinarello (1991). Matsushime et al (1991b) also demonstrated that CSF-1 induces the expression of murine IL-1ra mRNA within an hour in BAC1.2F5 cells and the level of this message continues to increase for five hours. This induction is unaffected by cycloheximide indicating that the induction is independent of *de novo* protein synthesis. In BMDM, expression of IL-1ra mRNA was induced within 45 minutes by CSF-1. The level of the mRNA continued to rise with time and peaked at around ten hours after which they declined. Messenger RNA for IL-1 α and IL-1 β , on the other hand, was induced early and peaked at around 6 hours and had returned to undetectable levels by twenty hours. Induction of IL-1 α was inhibited by cycloheximide whereas it increased the level of IL-1 β induced by CSF-1. In this study, all three of the mRNAs were also induced in BMDM by LPS. These data would put place expression of IL-1 β and IL-1 ra in the immediate early category whereas the expression of IL-1 α mRNA may be dependent on the expression of other genes.

Urokinase-type Plasminogen Activator

The expression of mRNA and protein for urokinase-type plasminogen activator (u-PA) has also been studied in BMDM (Hamilton et al 1991). Plasminogen activators are serine proteases that cleave plasminogen to create plasmin, another serine protease. They are expressed by a large number of cell types and appear to be

associated with processes such as tissue remodelling and cell migration (for review see Saskela et al 1985).

Expression of u-PA mRNA and activity by BMDM deprived of a source of CSF-1 for 18 hours was negligible. However, CSF-1 induced expression of u-PA mRNA after one hour and peak levels were seen between two and eight hours. By thirteen hours u-PA mRNA had declined to a lower, but still detectable, level and was still at this level twenty four hours after stimulation. The level of u-PA mRNA expressed after three hours was dependent on the dose of CSF-1 as was the production of u-PA activity in the culture medium. The CSF-1-mediated production of u-PA activity was abrogated by the addition of the transcriptional inhibitor actinomycin D to the culture which suggests that transcription is necessary for CSF-1 induced expression of this gene. U-PA mRNA and activity was also induced by treating BMDM with GM-CSF or IL-3 but G-CSF was ineffective at inducing u-PA activity in the culture medium. The macrophage activator ConA also induced u-PA mRNA; therefore it seems that u-PA expression can be induced both by specific cytokines and by non-specific macrophage activating agents. The synthetic glucocorticoid dexamethasone also inhibited CSF-1 induced production of mRNA and u-PA activity. A direct causal relationship between macrophage proliferation and u-PA gene expression has been proposed by both Hume and Gordon (1984) and Grimaldi et al (1986). However, despite the fact that the gene is expressed as part of the early response to growth factors, Hamilton et al (1991) identified conditions where gene expression was induced in the absence of proliferation (such as induction by ConA) and those in which proliferation took place in the absence of u-PA expression as the down-regulatory effect of dexamethasone on BMDM proliferation but not on u-PA mRNA expression could be overcome by high levels of CSF-1. Therefore, it appears that, like the expression of JE and KC, the expression of this gene can be induced separately from the induction of proliferation.

Taken together, the work discussed in this and the last section indicate that bone marrow-derived macrophages constitute a system suitable for the study of the regulation of gene expression in primary macrophages.

Aims of Thesis and Plan of Investigation

Identifying and understanding the signals and mechanisms underlying the production of negative and positive regulators of haemopoietic stem cell proliferation is essential for a full appreciation of the role played by these factors in the regulation of haemopoiesis and in other processes.

The central aim of this thesis was to study the regulation of expression of the gene for a negative regulator of haemopoietic stem cell proliferation, Stem Cell Inhibitor/ Macrophage Inflammatory Protein-1 α (SCI/MIP-1 α), which has recently shown to be functionally and antigenically identical to a previously described regulatory activity, Manchester Inhibitor, and that for a closely related molecule Macrophage Inflammatory Protein-1 β (MIP-1 β) in the context of haemopoietic regulation and other processes. The objectives were: a) to identify the characteristics of expression and to determine whether the expression of these genes was capable of being regulated by physiological signals, b) to identify the likely mechanisms underlying the observed behaviour, c) to identify putative physiological regulators of the expression of these genes, d) to investigate the relationship between the expression of these genes and the regulation of haemopoiesis and other processes.

The main approach taken was to study the regulation of expression of mRNA for SCI/MIP-1 α and MIP-1 β in a cell population approximating as closely as possible to the putative producers of the Manchester Inhibitor in the bone marrow.

The first step in this approach was to set up an *in vitro* cell culture system consisting of a defined population of macrophages cultured directly from normal murine bone marrow. This population was demonstrated by Northern and Western blotting, to express mRNA for SCI/MIP-1 α and MIP-1 β and SCI/MIP-1 α protein, respectively. Bacterial endotoxin was used as a model inducer, and hydrocortisone as a model downregulator, of the accumulation of both mRNAs. It was observed that the induction of accumulation of both SCI/MIP-1 α and MIP-1 β mRNA have

characteristics typical of genes expressed as part of the early phase of the response of macrophages to stimulation. These experiments, which helped define the parameters of the system, led to further studies aimed at testing candidate regulators of SCI/MIP-1 α and MIP-1 β gene expression in vitro. These experiments also allowed the relationship between the expression of SCI/MIP-1 α and MIP-1 β and the proliferation of this population to be investigated.

A complementary study was also carried out to investigate the relationship between SCI/MIP-1 α gene expression in vivo and the regulation of haemopoiesis by comparing the accumulation SCI/MIP-1 α mRNA in normal, unperturbed murine bone marrow to that in the marrow of animals treated with drugs demonstrated to have profound effects on the proliferation of haemopoietic stem cells.

Chapter 2

Materials and Methods

2.1 Mice.

All mice were housed within the animal facility of the Beatson Institute, twelve to a cage, and were fed water and mouse chow ad libitum. Female mice were used at age 8-12 weeks for all experimental procedures. For the culture of macrophages, bone marrow and spleens from normal untreated mice of the outbred strain CD1 (Charles River, UK) were used. For experiments involving drug treatment of animals, strain B6D2F1 mice, an F1 hybrid of C57Bl/6 (females) and DBA2 (males) strains (Harlan^{ol}-Olac, UK) were used.

2.2 Harvesting of Bone Marrow and Spleen Cells.

Bone marrow was obtained from the femora of mice. Mice were killed by either cervical dislocation or by suffocation with carbon dioxide. Femora were removed from the animal and cleaned of surrounding muscle tissue using a tissue soaked in 75% ethanol. Both ends of the femur were removed using scissors and the bone marrow plug expelled from the central cavity by inserting a 21 gauge needle into one end of the femur and flushing through 2ml of SLM/FCS (Table 2.1). The bone marrow plug was collected in a sterile container. The cells were then dispersed into a single cell suspension by vigorous pipetting and nucleated cells counted using a haemocytometer. Cells were diluted 1 in 10 with 0.23% methylene blue in 22% ethanol to enable discrete visualisation of nucleated cells. Ten to twenty million nucleated bone marrow cells were routinely obtained per femur. For experimental estimates of marrow cellularity, the cells from at least three pooled femurs were electronically counted using a Coulter Counter (Coulter Electronics, England).

The adherent fraction of normal bone marrow was prepared according to the method of Wright et al (1980a). Bone marrow cells were suspended in Fischer's medium (Table 2.1) at 5.10^6 cells/ml and allowed to adhere to plastic for 2 hours at 37°C . The supernatant was then removed and fresh Fischer's medium added to the plate, after which the adherent cells were detached using a disposable cell scraper and resuspended in the medium.

Spleens were carefully removed under sterile conditions using scissors and forceps. Spleens were disaggregated in SLM/FCS by passage through a 0.1mm, sterilised, stainless steel gauze and a single cell suspension obtained after repeated vigorous pipetting steps. Nucleated cells were counted as for bone marrow. Approximately 1.10^8 cells were obtained per normal spleen. For experimental determinations of spleen weight, spleens were weighed on an electronic balance and data presented as the mean of at least four spleens \pm 1 standard deviation.

2.3 Maintenance of Cell Lines and Production of Conditioned Media.

All cell lines were obtained from the frozen stocks of the Beatson Institute and were maintained in SLM/FCS. Cells were maintained in T75 tissue culture flasks (Nunc, Denmark). On reaching semi-confluence, cells were replated in fresh medium at one-fifth of the number at semi-confluence. Fibroblastic cell-lines were detached from the surface of the flask using trypsin (Gibco) diluted 1:10 in PE (Table 2.1). Macrophage- and monocyte-like cell lines were detached using a disposable cell scraper (Costar). For the production of conditioned media, the L929 (Stanley and Heard 1977), WEHI 3B (Ihle et al 1982) and AF1-19T (Franz et al 1985) cell lines were grown in roller bottles in SLM/FCS to half-confluence. Spent medium was then removed, replaced with fresh medium, and the cultures allowed to grow for another three days. The conditioned medium was then removed, passed through 0.45 and $0.22\mu\text{m}$ filters and stored at -20°C .

2.4 Culture of Monocyte/Macrophages from Normal Bone Marrow and Spleen.

The methods used in this section were adapted from Pojda et al (1988) and Tushinski et al (1982). For the culture of bone marrow-derived macrophages (BMDM) and spleen-derived macrophages (SDM), a single cell suspension was obtained from the appropriate tissue of female CD1 mice as described in section 2.2.

Normal bone marrow cells were plated at a concentration of $5 \cdot 10^5$ /ml in alpha-MEM medium (Table 2.1) containing 25% Donor Horse Serum (DHS) and 20% L929 conditioned medium (L929CM). Two methods were used for culture after this initial step. 1) Cells were cultured under these conditions for seven days. After seven days non-adherent cells were removed by aspirating the spent medium and washing the adherent cells twice in warm PBS (Table 2.1). The cells remaining attached were then used as BMDM. 2) Alternatively, the non-adherent cells were removed after three days of culture and replated in fresh alpha medium as before. The adherent fraction present after a further four days in culture was then used as BMDM.

Normal spleen cells were plated at a concentration of $1 \cdot 10^6$ /ml in the same culture conditions. The adherent fraction present after seven days of culture was used as SDM. The cells were identified as monocyte/macrophages by morphology and by positive staining for the enzyme alpha-naphthyl acetate esterase (as described in chapter 3).

2.5 Cytospin Preparations and Non-Specific Esterase Assay.

Cultured and sorted bone marrow cells were prepared for morphological examination by centrifugation onto glass slides. Approximately $2.5 \cdot 10^5$ cells were placed in a Cyto-tek chamber (Cyto-tek, France) and centrifuged at 500rpm for 5 minutes in a Cyto-tek cytocentrifuge. The slides were air-dried and fixed with methanol for ten minutes. After air-drying, the slides were stained in Giemsa's stain (BDH, UK) (10%

v/v in distilled water) for ten minutes and then rinsed under running tap water until the water ran clear. After drying, the slides were mounted with DPX mountant (BDH, UK) and glass coverslips (BDH, UK) and the slides examined by microscopy. Morphological observations were confirmed by an experienced histologist. Non-specific esterase assays were performed using a kit (diagnostic kit 91-A) supplied by Sigma (UK) and were performed as recommended by the manufacturer. Immediately prior to fixation, 1ml of sodium nitrite solution was added to 1ml of Fast Blue BB solution in a test tube. The contents were mixed gently and allowed to stand for 2 minutes. The resulting solution was then added to 40ml of distilled water prewarmed to 37°C. To the solution was added 5ml TRIZMAL 7.6 buffer concentrate (Sigma) and 1ml α -naphthyl acetate solution. The solution was then mixed well and poured into a Coplin jar. Cells were cytocentrifuged and fixed for 30 seconds in Citrate/ Acetone/ 37.5% Formaldehyde (25:65:8) fixative at room temperature. The slides were then rinsed for 60 seconds in running distilled water and placed, without drying, in the Coplin jar. The Coplin jar was then incubated in a 37°C water bath, protected from light. After incubation, the slides were removed, rinsed in tap water and air dried. Cells of the monocytic lineage were identified by brown/red staining of the cytoplasm. A macrophage cell line (J774.2) and a fibroblastic cell line (NRK) were used as positive and negative controls, respectively. On all occasions, BMDM were found to be greater than 95% positive for non-specific esterase. Non-specific esterase assays on the BMDM population derived in this study were also performed independently by the Department of Haematology of Glasgow University School of Veterinary Medicine and confirmed the observations of the author.

2.6 BMDM Growth Curves.

For the assessment of their growth properties, BMDM were replated at a concentration of $2.5 \cdot 10^5$ /ml in alpha-MEM medium containing 25% DHS in the presence or absence of either L929CM or WEHICM in T25 (5ml) Flasks (Nunc, Denmark). Cells were allowed to adhere to the surface of the culture flask for 48 hours and after this time were counted either daily or every two days. For counting, medium was aspirated and the remaining adherent cells washed twice with warm PBS to remove any remaining non-adherent cells. The adherent cells were then detached from the culture flask using a disposable cell scraper (Costar, USA) and counted using a haemocytometer. Six counts were performed per flask and the contents of four flasks were counted per time point. Data was presented as the average count from four flasks.

2.7 DNA Synthesis Assays.

DNA synthesis was measured by incorporation of ^3H -thymidine (^3H -TdR) into trichloroacetic acid precipitable material. The method used in this section was adapted from Vairo et al (1990). BMDM, in T25 (5ml) flasks were "starved" overnight in alpha-MEM containing 25% DHS in the absence of any additional growth factors to reduce proliferation to a minimum. The quiescent BMDM were then cultured in fresh alpha-MEM in the presence or absence of the putative mitogenic stimulus at 37°C . Thirty minutes before the appropriate time point, the medium was removed and the cells washed twice with SLM/FCS. Two ml SLM/FCS supplemented with ^3H -TdR ($10 \mu\text{Ci}/\text{ml}$) (NEN, UK) were then added to the flask and the flask incubated at 37°C for thirty minutes. After this, the medium was removed and the cells washed once with ice cold PBS. Three ml of ice cold trichloroacetic acid (10%) was then added and the flask left on ice for 60 seconds. The cells were then detached from the culture flask using a disposable cell scraper and the samples

filtered through a Millipore harvester using cellulose filters (Whatman GF/A). The filters were washed twice with ice cold 10% trichloroacetic acid and once with ethanol before β scintillation counting in an LKB 1215 Rackbeta scintillation counter. For a single time-point, ^3H -TdR incorporation was measured after twenty hours. Data were presented as the mean of triplicate cultures +/- 1 standard deviation.

2.8 Metabolic Labelling of Cysteine-Containing Proteins in BMDM and RAW264.7 Cells.

For the metabolic labelling of cells with ^{35}S -cysteine, BMDM or the cell line RAW264.7 were replated in 100mm tissue culture dishes (Falcon UK) at a concentration of 10^6 per ml in alpha-MEM medium plus 20% L929CM or SLM/FCS, respectively and the cultures incubated for 24 hours. Medium was then aspirated and the plates washed once with 7ml PBS, after which the plates were incubated for twenty minutes at 37°C for 20 minutes in 7ml PBS. Medium was then aspirated and 1ml of cysteine-free medium supplemented with $300\mu\text{Ci}$ ^{35}S -cysteine was added to each plate. The plates were then placed in a closed equilibrated box at 37°C and rocked every 30 minutes to ensure even distribution of medium. After four hours, the medium was removed and both cells and medium stored at -20°C for analysis (see section 2.20).

2.9 Clonogenic Assays.

a) CFU-A Primitive Progenitors (Pragnell et al 1988).

For the detection of CFU-A, 10^4 normal bone marrow cells in alpha-MEM medium containing 25% DHS and 0.3% agar (Difco, USA) were seeded on top of an underlayer of the same medium containing 0.6% agar, 10% L929CM and 10% AF1CM in a 60mm petri dish (Sterilin, UK). Cultures were incubated at 37°C in a fully humidified atmosphere of 10% CO_2 , 5% O_2 and 85% N_2 for 11 days. Colonies

were stained with INT (2-(4 Iodophenyl)-3-(nitrophenyl)-5-phenyltetrazolium chloride) [Table 2.1] overnight (Bol et al 1977). Colonies with a diameter greater than 2mm were scored as CFU-A.

b) CFU-M Progenitor Assay (Pragnell et al 1988).

For the detection of CFU-M, $7.5 \cdot 10^4$ normal bone marrow cells in 1ml alpha-MEM medium containing 25% DHS and 0.3% agar were seeded on top of an underlayer of the same medium containing 0.6% agar and either L929CM or recombinant human CSF-1 (rhCSF-1). Cultures were incubated as for CFU-A. After seven days the colonies were stained with a solution containing 0.13% methylene blue and 20% glutaraldehyde in PBS (Table 2.1). Colonies with greater than 50 cells were scored as CFU-M.

c) Cytosine-Arabinoside Suicide Assay (Dunlop et al 1992).

The proportion of CFU-A cells in S-phase was estimated using a "suicide" assay. Before plating bone marrow cells in a CFU-A assay, cells were divided into two aliquots of $2 \cdot 10^6$ to $5 \cdot 10^6$ cells each in 1ml Fischer's medium containing 20% DHS. Cytosine arabinoside (Ara-C) (Sigma, Poole, UK) was then added to one tube to a concentration of 10^{-3} M and both tubes gassed with 5% CO₂/ air and incubated for 1hour at 37°C. After incubation the cells were washed four times in 2ml fresh Fischer's medium containing 20% DHS to remove any remaining Ara-C, resuspended to appropriate cell concentrations and plated as for CFU-A assay. CFU-A numbers were calculated by averaging the counts from ten plates. The CFU-A proliferative status was derived by comparison of the plus or minus Ara-C data to calculate the proportion of progenitors sensitive to Ara-C and hence the proportion in S-phase at the time of assay (Dunlop et al 1992). For time courses of CFU-A cycling a normal,

untreated control was assayed in parallel for each time point to take into account the large daily fluctuations seen in CFU-A cycling with normal untreated animals.

2.10 5-Fluorouracil and Phenylhydrazine Treatment of Mice.

5-fluorouracil (Sigma, USA) was dissolved in PBS to a concentration of 6mg/ml. Each B6D2F1 mouse was then given a single intravenous injection of 0.2ml (a dose of 150mg/kg in accordance with the method of Harrison and Lerner 1991). Phenylhydrazine (PHZ) (Sigma, USA) was used according to the method of Rencricca et al (1970). B6D2F1 mice were injected subcutaneously with PHZ (60mg/kg) in PBS on days 0, 1 and 3 of experiments.

2.11 Polymixin B.

Carrier-fixed polymixin B (Boehringer Mannheim, UK), used to remove LPS from culture media, was used according to the method of Issekutz et al (1983). Polymixin B was added to media under sterile conditions to a concentration of 2mg/ml and the solution stirred at room temperature for twenty four hours. The polymixin B was then removed by centrifugation and the media used as normal.

2.12 Hybridoma Culture and Antibody Production.

The RA3-3A1 hybridoma cell line (Coffman and Weissman 1983) was used as a source of monoclonal antibody against the B220 antigen. The cells were grown in RPMI 1640 (Table 2.1) containing 15% FCS and insulin, transferrin, selenium supplement (Sigma, UK) plus penicillin (30 μ g/ml) and streptomycin (30 μ g/ml). A growth curve was plotted to find the concentration at which the cells would be growing exponentially (data not shown). Logarithmically growing cells were then centrifuged at 1500rpm for 30 minutes and the cells resuspended in 3-4 times their original volume in serum-free RPMI. The cells were harvested 4 days later and centrifuged at 10,000rpm for 15 minutes. The supernatant was decanted and

powdered ammonium sulphate added to 50% (331 grams/litre) and the solution stirred overnight at 4°C. The next day the solution was centrifuged at 10,000rpm for 60 minutes. The pellet was then resuspended in the smallest volume of PBS in which it would dissolve and dialysed overnight against PBS in dialysis tubing with a molecular weight cut-off of 12.5 KDa.

2.13 Bead Separation of Cells.

All manipulations were carried out in Falcon 2097 centrifuge tubes (Beckton Dickinson, USA). Magnetic beads with attached goat anti-rat Fc antibody were purchased from Metachem Diagnostics (England).

Bone marrow cells were suspended in SLM/FCS at a concentration of $5 \cdot 10^6$ /ml and either an appropriate dilution of primary antibody or an appropriate concentration of Bovine Serum Albumin (as a control) added. The tubes were then gassed with 5% CO₂ in air and incubated at 37°C for 30 minutes with agitation. Next, the secondary (bead-attached) antibody was added to the tube and the tube incubated as before.

After this step, the tubes were exposed to a powerful magnetic field (Magnetic Separator, Metachem Diagnostics) which caused the beads to adhere to the side of the tube. According to the nature of the experiment, either the supernatant or the bead-bound fraction was harvested (see chapter 3).

2.14 Isolation of Total RNA.

For RNA isolation from adherent cells, cells were washed twice with PBS and 5ml RNazol B (Biogenesis, England) added directly to the flask. The resulting mixture was then decanted to Falcon 1059 polypropylene centrifugation tubes. Non-adherent cells were transferred to a sterile universal container, pelleted by centrifugation, washed once in PBS, re-pelleted and resuspended in 250µl PBS and the contents transferred to Falcon 1059 tubes. Five ml of RNazol B were then added to tube and

the solution pipetted vigorously and 1ml of chloroform added to tube and the contents again pipetted vigorously. The tops of the tubes were then covered with parafilm (American National Can, USA) and left on ice for at least ten minutes to allow phase separation to take place. After this the tubes were spun at 10,000rpm for 15 minutes in an HB-4 rotor in a Sorvall RC-5B superspeed centrifuge. After centrifugation, the upper aqueous phase was removed from the tube and placed in a fresh identical tube to which an equal volume of propan-2-ol was added. The tube was then shaken using a whirlymixer before being stored for at least one hour at -20°C to allow precipitation of RNA. After precipitation, the tube was centrifuged as before and the pellet rinsed with 6ml ethanol (75% in DEPC-treated water [Table 2.2]) and centrifuged as before. The pellet was then resuspended in DEPC-treated water to give an RNA concentration of approximately 2mg/ml as estimated by optical density. An equal volume of ethanol was then added to give a stock solution of 1mg/ml and the sample stored at -20°C.

2.15 Isolation of Poly(A)+ and Poly(A)- Fraction of Total RNA.

Total RNA was isolated as described in the previous section by the author and the poly(A)+ and poly(A)- fractions isolated by Dr.E.Barone of the Beatson Institute. This was done using a kit manufactured by Promega (USA). Total RNA was incubated in an eppendorf tube with a buffer containing magnetic beads with attached poly(T) moiety, which specifically binds the poly(A)+ fraction, which was then adhered to the side of the tube by application of a strong magnetic field. The poly(A)- fraction was then decanted and the poly(A)+ fraction eluted by incubation in water at 65°C. RNA yield was determined by spectrophotometry.

2.16 Agarose Gel Electrophoresis.

Agarose gel electrophoresis was used to check the integrity of prepared RNA samples and to prepare RNA for Northern blot transfer. 1.4% (w/v) agarose gels (150ml total volume) were prepared by dissolving 2.8g agar (Gibco, Scotland) in 160ml water in a microwave oven. When cooled to 60°C, 20.4 ml of formaldehyde (37.5% solution) and 20ml 10xMOPS (Table 2.2) buffer were added and the gel poured under a chemical fumehood.

RNA samples were freeze-dried and redissolved in 15µl of RNA sample loading buffer (Table 2.2) plus 1µl of ethidium bromide (1mg/ml), heated to 65°C for 15 minutes, chilled on ice for 1 minute and then loaded on the gel. For determination of the size of mRNA species detected, 2µg of an RNA ladder (BRL, UK) containing RNA molecules of sizes 0.24, 1.4, 2.4, 4.4, 7.5 and 9.5kb in 15µl of RNA sample loading buffer plus 1µl of ethidium bromide (1mg/ml) was also run in on the agarose gel.

Electrophoresis was carried out with the gel fully submerged in 1xMOPS electrophoresis buffer at 50V (250mA) for 3-4 hours until the dye-front had travelled approximately half the length of the gel.

2.17 Northern Blot Transfer of RNA.

RNA samples were size separated as described in the previous section. The loading and integrity of the RNA was checked by visualisation under U.V. light and a photograph of the gel taken.

The gel was washed twice in 10xSSC (Table 2.3) for 15 minutes. Transfer was performed overnight in 10xSSC on to a Genescreen (Dupont NEN, USA) membrane which had previously been hydrated in DEPC-treated water and soaked once in 10xSSC for 15 minutes. After transfer, the membrane was removed and the gel

visualised under U.V. light to ensure efficient transfer. The membrane was then baked at 80°C for 2-3 hours to fix the RNA.

2.18 Preparation of Radiolabelled Probes.

The SCI/MIP-1 α -specific probe was generated as an in vitro transcription product of the coding sequence of SCI/MIP-1 α cDNA sequence cloned into the pBluescript II SK⁻ (pSK⁻) plasmid. The cDNA was generated as a PCR product whose synthesis involved the generation of additional XhoI and EcoRI restriction sites. This product was then cloned into the multiple cloning site in the pSK⁻ plasmid in the appropriate orientation to allow the generation of an antisense SCI/MIP-1 α RNA sequence by in vitro transcription from the T3 promoter contained within the plasmid. Before the in vitro transcription reaction was carried out, the plasmid was cut with Xho I to enhance the efficiency of reaction. A plasmid allowing the generation of a MIP-1 β -specific anti-sense RNA was generated in an identical fashion. Both of these plasmids were obtained from Dr. Mark Plumb, Institute for Cancer Studies, Leeds.

For riboprobes, approximately 0.5 μ g of the appropriate DNA transcription template was added to a reaction mixture containing 2 μ l 10xT3 buffer (400nM Tris, 50mM MgCl₂, pH8), 2 μ l DTT (50mM), 2 μ l spermidine (40mM), 2 μ l ATP,CTP,GTP (5mM), 1 μ l RNAGuard, 0.5 μ l T3 polymerase, 50 μ Ci ³²P UTP (Amersham, UK code PB20383), and 5 μ l DEPC-treated water. The mixture was agitated gently and incubated at 37°C for one hour.

The 7S RNA-specific probe is a 0.4kb Hinf I fragment of the plasmid pAT153 containing 150bp of a 190bp portion of of the 7S cDNA which had been cloned into the plasmid at EcoRI site 375. The plasmid was obtained from Frances Fee (Beatson Institute). This probe hybridises with an RNA message of approximately 280bp, which is the size of the 7S RNA (reference for plasmid Balmain et al 1982).

For DNA probes, approximately 100ng of DNA fragment was added to 20µl of water, boiled for 10 minutes to denature the DNA and then cooled on ice. To the tube was added 1µl each of dATP, dGTP, dTTP (0.5mM), 2µl reaction mixture (hexanucleotide mixture in 10x reaction buffer), 1µl Klenow enzyme and 50µCi ³²P dCTP (Amersham, UK code PB10205). The mixture was agitated gently and incubated at 37°C for one hour.

For both types of reaction, unincorporated nucleotides were removed by size fractionation on a NICK column (Pharmacia, Sweden). The specific activity of the probe was calculated after scintillation counting and sufficient probe added to the hybridisation buffer to give a concentration of 1.10^6 cpm/ml. DNA probes were always boiled for five minutes and chilled quickly on ice before use.

2.19 Hybridisation Analysis of RNA.

The solutions used for hybridisation are shown in table 2.3. Membranes were pre-hybridised in hybridisation buffer in the absence of probe in plastic boxes with agitation for at least six hours. Then radiolabelled probe was added and hybridisation carried out overnight with agitation. For riboprobes the hybridisation was carried out at 65°C, while hybridisation with DNA probes was carried out at 42°C. The following day, membranes were washed twice for 30 minutes with agitation at room temperature and then washed twice for 30 minutes at the probe hybridisation temperature with the appropriate wash buffers (Table 2.3). Membranes were dried on Whatman 3MM paper and sealed in thin polythene bags.

Sealed membranes were then exposed to Kodak X-OMAT AR film at -70°C and the films processed in an X-ray processor.

Membranes were stripped of bound probe by boiling for ten minutes in either Northern blot wash buffer (A) (Table 2.3) or in distilled water.

2.20 SDS-Polyacrylamide Gel Electrophoresis.

A 15% agarose gel with a 5.5% stacking gel (Table 2.4) was poured in a minigel system (Atto, UK). Protein samples were boiled for ten minutes in protein sample buffer (Table 2.4) and chilled on ice immediately prior to loading. Radiolabelled (^{14}C) molecular weight markers of 200, 97, 69, 46, 30, 21 and 14kDa were also loaded (Amersham UK). Electrophoresis was carried out in SDS-PAGE Electrophoresis Buffer (Table 2.4) at 150V until the dye-front was close to the end of the gel.

For the analysis of the media conditioned by ^{35}S -cysteine labelled cells, the gel was fixed in SDS-PAGE Gel Fixative (Table 2.4) for 30 minutes and then washed in Amplify (Amersham, UK) for 20 minutes to increase the sensitivity of detection of radioactivity. The gel was then placed on Whatman 3MM paper, covered in Saran Wrap (Dow, USA) and dried on a gel drier. The dried film was then exposed to Kodak X-OMAT AR film at -20°C .

2.21 Western Blotting.

SCI/MIP-1 α content of media conditioned by BMDM or RAW264.7 cells was analysed by Western blotting. Twenty μl of conditioned medium was boiled in an equal volume of protein sample buffer and separated by SDS-PAGE (3% stacker, 17.5% gel). Ten ng of pure recombinant SCI/MIP-1 α protein was also run as a positive control as described in section 2.20.

The contents of the gel were then transferred on to a nitrocellulose membrane (BA 85; Schleicher and Schuell) using a minigel electroblotting apparatus (Biotech Instruments Ltd., UK) driven by a current of 50mA for 30 minutes. The membrane was then removed and washed in multiple changes of "blotto" (PBS, 5% dried milk, 0.1 % NP40) for 1 hour to block non-specific binding sites. The membrane was then incubated in blotto containing a 1 in 1000 dilution of goat anti-mouse SCI/MIP-1 α

antibody (R and D Systems, USA). The membrane was then washed in blotto for 30 minutes, with multiple changes before the addition of a horse radish peroxidase-conjugated horse anti-goat antibody (Vector Labs, UK) in blotto. The membrane was then washed for 15 minutes in blotto before multiple washes in PBS/1% Tween. Antibody binding was visualised using an Enhanced Chemiluminescence (ECL) kit (Amersham, UK). The membrane was incubated in ECL solutions for 60 seconds and then exposed to X-OMAR AR X-ray film.

Reagent Tables.

Table 2.1 Tissue Culture

Special Liquid Medium (SLM)

Supplemented modified Eagles medium (SLM, Gibco cat. no. 043-01136M)
10% fetal calf serum (FCS, Gibco).
2mM L-Glutamine

Dulbecco's/SF12 (for 1 litre)

10x Dulbecco's medium	50ml	(Gibco cat. no. 042-0251 M)
10x SF12 medium	50ml	(Gibco cat. no. 041-01765M)
L-Glutamine (200mM)	10ml	
Pyruvate (100mM)	5ml	
Penicillin (750µg/ml)	5ml	
Double distilled water	840ml	
Nutridoma	10ml	
Sodium bicarbonate (7.5%)	41ml	

MEM Alpha Stock (Gibco)

Dissolve 5 litre pack (Gibco) in water, add 50ml MEM x100 vitamins (Gibco) and 100mg gentamycin sulphate. Make up to 1500ml and filter sterilise.

MEM Alpha x2 (for 100ml)

Alpha stock	21ml
L-Glutamine (200mM)	1ml
Sodium bicarbonate (7.5%)	3ml
Donor Horse Serum	25ml

(For MEM Alpha x1 add an equal volume of distilled water and check osmolarity in osmometer [range 300+/- 10 mOs] or add to equal volume of agar for clonogenic assays.)

Fischer's Medium

Distilled water	87ml	
10x Fischer's Stock	10ml	(Gibco cat. no. 042-01735M)

L-Glutamine (200mM)	1ml
Sodium bicarbonate (7.5%)	1.5ml
Donor Horse Serum	20ml

RPMI 1640

Distilled water	860ml	
RPMI x10 stock	100ml	(Biol. Industries cat. no. 06-1100-01-5A)
L-Glutamine (200mM)	10ml	
Sodium bicarbonate (7.5%)	1.5ml	

Dulbecco's phosphate-buffered saline, solution A (PBS).

KCl	2.68mM
KH ₂ PO ₄	1.47mM
NaCl	0.137mM
Na ₂ HPO ₄ ·7H ₂ O	8.06mM

Trypsin diluent, PBS/EDTA (PE)

PBS
1mM EDTA

Trypsin

0.25% trypsin in PE

INT Stain.

For the preparation of INT stain, 50mg 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) (BDH, UK) was added to 50ml PBS and the mixture heated to allow dissolution of the solid. After cooling, the INT solution was filtered to remove any undissolved solid and sterilised through a 0.22µm filter.

Methylene Blue Progenitor Stain (for 23ml).

Methylene Blue (0.23%)	1ml
Glutaraldehyde (25%)	4.6ml
PBS	17.4ml

Table 2.2 Agarose Gel Electrophoresis.

MOPS buffer x10 pH7 (for 1 litre)

MOPS (Sigma)	41.8g	(0.4M)
Sodium Acetate	6.8g	(0.1M)
EDTA	3.72g	(0.01M)

RNA Sample Loading Buffer

Formamide	55%
10x MOPS	11%
Formaldehyde	17.9%
Water	7.5%
Glycerol	7.5%
Bromophenol Blue to colour	

DEPC-treatment of H₂O (for 400ml)

Distilled H ₂ O	500ml
Diethyl Pyrocarbonate (DEPC)	500µl

Mixture was left at room temperature overnight and then autoclaved.

Table 2.3 Transfer and Hybridisation Buffers

Northern Blot Hybridisation Solution (for 50ml).

Formamide	25ml
50x Denhardt's Solution	2.5ml
20xSSPE	12.5ml
polyA (1mg/ml)	50µl
polyC (1mg/ml)	50µl
Herring Sperm DNA (10mg/ml)	0.5ml
10% SDS	0.5ml
Complete to 50mls with dH ₂ O	

20x SSC pH7. (for 1 litre)

Sodium chloride	175.3g (3M)
Sodium citrate	88.4g (0.3M)

20x SSPE pH7.4. (for 1 litre).

Sodium chloride	175.3g (3M)
Sodium citrate	88.2g (0.3M)
EDTA	7.4g (0.02M)

50x Denhardt's Solution.

Ficoll-400	1% (w/v)
Polyvinylpyrrolidone	1% (w/v)
Bovine Serum Albumin (Pentax Fraction V)	1% (w/v)

Northern Blot Wash Buffers

A) 25°C Wash:	2x SSC, 0.1% SDS.
B) Hybdn. Temp. Wash	0.1x SSC, 0.1% SDS

Table 2.4 SDS Polyacrylamide Gel Electrophoresis

15% Acrylamide Running Gel.

30% Acrylamide	7.5ml
1% Bis-acrylamide	1.3ml
Tris (1.5M)	3.75ml
SDS (20%)	0.075ml
Distilled Water	2.2ml
Ammonium persulphate	0.1ml
TEMED	0.01ml

5.5% Acrylamide Stacking Gel

Acrylamide (30%)	3.3ml
Bis-acrylamide (1%)	2.9ml
Tris (1M)	2.5ml
SDS (20%)	0.1ml
Distilled Water	8.8ml
Ammonium persulphate (10%)	0.1ml
TEMED	0.01ml

Protein Sample Buffer for SDS-PAGE (for 20ml).

Tris-HCl 0.5M, pH6.8	2ml
Glycerol	6ml
SDS (10%)	5ml
Distilled Water	7ml
β -mercaptoethanol	2% (immediately prior to use)

SDS Gel Fixative (for 100ml)

Propan-2-ol	25ml
Distilled Water	65ml
Acetic Acid	10ml

5x SDS PAGE Electrophoresis Buffer (for 1 litre)

Glycine	144g
Tris.HCl	30g
10% SDS	50ml
Make up to 1 litre with dH ₂ O.	

Table 2.5 Sources and Storage of Selected Reagents

Purified LPS from *Salmonella Typhinurium* was obtained from Sigma (UK) and was dissolved in PBS to a concentration of 2mg/ml, aliquoted and stored at -20°C.

Recombinant murine IFN γ and recombinant human CSF-1 were obtained from Genzyme (UK), aliquoted and stored at -20°C in alpha-MEM containing 25% DHS.

Hydrocortisone was obtained from Sigma (UK) dissolved in 5% alcohol in PBS to a concentration of 10⁻³M, aliquoted, and stored at -20°C.

Actinomycin D and cycloheximide were obtained from Sigma (UK) and stored at -20°C in distilled water at a concentration of 5mg/ml.

Anti-F4/80 antibody was obtained as a hybridoma tissue culture supernatant from Serotec (UK), aliquoted and stored at -20°C.

Chapter 3

Culture, Growth Properties and Demonstration of SCI/MIP-1 α mRNA and Protein Production by Bone Marrow-Derived Macrophages.

3.1 Attempted Sorting of Macrophages

Understanding the expression of the gene for SCI/MIP-1 α is important for a full appreciation of the role played by the Stem Cell Inhibitor/Macrophage Inflammatory Protein-1 α in the control of haemopoiesis and in other biological processes. The central aim of this thesis was to investigate SCI/MIP-1 α gene expression in an in vitro model system representing as far as possible the SCI/MIP-1 α -producer cell of the marrow. It was first necessary, therefore, to devise and then characterise that system.

It was reasoned that the best in vitro model for the in vivo SCI/MIP-1 α producer cell would be to use macrophages isolated directly from murine bone marrow.

The Manchester Inhibitor-producing cell had already been shown to be most probably a macrophage/monocyte by Wright et al (1980a); however, although Wright et al characterised the cell to a great extent and enriched for it greatly, they did not actually obtain a pure population.

Simmons and Lord (1985) sorted a population of monocyte/macrophages from normal murine bone marrow, using density fractionation and fluorescence activated cell sorting on the basis of expression of the macrophage-specific cell surface marker F4/80, which produced the Manchester Inhibitor. After an input of 40 femora (approximately $4-8 \cdot 10^8$ bone marrow cells), Simmons and Lord sorted a total of fifty thousand F4/80-positive cells (or 0.15% of the total starting population). This approach was considered but it was concluded that it would prove to be impractical as the numbers of producer cells generated per sort was incompatible with the

magnitude of some of the experiments envisaged. For example, it was envisaged that multiple time and concentration points would be necessary for a full analysis of SCI/MIP-1 α gene expression and an assay population of tens of thousands of cells would be insufficient for any one experiment. In addition, it was considered that the extremely lengthy, labour intensive process of electronic sorting would have limited the number of experiments able to be completed.

A related experimental approach, immunomagnetic sorting, was therefore initially adopted. The basis of this approach, in this study, is to bind antibodies raised against a lineage-specific antigen to bone marrow cells. A second anti-Fc antibody, which is covalently linked to a magnetic bead is then attached to the Fc region of the primary antibody. The secondary antibody is attached to a magnetic bead. This allows the separation of the bound and unbound cells by exposure to a strong magnetic field. At first, a negative selection process was attempted, i.e. antibodies specific for non-monocytic cells were used as the primary antibody, in the same fashion as the CFU-S separation methods of Sprangude et al (1988). A monoclonal IgM antibody specific for the surface determinant B220, which is produced by the hybridoma RA3 3A1 (Coffman and Weissman 1981) was employed. The B220 glycoprotein is restricted in its expression to cells of the B lymphoid lineage and is expressed on approximately 30% of bone marrow cells (Coffman and Weissman 1981). It was considered that removal of cells bearing the B220 surface antigen might provide a reasonable first enrichment step towards isolating SCI-producing macrophages from bone marrow. Using a rat anti-B220 antibody produced by the hybridoma RA3-3A1 in conjunction with a goat anti-rat Fc antibody linked to magnetic beads on unseparated normal bone marrow it was possible to remove 30-40% of the total marrow cells as illustrated in figure 3.1. It was not possible to determine by morphology whether B-lineage cells had actually been removed as they were obscured by being linked to the beads. Attempts to remove the beads by culture and by mild protease (trypsin) treatment

Figure 3.1 Reduction in bone marrow cell number using anti-B220 antibody.

Normal, unfractionated bone marrow was manipulated as described in chapter 2. Additions to tubes were as follows:

- 1) Nil no additions
- 2) 1/40AbNB anti-B220 diluted 1 in 40;
 no beads
- 3) 100ulB no antibody; 100ml beads;
 100µg/ml BSA
- 4) 1/40AbB anti-B220 diluted 1 in 40;
 100µl beads
- 5) 1/80AbB anti-B220 diluted 1 in 80;
 100µl beads
- 6) 1/160AbB anti-B220 diluted 1 in 160;
 100µl beads

Cells were counted using a haemocytometer and data presented as the mean determination of two tubes.

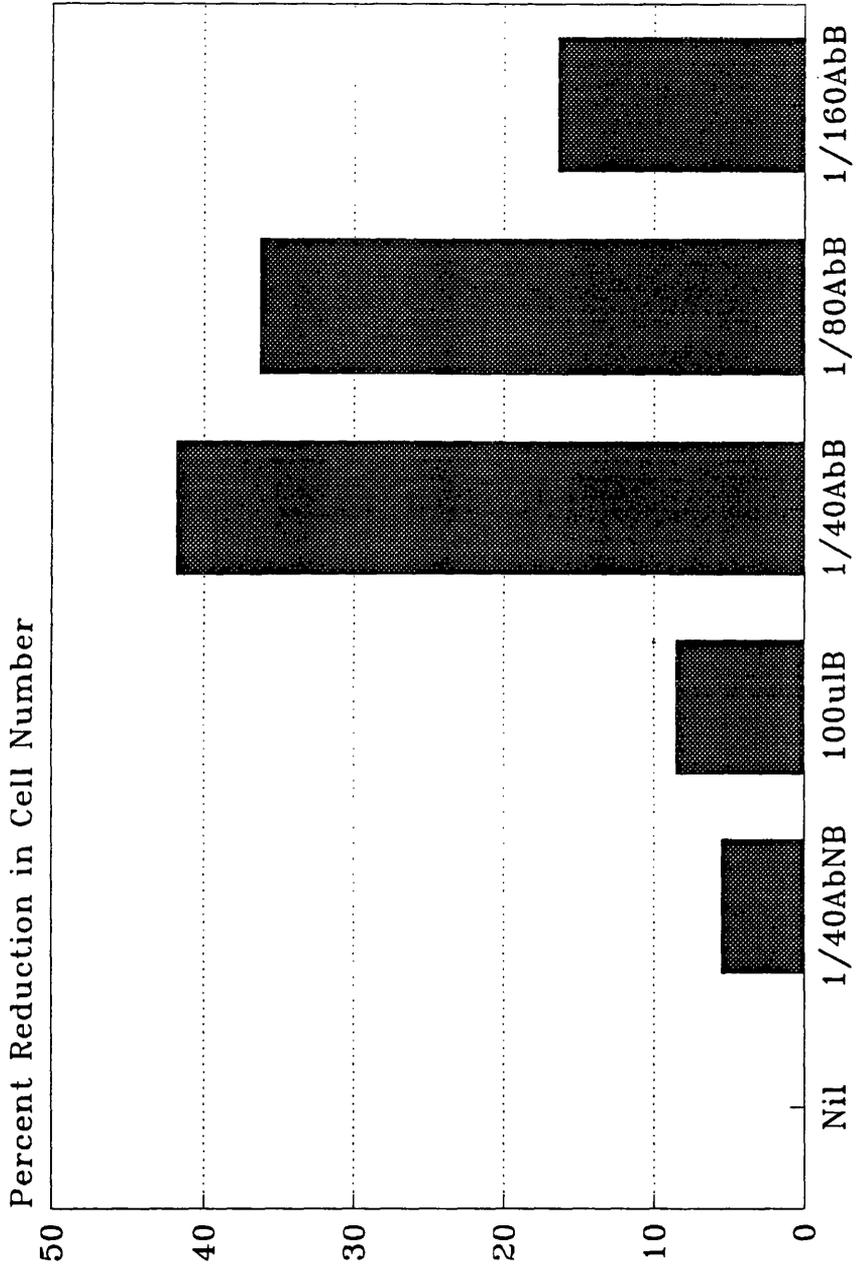


Figure 3.1

were unsuccessful. As fairly standard culture conditions (Special Liquid Medium and 10% fetal calf serum, SLM/FCS), not specially suited to B cells, were employed it is highly likely that attached cells did not stay viable long enough for the removal of beads to be effective and subsequent culture to take place.

As indicated, a general problem with these experiments was that it was not determined whether the population removed by the beads was specific. This was an important consideration as it was possible that cells could be removed via a non-antigen-specific interaction with the primary antibody such as binding to Fc receptors which are expressed on both lymphocytes and macrophages (Mellman et al 1988). Attempts to assess whether the unbound population in these experiments was specifically depleted of B220-positive cells compared to unfractionated bone marrow by immunofluorescence were uninformative due to technical difficulties.

Attempts were made to increase the depletion using antibodies directed against antigens expressed specifically on other lineages (such as the granulocytic lineage-specific antigen Gr-1) in combination with anti-B220. Unfortunately, these attempts did not result in an increase in the numbers of cells removed (data not shown), and the problems of positive identification of the sorted populations continued. It was concluded that this approach would not be suitable for the isolation of bone marrow macrophages.

It was considered that positive selection of macrophages might constitute an alternative method of obtaining the desired population. Attempts were made to do this using the immunomagnetic depletion with a commercially obtained IgG2b monoclonal antibody versus the macrophage-specific antigen F4/80.

The surface determinant F4/80 is a 160 KDa glycoprotein which is specifically expressed on cells of the mononuclear phagocyte system (Hume et al 1984).

It was found that use of this antibody in conjunction with magnetic beads resulted in a depletion of about 40% of the cells in the plastic-adherent fraction of bone marrow

cells as illustrated in figure 3.2. Approximately 10-20% of unfractionated bone marrow cells adhere to plastic and indeed Wright et al (1980a) had demonstrated that the Manchester Inhibitor-producing cells were confined to the adherent fraction of normal bone marrow; this therefore serves as a pre-enrichment step for macrophages which are readily adherent to plastic. However, it was again impossible to identify the selected population due to inability to remove it from the beads despite the fact that culture conditions suitable for the survival of monocytes and macrophages (alpha MEM medium containing 25% donor horse serum and a source of macrophage colony stimulating factor) were used.

It was clear that sorting the macrophage population straight from the marrow was not a feasible option as the results obtained with the antibody and magnetic beads approach were highly variable, it was not possible at that time to verify the specificity of removal and on reflection it had become apparent that the problem concerning the small number of producer cells generated by any direct isolation method had not actually been addressed by this particular approach. It was concluded, therefore, that an alternative strategy would have to be employed.

3.2 Culture of Macrophages from Normal Bone Marrow and Spleen.

Pojda et al (1988) and Wright and Lorimore (1987) had previously demonstrated that macrophages cultured from murine bone marrow and spleen were good producers of the Manchester Inhibitor and therefore it was thought possible that a similar strategy could be employed to provide a model for studying the expression of the SCI/MIP-1 α gene.

It should be noted that using macrophages cultured from haemopoietic tissues is in many respects a compromise as any cultured population is almost certain to be derived in vitro from progenitors of the monocyte/macrophage lineage and exposed to exogenous growth factors and can not therefore, by definition, be truly

Figure 3.2 Reduction in bone marrow cell number using anti-F4/80 antibody.

Normal bone marrow was adhered to plastic in Fischer's medium and manipulated as described in chapter 2. Additions to tubes were as follows:

- | | |
|------------|--------------------------------------------|
| 1) Nil | no additions |
| 2) 1/5AbNB | anti-F4/80 diluted 1 in 5; no beads |
| 3) 100ulB | no antibody; 100µl beads;
100µg/ml BSA |
| 4) 1/5AbB | anti-F4/80 diluted 1 in 5; 100µl
beads |
| 5) 1/10AbB | anti-F4/80 diluted 1 in 10; 100µl
beads |
| 6) 1/20AbB | anti-F4/80 diluted 1 in 20; 100µl
beads |

Bone marrow cells were counted using a haemocytometer and data presented as the mean percentage of adherent bone marrow cells remaining from three tubes plus or minus one standard deviation.

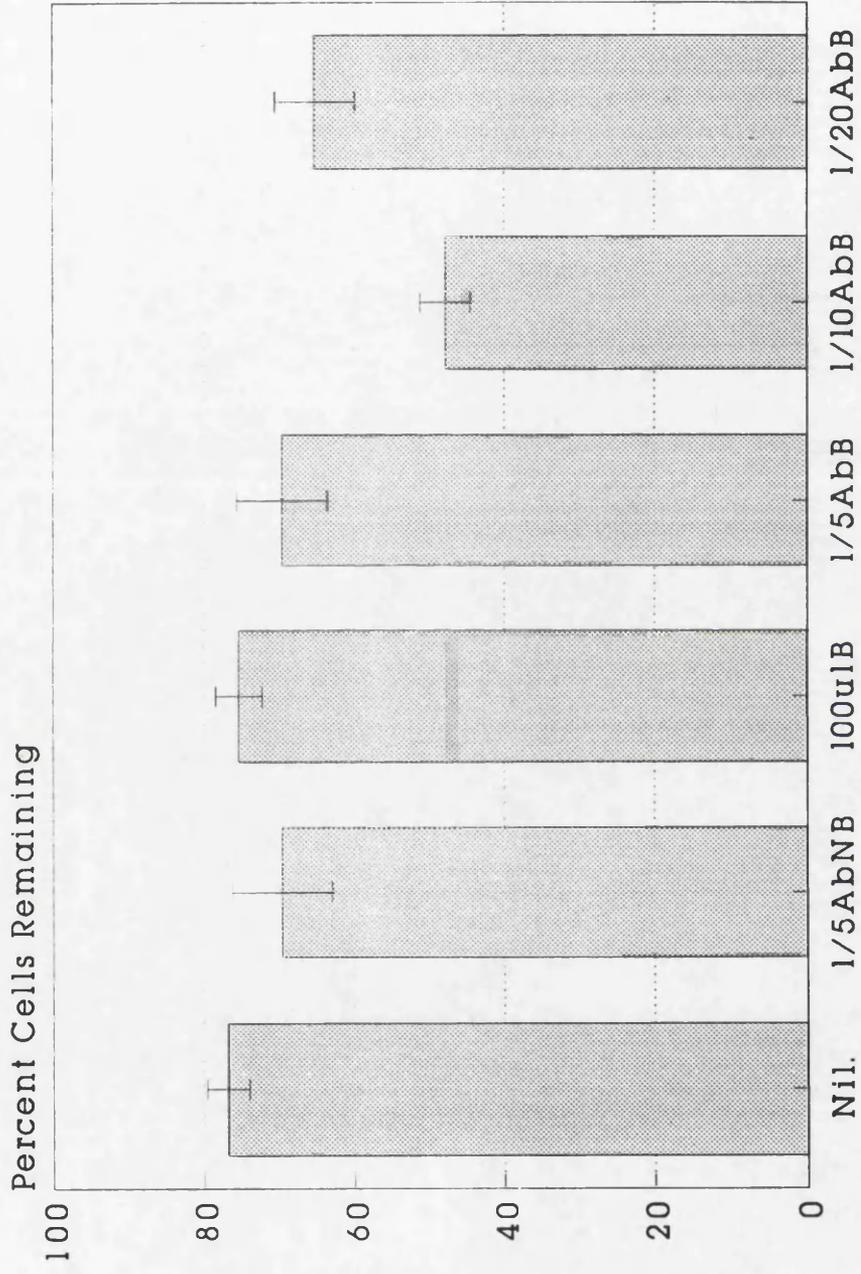


Figure 3.2

representative of the bone marrow stromal population or resident bone marrow macrophage (RBMM) population or any other macrophage population in the bone marrow. RBMM have been studied morphologically and are usually found associated with developing haemopoietic cells. They are apparently widespread throughout the marrow and are present at about 20% of the incidence of committed monocytic progenitors (Crocker et al 1988).

A major difference in the behaviour of the two populations is the fact that RBMM appear to be a non-proliferating end cell population as no mitotic figures or thymidine incorporation can be demonstrated for these cells whereas the monocytic progenitor cells are a rapidly dividing and differentiating population (Crocker and Gordon 1985). Crocker et al (1988) have argued that subtle but important aspects of the phenotypes of RBMM are conferred by virtue of their interactions with developing haemopoietic cells. Obviously, a cultured monocyte/macrophage population would not be exposed to these influences.

It is clear, therefore, that the differences between cultured and *in vivo* macrophages have to be borne in mind when discussing the haemopoietic implications of any experimental results obtained with cultured macrophages. Evidence will be presented in this section, however, to show that the behaviour of the cultured macrophages used in this study is very similar to that of the Manchester Inhibitor-producing population sorted by Simmons and Lord (1985) which validates the use of this population in the light of previous studies.

Murine bone marrow and spleen were cultured in conditioned media from three different cell lines; L929, a fibroblastic line and a source of M-CSF (Stanley and Guilbert 1981); WEHI 3B, a myelomonocytic line and a source of M-CSF and IL-3 (Bazill et al 1983) and AFI-19T, a retrovirally-transformed variant of the NRK (normal rat kidney) cell line which is a source of, at least, M-CSF and GM-CSF (Franz et al 1985; Pragnell et al 1988).

Normal, unfractionated bone marrow and spleen cells were cultured in the presence of various concentrations of these conditioned media (CM) in alpha-MEM containing 25% donor horse serum for seven days.

L929CM has been used widely in the culture of macrophages and therefore was an obvious choice; for example Tushinski et al (1982) and Gordon and Hume (1984) have used this conditioned medium to culture bone marrow derived macrophages (BMDM).

Wright et al (1980a, 1982) had demonstrated that the Manchester Inhibitor-producing cell was confined to a distinct subset of adherent bone marrow cells (on the basis of buoyant density) and an attempt was therefore made to culture macrophages of slightly different phenotypes using AFICM and WEHICM to ascertain whether this would affect SCI/MIP-1 α gene expression.

On morphological examination, after staining with Giemsa's stain, it was found that both the bone marrow and spleen cultures in which L929CM was used consisted almost entirely of cells with the characteristic appearance of mature monocyte/macrophages. Figure 3.3 (A and B) shows cytopspin slides of preparations of both bone marrow- and spleen-derived macrophages. In both cases, the overwhelming majority of the cells display the round nucleus and high cytoplasmic to nuclear ratio characteristic of mature cells of the monocytic lineage. In culture, under light microscopy, the cells also display the stellate morphology characteristic of mature monocyte/macrophages.

Tushinski et al (1982) have used a slightly different culture technique in that bone marrow was cultured in L929CM-containing medium for three days and then the non-adherent cells transferred to a fresh identical culture. This was done to produce as uniform a culture as possible and presumably to minimise any possible contamination by other adherent cell types such as fibroblasts. Macrophages were also cultured in this way for comparison. It was found that the adherent cells produced in these

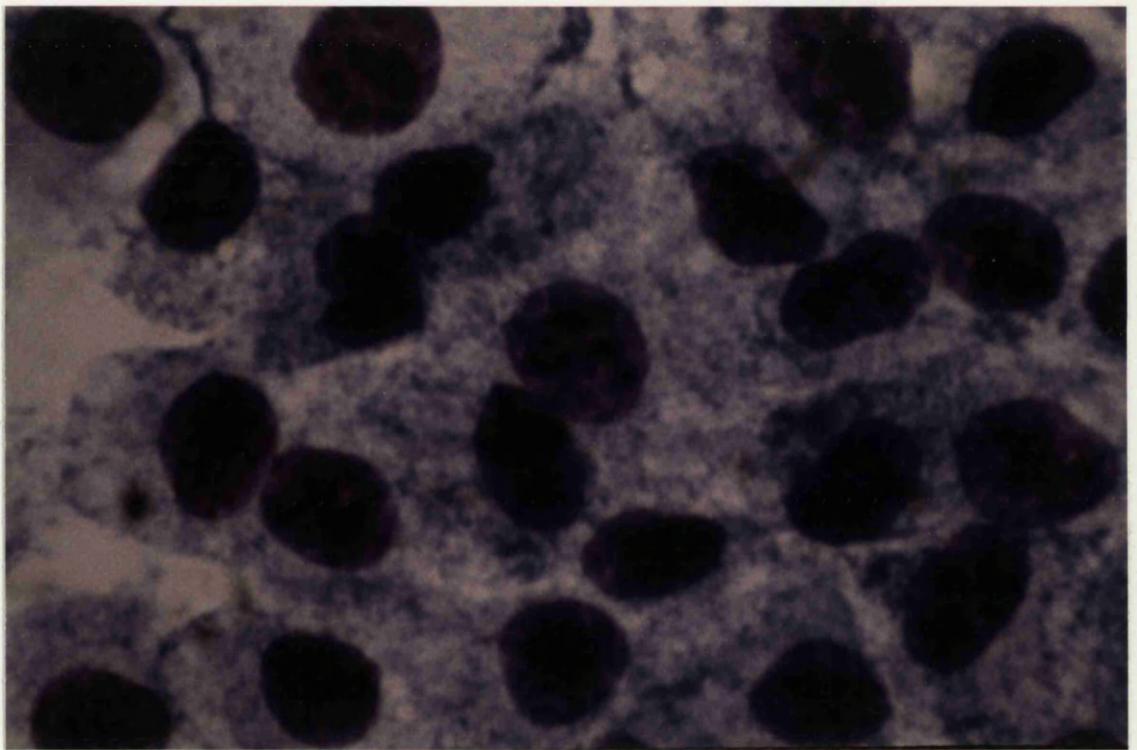


Figure 3.3A Morphology of normal murine bone marrow cultured for 7 days in alpha-MEM medium containing 25% DHS plus 20% L929CM (Bone Marrow-Derived Macrophages).

Normal, unfractionated murine bone marrow was cultured at a concentration of $5 \cdot 10^5$ cells/ml in alpha-MEM medium containing 25% DHS plus 20% L929CM. After 7 days, non-adherent cells were removed and adherent cells (Bone Marrow-Derived Macrophages) fixed with methanol and stained with 10% Giemsa's. Figure shows 100X magnification.

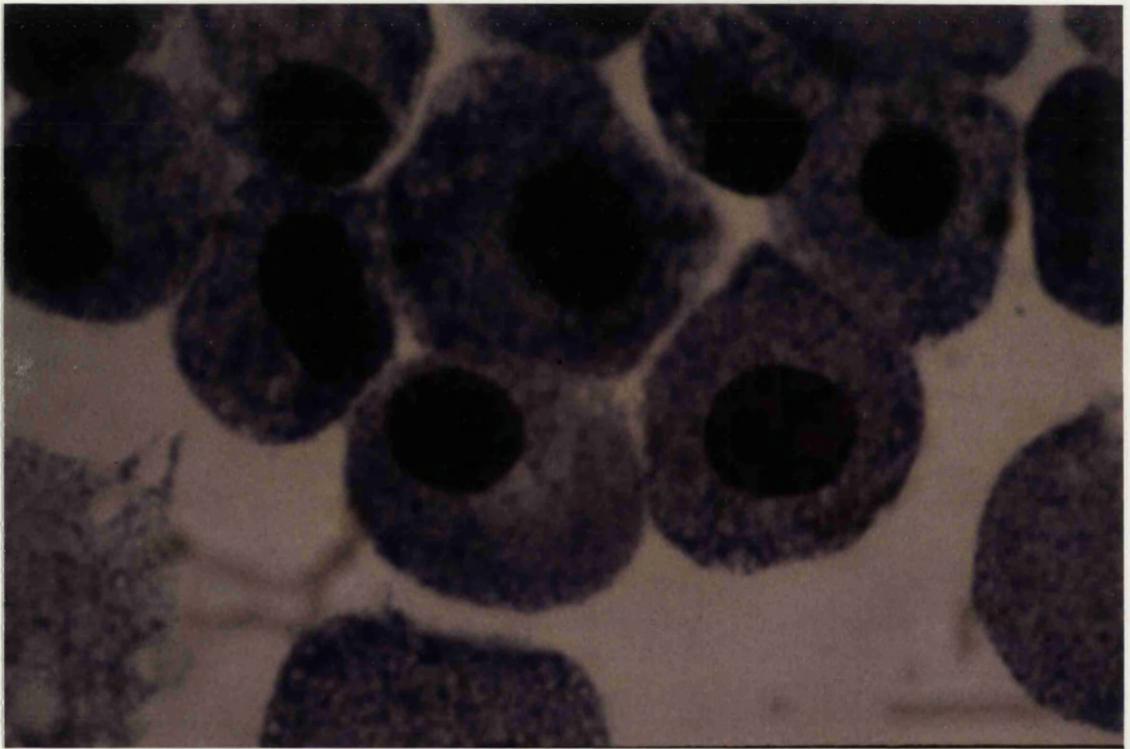


Figure 3.3B Morphology of normal murine spleen cultured for 7 days in alpha-MEM medium containing 25% DHS plus 20% L929CM (Spleen-Derived Macrophages).

Normal, unfractionated murine spleen was cultured at a concentration of $5 \cdot 10^5$ cells/ml in alpha-MEM medium containing 25% DHS plus 20% L929CM. After 7 days non-adherent cells were removed and adherent cells (Spleen-Derived Macrophages) fixed with methanol and stained with 10% Giemsa's. Figure shows 100X magnification.

cultures were morphologically indistinguishable from the cultures in which the replating step was not performed. In the present study the former technique was used for initial gene expression experiments (chapter 4) and the latter used for later experiments (chapters 5 and 6). It is unlikely that there was any significant fibroblast contamination in the cultures used in chapter 4 as they were morphologically indistinguishable from the cultures derived from the three day non-adherent cells; furthermore it is unlikely that any contaminants would contribute to data obtained on the expression of the SCI/MIP-1 α or MIP-1 β gene as no mRNA for either of these genes has been detected in fibroblasts and furthermore Plumb et al (1991) have reported structural analysis of the SCI/MIP-1 α gene which suggests that the gene is in an inactive conformation in epithelial and fibroblastic cell lines. In addition to this, no SCI/MIP-1 α mRNA could be detected in two fibroblastic cell lines analysed for SCI/MIP-1 α expression in this study (figure 3.14).

Positive staining for the monocytic lineage-specific enzyme alpha-naphthyl acetate esterase (non-specific esterase) identified the cells derived from bone marrow using both culture techniques as being overwhelmingly (>95%) monocytic (figure. 3.4). The spleen and bone marrow cultures incubated with AF1CM and WEH1CM contained cells with a variety of morphologies representing both monocytic and granulocytic lineages and so were not considered further as sources of macrophages. Figures 3.5 and 3.6 show the morphology of normal bone marrow cells after culture for seven days in medium containing 20% AF1CM or WEH1CM, respectively. The morphological heterogeneity of the cultures can be seen clearly and cells of an immature appearance can be seen in both cultures.

It was concluded that L929CM, as expected, was the most suitable conditioned medium for promoting the growth of macrophages *in vitro* from bone marrow. The monocyte/macrophage cells cultured using L929CM will be referred to as Bone

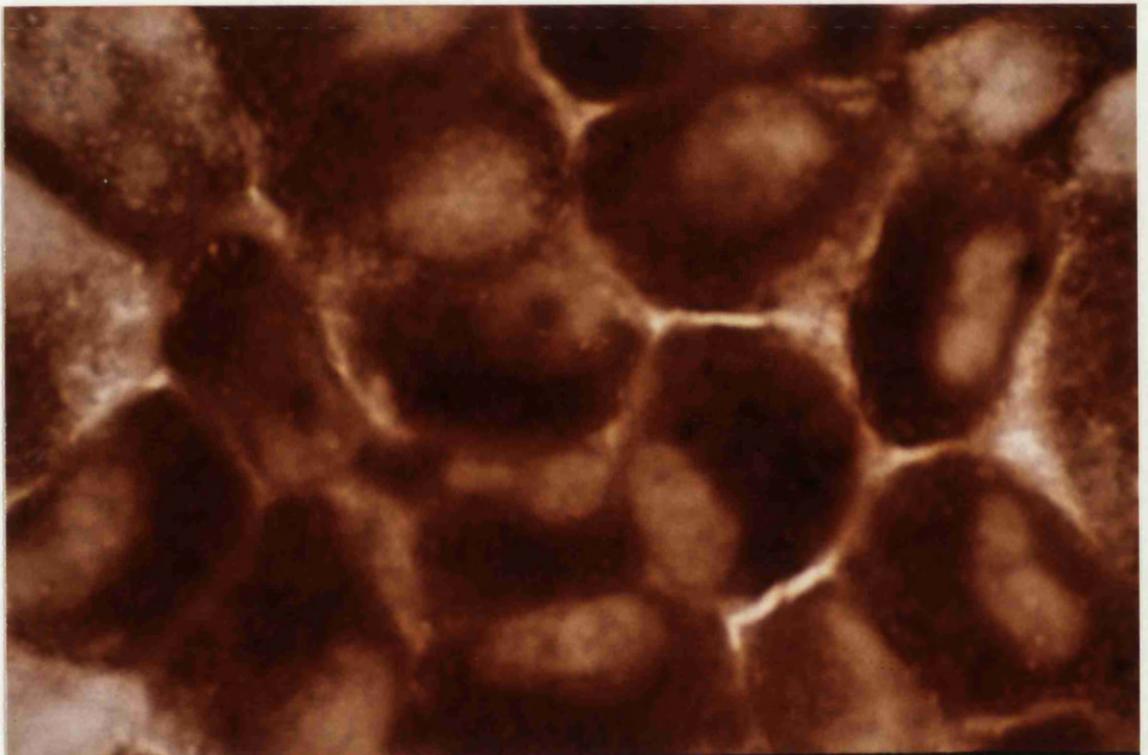


Figure 3.4 Alpha-naphthyl acetate esterase (non-specific esterase) staining of bone marrow-derived macrophages.

Bone marrow-derived macrophages were stained as described in chapter 2. Figure shows 100X magnification.

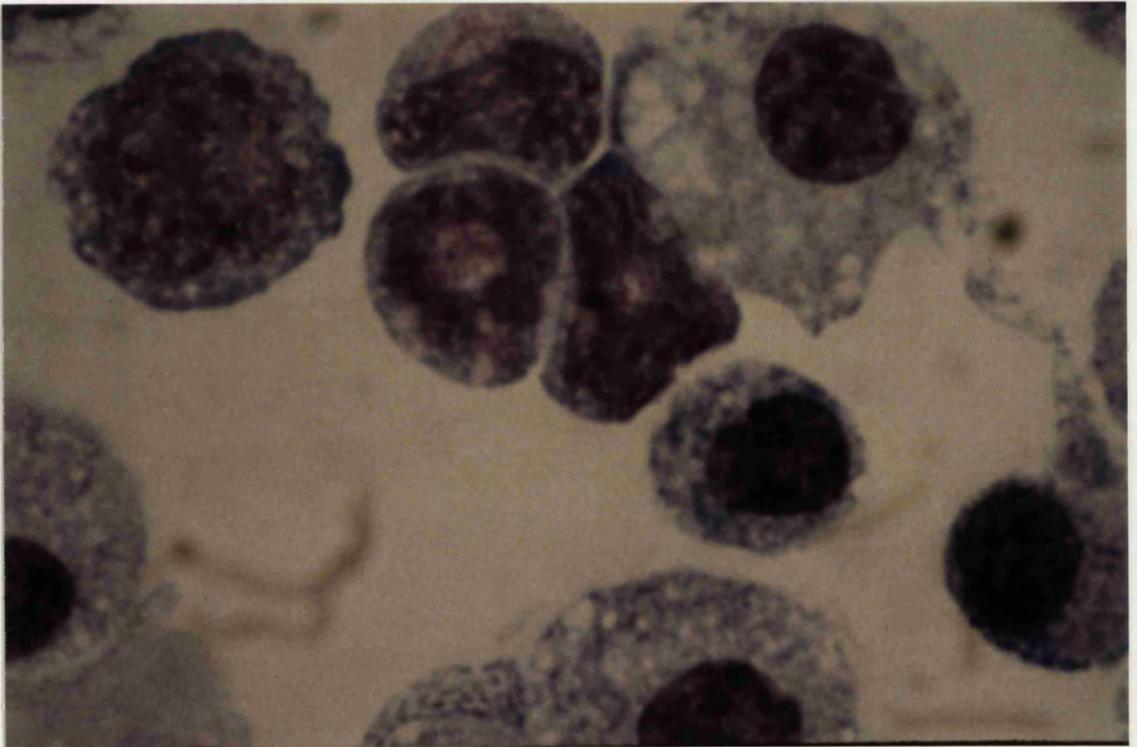


Figure 3.5 Morphology of normal bone marrow cultured for 7 days in alpha-MEM medium containing 25% DHS plus 20% AF1CM.

Normal, unfractionated murine bone marrow was cultured at a concentration of $5 \cdot 10^5$ cells/ml in alpha-MEM medium containing 25% DHS plus 20% AF1CM. After 7 days non-adherent cells were removed and adherent cells fixed with methanol and stained with 10% Giemsa's. Figure shows 100X magnification.

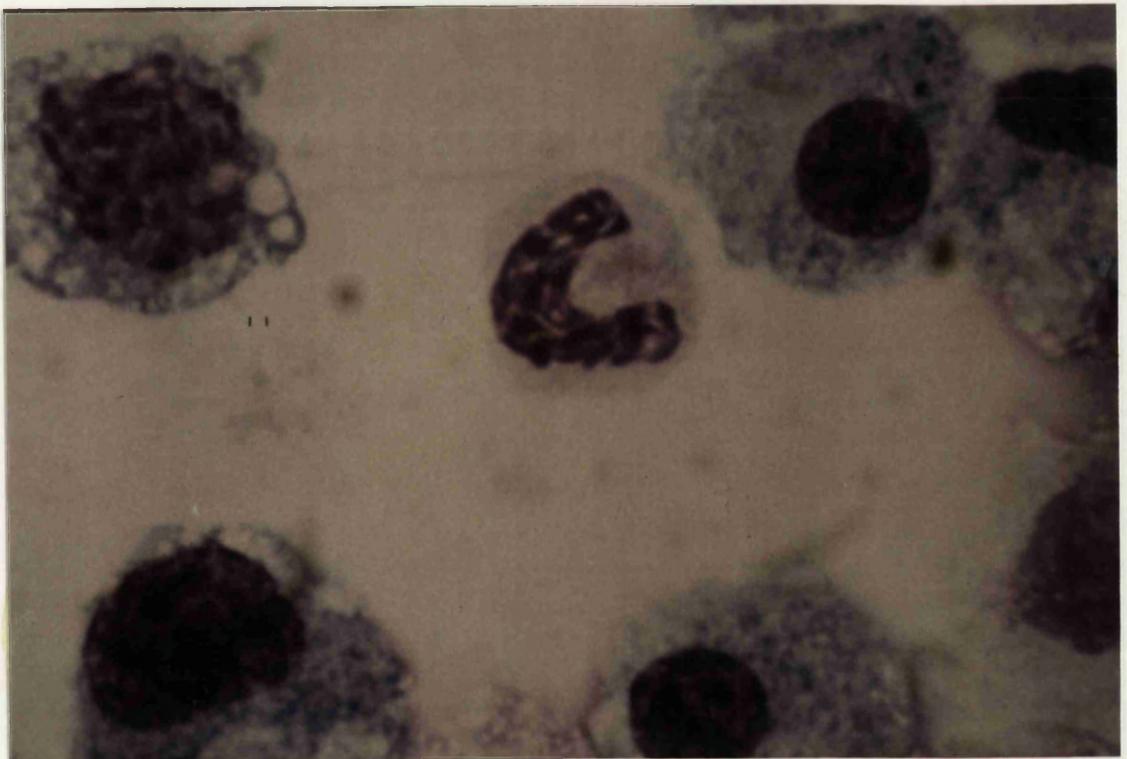


Figure 3.6 Morphology of normal murine bone marrow cultured for 7 days in alpha-MEM medium containing 25% DHS plus 20% WEHICM.

Normal, unfractionated murine bone marrow was cultured at a concentration of $5 \cdot 10^5$ cells/ml in alpha-MEM medium containing 25% DHS plus 20% WEHICM. After 7 days non-adherent cells were removed and adherent cells fixed with methanol and stained with 10% Giemsa's. Figure shows 100X magnification.

Marrow Derived Macrophages (BMDM) or Spleen Derived Macrophages (SDM) in the rest of this manuscript.

Parenthetically, morphological examination of L929CM-stimulated cultures at day three contained cells with the characteristic appearance of primitive progenitor cells of the monocyte/macrophage lineage. In retrospect it may have been possible to culture macrophages of a different phenotype by culturing normal bone marrow in L929CM for three days and then replating in either AF1CM or WEHICM.

3.3 Growth Characteristics of Bone Marrow-Derived Macrophages

The growth characteristics of the BMDM were investigated. This was done for three reasons: 1) While the proliferative capacity of cells of the monocytic lineage has long been a subject of much controversy, it appears that the resident macrophages of the bone marrow, which are potential producers of SCI/MIP-1 α *in vivo* do not proliferate (Crocker et al 1988). If the BMDM population was to act as a surrogate for these cells it was necessary to characterise the differences between these populations as fully as possible so that any data obtained on gene expression could be interpreted in the light of these. 2) Simmons and Lord (1985) had reported some observations on the growth properties of the F4/80-positive Manchester Inhibitor-producing population which they had sorted from density fractionated bone marrow and it was considered that the ability to compare the growth characteristics of these two populations would be informative in allowing comparison of the data from that study with the present one. 3) A population of macrophage/monocyte cells cultured using L929CM and recombinant human Colony Stimulating Factor 1 (rhCSF-1) had been described previously by Tushinski et al (1982) and their proliferative characteristics and their relationship to gene expression investigated in a series of publications (for review see Vairo and Hamilton 1991). A comparison of the growth characteristics of the two populations was, therefore, considered to be essential if the

characteristics of gene expression in the BMDM population used in this study was to be discussed in the context of previous studies.

Simmons and Lord had reported that the sorted F4/80 positive population would proliferate in L929CM and WEHICM while Hume and Gordon (1984) had observed that both L929CM and recombinant CSF-1 could induce DNA synthesis in BMDM as assessed by incorporation of tritiated thymidine.

The response of the BMDM population cultured in this study to L929CM and WEHICM were investigated. Initial experiments had indicated a high degree of variability in the growth properties of SDM and so this aspect of these cells was not investigated further.

Bone marrow-derived macrophages were re-plated at a density of $2.5 \cdot 10^5$ cells/ml in alpha-MEM medium containing 25% donor horse serum (DHS) in the presence or absence of either 20% L929CM or WEHICM.

Without a source of growth factor (other than those contained in DHS) the BMDM did not adhere to the surface of the plastic culture dish and by 72 hours very few cells could be observed in the culture dish, indicating cell death. When an established culture was refed with alpha-MEM medium (containing 25% DHS) without any other source of growth factor, the vast majority of the BMDM had become detached from the surface of the culture dish within forty-eight hours, again indicating cell death. It was concluded, therefore, that BMDM can not survive in alpha-MEM medium containing 25% DHS alone and hence possibly require an additional source of growth factor(s) to be present.

Tushinski et al (1982) had reported that the BMDM cultured by them could survive and proliferate in the presence but not the absence of either CSF-1 or L929CM, therefore the ability of the BMDM population cultured in this study to survive and proliferate in L929CM, and also in WEHICM, was assessed.

When BMDM were replated at a concentration of $2.5 \cdot 10^5$ cells/ml in alpha-MEM medium containing 25% DHS plus 20% L929CM, containing either 20% L929CM or 20% WEHICM, the cells were observed to have adhered to the bottom of the culture dish within twenty-four hours. As shown in figure 3.7 the growth kinetics of BMDM in 20% L929CM as monitored over seven days demonstrated exponential growth at a concentration of $1.25 \cdot 10^5$ - $1 \cdot 10^6$ cells/ml with a doubling time of approximately 24 hours after a lag phase of about three days. Twenty percent WEHICM, on the other hand, only had a modest effect on the growth of BMDM with the number of cells in the culture on day seven after replating being typically one and a half to two times initial number. This is consistent with the results of Simmons and Lord (1985) who found that sorted macrophages grew well in L929CM and that WEHICM could also support growth but at a slower rate. They did not, however, report the ability of these cells to survive and proliferate in absence of conditioned medium.

Interestingly, Simmons and Lord (1985) reported that many of the F4/80-positive cells "disintegrated" on replating in 20% L929CM and did not begin to proliferate until three days later. This effect was also observed on every occasion in this study (four experiments) in which a growth curve for BMDM in L929CM was performed. The reason for this behaviour is not clear and can not be due merely to experimental manipulation as BMDM replated in 20% WEHICM did not exhibit the same initial decline.

The BMDM could be scraped and replated in 20% L929CM at $2.5 \cdot 10^5$ cells/ml and a healthy confluent culture re-established three to four times before the proliferative ability of the cells was markedly compromised. The population sorted by Simmons and Lord (1985) behaved similarly.

The ability of BMDM to respond to the growth-promoting activity of L929CM was further confirmed by measuring the ability of L929CM to stimulate DNA synthesis in these cells. This was measured by the ability of BMDM to incorporate tritiated

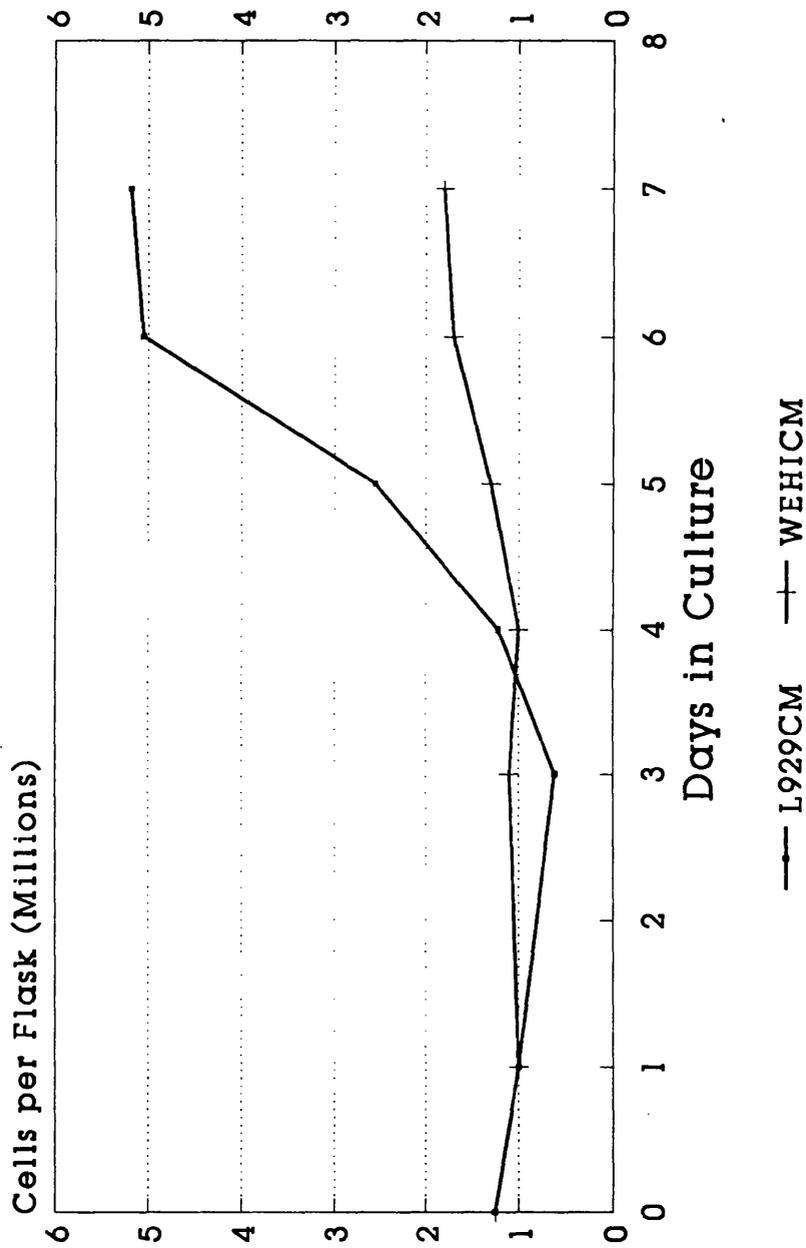


Figure 3.7

Figure 3.7 Growth of BMDM in medium containing L929CM and WEHICM.

BMDM were plated at a concentration of $1.25 \cdot 10^6$ per 5ml flask in alpha-MEM containing 25% DHS plus either 20% L929CM or 20% WEHICM as indicated. Adherent cells were counted using a haemocytometer and data presented as the mean determination from four flasks.

thymidine into trichloroacetic acid precipitable material. Figure 3.8 shows thymidine incorporation in BMDM after twenty hours in the presence or absence of various concentrations of L929CM or WEHICM. It can be seen that L929CM induces thymidine incorporation in a dose-dependent manner while no incorporation above background is detectable both in the absence of conditioned medium and with WEHICM even at a concentration of 40%. These data can be compared to those of Tushinski and Stanley (1982), Hume and Gordon (1984) and Hamilton et al (1988) who demonstrated that DNA synthesis in BMDM can be induced by L929CM and CSF-1.

The inability of WEHICM to promote the incorporation of thymidine during the time of exposure in this experiment (30 minutes) is consistent with the slow rate of proliferation observed with this conditioned medium (figure 3.7). Fetal calf serum could substitute for donor horse serum in this system; however, the magnitude of tritiated thymidine incorporation induced by L929CM in this system was consistently less than that observed with donor horse serum, as illustrated in figure 3.8, and hence donor horse serum was used in all subsequent experiments, unless otherwise indicated.

The kinetics of the induction of proliferation of BMDM in response to 20% L929CM was also investigated. It had been observed in initial experiments that the BMDM in untreated cultures after seven days could be proliferating moderately as assessed by tritiated thymidine incorporation (data not shown). In order to make the interpretation of data as straightforward as possible, BMDM were starved of L929CM for 12-16 hours to reduce proliferation to a minimum and then refed with alpha-MEM (containing 25% DHS) in the presence or absence of 20% L929CM and rates of DNA synthesis measured at various times after refeeding. It can be seen from figure 3.9 that there is a synchronous increase in tritiated thymidine incorporation from background to approximately fifty-thousand counts per minute between 12 and 16

Figure 3.8 Tritiated thymidine incorporation in BMDM following treatment with L929CM and WEHICM.

BMDM were starved overnight in alpha-MEM medium containing 25% DHS in the absence of any other additions. Medium was then removed and BMDM refed with alpha-MEM containing either 25% DHS (columns 1, 2, 3, 4, 5, 8 and 9) or 25% FCS (columns 6 and 7) plus the following additions:

- | | |
|----------|--------------|
| 1) Nil | no additions |
| 2) 20LCM | 20% L929CM |
| 3) 10LCM | 10% L929CM |
| 4) 5LCM | 5% L929CM |
| 5) 1LCM | 1% L929CM |
| 6) Nil/F | no additions |
| 7) 20L/F | 20% L929CM |
| 8) 20WCM | 20% WEHICM |
| 9) 40WCM | 40% WEHICM |

Tritiated thymidine incorporation was measured after 20 hours and the data presented as the mean determination of triplicate cultures plus or minus one standard deviation.

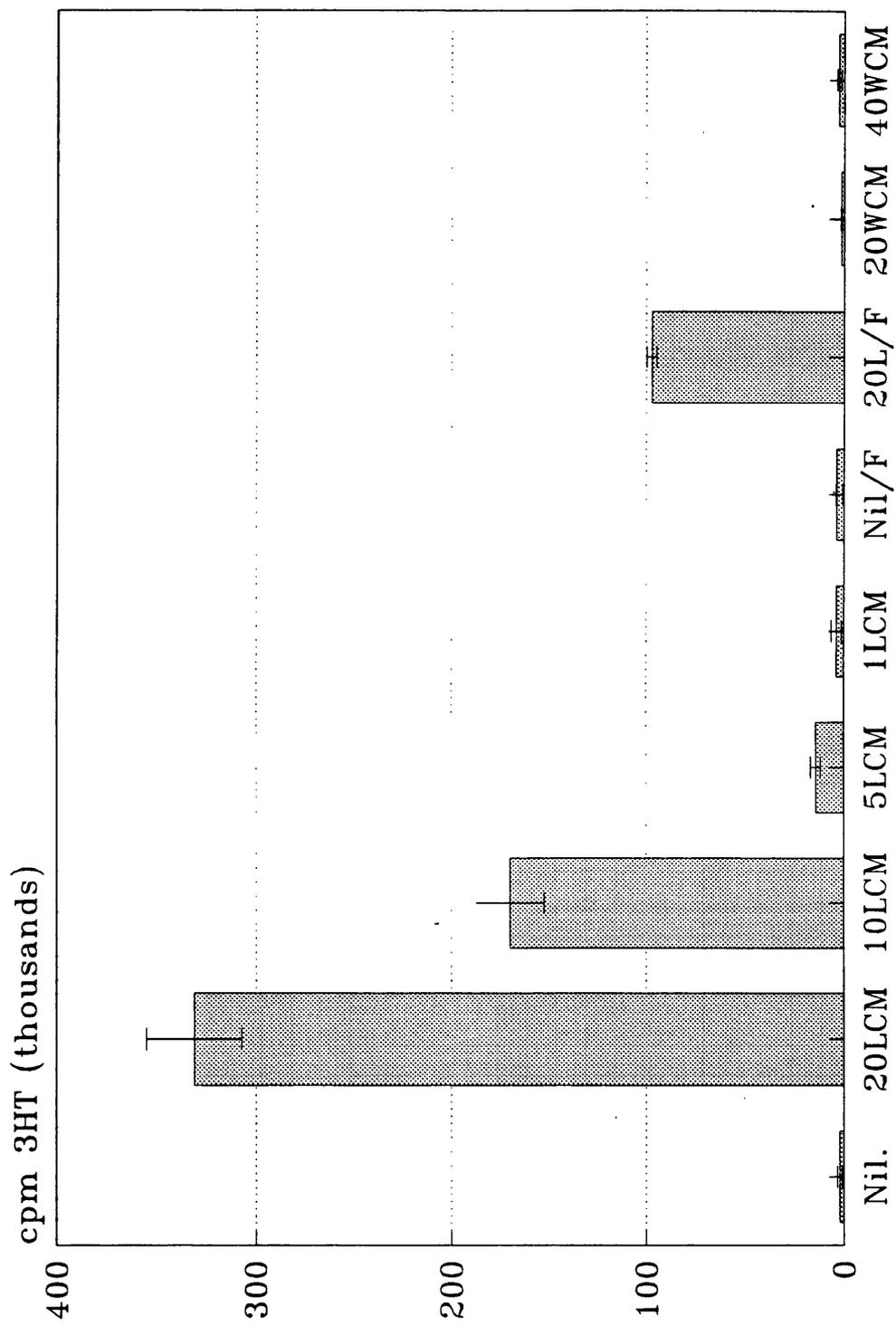
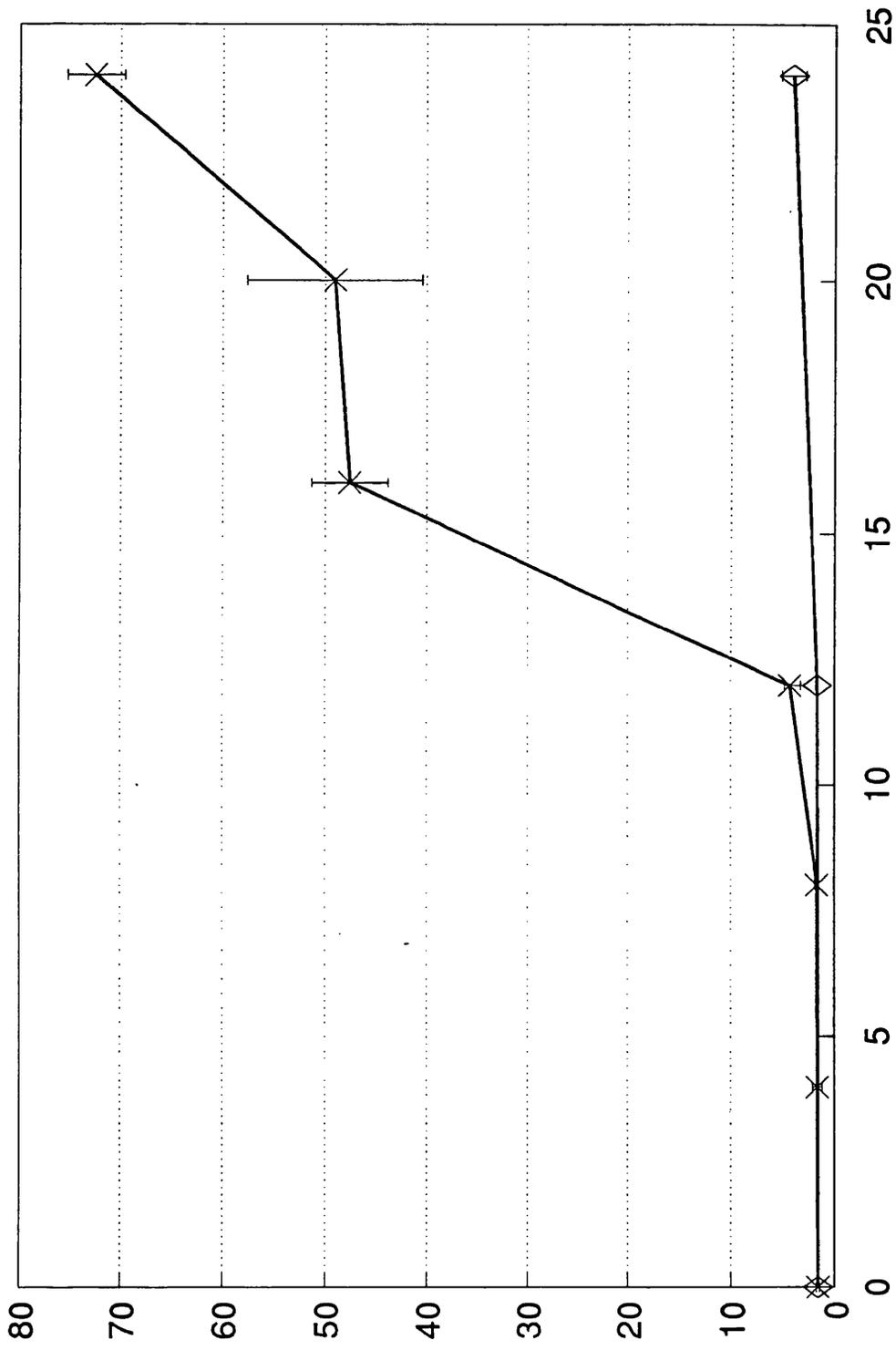


Figure 3.8

Figure 3.9 Time course of tritiated thymidine incorporation in BMDM following refeeding with alpha-MEM containing 25% DHS plus or minus 20% L929CM.

BMDM were starved overnight in alpha-MEM medium containing 25% DHS in the absence of any other additions. Medium was then removed and the cultures refeed with alpha-MEM medium containing 25% DHS plus (crosses) or minus (diamonds) 20% L929CM. Tritiated thymidine incorporation was measured at the appropriate time points and data presented as the mean determination of triplicate cultures plus or minus one standard deviation.

Tritiated Thymidine Incorporation: cpm (thousands)



Hours post refeed (+/- L929CM)

hours, compared to control cultures, in the absence of L929CM, where thymidine incorporation did not rise above background. The kinetics of the increase in tritiated thymidine incorporation suggest a synchronous entry of the BMDM into the S-phase of the cell cycle on refeeding with L929CM after starvation.

Although BMDM have been shown to be dependent on a source of CSF-1 for both survival and proliferation, at least two reports have demonstrated that both L929CM- and CSF-1-induced proliferation is affected by the presence or absence of serum. Hume and Gordon (1984) demonstrated that L929CM- and CSF-1-induced BMDM DNA synthesis was dose-dependent on the presence of (fetal calf) serum with proliferation being negligible even in the presence of a concentration of CSF-1 or L929CM which was optimal for proliferation. Tushinski and Stanley (1985) had also observed that incorporation of thymidine into BMDM nuclei was reduced by approximately 60% in the absence of fetal calf serum.

Accordingly, in order to further characterise the growth requirements of the BMDM population used in this study, the effect of serum on the ability of the BMDM population in this study to proliferate in response to L929CM, as measured by tritiated thymidine incorporation, was investigated. BMDM cells were cultured in a prepared serum-free medium (Dulbecco's/SF12 medium or SF12) in the presence or absence of donor horse serum (DHS) and/or 20% L929CM and DNA synthesis assessed by measuring the incorporation of tritiated thymidine by the cells after twenty-four hours. From figure 3.10A it can be seen that tritiated thymidine incorporation is negligible in serum-free medium alone and in alpha MEM plus 25% DHS as was expected. A level of thymidine incorporation comparable to that achieved with alpha MEM plus 25% DHS plus 20% L929CM was only seen after addition of both 20% L929CM and 25% DHS to SF12 medium and not with either of these alone. It was therefore concluded that the presence of both DHS and 20% L929CM was required for proliferation of the BMDM population used in this study.

Figure 3.10 Tritiated thymidine incorporation in BMDM in SF12 medium.

Figure 3.10A: BMDM were taken as a healthy growing culture 6 days after initial bone marrow inoculation, washed twice with PBS and refed with either SF12 medium (columns 1-4) or alpha-MEM medium (columns 5 and 6) plus the following additions:

SF	No additions
SF+L	20% L929CM
SF+D	25% DHS
SF+D+L	20% L929CM plus 25% DHS
MEM+D+L	20% L929CM plus 25% DHS
MEM	25% DHS

Tritiated thymidine incorporation was measured after 24 hours and data presented as the mean determination of triplicate cultures plus or minus one standard deviation.

Figure 3.10B: Cultures, treated as in figure 3.10A, had medium removed after 24 hours and all were refed with alpha-MEM containing 25% DHS plus 20% L929CM.

Tritiated thymidine incorporation was measured after 24 hours and data presented as the mean of triplicate cultures plus or minus one standard deviation.

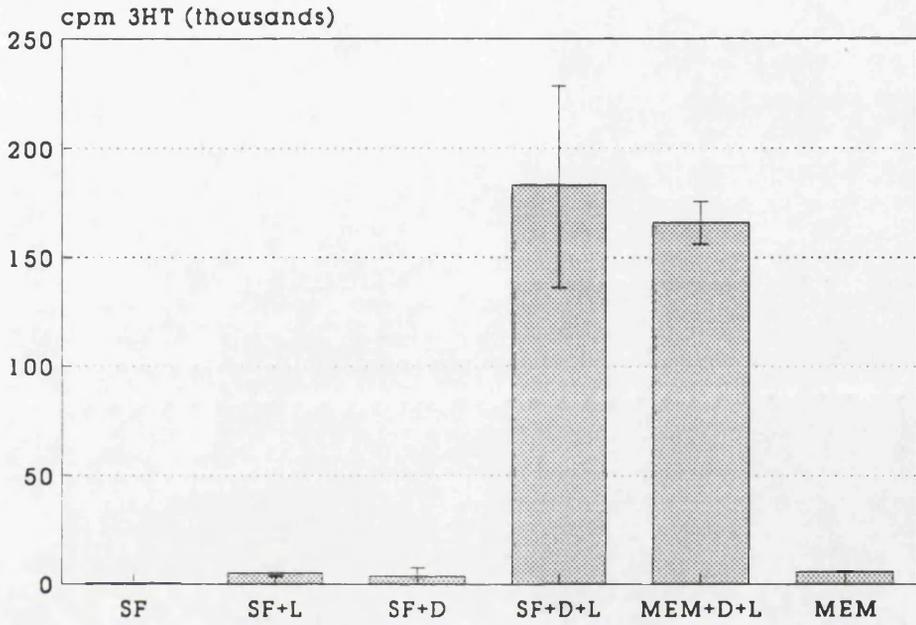


Figure 3.10A

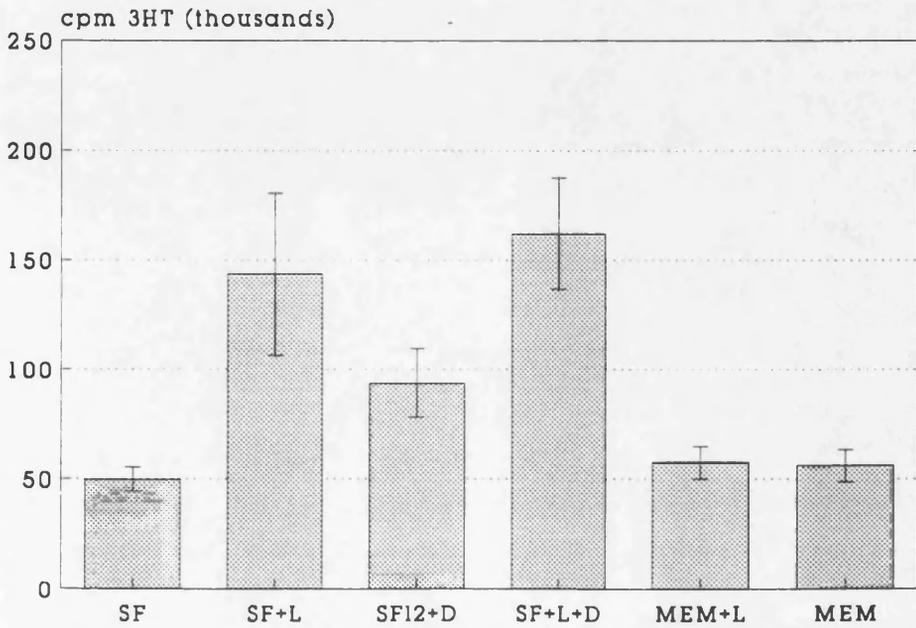


Figure 3.10B

The ability of these cells to survive in the various culture conditions was assessed in the same experiment by their subsequent ability to incorporate tritiated thymidine in response to 25% DHS-containing alpha-MEM medium with 20% L929CM. It can be seen (figure 3.10B) that while thymidine incorporation is reduced in all cases, this is more likely to be due to experimental manipulation than cell death as even those cells which had been cultured in the conditions optimal for growth had reduced thymidine incorporation on the second refeed. However, incubation in SF12 with either 20% L929CM or 25% DHS alone clearly enhances cell survival compared to that achieved with SF12 alone. This effect was seen in several separate experiments.

3.4 Detection of SCI/MIP-1 α mRNA in Bone Marrow Derived Macrophages, Normal Bone Marrow and Cell Lines.

An essential consideration with setting up the BMDM system was that SCI/MIP-1 α gene expression should be measured in some way.

One approach would have been to measure the ability of conditioned media from BMDM to inhibit the proliferation of CFU-A or CFU-S cells, as had been done with other cultured macrophage populations by Simmons and Lord (1985) and Pojda et al (1988), but this idea was rejected for two reasons. Firstly, these are both cumbersome assays and are not suitable for screening a large number of samples. Secondly, many molecules which could have an effect on primitive haemopoietic cells may be secreted by BMDM; Matsushime et al (1991b) have, for example, demonstrated that BMDM express IL-1 α , IL-1 β and the IL-1 receptor antagonist. The purpose of the present study, on the other hand, was to specifically study the expression of the SCI/MIP-1 α , as opposed to a haemoregulatory activity, in the context of haemopoiesis.

One way of ensuring that any assayed inhibitory activity from BMDM was in fact SCI/MIP-1 α would have been to block its activity by the use of a specific neutralising

antibody or antisera. Unfortunately no such reagent was available at any time during this study.

As Davatelis et al (1988) had previously isolated a cDNA sequence for SCI/MIP-1 α , it was clear that one way of evaluating expression of the SCI/MIP-1 α gene would be to measure levels of its messenger RNA by Northern blot analysis.

A radioactive antisense RNA molecule (riboprobe) was generated by using an in vitro run off transcription method. The template for this transcription reaction was an XhoI cut plasmid with the SCI/MIP-1 α cDNA sequence linked to a promoter from the bacteriophage T3. This allowed the production of an α -³²P-UTP-labelled riboprobe complementary to the mRNA for SCI/MIP-1 α which could be used to detect steady-state SCI/MIP-1 α levels by Northern blot analysis.

The SCI/MIP-1 α riboprobe was hybridised to total RNA (extracted from BMDM, SDM and the murine macrophage-like cell line J774.2) which had been separated by denaturing agarose gel electrophoresis and transferred and fixed onto a nylon membrane (Northern blotting). The J774.2 cell line had been used as a source of SCI/MIP-1 α protein by Graham et al (1990) and was therefore used as a positive control.

The integrity of the RNA loaded onto the gel was assessed by inspecting the ribosomal RNA (rRNA) bands, which could be visualised by staining with the fluorescent nucleic acid intercalating agent ethidium bromide under ultra violet light. Figure 3.11 shows a Northern blot demonstrating that BMDM, SDM and J774.2 cells contain an RNA species which hybridises to the SCI/MIP-1 α riboprobe. This transcript has a size of about approximately 800 nucleotides (as measured against RNA size markers) which corresponds to the size of the SCI/MIP-1 α transcript previously detected by Davatelis et al (1988). Furthermore, the levels of this transcript are increased approximately 50-fold (as measured by scanning densitometry) by treatment of both BMDM and SDM with 2 μ g/ml endotoxin from

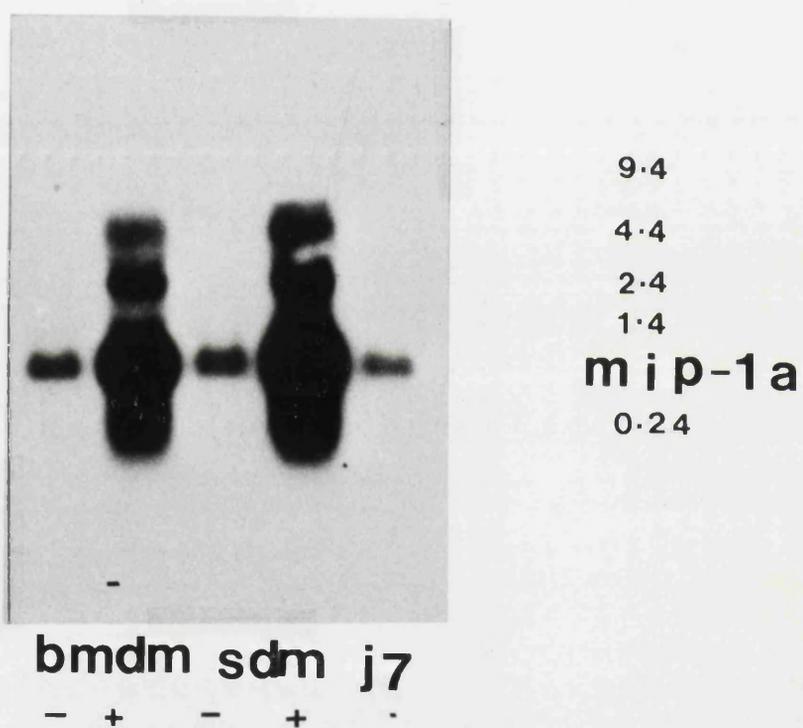


Figure 3.11 Northern analysis of the expression of SCI/MIP-1 α mRNA in BMDM, SDM and J774.2 cells.

Total RNA was isolated and blotted in equal amounts (20 μ g) onto nylon membrane. Lane 1, untreated BMDM; lane 2, BMDM treated for 6 hours with LPS (2 μ g/ml); lane 3, untreated SDM; lane 4, SDM treated for 6 hours with LPS (2 μ g/ml); lane 5, untreated J774.2 cells. Equal loading was ensured by ethidium bromide staining of the gel.

S. Typhimurium for six hours. This observation is in agreement with the work of Davatellis et al (1988) with the macrophage-like cell line RAW264.7.

To ascertain whether the RNA species detected in BMDM was in fact a messenger RNA, the poly(A)⁺ fraction of total RNA prepared from BMDM before and after treatment with LPS for four hours was analysed by Northern blot. From figure 3.12 (lane 1) it can be seen that a specific band hybridising to the SCI/MIP-1 α riboprobe can be detected in BMDM poly(A)⁺ RNA, indicating that the species detected in total RNA is in fact SCI/MIP-1 α mRNA. Lane 2 of the same figure shows that the extent of this hybridisation is increased by treating BMDM with LPS, demonstrating that LPS treatment increases the representation of SCI/MIP-1 α mRNA in BMDM.

Much of the RNA hybridising to the SCI/MIP-1 α -specific riboprobe in lanes 2 and 4 of figure 3.11 lies outside the 0.8kb size level. This is probably due to the fact that the level of SCI/MIP-1 α mRNA in the samples from cells treated with LPS is many times greater than that in control samples, where a discrete band of the expected size is seen; presumably due to the fact that only a certain amount of RNA can be contained at a particular level in a gel. In addition to this, two extra bands of approximately 5 and 2kb can also be seen in lanes 2 and 4. These sizes correspond to those of the two higher molecular weight ribosomal RNA (rRNA) bands. Non-specific hybridisation to rRNA is a common problem with riboprobes; however, it is unlikely that this is the case in these particular experiments for two reasons: 1) The hybridisation conditions for these experiments are extremely stringent and this should preclude non-specific effects. 2) The additional bands are only seen in the lanes containing RNA from cells in which the level of SCI/MIP-1 α -specific hybridising sequences has been greatly elevated. If non-specific hybridisation to rRNA was taking place these bands would have been seen with all samples as ethidium bromide staining of the gel showed that equal amounts of rRNA was loaded. A more likely explanation is that the level of SCI/MIP-1 α mRNA in samples from the LPS-treated

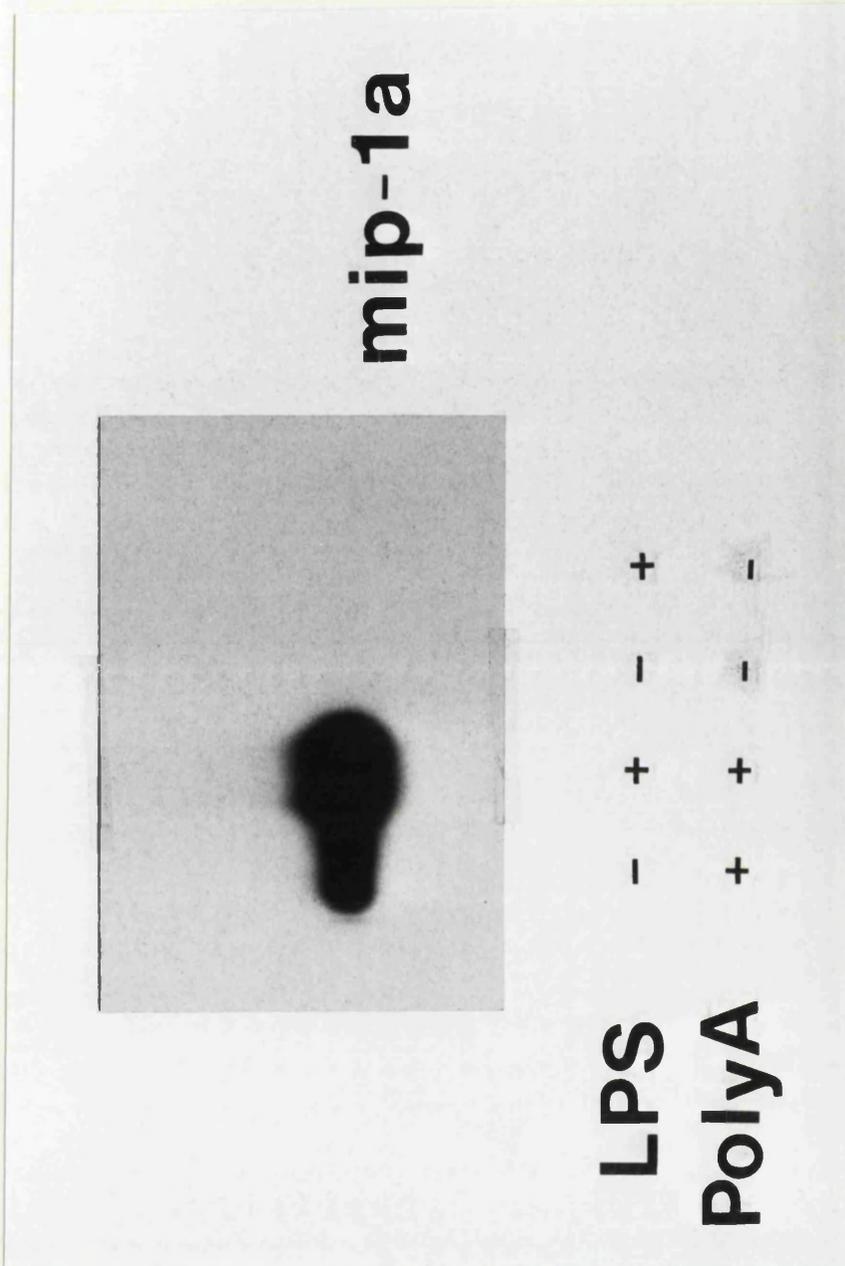


Figure 3.12 Northern analysis of the distribution of SCI/MIP-1 α -specific hybridising sequences in the poly(A)⁺ and poly(A)⁻ fractions of total cellular RNA from BMDM.

Total cellular RNA was isolated and separated into poly(A)⁺ and poly(A)⁻ fractions and blotted onto nylon membrane. Lane 1, poly(A)⁺ RNA from untreated BMDM; lane 2, poly(A)⁺ RNA from BMDM treated for 4 hours with LPS (2 μ g/ml); lane 3, poly(A)⁻ RNA from untreated BMDM; lane 4, poly(A)⁻ RNA from BMDM treated for 4 hours with LPS (2 μ g/ml).

BMDM and SDM is so great that some of it is complexed in some way with the hugely abundant ribosomal bands, due to incomplete denaturation. Hence the hybridisation to the additional higher molecular weight bands is specific for SCI/MIP-1 α mRNA; it is merely the position of hybridisation which is artefactual. Evidence to support this explanation can be gained by Northern blot analysis of both the poly(A)⁺ and poly(A)⁻ RNA fractions of LPS-treated BMDM. It can be seen from figure 3.12 that the SCI/MIP-1 α -specific riboprobe hybridises to only one discrete band in the poly(A)⁺ fraction (lane 2) indicating that only one size of SCI/MIP-1 α mRNA is expressed on LPS treatment. In addition, it can be seen from lane 3 that no SCI/MIP-1 α -specific sequences are contained in the poly(A)⁻ fraction, nor are any induced on treatment of cells with LPS, as seen in lane 4 of figure 3.12, indicating that the SCI/MIP-1 α -specific riboprobe only hybridises to mRNAs.

Hence, the most likely explanation for the presence of the additional bands hybridising to the SCI/MIP-1 α -specific riboprobe on LPS-treatment of BMDM and SDM is the complexing of SCI/MIP-1 α mRNA with rRNA.

It was concluded, therefore, that Northern analysis of total cellular RNA was a suitable method for the determination of SCI/MIP-1 α mRNA levels in BMDM (and SDM).

As the main aim of this project was to investigate the expression of the SCI/MIP-1 α gene in relation to its possible role in *in vivo* haemopoiesis, the ability to detect the expression of SCI/MIP-1 α mRNA in normal bone marrow was also investigated. Figure 3.13 shows a Northern blot in which total RNA from normal unfractionated bone marrow and the J774.2 cell line was hybridised to the SCI/MIP-1 α -specific riboprobe. It can be seen that a low level of SCI/MIP-1 α mRNA, approximately one twentieth of the level of that seen in unstimulated J774.2 cells (as assessed by densitometry), is detectable in normal, unfractionated bone marrow. This is an interesting finding in that mRNA for several other cytokines has not been reported as

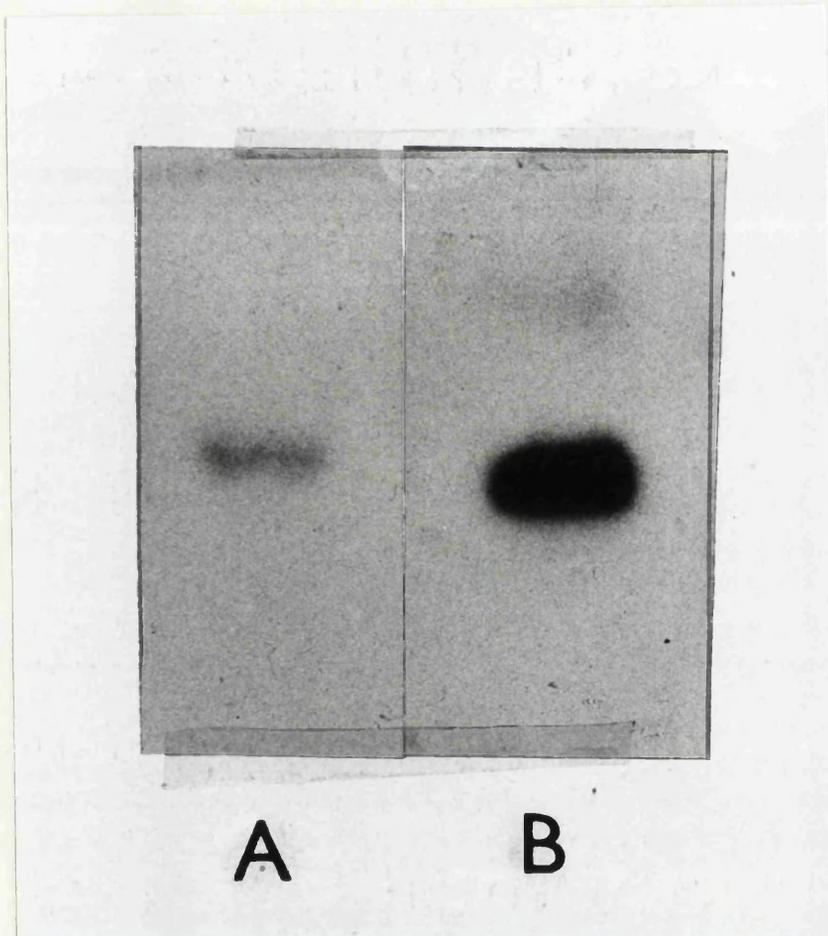


Figure 3.13 Northern analysis of the expression of SCI/MIP-1 α mRNA in normal murine bone marrow and in the J774.2 cell line.

Total cellular RNA was isolated and blotted in equal amounts (20 μ g) onto nylon membrane. Lane 1, normal murine bone marrow; lane 2 untreated J774.2 cells.

being detectable in normal untreated marrow (Troutt and Lee 1989) and Fahey et al (1990) have reported inability to detect mRNA for SCI/MIP-1 α in unstimulated murine peripheral blood leukocytes. This observation demonstrates expression of the SCI/MIP-1 α gene in normal, unperturbed murine bone marrow and hence is strongly suggestive of a role for SCI/MIP-1 α in the biology of normal, unperturbed marrow. In addition to this, and in an attempt to assess the cellular specificity of expression, the ability to detect SCI/MIP-1 α mRNA in cell lines representing the monocytic (RAW264.7, J774.2, WEHI3B, P388.1), fibroblastic (STO, L929), erythroid (F4), B lymphocytic (RA3 3A1) and T lymphocytic (DA2) lineages was assessed. It can be seen from figure 3.14 that mRNA could only be detected in the four cell lines representing the monocytic lineage and in a Moloney murine leukemia virus-transformed T cell line (DA2) (Ihle et al 1990), which was not unexpected considering that SCI/MIP-1 α mRNA has been demonstrated to be present in activated murine T cell lines by Brown et al (1989). No mRNA for SCI/MIP-1 α was detectable in any of the other cell lines even on prolonged exposure (six weeks) of the blots to X-ray film.

The ability to readily detect SCI/MIP-1 α mRNA in monocytic cell lines and in BMDM suggests, but not fibroblastic cells that macrophages are the main producers of SCI/MIP-1 α in the bone marrow. Although T cells and mast cells have been shown to express SCI/MIP-1 α mRNA, both of these cell-types are present at extremely low levels in the marrow (Kincaide 1990, Gordon et al 1990) and have to be specifically activated before SCI/MIP-1 α mRNA can be detected (Brown et al 1989, Burd et al 1989); it is therefore likely that they make little or no contribution to the level of SCI/MIP-1 α mRNA detectable in normal murine bone marrow, which indicates that the regulation of expression of SCI/MIP-1 α gene expression in the macrophage is most pertinent to marrow haemopoiesis.

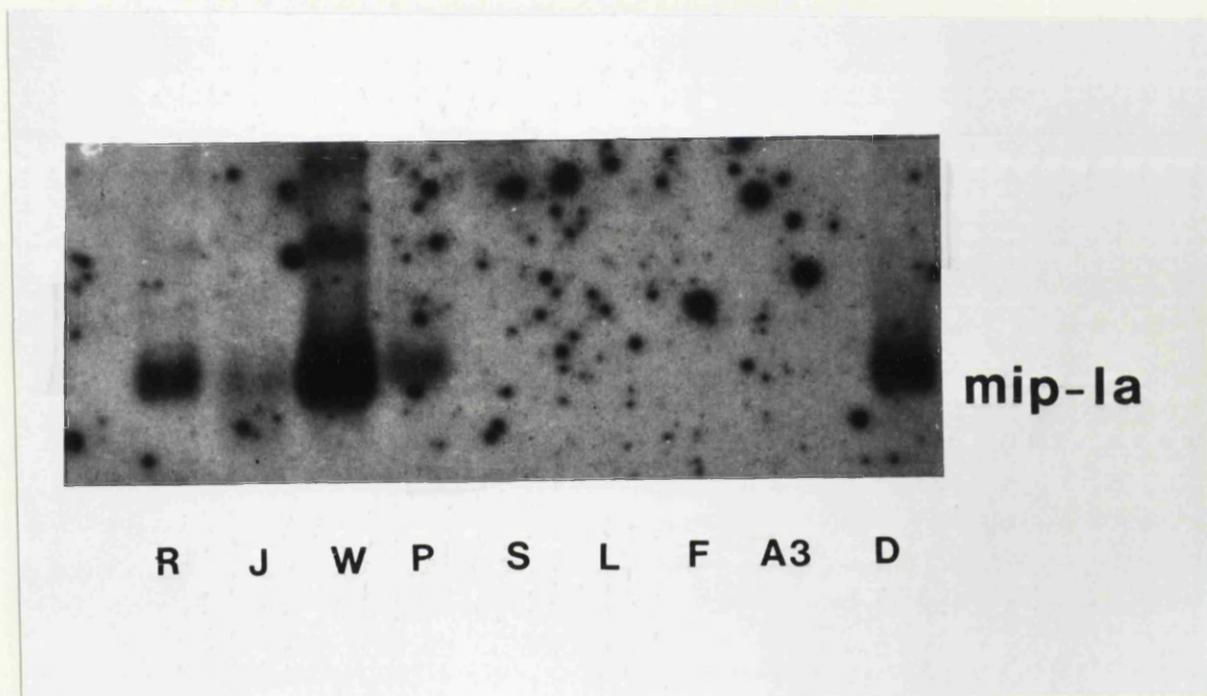


Figure 3.14 Northern analysis of the expression of SCI/MIP-1 α mRNA in 9 cell lines.

Total cellular RNA was isolated from nine different cell lines and blotted onto nylon membrane. Lane 1, RAW264.7 (R); lane 2, J774.2 (J); lane 3, WEHI 3B (W); lane 4, P388.1 (P); lane 5, STO (S); lane 6, L929 (L); lane 7, F4 (F); lane 8, RA3-3A1 (R); lane 9, DA-2 (D). Different amounts of RNA were added to each lane; therefore the level of mRNA is not representative of the relative levels expressed by the cell lines but does demonstrate the presence or absence of expression.

3.5 Detection of SCI/MIP-1 α Protein Secretion by Bone Marrow-Derived Macrophages.

Although mRNA for SCI/MIP-1 α had now been detected in BMDM it was also necessary to demonstrate that the cells translated and secreted mature SCI/MIP-1 α to gain a complete picture of SCI/MIP-1 α gene expression.

For most of the time of this investigation no specific tools were available for the detection of SCI/MIP-1 α protein, such as an antibody or a simple specific bioassay; therefore, an alternative method was sought.

Brown et al (1989) and Chang and Reinherz (1989) had isolated the cDNA sequences for SCI/MIP-1 α and human MIP-1 β ignorant of their function. Both of these investigators demonstrated that these were secreted proteins by exploiting the fact that both proteins contain cysteine. The proteins were detected in conditioned medium of fibroblastic cell lines which had been transfected with the appropriate cDNA in an expression vector and grown in culture media containing radioactive (^{35}S) cysteine. The presence of the secreted ^{35}S -labelled protein was then detected by SDS-polyacrylamide gel electrophoresis and autoradiography.

A similar approach was attempted to detect the presence of SCI/MIP-1 α (or MIP-1), expressed from the endogenous gene, in the media conditioned by BMDM. BMDM cells were cultured for four hours in cysteine-free medium supplemented with ^{35}S -labelled cysteine in the presence and absence of LPS. The proteins secreted into the medium by the cells were fractionated on an SDS-PAGE gel and the presence of ^{35}S -containing proteins resolved by autoradiography.

Figure 3.15 shows the ^{35}S -cysteine-containing proteins secreted by both RAW264.7 cells and BMDM in the presence or absence of LPS (2 $\mu\text{g/ml}$). LPS was added four hours before the culture medium was changed for medium supplemented with ^{35}S -cysteine (as this had been shown to be the time when SCI/MIP-1 α mRNA is maximally induced by LPS, see figure 4.1) and additional LPS was added with the

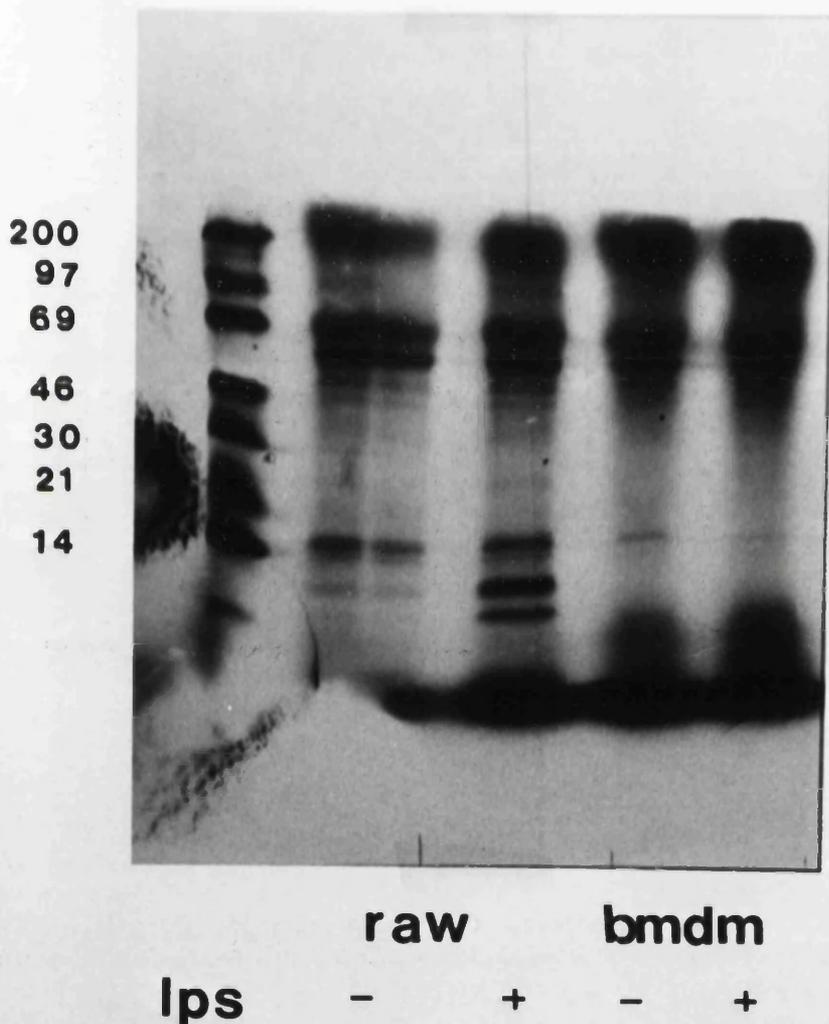


Figure 3.15 SDS-PAGE of ^{35}S -labelled proteins in medium conditioned by BMDM and RAW264.7 cells with and without stimulation by LPS ($2\mu\text{g/ml}$).

Lane 1, ^{14}C -labelled molecular weight markers; lane 2, untreated RAW264.7 cells; lane 3, RAW264.7 cells treated with LPS for 4 hours; lane 4, untreated BMDM; lane 5, BMDM treated with LPS for 4 hours.

new medium. MIP-1 protein migrates with an apparent molecular weight of 8kDa on SDS-PAGE (Wolpe et al 1988). There are a few bands which are induced by LPS and secreted by RAW264.7 which could correspond to MIP-1. However, the same region in the lanes containing medium conditioned by BMDM with or without being treated with LPS is very blurred, possibly due to protein degradation, and contains a large number of minor bands situated very close together and is therefore impossible to interpret. This culture technique was repeated twice and the samples run on SDS-PAGE several times.

Wolpe et al (1988) and Graham et al (1990) had demonstrated that MIP-1 protein binds heparin; therefore a way of improving this detection method may have been to enrich for heparin-binding proteins before electrophoresis. Alternatively, the timing of the endotoxin treatment (when mRNA levels were maximal) may not have been optimal for the translation or secretion of MIP-1 protein.

Extracts of intracellular proteins from these experiments were also made and run on SDS-PAGE in case MIP-1 was translated by the BMDM but not secreted into the culture medium. However, no discrete bands could be detected on autoradiography, presumably because of the large number of cysteine-containing intracellular proteins. One of the main problems with using this technique may be related to the fact that macrophages secrete over three hundred proteins (Nathan 1987) which means that the conclusive identification of a distinct band is difficult. Although this technique could possibly have been refined using perhaps chromatography, or a detailed analysis of the kinetics of protein translation and secretion, the technique was abandoned due to constraints of time.

Toward the end of this study, a polyclonal anti-sera raised against murine SCI/MIP-1 α protein in the goat became available. When used at a high titre in conjunction with the luminol detection kit this allowed sensitive detection of SCI/MIP-1 α protein by Western Blot. Figure 3.16A shows a Western Blot of the proteins secreted by both



Figure 3.16 Western analysis of SCI/MIP-1 α protein levels in media conditioned by BMDM and RAW264.7 cells in the presence or absence of LPS (2 μ g/ml).

Panel A Western analysis: Cells were plated at a concentration of 10⁶/ml; BMDM in alpha-MEM medium containing 25% DHS plus 20% L929CM, RAW264.7 in SLM/ 10% FCS. After 24 hours cells were refed with the appropriate medium plus or minus LPS (2 μ g/ml) for a further 24 hours, after which conditioned medium was harvested and analysed as described in chapter 2.

Lane 1, pure recombinant SCI/MIP-1 α (10ng); lane 2, untreated NBM; lane 3, BMDM treated with LPS; lane 4, untreated RAW264.7 cells; lane 5, RAW264.7 cells treated with LPS (2 μ g/ml).

Panel B Northern analysis of the same cultures (Note that the lanes do not correspond): Total cellular RNA was isolated and loaded in equal amounts (20 μ g) onto nylon membrane.

Lane A, untreated RAW264.7 cells; lane B, RAW264.2 cells treated with LPS (2 μ g/ml) for 24 hours; lane C, untreated BMDM; lane D, BMDM treated with LPS (2 μ g/ml) for 24 hours.

RAW264.7 cells and BMDM with or without treatment with 2 μ g/ml endotoxin from *S.Typhimurium* for twenty four hours. It can be seen that unstimulated RAW264.7 cells secrete detectable SCI/MIP-1 α protein and that this level can be increased 3-4-fold (as estimated by laser densitometry) by treating the cells with LPS for twenty-four hours. On the other hand, no SCI/MIP-1 α can be detected from BMDM until they are treated with LPS for twenty-four hours.

Changes in SCI/MIP-1 α mRNA levels of the cultures described in figure 3.16A were also assessed by Northern blotting as seen in figure 3.16B. It can be seen that the level of SCI/MIP-1 α mRNA is increased greater than fifty-fold in RAW264.7 cells twenty-four hours after LPS (lanes A and B), demonstrating that induction of SCI/MIP-1 α mRNA accumulation in BMDM by LPS is mirrored in protein secretion. It can be seen from lanes C and D of the same figure that the level of SCI/MIP-1 α mRNA in BMDM is the same before and after the same treatment; however, this does not mean that LPS-induced secretion of SCI/MIP-1 α protein by BMDM is unaccompanied by changes in SCI/MIP-1 α mRNA levels as will be discussed in chapter 4.

3.6 Summary of Chapter 3

Attempts were made to isolate macrophages directly from murine bone marrow using immunomagnetic depletion. This approach was discontinued as: 1) the reproducibility of the technique was highly variable, 2) positive identification of isolated populations was difficult due to practical problems, 3) the yield of separated populations was inadequate for the experiments envisaged.

An alternative approach was followed by culturing macrophages from normal bone marrow and spleen using medium conditioned by the fibroblastic cell line L929, which is a source of CSF-1.

These culture conditions produced an essentially homogeneous population of macrophages as identified by morphology and by positive staining for the macrophage-specific enzyme alpha-naphthyl acetate esterase.

The growth properties of the bone marrow-derived macrophage (BMDM) population were investigated and were found to be extremely similar to a macrophage population sorted directly from murine marrow which is a producer of the Manchester Inhibitor and to previously cultured macrophage populations.

In the absence of a method for readily detecting SCI/MIP-1 α protein, Northern blotting was used to detect expression of mRNA for SCI/MIP-1 α in BMDM (and also in spleen-derived macrophages). This level was found to be increased greatly by exposure of these cells to bacterial endotoxin. Fractionation of total cellular RNA from BMDM into poly(A)⁺ and poly(A)⁻ pools demonstrated that the SCI/MIP-1 α -specific hybridising sequence was a messenger RNA.

A low level of SCI/MIP-1 α mRNA was also detectable in total cellular RNA prepared from normal, unfractionated murine bone marrow, demonstrating expression of the SCI/MIP-1 α gene in normal, unperturbed murine bone marrow.

SCI/MIP-1 α mRNA was also readily detectable in monocytic cell lines and in a transformed T cell line but not in cell lines representing other haemopoietic lineages or fibroblasts.

Metabolic labelling of proteins secreted by BMDM did not prove to be a suitable method for the detection of the SCI/MIP-1 α protein.

Towards the end of the study it was demonstrated that SCI/MIP-1 α protein could be detected in media conditioned by BMDM after treatment with bacterial LPS by Western Blotting using a specific goat polyclonal anti-SCI/MIP-1 α antiserum.

Chapter 4

Induction of mRNA for SCI/MIP-1 α and MIP-1 β in BMDM by Bacterial Endotoxin (Lipopolysaccharide): Kinetics of Expression and Initial Mechanism Studies.

4.1 Introduction.

It had been demonstrated in chapter 3 that the level of SCI/MIP-1 α mRNA expressed in BMDM could be greatly increased by treating the cells with bacterial endotoxin. In order to form a clearer picture of the ways in which SCI/MIP-1 α mRNA levels are modulated, this induction was investigated in more detail. To this end, bacterial lipopolysaccharide (or endotoxin), which had been demonstrated in chapter 3 to induce accumulation of SCI/MIP-1 α mRNA in BMDM and the glucocorticoid hormone hydrocortisone were used a model inducer and downregulator of SCI/MIP-1 α gene expression, respectively.

Expression of the closely-related Macrophage Inflammatory Protein-1 β (MIP-1 β) gene, whose protein product has no detectable effect on the proliferation of haemopoietic stem cells *in vitro* (Graham et al 1990) but is apparently able to modulate the activity of SCI/MIP-1 α (Broxmeyer et al 1991) was also investigated for comparison. Messenger RNA for MIP-1 β is approximately the same size as that for SCI/MIP-1 α (Sherry et al 1988). It was reasoned that any differences in the expression of the two genes which could be demonstrated may be informative and make a contribution to both our understanding of the regulation of expression of these genes and the relationship of this to the biology of SCI/MIP-1 α and MIP-1 β .

Endotoxin, or Lipopolysaccharide (LPS), is a complex glycolipid which is the major component of the outermost coat of Gram-negative bacteria. The biologically active component of LPS is the lipid A moiety, which is highly conserved between the LPS

of many bacterial species (for review see Raetz 1990). LPS has pleiotropic effects on leukocytes, especially monocytes/macrophages, including adherence to endothelial cells (Sporn et al 1990) and priming for oxidative burst (Hamilton et al (1989) and has also been shown to induce the expression of the genes for, and promote the release of, a number of cytokines including IL-1 α , IL-1 β and TNF α as discussed in chapter 1.

It is generally agreed that LPS stimulates cells by binding to specific receptors, thereby initiating a signal transduction event.

Several LPS-binding proteins have been identified on macrophages and evidence suggests that these may be involved in several functions involving the recognition of LPS including the activation of macrophages, the uptake and detoxification of free LPS and the attachment of whole Gram-negative organisms prior to phagocytosis (for review see Lynn and Golenbock 1992). LPS can also prime macrophages for more complex cytotoxic behaviour such as tumour cell killing (Adams and Hamilton 1984). Candidate LPS receptors responsible for the activation of macrophages include the cell surface determinant CD14, a 55KDa phosphoinositol-linked protein which binds LPS complexed with a serum-binding protein (Schumann et al 1990, Wright et al 1990) and a 55KDa/65KDa protein dimer found on the surface of the J774.1 macrophage cell line (Hara-Kuge et al 1990) which appears to be essential for activation of these cells by LPS.

The signalling pathways activated by LPS in macrophages are, at the moment, unclear (Raetz 1990); however, the ability of LPS to activate inducible transcription factors, such as NF κ B (Baeurle 1991, Vincenti et al 1992)) and to alter mRNA stability (Thorens et al 1987, Koerner et al 1987) and to induce secretion of cytokines (Han et al 1990) in macrophage cell lines has been demonstrated.

The presence of MIP-1 β mRNA was detected by Northern blotting using a specific cDNA riboprobe in the same manner as SCI/MIP-1 α mRNA. As before, the integrity

of the total RNA and equal loading between lanes was ensured by ethidium bromide staining of the RNA samples. As an additional control to ensure equal loading of RNA, Northern blots were hybridised to a radioactive DNA probe specific for the murine 7S RNA species. 7S RNA is a component of the signal recognition particle of the ribosome and is highly conserved among eukaryotes (Lewin 1987). It has a size of 280 nucleotides (Balmain et al 1982).

Probes for RNA Polymerase II-transcribed "housekeeping" genes such as the gene for the cytoskeletal component actin were also considered as loading controls; however, Mufson (1990) has recently demonstrated that level of β -actin mRNA changes on stimulation of human peripheral blood macrophages when measured in relation to the level of ribosomal RNA visualised by ethidium bromide staining under ultraviolet light. In the light of this, it was considered that expression of the 7S mRNA would be less likely to fluctuate, with it being a component of the nuclear RNA splicing apparatus.

A probe for the β_2 -microglobulin gene was initially used as a loading control, however this probe hybridises to a mRNA which is approximately the same size as the SCI/MIP-1 α and MIP-1 β mRNA transcripts which meant that reprobing for β_2 -microglobulin necessitated stripping the membrane at high temperature and low salt which might result in the loss of some of the transferred RNA. In addition to this it has recently been demonstrated that levels of β_2 -microglobulin mRNA can be altered by interferon- γ (Sen and Lengyel 1992). The difference in size between the RNA transcripts of 7S and the two MIP-1 mRNAs was useful as it allowed hybridisation membranes to be sequentially probed without stripping. In all experiments in this study for which the level of 7S RNA was assessed differences in levels between samples corresponded to differences between the levels of ribosomal RNA visualised by ethidium bromide staining; hence it was concluded that hybridisation to 7S RNA was a suitable loading control for this study.

4.2 Kinetics of Expression of SCI/MIP-1 α and MIP-1 β mRNA.

If analysis of the modulation of SCI/MIP-1 α and MIP-1 β mRNA levels was to be carried out it was essential that the kinetics of accumulation and turnover of the mRNA transcripts be established given that lymphokine genes tend to be expressed in fairly short time frames in response to stimuli (Taniguchi 1988; Arai 1990). It was also necessary to demonstrate that BMDM expressed mRNA for MIP-1 β .

Northern blot analysis of the expression of both SCI/MIP-1 α and MIP-1 β mRNA transcripts in BMDM at various times after stimulation with 2 μ g/ml LPS was therefore carried out. Figure 4.1 shows the results of one of four different experiments. It can be seen that the levels of both mRNA transcripts are greatly elevated at four hours post treatment with LPS. This increase was found to be approximately 50-150-fold, for both mRNAs, by scanning laser densitometry. Levels of both mRNAs peak at 4-8 hours and decline thereafter. By twenty four hours the level of MIP-1 β mRNA has returned to the same level as the time zero control while that of SCI/MIP-1 α mRNA is still at approximately 5-10 times the level of the controls (as estimated by densitometry). In the experiment shown (figure 4.1), MIP-1 β mRNA levels did not decline as rapidly as those of SCI/MIP-1 α mRNA but this feature did vary between experiments. It can also be seen from lanes 1 and 2 of figure 4.3 that the levels of both SCI/MIP-1 α and MIP-1 β mRNA are elevated by one hour after addition of LPS which indicates that the onset of accumulation of these transcripts after stimulation is extremely rapid.

One explanation for the difference in the levels of the two transcripts at the later time points might be related to the fact that the 3' untranslated sequence of MIP-1 β mRNA has a complete copy (Sherry et al 1988) of the AU-rich sequence (consensus sequence TTATTTAT) which is present in many rapidly induced mRNA transcripts (Caput et al 1986) and are thought to confer instability (Shaw and Kamen 1986) and

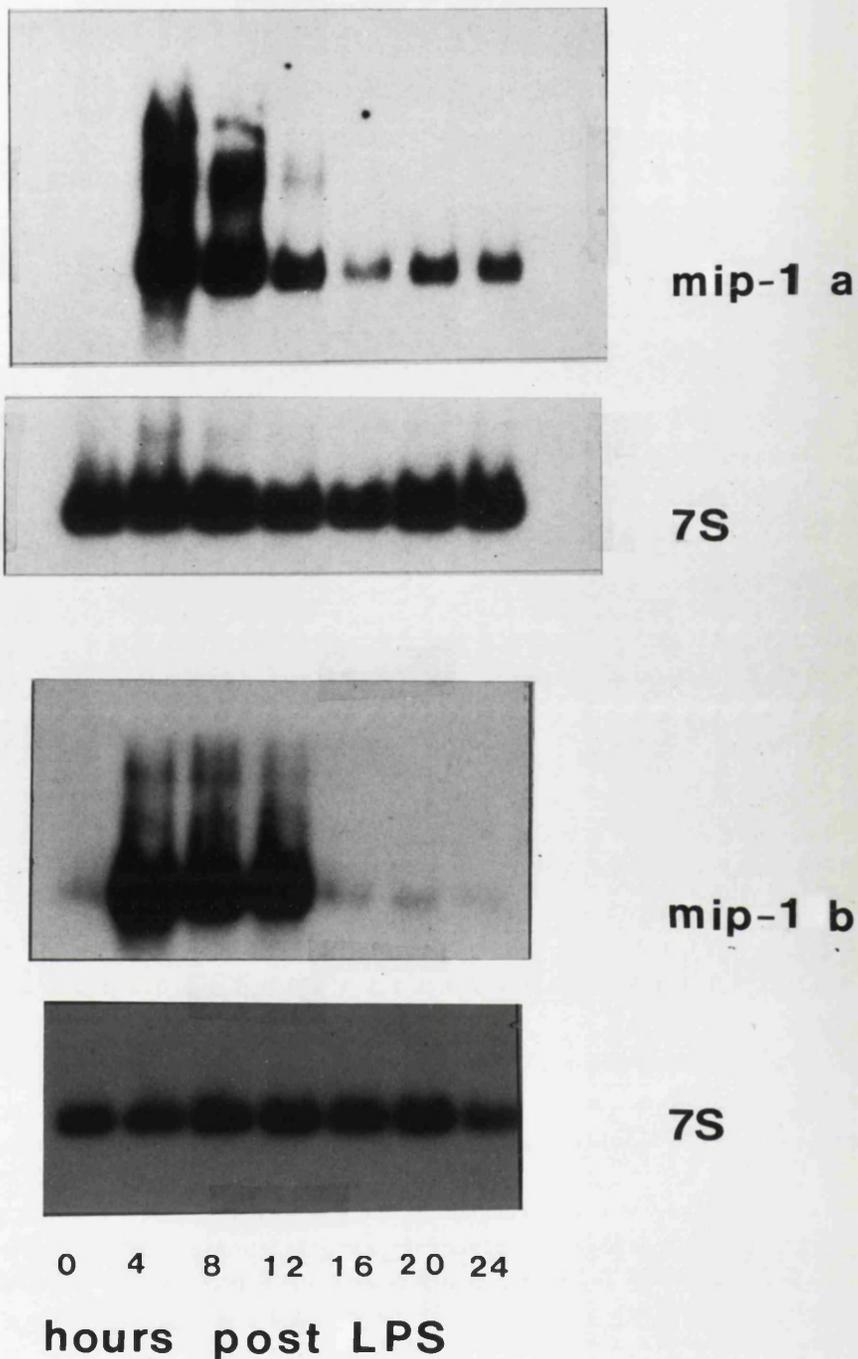


Figure 4.1 Northern analysis of the time course of accumulation of SCI/MIP-1 α and MIP-1 β mRNA in BMDM treated with LPS (2 μ g/ml).

Total cellular RNA was isolated and blotted in equal amounts (20 μ g) onto nylon membrane. Lanes 1-7, BMDM treated with LPS for 0, 4, 8, 12, 16, 20 and 24 hours. Equal loading was confirmed by hybridisation with a 7S probe.

may also be necessary for the modulation of that instability (Akashi et al 1991); MIP-1 α only has incomplete copies of this consensus sequence (Davatellis et al 1988; Wolpe and Cerami 1988; M.Plumb personal communication). It is possible that the differences in the AU-rich sequences confer different turnover rates on SCI/MIP-1 α and MIP-1 β mRNA at these later times.

It should be borne in mind, however, that the decay of several labile mRNA species has been attributed to other poorly-defined sequence components and indeed the 3' AU-rich sequence may only be a minor component (Kabnick and Houseman 1988; Shyu et al 1989; Han et al 1991a).

These expression patterns are different to those exhibited by the same transcripts in RAW264.7 cells in response to LPS where both transcripts are elevated rapidly but remain elevated at the peak level even up to 48 hours later (Davatellis et al 1989; Orlofsky et al 1991; Widmers et al 1991). In this present study it was found that levels of MIP-1 α and MIP-1 β mRNA were still highly elevated in RAW264.7 cells twenty-four hours after treatment with LPS (figure 4.2). However, rapid and transient expression in response to stimulation of the human equivalent of SCI/MIP-1 α has been reported in both monocytic and T lymphocytic cell lines (Yamamura et al 1989; Nakao et al 1990; Schall et al 1992). The same pattern of expression has also been reported for human MIP-1 β in isolated human monocytes, peripheral blood lymphocytes, peripheral blood mononuclear cells and human T and B lymphocytic cell lines (Sporn et al 1990; Chang and Reinherz 1989; Lipes et al 1988; Schall et al (1992).

This pattern of expression is common for many lymphokine genes (Taniguchi 1986, Arai et al 1990) and may relate to their role in the response to inflammatory and immune stimuli. The confinement of the expression of these genes to discrete bursts may be one way of ensuring that they are not expressed at inappropriate times as there is a tight coupling between stimulation and response.

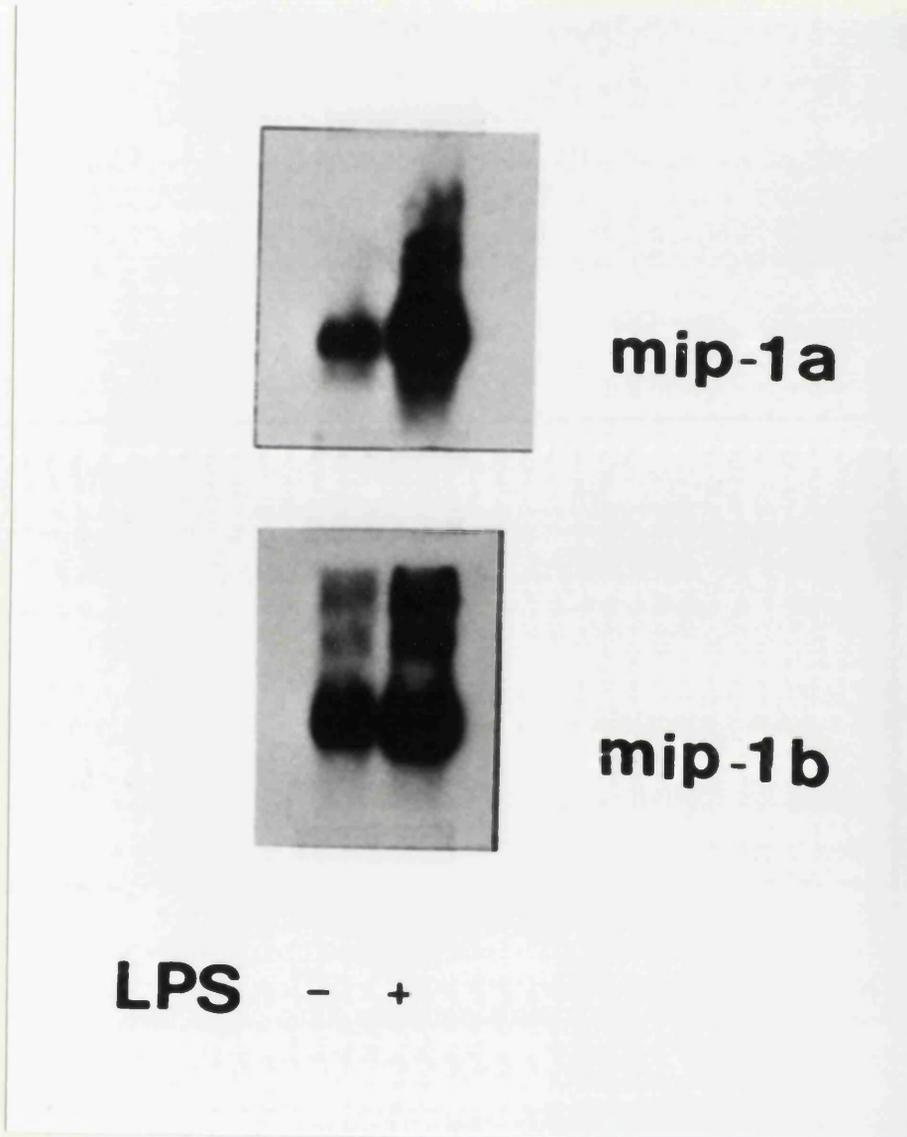


Figure 4.2 Northern analysis of the accumulation of SCI/MIP-1 α and MIP-1 β mRNA in RAW264.7 cells in the presence or absence of LPS (2 μ g/ml).

Total cellular RNA was isolated and blotted in equal amounts (20 μ g) onto nylon membrane. Lane 1, untreated RAW264.7 cells; lane 2, RAW264.7 cells treated with LPS for 24 hours. Equal loading was ensured by ethidium bromide staining of the gel.

4.3 Effect of Actinomycin D on Expression of SCI/MIP-1 α and MIP-1 β mRNA.

Macrophages respond to external stimuli by expressing genes in several different time frames (Adams and Hamilton 1984) which may be triggered by several different pathways (Vairo and Hamilton 1991). The time course of expression of mRNA for SCI/MIP-1 α and MIP-1 β in response to LPS suggested that this response may be a part of the early response to stimulation. It has already been stated in chapter 1 that rapid accumulation of most cytokines mRNAs is achieved by a burst of transcriptional activity, although the induction of some transcripts can be achieved completely by posttranscriptional mechanisms such as mRNA stabilisation; for example, the induction of GM-CSF mRNA in peritoneal macrophages (Thorens et al 1987) by a variety of inflammatory stimuli. An understanding of the mechanisms by which the induction of these genes is controlled is important for understanding how this fits in with the overall coordinated response of the monocyte/ macrophage and its response to activation and proliferation stimuli. This, in turn, contributes to our understanding of the biology of the macrophage and its activation.

No studies had been carried out at that time on the mechanisms of expression of the MIP-1 genes in untransformed primary murine macrophages or in murine macrophage cell lines; therefore, simple experiments, using metabolic inhibitors in vitro, were designed to attempt to elucidate the molecular mechanism of induction in BMDM.

The transcriptional inhibitor actinomycin D was used to test whether or not the stimulation of transcription was necessary for the LPS-induced accumulation of SCI/MIP-1 α and/or MIP-1 β mRNA. Actinomycin D specifically inhibits RNA Polymerase II which is responsible for the synthesis of mRNA by binding to DNA and blocking the movement of the polymerase enzyme and hence prevents mRNA elongation (Alberts et al 1989; Sambrook et al 1989) It was reasoned, therefore, that

if addition of actinomycin D to BMDM suppressed the LPS-induced accumulation mRNA for either of these genes, this would indicate that stimulation of transcription was a necessary regulatory component.

Figure 4.3 shows Northern blot analyses of both SCI/MIP-1 α and MIP-1 β mRNA levels at different times after treatment with LPS (2 μ g/ml) with or without the subsequent addition of actinomycin-D (10 μ g/ml) one hour after treatment. It can be seen that the LPS-mediated rise in the levels of both transcripts is inhibited when actinomycin D was added one hour after the LPS, indicating that the stimulation of transcription is necessary for the accumulation both mRNAs to take place. As expected, the levels of 7S mRNA were unaffected by the treatments.

If lanes 1, 2 and 6 of figure 4.3 are compared, it can be seen that the level of both mRNAs is reduced to level of controls, from an elevated level, within an hour of the addition of actinomycin D. This suggests that the LPS-induced accumulation of both SCI/MIP-1 α and MIP-1 β mRNA is dependent on continuous transcription for at least the first two hours after LPS stimulation.

Interestingly, the basal level of SCI/MIP-1 α mRNA is maintained throughout the five hours of treatment with actinomycin D while the level of MIP-1 β mRNA had declined to below basal levels at the end of the experiment. This suggests that SCI/MIP-1 α mRNA is more stable than that of MIP-1 β in the presence of actinomycin D and LPS.

Accordingly, an experiment was designed, again using actinomycin D, to test whether the stabilities of SCI/MIP-1 α and MIP-1 β mRNA are different both before and after treatment with LPS.

Firstly, to assess the stability of SCI/MIP-1 α and MIP-1 β mRNA in unstimulated BMDM, the levels of both mRNA transcripts (after refeeding of untreated BMDM) were monitored over a six hour time period in the presence or absence of actinomycin D. It can be seen from lanes 8-14 of figure 4.4 that the level of mRNA for both

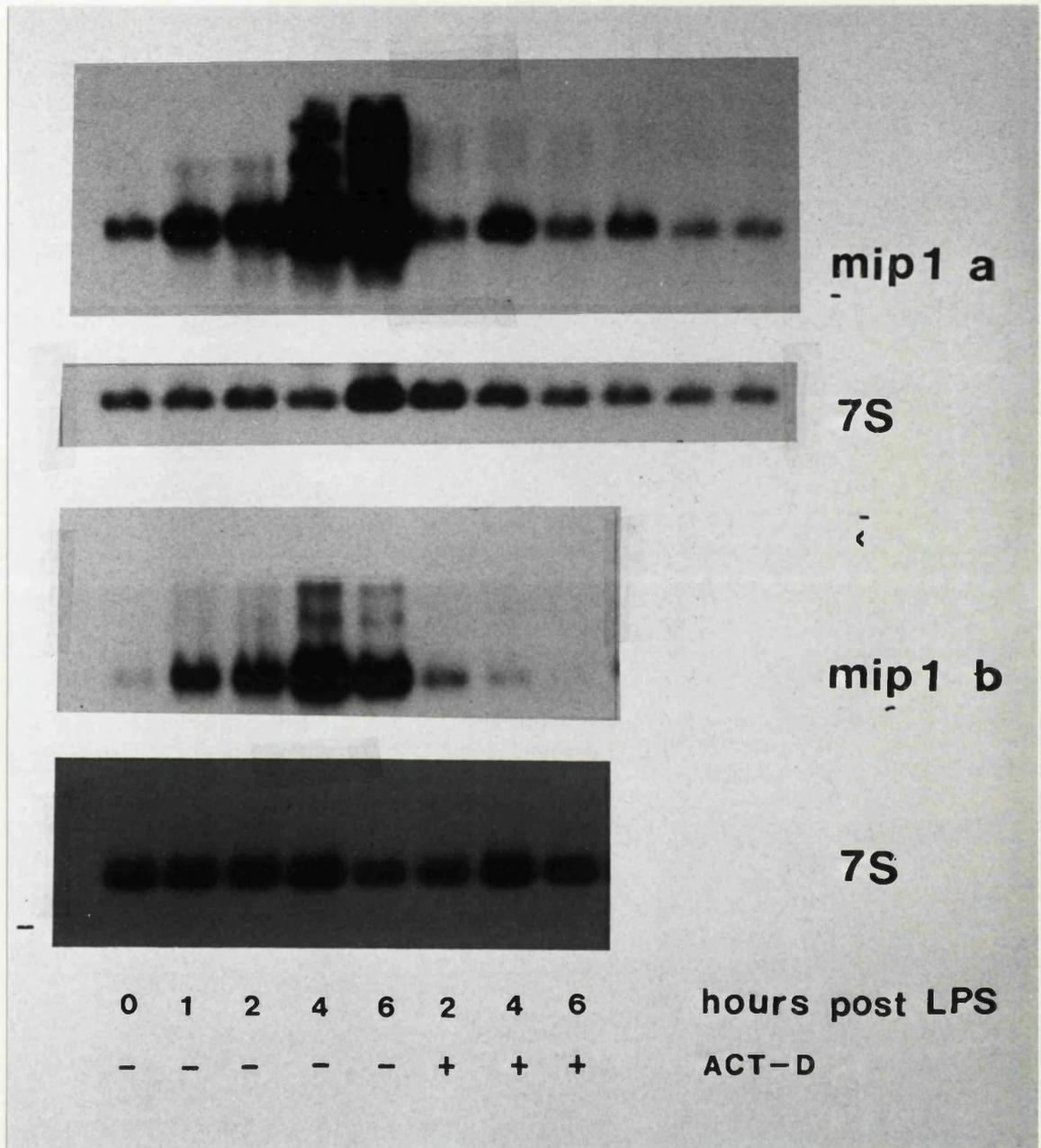


Figure 4.3 Northern analysis of the time course of accumulation of SCI/MIP-1 α and MIP-1 β mRNA in BMDM treated with LPS (2 μ g/ml) in the presence or absence of actinomycin D (10 μ g/ml).

Total cellular RNA was isolated and blotted in equal amounts (20 μ g) onto nylon membrane. Lanes 1-5, BMDM treated with LPS for 0, 1, 2, 4 and 6 hours; lanes 6-8, BMDM treated with LPS for 2, 4 and 6 hours with the addition of actinomycin D after 1 hour. Lanes 9-11 of upper panel are repeats of lanes 6-8. Equal loading was confirmed by hybridisation with a 7S probe.

SCI/MIP-1 α and MIP-1 β declines to undetectable levels in the presence but not the absence of actinomycin D. This suggests that the expression of the basal levels of both transcripts is dependent on continuous transcription. It is evident, therefore, that the stimulation of transcription is necessary for both the expression of a basal level of SCI/MIP-1 α and MIP-1 β mRNA and for the elevation of these levels of upon treatment of BMDM with LPS. Interestingly, the ability of actinomycin D to abolish expression of both SCI/MIP-1 α and MIP-1 β mRNA within six hours indicates that the half-lives of these transcripts would be measured in the range of a few hours, which is not inconsistent with the transient patterns of accumulation of these mRNAs seen upon stimulation of BMDM with LPS.

One explanation for the differential decay seen in figure 4.3 (and possibly also in figure 4.1) is that LPS has a stabilising effect on SCI/MIP-1 α but not MIP-1 β mRNA. This possibility was tested by first treating BMDM with LPS and then monitoring the decay of mRNA levels after four hours (the peak of mRNA accumulation) in the presence or absence of actinomycin D. The rationale behind this approach being that the rate of decay after the peak of expression for SCI/MIP-1 α mRNA might be retarded in comparison to that for MIP-1 β . The results of one such experiment can be seen in figure 4.4.

No difference was detected in the decay of the two molecules at the gross level. In figure 4.4 the decay of SCI/MIP-1 α mRNA levels in the presence of actinomycin D (lanes 5, 6 and 7) appears to be retarded; however, this pattern was not borne out by repetition; therefore, no evidence for modulation of stabilisation of either of the MIP-1 mRNAs was obtained in these experiments.

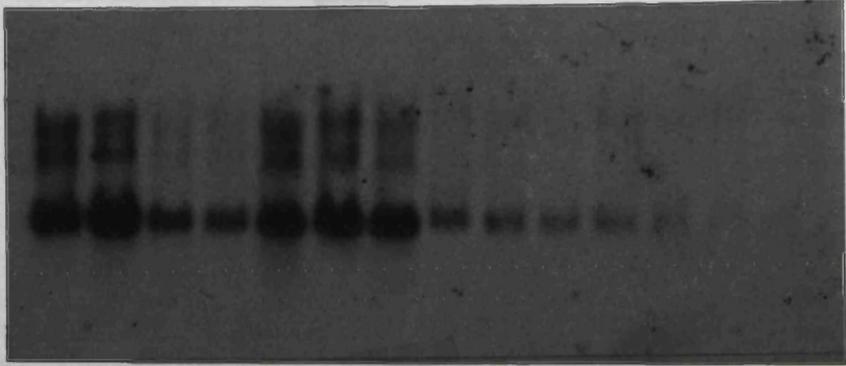
It is possible that a more detailed analysis of this phenomenon, involving the quantitative analysis of several experiments, might reveal subtle differences.

Unfortunately, only two runs of this experiment yielded interpretable results and therefore no such analysis can be carried out.

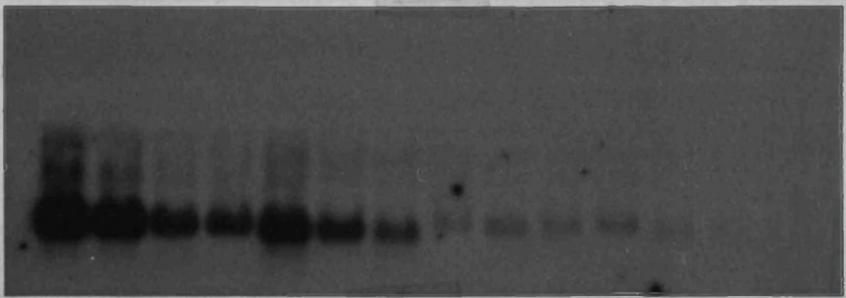
Figure 4.4 Northern analysis of the steady-state levels of SCI/MIP-1 α and MIP-1 β mRNA in BMDM with or without the addition of LPS (2 μ g/ml) plus or minus the addition of actinomycin D (10 μ g/ml).

Total cellular RNA was isolated and blotted in equal amounts onto nylon membrane. Lanes 1-4, BMDM treated with LPS for 4, 6, 8 and 10 hours. Lanes 5-7, BMDM treated with LPS for 6, 8 and 10 hours with addition of actinomycin D after 4 hours. Lanes 8-11, BMDM refed with alpha-MEM medium containing 25% DHS for 4,6,8 and 10 hours; lanes 12-14, BMDM refed with alpha-MEM medium plus 25% DHS for 4, 6, 8 or 10 hours with the addition of actinomycin D after 4 hours.

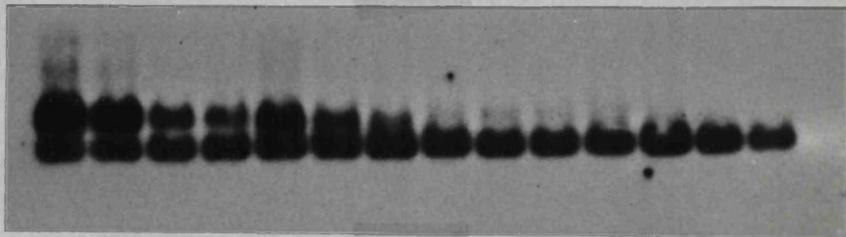
The membrane was hybridised first with a SCI/MIP-1 α probe, then stripped and rehybridised with a MIP-1 β probe. Equal loading was confirmed by hybridisation with a 7S probe. The lower panel of the figure shows hybridisation with both MIP-1 β and 7S probes; however, the equal hybridisation to the 7S probe can be seen clearly.



a



b



7S

	4	6	8	10	6	8	10	4	6	8	10	6	8	10
LPS	+	+	+	+	+	+	+	-	-	-	-	-	-	-

It is interesting to note that the presence of actinomycin D did not markedly accelerate the decay of either SCI/MIP-1 α or MIP-1 β mRNA 4-8 hours post treatment with LPS, which suggests that the contribution of transcription to the increased levels of these mRNAs has ceased by these times.

These results therefore suggest that the rate-limiting determinant for the LPS-induced increases in both SCI/MIP-1 α and MIP-1 β could be a burst of transcriptional activity between zero and around four hours after stimulation, and thereafter the modulation of the rate of mRNA turnover, although a role for mRNA stabilisation in the induction of either of these mRNAs by LPS has not actually been demonstrated by these experiments. ?

It should be stressed that these data are merely indicative and there are other approaches, which were outside the scope of this project might have been more informative. For example, the specific time-frame during which transcription of SCI/MIP-1 α and MIP-1 β mRNA is stimulated could have been assessed by "nuclear run-on" analysis or, as indicated above, quantitative analysis of the turnover of both mRNAs could have been carried out. Nevertheless, it is interesting to compare these results to those reported by Koerner et al (1987) who studied the mechanism of elevation of mRNA for the MIP-1 family member JE and the PF4-family member KC in murine peritoneal macrophages stimulated with LPS. While the levels of both mRNAs were elevated after 30 minutes the kinetics of expression observed thereafter were different. The level of KC mRNA peaked at two to four hours and had begun to decline by six hours, whereas the accumulation of JE mRNA is stable and stays at a constant level after the peak is reached. Using a nuclear "run-on" technique it was demonstrated that LPS elevated the transcription of the KC gene within 15 minutes with a peak at 1-2 hours and had ceased by six hours. The transcriptional activity of the JE gene, on the other hand, was unaffected by treatment with LPS. This would indicate that the accumulation of KC mRNA in murine peritoneal macrophages is

dependent on the stimulation of transcription while the elevation of JE mRNA is most likely to be due to a posttranscriptional mechanism such as mRNA stabilisation. It is important to realise that the elevation of transiently expressed genes such as these may be regulated by different mechanisms in different cell types as Koerner et al (1987) and Hall and Stiles (1987) have demonstrated that serum-induced expression of JE in fibroblasts is mediated by an increase in the transcriptional activity of the gene. This means that any information obtained about the mechanism of accumulation of SCI/MIP-1 α or MIP-1 β mRNA in BMDM can not be directly extrapolated to other cell types.

No other studies, of the type described above, have been carried out for the murine SCI/MIP-1 α or MIP-1 β genes. However, Yamamura et al (1990) have demonstrated that actinomycin D abolished the basal human MIP-1 α (LD78) mRNA levels in the human promyelocytic cell line HL60 but not in the HTLV-1-infected human T cell line MJ, suggesting that ongoing transcription is necessary for human MIP-1 α (LD78) mRNA expression in the former but not the latter cell line.

4.4 Effects of Cycloheximide on Expression of SCI/MIP-1 α and MIP-1 β mRNA.

A common feature of rapidly induced genes (in particular the so-called "immediate early" or "early response" genes) is that *de novo* protein synthesis is not necessary for the stimulation of transcription (Rollins and Stiles 1989). The fact that transcription can take place in the absence of new protein synthesis allows the induction of accumulation of mRNA to take place rapidly after cellular stimulation.

As LPS induces the accumulation of SCI/MIP-1 α and MIP-1 β mRNAs within one hour it seemed possible that the transcriptional induction of either or both of these genes could share this characteristic of the immediate early genes. Therefore, the ability of LPS to induce the accumulation of SCI/MIP-1 α and MIP-1 β mRNA in BMDM in the presence of the protein synthesis inhibitor cycloheximide was tested.

Cycloheximide had slightly different effects on the LPS-mediated induction of SCI/MIP-1 α and MIP-1 β mRNA.

In figure 4.5 (lower panel) it can be seen that LPS induces accumulation of MIP-1 β mRNA after 4 hours to a level which is much greater than that induced by cycloheximide alone. The LPS-induced level is not affected by the presence of cycloheximide, suggesting that LPS can cause an increase in the level of this messenger RNA in the absence of new protein synthesis. The row containing the signals produced on hybridisation to MIP-1 β mRNA is underexposed as the level of increase seen with LPS in this particular experiment is so great as to obscure the other lanes on a longer exposure.

The result obtained in these experiments with SCI/MIP-1 α mRNA were not as straight-forward to interpret. In the experiment seen in the upper panel of figure 4.5 it can be seen that, in this particular experiment, cycloheximide and LPS induce accumulation of SCI/MIP-1 α mRNA to a level which is approximately equal. However, when the two agents are added together, the level of SCI/MIP-1 α mRNA which accumulates after four hours is greater than that seen with each agent alone, indicating that the LPS-mediated rise in SCI/MIP-1 α mRNA is not blocked by inhibition of protein synthesis.

These results would again suggest the possibility that the main determinant for the rapid induction of both of the MIP-1 mRNAs could be the transient stimulation of transcription between zero and approximately four hours. The independence of this process from *de novo* protein synthesis again emphasises that accumulation of both SCI/MIP-1 α and MIP-1 β mRNA is part of the early phase of the response of BMDM to stimulation.

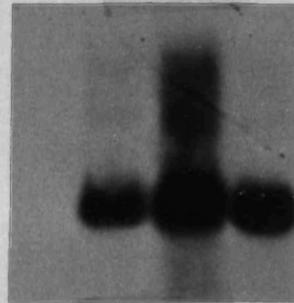
It is possible, however, that mRNA stabilisation could play some role in determining the actual level of the mRNAs and may be responsible for the elevated levels of SCI/MIP-1 α mRNA in relation to unstimulated cells at later time points.

Figure 4.5 Northern analysis of accumulation of SCI/MIP-1 α and MIP-1 β mRNA in BMDM treated with LPS (2 μ g/ml) and/or cycloheximide (10 μ g/ml).

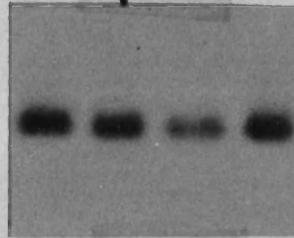
Total cellular RNA was isolated after 4 hours and blotted in equal amounts (20 μ g) onto nylon membrane.

Upper panel, SCI/MIP-1 α mRNA. Lane 1, untreated BMDM; lane 2, BMDM treated with cycloheximide; lane 3, BMDM treated with LPS plus cycloheximide; lane 4, BMDM treated with LPS. Equal loading was confirmed by hybridisation to a 7S probe.

Lower panel, MIP-1 β mRNA. Lane 1, untreated BMDM; lane 2, BMDM treated with LPS; lane 3, BMDM treated with cycloheximide; lane 4, BMDM treated with LPS plus cycloheximide. Equal loading was confirmed by hybridisation to a 7S probe.

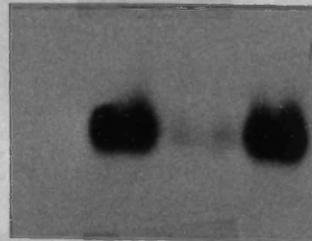


mip-1a

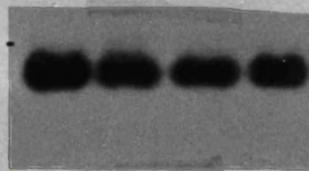


7S

LPS	-	-	+	+
CHX	-	+	+	-



mip-1b



7S

LPS	-	+	-	+
CHX	-	-	+	+

Protein synthesis inhibitors, especially cycloheximide, have been shown to cause elevation of mRNA levels for several rapidly induced genes (Greenberg et al 1986, Subramaniam et al 1989, Mahadevan and Edwards 1991, Collart et al 1986).

Figure 4.6 shows a time course of the expression of MIP-1 α and MIP-1 β mRNAs in BMDM after treatment with cycloheximide. It can be seen that the level of SCI/MIP-1 α mRNA is elevated at one hour after addition and stays elevated for at least eight hours; samples were not taken at any times after this as the cells begin to die after prolonged exposure to cycloheximide.

It can also be seen that the level of MIP-1 β mRNA is increased at one hour, but has begun to decline by six hours and by eight hours is almost at the level of unstimulated controls.

The interpretation of the results of this experiment must be guarded with caution.

The levels of mRNA for several genes such as c-fos, c-myc and actin (Greenberg et al 1986, Subramaniam et al 1989, Mahadevan and Edwards 1991) and TNF α , IL-1 α and urokinase-plasminogen activator (u-PA) (Collart et al 1986) are increased by cycloheximide and other inhibitors of protein synthesis. Conventionally, this has been attributed to stabilisation of the mRNA as a consequence of translational arrest (and hence be indicative of a role for the modulation of mRNA stability in the control of gene expression). Indeed, Wilson and Treisman (1988) have demonstrated that after treatment with cycloheximide, the half-life of c-fos mRNA increases from approximately thirty minutes to several hours. Hence, elevation of a messenger RNA by cycloheximide might be used as evidence for the possibility that modulation of mRNA stabilisation might be involved in the control of the expression of that gene. However, it has also been demonstrated that the transcription of several genes can be affected by cycloheximide; for example Collart et al (1986) have demonstrated that cycloheximide increases the rate of transcription of the genes for TNF α , IL-1 α and u-PA in elicited peritoneal macrophages. In addition to this, Mahadevan and Edwards

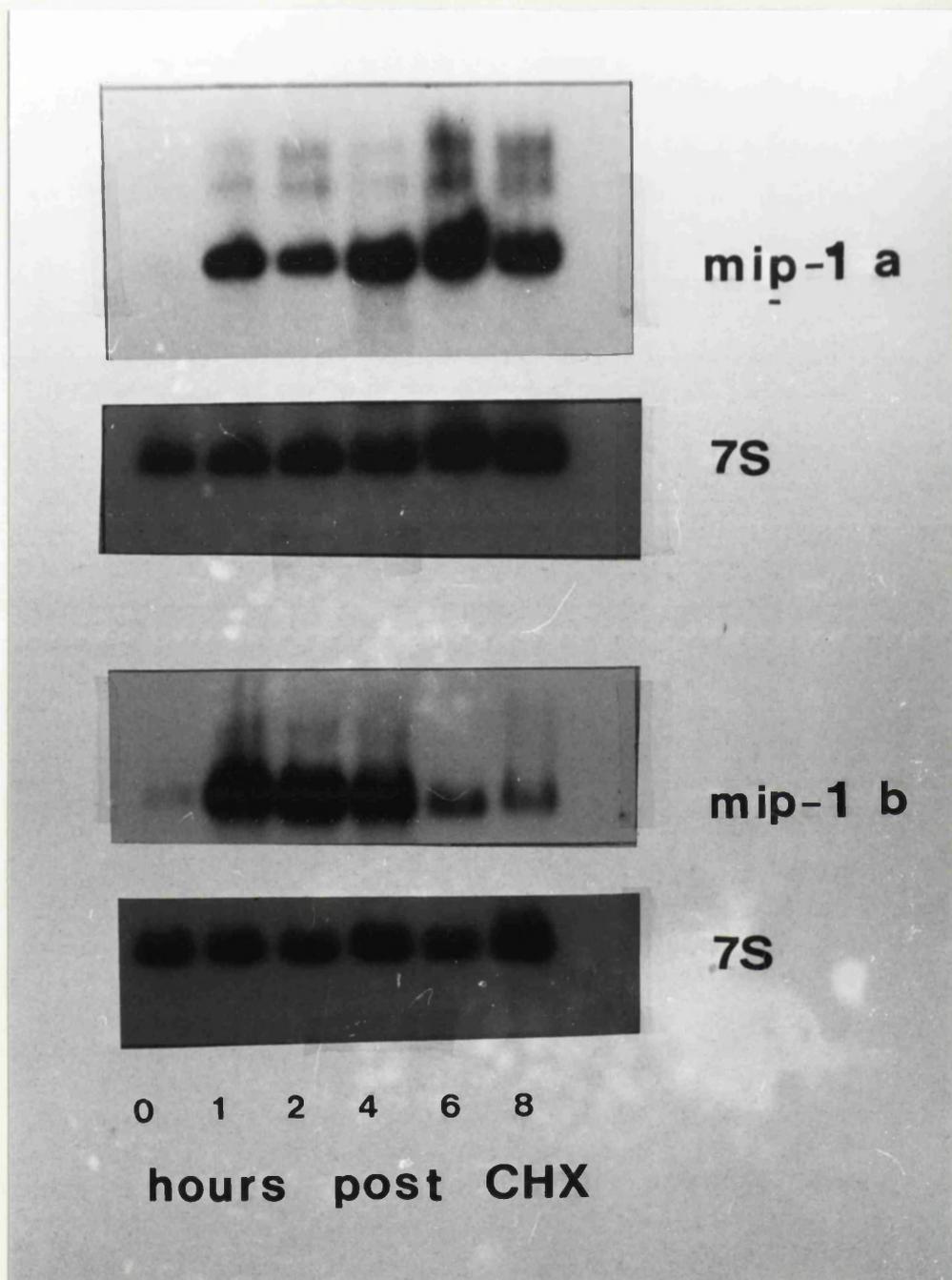


Figure 4.6 Northern analysis of the time course of accumulation of SCI/MIP-1 α and MIP-1 β mRNA in BMDM treated with cycloheximide (10 μ g/ml).

Total RNA was isolated from BMDM 0, 1, 2, 4, 6 and 8 hours after treatment (lanes 1-6) and blotted in equal amounts (20 μ g) onto nylon membrane. Equal loading was confirmed by hybridisation with a 7S probe.

(1991) and Edwards and Mahadevan (1992) have demonstrated that cycloheximide is able to act as a nuclear signalling agonist in fibroblasts. Edwards and Mahadevan (1992) have argued that this process may be making a major contribution to the induction of gene expression.

One interpretation of the data from the experiment shown in figure 4.6 could be that cycloheximide perhaps induces a transient burst of transcription from the MIP-1 β gene. This burst, however, ends around four hours after addition of cycloheximide and the level of the labile mRNA then declines, whereas the SCI/MIP-1 α mRNA is comparably stable which would account for the unaltered levels of this mRNA beyond four hours.

Interestingly, Yamamura et al (1989) reported a transient elevation of hMIP-1 α (LD78) mRNA in the human promyelocytic cell line HL60 after treatment with cycloheximide, with levels being elevated as early as one hour and returning to basal levels by six hours. The addition of actinomycin D along with cycloheximide diminished but did not completely abolish the extent of elevation which suggests that both transcription and mRNA stability may be making a contribution.

Accordingly experiments were also performed using actinomycin D in conjunction with cycloheximide at early times to test whether transcription was necessary for the inductive effect of cycloheximide on SCI/MIP-1 α and/or MIP-1 β levels and at later times to test whether the persistence of the elevated levels of SCI/MIP-1 α could be sustained in the absence of transcription. Unfortunately no consistent or interpretable results could be obtained in these experiments possibly because the insult sustained by the cells in these experiments is too severe.

4.5 Effect of Hydrocortisone on accumulation of SCI/MIP-1 α and MIP-1 β mRNA.

The induction of many genes which are involved in the inflammatory response, including members of the MIP-1 family, have been shown to be down-regulated by

glucocorticoid hormones. In particular, Kawahara et al (1991) and Poon et al (1991) have demonstrated that the induction of the MIP-1 family member JE can be blocked by glucocorticoids in fibroblasts and vascular smooth muscle cells, respectively. In monocytic cells, Thorens et al (1987) have demonstrated that the accumulation of GM-CSF mRNA in mouse peritoneal macrophages in response to various factors is prevented by dexamethasone and Hamilton et al (1991) have demonstrated that the elevation of u-PA mRNA by rhCSF-1 in BMDM is prevented by dexamethasone. It seemed likely that the expression of gene products with such potent biological effects as SCI/MIP-1 α and MIP-1 β might be subject to negative, as well as positive, regulation. Accordingly, the effect of hydrocortisone on SCI/MIP-1 α and MIP-1 β mRNA levels was assessed.

BMDM were placed in serum-free (SF12) medium or serum-containing medium (alpha-MEM plus 25% DHS) for four hours in the presence or absence of concentrations of hydrocortisone ranging from 10^{-6} to 10^{-9} M.

It can be seen from figure 4.7 that the levels of both SCI/MIP-1 α and MIP-1 β mRNA are reduced by hydrocortisone at both 10^{-6} and 10^{-7} M in SF12 medium. In alpha-MEM medium, containing 25% DHS, hydrocortisone had no effect on the level of SCI/MIP-1 α mRNA, but did appear to have a modest effect on the levels of MIP-1 β mRNA. This may reflect a differential sensitivity of SCI/MIP-1 α and MIP-1 β mRNA to hydrocortisone. Interestingly, a basal level of both mRNAs is still seen, even at the highest concentration (10^{-6} M) of hydrocortisone (figure 4.7).

The different effect seen in the two media is most likely due the fact that hydrocortisone is usually able to oppose rises in levels of mRNA rather than diminish basal levels as culturing BMDM in serum-free (SF12) induces accumulation of mRNA for both SCI/MIP-1 α and MIP-1 β as will be discussed in chapter 6.

Preliminary experiments with other steroids indicated that this effect was specific to glucocorticoids as neither testosterone propionate (androgen), 17 β -oestradiol

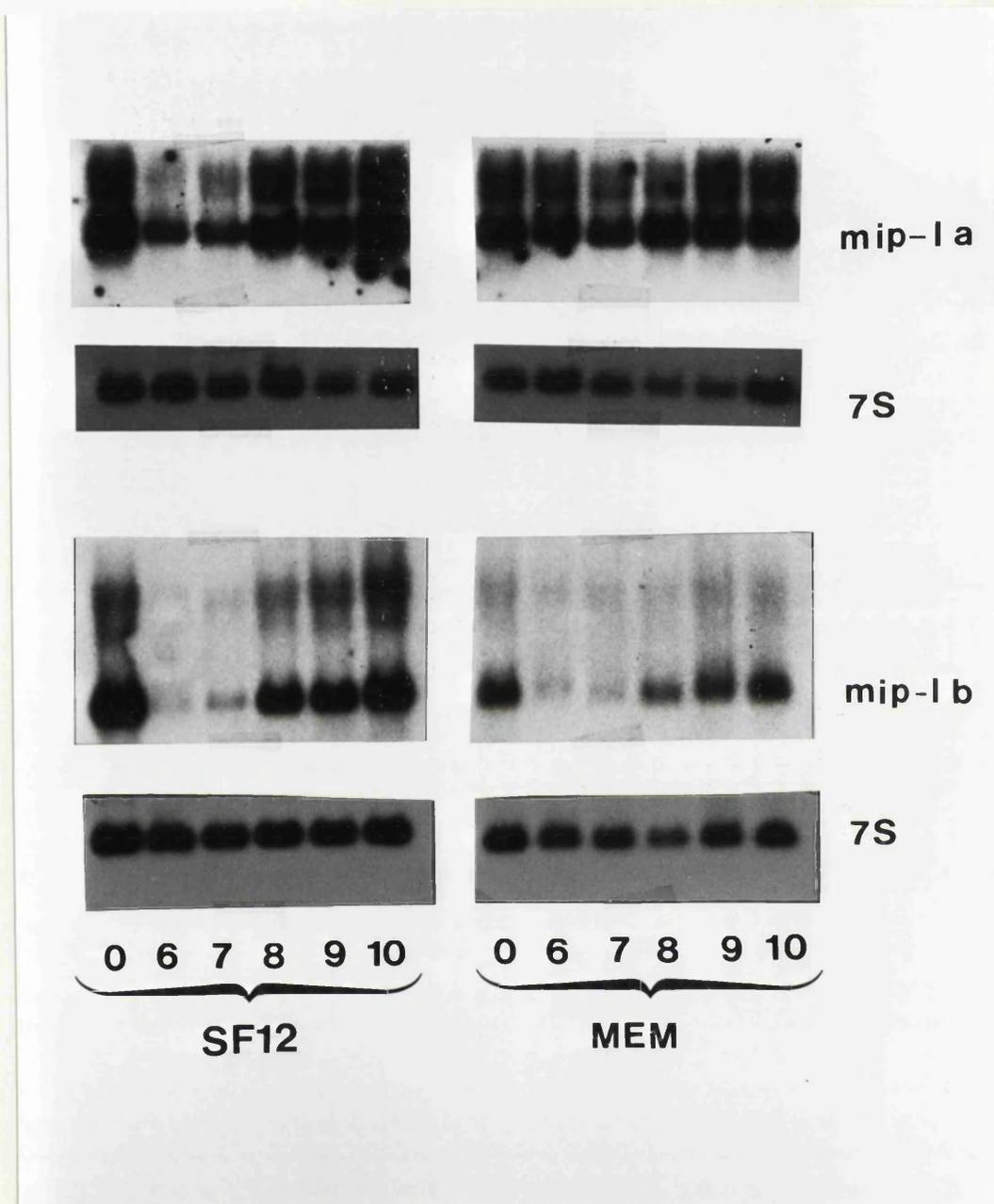


Figure 4.7 Northern analysis of the effect of hydrocortisone on the accumulation of SCI/MIP-1 α and MIP-1 β mRNA in BMDM in both SF12 and alpha-MEM media.

Total cellular RNA was isolated after 4 hours and blotted in equal amounts (20 μ g) onto nylon membrane.

Lanes 1-6, BMDM refed with SF12 medium plus 0, 10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹ and 10⁻¹⁰M hydrocortisone; lanes 7-12, BMDM refed with alpha-MEM medium plus 0, 10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹ and 10⁻¹⁰M hydrocortisone.

(oestrogen) or retinoic acid (retinoid) at 10^{-6} M had any effect on the accumulation of SCI/MIP-1 α mRNA in BMDM in either serum-free or serum-containing medium (data not shown).

As it had already been demonstrated that LPS dramatically increased the levels of SCI/MIP-1 α and MIP-1 β mRNA it seemed likely that there might be a counteracting effect of an anti-inflammatory agent on the expression of these genes.

Accordingly, levels of SCI/MIP-1 α and MIP-1 β were assessed in BMDM four hours after the addition of either LPS, 10^{-6} M hydrocortisone, or both agents together in both SF12 medium and alpha-MEM medium containing 25% DHS. It can be seen from figure 4.8 that the LPS-mediated rise in both mRNAs is opposed by hydrocortisone in both the serum-free and serum-containing medium; this downregulation was found to be between approximately 40 and 60%, for both mRNAs, (by laser densitometry) over three experiments, which is similar to the downregulation by PGE1 of these mRNAs seen by Martin and Dorf (1991) in the J774A.1 macrophage-like cell line.

In addition, the observations made in figure 4.7 are confirmed by these experiments. From these experiments it can be seen that hydrocortisone opposes rises in SCI/MIP-1 α and MIP-1 β mRNA above the basal level, which is characteristic of the repressive action of glucocorticoid hormones on the expression of cytokine genes. It is interesting that most other investigators have demonstrated downregulation of the expression of cytokine gene expression using dexamethasone which is approximately ten times as potent a glucocorticoid as hydrocortisone (Malan & Gould 1982). This means that downregulation of accumulation of mRNA for both SCI/MIP-1 α and MIP-1 β can be achieved by the equivalent of 10^{-8} M dexamethasone, which suggests that the expression of both of these genes is extremely sensitive to glucocorticoids. As discussed in chapter 1, hydrocortisone can downregulate gene expression at the level of both transcription and mRNA stability. The downregulation of expression of

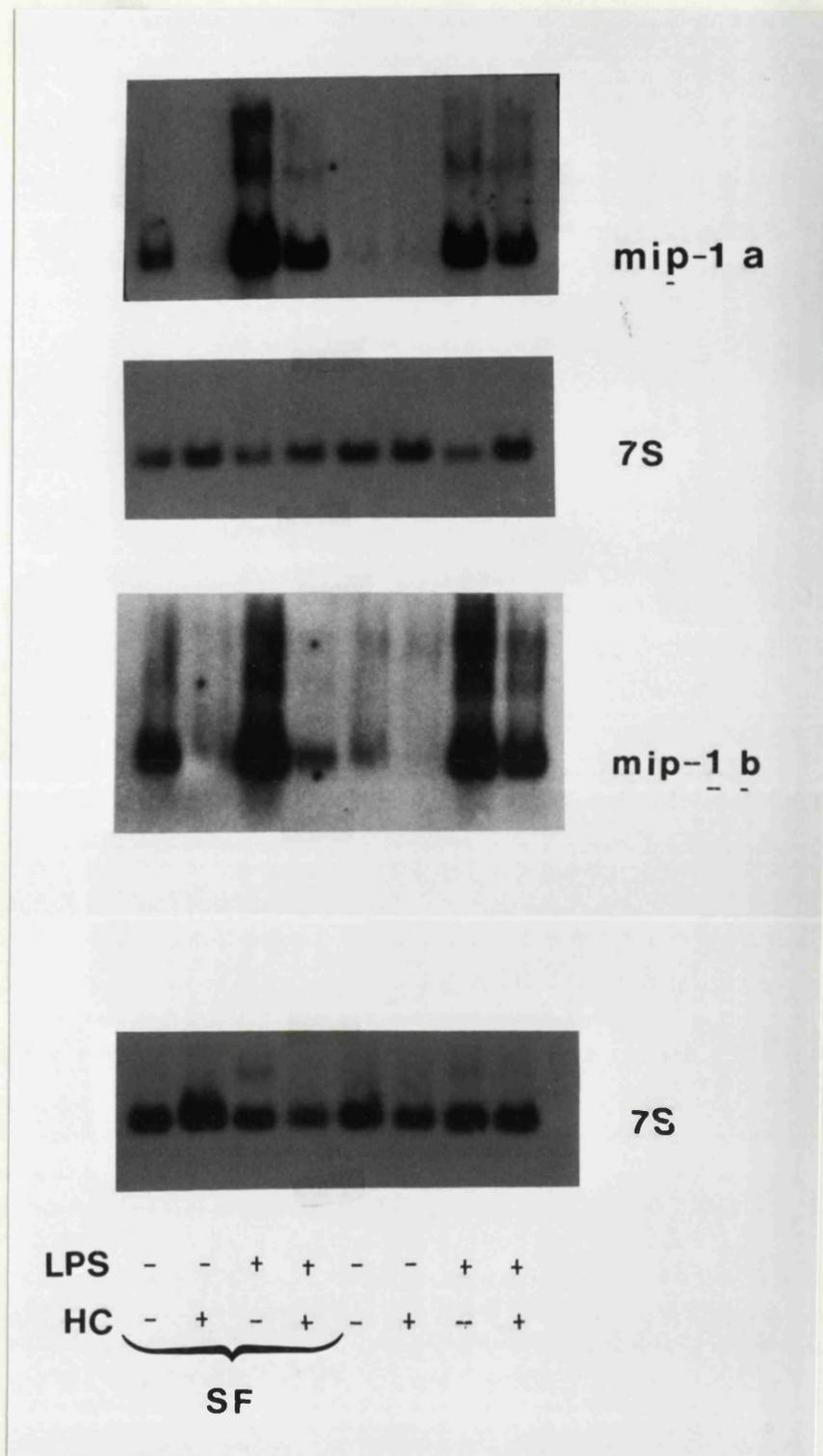


Figure 4.8 Northern analysis of the accumulation of SCI/MIP-1 α and MIP-1 β mRNA in BMDM in SF12 and alpha-MEM medium in the presence or absence of LPS (2 μ g/ml) and/or hydrocortisone (10 $^{-6}$ M).

Cells were refed with either SF12 (lanes 1-4) or alpha-MEM medium (lanes 5-8). Total cellular RNA was isolated after 4 hours and blotted in equal amounts onto nylon membrane. Lanes 1 and 5, no additions; lanes 2 and 6, hydrocortisone; lanes 3 and 7, LPS; lanes 4 and 8, LPS plus hydrocortisone.

SCI/MIP-1 α and MIP-1 β by hydrocortisone could be mediated at either, or both, of these levels.

4.6 Summary of Chapter 4.

The accumulation of mRNA for SCI/MIP-1 α and MIP-1 β is greatly increased in a rapid and transient fashion by stimulation of BMDM with bacterial endotoxin (LPS). These inductions can take place in the absence of new protein synthesis and this indicates that upregulation of the expression of these genes is a part of the early response of BMDM to stimulation by LPS and allows SCI/MIP-1 α and MIP-1 β to be designated as belonging to the "immediate-early" group of genes.

Early stimulation of transcription is necessary for the accumulation of both SCI/MIP-1 α and MIP-1 β mRNA in BMDM but it is possible that mRNA stabilisation may also play a role in determining the overall level of mRNA induced, although this has not actually been demonstrated in this chapter. Cycloheximide on its own is able to induce the accumulation of SCI/MIP-1 α and MIP-1 β mRNA and the implications of this were discussed. In common with many other cytokine genes, accumulation of SCI/MIP-1 α and MIP-1 β mRNA can be downregulated by hydrocortisone. The features described above are typical of the many cytokine genes whose products are involved in mediating inflammation.

The experiments in this chapter also demonstrate that BMDM are a satisfactory in vitro system for studying the modulation of expression of both SCI/MIP-1 α and MIP-1 β as mRNA levels for both of these genes can be regulated both positively and negatively.

Chapter 5

Effect of L929CM, rhCSF-1 and rmIFN γ on Expression of SCI/MIP-1 α and MIP-1 β mRNA in Bone Marrow-Derived Macrophages.

5.1 Introduction.

In chapter 4, the characteristics of the induction of mRNA for SCI/MIP-1 α and MIP-1 β by LPS, which is a potent activator of macrophages (Adams and Hamilton 1984) had been investigated. To further investigate the biology of SCI/MIP-1 α and MIP-1 β gene expression, and in an attempt to identify candidate regulators of SCI/MIP-1 α and MIP-1 β gene expression *in vitro*, the effects of three other agents, L929CM, colony stimulating factor 1 (CSF-1) and interferon- γ (IFN γ), was investigated.

In the BMDM culture system described in chapter 3, L929CM was used as a source of colony stimulating factor 1, the lineage-specific growth factor, which stimulates the proliferation, differentiation and survival of cells of the mononuclear phagocyte series (Sherr 1991). In BMDM, CSF-1 has been shown to affect the expression of a number of genes, as discussed in chapter 1, without causing macrophage activation (Phillips and Hamilton 1989, Hamilton et al 1989). Therefore, it was of interest to determine whether gene expression could be upregulated by a growth factor as well as by a macrophage activating factor as this might give a clearer indication of the range of situations in which the induction of expression of these genes might play a role. In particular, since bone marrow haemopoiesis has been proposed to be directed by interacting networks of cytokines produced by stromal cells it was thought that studying the effect of cytokines on the expression of SCI/MIP-1 α and MIP-1 β mRNA in BMDM might make a contribution towards understanding the role of these molecules in marrow haemopoiesis.

Interferon- γ (IFN γ) can affect gene expression in a wide variety of cell types (Sen and Lengyel 1992) and is believed to play a role in priming macrophages for antigen

presentation, as evidenced by its ability to upregulate expression of MHC class II molecule genes in both the human (Pestka et al 1987) and the mouse (Yi and Willman 1989). It was, therefore, of interest to determine the effect, if any, of IFN γ on the accumulation of mRNA for the two genes to determine whether expression of SCI/MIP-1 α and/or MIP-1 β in the context of the specific response to infection. IFN γ has also been demonstrated to reversibly oppose the CSF-1-induced proliferation of BMDM (Vairo et al 1991, Knight et al 1992).

5.2 Effect of L929CM on Expression of SCI/MIP-1 α and MIP-1 β mRNA.

The effects of L929CM and CSF-1 on the expression of both MIP-1 mRNAs was investigated for the following reasons:

1) The mRNA for many inducible genes, including MIP-1 family members, as discussed in chapter 1, have been shown to be increased in BMDM or related cell lines as part of the early response to CSF-1 (Orlofsky and Stanley 1987, Hamilton et al 1989, Matsushime et al 1991b, Sherr 1991). Given that the characteristics of accumulation of SCI/MIP-1 α and MIP-1 β mRNA in BMDM in response to LPS were indicative of this being part of the early response to stimulation, it seemed possible that this might also be the case for stimulation by a growth factor. As the ability of L929CM to induce proliferation of BMDM had already been established, it was reasoned that this might also allow assessment of any relationship between BMDM proliferation and expression of SCI/MIP-1 α and/or MIP-1 β mRNA.

2) Simmons and Lord (1985) had demonstrated that culture, in 20% L929CM, of macrophages, sorted on the basis of the expression of the F4/80 antigen, greatly increased the production of the Manchester Inhibitor. This increase could have been due to either increased production from the cells themselves or to an increase in cell number in the culture. It was considered that assessment of the effect of L929CM on the expression of SCI/MIP-1 α mRNA levels over a short-time span in which the

growth properties of BMDM were well characterised would go some way to resolving this issue.

To assess whether L929CM might affect the accumulation of mRNA for SCI/MIP-1 α and MIP-1 β in BMDM, and to determine appropriate conditions for this assessment, mRNA levels were measured at six and twenty-four hours after reculturing in alpha-MEM medium containing 25% DHS or FCS plus or minus 20% L929CM by Northern blotting. These time points were chosen in the light of the pattern of accumulation of these mRNA transcripts in response to LPS. Hamilton et al (1989) had used FCS in their BMDM culture system and therefore the suitability of both DHS and FCS for use in the assessment of the effects of L929CM on gene expression was assessed. As a control, levels in the treated cultures were compared to those in an untreated seven day culture.

In figure 5.1 it can be seen that when BMDM were cultured in alpha-MEM containing 25% DHS in the absence of L929CM the level of SCI/MIP-1 α mRNA is reduced to a level which is lower than that of the untreated control culture at both six and twenty-four hour hours (lanes 1 and 2). With FCS, a dramatic increase in the level of SCI/MIP-1 α mRNA (approximately 40 to 50 fold as assessed by densitometry) at six hours is seen, which appears to be augmented slightly by L929CM (lanes 4 and 5). However, this effect is not seen at twenty-fours (lanes 8 and 9) and indeed, mRNA levels are lower than the control, suggesting that the effect of FCS on SCI/MIP-1 α and MIP-1 β mRNA accumulation, like that of LPS, is transient. The effect of FCS on the level of SCI/MIP-1 α mRNA levels is very similar to that observed for GM-CSF in murine peritoneal macrophages by Thorens et al (1987). The effects of these treatments on MIP-1 β mRNA levels are essentially identical, although the overall extent of hybridisation to MIP-1 β is less than that to SCI/MIP-1 α . This is most likely to be due to the fact that, in the experiment shown in figure 5.1, the same membrane was first probed for SCI/MIP-1 α and then stripped before

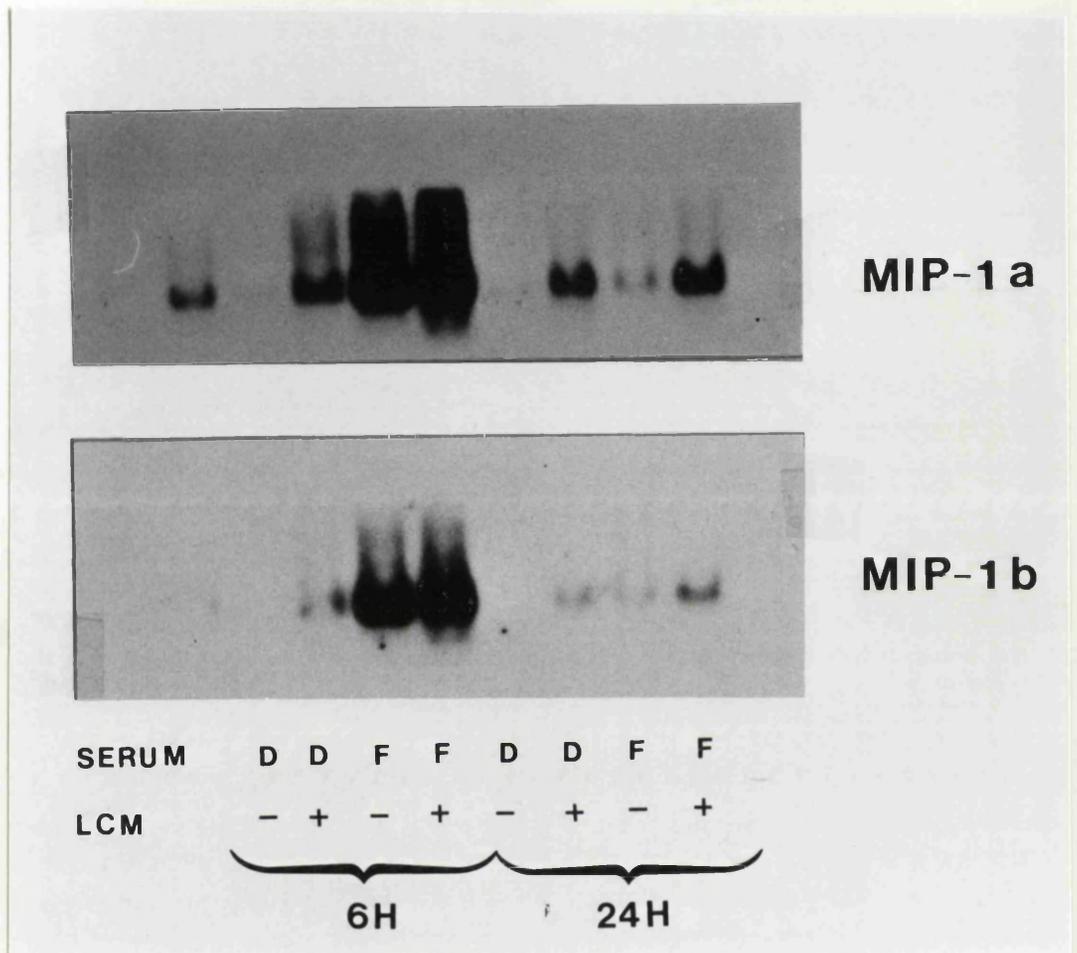


Figure 5.1 Northern analysis of the accumulation of SCI/MIP-1 α and MIP-1 β mRNA in BMDM after refeeding with alpha-MEM medium in the presence or absence of various additions.

Total cellular RNA was isolated 6 hours (lanes 2-5) or 24 hours (lanes 6-9) after refeeding and blotted in equal amounts (20 μ g) onto nylon membrane. Lane 1, untreated BMDM; lanes 2 and 6, BMDM refed plus 25% DHS; lanes 3 and 7, BMDM refed plus 25% DHS plus 20% L929CM; lanes 4 and 8, BMDM refed with alpha-MEM plus 25% FCS; lanes 5 and 9, BMDM refed with alpha-MEM medium plus 25% FCS plus 20% L929CM. Equal loading was ensured by ethidium bromide staining of the gel. The membrane was probed first for SCI/MIP-1 α and then stripped and rehybridised for MIP-1 β mRNA.

reprobing for MIP-1 β . Twenty percent L929CM does appear to have a positive effect on the level of both mRNAs as the level seen on refeeding with medium containing 20% L929CM is greater than that seen in its absence (lanes 2 and 3). This experiment provided evidence, therefore, that the levels of SCI/MIP-1 α and MIP-1 β mRNA in BMDM could indeed be affected (positively) by L929CM and it was concluded that these effects were worthy of further investigation. An interesting feature of this induction was the fact that the upregulation seen in response to 20% L929CM was still evident after 24 hours which had not been found with any other mediator and it seemed possible that L929CM might be inducing a pattern of expression different from that induced by LPS, i.e. a sustained, as opposed to a transient, increase. The observed increase raised the possibility that L929CM might be affecting the accumulation of SCI/MIP-1 α and MIP-1 β mRNA through a different mechanism to that of LPS.

An additional consideration was that it was not possible to tell from these experiments whether the effect of L929CM was to increase the accumulation of SCI/MIP-1 α and MIP-1 β mRNA or merely to prevent the downregulation of mRNA levels by DHS. Accordingly, experiments were designed to investigate whether L929CM was able to induce the accumulation of SCI/MIP-1 α and MIP-1 β mRNA and, if so, the kinetics of this expression.

For these experiments, the cells were "starved" overnight by culturing in alpha-MEM medium containing 25% DHS in the absence of any other additions. Starvation ensured that the starting levels of SCI/MIP-1 α and MIP-1 β mRNA were as low as could be obtained in the culture system, greatly simplifying the analysis of the effects of L929CM. In addition to this, overnight starvation, as illustrated in chapter 3, has the effect of arresting the proliferation of BMDM which means that the cells would respond to the proliferation-inducing effects of L929CM in as uniform a manner as could be achieved in the culture system. The level of expression of mRNA for

SCI/MIP-1 α and MIP-1 β was then assessed in the starved cultures and at times after refeeding with fresh medium plus 20% L929CM (figure 5.2).

Figure 5.2 shows that the levels of both SCI/MIP-1 α and MIP-1 β mRNAs are increased approximately 10 to 20-fold (as assessed by laser densitometry) by treatment with 20% L929CM. This, in contrast to the observations in the experiment described immediately above, is a rapid and transient induction with a peak of expression at four hours. Similarly to the LPS-mediated induction (see figure 4.1), the levels of mRNA for SCI/MIP-1 α , but not MIP-1 β , are elevated at later times (12 to 24 hours) in comparison to untreated controls. This experiment was repeated several times and the rapid and transient pattern of induction was most commonly seen, however sustained elevation of both transcripts, for up to 24 hours, was seen a number of times.

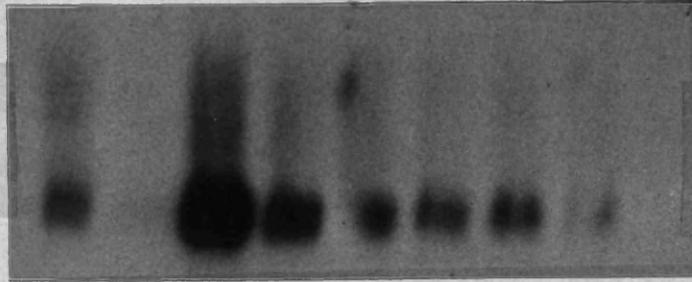
Increases in the expression of many inducible genes, including some oncogenes and MIP-1 family members have been demonstrated to be increased within one hour of stimulation which suggests that the pathways leading to the induction of their expression are activated almost immediately on cellular stimulation (as was discussed in chapter 1). To investigate whether this was also true of SCI/MIP-1 α and MIP-1 β gene expression, BMDM cells were treated as before and levels of mRNA assessed at earlier times following refeeding with L929CM.

Figure 5.3 shows the results of an experiment carried out in an identical manner to that just described except that the levels of mRNA for both SCI/MIP-1 α and MIP-1 β were assessed at times between 20 minutes and six hours after stimulation. It can be seen that the level of both mRNAs has begun to increase within one to two hours of stimulation which suggests that the induction of expression of both genes with L929CM, as with LPS, is extremely rapid and is in the same time frame as that of other lymphokine genes expressed as part of the early response to stimulation. This pattern was seen in several experiments, although the exact timing of the induction

Figure 5.2 Northern analysis of accumulation at four hourly intervals of SCI/MIP-1 α and MIP-1 β mRNA in BMDM treated with 20% L929CM.

Upper Panel, SCI/MIP-1 α . Lane 1, untreated seven day culture of BMDM; lane 2 BMDM starved overnight in alpha-MEM medium containing 25% DHS; lanes 3-8, BMDM starved overnight and refed with alpha-MEM medium containing 25% DHS plus 20% L929CM for 4, 8, 12, 16, 20 and 24 hours. Total cellular RNA was isolated and blotted in equal amounts onto nylon membrane. Equal loading was confirmed by hybridisation with a 7S probe.

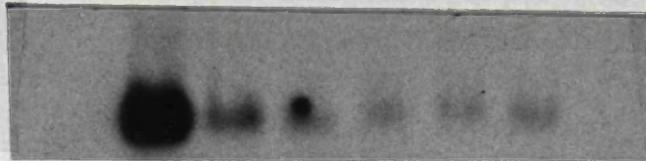
Lower Panel, MIP-1 β . Lane 1, BMDM starved overnight in alpha-MEM medium containing 25% DHS; lanes 2-7, BMDM starved overnight and refed with alpha-MEM medium containing 25% DHS plus 20% L929CM for 4, 8, 12, 16, 20 and 24 hours. Total cellular RNA was isolated and blotted in equal amounts onto nylon membrane. Equal loading was confirmed by hybridisation with a 7S probe.



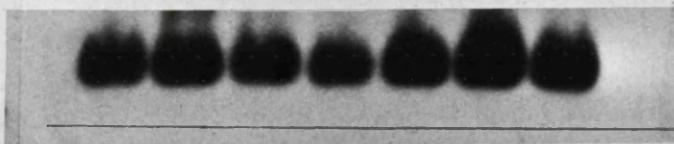
mip1 a



7S



mip 1 b



7S

0 4 8 12 16 20 24

Hours post refeed

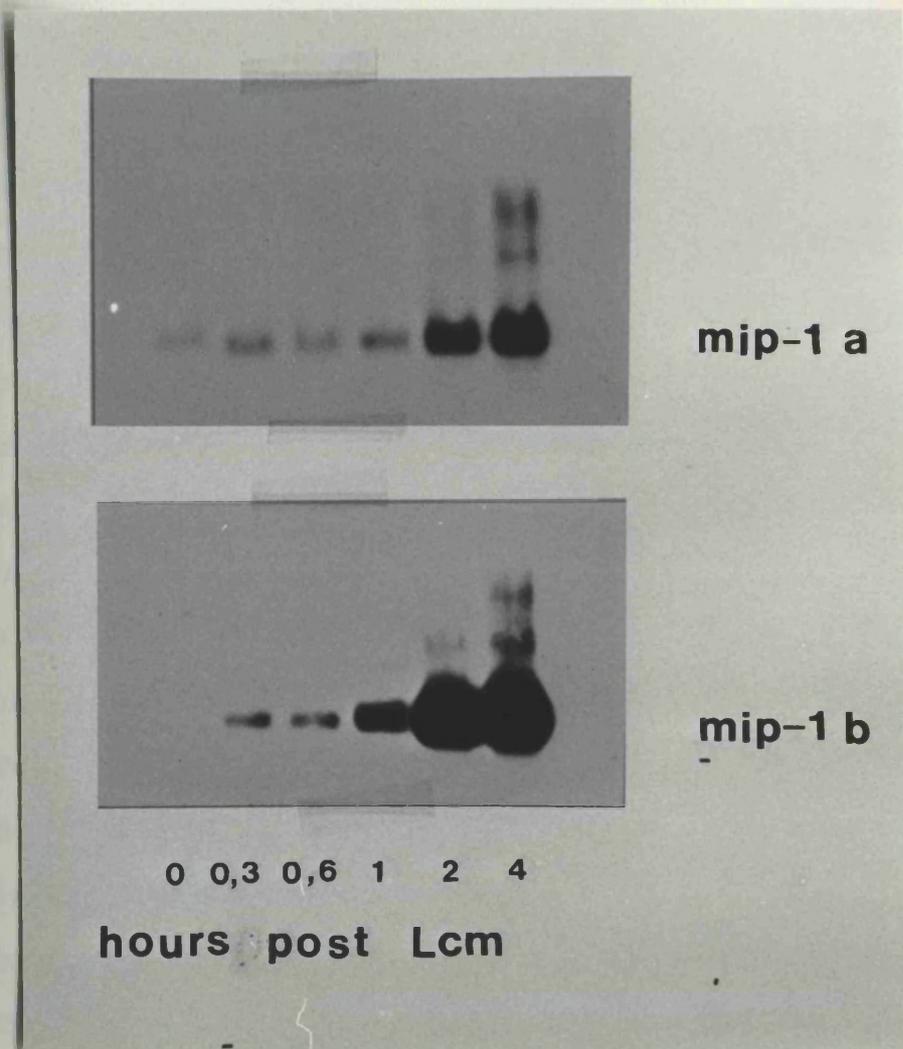


Figure 5.3 Northern analysis of the time course of accumulation of SCI/MIP-1 α and MIP-1 β mRNA in BMDM after treatment with 20% L929CM.

Total cellular RNA was isolated after 0, 0.3, 0.6, 1, 2 and 4 hours and blotted in equal amounts (20 μ g) onto nylon membrane. Equal loading was confirmed by hybridisation with a 7S probe.

did vary between experiments which is consistent with the observations of Orlofsky and Stanley (1987) on the CSF-1-induced accumulation of JE and KC mRNA in the BMDM-like BAC1 25F cell line.

It can also be seen in lanes 1 to 5 of figure 5.5 that the action of L929CM in inducing the accumulation of both SCI/MIP-1 α and MIP-1 β mRNA in BMDM after 4 hours is dose-dependent.

The kinetics of and dose-dependency of L929-induced SCI/MIP-1 α and MIP-1 β expression is very similar to that of the CSF-1-induced expression of u-PA (Hamilton et al 1991) and IL-1 α (Matsushima et al 1991b) mRNAs in BMDM and suggests that the CSF-1 component of L929CM may be making a contribution to this induction.

In common with the expression of these genes is also the fact that the peak of expression precedes the onset of DNA synthesis in BMDM as demonstrated in chapter 3, figure 3.11.

The coincidence of the stimulation of proliferation and accumulation of mRNA for SCI/MIP-1 α and MIP-1 β also raised the possibility that these two processes might be linked as has been proposed for the expression of the urokinase-plasminogen activator protein and mRNA by macrophages (Hume and Gordon 1984) and fibroblasts and keratinocytes (Grimaldi et al 1986), respectively.

A problem with the interpretation of these experiments, however, lies in the heterogeneous composition of L929CM. Even though L929CM is a potent source of CSF-1 (Stanley and Guilbert 1981) it also contains other components which could have an effect on the accumulation of mRNA from inducible genes such as fetal calf serum. It is apparent from figure 5.1 (lanes 3 and 4) that 20% fresh FCS could greatly upregulate the expression of mRNA for both SCI/MIP-1 α and MIP-1 β in BMDM making it possible that the FCS component of L929CM could be making a contribution to the upregulation of the MIP-1 mRNAs; however, it is difficult to

compare the effect of 20% fresh FCS to that of a final concentration of 2% in medium in which cells have been cultured.

In addition, although none of the components of this culture system contained significant levels of endotoxin (manufacturers' data) it was a concern that this may have been introduced during the production of L929CM and hence had an effect on the expression of mRNA for SCI/MIP-1 α and MIP-1 β . However, it is unlikely that the accumulation of these mRNAs is due to LPS contamination as introducing the antibiotic polymixin B, which binds LPS and prevents it interacting with cellular LPS receptors (Issekutz 1983), into the culture system had no effect on the level of SCI/MIP-1 α mRNA induced by 20% L929CM (figure 5.4).

As L929CM is a potent source of CSF-1, this cytokine still appeared to be the most likely candidate for the inducing agent. There are several ways that this could be tested, including the use of neutralising antibodies. However, the most straight forward way is to use recombinant CSF-1.

5.3 Effect of rhCSF-1 on the Expression of SCI/MIP-1 α and MIP-1 β mRNA

The ability of CSF-1 to affect the accumulation of mRNA for SCI/MIP-1 α and MIP-1 β in BMDM was assessed in several experiments using material from two different sources (see Chapter 2) and using a range of concentrations from 10U/ml to 10,000U/ml. The levels of the two transcripts were assessed four hours after refeeding with alpha-MEM medium containing 25% DHS with or without the addition of recombinant human CSF-1 (rhCSF-1). The potency of these preparations was also assessed by their ability to induce thymidine incorporation in BMDM and to promote the formation of CFU-M colonies in semi-solid agar cultures of normal murine bone marrow. The effect of L929CM was also assessed in all of these experiments as a positive control.

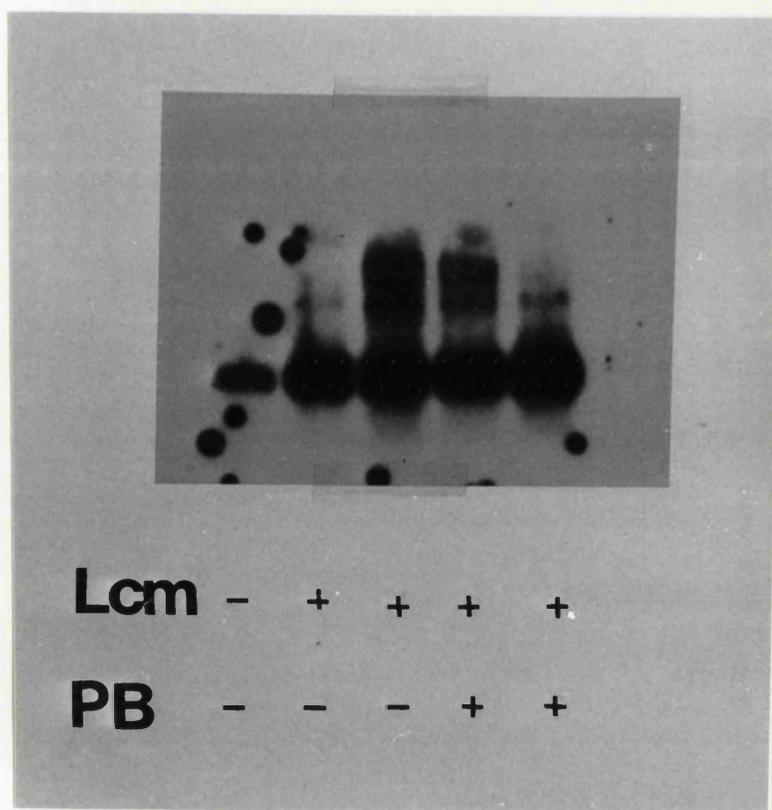


Figure 5.4 Northern analysis of the effect of polymixin B on the L929-induced accumulation of SCI/MIP-1 α mRNA in BMDM.

Total cellular RNA was isolated after 4 hours and blotted in equal amounts onto nylon membrane. Lane 1, untreated BMDM; lanes 2 and 3, BMDM treated with 20% L929CM; lanes 4 and 5, BMDM treated with 20% L929CM treated previously with polymixin B (2mg/ml). Equal loading was ensured by ethidium bromide staining of the gel.

Figure 5.5 shows the results of an experiment in which the ability of rhCSF-1 at 500 and 1000U/ml to affect the levels of mRNA for SCI/MIP-1 α and MIP-1 β were assessed. The activity of the rhCSF-1 was also assessed, in parallel with L929CM, for its ability to induce proliferation of BMDM after 20 hours (figure 5.6) and its ability to promote colony formation in normal murine bone marrow (figure 5.7).

Perhaps surprisingly, the rhCSF-1 failed to have a profound effect on the levels of either transcript, although in one instance rhCSF-1 caused an apparently 2 to 3-fold increase in the levels of both mRNAs (as assessed by laser densitometry, as seen in figure 5.5). L929CM, on the other hand, was able to induce accumulation of both of these in a dose-dependent manner.

Hamilton et al (1989, 1991) had demonstrated that the levels of c-fos, c-myc and urokinase-plasminogen activator mRNA could be markedly elevated by as little as 125 U/ml rhCSF-1, so the inability of the preparation at 500 and 1000 U/ml to have a great effect on SCI/MIP-1 α and MIP-1 β mRNA after four hours was surprising.

It was interesting that in these experiments 1000 U/ml rhCSF-1 had an ability to induce thymidine incorporation in BMDM which was approximately equal to that of 5% L929CM. It seemed possible, therefore, that the concentration of rhCSF-1 used was too low for changes in gene expression to be detected in this particular system.

When the potency of this preparation of rhCSF-1 was assessed by its ability to promote the formation of normal bone marrow colonies it was found that this was modest. According to information supplied by the manufacturer, one unit of rhCSF-1 should cause the formation of one CFU-M colony under the conditions of the experiment. However, it can be seen from figure 5.7 that this particular preparation had less than one tenth of the activity ascribed to it.

An alternative source of rhCSF-1 was obtained as a gift from Genetics Institute and was used at a concentration of 10,000 U/ml. Unfortunately this concentration had no effect on the level of either transcript in a single experiment, while L929CM was

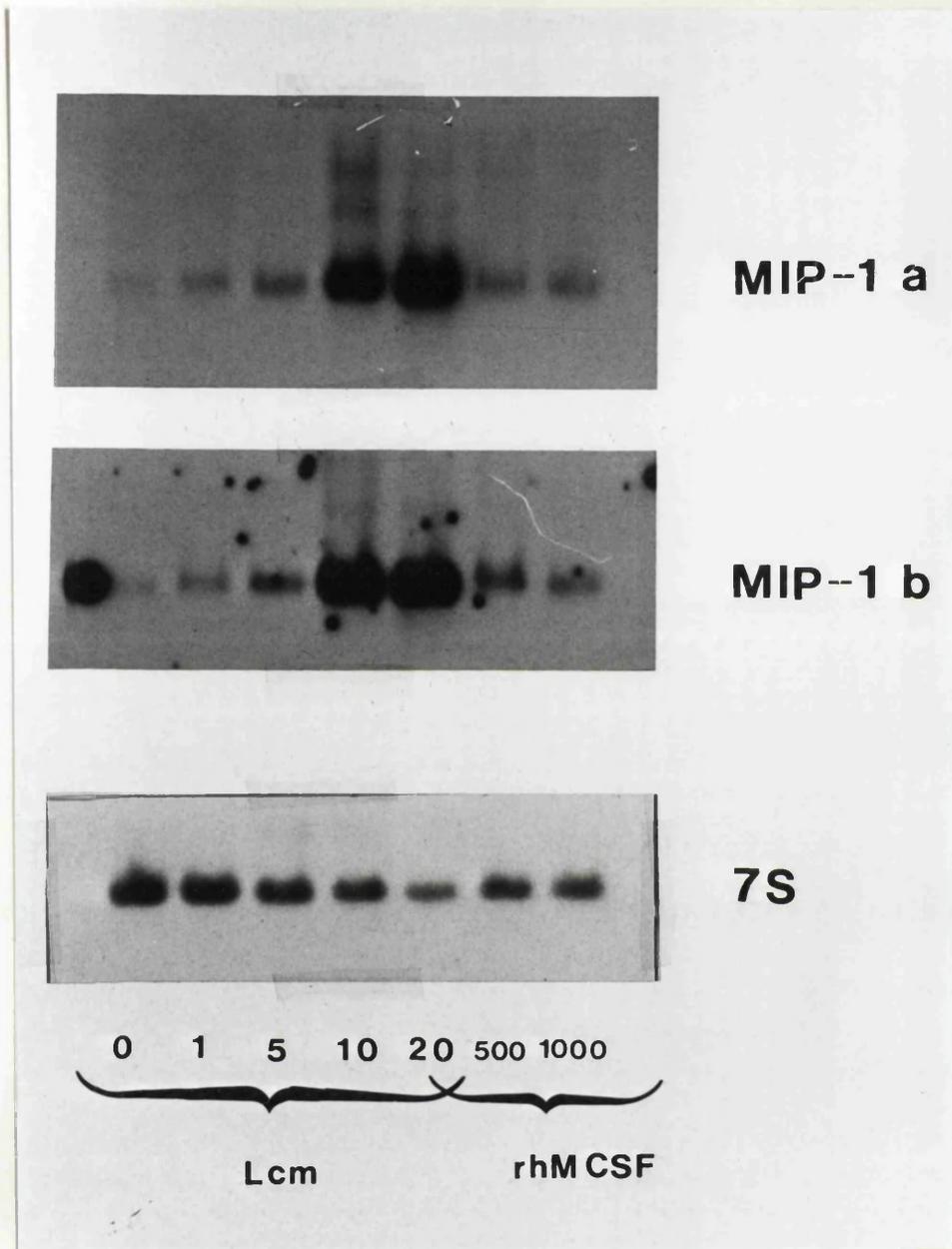


Figure 5.5 Northern analysis of the effect of recombinant human CSF-1 and L929CM on accumulation of SCI/MIP-1 α and MIP-1 β mRNA in BMDM.

Total cellular RNA was isolated after 4 hours and blotted in equal amounts (20 μ g) onto nylon membrane. Lanes 1-5, BMDM treated with 0, 1, 5, 10 and 20% L929CM; lanes 6 and 7, BMDM treated with 500 and 1000 U/ml recombinant human CSF-1. The membrane was hybridised first for SCI/MIP-1 α , then was stripped and reprobed for MIP-1 β . Equal loading was confirmed by hybridisation with a 7S probe.

Figure 5.6 Tritiated thymidine incorporation in BMDM following treatment with L929CM or recombinant human CSF-1.

BMDM were starved overnight in alpha-MEM medium containing 25% DHS in the absence of any other additions. Medium was then removed and BMDM refed with alpha-MEM medium containing 25% DHS plus the following additions:

- | | |
|---------------|------------------|
| 1) Nil | no additions |
| 2) 20% LCM | 20% L929CM |
| 3) 10% LCM | 10% L929CM |
| 4) 5% LCM | 5% L929CM |
| 5) 500U CSF-1 | 500 U/ml rhCSF-1 |

Tritiated thymidine incorporation was measured after 20 hours. Data is presented as the mean determination of triplicate cultures plus or minus one standard deviation.

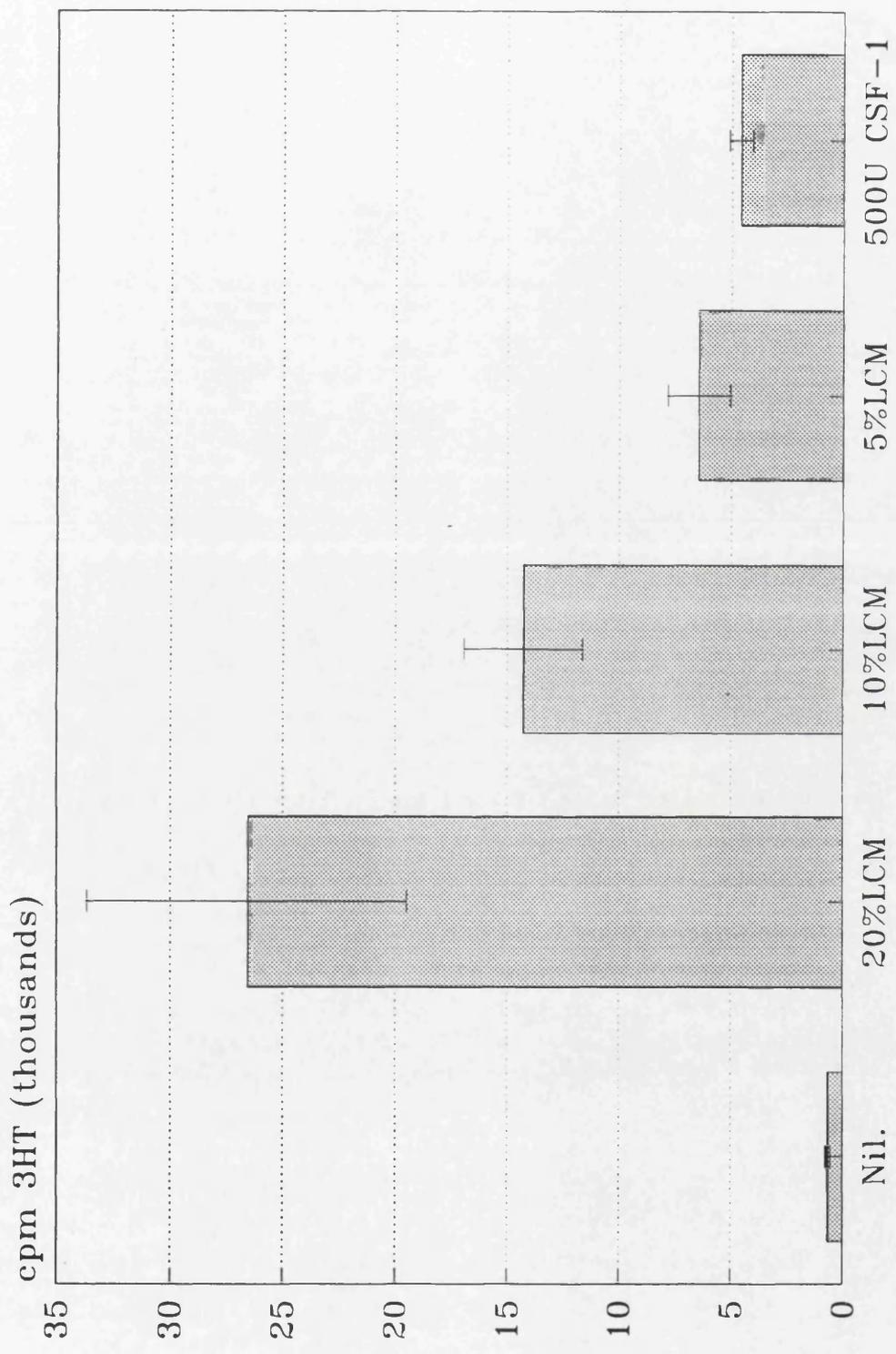
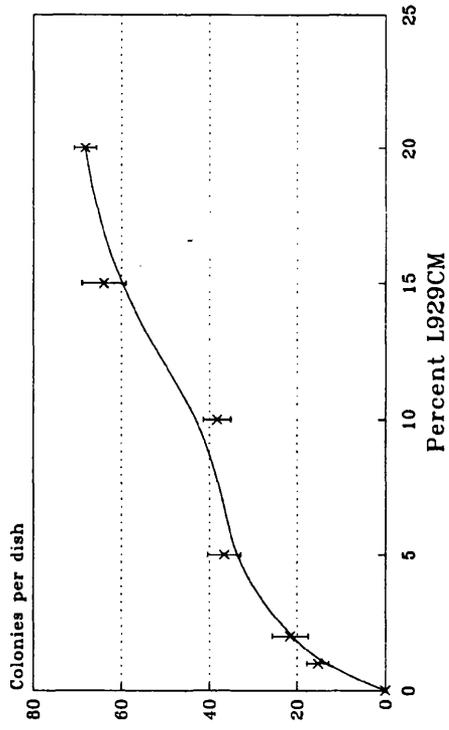


Figure 5.6

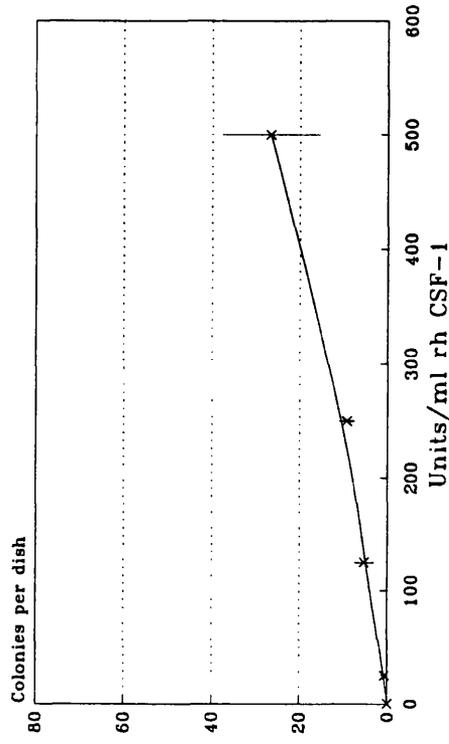
Figure 5.7 Effect of L929CM and recombinant human CSF-1 on colony formation by normal murine bone marrow.

$75 \cdot 10^3$ normal bone marrow cells from B6D2F1 mice were plated in 1ml plates in semi-solid alpha-MEM medium containing 25% DHS plus the concentration of L929CM or rhCSF-1 indicated in the figure and the cultures incubated for 7 days in the conditions detailed in chapter 2. Colonies containing greater than 50 cells were scored and data presented as the mean determination of triplicate cultures plus or minus one standard deviation.

NBM Colony Formation in L929CM



NBM Colony Formation in rhCSF-1



again effective in a dose-dependent manner (data not shown). The same concentration induced proliferation in BMDM equal to that of 5% L929CM (data not shown), which again in this experiment had little effect to on the expression of SCI/MIP-1 α and MIP-1 β mRNA. This experiment could only be performed once due to scarcity of material.

Therefore attempts to demonstrate an effect of rhCSF-1 on the levels of these two mRNAs were only marginally successful. This could have been for several reasons:

1) the recombinant protein was sticking to the plastic culture dish and therefore was prevented from acting on the cells, although this seemed unlikely as all assays were performed in the presence of 25% DHS which should be an effective carrier.

2) the constitutive level of SCI/MIP-1 α and MIP-1 β mRNA expressed by the BMDM masked any effect which the rhCSF-1 was having on the expression of these genes.

This seems unlikely as Hamilton et al (1989) were able to demonstrate a change above a constitutive level of c-fos in BMDM in response to as little as 125 U/ml rhCSF-1.

3) The expression of SCI/MIP-1 α and MIP-1 β mRNA is not affected by rhCSF-1.

This possibility could have been explored by measuring the levels of a gene known to be induced by CSF-1 in BMDM. An attempt was made to measure the levels of c-myc in the experiments described above but unfortunately this attempt was unsuccessful due to the inability to radioactively label the DNA probe.

4) The mRNA transcripts for SCI/MIP-1 α and MIP-1 β are induced by rhCSF-1 but in a later time-frame from that induced by LPS and L929CM. Unfortunately, investigating this possibility would require assessment of expression at multiple time points which was not possible due to scarcity of material.

However, the most likely explanation is still that the potency of the rhCSF-1 preparations used in these experiments were inadequate to affect gene expression. It is possible, therefore, that accumulation of SCI/MIP-1 α and MIP-1 β mRNA might be

induced by different rhCSF-1 preparations. This work was unable to be carried out, however, due to constraints of time. It is also possible, in addition to this, that the culture conditions used to assess the effects of rhCSF-1 were not optimal; for example all of the experiments involving this cytokine were carried out in 25% DHS, which is likely to be a rich source of growth factors and biological effector molecules. It is possible that the apparent ability of DHS to downregulate the accumulation of both SCI/MIP-1 α and MIP-1 β mRNA in BMDM (seen in figure 5.1) could mask the inductive effect of cytokines such as rhCSF-1.

The effect of CSF-1 on the expression of the SCI/MIP-1 α and MIP-1 β genes has not been reported by any other investigator.

Attempts were also made to investigate the effects of interleukins -1 and -6 on the expression of the two genes. The effect of these cytokines was assessed in two separate experiments and no significant difference, either positive or negative, in the levels of the two transcripts was detected (data not shown). These cytokines were chosen as they have been shown to be positive regulators of haemopoietic stem cell proliferation (Ikebuchi et al 1988) and it was thought possible that one of the effects of positive stem cell regulators might be to down-regulate the expression of negative regulators. Interestingly, however, Martin and Dorf (1991) have also recently reported the inability of IL-1 and TNF α to affect the expression of mRNA for SCI/MIP-1 α and MIP-1 β in the macrophage-like cell line J774A.1.

5.4 Effect of rmIFN γ on Expression of SCI/MIP-1 α and MIP-1 β mRNA

The interferons are proteins which exert multiple biological activities on the immune and other systems, primarily by inducing the synthesis of other biologically important proteins (Samuel 1991, Sen and Lengyell 1992). Vairo et al (1991) have demonstrated reversible inhibition of CSF-1-stimulated proliferation in BMDM by IFN γ .

The effect of IFN γ on L929-induced proliferation and on the expression of mRNA for SCI/MIP-1 α and MIP-1 β in BMDM was therefore assessed as a comparison to L929CM and CSF-1.

IFN γ (20ng/ml), as expected, as well as LPS (2 μ g/ml), was able to block the proliferation of BMDM stimulated with 20% L929CM while having no effect on thymidine incorporation when used alone, as can be seen from figure 5.8, confirming the results of Vairo et al (1991) and Knight et al (1992). The ability of IFN γ to block BMDM proliferation was blocked by boiling the preparation prior to use, indicating that the activity seen in this preparation is not due to contamination due to LPS, which is resistant to boiling (data not shown).

From figure 5.9 it can be seen that recombinant murine IFN γ (used at 20ng/ml) was able to increase expression of mRNA for both SCI/MIP-1 α and MIP-1 β at four hours to a level approximately equal to that induced by 20% L929CM. By twenty-four hours the levels of mRNA had returned to control levels. This again suggests that the induction of accumulation of SCI/MIP-1 α and MIP-1 β mRNA, upon cellular stimulation, is transient.

This finding is in agreement with the observations of Martin and Dorf (1991) who found that IFN γ increased the level of mRNA for JE SCI/MIP-1 α and MIP-1 β in the macrophage cell line J774A.1 six hours after treatment.

The fact that rmIFN γ could increase the accumulation of mRNA for SCI/MIP-1 α and MIP-1 β in BMDM was informative for three reasons:

- 1) It demonstrates that the accumulation of SCI/MIP-1 α and MIP-1 β mRNA in BMDM can be induced by a cytokine as well as a macrophage activating factor (LPS).
- 2) It demonstrates that accumulation of mRNA for SCI/MIP-1 α and MIP-1 β can take place in the absence of proliferation, providing evidence that the two processes can be regulated independently of each other.

Figure 5.8 Inhibition of L929CM-induced tritiated thymidine incorporation in BMDM by LPS (2µg/ml) and recombinant murine IFNγ (20ng/ml).

BMDM were starved overnight in alpha-MEM medium containing 25% DHS in the absence of any other additions. Medium was then removed and BMDM refed with alpha-MEM medium containing 25% DHS plus the following additions:

- | | |
|----------------------|------------------------------------------|
| 1) Nil | No additions |
| 2) 20%LCM | 20% L929CM |
| 3) LPS | LPS (2µg/ml) |
| 4) LPS+LCM | 20% L929CM plus LPS (2µg/ml) |
| 5) IFN γ | rmIFN γ (20ng/ml) |
| 6) IFN γ +LCM | 20% L929CM plus rmIFN γ (20ng/ml) |

Tritiated thymidine incorporation was measured after 20 hours and data presented as the mean of triplicate cultures plus or minus one standard deviation.

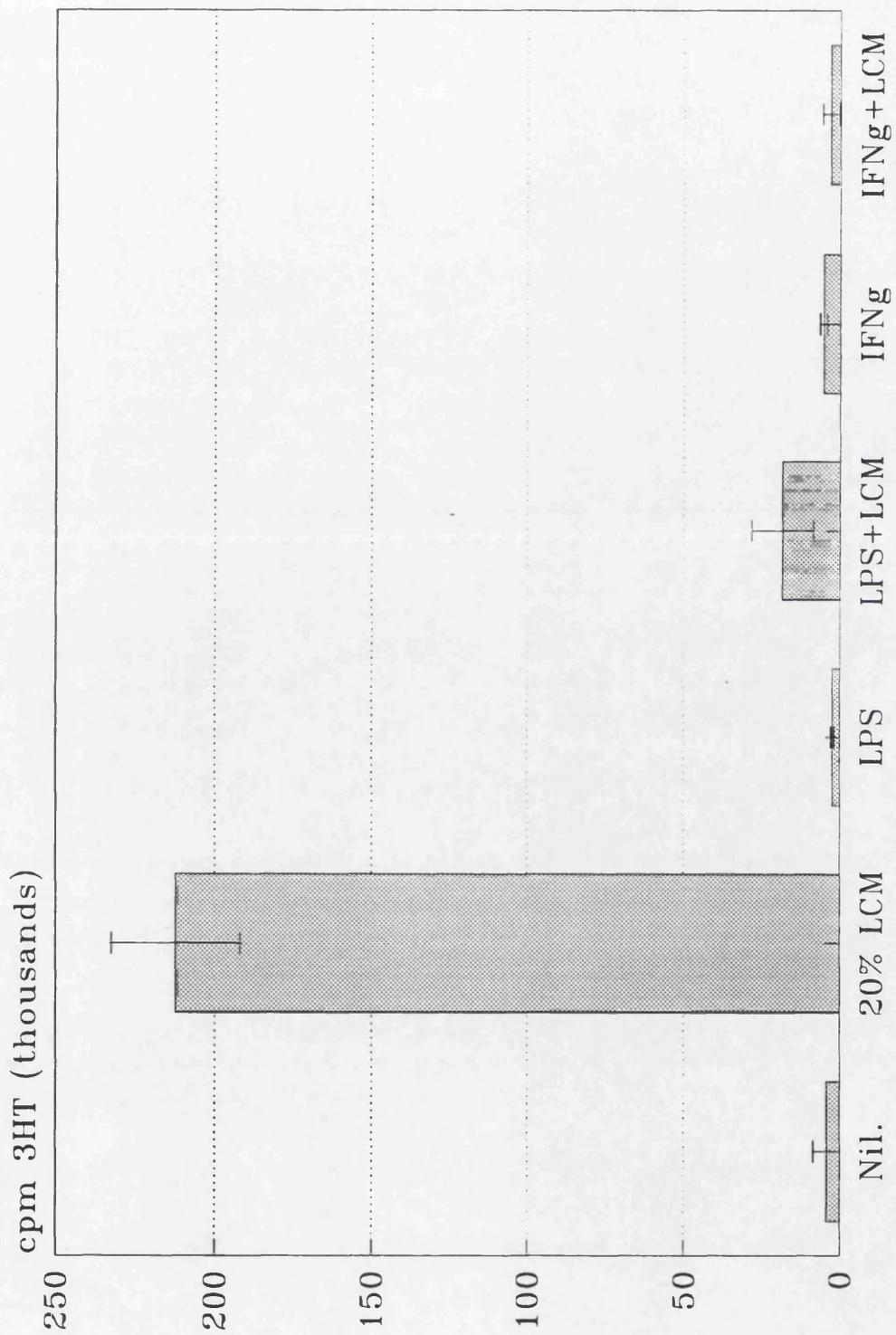


Figure 5.8

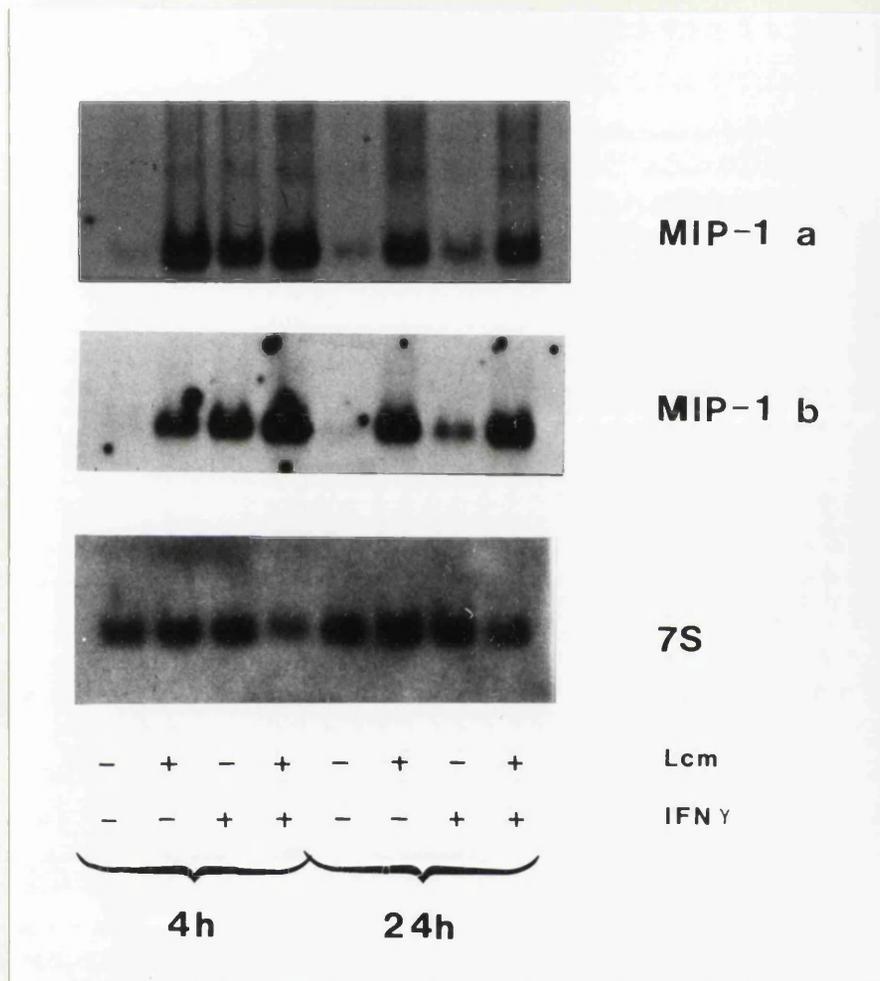


Figure 5.9 Northern analysis of the effect of recombinant murine IFN γ (20ng/ml) and 20% L929CM on the accumulation of SCI/MIP-1 α and MIP-1 β mRNA in BMDM.

Total cellular RNA was isolated after 4 hours (lanes 1-4) or 24 hours (lanes 5-8) and transferred in equal amounts (10 μ g) onto nylon membrane. Lanes 1 and 5, untreated BMDM; lanes 2 and 6, BMDM treated with 20% L929CM; lanes 3 and 7, BMDM treated with IFN γ ; lanes 4 and 8, BMDM treated with 20% L929CM plus IFN γ . The membrane was probed first for SCI/MIP-1 α , then stripped and reprobed for MIP-1 β . Equal loading was confirmed by hybridisation with a 7S probe.

3) It raises the possibility that expression of SCI/MIP-1 α and MIP-1 β may be involved in the specific immune response as well as in the non-specific response as evidenced by the effect of LPS.

5.7 Summary of Chapter 5

The accumulation of mRNA for SCI/MIP-1 α and MIP-1 β in BMDM by L929CM, recombinant human (rh) CSF-1 and recombinant murine (rm) IFN γ was assessed as part of a study to investigate candidate regulators of the expression of the SCI/MIP-1 α and MIP-1 β genes. The effects of two different sera, donor horse (DHS) and fetal calf (FCS), were also assessed.

Refeeding cells with medium containing 25% FCS lead to a profound, although transient increase in the levels of both mRNAs above the level in untreated control cultures. Replacing the FCS with DHS, on the other hand, lead to a marked decrease. Twenty percent L929CM induced accumulation of both mRNA transcripts approximately 10 to 20-fold, in a rapid and transient manner. Increases in the levels of both transcripts could be detected by one hour after stimulation. These patterns of expression are extremely similar to that seen with LPS, further emphasising that accumulation of mRNA for SCI/MIP-1 α and MIP-1 β is a component of the early response to stimulation of BMDM. With L929CM, accumulation of SCI/MIP-1 α and MIP-1 β mRNA was dose-dependent when measured 4 hours after treatment and preceded the onset of DNA synthesis. This may suggest that the signal transduction pathways and/or genetic programmes which control the induction of accumulation of SCI/MIP-1 α and MIP-1 β mRNA and the proliferation of BMDM may share elements in common.

Recombinant human CSF-1 had only a slight effect, if any, on the expression of the mRNAs, although this most likely relates to the activity of the preparations used as evidenced by the assays for activity performed in parallel with the gene expression

experiments; however, the culture conditions used may play a part. Further experiments are required if this issue is to be pursued.

Recombinant murine IFN γ , which opposed the L929CM-induced proliferation of BMDM, also increased accumulation of mRNA for both SCI/MIP-1 α and MIP-1 β after four hours, again in a transient manner. This result demonstrates that SCI/MIP-1 α and MIP-1 β mRNA accumulation can be induced by a cytokine and also that this induction and BMDM proliferation can be regulated independently.

Chapter 6.

Accumulation of mRNA for SCI/MIP-1 α and MIP-1 β in Bone Marrow-Derived Macrophages in Serum-Free Medium.

6.1 Introduction

The investigation of changes in the accumulation of SCI/MIP-1 α and MIP-1 β mRNA induced by cytokines in alpha-MEM medium containing 25% DHS (in chapter 5) had yielded some useful data. However, the minimal efficacy of recombinant human CSF-1, especially in inducing BMDM DNA synthesis, suggested that the culture conditions employed might not be optimal for assessing the effects of recombinant material on the accumulation of SCI/MIP-1 α and MIP-1 β mRNA in BMDM. In particular, the apparent ability of DHS to affect the accumulation of both SCI/MIP-1 α and MIP-1 β mRNA suggested that inclusion of this in the culture system might be masking the effects of recombinant proteins such as CSF-1.

In addition, experiments with the SF12 serum-free medium (in chapter 3) had suggested that the survival of BMDM could be enhanced in this medium in comparison to alpha-MEM.

In an attempt to determine whether modulation of the expression of SCI/MIP-1 α and MIP-1 β genes might be more effectively studied under different, and more defined, culture conditions and to assess the effects on SCI/MIP-1 α and MIP-1 β gene expression of different components of the culture conditions used in chapters 3, 4 and 5, the accumulation of SCI/MIP-1 α and MIP-1 β mRNA was assessed on culturing BMDM in SF12 medium in the presence and absence of DHS, L929CM and hydrocortisone. SF12 medium was used in preference to reducing the level of DHS in alpha-MEM as SF12 has been specially formulated for the culture of primary haemopoietic cells.

6.2 Accumulation of SCI/MIP-1 α and MIP-1 β mRNA in Bone Marrow-Derived Macrophages in SF12 Medium.

As a pilot experiment, SCI/MIP-1 α mRNA accumulation was assessed before and after culturing in SF12 medium in the presence or absence of either 20% DHS or 20% L929CM. Levels were measured at one, three and six as well as twenty-four hours, as if accumulation of SCI/MIP-1 α mRNA was going to be affected by this treatment it was likely that this would happen within a few hours.

It can be seen from figure 6.1 that the relative level of SCI/MIP-1 α mRNA in BMDM after 24 hours in serum-free medium is approximately the same as that seen in the unstimulated cells in serum-containing medium (figure 6.1 upper panel, lanes 1 and 6). However, the level of SCI/MIP-1 α mRNA is increased relative to control levels at three and six hours after refeeding, with the highest level observed after 6 hours (figure 6.1 upper panel, lanes 4 and 5). The addition of donor horse serum (DHS) appears to block this induction, suggesting that DHS is a downregulator of SCI/MIP-1 α (and hence possibly MIP-1 β) mRNA accumulation. In this particular experiment, addition of L929CM at the same time as the medium change appears to augment the induction of SCI/MIP-1 α mRNA but this was found to vary between experiments. It was decided that, in order to further assess the suitability of SF12 medium for investigating the expression of SCI/MIP-1 α and MIP-1 β mRNA, this induction would be further investigated.

Figure 6.2 shows the effect of refeeding with SF12 alone on levels of both SCI/MIP-1 α and MIP-1 β mRNA in greater detail. It can be seen that the accumulation of both transcripts is rapid and transient with the peak of accumulation occurring at around four hours. The similarity between this pattern and that seen after stimulation with LPS or L929CM is obvious; this pattern again indicates that accumulation of

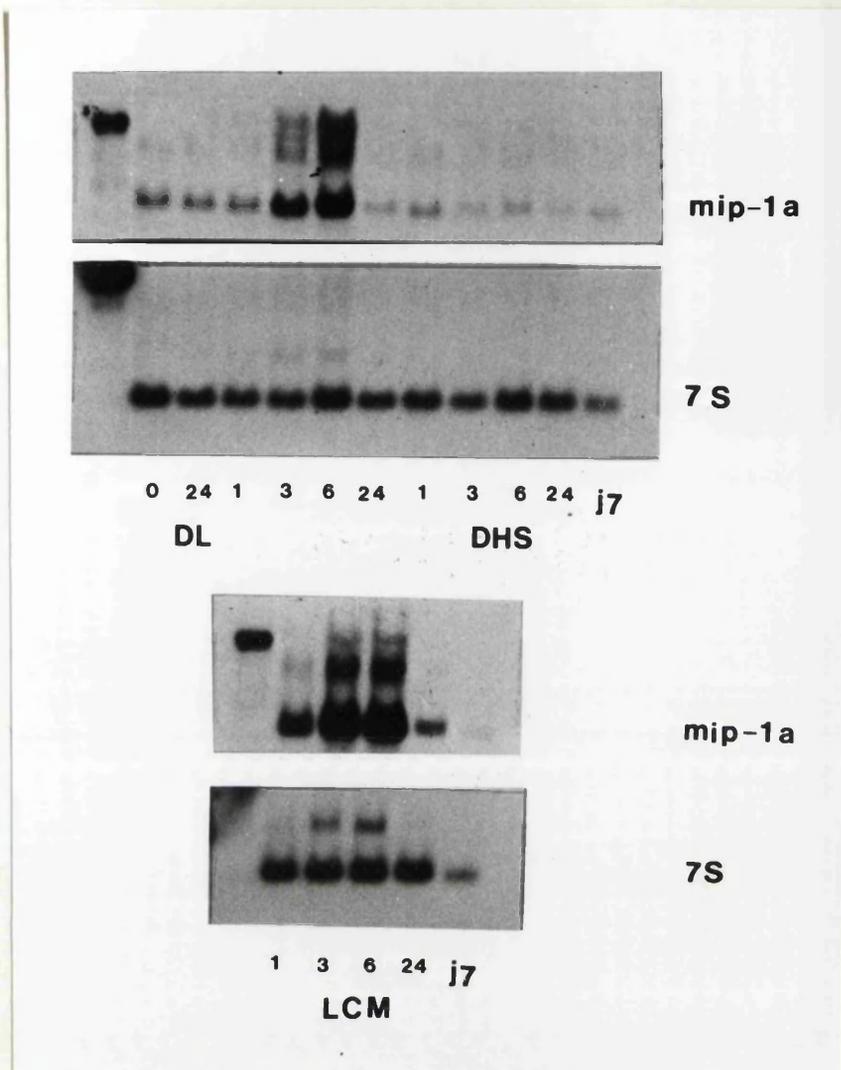


Figure 6.1 Northern analysis of the accumulation of SCI/MIP-1 α mRNA in BMDM on refeeding with SF12 medium.

Total cellular RNA was isolated at various times after refeeding with SF12 medium plus or minus 20% L929CM and/or DHS (25%) and blotted in equal amounts (20 μ g) onto nylon membrane.

Upper panel: Lane 1, RNA ladder; lane 2, untreated BMDM; lane 3, BMDM refed with SF12 containing 25% DHS plus 20% L929CM for 24 hours; lanes 4-7, BMDM refed with SF12 medium for 1, 3, 6 and 24 hours; lanes 8-11, BMDM refed with SF12 medium plus 25% DHS for 1, 3, 6 and 24 hours; lane 12, untreated J774.2 cells. Equal loading was ensured by hybridisation to a 7S probe.

Lower panel: Lane 1, RNA ladder; lanes 2-5, BMDM refed with SF12 containing 20% L929CM, lane 6, untreated J774.2 cells. Equal loading was confirmed by hybridisation to a 7S probe.

SCI/MIP-1 α and MIP-1 β mRNA is an early response to stimulation; in this case stimulation consists of changing the medium.

The effect of DHS on the accumulation of SCI/MIP-1 α and MIP-1 β mRNA was of interest as it was reasoned that investigating the effects of DHS on accumulation of mRNA for SCI/MIP-1 α and MIP-1 β in SF12 medium might contribute to understanding the role played in this respect by DHS in alpha-MEM medium and hence enhance the interpretation of the results of chapter 5.

The effect of L929CM on MIP-1 mRNA accumulation in SF12 medium was also investigated to allow direct comparison between SF12 and alpha-MEM cultures.

Accordingly, the levels of SCI/MIP-1 α and MIP-1 β mRNA were assessed in BMDM four hours after culturing in SF12 medium with the addition of various concentrations of DHS in the presence or absence of 20% L929CM. The four hour time point was chosen as this represented the peak of accumulation of both SCI/MIP-1 α and MIP-1 β mRNA transcripts on refeeding with SF12 medium, as illustrated in figure 6.2.

Figure 6.3 shows the results of a representative of one of two experiments. It can be seen that DHS reduces the level of mRNA for both SCI/MIP-1 α and MIP-1 β mRNA after four hours in a dose-dependent manner. In this experiment, the presence of 20% L929CM appears to have a positive effect on the levels of both transcripts, however, this effect was not seen in all of these experiments; as stated above, this inconsistency in the effect of L929CM was a persistent feature of experiments carried out in SF12 medium.

To assess the specificity of the effect of DHS on the induction in SF12 medium and to assess the suitability of using FCS in this system, the effect of various concentrations of FCS on the accumulation of SCI/MIP-1 α mRNA on refeeding BMDM with SF12 medium was assessed. It was found that FCS was unable to cause the same down-regulation in the level of SCI/MIP-1 α mRNA as DHS even when used at 20% of the total volume of the medium (figure 6.4), mirroring the differential

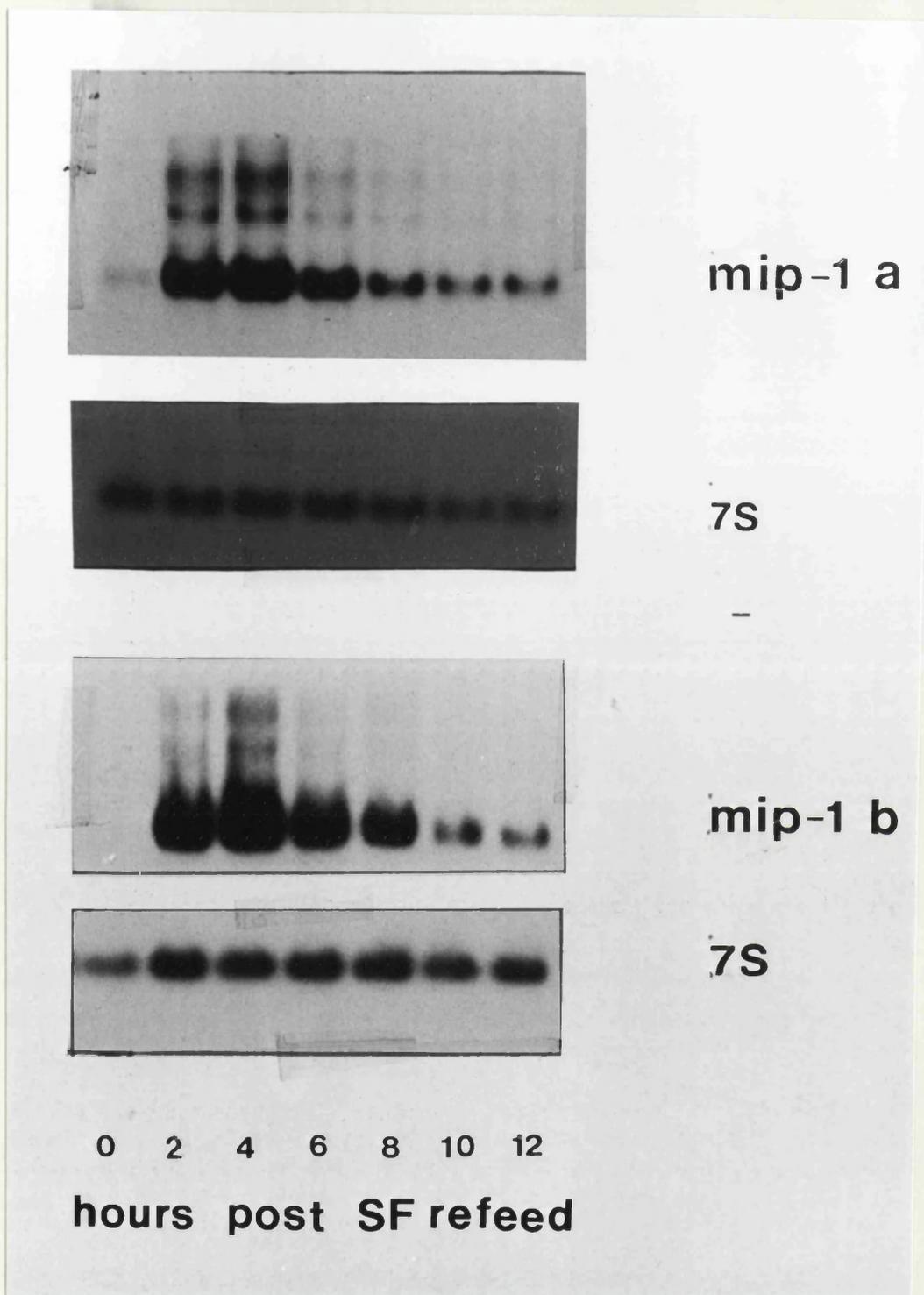


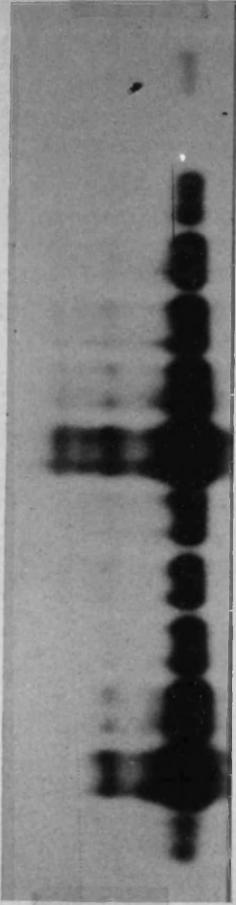
Figure 6.2 Northern analysis of the time course of accumulation of SCI/MIP-1 α and MIP-1 β mRNA in BMDM refed with SF12 medium.

Total cellular RNA was isolated from BMDM 0, 2, 4, 6, 8 and 10 hours after refeeding with SF12 medium and blotted in equal amounts (20 μ g) onto nylon membrane. Equal loading was confirmed by hybridisation with a 7S probe.

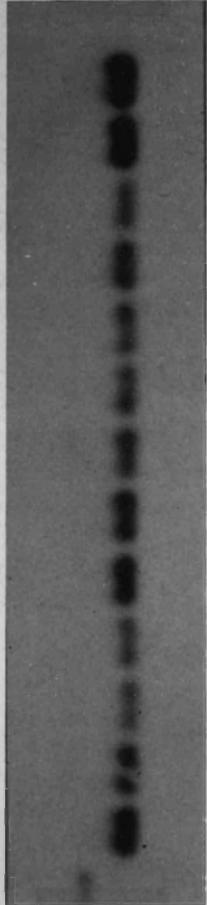
Figure 6.3 Northern analysis of accumulation of SCI/MIP-1 α and MIP-1 β mRNA in BMDM after refeeding with SF12 medium in the presence and absence of various concentrations of DHS plus or minus 20% L929CM.

Upper panel, SCI/MIP-1 α mRNA. Lane 1, untreated BMDM; lanes 2-6, BMDM refed with SF12 medium in the presence of 0, 5, 10, 15 and 20% DHS; lanes 7-11, BMDM refed with SF12 medium containing 20% L929CM in the presence of 0, 5, 10, 15 and 20% DHS; lane 12, untreated STO cells; lane 13, untreated J774.2 cells. Equal loading was confirmed by hybridisation with a 7S probe.

Lower panel, MIP-1 β mRNA. Lane 1, untreated BMDM; lanes 2-6, BMDM refed with SF12 medium in the presence of 0, 5, 10, 15 and 20% DHS; lanes 7-11, BMDM refed with SF12 medium containing 20% L929CM in the presence of 5, 10, 15, 20 and 0% DHS. Equal loading was ensured by ethidium bromide staining of the gel.



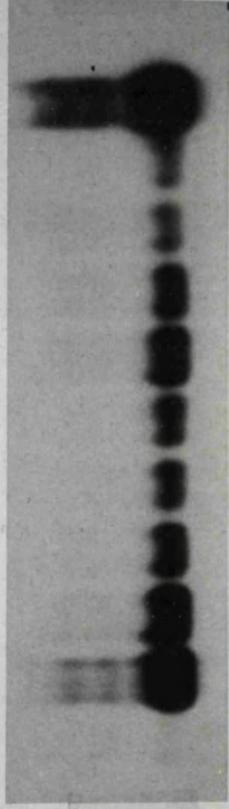
mip-1 a



7S

DHS

C 0 5 10 15 20 0
 Lcm



mip-1 b

DHS

C 0 5 10 15 20 0
 Lcm

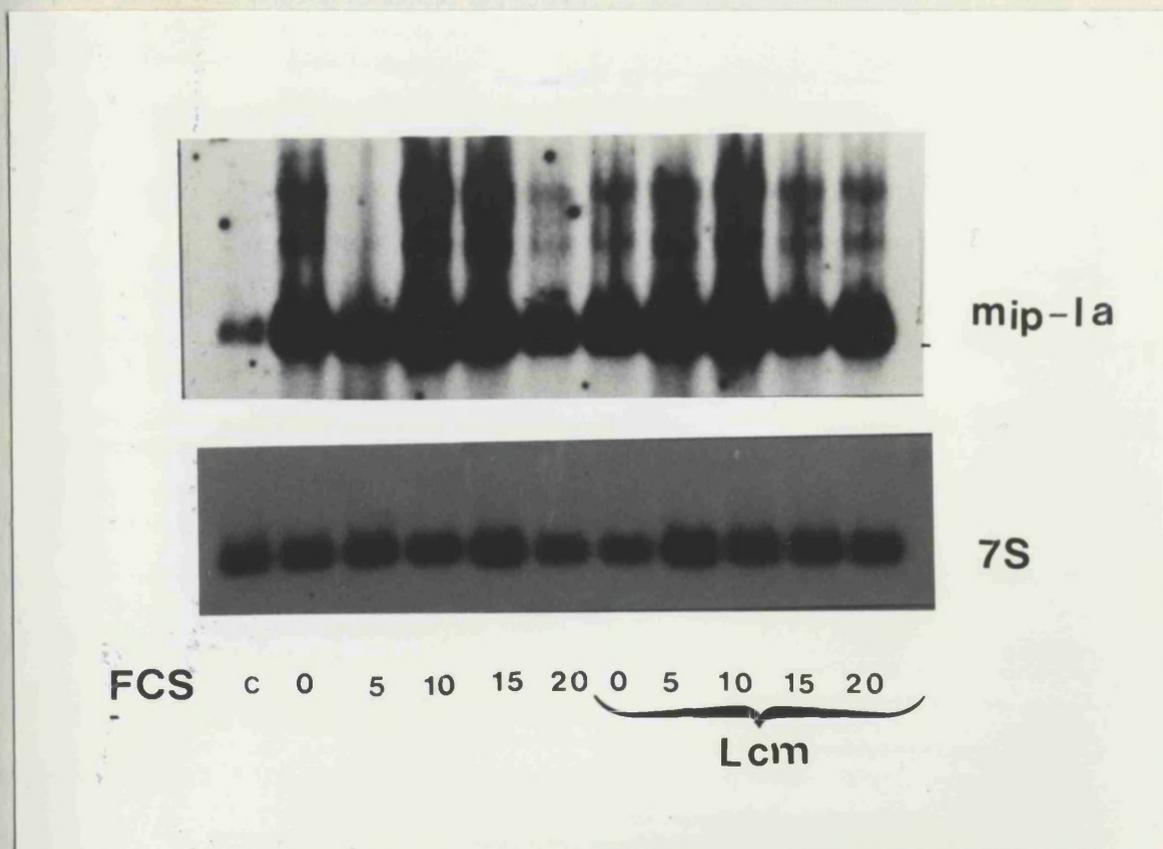


Figure 6.4 Northern analysis of the accumulation of SCI/MIP-1 α mRNA in BMDM in the presence or absence of various concentrations of FCS.

Total cellular RNA was isolated after 4 hours and blotted in equal amounts onto nylon membrane. Lane 1, untreated BMDM; lanes 2-6, BMDM refed with SF12 medium in the presence of 0, 5, 10, 15 and 20% FCS; lanes 7-11, BMDM refed with SF12 medium containing 20% L929CM in the presence of 0, 5, 10, 15 and 20% FCS.

effects of the two sera on the accumulation of SCI/MIP-1 α and MIP-1 β mRNA in alpha-MEM medium.

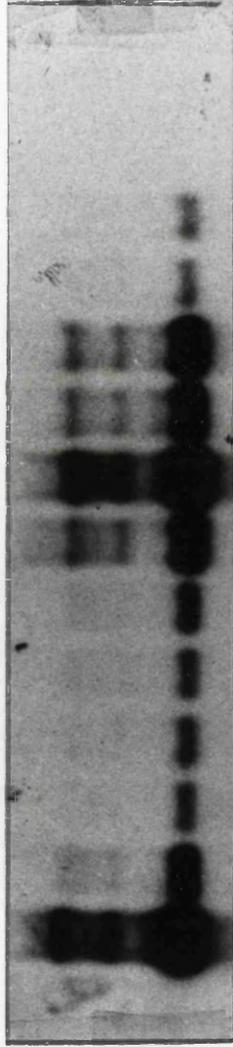
The ability of DHS to oppose the accumulation of SCI/MIP-1 α and MIP-1 β mRNA was further investigated by comparing this with the effect of hydrocortisone, which had been shown to have a similar effect (as discussed in chapter 4). Again the four hour time point was chosen to assess the effects of these agents. It can be seen from figure 6.5 that both DHS and hydrocortisone are able to reduce the levels of both SCI/MIP-1 α and MIP-1 β mRNA.

When the combined effects of DHS and hydrocortisone on the levels of SCI/MIP-1 α and MIP-1 β in serum-free medium were assessed (figure 4.4) it was found that these were additive and that the two agents in combination reduced the level of both transcripts to approximately control levels. However, even when both of the repressive factors were combined a basal level of both SCI/MIP-1 α and MIP-1 β mRNA could still be detected. This makes it possible that both DHS and hydrocortisone exert their effects on the level of mRNA for both SCI/MIP-1 α and MIP-1 β at the level of mRNA stability rather than transcription as it had already been demonstrated (in chapter 4, figure 4.4) that the basal levels of these mRNA transcripts expressed in resting BMDM was sensitive to actinomycin D, from which it was inferred that the expression of the basal levels was dependent on ongoing transcription. If either or both of the above-mentioned agents had been negatively affecting transcription of these genes it is unlikely that a basal level of transcript could have been detectable when both agents were used in combination, considering the profound effects of both agents in downregulating the levels of both SCI/MIP-1 α and MIP-1 β mRNA. This assumes, of course, that the mechanisms underlying the expression of a basal level of SCI/MIP-1 α and MIP-1 β mRNA are the same in SF12 as in alpha-MEM medium.

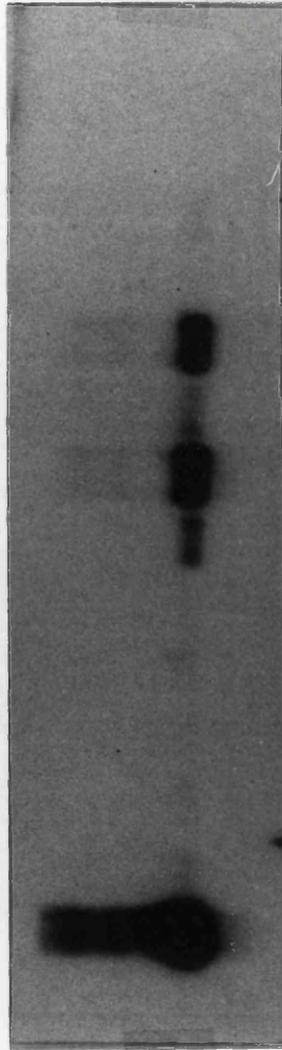
Figure 6.5 Northern analysis of the accumulation of SCI/MIP-1 α and MIP-1 β mRNA in BMDM in SF12 medium in the presence or absence of DHS (25%) and/or hydrocortisone.

Total cellular RNA was isolated after four hours and blotted in equal amounts (20 μ g) onto nylon membrane. Lane 1, BMDM refed with SF12 medium; lane 2, BMDM refed with SF12 medium plus 10⁻⁶M hydrocortisone; lanes 3 and 5, BMDM refed with SF12 medium plus 25% DHS plus 10⁻⁶M hydrocortisone; lanes 4 and 6, BMDM refed with SF12 medium plus 25% DHS plus 10⁻⁷M hydrocortisone; lane 7, BMDM refed with SF12 plus 25% DHS; lane 8, BMDM refed with SF12 medium plus 20% L929CM; lane 9, BMDM refed with SF12 medium plus 20% L929CM plus 10⁻⁶M hydrocortisone; lane 10, BMDM refed with SF12 medium plus 25% DHS plus 20% L929CM; lane 11, BMDM refed with SF12 medium plus 25% DHS plus 20% L929CM plus 10⁻⁶M hydrocortisone; lane 12, untreated BMDM. Equal loading was confirmed by ethidium bromide staining of the gel.

MIP-1 a



MIP-1 b



HC 0 6 6 7 6 6 7 0 0 6 0 6 C
DHS L DL

In this experiment L929CM does not appear to have any effect, apart from the fact that it seems to have reduced the accumulation of MIP-1 β mRNA upon refeeding with SF12 medium. (compare lanes 8 and 1). This is in contrast to the effects seen with L929CM in alpha-MEM medium containing 25% DHS (as demonstrated in chapter 5) and also to the effect seen in SF12 medium in figures 6.1 and 6.3).

The transient accumulation of SCI/MIP-1 α and MIP-1 β mRNA in BMDM on culture in serum-free medium was unexpected as this medium contains no growth factors, mitogens or hormones other than insulin (which is supplied by the serum-free supplement). It is possible that the rise was due to the effects of non-specific cellular stress but this was thought to be unlikely for several reasons. Firstly, it had already been demonstrated in chapter 3 (figure 3.10) that the survival of BMDM in serum-free medium is not inferior to that seen with the growth medium (alpha MEM containing 20% L929CM), indeed it was apparently enhanced on addition of either L929CM and/or DHS to SF12 medium. Secondly, the induction is transient, which implies control at both induction and in termination of expression. It is unlikely that an increase due to non-specific effects would display such characteristics. The fact that hydrocortisone downregulated the expression of both transcripts in SF12 medium both in the presence and the absence of 25% DHS also suggested that the gene expression machinery of the cell was intact, again arguing against a non-specific inductive effect of culturing in SF12 medium. In addition to this, the elevation of both the mRNA transcripts studied is specifically down-regulated by one type of serum but not another, which argues against the possibility that the down-regulation seen with DHS is not due to an increase in cell viability due to non-specific trophic effects. This fact also argues that the induction in serum-free medium was not due to non-specific effects of the absence of serum such as changes in pH or osmolarity. Nevertheless, the pH and osmolarity of SF12 medium was checked and found to be within the normal range (data not shown). Furthermore, the level of 7S RNA is not affected by

this treatment which argues against a non-specific effect on general RNA metabolism. It may have been instructive, however, to follow changes of the mRNA for another lymphokine gene in the same cells to gauge whether this effect was specific to the MIP-1 transcripts or was general for other inducible genes.

6.3 Summary of Chapter 6.

The expression of SCI/MIP-1 α and MIP-1 β mRNA in BMDM on culturing in a specially formulated serum-free medium (SF12) was assessed. In SF12 medium alone, it was found that the level of both SCI/MIP-1 α and MIP-1 β mRNA was increased in a rapid and transient manner, suggestive of this being part of the early response to stimulation, in a similar way to that seen by treatment of the BMDM with either LPS, L929CM or FCS.

Both DHS and hydrocortisone downregulated the level of SCI/MIP-1 α and MIP-1 β mRNA in BMDM in SF12 medium. The effects of these two agents was additive; therefore the accumulation of SCI/MIP-1 α and MIP-1 β mRNA accumulation in BMDM in SF12 medium was demonstrated to be able to be both induced and down-regulated. However, the effects of L929CM on the accumulation of these transcripts was highly variable between experiments which suggested that this culture system was unsuitable for studying the effects of growth factors on accumulation of SCI/MIP-1 α and MIP-1 β mRNA.

The marked ability of DHS to downregulate the accumulation of both SCI/MIP-1 α and MIP-1 β mRNA may mean that the effects of some inducing agents might be masked when DHS is included in the culture system, especially if the induction is modest; this suggests that DHS may not be the most suitable serum for use in the study of SCI/MIP-1 α and MIP-1 β gene expression.

Taken together, the data presented in this chapter indicate that SF12 is not a suitable culture medium for the assessment of candidate cytokine regulators of SCI/MIP-1 α and MIP-1 β gene expression.

However, although the experiments detailed immediately above and in chapter 5 describe the use of an experimental system for assessing the effects of various agents on the accumulation of SCI/MIP-1 α and MIP-1 β mRNA, the data are sufficient to allow conclusions to be drawn about other aspects of the expression of SCI/MIP-1 α and MIP-1 β mRNA in BMDM especially with respect to its relationship to cellular proliferation.

It has often been speculated that the induction of expression of many cytokine genes in the process of macrophage and T lymphocyte activation or proliferation is directly coupled to the mitogenic response and subsequent DNA synthesis; for example Hume and Gordon (1984) and Grimaldi et al (1986) in the case of u-PA expression in macrophages and keratinocytes and fibroblasts, respectively. Siderovski et al (1990) have argued for a direct link between the induction of accumulation of mRNA for human MIP-1 α (GOS 19-1) in activated human T peripheral blood mononuclear cells and G0 to G1 transition of the cell cycle. This is a particularly attractive hypothesis for the expression of cytokine genes given that they are generally expressed within a similar time frame after stimulation as proto-oncogenes believed to be involved in the initial commitment to proliferation. These two types of genes often have 5' regulatory elements in common such as consensus sequences for the binding of the transcriptional regulator AP-1 and elements shown to be involved in the transcriptional response to serum and oestrogen (for review see Hall and Stiles 1989) which suggests that it is likely that their expression is controlled by similar genetic programmes.

Plumb et al (1991) have also postulated that such a coupling, in the case of the expression of SCI/MIP-1 α , could be directly relevant to its role as an inhibitor of

haemopoietic stem cells. They postulate that production of SCI/MIP-1 α from proliferating cells at the mature end of the monocyte/macrophage lineage could constitute a feedback signal to proliferating stem cells which is independent of the resident bone marrow macrophage population. The fact that the induction of accumulation of SCI/MIP-1 α and MIP-1 β mRNA has been shown in this study to have properties characteristic of "immediate early" genes strengthens this hypothesis. If the thymidine incorporation and mRNA expression data of chapters 3 and 5 and the results of this chapter are compared it is possible to comment on this idea with respect to the expression mRNA for SCI/MIP-1 α and MIP-1 β in BMDM.

Figure 6.6 shows a summary table of the effects of different culture conditions on both the proliferation of BMDM and their expression of mRNA for SCI/MIP-1 α and MIP-1 β .

An increase in the expression of SCI/MIP-1 α and MIP-1 β mRNA is coincident with the induction of proliferation when BMDM are cultured in the presence of 20% L929CM in alpha MEM medium containing either DHS or FCS, which suggests a coupling between the two processes. However, the rest of the data demonstrates that these two processes can be regulated independently of each other. For example, the level of both transcripts can be increased in the absence of subsequent proliferation on culturing BMDM in SF12 medium alone or in alpha MEM medium containing 25% FCS in the absence of L929CM. It also seems to be possible to have a reduction in the level of both SCI/MIP-1 α and MIP-1 β mRNA in BMDM which will subsequently proliferate as BMDM cultured in the presence of SF12 plus 20% L929CM, which do not proliferate, express a higher level of both SCI/MIP-1 α and MIP-1 β mRNA than those cultured in SF12 with both 20% L929CM and 20% DHS which have been demonstrated to be proliferating rapidly after twenty hours. Therefore, although coincident expression of SCI/MIP-1 α and MIP-1 β can be induced by one set of conditions, the coupling between these two processes is not

MIP mRNA v BMDM Proliferation

<u>culture conditions</u>	<u>proliferation</u>	<u>MIP mRNA</u>
MEM (25%DHS)	-	+
MEM (25%DHS,20% LCM)	+	+++
MEM (25%FCS)	-	++++++
MEM (25%FCS,20%LCM)	+	++++++
SF12 alone	-	++++++
SF12 (20%LCM)	-	++++++
SF12 (25%DHS,20%LCM)	+	++

Figure 6.6 The accumulation of SCI/MIP-1 α and MIP-1 β mRNA versus proliferation (DNA synthesis) in bone marrow-derived macrophages.

This figure summarises several sets of experiments with regards to both the accumulation of SCI/MIP-1 α and MIP-1 β mRNA and the induction of DNA synthesis in BMDM in several different culture conditions.

Induction of proliferation (DNA synthesis) is denoted by (+), while its absence is denoted by (-).

Relative levels of the accumulation of SCI/MIP-1 α and MIP-1 β mRNA (arbitrary units) are denoted by the number of crosses (+).

absolute. Hence, it would then follow that the programmes regulating BMDM proliferation and the expression of SCI/MIP-1 α and MIP-1 β are may be overlapping but are not identical.

Chapter 7

Expression of SCI/MIP-1 α mRNA in Murine Bone Marrow.

7.1 Introduction.

In chapters 3 to 6, the use of a system which allows the expression of mRNA for both SCI/MIP-1 α and MIP-1 β in primary murine macrophages to be studied was described. However, as with most cytokines, the role which this expression plays in marrow homeostasis, if any, is unclear, although observations by several investigators, as discussed in chapter 1 have strongly suggested the ability of SCI/MIP-1 α and/or the Manchester Inhibitor to act as a physiological regulator of CFU-S proliferation in vivo.

Initial experiments in this study (chapter 3, figure 3.13) had shown that mRNA for SCI/MIP-1 α could be readily detected by Northern blotting, demonstrating expression of the gene in normal, unperturbed marrow. It was reasoned that it might be possible to exploit this situation to study changes in the level of expression of SCI/MIP-1 α in the bone marrow in relation to changes in the cycling of stem cells. If so, it might be possible to generate evidence for or against a role for SCI/MIP-1 α gene expression in stem cell proliferation regulation in vivo and also set up a model which would allow for further analysis of the mechanisms and signals underlying this expression in vivo. Two model systems utilising drugs with well characterized effects on the haemopoietic system were used for this purpose.

5-fluorouracil (5-FU) is an agent which kills proliferating cells through its ability to inhibit thymidylate synthetase activity and, hence, DNA synthesis. 5-FU may also affect some non-proliferating cells as it can affect the metabolism of RNA (Parker and Cheung 1991).

This drug has been shown to have profound effects in both the short- and the long-term on the haemopoietic compartment. Marrow cellularity is reduced by between 80 and 95% by four days after a single injection of 5-FU and has regenerated to approximately normal levels by days twelve to fourteen. A transient overshoot of cell number is often seen at this time (Hodgson and Bradley 1979; Lerner and Harrison 1990; Harrison and Lerner 1991; Rich 1991).

Lorimore et al (1990) observed that both the CFU-S and CFU-A compartments are extremely sensitive to a single injection of 5-FU with all three compartments being reduced by approximately 98% within twenty-four hours of a single injection and that these primitive cells display characteristic recovery patterns. CFU-S recover from the drug-induced nadir to approximately control levels at day 7-8. This is followed by an overshoot in CFU-S numbers which reaches a peak of four-fold over control by day 15. CFU-S numbers have returned to control levels by day 18. Lorimore et al (1990) also observed that CFU-A exhibit a biphasic pattern of recovery with numbers returning to 2.5 times control by day 8, decline over the next five days then reach another peak at twice control values at day 15 before returning to control values by 18.

Pluripotential Haemopoietic Stem Cells (PHSC), as defined by the repopulation studies of Harrison et al (1980), on the other hand, are resistant to a single injection of 5FU (Lerner and Harrison 1990). However, they are sensitive to a second injection, the effect of which is dependent on its time after the first injection. From these studies it can be concluded that the PHSC cycle rapidly during the initial recovery phase with the greatest proliferation at days 3-5. Proliferation has returned to control levels by day 8 (Harrison and Lerner 1991).

5-fluorouracil has also been demonstrated to have characteristic effects on lineage-committed cells. Vetvicka et al (1986) have demonstrated that B lineage cells in the

marrow are extremely sensitive to 5-FU with a profound depletion in their numbers by 24 hours which do not return to control levels for 4-5 weeks.

The effects of 5-FU on the erythropoietic compartment have also been documented (Rich 1991). A single injection severely reduces all of the mature erythroid and in vitro erythropoietic progenitors. A transient overshoot of the BFU-E and CFU-E populations on days 6 and 7 leads to a profound reticulocytosis after day 9.

Phenylhydrazine (PHZ) is another drug with well documented effects on haemopoiesis, causing a severe anaemia through erytholysis (Rencricca et al 1970). Rencricca et al (1970) demonstrated that CFU-S migrate from the marrow to the spleen five days after initiation of a course of phenylhydrazine. The CFU-S remaining in the marrow enter cell cycle on day 7, presumably to self-renew in order to restore marrow CFU-S numbers to a full complement. The proliferation of CFU-S returns to normal by day 9. A slight overshoot of CFU-S numbers is observed between days twelve and sixteen after drug treatment.

Interestingly, Lord and Wright (1982) had demonstrated that CFU-S inhibitory activity (Manchester Inhibitor) could not be detected in marrow from mice seven days after the beginning of a course of phenylhydrazine but could be readily detected on day nine. This is strong evidence for the Manchester Inhibitor playing a role in the control of CFU-S proliferation.

As mRNA for SCI/MIP-1 α could be detected in normal untreated bone marrow it was reasoned that any alterations in the level of expression of this transcript which were associated with disruption of the haemopoietic system would be detectable by Northern blotting and that it might be possible to relate these to changes in stem cell physiology.

7.2 Expression of SCI/MIP-1 α mRNA in Post 5-FU Bone Marrow.

The effects of a single injection of 5-fluorouracil on the level of SCI/MIP-1 α mRNA expressed in the marrow of female B6D2F1 mice was assessed by Northern blotting at various times after treatment.

To ensure that 5-FU was having an effect on the marrow and to monitor changes in stem cell cycling, both marrow cellularity and the cycle status of CFU-A were followed during the course of the experiment. These data are presented in figure 7.1. It can be seen that on day 8 following a single injection of 5-FU, the numbers of cells per femur were between 10 and 20% of normal controls counted on the same day. By day 24 numbers had returned to normal. A similar pattern was observed in two other experiments. These figures were in general agreement with those of Lerner and Harrison (1990) and other reports in the literature.

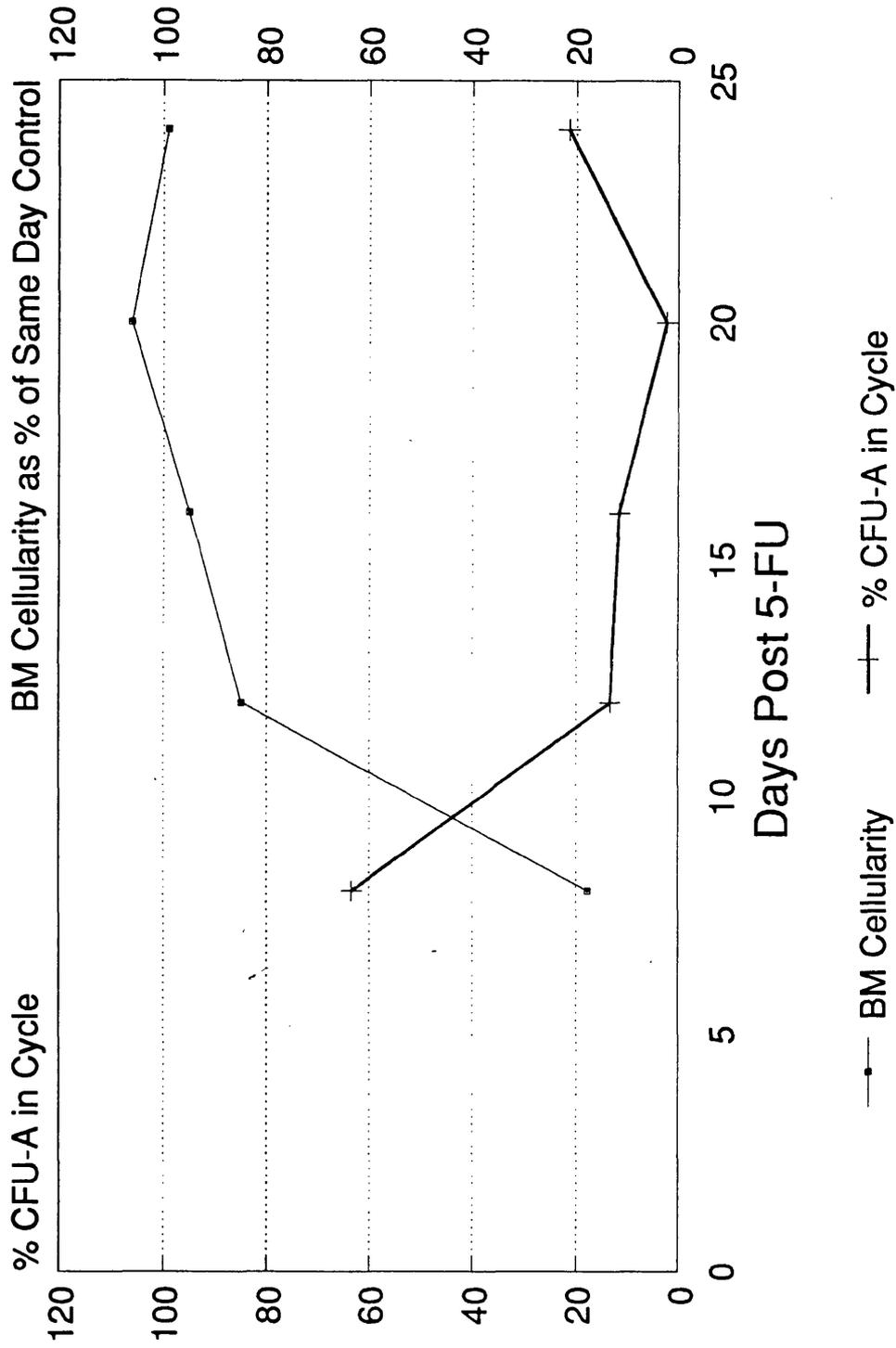
The cycling status of CFU-A in the marrow at times after 5-FU was assessed by cytosine arabinoside suicide assay. From figure 7.1 it can be seen that at day 8 post 5-FU approximately 60% of CFU-A are proliferating. By day 12, cycling has returned to control values. It should be noted that these are the results of a single experiment and that several repetitions would be required to constitute a thorough investigation of CFU-A proliferation in response to 5-FU. Nevertheless, these values do agree with results published for CFU-S (Shibagaki et al 1986) and recently for CFU-A (Dunlop et al 1992).

When the expression of SCI/MIP-1 α mRNA in post 5-FU bone marrow was evaluated an unexpected pattern of expression was obtained. It can be seen from figure 7.2 (panel B) that at days 8 and 11 post 5 FU the marrow expresses two readily detectable transcripts; this level is approximately equal to that of the untreated control. However, on days 18, 20 and 22 the intensity of these two bands increases. By day 28 the intensity of hybridisation has declined, but is still at an elevated level.

Figure 7.1 Bone marrow cellularity and CFU-A cycling following treatment of B6D2F1 mice with 5-fluorouracil (150mg/kg).

Bone marrow cells were counted electronically. Data is presented as the mean count from three femora from experimental animals expressed as a percentage of the mean count from three femora from control animals counted on the same day.

Percentage of CFU-A in cycle was determined by cytosine arabinoside "suicide" assay as described in chapter 2.



Normal Control % CFU-A = 18.6+/-10.5

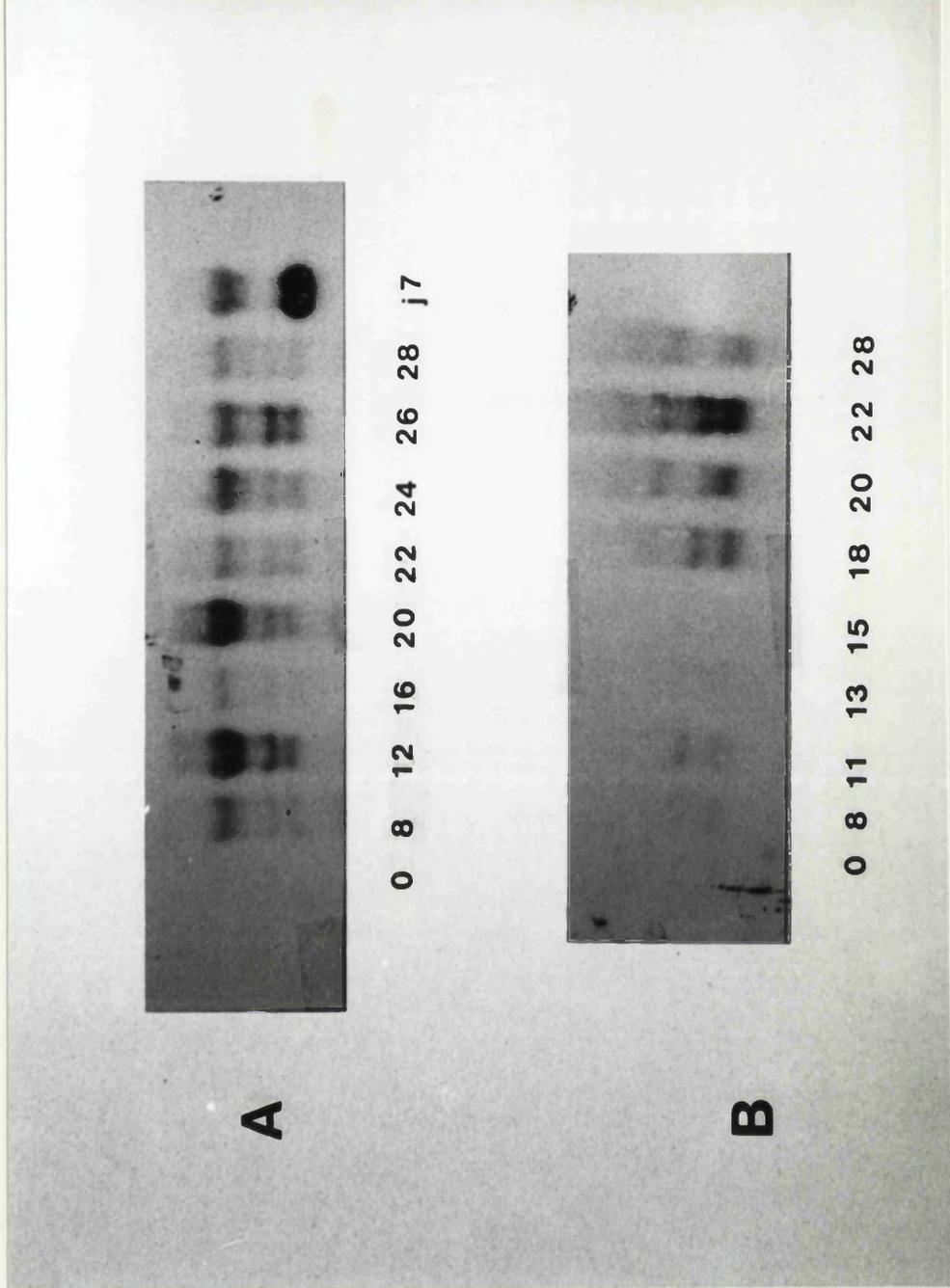


Figure 7.2 Northern analysis of the accumulation of SCI/MIP-1 α mRNA in total murine bone marrow following treatment of animals with 5-FU (150mg/kg).

Panel A: Total cellular RNA was isolated and blotted in equal amounts (5 μ g) onto nylon membrane 0, 8, 12, 16, 20, 22, 24, 26 and 28 days after 5-FU treatment (lanes 1-9). Lane 10, 10 μ g total RNA from untreated J774.2 cells. Equal loading was ensured by ethidium bromide staining of the gel.

Panel B: Total cellular RNA was isolated and blotted in equal amounts (5 μ g) onto nylon membrane 0, 8, 11, 13, 15, 18, 20, 22 and 28 days following 5-FU treatment. Equal loading was ensured by ethidium bromide staining of the gel.

A similar, though less marked pattern of accumulation is seen in panel A of figure 7.2 which represents a separate experiment.

In panel A an additional higher molecular weight band is also seen, although this appears to be artefactual and produced by non-specific hybridisation, suggested by the fact that this band is also seen in the positive control. The loading of RNA on the formaldehyde gel was approximately equal and could not account for the differences in intensity of hybridisation, so the increase in the amount of RNA hybridising to the SCI/MIP-1 α probe appears to be a genuine effect. A similar pattern of expression was found in one other experiment. It is unlikely that the additional band, seen at the same level as that of SCI/MIP-1 α mRNA arose as a result of non-specific hybridisation, which, as mentioned in chapter 3, is a common problem with riboprobes, for two reasons:

- 1) If the additional band was seen as a result of non-specific hybridisation, the band would be present in the lane containing the positive control, but this was not the case.
- 2) The RNA samples in both of the experiments illustrated in figure 7.2 were reblotted and hybridised with separate probes three times and one of these occasions were washed at 70 rather than 65 $^{\circ}$ C, to reduce the possibility of non-specific binding and still gave the same pattern of hybridisation.

Two aspects of this expression pattern, were of potential interest as regards possible roles of SCI/MIP-1 α in haemopoietic stem cell regulation.

Firstly, the timing of the increase in the level of expression of SCI/MIP-1 α at later times may indicate a role for SCI/MIP-1 α in inducing or maintaining quiescence of the CFU-S/CFU-A compartment of the marrow as the upregulation corresponds roughly with the descent from the peak of post 5-FU-induced cycling.

The double band was also of great interest as the soluble and matrix-associated forms of LIF identified by Rathjen et al (1990) are translated from two mRNA transcripts which are generated by alternative promoter usage. It seemed possible, therefore, that

the Northern blotting analysis of post 5-FU bone marrow could have been detecting mRNA encoding two distinct forms of the SCI/MIP-1 α protein.

Alternatively, the additional band may have represented a slowly-spliced precursor of the mature SCI/MIP-1 α mRNA, which raised the possibility that mRNA processing might be a point of control, or at least a rate-limiting step, in the production of SCI/MIP-1 α in the marrow.

It was, therefore, decided that the nature of the additional transcript observed in the initial experiments was worth pursuing because of the potentially important ramifications for our understanding of the biology of SCI/MIP-1 α .

An attempt was made to determine whether the upper band might contain intronic sequence which would indicate that this was a precursor of SCI/MIP-1 α mRNA.

The approach taken was to generate, as a polymerase chain reaction product, a probe specific for the first intron of the SCI/MIP-1 α gene. It was reasoned that if spliced intermediates were represented in the extra bands expressed post 5-FU, then their presence would be detected by hybridising with a DNA probe against an intron sequence as introns do not constitute a part of the mature messenger RNA. The first intron was chosen because comparison of the relative sizes of the two SCI/MIP-1 α -specific bands indicated that the difference in size was in approximately the same range as the length of this intron.

The radioactive intron 1 probe was hybridised against total cellular RNA from unfractionated post 5-FU bone marrow. Unfortunately, the signal to noise ratio in this experiment was too low for any discrete hybridisation bands to be detected and no useful information was generated by this experiment. In hindsight a much more efficient probe could have been generated by cloning the intron 1 sequence into a riboprobe vector. In addition, this experiment had a basic design fault in that a negative result was uninterpretable. As using the technique described above proved unfruitful, the problem was approached from a different angle.

The poly A-positive (poly (A⁺)) fraction of cellular RNA contains, in addition to the mature messenger RNA molecules, the intermediate nuclear processing forms of genes transcribed by RNA polymerase II. However, it does not contain any of the structural or ribosomal RNA molecules. It was reasoned that hybridisation of the SCI/MIP-1 α riboprobe to poly (A⁺) RNA from post 5-FU mice would only lead to the detection of RNA molecules associated with the production of mature RNA coding for the SCI/MIP-1 α protein.

Total cellular RNA was extracted from the femoral bone marrow of mice at various times after treatment with 5-FU and the poly (A⁺) fraction of these samples isolated. When the level of SCI/MIP-1 α mRNA was assessed by Northern blotting analysis, a similar but non-identical pattern of hybridisation was seen (figure 7.3).

A single transcript was expressed in normal, untreated marrow (as before) and the level of expression increased dramatically (at least 20-fold as assessed by laser densitometry) at day 12, had begun to decline by day 20 and had disappeared by day 24. The double band pattern of hybridisation seen at later times after 5-FU with total RNA was not evident in this experiment which suggests that these transcripts are not involved in the production of the mature SCI/MIP-1 α mRNA.

Nevertheless, the rise in the level of SCI/MIP-1 α mRNA around day 12 post 5-FU is borne out by using poly (A⁺) RNA which demonstrates that this is a genuine effect. It is important to note, however, that the timing of the rise of SCI/MIP-1 α mRNA is extremely variable between experiments which should be borne in mind when interpreting the data.

This data suggests several possibilities. Firstly, it could be argued that there is a connection between the late rise in SCI/MIP-1 α mRNA and the return of the CFU-A/CFU-S compartment to the quiescent state. In this case, upregulation of expression of SCI/MIP-1 α mRNA might be associated either with inducing or maintaining a quiescent state in the myeloid stem cells. The variation in the timing of the

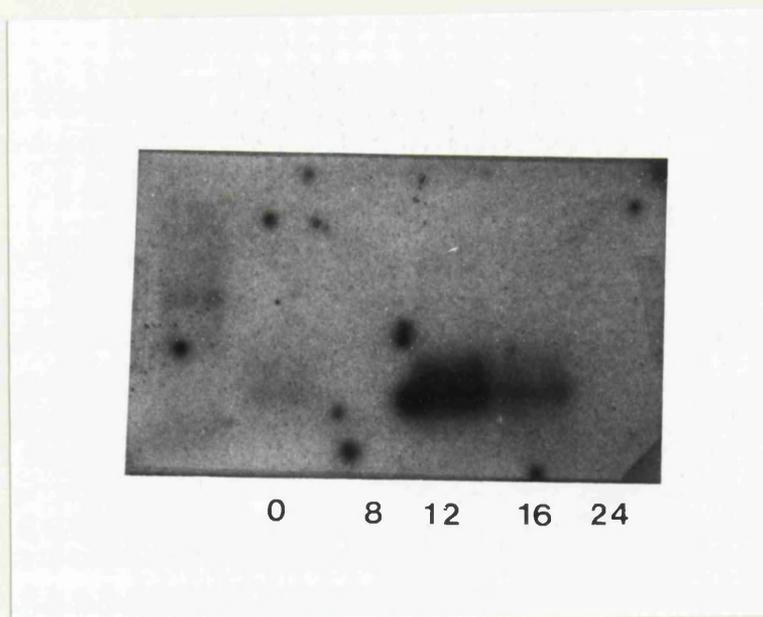


Figure 7.3 Northern analysis of the accumulation of SCI/MIP-1 α mRNA in the poly(A)+ fraction of RNA from total murine bone marrow after treatment of animals with 5-FU (150mg/kg).

Total cellular RNA was isolated and blotted in equal amounts (1 μ g) onto nylon membrane 0, 8, 12, 16 and 24 days after injection. Equal amounts of RNA were loaded according to spectrophotometric measurement.

upregulation of SCI/MIP-1 α mRNA makes it unlikely that this is a critical event for inducing quiescence.

It is, however, possible to envisage that a strong inhibitory signal is necessary to overcome the extremely powerful proliferative signals which must be part of the regeneration process; therefore the data is not inconsistent with a role for SCI/MIP-1 α in the maintenance of myeloid stem cell quiescence.

On the other hand, it may be more useful to think of the increase in expression as part of macrophage-borne wound and/or tissue remodelling response to the profound changes in marrow architecture which must accompany the action of 5-FU and the response which it elicits.

It should also be borne in mind that 5-FU has been demonstrated to have profound effects on RNA metabolism (Parker and Cheung 1991).

The nature of the additional RNA band hybridising to the SCI/MIP-1 α -specific probe is unclear. The data indicate that it is not a precursor or mature mRNA but is probably not seen as a result of non-specific hybridisation. One possibility is that it is an unusual metabolite of SCI/MIP-1 α mRNA which is induced by the drug treatment. Wilkenson and Pitot (1973) have demonstrated that incubation of cells with 5-FU inhibits the maturation of the 45S ribosomal RNA (rRNA) precursor to the 18 and 28S molecules. Will and Dolnick (1987) have shown that 5-FU causes an increase in the level of an unspliced precursor of the dihydrofolate reductase (DHFR) mRNA. In addition, Dolnick and Pink (1983) have shown that treatment of KB cells can increase the accumulation of the mature DHFR mRNA transcript by 3-fold. Therefore, it can be seen that both the metabolism and the accumulation of mRNA can be affected by 5-fluorouracil. However, it seems unlikely that interference with the metabolism of SCI/MIP-1 α mRNA could account for the pattern of expression seen in regenerating marrow two weeks after injection of 5-FU as clearance of 5-FU (via the liver) is an

extremely rapid process, and happens within a few days at the most (Parker and Cheung 1991).

7.3 Expression of SCI/MIP-1 α mRNA in Post-Phenylhydrazine Bone Marrow.

A salient feature of the 5-FU model is that marrow regeneration is dependent on increased CFU-S proliferation which means that these two processes can not be studied separately in this model. In an attempt to differentiate between the these two possibilities, the expression of SCI/MIP-1 α mRNA in bone marrow at various times after mice had been treated with phenylhydrazine (PHZ) was assessed. For this study, the advantage with using PHZ to alter CFU-S proliferation is that this can be achieved without a profound change in marrow cellularity which means that any changes in gene expression in the marrow as a result of tissue remodelling will be kept to a minimum. In addition to this, any changes in the level of SCI/MIP-1 α mRNA observed during the course of the experiment could be related to the observations of Lord and Wright (1982) on changes in the level of the Manchester Inhibitor, as discussed above.

Phenylhydrazine was administered according to the method of Rencricca et al (1970). Spleen weight and marrow cellularity were followed throughout the course of treatment to ensure that the drug was acting. In the expected manner, Marrow cellularity was not greatly affected by the treatment (figure 7.4) as previously observed by Rencricca et al (1970). The average wet splenic weight was increased by approximately 500% over control values on days seven and nine after treatment with drug but had returned to control values by day 10 (figure 7.5). It should be noted that wet spleen weight is not a direct measure of spleen cellularity as the spleen vasculature and its associated blood will be making a contribution to the overall weight. Nevertheless, the data in figure 7.5 is indicative that the phenylhydrazine treatment is inducing the "emergency" haemopoiesis which is a characteristic reaction

Figure 7.4 Bone marrow cellularity in murine femora following treatment with phenylhydrazine (60mg/kg).

Bone marrow cells were counted electronically and data presented as the mean determination from three femora.

Figure 7.5 Mouse splenic weight following treatment with phenylhydrazine (60mg/kg).

Splenic weight was measured using an electronic balance. Data is presented as the mean weight of 4 spleens plus or minus one standard deviation.

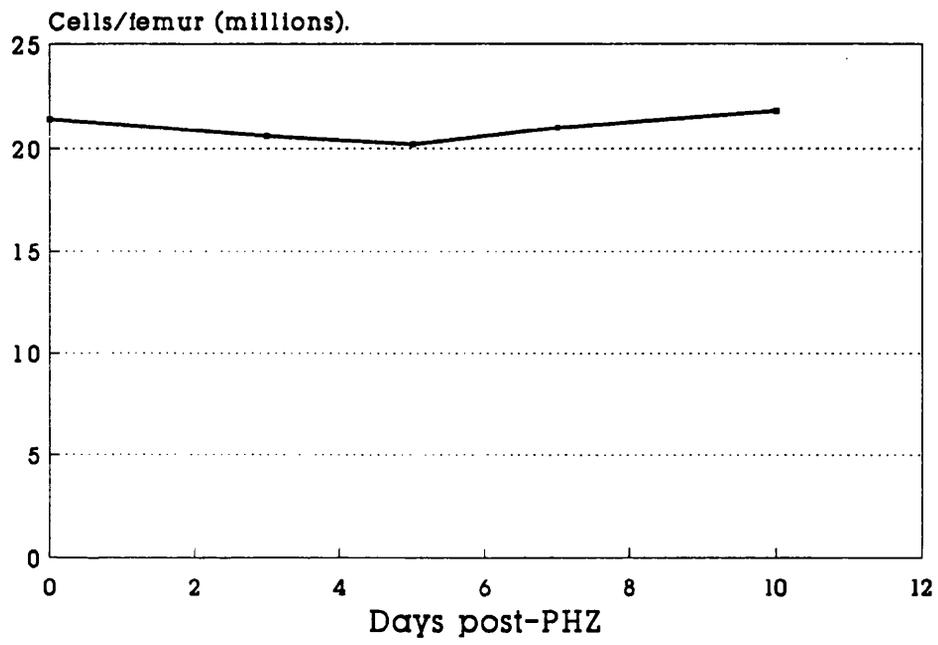


Figure 7.4

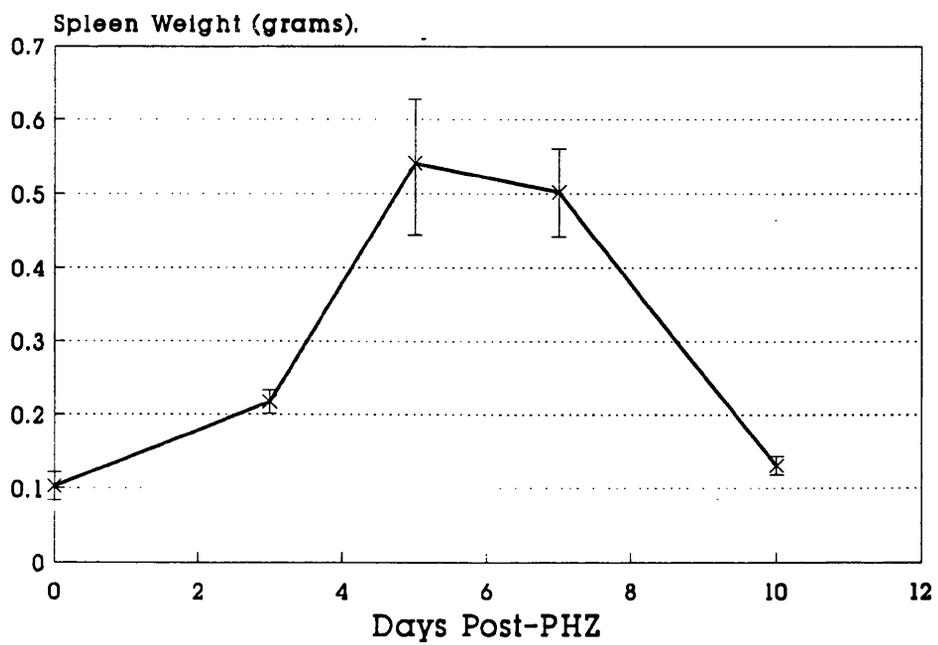


Figure 7.5

to the anaemia induced by the drug. A deepening in the red colour of the spleens at times following drug administration was also indicative of splenic erythropoiesis. Attempts were also made to measure the proportion of CFU-A in cell cycle at different times after phenylhydrazine, in the same manner as for post 5-FU marrow. Unfortunately, these experiments did not yield any interpretable results.

The expression of SCI/MIP-1 α mRNA in post-PHZ marrow was first assessed by Northern analysis of total RNA samples prepared from femoral marrow of mice at various times after treatment. It can be seen from figure 7.6 that no changes in the expression of SCI/MIP-1 α mRNA could be detected, indeed no message could be detected even in samples from untreated mice. A band corresponding to the correct size could, however, be detected in total RNA prepared from the macrophage cell line J774.1 and each lane was shown to contain approximately equal amounts of RNA by hybridisation to a probe for the 7S RNA which demonstrates that the hybridisation and transfer of the RNA was efficient. This experiment was repeated twice and the samples from each experiment reblotted and hybridised twice with identical results. Even on exposure of the filters for periods up to 4 weeks no bands hybridising to any lanes other than that containing the positive control sample could be detected. This was a surprising finding as SCI/MIP-1 α mRNA had been readily detectable in normal, murine bone marrow in previous experiments (chapter 1, figure 3.13). There had been no change in the experimental conditions or method which suggests that the difference lay in the experimental animals.

It has been previously documented (Metcalf 1984) that bone marrow colony stimulating activity in murine serum disappears on housing mice in strict pathogen-free conditions. A change in the living-conditions of the strain of mice used throughout this study could be one explanation for the change in ability to detect SCI/MIP-1 α mRNA given that it is expected that the gene product plays a role in the

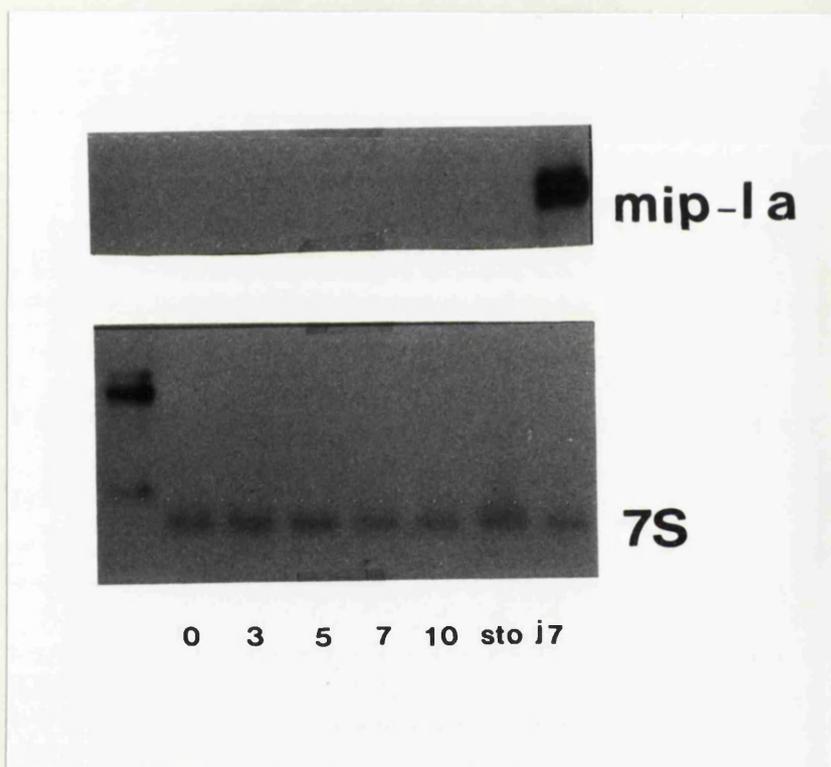


Figure 7.6 Northern analysis of time course of accumulation of SCI/MIP-1 α mRNA in total murine bone marrow after treatment of animals with phenylhydrazine (60mg/kg).

Total cellular RNA was isolated and blotted in equal amounts (10 μ g) onto nylon membrane. Lane 1, RNA ladder; lanes 2-6, total cellular RNA isolated from total murine bone marrow 0, 3, 5, 7 and 10 days after phenylhydrazine; lane 7, untreated STO cells; lane 8, untreated J774.2 cells. Equal loading was confirmed by hybridisation with a 7S probe.

immune response. However, there was no obvious reason to suspect that this might be the case for the mice used in this study.

It should also be noted that on administration of bacterial endotoxin to mice, Troutt and Lee (1989) observed induction of accumulation of mRNA for IL-1 α and IL-6 in lung and heart but failed to observe accumulation of any of a number of haemopoietic growth factor mRNAs in bone marrow, so bacterial infection may not necessarily be associated with changes in the expression of cytokine mRNAs in the marrow.

The inability to detect SCI/MIP-1 α mRNA by Northern blotting of total RNA did not necessarily mean that the gene was not being expressed at all as it was possible that the message was still present in bone marrow but at a level too low for detection using this technique; thus any changes in the level of SCI/MIP-1 α mRNA would remain undetected.

In order to overcome this problem, more sensitive methods for detecting mRNA were employed.

Firstly, the poly(A)+ fraction of total RNA from post-PHZ marrow was isolated and subjected to Northern blotting analysis; this allows a greater amount of RNA to be analysed at one time. As approximately 1-5% of total RNA is poly(A)+ (Sambrook et al 1989), this means that the representation of specific messenger RNAs in these samples should be greatly increased. Unfortunately no expression could be detected using this method (data not shown).

Next, the polymerase chain reaction (PCR) was employed to detect expression of SCI/MIP-1 α mRNA in post-PHZ marrow. Marrow samples were extracted by the author and PCR reactions and Southern blotting and hybridisations of the products was performed by Dr.E. Barone of the Beatson Institute.

Preliminary experiments indicated that the level of SCI/MIP-1 α mRNA in the marrow is reduced on day seven of the treatment regime (data not shown) when CFU-S should be proliferating rapidly, compared to controls and day five and day nine

marrow, in which CFU-S are only proliferating minimally. This is strong circumstantial evidence of an involvement of SCI/MIP-1 α in the control of bone marrow CFU-S proliferation and mirrors the changes in the levels of the Manchester Inhibitor observed by Lord and Wright (1982).

7.4 Summary of Chapter 7.

The level of mRNA for SCI/MIP-1 α in the femoral bone marrow of female B6D2F1 mice was investigated before and after treatment with two drugs which have profound effects on the haemopoietic system.

The level of SCI/MIP-1 α mRNA is greatly increased above control levels at variable times between twelve and approximately twenty-four days after treatment with a single injection of the marrow ablative agent 5-fluorouracil and later declines to normal levels. The marrow cellularity and proliferative status of marrow CFU-A were also followed over the same treatment period. Femoral marrow cellularity was decreased by 80-90% on day eight after treatment and had returned to almost control values after twelve days. Reflecting this regenerative process, approximately 60% of femoral marrow CFU-A were found to be in cycle on day 8 but had returned to control (quiescent) levels by day 12.

The fact that the upregulation of SCI/MIP-1 α mRNA levels in femoral marrow corresponds approximately with the return of the CFU-A compartment to its normal, minimally proliferative, status suggests the possibility of an association between the two events. However, this is only one explanation for the observed phenomenon; it is equally as likely that the upregulation in the levels of this transcript could be associated with the extensive tissue remodelling which must accompany haemopoietic regeneration.

In an attempt to differentiate between these two possibilities, the level of SCI/MIP-1 α mRNA was assessed in femoral marrow before and after treatment with the

erythrolytic agent phenylhydrazine. This agent also has a well-documented effect on the proliferation of marrow CFU-S without greatly affecting marrow cellularity; hence extensive marrow regeneration is not a feature of the recovery from treatment with this agent. Unfortunately, SCI/MIP-1 α mRNA could not be detected in any of these experiments by Northern blotting but polymerase chain reaction (PCR) proved to be a sensitive enough technique to detect the presence of SCI/MIP-1 α mRNA in experimental samples.

PCR analysis detected a lower level of mRNA for SCI/MIP-1 α in post-PHZ marrow than controls at a time when CFU-S should be proliferating rapidly but levels were restored at times when CFU-S cycling has ceased.

The data presented in this chapter is not inconsistent with changes in SCI/MIP-1 α gene expression playing a role in the control of CFU-A/CFU-S proliferation.

Chapter 8

Discussion.

8.1 Introduction.

This chapter will be divided into two main sections. Firstly, the expression of SCI/MIP-1 α and MIP-1 β mRNA and the possible underlying mechanisms, will be discussed and then the implications of this data for understanding the role of SCI/MIP-1 α and MIP-1 β in haemopoietic regulation and other processes will be considered.

8.2 Expression of SCI/MIP-1 α and MIP-1 β in Bone Marrow-Derived Macrophages.

8.2.1 The use of bone marrow-derived macrophages.

Like all in vitro experimental systems, using BMDM as a surrogate for in vivo bone marrow macrophages is a compromise. However, using BMDM has definite advantages over the more conventional use of macrophage-like cell lines. The first and most obvious advantage is that these are an easily obtainable essentially homogeneous, primary population and therefore should represent a better approximation to "normal" macrophages than cell lines. The difference in the length of the period of accumulation of SCI/MIP-1 α and MIP-1 β mRNA in BMDM and in the RAW264.7 cells described in chapter 4 (figures 4.1 and 4.2) of this thesis is one example of how the expression of these genes might be qualitatively different in primary and transformed cells. The apparent dysregulation of expression of the human MIP-1 α gene in fresh leukemic samples and leukemic cell lines (Yamamura et al 1989, Nakao et al 1990) is another. The BMDM population also has characteristic proliferative

properties (figures 3.7 to 3.10) and this allows the expression of genes by BMDM to be related to DNA synthesis.

In addition, previous investigators (Wright and Lorimore 1987, Pojda et al 1988) have used macrophages cultured from marrow as sources of the Manchester Inhibitor, allowing a degree of continuity between this study and previous work on the production of negative regulators of CFU-S proliferation by macrophages.

An obvious disadvantage with using macrophages derived *in vitro* from monocytic progenitors, is that they have not been exposed to exactly the same environment as the macrophages of the marrow stroma. The BMDM population has growth properties which are very similar to isolated human monocytes (Tushinski and Stanley 1985, Hamilton et al 1988, Cheung and Hamilton 1992), which suggests that this population may be more representative of blood-borne monocytes than macrophages of the bone marrow stroma. The minimal mitotic activity of the resident macrophages of the marrow (Crocker et al 1988) might be cited to support this point.

However, this argument may not carry as much weight as first anticipated. The Manchester Inhibitor-producing population sorted directly from normal marrow by Simmons and Lord (1985) on the basis of the expression of the F4/80 antigen, which is present on resident macrophages in the marrow stroma, proliferate readily. The BMDM population used in this study has very similar growth properties to this sorted population (as was demonstrated in chapter 3, figure 3.7) which indicates the suitability of using BMDM for studying the expression of SCI/MIP-1 α and MIP-1 β in the context of haemopoietic regulation.

Adams and Hamilton (1984) have argued that the resident phenotype is conferred on macrophages only in the context of the tissue and may be

reversible. It follows, then, that resident macrophages may actually be monocytes upon which a particular phenotype has been conferred by geographical location; hence, this would mean that even if a pure population of resident marrow macrophages could be sorted directly from bone marrow, they would be likely to behave like isolated monocytes in culture, so there may, in fact, be no added advantage to be had by studying gene expression in a sorted population.

8.2.2 Characteristics of accumulation of SCI/MIP-1 α and MIP-1 β mRNA in BMDM.

The characteristics of expression of mRNA for SCI/MIP-1 α and MIP-1 β in BMDM are extremely similar. The experiments presented in chapter 4 demonstrated that both the SCI/MIP-1 α and MIP-1 β genes behave like typical cytokine genes in that they display the rapid and transient, protein synthesis-independent induction of "immediate early"-type genes (figures 4.1 and 4.5). Early stimulation of transcription was also demonstrated to be necessary for the induction of accumulation of both SCI/MIP-1 α and MIP-1 β mRNA by LPS (figures 4.3 and 4.4) indicating that the rapid induction of transcription is likely to be the primary control point for the expression of these genes. In addition, the accumulation of both SCI/MIP-1 α and MIP-1 β mRNA is downregulated by the glucocorticoid hormone hydrocortisone (figures 4.4.7 and 4.8), which is again typical of genes whose products have a role to play in the inflammatory response and demonstrates that the expression of both of these genes can be efficiently downregulated as well as induced.

These characteristics are perhaps not surprising considering that SCI/MIP-1 α and MIP-1 β are members of a cytokine family, although it was important that this be established in primary cells as it should be noted that not all of the

MIP-1 family cytokines display these characteristics of expression (Schall et al 1988, 1992, Orlofsky et al 1991).

The rapid and transient nature of the induction, which was seen with all cases of the induction of both SCI/MIP-1 α and MIP-1 β mRNA in BMDM in this study, allows a close coupling between stimulus and response. Given the ability of these molecules to alter cell behaviour at very low concentrations and in view of the possible role of over-production of SCI/MIP-1 α and MIP-1 β in diseases involving chronic inflammation (Wilcox et al 1990, Schall 1991, Graves et al 1989) and myelosuppression (I.Pragnell personal communication), this "quantal" pattern of expression is likely to be an extremely important aspect of the biology of these molecules.

A basal level of mRNA for both molecules is detected in unstimulated BMDM which appears to be dependent on ongoing gene transcription, as evidenced by the ability of actinomycin D to abolish the basal level of both mRNAs (figure 4.4). This basal level was observed, in several different experiments, to be refractile to agents which are potent downregulators of SCI/MIP-1 α and MIP-1 β mRNA induction (figures 6.3 and 6.5), which makes it possible that the mechanisms underlying the regulation of expression of the basal and induced levels of SCI/MIP-1 α and MIP-1 β mRNA are different. The two mRNAs appear to be co-induced in all the situations examined in this study.

Widmer et al (1991) have identified elements in the 5' regulatory region of the SCI/MIP-1 α gene which are conserved in a number of inducible lymphokine genes. For example, the promoter region contains two decanucleotide elements, CLE1 and CLE2, which are conserved in the promoters of several lymphokine genes, including IL-2, IL-3, GM-CSF and G-CSF (Arai et al 1990). Recent work by M.Grove (personal communication) has indicated that

the 5' regulatory sequences of the SCI/MIP-1 α gene bind proteins related to the inducible transcriptional activators NF κ B and AP-1 upon LPS stimulation of the macrophage cell line RAW264.7, again emphasising the designation of these genes as belonging to the set induced as part of the early response to stimulation and indicating the rapidity of transcriptional activation of both of these genes.

Although stimulation of transcription is necessary for the induction of accumulation of both of these mRNAs it is possible that stabilisation of the mRNA product plays a significant role in determining the exact level of mRNA induced as seen for the induction of mRNA for erythropoietin (Goldberg et al 1991) and TNF α (Beutler and Cerami 1988). Further studies will be needed to determine whether stabilisation of SCI/MIP-1 α and MIP-1 β mRNA plays such a role.

The genomic sequence of the MIP-1 β gene has recently been obtained (M.Plumb personal communication). Interestingly, the promoter sequence contains no significant homology with that of SCI/MIP-1 α , which makes it likely that the expression of these genes is controlled by very different mechanisms. This is perhaps surprising, given that the two genes may have evolved through duplication and subsequent divergence of a common ancestral sequence (Wolpe and Cerami 1989, Schall 1991) and the observed coinduction of SCI/MIP-1 α and MIP-1 β mRNA seen in this study. This also raises the possibility of differential regulation of the two genes in situations other than those employed in this study.

Interestingly, Schuler et al (personal communication) have observed accumulation of mRNA for SCI/MIP-1 α , but not MIP-1 β , on *in vitro* culture of purified epidermal Langerhan's cells.

Differential control of expression of the two genes is an important question in understanding the biology of both SCI/MIP-1 α and MIP-1 β , given that the biological properties of the two molecules are overlapping but not identical and the possible interactive effects of the two molecules. Further studies on the incidence, and the underlying mechanisms, of SCI/MIP-1 α and MIP-1 β expression in a wide variety of circumstances will allow this question to be answered.

The relationship between the accumulation of SCI/MIP-1 α and MIP-1 β mRNA and the proliferation of BMDM has been discussed in detail in the summary of chapter 6. It was demonstrated (and summarised in figure 6.6) that while accumulation of both MIP-1 mRNAs can be concomitant with the stimulation of proliferation, the two processes can be regulated independently. This indicates that while the signals and mechanisms directing the two processes may be overlapping, they are not identical. These results are consistent with the work of Vairo et al (1990) who demonstrated that the proliferation of BMDM could be inhibited independently of a variety of early responses including immediate-early gene expression.

Another important issue concerning the expression of SCI/MIP-1 α and MIP-1 β in BMDM is whether changes in mRNA levels are reflected in the production of protein by these cells. In chapter 1 of this study (figure 3.16) it was demonstrated that upon stimulation with LPS, BMDM can secrete sufficient levels of SCI/MIP-1 α protein to be detected by Western blotting. The inability to detect SCI/MIP-1 α protein in media conditioned by unstimulated BMDM by Western blot may suggest that no protein is produced in the absence of a specific stimulus. However, it is possible that protein is produced by these cells but at a level below the sensitivity of the technique used. As demonstrated recently by Kittler et al (1992) such low levels of

cytokines may still be biologically relevant. Studies with more sensitive protein-detection techniques, such as an ELISA assay specific for SCI/MIP-1 α may allow this question to be answered. It should be noted, however, as discussed in chapter 1, that uncoupling between the synthesis of mRNA and its translation into protein, in some circumstances, has been demonstrated for several cytokines including, recently, human MIP-1 α and MIP-1 β (Schall et al 1992).

Martin and Dorf (1991) have reported that the accumulation of mRNA for IL-6 and TNF α induced by IFN γ in the J774A.1 cell line is not accompanied by an increase in levels of protein secreted. It is conceivable that the IFN γ -mediated increase in SCI/MIP-1 α and MIP-1 β mRNA levels in BMDM has similar characteristics. It may be that a second signal (such as LPS) or more intense stimulation is required to induce translation and/or secretion of the cytokine. The question of whether macrophages have the ability to store SCI/MIP-1 α and MIP-1 β mRNA in an untranslated form or protein in lieu of subsequent secretion is obviously important for a full appreciation of the expression of these molecules and is worthy of investigation. This possibility also means that caution should be employed when inferring biological activity from mRNA expression data. Further studies with antisera which specifically recognise SCI/MIP-1 α and MIP-1 β , which are now available, will enable this issue to be addressed.

8.3 Expression of SCI/MIP-1 α mRNA in Murine Bone Marrow.

A low level of mRNA for SCI/MIP-1 α was detectable in normal, untreated, unfractionated marrow by Northern blotting (chapter 3, figure 3.13).

The inability of other investigators to detect mRNA for other cytokines and CSFs in the marrow from normal and LPS-treated mice (Troutt and Lee

1989), and of Fahey et al (1990) to detect expression of SCI/MIP-1 α and MIP-1 β mRNA in murine peripheral blood leukocytes, even when using techniques more sensitive than Northern blotting is strongly suggestive of a role for SCI/MIP-1 α in the marrow.

When the ability to detect mRNA for SCI/MIP-1 α in cell lines representing several cell lineages, including fibroblasts, erythroid cells and lymphocytes, was tested, it was found that SCI/MIP-1 α mRNA could only be detected in monocytic cells and in a transformed T cell line (chapter 3, figure 3.14).

Given that a basal level of SCI/MIP-1 α mRNA is expressed in BMDM and since cells of the T lymphocyte and mast lineage are extremely rare in the marrow (Kincaide et al 1990, Gordon et al 1990) and have to be activated by specific immune stimuli to express SCI/MIP-1 α and/or MIP-1 β mRNA (Burd et al 1989, Brown et al 1989), it is likely that cells of the monocyte/macrophage lineage are the main producers of SCI/MIP-1 α mRNA in the marrow. The fact that the Manchester Inhibitor can be prepared merely by washing unfractionated murine bone marrow suggests that this mRNA level is reflected in the production of protein.

The level of SCI/MIP-1 α mRNA detectable in unfractionated murine bone marrow increases dramatically in the recovery period following marrow ablation by treatment of mice with the cytotoxic agent 5-fluorouracil (5-FU) (figures 7.2 and 7.3). It is unlikely that this increase is a direct consequence of the effects of 5-FU on RNA metabolism as discussed in chapter 7. The possible biological significance of this increase will be dealt with in the next section.

One striking feature of the expression of SCI/MIP-1 α in total cellular RNA extracted from post-5-FU bone marrow was the appearance of two bands specifically hybridising to the SCI/MIP-1 α probe (figure 7.2). The additional

band suggested that an alternative form of the molecule might be expressed in murine bone marrow. However, analysis of poly(A)+ RNA from post-5FU marrow (figure 7.3) suggested that the additional band is not polyadenylated and hence it is unlikely, but not impossible, that it is representative of a messenger RNA. However, the stringency of hybridisation employed in these experiments still makes it unlikely that the additional band is an artefact. Further experiments will aid the understanding of this potentially interesting aspect of the molecular biology of marrow regeneration.

8.4 The Possible Roles of the Control of Gene Expression in the Biology of SCI/MIP-1 α and MIP-1 β .

8.4.1 Inflammatory Situations.

Upon injury or infection, organisms initiate synthesis of a complex set of mediators which lead to the induction of inflammation at both a local tissue and a systemic level (Stadnyck and Gauldie 1991).

Nearly all inflammatory responses to host invasion involve the activation of tissue macrophages and infiltrating blood monocytes (Titus et al 1991). The ability of macrophages and monocytes to recognise and respond to the presence of bacteria, viruses and parasites by the rapid synthesis of cytokines such as TNF α ,

1 α and IL-6, which play major roles in inducing and sustaining an acute phase

inflammatory response, makes it likely that macrophage activation plays a pivotal role in this process.

TNF α , IL-1 α and IL-6 have overlapping but non-identical activities in mediating the inflammatory response which include the induction of fever, the elicitation of specific acute phase response proteins from hepatocytes and the differentiation and/or activation and augmentation of the effector functions of T cells, B cells and/or macrophages. These cytokines can also induce the local

synthesis of other cytokines from tissue fibroblasts and endothelial cells. In addition, TNF α and IL-1 α can induce the synthesis of each other and IL-6. The concentration of cortisol (hydrocortisone) in the circulation is greatly increased as part of this process and may be important as a general downregulatory signal (Stadnyck and Gauldie 1991).

An early response-type expression pattern would be expected of the genes for proteins with such properties and indeed this pattern of expression is exhibited in macrophages by these three genes (Dinarello 1991, Beutler and Cerami 1988).

Evidence is accumulating which associates the MIP-1 proteins with inflammatory situations (Fahey et al 1990) and indicating the ability of these proteins to induce leukocyte mobilisation (Wolpe and Cerami 1989, Schall 1991, Saukonnen et al 1991, Fahey et al 1992) and fever (Davatelis et al 1989, Minano et al 1990) as discussed in chapter 1.

Since it has been demonstrated in this study that the accumulation of SCI/MIP-1 α and MIP-1 β mRNA (figures 4.1 to 4.6), and secretion of SCI/MIP-1 α protein (figure 3.16) are induced as part of the early macrophage response to challenge by the bacterial cell wall product lipopolysaccharide (LPS), it is possible that SCI/MIP-1 α (and MIP-1 β) might also play an important role in the initiation of the acute phase response. The ability of SCI/MIP-1 α to induce the synthesis of TNF α , IL-1 α and IL-6 (Fahey et al 1992) and the inability of TNF α or IL-1 α to induce accumulation of SCI/MIP-1 α mRNA (Martin and Dorf 1991 and this study, chapter 5) suggests that secretion of SCI/MIP-1 α could be a part of the primary wave of cytokine production associated with acute inflammatory defence. It should be borne in mind, however, that accumulation of SCI/MIP-1 α and MIP-1 β mRNA in BMDM can be induced by IFN γ (figure 5.9), so it is possible that

SCI/MIP-1 α production from macrophages may be induced from macrophages at several points during the acute response.

Fahey et al (1990) failed to detect mRNA for SCI/MIP-1 α and MIP-1 β in murine peripheral blood leukocytes implying that these proteins do not play a role in the circulation in the absence of a challenge to the organism. The ability of IFN γ , which primes macrophages for antigen presentation (Roitt et al 1985) to induce accumulation of both SCI/MIP-1 α and MIP-1 β mRNA observed in this study in BMDM (chapter 5) and in the J774.1 cell line by Martin and Dorf (1991) may suggest a role for the expression of these genes in the specific, as well as the non-specific, immune response.

A major difference in the biology of SCI/MIP-1 α and that of TNF α , IL-1 α and IL-6 is that expression of SCI/MIP-1 α appears to be restricted to haemopoietic cells whereas the other genes are expressed in a wide variety of cell types (Dinarello 1991, Beutler and Cerami 1988). This may suggest a divergence in the roles of SCI/MIP-1 α and TNF α , IL-1 α and IL-6 in the secondary part of the acute response.

The heparin-binding properties of SCI/MIP-1 α and MIP-1 β makes it likely that these molecules will be sequestered in the immediate vicinity of the producer cells. The confinement of the expression of these genes to macrophages, and other haemopoietic cells, may be a way of ensuring that SCI/MIP-1 α and MIP-1 β proteins are only deposited at sites of active inflammation.

The role played by MIP-1 β in inflammation is not clear at the moment.

Recombinant MIP-1 β has been shown to be ineffective in a variety of systems in which SCI/MIP-1 α has a potent activity (Graham et al 1990, Saukonnen et al 1991, Broxmeyer et al 1990). The only activity ascribed to MIP-1 β ,

independent of its association with SCI/MIP-1 α , is that of a specific inhibitor of the action of SCI/MIP-1 α (Broxmeyer et al 1991, Fahey et al 1992).

It is now becoming clear that the activities of many cytokines can be controlled post-secretion by mechanisms including specific antagonists of cytokine receptors (for review see Dinarello 1991). Specific inhibition of SCI/MIP-1 α activity by MIP-1 β may be one way of controlling its activity.

The coordinate expression of mRNA for both genes in BMDM seen throughout this study indicates that SCI/MIP-1 α and MIP-1 β would be secreted by BMDM simultaneously. For MIP-1 β to be effective as an antagonist it has to be present in excess of SCI/MIP-1 α ; regulation of the relative levels of production of these two molecules, perhaps at the level of gene expression, could be one way of controlling this interaction.

The ability of hydrocortisone to downregulate the expression of both SCI/MIP-1 α and MIP-1 β mRNA (as demonstrated in chapter 4, figures 4.7 and 4.8) in addition to that of TNF α , IL-1 α and IL-6 (Dinarello 1991, Beutler and Cerami 1988) may represent a "main switch" mechanism for down-regulating the acute phase response. It seems that expression of SCI/MIP-1 α and MIP-1 β can be downregulated by immunosuppressants acting both locally, in the case of prostaglandin E1 (Martin and Dorf 1991) and systemically. When this is considered along with the transient nature of accumulation, it seems clear that the ability for an organism to downregulate the expression of these gene is likely to be just as important as the ability to induce expression.

8.4.2 Haemopoietic Regulation.

Steady-State Haemopoiesis.

Assuming, and there is no definite reason not to, that the expression of SCI/MIP-1 α in BMDM in vitro and in macrophages in the marrow display the same characteristics it is pertinent to ask how this might relate to the possible role of SCI/MIP-1 α in steady-state haemopoiesis.

It is possible that the low level of SCI/MIP-1 α mRNA seen in unstimulated BMDM is translated into protein and that the production of SCI/MIP-1 α mRNA by unstimulated macrophages in the marrow is sufficient for the level of SCI/MIP-1 α mRNA detected in normal, unstimulated bone marrow.

Therefore, it might be possible to propose a role for a constant level of SCI/MIP-1 α in maintaining myeloid stem cells in the marrow in a state of minimal proliferation.

This might be achieved by a continuous production and destruction of the molecule in the marrow which would allow for rapid modulation of levels to control the rate of myeloid stem cell proliferation. The changes in the level of SCI/MIP-1 α mRNA in post-phenylhydrazine marrow detectable by PCR, as discussed in chapter 7, might indicate such an association between SCI/MIP-1 α mRNA expression and CFU-A proliferation. However, this data is very preliminary and should be considered in this light.

On the other hand, the proliferative status might be regulated by the relative level of positive to negative regulators and SCI/MIP-1 α may only be produced at a constant level. Such an arrangement removes the need for modulation of the levels of SCI/MIP-1 α production in normal marrow as stem cell proliferation could be regulated by changes in the levels of positive regulators.

It is pertinent to ask what factors might regulate the production of the SCI/MIP-1 α and MIP-1 β in the marrow stroma.

It was demonstrated in chapter 5 that the expression of mRNA for both SCI/MIP-1 α and MIP-1 β mRNA can be upregulated by the cytokine IFN γ . A role for cytokine stimulation of SCI/MIP-1 α and MIP-1 β expression in the inflammatory response has already been argued; it is also possible that stromal cell-derived cytokines may trigger the expression of SCI/MIP-1 α mRNA. This might be a feasible basis for the negative regulation of primitive cell proliferation by SCI/MIP-1 α in that both a negative stem cell regulator and a stimulatory growth factor would be present at the same location in the marrow at the same time; as the marrow is an extremely active tissue in terms of cellular kinetics this is highly likely to be the case. The specificity of the negative regulatory activity of SCI/MIP-1 α for primitive cells, would ensure that only the division of primitive cells would be inhibited while the proliferation of the more mature progenitors would be augmented. This could represent the "normal" situation in unstressed marrow.

The expression of both SCI/MIP-1 α and MIP-1 β mRNA has been demonstrated, in this study (chapter 5, figure 5.1) to be induced by (fetal calf) serum and on refeeding with SF12 medium (chapter 6, figures 6.1 to 6.4). This may be seen as a tissue culture artefact and merely a function of cellular refeeding. However, Caldwell et al (1991) have calculated that the rate of delivery of plasma proteins and nutrients to bone marrow is far higher in vivo than in, for example, long-term bone marrow culture given a medium change once weekly. It may be, therefore, that nutrient delivery is a relevant experimental parameter when considering conditions affecting the production of a molecule in the marrow. It might be argued, then, that the production of

SCI/MIP-1 α in the marrow could be maintained simply by the trafficking of blood components.

In the model of the control of proliferation of CFU-S cells by the Manchester Inhibitor and Stimulator, discussed in chapter 1, an essential component is the ability of Stimulator to block production of the Inhibitor by stromal macrophages. This could be tested at the level of gene expression by assessing the ability of Stimulator to downregulate the expression of SCI/MIP-1 α mRNA by BMDM. This work would also be enhanced by the molecular characterisation of the Stimulator. However, whatever the outcome of such studies, it is clear from the work presented in this thesis that the model proposed by Wright and Lord (1982) and Lord (1988) is incomplete as the expression of the SCI/MIP-1 α gene can be regulated by a variety of agents, rather than being determined solely by the action of one opposing factor.

In the marrow stromal layer, heparin-binding SCI/MIP-1 α might be associated with the extracellular matrix which could allow the localisation of expression of the molecule to discrete areas of the marrow. If this were the case, however, it is unlikely that mRNA for SCI/MIP-1 α mRNA could be detected in normal marrow; levels of molecule necessary to fulfil such a function are more likely to be expressed at the "subliminal" level demonstrated by Kittler et al (1992) for IL-3 and GM-CSF in long-term bone marrow culture stroma.

Lord and Wright (1984) reported some evidence to support the idea of spatial distribution of the Manchester Inhibitor and Stimulator across the murine femur, so it remains possible that the level of SCI/MIP-1 α mRNA detected in normal bone marrow is due to very high, continuous expression by geographically distinct subset of macrophages. In situ hybridisation studies with SCI/MIP-1 α mRNA may be informative in this respect, although it is

possible that SCI/MIP-1 α is produced by most macrophages in the marrow and the specific deposition of the protein is responsible for its localisation. The experiments described in chapter 5 also indicated that it is possible that the mononuclear phagocyte lineage-specific growth factor CSF-1 also upregulates SCI/MIP-1 α mRNA expression and that, although BMDM proliferation is not necessary for increased accumulation of SCI/MIP-1 α and MIP-1 β mRNA, the two processes can be induced by the same agent, L929-conditioned medium. It is, therefore, possible that proliferating, maturing monocytes might secrete SCI/MIP-1 α during the final stages of their differentiation before leaving the marrow for the blood. SCI/MIP-1 α would then act to inhibit the proliferation of the myeloid stem cells in the marrow in the manner of a classical feedback loop, as discussed in chapter 1.

The major problem with such a model is that the myeloid stem cell, by definition, gives rise to at least four blood cell lineages. It is unlikely, therefore, that the most important control point for the production of these cells could be dictated solely by the status of the monocytic compartment. In order for such a mechanism to be feasible, complex interactions between several lineage-specific feedback factors would have to be invoked. This idea might have more weight if expression of the SCI/MIP-1 α and MIP-1 β genes was dependent on concurrent macrophage proliferation; however, it has been clearly demonstrated in this study that the accumulation of SCI/MIP-1 α and MIP-1 β mRNA can take place in its absence which means that fixed resident bone marrow macrophages should not be restricted in their ability to express these genes.

Wdzieczak-Bakala et al (1990) have reported constitutive production of the tetrapeptide AcSDKP in normal murine bone marrow. The ability of a specific anti-serum against this molecule to greatly increase CFU-S cycling *in vivo*

(Lauret et al 1988, Frindel and Monpezat 1989) suggests that this molecule may be necessary, though not necessarily sufficient, for the maintenance of CFU-S quiescence.

A similar approach might be used to ascertain whether SCI/MIP-1 α displays a similar activity. It should be borne in mind, however, that the interpretation of such experiments may be difficult when inhibiting the activity of a molecule with such pleiotropic effects.

The potential role of other inhibitors of haemopoietic stem cell proliferation should also be borne in mind when considering the biology of SCI/MIP-1 α . The redundancy of the action of haemopoietic growth factors in many aspects of their biology is now well-established. This also seems to be the case for the positive control of haemopoietic stem cell proliferation as discussed in chapter 1. It seems unlikely, given the primacy of the stem cell proliferation in the regulation of haemopoiesis, that negative regulation of these cells is controlled by only one switch, although the results of Lauret et al 1988 and Frindel and Monpezat 1989 would seem to indicate this. It is possible also that the roles played by the different negative regulators are overlapping but not identical, which would introduce a high degree of flexibility into the system. For example, studies on the mechanism of action of the tetrapeptide AcSDKP indicate that while it is effective at opposing proliferation of CFU-S, it is incapable of recruiting proliferating CFU-S into quiescence (Monpezat and Frindel 1989). On the other hand, SCI/MIP-1 α has been shown to render proliferating CFU-S quiescent (Graham et al 1990, Dunlop et al 1992). It is possible, therefore, that AcSDKP may be primarily responsible for the maintenance of CFU-S quiescence while the main role of SCI/MIP-1 α might be to recruit inappropriately cycling CFU-S back to minimal proliferation.

The exact cellular populations within the stem cell compartment affected by the different negative regulators also appear to be overlapping but not identical. For example, TGF β appears capable of inhibiting the proliferation of a wide range of primitive haemopoietic progenitors (Keller et al 1992), while the action of SCI/MIP-1 α and AcSDKP appears to be centred largely around the CFU-S compartment (Monpezat and Frindel 1989, Graham et al 1990). It may be possible, therefore, to define the subset of the stem cell compartment which is capable of responding to a positive growth regulator within a very narrow range by differential regulation of the expression of these molecules within the marrow. In terms of gene expression studies it may be of interest to determine whether, and under what circumstances the expression of the genes for SCI/MIP-1 α , MIP-1 β and TGF β might be regulated differentially in the bone marrow. Studies of the regulation of production of AcSDKP and the hemoregulatory peptide await identification of the producing cells.

Emergency Haemopoiesis and Marrow Regeneration.

One of the features of the acute phase response is the need for the rapid delivery of a large number of functional, mature haemopoietic cells. This is achieved in part by migration, however, it is possible that marrow-independent haemopoiesis also plays a role. Activated macrophages and infected tissue cells such as fibroblasts and endothelial cells secrete a number of haemopoietically active cytokines including IL-3 and GM-CSF (Morstyn and Burgess 1988). The elevation of the levels of these cytokines in the blood might be capable of mobilising haemopoietic progenitors from storage sites, including possibly the marrow, and induce extensive proliferation and differentiation so as to provide a sufficient number of activated cells to

counter the emergency state. The increased number of activated mature haemopoietic cells are, in turn, a source of CSFs. This would lead to a considerable concentration of haemopoietic growth factors in the bloodstream. It is a possibility that the primary function of negative regulatory cytokines produced by activated macrophages, such as SCI/MIP-1 α may be to feedback and prevent the extinction of stem cells through proliferation and differentiation induced by the elevated levels of growth factors with the ability to act on primitive cells. Such a role for negative regulators has been postulated by Zipori (1991). However, the blood-bone marrow barrier formed by the basal lamina of the marrow venous sinus may be sufficient to prevent the exposure of stem cells to abnormal concentrations of growth factors in most cases; it is likely, therefore, that the protection of stem cells by SCI/MIP-1 α secreted by blood-borne monocytes is only relevant during abnormally advanced infections such as, for example, severe bacteraemia. However, the localisation of SCI/MIP-1 α by binding to the extracellular matrix may in fact limit the ability of SCI/MIP-1 α produced in the blood to affect the marrow. It is possible, however, that the ability of SCI/MIP-1 α to augment the proliferation of haemopoietic progenitors (Broxmeyer et al 1990) and mature macrophages (Fahey et al 1992) could make a contribution to the enhancement of the number of immune effector cells at inflammatory sites infiltrated by macrophages.

G.Graham and K.Parkinson (personal communication) have recently demonstrated the ability of SCI/MIP-1 α to inhibit the proliferation of primitive epidermal cells *in vitro*. In addition to the potential role of SCI/MIP-1 α (produced by epidermal Langerhan's cells) in regulating the production of keratinocytes, it is possible that SCI/MIP-1 α , produced by activated macrophages may protect primitive epidermal stem cells from extinction by

differentiation induced by positive regulatory factors in situations such as skin wounding.

As discussed in chapter 1 it is likely that there are specific regulatory signals which control the rapid, marked increase in proliferation and the subsequent return to quiescence of stem cells in regenerating marrow. The dramatic increase in SCI/MIP-1 α mRNA seen in murine bone marrow in the later stages of the recovery period following treatment with 5-FU suggests a role for SCI/MIP-1 α in this process.

The rise in SCI/MIP-1 α mRNA levels corresponds roughly, although not exactly, with the return of marrow cellularity to normal levels and of CFU-A to the quiescent state. The presumed increased level of SCI/MIP-1 α protein accompanying the mRNA increase could be responsible for either causing the reduction in myeloid stem cell proliferation or for maintaining the cells in that state or both.

It could be that the main role of SCI/MIP-1 α in haemopoietic regulation concerns the maintenance of myeloid stem cells in a quiescent state in the face of powerful proliferative stimuli. This would be directly analogous to its activity in vitro in inhibiting the growth of colonies derived from CFU-A cells. The demonstration by Dunlop et al (1992) and Lord et al (1992) of the efficacy of recombinant human MIP-1 α in inducing quiescence in rapidly cycling CFU-S and CFU-A in vivo lends weight to this hypothesis. This possibility could also be tested with the use of antisera specific for SCI/MIP-1 α ; failure of CFU-S/ CFU-A to return to quiescence in the presence of a specific blocking agent would indicate the necessity of SCI/MIP-1 α for the return to quiescence. If, as discussed above, the role of SCI/MIP-1 α is to maintain minimal proliferation in the myeloid stem cell compartment in unperturbed marrow, the burst of expression seen in the regeneration period

may be part of a process in which components of the microenvironment, disrupted during marrow ablation, are replaced. For instance, the deposition of secreted SCI/MIP-1 α protein on extracellular matrix components. Therefore, the observed increase in SCI/MIP-1 α mRNA seen in regeneration may relate to tissue "refurbishment" rather than correlate directly with the control of stem cell proliferation *per se*. Further studies correlating SCI/MIP-1 α mRNA and protein expression in murine bone marrow and stem cell cycling will allow this issue to be addressed.

8.5 Concluding Remarks.

This thesis represents the first study of the expression of the murine SCI/MIP-1 α and MIP-1 β genes in primary, untransformed cells. The characteristics of expression of these genes in bone marrow-derived macrophages establish these as belonging to the "immediate early" group of genes whose expression is induced as part of the early phase of the response of macrophages to stimulation. This must be taken into account in all future models concerning the involvement of SCI/MIP-1 α and MIP-1 β and the expression of their genes in both the regulation of haemopoiesis and in the inflammatory response. Bone marrow-derived macrophages also appear to be a suitable model system for identifying candidate regulators of SCI/MIP-1 α and MIP-1 β gene expression, although further refinement of the culture conditions used may be helpful given the demonstration in this study of the ability of sera and certain culture media to affect accumulation of mRNA for these genes.

In this study, the expression of the SCI/MIP-1 α gene in normal, unperturbed bone marrow was demonstrated. The level of SCI/MIP-1 α mRNA expressed in normal bone marrow was seen to be altered by the treatment of animals with the drug 5-fluorouracil. While this data is not inconsistent with a role for

the modulation of SCI/MIP-1 α gene expression in the control of myeloid stem cell proliferation, further studies will be required to clarify this issue.

Taken together, the studies presented in this thesis represent a strong basis for future work on the expression of the genes for SCI/MIP-1 α and MIP-1 β in both macrophages and bone marrow.

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