THE INVESTIGATION OF MACROPHAGE INFLAMMATORY PROTEIN- 1α AS A MYELOPROTECTIVE AGENT

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CONTENTS

ABSTRACT

This thesis describes a programme of investigation designed to evaluate the potential myeloprotective properties of BB-10010, a stable variant of human Macrophage Inflammatory Protein -1α.

In part I, a series of experiments are described in which the effects of BB-10010 were evaluated in mice receiving either cytotoxic chemotherapy or radiation treatment. A 7 day schedule of BB-10010 was developed and attenuated the cumulative bone marrow toxicity associated with repeated cycles of sublethal irradiation; a potential mechanism of action is discussed. An optimal model of chemotherapy-induced bone marrow damage was not defined, however, when administered simultaneously with cytotoxic chemotherapy (cyclophosphamide or BCNU), BB-10010 resulted in a similar, but less pronounced effect as was seen in the radiation experiments. BB-10010 enhanced peripheral blood mobilisation following cyclophosphamide treatment and augmented the mobilising effect of granulocyte colony stimulating factor(G-CSF).

During the myeloprotection experiments, an effect of BB-10010 on gut stem cell regeneration was observed. A number of further studies were therefore designed to define any growth modulating effects of BB-10010 on cytotoxic- and radiation-induced gut damage (mucositis). The results of these preliminary experiments are presented and discussed.

The design and results of the early clinical trials programme of BB-10010 are discussed in part II of the thesis. Three phase I studies and 2 phase I/II studies have been completed in collaboration with British Biotech Pharmaceuticals LTD. (BBL) These studies evaluated BB-10010 in normal healthy volunteers and in advanced cancer patients receiving non-cycle-active chemotherapy. The results of these studies are presented and further routes of investigation are discussed.

DECLARATION

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning;

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INTRODUCTION

1. Feedback Inhibition and Historical Perspectives

The stability of the "Milieu Interieur" is recognised to be an essential requirement of all living organisms and ensures a state of permanent mass and function. At its most basic it defines a steady state between the rate of cell loss and cell gain and is particularly relevant when discussing control at a supracellular level such as temperature and blood glucose regulation. Despite the term, however, homeostasis is not a static process but rather a dynamic interplay in which the organism must adapt to changing requirements. This adaptation is made possible by the development of numerous complex sensory monitoring systems that utilise a series of feedback loops and thus allow fine tuning and resetting of the equilibrium.

Our understanding of the underlying mechanisms remains in its infancy but considerable insight has come from the investigation and observation of the regulatory pathways governing haemopoiesis and epithelial cell proliferation. Within these tissues considerable emphasis has been placed on the role of stimulatory polypeptides in normal growth regulation. Until relatively recently, and despite their obvious importance, the counterbalancing inhibitory factors have been largely neglected.

The basic cybernetic principle of negative feedback has been exploited by mankind for many centuries in both Science and Technology. Despite this, the concept was not considered seriously, in physiological terms, until 1957 when Weiss and Kavanau published their general theoretical growth model. The theory, illustrated in Fig 1,

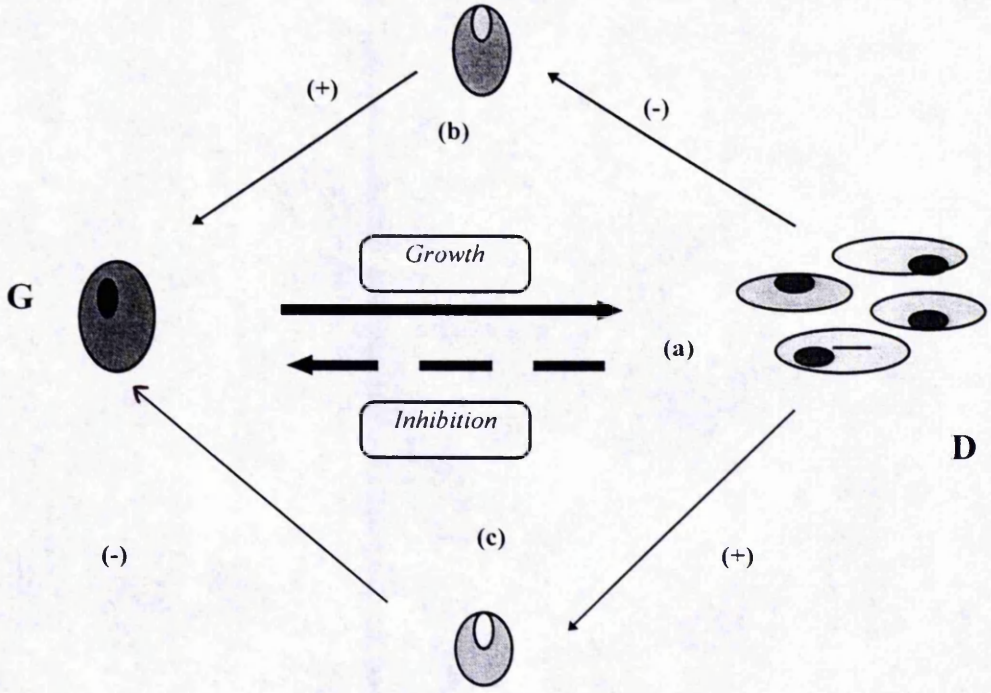


Figure 1. Principles of feedback inhibition. A generating mass (G) gives rise to a larger differentiated mass (D) which itself limits the growth of G via an inhibitory feedback signal. The feedback signal may be direct (a) or occur via an intermediary cell population (b and c). In 'b' an inhibitory factor (-) blocks the production of a stimulatory factor (+). In 'c' a stimulatory factor increases the production of an inhibitory factor.

outlined a system of templates (stimulatory factors) and antitemplates (inhibitory factors) which regulate growth by a negative feedback mechanism.

" Each specific cell type reproduces its protoplasm by a mechanism in which key compounds (templates) characteristic of the particular cell type act as catalysts. Each cell also produces specific freely diffusable compounds antagonistic to the former (antitemplates) which can block and thus inhibit the reproductive activity of the corresponding templates. The antitemplate system acts as a growth regulator by a negative feedback mechanism in which increasing populations of antitemplates render ineffective an increasing proportion of homologous templates, resulting in a corresponding decline of the growth rate."

The hypothesis, initially formulated to explain the growth curve of chickens, was taken up enthusiastically by several investigators and proved particularly constructive when applied to the field of epithelial growth regulation. In 1960 Iverson developed the idea further and published corroborating evidence from his work on skin carcinogenesis. In the same year, Bullough and Laurence (1960) devised a particularly innovative model based on the mouse ear (Fig 2). In this experiment, a $3mm^2$ area of superficial epidermis and dermis were removed and the mitotic activity of the undamaged, contralateral epidermis was analysed. If a stimulating wound hormone were produced by the damaged epidermis (figure 2, upper diagram), two regions of increased mitotic activity corresponding to the damaged edges would develop. Alternatively, if the concentrations of epidermal inhibitor were reduced (lower diagram), a continuous zone would be expected. The result, confirmed in Iversons experiment, was consistent with the hypothesis that epithelial proliferation

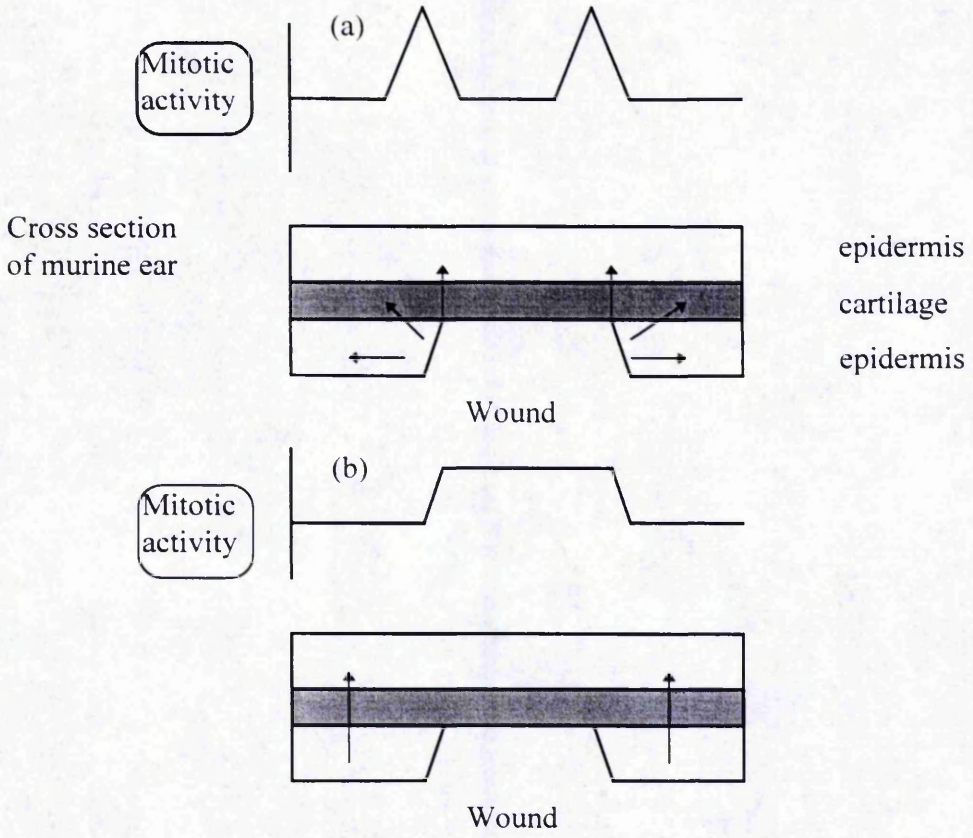


Figure 2. Bullough's ear model (Bullough and Laurence, 1960) showing the hypothetical mitotic activity in undamaged ear epidermis opposite a $3mm^2$ area from which the epidermis and superficial dermis have been removed. The upper diagram (a) shows the proliferative activity assuming the damaged tissue releases a stimulator. Release of stimulator at the regenerating boundries would lead to corresponding peaks of mitotoic activity on the contralateral side.The lower diagram (b) shows the proliferative response assuming that an epidermal inhibitor is reduced in the neighbourhood of the wound. Levels of inhibitor in the undamaged area maintain low mitotic activity on the contralateral side. Its absence in the damaged area would allow increased mitotoic activity over the whole region of the wound.

was controlled by a locally acting, freely diffusable negative feedback inhibitory regulator.

In 1962 Bullough introduced the term chalone for substances acting as tissue specific mitotic inhibitors. ('Chalone'- derived from the Greek word originally meaning "to slow down the speed" or "to reef in the sails"). Such molecules were assumed to be synthesised locally, possessed lineage and tissue specificity and were conserved through evolution, thus showing cross species activity. Following the demonstration of the epithelial inhibitor in tissue extracts, a variety of other chalones were soon being discussed (Voaden and Leeson, 1969; Forcher and Houck, 1973). Rytomaa and Kiviniemi (1968a) were the first to report a haemopoietic chalone, produced from a granulocyte extract, which inhibited myelocyte proliferation. This culminated in the characterisation of an acidic pentapeptide, pEEDCK (Paukovits and Laerum., 1982).

The model described by Wiess and Kavanau (1957), and illustrated in figure 1a, represents the simplest form of negative feedback regulation. An expanding, differentiated mass (D) elaborates an inhibitory factor (I) which acts directly on the generating mass (G), thus limiting its output. In a more sophisticated arrangement the feedback inhibitory factor may act via intermediary cell populations. In this scenario the feedback inhibitory signal may suppress production of a growth factor, that acts on the generating mass (fig.1b). Alternatively, the differentiating population may release a stimulatory factor that enhances an intermediary cell-produced inhibitor (fig.1c). It is important to note that in all cases the principal regulatory mechanism must involve an inhibitory signal and that positive feedback, when viewed in isolation, is not a

tenable concept, as it would rapidly lead to instability and ultimately, exhaustion of the system.

1.1 Haemopoiesis

The diversity and amplification of haemopoietic progenitor cells is made possible by factors that regulate the cellular options of self renewal, differentiation and proliferation. The role of the haemopoietic growth stimulatory factors has, historically, been emphasised in dictating these proliferation and differentiation decisions (Metcalf, 1990). More recently, the additional role of haemopoietic inhibitory feedback factors has been appreciated and our understanding, particularly of stem cell regulation, has expanded dramatically.

Haemopoiesis can be viewed as a three-part structure of developing cell populations (see fig. 3). A relatively small number of self-renewing and pluripotent stem cells give rise to an increasingly lineage committed progenitor cell population and ultimately the mature effector cells that are morphologically recognisable. The murine Spleen Colony Forming Unit (CFU-S) assay described by Till and McCullough (1961) has traditionally been used to define a multipotent, self-renewing cell population in the mouse. A more primitive pre-CFU-S is now well recognised [variously termed "marrow repopulating cells"(MRA), "long-term reconstituting cells"(LTR), "long-term culture initiating cells"(LTC-IC)] and the stem cell is more appropriately visualised as a continuum of cells with an age distribution and decreasing self-renewal capacity (Schofield, 1978; Hodgson and Bradley, 1979). The human stem cell remains particularly elusive, although quantitative and qualitative data can be obtained from the in vitro growth of bone marrow cells in Long Term

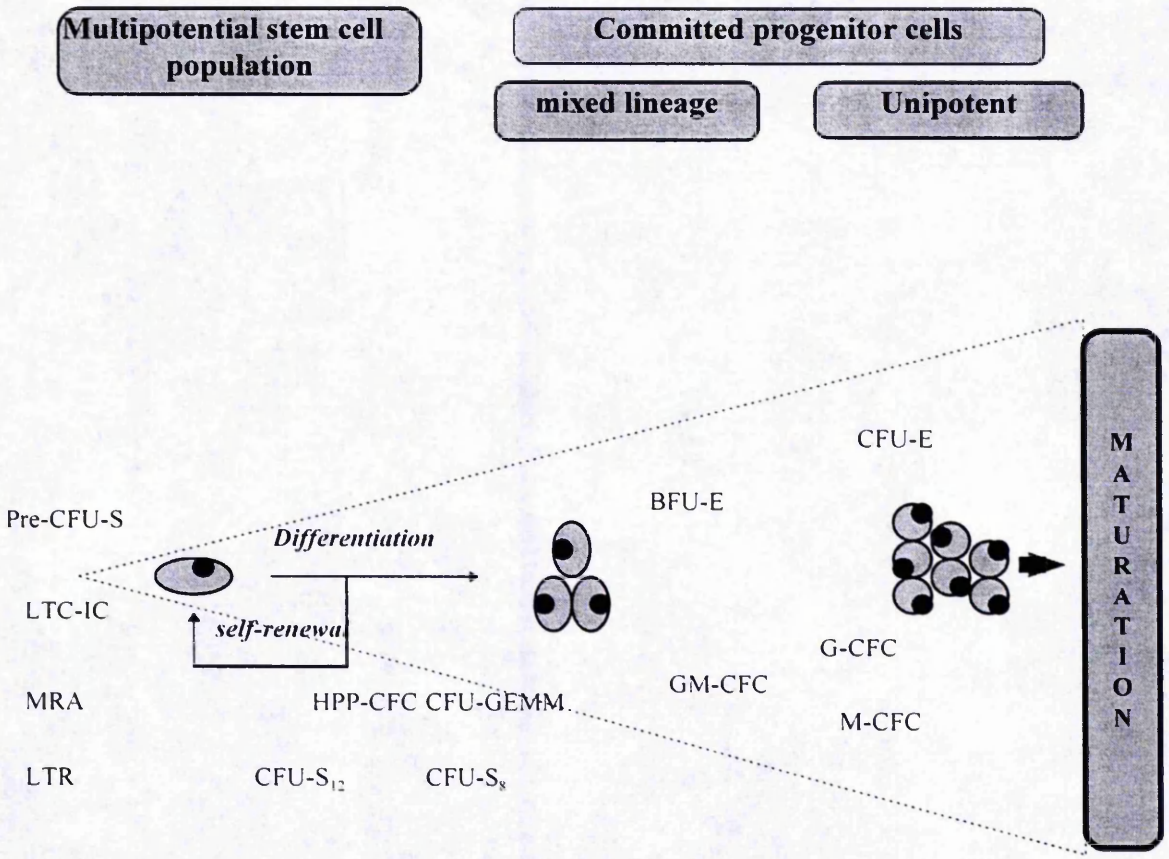


Figure 3. The haemopoietic hierarchy.

Bone Marrow Culture (LTBMC) (Dexter et al., 1978). Using this system, a long-term culture initiating cell (LTC-IC) has been described that gives rise to multipotential and unipotential clonogenic cells for as long as 5-8 weeks in culture (Sutherland et al., 1990). Limiting dilution analysis has shown that the frequency of LTC-IC's in unfractionated bone marrow is approximately 1-2 per 10^4 cells (Sutherland et al., 1990).

Immunophenotypic analysis has been used in an attempt to purify and quantitate primitive haemopoietic stem cells. Monoclonal antibodies to cell surface antigens such as CD34, HLA and Sca-1 are important markers in several enrichment protocols but none of these completely distinguishes the most primitive stem cell from CFU-S and other progenitor cells (Sprangrude et al., 1988, Andrews et al., 1989). The maturing progenitor populations remain morphologically indistinguishable but can be assessed in colony assays under appropriate in vitro conditions. The lineage and age characteristics of these colonies depend upon the culture conditions, the growth factors present (and their source) and the timing of read out. In semi-solid media, some bone marrow cells can form multilineage colonies. The CFC-mix or CFU-GEMM (human) identifies a cell with the potential to form granulocytes, erythrocytes, monocytes and megakaryocytes. The cells detected in this assay share some properties with the day-8 CFU-S. A CFC with a high proliferative potential can also be detected in murine and human bone marrow (McNeice et al.,1989) and is considered to be amongst the most primitive of progenitor cells grown in vitro, being closely related to the day-12 CFU-S [sometimes also equated with colony-forming unit-A (CFU-A) colonies grown in vitro (Lorimore et al., 1990)]. In addition to the multipotential

progenitor cells, a number of bipotential- and unipotential-committed progenitor cells can also be recognised including the granulocyte-macrophage CFC, the erythroid burst forming unit (BFU-E) and the more mature erythroid progenitor cell (CFC-E).

Rytomaa and Kiviniemi first described a granulocytic extract that regulated myelocyte proliferation (1968a). Subsequently, inhibitors were reported for other lineage committed haemopoietic cells including both granulopoietic and erythropoietic compartments (Fetsch and Maurer, 1987, Kivilaaska and Rytomaa, 1971, Axelrad et al., 1987, Guigon et al., 1990). These molecules limit the proliferation rates of the differentiated and maturing progeny and presumably appropriate inhibitors exist at all levels of cellular differentiation and in all haemopoietic lineages.

The recognition that haemopoietic stem cells are proliferatively quiescent (Becker et al., 1965), as opposed to the proliferatively active progenitor cell population, suggests the major control point for proliferation regulation exists within the former compartment. Utilising an ingenious experiment in which mice were irradiated with one hind limb shielded, stem cell regulation was found to be locally controlled (Gidali and Lajtha, 1972). The proliferative behaviour of the CFU-S in the shielded limb was noted to be independent of that in the unshielded limb and suggested localised CFU-S proliferation control. As a consequence of these experiments Lord et al (1976) obtained a conditioned medium from normal bone marrow (NBME-IV) that blocked entry of CFU-S into DNA synthesis. Furthermore, it was demonstrated that in regenerating haemopoietic tissues the inhibitor was replaced by a proliferation stimulator and that the two factors were necessary for regulating cell proliferation. Further analysis of the stimulator to inhibitor interaction revealed that the two

activities function via an on/off switch mechanism with the presence of the opposing factor necessary to effect the proliferation reversal (Lord et al., 1977). This contrasts with the transit cell proliferation inhibitors (eg. granulocyte extract-chalone) whose effects can be reversed simply by washing (Lord et al., 1975) Both of these factors proved to be specific for CFU-S with no effects on lineage committed progenitor cells. In addition to tissue specificity (CFU-S inhibition with no effects seen on in vitro assays for mixed and lineage-committed CFCs) the inhibitor also showed cross species activity with marrow preparation from human, pig, rabbit, rat and mouse all inhibiting mouse CFU-S (Lord et al., 1976, Tejero et al., 1984).

Using a variety of sorting techniques, a subpopulation of marrow macrophages was found to be the source of the inhibitory activity (Wright et al., 1980) whilst a second subpopulation of macrophages synthesised the CFU-S stimulator (Wright et al., 1982). It required 14 years of research, however, before the active component of NBME-IV was isolated and characterised as the previously described chemokine Macrophage Inflammatory Protein 1α (MIP−1α) (Graham et al., 1990), the clinching fact being the neutralisation of NBME-IV by antibody to MIP-1α.

Three further inhibitors of haemopoietic stem cell proliferation have been recognised, sequenced and synthesised, however, their interrelationships have still to be elucidated. All appear to share common actions, suggesting a degree of overlap and redundancy in a manner analogous to the functional utilisation of haemopoietic growth factors (Metcalf, 1993). Two inhibitors, AcSDKP (Goralatide) and pEEDCK, have recently entered clinical trial protocols, the third, Transforming Growth Factor-β (TGF-β) is in the latter stages of preclinical studies. These factors and other putative inhibitors are discussed later in section 2.2.

1.2 Microenvironmental Influences and Self - Renewal

Despite an apparent random distribution of cells in the bone marrow it has become increasingly evident that the haemopoietic system is organised spatially along similar lines to other self-renewing tissues eg.epithelium (Lord and Hendry, 1972, Lord and Schofield, 1980, Cui et al, 1996). This organisation ensures that the more primitive haemopoietic progenitor cells will lie in close proximity to the appropriate stromal components. The relationship between haemopoiesis and its microenvironment has been highlighted in the LTBMC model (Dexter et al., 1978) in which haemopoiesis can be maintained in the absence of exogenously added growth factors. In this system, the cultured marrow first develops an adherent stromal layer in which the more primitive haemopoietic progenitors cells reside and undergo cyclical changes in their proliferative status under the influence of locally produced feedback regulators, including MIP-1α and TGF-β and stromal-derived growth factors (Eaves et al., 1993a, Cashman et al., 1990). Movement of cells away from this local environment is associated with enhanced proliferation and differentiation.

The importance of the bone marrow microenvironment for growth regulation is not unexpected given the known relationship and interactions of growth factors with extracellular matrix (ECM). This interaction is even more apparent with the proliferation inhibitor, TGF-β, which enhances ECM formation and upregulates cell adhesion receptors (Ignotz and Massague, 1987, Ignotz et al., 1989). Some insight into the link between the microenvironment and stem cell inhibition has come from experimental work on Chronic Myeloid Leukaemia cells grown in LTBMC. In this culture system primitive normal haemopoietic progenitor cells are proliferatively

quiescent and adhere to the extracellular matrix component fibronectin, partly through the integrin family of adhesion molecules (Verfaille, 1994). In contrast, CML progenitor cells proliferate rapidly with reduced stromal adherence despite a normal complement of adhesion factors. Verfaille (1994) has suggested that dysfunctional activity of one of these molecules may account for the defective adhesive properties and enhanced proliferation. Upregulation of the β1 integrin restores adhesion and interestingly, appears to be associated with reduced proliferation of the CML progenitor cell population. Furthermore, adhesion of CML progenitor cells can be enhanced by the addition of TGF-β or MIP-1α (Bhatia et al., 1994), suggesting that proliferation regulation of primitive haemopoietic progenitor cells occurs as a consequence of stem cell-stromal cell interactions. Both MIP-1α and TGF-β inhibit purified bone marrow progenitor cell subsets, in the absence of stromal cells (Keller et al.,1994), suggesting that both these factors are products of the stromal cell population. However although MIP-1α may restore adhesive properties to CML cells Eaves et al (1993a) showed that these are resistant to the proliferation inhibitory actions of MIP-1α.

The interactions outlined above are particularly relevant when considering self renewal as a mechanism for growth control in stem cell populations. Under steady state conditions, the small population of stem cells must maintain its own numbers whilst also allowing a proportion of their progeny to differentiate and amplify. This self-renewal probability, p, must by definition be set at or above 0.5 in order to conserve sufficient stem cell numbers for the lifespan of the organism. Values of less than or greater than 0.5 will respectively, reduce and increase the stem cell population. Control of stem cell numbers can therefore take place by one of two

mechanisms. The first is cell cycle modulation thus varying the population growth fraction. The second is the ability to vary the number of stem cell daughters cells that remain stem cells i.e. alteration of p. The latter mechanism can, under certain conditions, represent the principle mechanism for governing the stem cell growth rate (e.g. hydra) without the necessity to vary the cycle length.

The proliferative activity of haemopoietic progenitor cell populations, is inversley related to its self renewal capabilities. It is of interest, therefore, to consider whether proliferation inhibition itself represents a mechanism to regulate self-renewal and differentiation. Proliferatively quiescent stem cell populations (in a G_0 state - see section 1.3) are associated with the greatest self-renewal capacity. Induced proliferation, during regeneration post cytotoxic therapy for example, results in ageing of the stem cell population and a corresponding reduction in marrow repopulating ability. Maintenance of a proliferatively quiescent stem cell population may therefore represent an evolutionary strategy to maintain the integrity of the genome. Under these circumstances the G_0 state may be viewed as a rest area, allowing cells to perform essential gene housekeeping functions and thereby reduce the risk of developing and propagating potentially damaging DNA mutations.

This possible relationship between proliferation inhibition and self-renewal suggests a further role for feedback regulators, which ultimately may control the switch between self-renewal and differentiation and not simply inhibit cell cycling. This has recently been highlighted in a cytotoxic model using MIP-1α in which haemopoietic recovery was enhanced, at least in part, as a result of an increased self-renewal capacity of the CFU-S population (Lord, 1995).

1.3 The Cell Cycle and its Regulation.

Manipulation of the cell cycle ultimately represents the common final pathway for all feedback inhibitors of haemopoiesis. However, the specific intracellular response(s) remain poorly defined.

In 1953 Howard and Pelc defined the four classical phases of the cell cycle. They are DNA synthesis (S) and mitosis (M) separated by two 'gaps', designated G_1 and G_2. In the majority of mammalian cells, S-phase typically lasts 6-10 hours, G_2 3-5 hours and mitosis, 1 hour. In contrast, the duration of G_1 shows great variability, for example, in culture, Chinese hamster lung cells do not have a definable G_1 (Robbins and Scharff, 1967), while in other quiescent populations there does not appear to be an upper limit on the interval of time between mitosis and next entering S phase. The great variability suggests that the main control point(s) - some of which represent arrest points - for cycle progression exist in this G_1 interval.

Observations on extremely proliferatively quiescent populations e.g., normal hepatocytes and uterine epithelium of oophorectomised mice, suggests that these cells normally reside in an out-of-cycle G_0 state (Lajtha, 1963). This is distinct from, G_1 growth arrest, since a small percentage of cells, even in populations of extreme proliferative quiescence, continues to move into cycle and through DNA synthesis and mitosis.

G_1 itself is not a single entity but more precisely a series of stages that a cell must complete before proceeding into DNA synthesis. Restriction points determine whether a cell will progress to S or enter quiescence. Once cells have reached a point late in G_1, referred to as the R point and analogous to START in yeast (Pardee, 1989), cells will automatically proceed to S, even in the absence of nutrients. DNA synthesis

is probably programmed late in G_1 and will normally proceed, in the absence of any artificial block, automatically to G_2 and mitosis. The decision whether to enter G_0 is dependent on the concentration of mitogens or inhibitors in the local environment. In this respect, cell concentration is recognised as an important factor which may directly or indirectly determine the levels of those mitogens and/or inhibitors.. Once a cell has entered G_0, it can remain in this state until it recognises an alteration in the feedback messages. Re-entry into the cell cycle occurs at some point before S but the precise position of reentry is unknown and may vary between different cell types. Kinetic studies on CFU-S show that movement of cells from G_0 to S phase is very rapid so that G_0 in this population must lie very close to the onset of DNA synthesis (Lord, 1981).

It is not surprising, given that control points exist predominantly in G_1, that inhibitory haemopoietic regulators are thought to act at this stage of the cell cycle. The inhibitor in normal bone marrow extract -fraction IV acts at the G_0/S switch with loss of sensitivity to the inhibitor as the cell approaches the G_1/S interface and complete loss of sensitivity in S-phase (Lord et al., 1979). Similar conclusions were drawn for the inhibitory tetrapeptide, AcSDKP, which acts on CFU-S only in G_0 or early G_1 to block entry into a proliferative phase (Frindel et al., 1992). In contrast, TGF-β inhibits keratinocytes when added at any time during G_1 up to the G_1/S boundary (Coffey et al., 1988, Pietenpol et al., 1990).

The downstream events that follow inhibitor/receptor binding, are poorly understood with very little published work concerning MIP-1α, AcSDKP or pEEDCK. These inhibitors may function directly or indirectly via the recently recognised tumour suppressor genes. TGFβ, for example, regulates phosphorylation

of the retinoblastoma susceptibility gene product (pRB), a known cell cycle regulatory factor (Chen et al., 1989, Goodrich et al., 1991). The addition of TGF-β to lung epithelial cells in mid to late G_1 prevents the phosphorylation of pRB and leads to cell cycle arrest in G_1 (Lahio et al., 1990). The addition of TGF-β in late G_1 when pRB is already phosphorylated or during S has, however, no effect (Lahio et al., 1990).

Whilst less well described, control points for feedback regulators may exist also in G2 (Forsburg and Nurse, 1991) and in DNA synthesis itself (Axelrad et al., 1981). Erythroid progenitor burst forming units (BFU-E) normally proliferate slowly. Washing to remove endogenous inhibitory molecules causes a rapid increase in the proportion synthesising DNA (Axelrad et al., 1981). Conversely, DNA synthesis falls equally precipitously (within 20 minutes) following the re-addition of the erythroid inhibitor, Negative Regulatory Protein (NRP)(Axelrad et al., 1983). The rapidity of the response in these experiments suggest that control of DNA synthesis may - at least in these cells- take place after they have already embarked on DNA synthesis.

In summary, inhibitory regulators, active in multipotential stem and progenitor cell populations appear, therefore, to act principally in the G_1 phase of the cell cycle. Within this period they probably act by switching cells from G_1 to G_0 and subsequently maintain them in this holding reservoir. This appears to be the case with MIP-1α, the active component of NBME-IV and probably AcSDKP. For the later maturing but still proliferative cell populations progression through G_1 to S may merely be delayed, with inhibition (prolonged cell cycle time) reversed simply by washing away the inhibitory factor. At present there is no evidence that inhibitory

haemopoietic regulators act in G_2 and, apart from the special case of NRP, there is no evidence for activity on cells already in DNA synthesis.

1.4 Assays of Proliferation

The low frequency of stem cell and early progenitor cell numbers, together with their lack of distinguishing morphological features, precludes the use of direct methods of analysis such as autoradiography. Recent advances in immunophenotyping and flow cytometry have improved our ability to purify stem cell populations. However, there remains considerable overlap in phenotype for cell morphology and surface markers. As a consequence, assays for inhibitory regulators of stem cells have been based on the property of colony formation and suicide techniques. However, some caution is always necessary when interpreting results of experiments designed to detect inhibition.

Haemopoietic colonies formed in vivo following BM transplantation into an irradiated recipient and known as spleen colony forming units (CFU-S) or in semi-solid culture media are clonal i.e., they are derived from a single cell. The number of colonies developing therefore becomes a measure of the progenitor cells in the sample being assayed. Theoretically, in the presence of an inhibitor the suppression of colony formation can be interpreted as evidence for inhibitory regulation, however, it would also be indistinguishable from cytotoxicity.

A more specific means of analysis is available from suicide assays that exploit cytotoxic agents such as hydroxyurea (HU), cytosine arabinoside, and tritiated thymidine ([^{3}H]TdR) which kill cells specifically in S-phase.With sufficient dose, the incorporation of [^{3}H]TdR into DNA of the test sample produces multiple double

strand breaks, thus rendering cells incapable of further proliferation and colony formation. With appropriate controls using the same test sample and 'cold' thymidine the difference in colony numbers becomes a measure of the proliferative status.The addition of a further control group, using the same test cells, but in the absence of the putative inhibitor, allows quantification of the inhibitory effect and includes an independent check that the effects are not due to any cytotoxic activity.

Despite the common usage of the suicide assay, a number of pitfalls have been recognised (Lord et al.,1974, Maurer, 1981) and not infrequently ignored. Since the calculation of kill depends on the difference between two colony counts, each with its own error, the error on the kill can be very large. This is readily apparent when observing the variability in haemopoietic progenitor cell cycling reported in the literature. Several other commonly used S-phase cytotoxics may be utilised in the suicide assay but may not produce results that are directly comparable with those using [^{3}H] thymidine (Lord et al., 1974). Caution is therefore required in extrapolating the data from one suicide technique to another. The large errors, inherent in the assay, are not always recognised and appropriate statistical analysis is essential. Contradictory results between laboratories are more likely to arise as a result of an inadequate database rather than methodological differences. An analysis that used hydroxyurea suicide on murine marrow colony forming cells in vitro, for example, confirmed the large number of individual experiments necessary to detect a specific difference (Quesenberry and Stanley, 1990). To detect a specific difference of 25-30% with a probability of less than 0.05, 6-9 experiments were necessary. In order to be sure that a 25-30% difference is not present, 15-21 experiments were required.

Despite the potential pitfalls, the suicide assay remains a valuable laboratory tool for investigating and quantifying growth inhibition. A more specific and sensitive assessment awaits advances in both the purification of pluripotent haemopoietic progenitor cells and a greater understanding of the cellular events that follow inhibitor/receptor binding.

2. Haemopoietic Stem Cell Proliferation Inhibitors.

2.1 Macrophage Inflammatory Protein - 1α

The active component of NBME - IV proved to be somewhat elusive and was not identified and characterised until 1990 (Graham et al., 1990). Difficulties with biochemical purification were exacerbated by the lack of a convenient in vitro assay system for the target cell(s). The search was greatly simplified following the development of the colony forming unit-A (CFU-A) assay (Lorimore et al., 1990) which is carried out in vitro. Preliminary experiments, by Graham et al (1990), confirmed the inhibitory effects of NBME-IV on colony growth in the CFU-A assay. A subpopulation of macrophages had previously been defined as the principal source of the inhibitory activity (Wright et al., 1980, Simmons and Lord, 1985) and subsequent screening of the conditioned media from several murine macrophage cell lines identified J774.2 as an effective source. Conditioned medium from these cells inhibited CFU-A growth and CFU-S proliferation and retained the cellular specificity demonstrated by NBME-IV by having no effect on proliferation of the more mature GM-CFCs (Graham et al., 1990). Chromatographic separation, Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) analysis and High Performance Liquid Chromatography (HPLC) of J774.2-conditioned medium

ultimately revealed the inhibitor to be identical to a previously characterised chemokine, MIP-1α, whose only known property was an ability to produce a localised inflammatory reaction (Sherry et al., 1988). MIP-1 (a doublet of MIP-1α and MIP-1β) and MIP-1α, but not MIP-1β, reversibly decreased the proliferative activity of CFU-A. The inhibitory activity of MIP-1α was confirmed in vivo on CFU-S proliferation and using a neutralising antibody, it was shown that MIP-1α is both functionally and antigenically identical to the proliferation inhibitor in NBME-IV.

MIP-1 had originally been recognised in 1988 by Wolpe et al., who observed a third protein with interesting physical properties during the purification and characterisation of cachectin/ tumour necrosis factor (TNF). The protein, obtained as a doublet, was isolated from another murine macrophage tumour cell line (RAW 264.7) and although having a molecular mass of 8kDa on SDS PAGE it readily formed large multimeric aggregates of $>2 \times 10^6$ Da on gel filtration, a property that was later to present some practical problems. The MIP-1 doublet was subsequently separated chromatographically with MIP-1α representing the major component (109 amino acids) and MIP-1β the minor component (69 amino acids) (Sherry et al., 1988).

The murine MIP-1α chemokine is a member of a large family of small, inducible and secreted cytokines (Wolpe and Cerami, 1989; Brown et al., 1989, Oppenheim et al., 1991). The family members are all basic heparin-binding polypeptides that possess proinflammatory and reparative activity. They are defined by the presence of four conserved cysteine residues and have been subdivided into two groups. The first two cysteine residues are adjacent in the c-c group but are separated by a single amino acid as a c-x-c grouping (Table I). Human MIP-1α (LD78) is approximately 75% homologous to murine MIP-1α at the amino acid level and comparison of the gene

sequences has revealed approximately 77% homology within the first 350 bp of proximal promotor sequences, suggesting that the protein and its gene regulatory sequences have been conserved through evolution (Yamamura et al., 1989, Blum et al., 1990, Widmer et al., 1991).

Table 1. The chemokine Family.

c-c structure		**c-x-c structure**	
murine	**human**	**murine**	**human**
MIP-1α	LD78	MIP-2	GRO
MIP-1β	ACT-2	Not known	NAP-1(IL-8)
Not known	RANTES	PF4 (rat)	PF4
JE	MCAF	Not known	βTG (PBP)
TCA-3	I-309	CRC-2	IP-10

The production of MIP-1α appears to be restricted to cells originating from lymphohaemopoietic tissues. Monocytes and macrophages represent the principal, but not exclusive, source. MIP-1α has been isolated as cDNA from human lymphocytes (Shiozaki et al., 1992, Zipfel et al., 1989), neutrophils (Kasama et al.,1993), and eosinophils (Costa et al.,1993). Neoplastic haemopoietic cells retain the capacity to produce MIP-1α but appear to be resistant to its actions (Yamamura et al., 1989, Eaves et al., 1993a/b). Epidermal Langerhans cells express MIP-1α (Heufler et al., 1992) suggesting a possible role for them in the regulation of epithelial proliferation.

This was not completely unexpected, however, as these cells are derived from the haemopoietic stem cell (Katz et al., 1979) and carry surface antigens that are similar to those on the monocyte/macrophage series (Stingl et al., 1978).

The macrophage origin of MIP-1α and its initial recognition as a product of endotoxin stimulation suggested a role as an inflammatory mediator (Wolpe et al., 1988). Early experiments appeared to confirm this suggestion and when MIP-1 was injected into the footpad of mice it resulted in an immediate inflammatory response characterised by neutrophil infiltration and mast cell degranulation (Wolpe et al., 1988). Increased levels of MIP-1α mRNA have been detected in diverse inflammatory states of both infective and non-infective aetiology. Mice inflicted with the parasitic infections, toxoplasmosis (Hunter et al.,1992a) and trypanosomiasis (Hunter et al., 1992b), develop a meningoencephalitis that is accompanied by an increase in MIP-1α transcripts in the brain. In addition, high levels of MIP-1α expression were detected in the lungs and joints of patients with chronic lung disease (Standiford et al., 1993) and rheumatoid arthritis, respectively (Koch et al., 1994). The pathogenic role of MIP-1α in these conditions is, however, poorly defined and blurred by the concomitant activation of many other cytokines including interleukin-1, interleukin-6, tumour necrosis factor, and γ-interferon (Hunter et al.,1992a, 1992b).

MIP-1α , like other chemokine relatives, may function as a chemotactic factor for specific leucocyte subsets. The acute inflammatory response is characterised by an initial infiltration of neutrophils followed by an accumulation of monocytes and macrophages. MIP-1α may play a distinct role in this shift in cellular response from neutrophil to mononuclear cells (Kasama et al., 1993). Conflicting reports exist concerning the chemotactic response of neutrophils following MIP-1α exposure.

Mycobacterial infections produce a chronic mobilisation of neutrophils to the site of infection. Injections of MIP-1 antibody in mice reduce the peritoneal neutrophilia suggesting a role for MIP-1α (or MIP-1β) in neutrophil recruitment (Appelberg 1992). McColl et al., (1993) reported that MIP-1α induces intracellular calcium mobilisation in human neutrophils but that this effect is not coupled to neutrophil effector functions such as degranulation or chemotaxis. The difference, perhaps, may reflect a species specific effect of MIP-1α. In addition to the proposed chemotactic effects on monocytes, MIP-1α was reported to be chemotactic for other leucocyte subsets including eosinophils (Rot et al., 1992), basophils (Alam et al., 1992) and mast cells (Alam et al.,1992). A role in the immune response is also suggested by the induction of MIP-1α gene expression in activated B- and T-cells (Schall et al., 1993, Taub et al., 1993).

Direct injection of MIP-1 into the thermoregulatory centre (anterior hypothalamic and preoptic areas) results in a monophasic fever that is not blocked by the cyclo-oxygenase inhibitors, ibuprofen or indomethacin (Minano et al., 1991, Davatelis et al., 1989) but is attenuated by addition of the protein synthesis inhibitor, anisomycin (Zawada et al., 1993). Analysing the specific effects of MIP-1 and its constituent parts, Myers et al., (1993) showed that MIP-1 and MIP-1β are the predominant inducers of fever and that MIP-1α plays a relatively minor role. A febrile response induced by MIP-1 is accompanied by loss of appetite, probably via independent mechanisms involving the ventromedial hypothalamus (feeding centre) although this is not associated with any significant weight loss. In contrast, these findings were not confirmed by Plata-Solomon and Borkoski (1994), who reported short term effects on

appetite suppression following the intracerebroventricular administration of various chemokines (IL-8, PF4, MCAF, and RANTES) but not including MIP-1α.

In summary, it has been suggested that MIP-1α may serve a role as an inflammatory mediator acting both as a chemotactic factor for specific leucocyte subsets and as a prostaglandin-independent pyrogen. This role, however, remains questionable. Increased expression of MIP-1α at sites of inflammation does not necessarily equate with activity and may represent a secondary or non-specific event. Parallel studies comparing MIP-1α with other chemokine members have shown that MIP-1α has a relatively modest effect on chemoattractance and that the biochemical response may not always be coupled to cellular function.The evidence for a pyrogenic effect of MIP-α is also weak as many of the earlier experiments do not differentiate between MIP-1 and its constituent parts and may, therefore, simply represent non-specific pharmacological rather than true physiological responses. Finally and most significantly, a large number of murine chemoprotection models have defined an optimum dose of MIP-1α, administered in vivo, that is remarkably non-toxic and devoid of any inflammatory response (Lord et al., 1992, Clements et al., 1992, Dunlop et al., 1992).

2.1a Polymerisation

A problem that rapidly came to light in the early work with MIP-1α was one of molecular aggregation. MIP-1α is a rather 'sticky' molecule and although its basic molecular weight is around 8 kDa, it has a strong tendency for non-covalent self aggregation displaying a wide range of molecular sizes. This initially caused some problems in obtaining reproducible dose/response results and satisfactory

interlaboratory comparisons. Aggregation appears to be greatly reduced in high ionic strength buffers and at least one commercial preparation is supplied in aceto-nitrile in order to maintain its monomeric form (Wolpe and Cerami, 1989).

The importance of this property is not clear. Mantel et al., (1993) reported that monomeric MIP-1α in vitro was 1000-fold more effective than polymerised material and, in spite of finding that polymerised MIP-1α does not interfere with suppression by monomeric MIP-1α, they came to the conclusion that polymerisation of MIP-1α might be a control mechanism that limits the myelosuppresive effects of the monomeric molecule. One year later, the same group confirmed that a 1000-fold lower dose of monomeric MIP-1α, injected in vivo, rapidly reduced the cycling and numbers of progenitor cells in the bone marrow and spleen (Cooper et al., 1994).

Graham et al., (1994) failed to confirm these findings and found that monomeric, dimeric and tetrameric mutant MIP-1α molecules were equipotent with each other and with wild type protein in stem cell and monocyte shape change assays. They suggested that both aggregated MIP-1α and the aggregated mutants spontaneously disaggregate under assay conditions and function as monomers, concluding that aggregation is a dynamic and reversible phenomenon that has little impact on bioactivity in vivo.

2.1b BB-10010

Biophysical characterisation of the polymerisation pathway has shown that high ionic strength buffers substantially reduce the multimerisation of MIP-1α beyond a tetramer (Hunter et al., 1995). This finding indicates that electrostatic forces play a major role in the formation and stabilisation of higher molecular weight complexes. To

dissociate MIP-1α to its monomeric subunits, the relatively harsh conditions of acidified acetonitrile are required, suggesting that the principle stabilising force for the tetrameric unit is hydrophobic (Patel et al., 1993). Using mutagenesis, a stable variant of MIP-1α was identified, carrying a single amino acid substitution of Asp26⇒Ala, with a reduced tendency to form large polymers at physiological pH and ionic strength (Hunter et al., 1995). This variant, BB-10010, retains similar potency to native human MIP-1α in receptor binding, calcium mobilisation, inhibition of colony formation (in vitro and in vivo) and thymidine suicide assays (Hunter et al.,1995).

2.1c Modification of Cell Proliferation.

The inhibitory effects of MIP-1α appear to be specific for multipotential haemopoietic precursor cells that are intermediate to late in the the stem cell hierarchy (see fig. 3). Day 12 CFU-S appear to be more sensitive to MIP1-α–induced inhibition than the later more mature day 8 CFU-S (Wright et al., 1985, Graham et al., 1990, Lord et al., 1992). In vitro, MIP-1α inhibits proliferation of primitive BFU-E (Broxmeyer et al., 1991), human CFU-GEMM (Broxmeyer et al., 1990, 1991) and colony formation in populations enriched for primitive haemopoietic progenitor cell subsets (Keller et al., 1994, Broxmeyer et al., 1990). Growth factor-stimulated proliferation of the multipotent, growth factor-dependent, haemopoietic cell line Paterson (FDCP-mix A4) is also inhibited (Clements et al., 1992). The most primitive haemopoietic stem cells, or preCFU-S appear more resistant. MIP1-α did not inhibit the primitive High Proliferative Potential-CFC when combinations of Interleukin-1 (IL-1), Stem Cell Factor (SCF) and Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) were used as growth promoters (Schneider and Moore, 1991). Cells

that possess LTR potential normally reside in a quiescent state and are resistant to cycle active drugs. They can, however, be triggered into proliferation by a single dose of 5-Fluorouracil (5FU). Using a murine model, Quesniaux et al., (1993) showed that MIP-1α administered subcutaneously twice daily from Day 0 to Day 7 was unable to prevent the depletion of LTR stem cells by 5FU despite apparent inhibition of later multipotential progenitor cells. The authors concluded that MIP-1α had no inhibitory effect on the proliferative regeneration of LTR stem cells in vivo. However, one might have anticipated that MIP-1α-induced CFU-S inhibition, would itself have indirectly blocked recruitment of those preCFU-S populations and thus maintained their numbers.

There have been conflicting reports concerning the in vitro effects of MIP-1α on the more mature, lineage-committed colony-forming cells. GM-CFC, induced to proliferate by a combination of growth factors, are inhibited by MIP-1α (Broxmeyer et al., 1990, 1991, Cooper et al., 1994) an effect reproduced using monomeric MIP-1α at a 1000-fold lower concentration (Cooper et al., 1994). Other groups have been unable to show any consistent inhibitory effect of MIP-1α on lineage committed cell populations (Graham et al., 1990, Keller et al., 1994, CM Heyworth-personal communication). In contrast, others have reported that MIP-1α may have a bidirectional effect on haemopoietic progenitor cell subsets (Broxmeyer et al., 1989, 1990, Keller et al., 1994). Broxmeyer et al., (1989, 1990) reported that the growth of the earlier progenitor cells, CFU-GEMM and subpopulations of BFU-E, was suppressed by MIP-1α. Paradoxically, the more mature GM-CFC and BFU-E were stimulated by MIP-1α in the presence of suboptimal concentrations of M-CSF and GM-CSF. Clements et al. (1992), however, observed stimulation in the presence of

GM-CSF but no effect with M-CSF or IL-3. To add to the confusion, Keller et al. (1994) showed that MIP-1α enhanced IL-3 and GM-CSF-induced colony formation of normal bone marrow progenitor cells and lineage negative (Lin^-) progenitors but had no effect on G-CSF or M-CSF-induced colony growth. The significance of these apparent differences is unclear but they most likely reflect subtle variations in assay conditions and perhaps indirect effects of MIP-1α via contaminating accessory cells. It is important to note that the combination of cytokines and, therefore, the maturational stage of the CFC's stimulated in the culture, is central to the observed response with MIP-1α and that no experiment has shown MIP-1α to have colony stimulating activity per se. It is noteworthy that the property of bidirectional haemopoietic growth regulation has been suggested for another putative inhibitor, TGF-β also (Keller et al., 1994).

From studies on highly enriched cell populations, it was concluded that the inhibitory actions of MIP-1α on its target cells are direct (Keller et al., 1994, Broxmeyer et al., 1990). To rule out any effects from contaminating accessory cells, however, Lu et al. (1993) investigated the effects of several members of the chemokine family on colony formation initiated by $CD34^+$ cells from bone marrow and cord blood. Sorted into single wells in the presence of a combination of Erythropoietin (Epo), stem cell factor (SCF), GM-CSF, and IL-3 in serum and serum-free conditions, proliferation of these cells was directly suppressed by MIP-1α. Inhibitory effects were also seen with the related chemokines MIP-2α, Platelet Factor 4 (PF4), IL-8 and Monocyte Chemotactic and Activating Factor (MCAF) (table II). The significance of this functional overlap is unclear because some of these related members bind different receptors and also span the structural families of the MIP-1

and MIP-2 groups (see table I). Single sorted $CD34^+$ cord blood cells were much less sensitive to inhibition by these cytokines, possibly as a result of their inherent lower cycling rates (Lu et al., 1993).

Table 2. Members of the chemokine family that may possess proliferation inhibitory properties against haemopoietic cells.

MIP-1 Family (c-c)	MIP-2 Family (c-x-c)
MIP-1α	*MIP-2α/β*
MCAF	*IL-8*
PF4	
RANTES	

The potential role of MIP-1α as a haemopoietic proliferation regulator has been particularly well demonstrated in LTBMC, an in vitro model that reproduces the [illegible] relationship between the bone marrow stromal cells and the primitive haemopoietic progenitor cells (Dexter et al., 1978). In this system, the primitive progenitor cells reside in the adherent layer and can be distinguished by their ability to generate very large colonies in vitro (more than 500 granulocytes and macrophages

and more than eight clusters of erythroblasts) (Cashman et al., 1985). The progenitor cells undergo cyclic oscillation in their proliferative status, each cycle triggered by a weekly change in culture medium (Toksoz et al., 1980, Cashman et al., 1985, Eaves et al., 1991), and as a direct consequence of the opposing actions of endogenous inhibitors and stimulators in this system (Toksoz et al.,1980, Cashman et al., 1985, 1990, 1994, Eaves et al., 1991, 1993a) (Figure 4).

Cashman et al. (1990) reported that in the primitive progenitor cell population, DNA synthesis was triggered by a factor(s) present in horse serum and that the return to quiescence occured under the influence of a proliferation inhibitor(s). MIP-1α mRNA had previously been identified in extracts of both primary and subcultured LTBMC adherent layers, confirming endogenous production and suggesting a possible role for the molecule in proliferation regulation in LTBMC (Otsuka et al., 1991). Exploiting the antagonistic properties of MIP-1β on MIP-1α, Eaves et al (1993a) reported that the addition MIP-1β 2-3 days after feeding prevents the primitive haemopoietic progenitors in the adherent layer, from returning to a quiescent state. Furthermore, the effect of exogenously added MIP-1β is itself overcome by the simultaneous addition of MIP-1α. The results using MIP-1β suggest that the return to quiescence of primitive progenitor cells occurs under the influence of endogenously produced MIP-1α. In agreement with the in vitro colony data, exogenously added MIP-1α does not block DNA synthesis in the more mature progenitors. Neither does it appear to be the only inhibitor present in the LTBMC system because both TGF-β (Cashman et al., 1990) and AcSDKP (Cashman et al., 1994) appear to act similarly. Addition of TGF-β antibody or MIP-1β to the LTBMC, results in an increase in cycling of primitive progenitor cells, suggesting that a combination TGF-β and MIP-

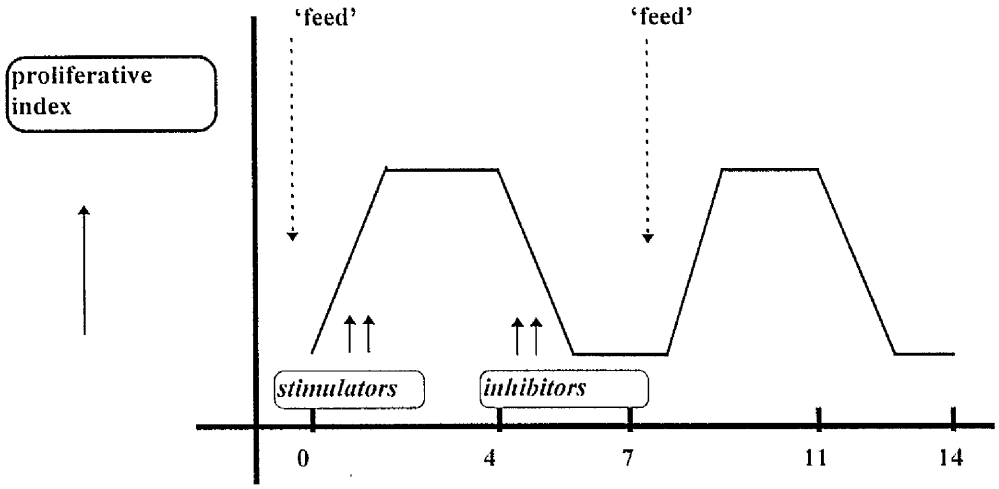


Figure 4. Schematic representation of the cyclical changes in LTBMC progenitor cell proliferation associated with a weekly change in culture medium ('feed'). Stimulators and inhibitors are generated endogenously within the adherent stromal layer of the culture.

1α is required to maintain progenitor cell quiescence (Cashman et al., 1990, Eaves et al.,1993a), a feature that potentially has important clinical implications.

The potential importance of inhibitor co-operation is highlighted in CML. In contrast to normal progenitor cells, the primitive CML progenitor cells are in a state of continuous turnover, irrespective of their location (marrow or blood) or differentiative potential (A.C. Eaves et al., 1986. C.J. Eaves et al., 1993b). This situation can be reproduced in LTBMC, thus permitting investigation of the underlying mechanisms. The results support the hypothesis that malignant transformation is primarily associated with escape from the normal "braking" influence of inhibitory factors and not necessarily excess stimulation. The addition of MIP-1α around the time of feeeding to CML-LTBMC does not produce any antiproliferative effect on the primitive CML granulopoietic and erythroid progenitor cells in the adherent layer (Eaves et al., 1993a). However, in parallel cultures an identical dose inhibits their normal counterparts. This loss of MIP-1α sensitivity is in marked contrast to that for TGF-β, which specifically and reversibly arrests those primitive (not mature) CML progenitor cells and normal progenitors equally well. This aberrant response of CML progenitor cells to MIP-1α suggests the presence of a signalling pathway that is presumably blocked or altered by the action of the BCR-ABL gene product. With the recognition that MIP-1α enhances self-renewal (Lord et al.,1995, Verfaille et al., 1994) it is tempting to speculate that the dramatic reduction in the self-renewing capacity of CML progenitors (Eaves et al., 1993b) is also perhaps, related to the loss of MIP-1α sensitivity.

2.1d. Cell Cycle Modification - Potential Clinical Models

The use of suicide techniques with S-phase cytotoxic drugs, as a means of evaluating potential proliferation inhibitory action, serendipitously provided a model of chemoprotection that has been both recognised and actively pursued. The practicality of this approach was first demonstrated in vivo by Lord and Wright (1980) in an experimental model using repeated treatments with hydroxyurea (HU). Hydroxyurea is a cytotoxic drug that specifically kills cells in DNA synthesis and blocks further entry of non-S-phase cells into DNA synthesis for about 4 hours before a semisynchronised cohort of cells is released into the next period of DNA synthesis (Hodgson et al., 1975). A single dose of HU kills relatively few of the quiescent CFU-S but the more mature cells that are rapidly proliferating are depleted, their loss resulting in recruitment of the CFU-S population into DNA synthesis. A second dose of HU in mid-S phase (about 7 hours) was then used to kill a large proportion of proliferating CFU-S with recovery to normal steady state numbers occurring in about 7 days (fig. 6) (Lord et al., 1992). The addition of MIP-1α, as a CFU-S-specific proliferation inhibitor, theoretically should prolong the HU-induced block and therefore protect the CFU-S population against the second dose of the cytotoxic drug. In mice administered MIP-1α the CFU-S recovery rate exceeded that observed in mice treated with HU alone (Lord et al., 1992). Recovery to normal or supranormal levels was complete in 5 days and the use of optimal doses offered full protection. A similar study by Dunlop et al. (1992) using cytosine arabinoside, another S-phase cytotoxic agent, confirmed this protective role over the progenitor cells. Furthermore,

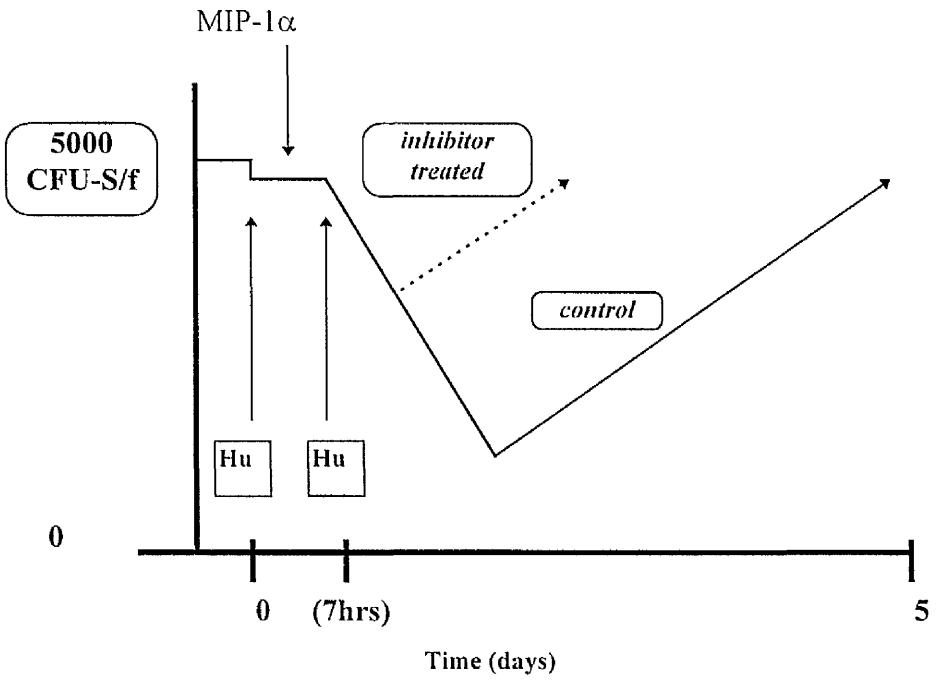


Figure 5. Schematic representation of the CFU-S (stem cell) loss after S-phase cytotoxic drug administration and the potential enhancement of recovery following the use of a stem cell proliferation inhibitor to hold the stem cells out of cycle and thereby protect them from subsequent cytotoxic treatment.

they also described an earlier neutrophil recovery, presumably led by the increased progenitor pool size.

The more rapid recovery of haemopoietic cells, following cytotoxic insult, should allow escalation of the delivered dose intensity which ultimately may improve tumour response and possibly patient survival.

The application of haemopoietic stem inhibitors to clinical practice offers exiting potential if the effects can be reproduced with all classes of cytotoxic drugs and not just S-phase cytotoxics. The intimate relationship between proliferation inhibition and stem cell self renewal has been outlined in an earlier section. Hints from the preliminary Ara-C/NBME-IV protection experiments in vitro suggested that during the recovery phase, inhibitor-treated cultures outperform the controls (Lord et al., 1987). Similarly, more rapid regeneration of the CFU-S population followed suboptimal protection treatment from Hydroxyurea in vivo and it was speculated that secondary effects on the self renewal and differentiation patterns of early stem cells might be involved (Lord et al., 1992). Subsequent experiments, measuring the generation of secondary CFU-S in spleen colonies, confirmed both that the improved self-renewal quality of a post-MIP-1α–treated, regenerating CFU-S population is improved (Lord, 1995) and that human LTC-IC's in LTBMC are maintained better when the cultures are treated with MIP-1α (Verfaille et al., 1994). These results provided the rationale for investigating the use of MIP-1α as a chemoprotectant and stem cell self-renewal enhancer following non-S-phase cytotoxics and radiation therapy.

2.1e MIP-1α : Dose and Duration of Activity

A number of studies have evaluated the dose and duration of action of MIP-1α in vivo. Clements et al., (1992) pretreated mice with phenylhydrazine for 7 days followed by an intravenous injection of MIP-1α 4.5 hours before BM was harvested for CFU-S cell cycle analysis. MIP-1α (5 to 20μg/mouse) reduced the percentage of CFU-S in DNA synthesis from 48.8 to 20.2-28.9%. No inhibition was observed using doses of less than 2.5μg/mouse. Lord et al. (1992) investigated the dose and schedule of MIP-1α in a murine model where HU was administered (t_0 and t_7 hours) with varying doses of MIP-1α (injected i.v. at t_3 and t_6 hours). Optimal CFU-S protection was observed using 5-15μg/mouse administered at both time points, however, injections of up to 15μg/mouse, at either t_3 or t_6 resulted in no protection or only intermediate protection, respectively. In a similar model utilising Ara C, another S-phase specific cytotoxic drug, Dunlop et al. (1992) confirmed 10μg/mouse to be an effective dose when injected i.p. at t_3 and t_6 hours - CFU-S survival at day 2 increased from 56% to 80.1%. The in vivo activity of MIP-1α was also assessed in 2 further models of BM regeneration following 5FU or phenylhydrazine (PHZ) (Dunlop et al., 1992). In these, MIP-1α (10μg/mouse) injected i.p., 4 hours before BM harvesting, reduced the percentage of CFU-S in S-phase from 48.1% to 9.5% and 38.1% to 2.1% following 5FU and PHZ, respectively.

Whilst consistent effects on CFU-S have been reported, the most primitive stem cells may not be sensitive to the inhibitory actions of MIP-1α. When administered subcutaneously (3μg/mouse/day, twice daily for 7 days), MIP-1α failed to prevent

the loss of LTR cells following repeated 5FU (injected day 0 and day 5)(Quesneaux et al, 1993), however, this dose and schedule was active as revealed by a 70-90% reduction in the number of BM CFC's and CFU-A at day 7.

In summary, these studies suggest that MIP-1α is active in vivo over the 3-20μg/mouse dose range and following the i.v, i.p and s.c. routes of administration . The regeneration models (Lord et al., 1992, Dunlop et al., 1992) suggest a minimum dose of 2.5μg/mouse and an optimum dose of 10μg/mouse. None of the studies confirmed an upper dose limit. The study reported by Quesneaux et al (1993) revealed a biological effect at the lower dose of 3μg/mouse using a repeated twice daily injection schedule although the target cell was less clear in that report and one might have anticipated a reduction in LTR recruitment if this dose schedule had inhibited CFU-S proliferation.

2.1f The MIP-1α Receptor(s)

The pleiotropic action of MIP-1α in inflammation, chemotaxis and haemopoietic cell cycle regulation has understandably led to considerable difficulties in isolating the MIP-1α receptor(s). The diverse effects of the molecule and overlapping actions with several related chemokine members suggests that several receptors may exist, each specific for a given effect.

Oh et al. (1991) were the first to identify a receptor for MIP-1α on the T-cell line, CTLL-R8 and LPS-stimulated RAW 267.7, a macrophage cell line. Scatchard analysis, indicated a single class of high affinity receptor with approximately 1200 binding sites per Con A-stimulated CTLL-R8 cell and approximately 380 binding sites/ RAW 264.7 cell. Additional receptors for MIP-1α have since been described on

human monocytes (Wang et al.,1993), polymorphonuclear leucocytes (Goa et al., 1993), basophils (Bischoff et al.,1993), eosinophils (Van Riper et al.,1994), and the HL60 leukaemia cell line (Van Riper et al., 1994). These receptors, however, do not appear to be unique for MIP-1α, having similar binding affinities with other members of the chemokine family including, RANTES and monocyte chemoattractant protein-1 (MCP-1/MCAF) (Wang et al., 1993, Goa et al., 1993, Bischoff et al., 1993, Van Riper et al., 1994). MIP-1β, the second component of the MIP-1 doublet, also shares a common receptor with MIP-1α (Wang et al., 1993), and it is this, together with similar binding affinities for both MIP-1α and MIP-1β, that presumably account for the reported antagonism of MIP-1β on MIP-1α's proliferation inhibitory properties.

The search for MIP-1α receptors on haemopoietic stem cells is limited by their extremely low concentration in the tissue and the heterogeneous nature of this population. The murine multipotent FDCP-MIX cell line, however, possesses MIP-1α receptors that appear to be specific for MIP-1α and its related inhibitory chemokines (Graham et al., 1993). In contrast, the MIP-1α receptor on the human myeloerythroleukaemic cell line, K562, has a much wider binding repertoire suggesting distinct receptors for inflammatory mediation and proliferation inhibition.

The lack of a readily available MIP-1α receptor bearing cell has hindered the flow of information on the signal transduction pathways that are linked to receptor binding. Furthermore, the elucidation of these pathways may be hampered by the variable response elicited by MIP-1α on different cell populations. Evidence is available implicating both Rb and c-myc, in the cellular response to TGF-β (Pietenpol et al., 1990) but there is currently no information concerning oncogene expression with MIP-1α. Preliminary work on anti CD3-stimulated T-cells indicates that MIP-

1α-induced inhibition is associated with decreased phosphorylation of MAP kinase, reduction of $p56^{ICK}$ autophosphorylation, and elevation of diacyl glycerol levels (Zhou et al., 1993). The significance of these findings is still to be determined.

The sparsity of data on mechanisms of inhibition with all the proliferation inhibitory molecules ultimately means the subject remains open to speculation. Growth factor antagonism, via receptor downregulation, or a reduction in the intracellular synthetic pathways for growth factors, may represent possible mechanisms. IL-2 is known to be a T-cell autocrine growth factor and downregulation of both IL-2 mRNA and receptors has been documented following MIP-1α binding to these cells (Zhou et al., 1993). The molecular interactions of the other proliferation inhibitors are equally vague and little information is available to account for the apparent functional overlap. Receptor sharing between these molecules, however, appears unlikely as all are structurally dissimilar. TGF-β was shown to downregulate MIP-1α receptor numbers of FDCP-MIX A4 cells without a change in the affinity of remaining receptors (Graham et al., 1993), suggesting that MIP-1α may merely be a weak contributor to the overall physiological inhibition of stem cells. However, this is difficult to justify in the light of LTBMC data that reveal a dramatic increase in progenitor cell proliferation with MIP-1β and anti MIP–1α antibodies despite the likely presence of physiological concentrations of TGF-β (Eaves et al., 1993a).

The cellular response triggered by MIP-1α binding is associated with a rapid change in cytosolic free Ca^{2+} (Goa et al., 1993, Bischoff et al., 1993, Van Riper et al., 1994, Sozzani et al., 1993) and that this is sensitive to pertussis toxin. This finding, indicating activation via a G-protein-coupled receptor(s) (Bischoff etal., 1993, McColl et al., 1993, Neote et al., 1993), was confirmed by Gao et al. (1993) who cloned the

cDNA for the human PMN receptor. The gene for the receptor was functionally expressed in xenopous oocytes and mapped to human chromosome 3p21. The receptor was characterised as a seven-transmembrane spanning receptor belonging to the superfamily of G-protein-linked receptors that includes the related IL-8 receptor. Interestingly, the open reading frame US28 of human cytomegalovirus (CMV) encodes a protein that is similar in sequence to the MIP-1α/RANTES receptor, hinting at a link with human CMV infection and viral replication (Goa et al., 1993).

2.1g Effects on Non-haemopoietic Tissues

The spatial organisation and growth regulatory mechanisms concerning the pluripotent stem cell are not unique to haemopoiesis, but are mirrored in other self renewing tissues including epidermis, gut mucosa, and seminiferous epithelium. Although the feedback systems are less well defined, there is some evidence that feedback regulators such as MIP-1α may function as pan-stem cell inhibitors without true tissue specificity. Several investigators have hinted at inhibitory effects of MIP-1α on colonic epithelial cells but this is largely unsubstantiated (Graham and Pragnell, 1992, Lord et al., 1993). The recognition that MIP-1α transcripts are present in epidermal Langerhan's cells suggests a further potential role in the regulation of keratinocyte proliferation (Parkinson et al., 1993). This hypothesis is strengthened further by the knowledge that Langerhans' cells originate from haemopoietic tissue and have a special spatial relationship to the keratinocyte stem cell of the epidermal proliferative unit (Parkinson et al., 1993, Breathnach, 1991). Parkinson et al. (1993) documented an inhibitory effect of MIP-1α on keratinocyte proliferation in vitro using partially purified recombinant murine MIP-1α, but it could not be reproduced using

pure bacterially produced MIP-1α. Furthermore, the addition of antimouse MIP-1α neutralising antibody did not stimulate the epidermal keratinocytes throwing into doubt a physiological role for MIP-1α in maintaining keratinocyte stem cell quiescence.

The seminiferous epithelium provides an alternative model to study mechanisms of stem cell proliferation and differentiation and is the only mammalian tissue that contains both mitotic and meiotic cell cycles. Work by Hakovirta et al. (1994) suggests that MIP-1α may be a local regulator of both mitotic and meiotic DNA synthesis during spermatogenesis. Once again the molecule appeared to have a bidirectional effect and, as with haemopoiesis, the most sensitive phases - intermediate spermatogonia - were inhibited. Paradoxically, the most primitive type A_{2-4} spermatogonia were stimulated.

At the present time, the role of MIP-1α in the regulation of non-haemopoietic stem cell proliferation is unclear. Further investigation is warranted, particularly as stem cell inhibition may represent a novel therapeutic option for ameliorating non-haemopoietic toxicity following cytotoxic therapy.

2.2 Other Inhibitors

The recognition that feedback inhibition represents the fundamental mechanism underlying growth regulation has shifted the emphasis from stimulatory factors to putative cell cycle inhibitors. Initial difficulties with isolation, characterisation and experimental confirmation have now been largely overcome. Consequently there has been a rapid escalation in the number of possible candidates that may act as physiological inhibitory regulators (see Table 3). Given the complexity of the

haemopoietic system, and the multiple levels of haemopoietic progenitor cell development, is it perhaps not unexpected that several factors are required to regulate proliferation and influence differentiation and self-renewal. A similar scenario is clearly evident with the haemopoietic growth factors (HGF's) in which multiple factors are involved in proliferation and differentiation decisions at the stem cell level. The increasing number of inhibitory molecules highlight the considerable functional overlap and suggest a certain degree of redundancy. Our understanding of these interactions is in its infancy and, in some ways, is compounded by the literary explosion that reveals paradoxical and conflicting effects of specific molecules. This confusion is partly due to experimental design. In many cases, it may simply be an in vitro phenomenon but, equally, it may represent the normal situation with target cell responses varying according to cell-cell and cell-cytokine interactions.

Several inhibitory factors have undergone extensive preclinical study and appear to be further candidates for physiological regulators of stem cell proliferation. Two of these, AcSDKP (Lenfant et al., 1989) and pEEDCK (Paukavits et al., 1982), are oligopeptides with high specificity for haemopoietic tissues, the third, TGF-β (Keller et al., 1990), is a glycoprotein that is both ubiquitous and pleiotropic in nature.

pEEDCK

The pentapeptide (pEEDCK) was first isolated in 1982 (Paukovits et al.) as a product of mature human leucocytes and as a natural development of the work that first described a granulocyte chalone (Rytomaa and Kiviniemi, 1968a). Its cellular specificity is wider than that of MIP-1α, extending at least to the more mature progenitors including GM-CFC (Laerum et al., 1984) in one direction and to the more

Table 3. Proposed physiological inhibitors of haemopoiesis.

- Macrophage inflammatory protein-1α
- Transforming growth factor-β
- Haemoregulatory pentapeptide (HP5b, pEEDCK)
- Haemoregulatory tetrapeptide (AcSDKP, Seraspenide, Gorolatide)
- Negative regulatory protein (superoxide dismutase)
- Tumour necrosis factor
- Interferon (α, β, γ)
- Glutathione
- Lactoferrin
- Isoferritins

primitive stem cells in the other direction (Paukovits et al 1991b). The peptide increases survival in mice following lethal doses of cytosine arabinoside (Paukovits et al.,1991a), and in a more clinically relevant model was associated with a reduced duration of neutropenia following cytotoxic therapy (Paukovits et al., 1991b). The pEEDCK monomer is easily oxidised to a dimer that paradoxically has colony stimulating activity (Paukovits et al., 1991a, Laerum et al., 1988). Combination experiments indicate that the sequential use of monomer and dimer can completely abrogate chemotherapy-induced neutropenia (Paukovits et al.,1991a, Laerum et al., 1988).

2.2b AcSDKP

In 1977, Frindel and Guigon isolated a stem cell inhibitor from fetal calf bone marrow. The inhibitor has subsequently been identified as the tetrapeptide, AcSDKP, and was the first member of this class of agent to enter clinical trial (Carde et al., 1992). AcSDKP is derived from thymosin β-4 (Grillon et al.,1990) and is synthesised endogenously in LTBMC (Wdzieczak-Bakala et al., 1990). Its inhibitory effect is thought to be indirect and may even function via MIP-1α (Cashman et al., 1994). Experimental models have confirmed protection against S-phase-specific drugs by preventing recruitment of stem cells into cell cycle (Guigon et al.,1980, 1990) in addition to survival benefits in cyclophosphamide-treated mice (Bogden et al., 1991) and in vitro protection against 3'-azido-3' deoxythymidine (AZT) (Grillon et al., 1994). The first phaseI/II study with AcSDKP (referred to as Seraspenide/Goralotide in clinical studies) was undertaken in 1991 in cancer patients receiving two

consecutive cycles of monochemotherapy (cytosine or ifosfamide) (Carde et al., 1992). Tolerance was excellent and a protective effect was demonstrated when comparing both leucocyte area under the curve (AUC) and the duration and depth of neutropenia. The study highlighted a number of problems with the introduction of these agents—that of trial design and endpoint. Given the excellent tolerance of these agents and specific effects on cell cycling, conventional phase I dose-finding studies may be relatively uninformative. Furthermore, investigation of efficacy using cross-over studies may be comprimised by the 'carry over' effect from the previous cycle.

2.1c Transforming Growth Factor-β

TGF-β has wide reaching effects that are determined by the target cell and local environment (Roberts et al., 1985). It arises ubiquitously in the body but in an inactive form. In addition to haemopoietic stem cell inhibition (Keller et al., 1990, Migdalska et al., 1991), TGF-β also plays a leading role in cellular differentiation, wound healing and extracellular matrix formation (Massague, 1990) presumably achieving specificity by local activation of the latent molecule at the site of action. TGF-β was shown to prevent stem cell exhaustion in a cytotoxic model using SCF (Molineux et al.,1994) but its toxicity and pleiotropic nature are likely to prove major obstacles in clinical development. A significant advantage arises from the extension of its inhibitory effects on other clinically relevant tissues. The ability to reduce oral mucositis (Sonis et al., 1994), if confirmed, will have considerable clinical inpact and, given the escalating use of peripheral blood stem cells for transplantation purposes, may prove far more rewarding than haemopoietic protection.

2.2d Comparisons

A number of investigators have attempted to compare several inhibitors using identical experimential conditions (Bonnet et al., 1995, Keller et al., 1994). Generally, these studies have confirmed a high degree of functional overlap between inhibitory factors but they have also highlighted subtle differences in dose-response and cell specificity, suggesting that each factor may have a very specific role. Several molecules, including TGF-β, Tumour Necrosis Factor α (TNFα) and Interferon (IFN) have profound effects on various tissues and at all levels of cell differentiation. These actions may well represent a non-specific activation of the cytokine cascade. Other inhibitiors, such as MIP-1α and AcSDKP have a more restricted spectrum of activity suggesting a more specific physiological role for these molecules in cell cycle regulation.

3. Modulation of Tumour growth by proliferation inhibitors

Even if neoplastic cells can still produce one or more of the feedback inhibitors the question arises as to whether they retain sensitivity to these molecules or, alternatively, develop resistance in the form of a novel mechanism that enables the transformed cells to maintain a growth advantage over their normal counterparts. Over 20 years ago Bichel (1972) showed that the growth of the Erlichs ascites tumour was limited by a factor in its own ascitic fluid. Cell-depleted ascitic fluid from a tumour-bearing mouse had a specific antiproliferative effect and was shown to block the entry of cells into mitosis. It would appear, therefore, that some tumours do continue to regulate their own growth rate. However, it is also possible that the inhibitory factor present in the ascites was of host origin and not tumour derived.

Despite Bichel's findings, it is likely that tumour cells are intrinsically more resistant to feedback inhibitory signals than their normal counterparts and that this, at least in part, accounts for the unregulated proliferation observed in malignancy. Rytomaa and Kiviniemi (1968b) found that Shay chloroleukaemia cells generate large quantities of the granulocyte chalone but are themselves considerably less sensitive to its effects. It remains to be seen whether the relatively tissue specific inhibitors such as MIP-1α have any growth modulating effect on tumours of varied histiogenesis.

A number of limited studies have evaluated the antiproliferative effects of the haemopoietic stem cell inhibitors on neoplastic cells. The majority of inhibitors have not resulted in any growth modulation. However, the conclusions have been based largely on in-vitro effects of inhibitors on a spectrum of haemopoietic and non-haemopoietic cell lines. The pEEDCK monomer had no effect on the MCF7 human breast carcinoma cell line or the GaMg human glioblastoma cell line (Laerum et al.,1990). A modest inhibition was seen with the Erhlichs ascites tumour using higher doses (10^{-7}M) than those required for haemopoietic progenitor cell inhibition. The pentapeptide also inhibited growth of the HL60 leukaemia cell line but, surprisingly, this did not modify the leukaemia's response to cytosine arabinoside which is supposedly S-phase specific (Paukovits et al., 1990). Similarly, the tetrapeptide, AcSDKP, does not appear to inhibit tumour cell growth in vitro, but once again the experimental evidence is extremely limited. Guigon et al. (1991) found AcSDKP had no effect on the leukaemia cell lines HL60 or K562. Neither was it inhibitory in LTBMC to leukaemic cells from CML patients (Cashman et al., 1994).

The effect of MIP-1α has been evaluated using solid tumour cell lines and leukaemia cells, with variable results. Lord et al. (1987) first reported that a

potentially leukaemic haemopoietic stem cell line (IL-3 independent A4-17H cells) was highly reistant to the inhibitory effects of NBME-IV. This was in contrast to clear inhibition of the corresponding IL-3-dependent line from which the independent line was derived. In a further model, L1210 lymphoid leukaemia cells were cocultured in LTBMC with normal bone marrow (Tsyrlova and Lord, 1989). Under these circumstances the L1210 cells dominated the culture, suppressing normal haemopoiesis. Concurrent treatment with NBME-IV and cytosine arabinoside resulted in specific cytotoxicity to the leukaemia cells and the remergence of normal marrow-like culture. More recently, leukaemia cells from patients with CML were shown to be MIP-1α resistant in LTBMC (Eaves et al., 1993a, 1993b, Holyoake et al., 1993). The differential toxicities on normal and leukaemia cells in the L1210 model and CML suggest that combined chemotherapy and inhibitor protocols may be benificial in clinical practice, MIP-1α providing protection for the normal stem cells while specifically targetting the malignancy with a cytotoxic agent. Despite the potential benefit in CML, a degree of caution is still required; MIP-1α resistance may not extend to all leukaemia subtypes. Recently Ferrajoli et al., (1994) reported that MIP-1α prevented AML progenitor cells (variable FAB subtypes) from entering DNA synthesis in a significant number of patients. While conflicting, these findings are perhaps not surprising given the marked heterogeneity in the response of leukaemic cells to growth factors and cytotoxic agents. Clearly, further experimental studies are necessary to elucidate fully the effects of MIP-1α on haemopoietic-derived malignancies.

Non-haemopoietic tumour cell lines do not appear to be sensitive to MIP-1α, at least in vitro. The clonal growth of a wide spectrum of solid tumour cell lines

remained unaffected by continuous exposure to MIP-1α (Korfel et al., 1994). The report, however, failed to confirm the activity of the MIP-1α on normal cells.

The mechanism(s) underlying differential sensitivity between normal and tumour cells are far from clear and presumably exist at multiple levels. Alterations in the delicate balance between stimulatory and inhibitory growth regulatory signals may represent one mechanism. Thus, protooncogene overexpression may act to enhance the growth stimulus and override the feedback inhibitory circuit. Alternatively, loss of tumour suppressor genes would result in a similar scenario. A major regulating step governing the action of TGF-β occurs at the level of activation of its latent, precursor molecule. Like the Shay Chloroleukaemia cells which continue to generate, but not respond to, their inhibitory factor, so some epithelial carcinoma cell lines exist that continue to secrete latent TGF-β, and bear receptors for the molecule, but they have lost the ability to activate the inactive molecule (Keski-Oja et al.,1987).

In addition to the variable effects of inhibitors on normal and transformed cells, neoplasms may also respond differentially to individual inhibitory molecules. CML is associated with a specific genetic rearrangement, BCR-ABL, that encodes a protein with a number of novel properties including tyrosine kinase activity (De Klein et al., 1982, Konopka et al., 1984). This fusion gene is specific for CML and almost certainly plays a primary role in the pathogenesis of the disease and its hyperproliferative state. It is therefore interesting to speculate on the link between MIP-1α resistance and the fusion gene product. The continued response to TGF-β exhibited in the same leukaemic cell suggests that the two inhibitors act through different signal transduction pathways. Alternatively, the BCR-ABL product may

interfere with the action of MIP-1α at a site proximal to its convergence with the pathway that delivers the antiproliferative signal initiated by TGF-β.

In summary, neoplastic transformation appears to be associated with loss of sensitivity to inhibitory molecules despite the continued production of these factors by the tumour cells. The mechanisms underlying this loss of sensitivity are far from clear but a greater understanding may allow the development of a more targetted approach towards malignancy. From a clinical viewpoint, the current evidence does suggest that inhibitors, with the possible exception of TGF-β, are unlikely to modulate the cytotoxic effect of chemotherapeutic agents on cancer itself but that differential sensitivities of normal and neoplastic tissue to them may allow them to be used as a means of protecting normal cell populations during periods of cytotoxic therapy.

4. Clinical Perspectives

Enhanced production of inhibitory factors may account for a number of clinical observations that are relevant to both malignant and non-malignant diseases. Under normal conditions, human bone marrow nucleated cells, express low levels of MIP-1α mRNA (Maciejewski et al., 1992). However, there is a very significant increase in the level of MIP-1α transcripts in patients with aplastic anaemia and myelodysplasia (Maciejewski et al., 1992). It is perhaps possible, therefore, that an exaggerated production of MIP-1α plays a role in the underlying pathophysiology of the bone marrow suppression. Similarly, it appears likely that certain inhibitors are responsible, at least in part, for malignancy-associated bone marrow failure, e.g. TGF-β and CLL (Stryckmans et al., 1988, Israels et al., 1991, Lagneaux et al., 1993).

4.1 Bone Marrow Protection

Experimental studies and clinical trial data have shown that a tumour response to chemotherapy is directly proportional to the delivered dose (Skipper, 1967, Frei and Canellos, 1980, Hryniuk, 1988). More precisely, the response is related to the *intensity* of delivery when expressed as per unit of time (dose/m^2/week). This concept of dose intensity is fundamental to treatment design and ultimately may represent the major variable in tumour response and survival. The major dose limiting toxicity of cancer chemotherapy, irrespective of mode of action, remains bone marrow damage. Neutropaenia and thrombocytopaenia may result in significant patient morbidity and potentially may prevent the delivery of curative chemotherapy regimens. Most of the clinically relevant chemotherapeutic agents produce reversible myelosuppression as a consequence of their toxicity on the rapidly dividing progenitor cell compartment. The reduction in progenitor cell numbers is reflected by an attenuated feedback inhibitory stimulus to the normally quiescent pluripotent stem cell pool. Recruitment of these latter cells into DNA synthesis then replenishes the maturing populations with ultimate normalisation of the blood picture. Unfortunately, the return to stem cell quiescence does not appear necessarily to mirror the recovery of mature cell numbers (Lord, 1988) - an index that historically has guided the decision for retreatment. At present, treatment programmes are based on multicyclic schedules that almost certainly produce incremental damage to the more primitive, self renewing haemopoietic precursor cells and this is borne out by the clinical observations of delayed neutrophil recovery and increasing toxicity on megakarycytopoiesis.

Ultimately this damage may produce a picture of delayed bone marrow failure, dysplasia or secondary acute myeloid leukaemia (Testa et al.,1990).

One approach to the problem was the introduction of haemopoietic growth factors to promote accelerated production of peripheral blood cells. This has allowed a modest increase in dose intensity but typically less than twofold. The ability to accelerate the delivery of chemotherapy with G-CSF or GM-CSF has resulted in a greater response rate but it is unlikely, given the limited improvement in dose intensity, that this will be reflected by a significant improvement in survival or remission duration. Furthermore, this approach is hampered by increasing thrombocytopaenia and additional dose-limiting toxicities, including mucositis. Experimental studies also suggest that the accelerated delivery of chemotherapy with growth factor support may increase stem cell damage with the attendant risk of late complications (Hornung and Longo, 1992).

The functional assessment of proliferation inhibitors is based on their ability to reduce cell death from S-phase-specific agents such as tritiated thymidine. This evaluation points to a potential role for stem cell inhibitors as chemoprotective agents. Based on the principle illustrated in figure 5, murine models, utilising MIP-1α in combination with hydroxyurea or cytosine arabinoside have confirmed this protective effect in vivo (Lord et al., 1992, Dunlop et al., 1992). MIP-1α–treated mice showed a faster recovery of the CFU-S population and an earlier normalisation of neutrophil numbers. This mode of therapy, therefore, not only provides an alternative method for escalating dose intensity but also has the advantage of maintaining stem cell numbers and viability, which if confirmed with other cytotoxics, could result in less

cumulative toxicity on bone marrow precursor cells and possibly reduce the risks of long-term bone marrow damage (and perhaps second malignancy).

Many of the clinically useful cytotoxic agents, however, are not specific for cells in DNA synthesis, but target cells largely irrespective of their cycle status. It therefore remains to be seen whether proliferation inhibition will have any useful therapeutic impact in this setting. Further experimental investigation is required but several observations suggest that there may be a therapeutic benefit for protection against all classes of cytotoxics. Non-S-phase- specific drugs such as doxorubicin and cyclophosphamide produce a greater cell kill when cells are synchronised in DNA synthesis (Dewys et al., 1970, Kim and Kim, 1972). Furthermore, the qualitative damage induced by alkylating species is potentially reparable (Sancar and Sancar, 1988) if the cell has sufficient time to carry out gene housekeeping functions before it enters DNA synthesis. While rather esoteric, these findings suggest that cell cycle inhibition may at least attenuate the toxicity of non-cycle specific agents and may limit the propagation of sublethal mutational damage.

At present there are only limited experimental data on the use of inhibitors and non-S-phase-specific drugs. The tetrapeptide, AcSDKP, has allowed a modest increase in survival of mice receiving lethal doses of doxorubicin (Ramirez et al., 1994) or cyclophosphamide (Bogden et al., 1991). The mechanism of this advantage has still to be elucidated, however, the recent observations that MIP-1α also increases stem cell self-renewal (Lord, 1995) is particularly relevant and may signify a novel mechanism (? for all inhibitors) for enhancing stem cell recovery post chemotherapy irrespective of the mode of cytotoxicity.

The increased use of intensified chemotherapy with haemopoeitic growth factor support has highlighted the problem of additional dose-limiting toxicities on other self renewing tissues. Ulcerative stomatitis or mucositis is a common, painful condition that frequently accompanies chemotherapy. Increasingly, this has become the dose limiting toxicity for a number of chemotherapy regimens. The aetiology of mucositis is probably related to the cytotoxic effect of chemotherapy on the rapidly dividing cells of the basal oral epithelium. The development of an epithelial proliferation inhibitor might therefore afford protection to the oral and gut mucosa in a fashion similar to that for the haemopoietic inhibitors. Sonis et al., (1994) found that topical application of TGF-β3 reduced the fraction of oral epithelial cells undergoing DNA synthesis so that when applied prior to chemotherapy, the inhibitor resulted in a significant reduction in the incidence, severity and duration of mucositis.

A limited number of observations suggest MIP1-α may also possess growth modulating properties on epithelial tissues (Graham and Pragnell, 1992, Lord et al., 1993). The recognition that MIP1-α may also influence DNA synthesis of spermatogonia (Hakovirta et al., 1994) hints at an intriguing mode of protection for limiting gonadal damage during cancer chemotherapy.

4.2 Mobilisation of Haemopoietic Progenitor Cells by MIP-1α

During the course of investigating the myeloprotective effects of the MIP-1α analogue BB-10010, Lord et al. (1995) observed an unexpected transient mobilisation of leucocytes giving a 4-fold increase over baseline cell numbers within 30 minutes. This was mirrored by progenitor cell release into the peripheral blood. A single subcutaneous administration of BB-10010 doubled the circulating CFU-$S_{(8)}$, and

$CFU\text{-}S_{(12)}$. When evaluated after 2 days of G-CSF priming, a single administration of BB-10010 increased circulating $CFU\text{-}S_{(8)}$, $CFU\text{-}S_{(12)}$ and MRA to 38,-33,-100-fold, respectively, again after 30 minutes. Current clinical practice for peripheral blood stem cell transplantation necessitates approximately 5 days of G-CSF for optimal harvesting of sufficient haemopoietic progenitor. Furthermore, the peak effect of this mobilisation is poorly defined, occurring between 24-30 hours post-final G-CSF (Sato et al., 1994). The report by Lord et al (1995) suggests that the combined use of BB-10010 and G-CSF may enhance progenitor cell numbers, improve the quality of the apheresis and allow a more rapid and predictable time course for harvesting.

The mechanism of progenitor release following BB-10010 is unclear but appears to be the result of mobilisation rather than sequestration. MIP-1α is thought to have chemotactic properties on several mature leucocyte subsets and may act via adhesion factor expression. Interestingly, many unrelated chemotactic factors also share this ability to produce an acute leucocytosis, possibly by producing morphological or cytoskeletal changes in the target cells or by modifying surface proteins involved in adhesion (Jagel and Hugli, 1992). Recently, a related chemokine, IL-8 has also been reported to produce acute mobilisation of progenitor cells with remarkably similar kinetics (Laterveer et al., 1995). At present there are no data showing a similar mobilisation potential for other haemopoietic inhibitory molecules.

In conclusion, the current evidence suggests that proliferation inhibitors such as MIP-1α may have a therapeutic role in ameliorating chemotherapy-induced cytotoxicity, at least on haemopoietic tissues. This has been confirmed using S-phase specific drugs but preliminary experiments revealing enhanced stem cell self-renewal with MIP-1α

suggest a greater spectrum of activity with all classes of cytotoxic drug. The lack of an inflammatory response in the myeloprotection models and the absence of any tumour inhibition also suggest an acceptable safety and tolerability profile for clinical investigation.

The objective of this thesis was to extend investigations of the MIP-1α analogue, BB-10010, defining a) the effects on chemotherapy- and radiation-induced myelosuppression using appropriate murine models and b) developing an early clinical trials programme based on those observations. Preliminary clinical trials investigating a) the safety and tolerability of BB-10010 in normal volunteers and cancer patients and b) its protective capacity in combination with cytotoxic chemotherapy.

PART I: LABORATORY STUDIES

Experimental protocols were designed around a series of murine models of BM damage and subsequent haemopoietic regeneration. These studies were undertaken to investigate a) the duration of action of BB-10010 in vivo, b) the myeloprotective properties of BB-10010 when used in combination with non-S-phase specific cytotoxic drugs or sublethal irradiation and c) to delineate any modulating effect of BB-10010 on gut epithelial regeneration following either cytotoxic- or radiation-induced mucositis.

The protocol design, rationale and methodology for each study is described and the results and their interpretation are discussed.

The non-aggregrating variant of MIP-1α was used in all of the preclinical studies and, therefore, the term 'MIP-1α' has been retained throughout and signifies the BB-10010 analogue.

MATERIALS AND METHODS

1. Source of bone marrow

Cell suspensions of murine bone marrow cells were prepared from individual femora and pooled. For standard assays, 3 mice were killed by cervical dislocation and 1 femur was dissected from each mouse. After attached muscle was removed, a minimal amount of bone was trimmed off from each end. A 21 gauge needle was then inserted through the epiphyseal cartilage plate and the marrow was flushed into an appropriate volume of Fischer's medium (typically, 1 femur/2ml). The suspension was then aspirated repeatedly through a 25 gauge needle to ensure a single cell suspension. A

mononuclear cell count was performed on the suspension using an automated cell counter (Sysmex) following application of an erythrocyte lysing solution.

2. Injection technique

2.1 Bone marrow.

Cells were injected into recipient mice (a standard inoculum volume of 0.2ml was used unless otherwise specified) via one of the two lateral tail veins while the mouse was held in a restraining device from which the tail projected.

2.2 Cytotoxic administration.

Unless otherwise specified, cytotoxic agents were administered at the appropriate concentration with a 25 gauge needle using 0.2ml aliquots injected via the intraperitoneal route. In all cases appropriate safety precautions were observed when handling cytotoxic drugs.

2.3 Cytokine administration.

MIP-1α was kindly supplied by British Biotech Pharmaceuticals limited (Oxford, U.K.) as a non-aggregating genetically engineered variant of human MIP-1α (LD78). It is currently known as BB-10010. It was administered either by bolus injection in 0.2ml aliquots (subcutaneously or intravenously) or by continous infusion using a mini osmotic pump (Alzet 2001, California, U.S.A.). Pumps were inserted subcutaneously, under a short acting anaesthetic, on the backs of mice and secured with surgical clips or sutures. Pumps were removed at the completion of the infusion and repeated cycles of treatment involved reinsertion of fresh pumps at the previous

incision site. Mice showing local signs of infection at the surgical site were killed and were excluded from any further analysis.

3. Blood Counts

Blood was collected either from the lateral tail vein or by terminal cardiac puncture under light anaesthesia (ethrane). Blood samples were pooled from the various groups of donor mice with heparin (25u/ml) used as an anticoagulant. Leucocyte counts were performed on an automated cell counter (Sysmex).

4. Spleen Colony Assays

The spleen colony assay was introduced in 1961 (Till and McCulloch) when it was found that bone marrow injected into a lethally irradiated recipient mouse generated colonies of haemopoietic cells in the spleen, each one being derived from a single multipotential progenitor cell. They were designated spleen colony-forming units - CFU-S (figure 6).

CFU-S and primitive cells with Marrow Repoulating Ability (MRA) were assayed as described in detail previously (Lord, 1993). Briefly, mice (groups of 10) were exposed to 15.25Gy ^{60}Co γ-ray irradiation at 0.85Gy/hour. They were then injected with a freshly prepared suspension of bone marrow or whole blood. For this, known fractions of donor femoral marrow were adjusted to generate approximately 10 colonies per spleen from an injected volume of 0.2ml. Similarly, venesected blood was diluted to a final volume such that 0.2mls again should generate approximately 10 colonies per spleen. Eight, ten or twelve days later the recipient mice were killed, their spleens fixed in Tellesniczky's fixative and 24 hours later the number of colonies counted

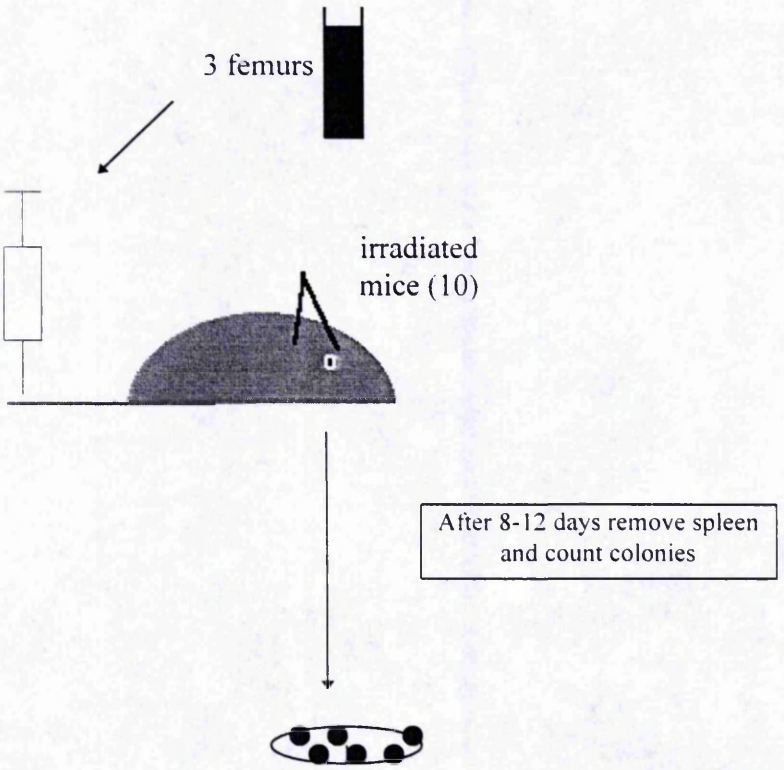
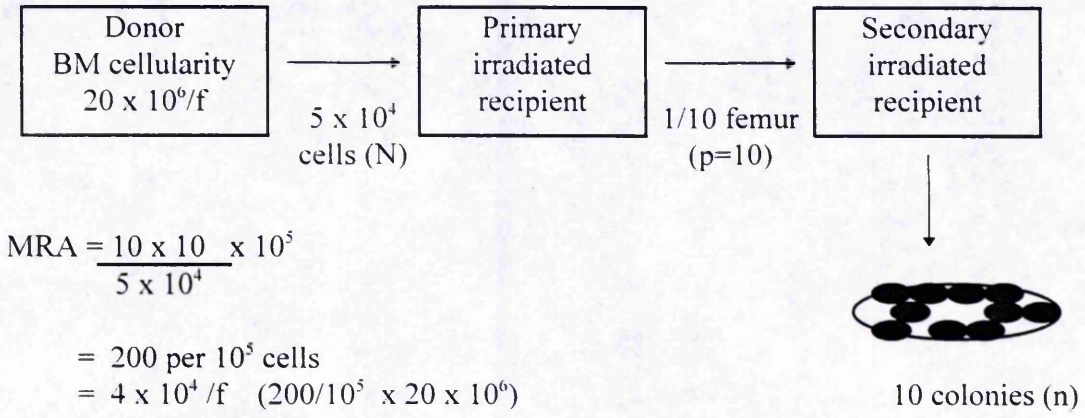


Figure 4. Spleen colony assay. A BM suspension from 3 treated donor mice is injected (0.2ml aliquots) into 10 recipient irradiated mice. After 8-12 days the mice are killed and their spleens fixed.Colonies per spleen are counted and expressed as the means ± 1 s.e.

using a dissecting microscope. Results are expressed as CFU-S/femur ± s.e or CFU-S/ml of whole blood ± s.e.

Marrow repopulating ability is a measure of the more primitive cells that possess the greatest self-renewal capacity (variously termed preCFU-S or MRA). These cells cannot generate a colony within the time limits of the spleen colony assay but can be quantified using a double transplantation technique. Cells with MRA were measured by transplanting haemopoietic tissue from the donor into an additional 5 primary irradiated recipient mice. The transplanted cells were allowed to engraft and develop in the marrow for 13 days before harvesting and transplanting into a second irradiated recipient mouse for a day 12 CFU-S assay. The MRA can be calculated using the formula $(n \times p/N) \times 10^5$, where n = colonies per spleen, 1/p = the fraction of a femur injected into the secondary recipients and N = the number of cells injected into the primary irradiated mice. The result calculated is then expressed as MRA per 10^5 donor cells. More usefully this can be expressed as MRA per femur (or per ml of blood) by multiplying the cellularity of the original donor tissue.

BM MRA

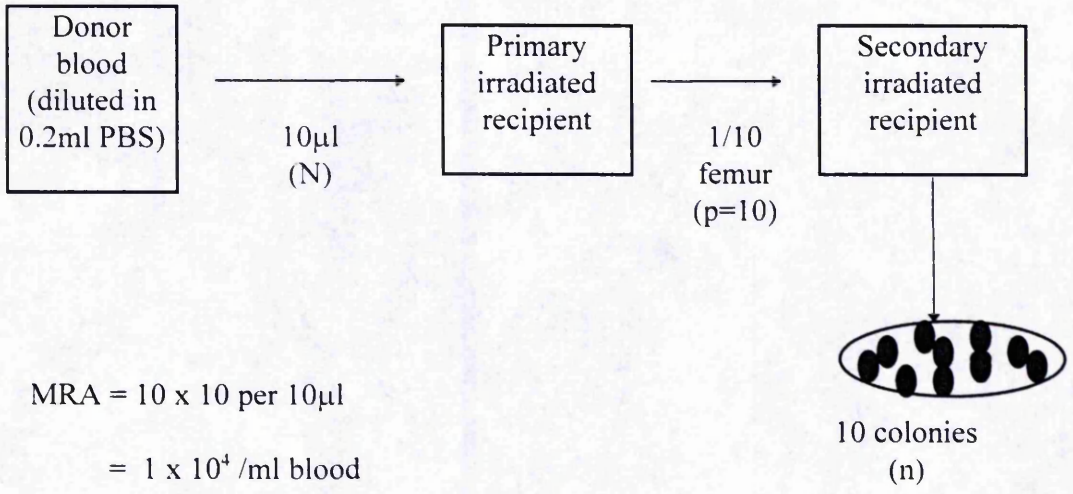


$$MRA = \frac{10 \times 10}{5 \times 10^4} \times 10^5$$

$= 200$ per 10^5 cells

$= 4 \times 10^4$ /f $(200/10^5 \times 20 \times 10^6)$

Blood



MRA = 10 x 10 per 10µl

$= 1 \times 10^4$ /ml blood

Figure 7. Examples of MRA calculation for BM and blood. Data used are guidline figures (not actual experiment) for normal BM and Blood.

5. CFU-S Proliferation.

CFU-S proliferation was assessed using an in vitro tritiated thymidine ([^{3}H] TdR) suicide assay (Lord, 1993). Thymidine is a specific precusor of DNA synthesis and is incorporated during this period. High doses of [^{3}H] TdR results in cell death by intranuclear irradiation thus causing a loss of colony forming potential that is equivalent to the proportion in S-phase.

The cell suspension under investigation was adjusted to a concentration of 5 x 10^6 cells/ml. Two 1ml aliquots were dispensed and then prewarmed at 37°C for 10 minutes. 0.2ml of [^{3}H]TdR (200μCi or 7.4 MBq at specific activity of 740 G8q/mmol in isotonic solution) was then added to one of the tubes and an equivalent amount of 'cold' TdR was added to the second tube. Both suspensions were incubated for 30 minutes and then placed on ice to prevent further [^{3}H]TdR incorporation. The suspensions were then diluted to the appropriate cell concentration and assayed for CFU-S. The percentage of CFU-S in DNA synthesis, i.e. the percentage killed by [^{3}H] TdR, was given by the calculation:

$$\frac{\text{Control colonies - } {}^3\text{HTdR Treated colonies}}{\text{Control colonies}} \times 100$$

Given that the calculation of kill depends on the difference of two colony counts each with a potential standard error of up to 10%, the error of the kill se_k can be very large and is calculated from the formula:

$$se_k = \frac{1}{C}\sqrt{se_T^2 + \frac{(T)^2 \times se_C^2}{(C)^2}}$$

With a standard error of ~ 10% on the colony counts, the kill only becomes significantly different from zero at approximately 12% (Lord , 1993). Kills of < 10% were taken to indicate non-significant proliferation, i.e. quiescence, and of > 20% to indicate a proliferating population.

6. The microcolony assay in mouse intestine.

In 1970 Withers and Elkind described the microcolony assay in mouse intestine, an assay that is analogous to the haemopoietic CFU-S assay but measures the clonal regeneration of gut epithelium. Mice exposed to total body irradiation develop dose related gut damage with early regenerative foci present from 2-4 days. These foci develop a structure that is similar to normal gastrointestinal crypts and are considered to originate from single pluripotent stem cells. The number of crypts thus gives a measure of the surviving clonogenic cells and is presented as an absolute number per circumference of intestine. The size of the regenerating crypt will vary with time after treatment and will influence the probability of a crypt being seen in any given section. A size correction factor is therefore applied with values normalised to take into account the intestinal crypt width of untreated control mice (Potten and Hendry, 1983).

Thus the treated crypt number x control crypt width/ treated crypt width = size corrected number.

Groups of 4 mice were killed by cervical dislocation and their intestines removed in whole. The intestine was first flushed with saline to remove the contents before fixing in Carnoy's fixative (alcohol: chloroform: acetic acid in ratio 6:3:1).The intestines

were then sectioned to produce approximately 10 small segments that were collected together in a bundle and secured with surgical tape. The edges were trimmed with a scalpel and then sectioned and processed using standard histological procedures thus providing 10 sections per animal. These sections then viewed using a high power microscope and the number of surviving crypts determined. Results were size corrected and are expressed as the number of crypts per circumference

7. In vitro colony-forming cell (CFC) Assay.

The in vitro CFC assay defines a range of committed haemopoietic progenitor cells and depends on the development of haemopoietic colonies during incubation in a sloppy agar medium supplemented with growth factors.

A cell suspension was prepared as previously described for the spleen colony assay. From this suspension 5×10^4 cells were added to the plating mixture shown in table 4. The primary mixture was then preheated to 37°C and 0.33ml of 3.3% agar was added. After repeated pipetting, 1ml aliquots were plated in triplicate into sterile petri dishes which were then cooled at 4°C for approximately 5 minutes to allow the agar to gel. The dishes were placed in a humidified incubator, gassed with 5% carbon dioxide and 5% oxygen, and incubated at 37^0C for 7 days. After 7 days, colonies (aggregates of 50 cells or more) were counted using a low power counting microscope and the number of granulocyte/macrophage (GM) -CFC expressed as the mean of the scores obtained from each of the triplicate plates per femur or per ml of whole blood.

Table 4. Constituents of the murine GM-CFC assay.

		% Volume	Volume (μl)
Primary mixture	**murine IL-3**	5%	165
	fetal calf serum	20%	660
	Fischer's medium	55%	1815
	cells	10%	330
	agar (3.3%)	10%	330
	Total	100%	3300

EXPERIMENTAL PROTOCOLS

1. MIP-1α : Duration of action in vivo

The duration of action of MIP-1α was investigated in vivo using a model of BM regeneration that followed a single exposure to total body irradiation. At a specified point during the regeneration phase MIP-1α was administered and the S-phase fraction of the CFU-S population was determined. Groups of 3 mice were irradiated (day 0) with 3.5Gy γ-rays from a caesium-137 source (dose rate 2.5 Gy/min). On day 3, mice were killed and their femoral marrow assayed to calculate the percentage of $CFU\text{-}S_{(8)}$ in DNA synthesis using the thymidine suicide technique. Control mice were irradiated only. Treatment groups received MIP-1α (10μg/mouse) as a single subcutaneous or intravenous injection, between 2 and 24 hours before their BM was assayed.

2. Models of BM Damage

a) Repeated Sublethal Irradiation.

Repeated exposure to sublethal irradiation results in a picture of incremental BM damage in mice with minimal non-haemopoietic toxicity (Hendry etal., 1972) and thus provides a useful model for investigating myeloprotection. A series of experiments was carried out in which mice received 4 cycles of irradiation with or without a protracted infusion of MIP-1α. In these experiments groups of 3 mice received MIP-1α (40μg/mouse/day) or phosphate buffered saline via implanted subcutaneous pumps which ran for 7 days. Pumps were inserted approximately 3-4

hours before mice were exposed to 4.5Gy γ-rays from a caesium-137 source (dose rate 2.5 Gy/min). Spent pumps were removed after 7 days. Groups of 3 mice were killed at 1, 7 and 14 days after irradiation and their BM assayed for cellularity, CFU-S and MRA. This 2 week cycle was repeated 3 more times with mice receiving a total of 4 cycles of irradiation.

In a separate series of experiments the schedule dependency of MIP-1α was also investigated. In these studies, observations were limited to two 14 day cycles in which MIP-1α was dispensed for 7 days from day 0 (3-4 hours before irradiation) to day 7, from day 1 to day 8 post-irradiation or from day 7 to day 14 post-irradiation .

b) Chemotherapy Studies.

The modulating effect of MIP-1α on haemopoietic cell regeneration following non-S-phase cytotoxic drug administration was investigated using the alkylating agents, cyclophosphamide and BCNU. Cyclophosphamide represents a common constituent of combination chemotherapy protocols and its myelosuppressive effects are well described in both humans and mice. Whilst BCNU has a more narrow spectrum of antitumour activity it results in a more specific stem cell defect and thus provides a useful model of chemotherapy-induced BM damage not unlike that described following irradiation.

Cyclophosphamide

2.1 Single cycle cyclophosphamide. Groups of 3 mice received a single intraperitoneal (i.p.) injection of cyclophosphamide (200mg/kg) either alone or with MIP-1α, G-CSF or a combination of both cytokines (figure 8). MIP-1α was

administered as a 7 day continuous infusion (40μg/mouse/day) via an subcutaneous mini osmotic pump inserted 3-4 hours pre-cyclophosphamide treatment. In some cases, recombinant human G-CSF (Amgen, Thousand Oakes) was also injected at a dose of 100μg/kg subcutaneously every 12 hours from day 3 to day 7. BM cellularity and CFU-S were assayed in groups of 3 mice between days 1 and 14. Peripheral white blood counts were made from day 1 to day 10 and the peripherally mobilised progenitor cells assayed from day 4 to day 7.

2.2 Correlation of cylophosphamide sensitivity to CFU-S proliferation. Groups of 3 mice received a single i.p. injection of cyclophosphamide (200mg/kg), or cylophosphamide preceeded 7 hours earlier by hydroxyurea (1g/kg). In a further group, mice received the hydroxyurea/cyclophosphamide schedule and MIP-1α administered as a 24 hour continous infusion (40μg/mouse/day) commencing approximately 3 hours before the HU dose. Femoral BM was assessed for CFU-S_{8+12} on day 1 only.

2.3 MIP-1α scheduling. Groups of 3 mice received a single i.p. injection of cyclophosphamide (200mg/kg) either alone or in association with a variable MIP-1α infusion schedule (40μg/mouse/day for 1, 3, 7, or 14 days). Femoral BM CFU-S_{8+12} were assayed on days 10 and 14. In a further series of experiments to study the same effect in proliferating cells, groups of 3 mice received a single i.p. injection of cyclophosphamide (200mg/kg) 7 hours after a single i.p. injection of hydroxyurea (1g/kg). Groups of mice received the same cytotoxic schedule with and without MIP-1α, administered as a 1 or 3 day infusion (40μg/mouse/day) or s.c. bolus injection

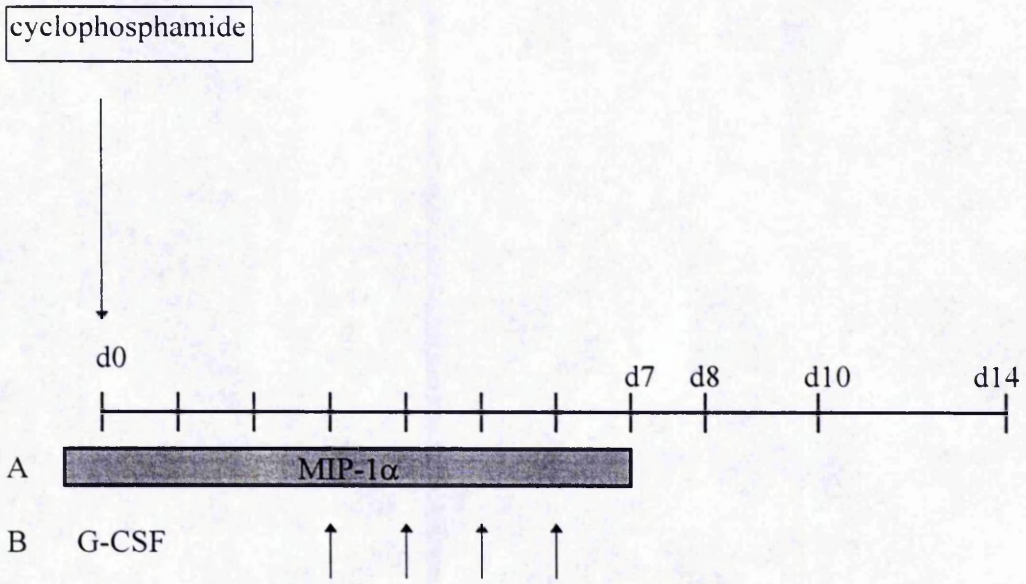


Figure 8. Experimental design for single cycle cyclophosphamide study. Mice received a single i.p. injection of cyclophosphamide with i) a 7 day schedule of MIP-1α, ii) twice daily G-CSF on days 3 to 6 or, iii) a combined A and B. Control mice received cyclophosphamide only.

(10μg/mouse) at 3, 6, 9, and 12 hours. Femoral BM CFU-S were assayed on days 1, 10, and 14. Mini osmotic pumps were inserted approximately 3-4 hours before chemotherapy.

BCNU

2.4 Single agent BCNU. BCNU was reconstituted with sterile water and absolute ethanol to produce a solution of 3.3mg/ml of BCNU in 10% ethanol with a pH of 5.6 to 6.0. Groups of 3 mice received i.p. single injections of BCNU (33mg/kg) in 0.2ml aliquots every 14 days for 4 cycles. MIP-1α (40μg/mouse/day) was administered via a previously (3 to 4 hours) implanted subcutaneously placed mini osmotic pump for 3 days. Control mice received cytotoxic therapy only. Mice were killed on days 1 and 14 of each cycle and their femoral BM assayed for cellularity, CFU-S, and MRA.

2.5 Combined BCNU and cyclophosphamide. Groups of 3 mice received cyclophosphamide (200mg/kg) and BCNU (33mg/kg) every 14 days for 3 cycles. MIP1-α (40μg/mouse/day) was administered for 7 days via mini osmotic pump. Control mice received cytotoxic therapy only. Mice were killed on days 1, 7, and 14 of each cycle and their femoral BM assayed for cellularity and CFU-S.

3. Gut Protection models

A number of authors have proposed that the wild type MIP-1α molecule has a wider spectrum of activity and may in fact act as a 'pan-stem cell inhibitor' (Graham and Pragnell, 1992, Lord 1993). In view of this possibility and the significant clinical

implications, the modulating effect of the disaggragated protein on gut regeneration was investigated using both radiation- and chemotherapy-induced gut damage models.

3.1. Irradiation model. 4 mice per group were exposed to whole body irradiation from a caesium-137 source at one of 3 dose levels, 12, 14 or 16Gy γ-rays (dose rate 2Gy/min). Control mice were injected i.p. with 0.2ml PBS at 24, 8 and 4 hours preceeding irradiation. Active treatment groups received a bolus i.p. injection of MIP-1α (10μg/mouse) at identical time points. At day 4, groups of 3 mice were killed and their small and large intestines removed en bloc. Sections of intestine were fixed, bound and processed as described in the methodology.

3.2. BCNU model. Groups of 4 mice received a single i.p.injection of BCNU (66mg/kg) with or without MIP-1α (40μg/mouse/day) administered for 7 days via a subcutaneous pump. Pumps were inserted approximately 3 hours before chemotherapy. Mice were killed on day 4 and day 7 and their intestines removed for assessment of crypt size and number.

3.3. 5-Fluorouracil model. Groups of 4 mice received 5-Fluorouracil with and without MIP-1α. 5-Fluorouracil was injected at 0 and 6 hours at one of 3 dose levels, 7.5mg, 11.25mg or 15mg/mouse. MIP-1α, (40μg/mouse/day) was administered for 7 days via a subcutaneous pump, inserted approximately 3 hours before chemotherapy. Control mice received chemotherapy only. On day 4 and day 7, mice were killed and their intestines removed for assessment of crypt size and number.

RESULTS

1. MIP-1α : Duration of action in vivo

Femoral BM cellularity measured 4 days after irradiation had recovered to about 50% of normal controls and was unaffected by MIP1-α, administration between 2 and 24 hours before assay (Table 5). The percentage of CFU-S in DNA synthesis was determined under the same conditions of MIP-1α treatment (Table 6). A comparison of the mode of delivery (intravenous route versus subcutaneous route) was also evaluated but in one experiment only. During recovery from radiation CFU-S proliferation in control mice was high with 29-65% of CFU-$S_{(8)}$ in DNA synthesis by day 4. Administration of MIP-1α between -4 and -6 hours consistently decreased CFU-S proliferation to about 18% ($p = 0.001$). When MIP-1α was injected at -2 hours, the response was more variable, perhaps in part depending on control levels for individual experiments, but overall, the apparent inhibition in proliferation was not significant. MIP-1α pretreatment between 8 and 24 hours had little effect on cell proliferation (experiments 1, 3 and 6). Both routes of administration appeared equally effective at 4 hours (and ineffective at 8 hours) although this was only assessed in a single experiment (experiment 6). In vivo therefore, MIP-1α was optimally effective in inhibiting CFU-S proliferation when administered 4-6 hours before assay.

2. Repeated Sublethal Irradiation Model.

The repeated irradiation treatment was conducted in 4 separate experiments with results shown in table 7 and figures 9 to 11. Following irradiation the BM cellularity fell to approximately 20% of normal within 24 hours but recovered to near normal

Table 5. BM cellularity (day 4) following whole body irradiation (3.5Gy γ-rays).

Expt. number	Cellularity(10^{-6}/femur)						
	control	MIP-1α pretreatment times(hours)					
		-2	-4	-6	-8	-12	-24
1	10	-	-	-	11	11.2	10.4
2	8	9.6	11.2	10.2	-	-	-
3	6.2	-	-	-	10.4	8.4	6
4	12.6	12.5	11.6	12.5	-	-	-
5	13.2	13	11.3	13.2	-	-	-
6	9.3	-	10.2(iv) 8.7(sc)	-	10.6(iv) 9.8(sc)	-	-

Control mice were exposed to irradiation only. MIP-1α-treated mice received 10μg/mouse at the specified times before BM was assayed for CFU-S proliferation. MIP-1α was administered s.c. in experiments 1-5.

Table 6. The % CFU-S in DNA synthesis 4 days after irradiation and following MIP-1α.

Timing of MIP-1α (hrs)	% CFU-S in DNA synthesis	Experiment 6 i.v	Experiment 6 s.c.	n
control	46 ± 6	45 ± 4	45 ± 4	6
2	23 ± 12	-	-	3
4	17 ± 1	22 ± 2	14 ± 1	5
6	19 ± 2	-	-	3
8	45 ± 6	53 ± 7	39 ± 3	4
12	48 ± 2	-	-	2
24	45 ± 4	-	-	2

MIP-1α was administered subcutaneously (s.c) at varying time points before the BM was harvested. n = total number of experiments. In experiment 6 MIP-1α administration was compared using the s.c and intravenous (i.v.) routes.

levels by day 14 (Table 7). Successive cycles of irradiation did not result in any progressive fall in cellularity either by day 1 following irradiation or after 14 days of recovery despite a severe and incremental CFU-S deficit (see below).

At day 1, BM CFU-S were reduced to less than 1% of their normal levels and in 14 days, CFU-S_8 and CFU-S_{12}, recovered to 40 and 20%, respectively (figure 9). Repeated cycles of irradiation increased the damage to the marrow CFU-S pool with surviving CFU-S numbers progressively reduced 1 day after irradiation and becoming particularly evident in the day 14 recovery values of each successive cycle (figures 9 and 10). After the fourth cycle of treatment, CFU-S_8 numbers reached only 10% and CFU-S_{12} reached 6% of their starting levels. Mice receiving MIP-1α showed enhanced recovery of CFU-S with an increasingly accentuated effect over successive cycles since, unlike the controls, that recovery was maintained in each cycle. Thus, the maximal recovery advantage with MIP-1α was seen in the final cycle when CFU-S_8 and CFU-S_{12} had recovered respectively to 1500 (compared to 200 in control) and 1200 (compared to 200 in control) per femur. MIP-1α did not appear to have have any direct radioprotective effect in these experiments with similar CFU-S depopulations being observed in both control and MIP-1α-treated groups. Nevertheless, delaying the exposure to MIP-1α until day 1 after irradiation did ameliorate the recovery significantly and delaying exposure until day 7 completely abrogated the advantage (Table 8).

The repeated irradiation schedule was responsible for a severe depletion of the most primitive cells that possess MRA (figure 11). Thirteen day recovery marrow in the third and fourth cycles of treatment yielded an MRA of less than 10% of normal in

Table 7. BM cellularity in mice subjected to repeated cycles of whole body sublethal irradiation.

Day	Cellularity (10^6/femur)	
	control	**MIP-1α**
0	20	20
1	5.1±0.5	4.2±0.4
7	15.8±2.0	20.0±2.5
14	17.0±1.7	18.1±2.3
15	7.2±1.6	8.2±5.6
21	23.2	20.0
28	16.5±2.1	23.3±2.7
29	4.0±1.4	4.1±0.7
35	13.4	24.5
42	16.0±2.4	18.9±1.6
43	4.1±0.3	5.0±0.4
49	20.8	14
56	14.7±2.5	22.3±3.1

Data are the mean of 4 experiments ± standard error. Data for day 0 are standardised norms for these mice.

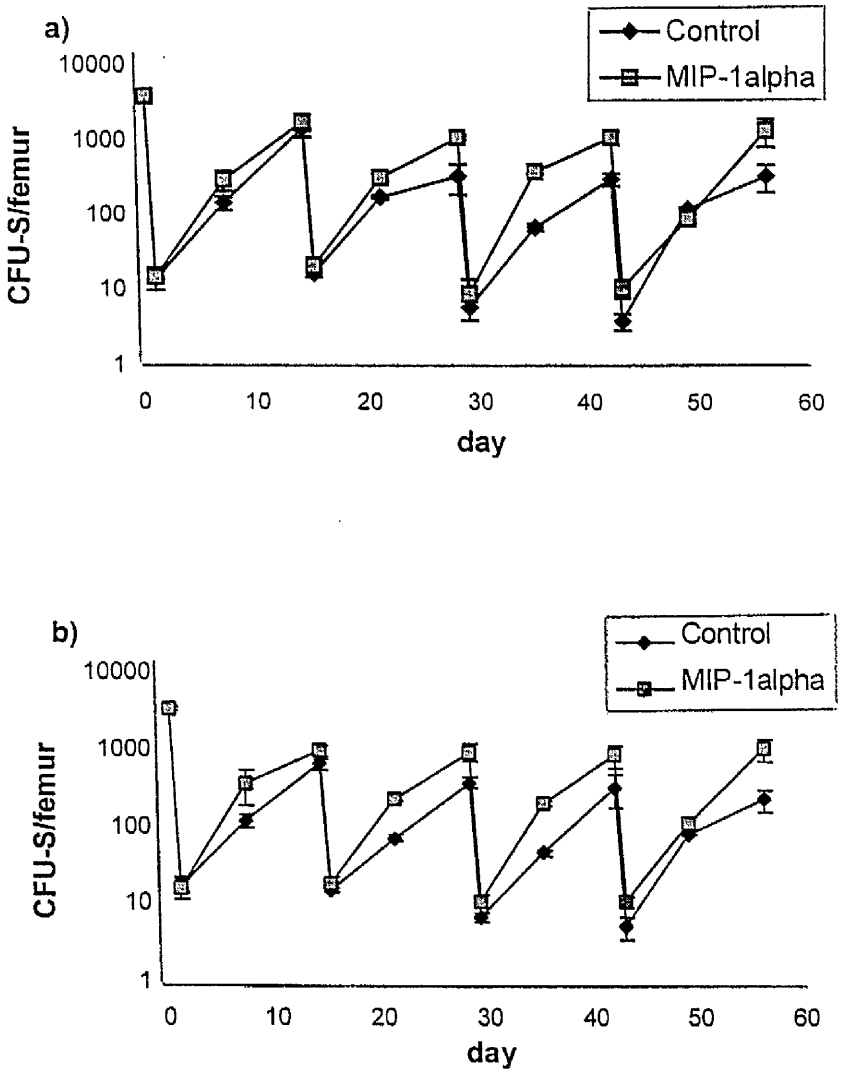


Figure 9. Day 8 (a) and day 12 (b) CFU-S after repeated sublethal irradiation ± MIP-1alpha. Results are the means ± s.e of 4 experiments.

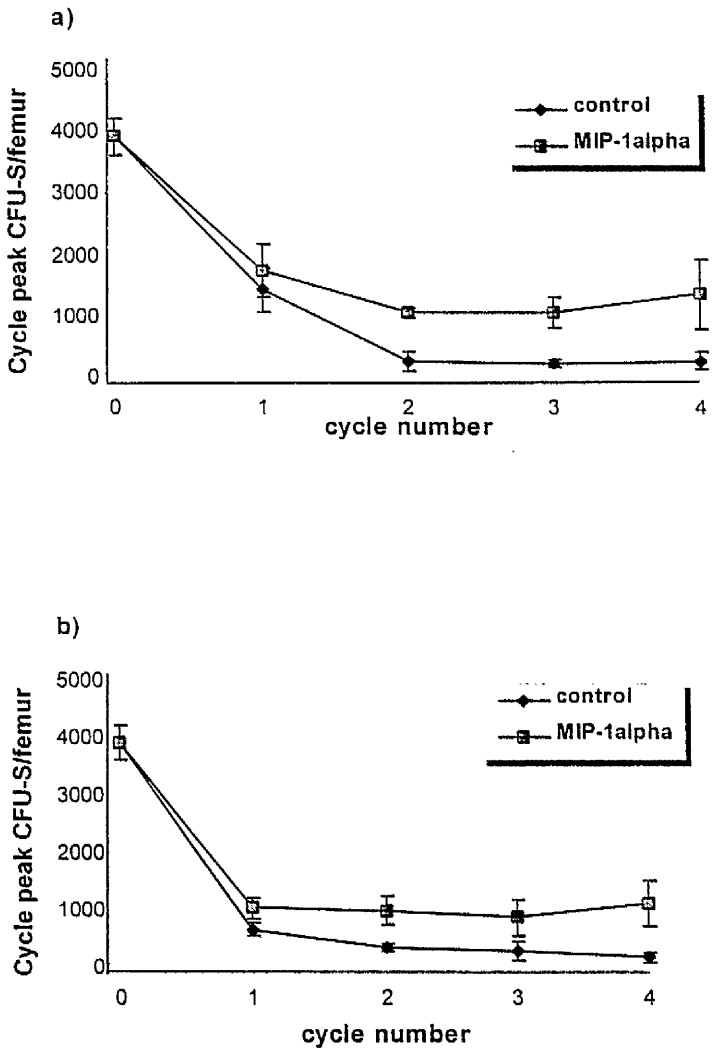


Figure 10. a) CFU-S_8 and b) CFU-S_{12} peak numbers over 4 cycles of repeated irradiation. Results are the means ± s.e. of 4 experiments.

Table 8. Recovery of bone marrow CFU-S following irradiation and variable dosing with MIP-1α.

MIP-1α Treatment	CFU-S per femur
4.5 G γ-rays only	730 ± 94
Days 0-7	1270 ± 127*
Days 1-8	960 ± 69
days 7-14	690 ± 64
days 0 - 3	799 ± 81
daily bolus	711 ± 67
twice daily bolus	550 ± 56

Data for CFU-S_8 and CFU-S_{12} combined. Results are the mean of 3 experiments ± s.e.

* $p < 0.05$.

control mice . Mice receiving irradiation only had 6533 ± 352 MRA/femur after 3 cycles and 7266 ± 688 MRA/femur after the fourth cycle. Treatment with MIP-1α increased the surviving fraction of MRA to 26.5% of normal (26600/f - $p < 0.001$) after 3 cycles and 11.2% (11206/f - $p < 0.02$) after 4 cycles (figure 11).

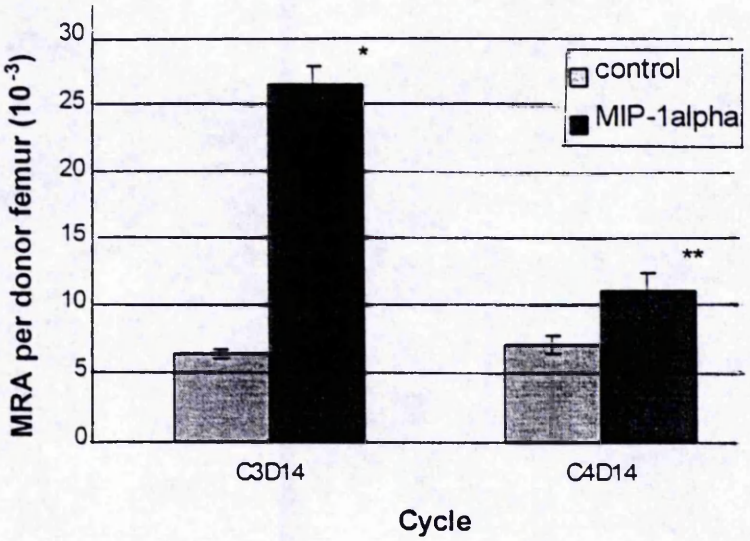


Figure 11. Percentage MRA survival on day 14 of cycles 3 (C3D14) and 4 (C4D14) following repeated sublethal irradiation ± MIP-1α. Results are the means ± s.e of 4 experiments. * $p < 0.001$. ** $p < 0.02$.

3. Cyclophosphamide Studies

3.1 Single cycle cyclophosphamide

3.1a Leucocyte recovery

Leucocyte recovery in the peripheral blood over 10 days following a single i.p. dose of 200mg/kg of cyclophosphamide is shown in figure 12. For the first 3 days, the WBC remained in the normal range after which cyclophoshamide treatment produced a short lasting leucopenia (days 4-6) with a mean nadir leucocyte count of $1.6 \pm 0.1 \times 10^9$/l. Leucocyte recovery was enhanced by the addition of either MIP-1α or G-CSF treatment (figure 12, Table 9). Mice receiving a continous infusion of MIP-1α developed a similar nadir ($2 \pm 0.5 \times 10^9$/l) but recovered to control leucocyte numbers one day earlier (day 6) ($p < 0.01$) and overshot twofold by day 7. G-CSF given, on its own on days 3-6, was more effective than MIP-1α, normalising leucocyte numbers by day 5 and increasing them 4-fold by day 6. In combination, however, MIP-1α and G-CSF accelerated recovery even further resulting in a significant overshoot to $14.8 \pm 4.7 \times 10^9$/l by day 5 and > 3-fold normal levels by day 6.

3.1b BM recovery

Cyclophosphamide suppressed the bone marrow cellularity (figure 13) to below 30% of normal controls by day 1, irrespective of MIP-1α treatment (8×10^6/f). Recovery, however, was accelerated. By day 4 control cellularity had further declined to $5.8 \pm 0.6 \times 10^6$/f . With continous administration of MIP-1α however it had risen to $12.7 \pm 2.9 \times 10^6$/f ($p = 0.05$).

The recovery patterns of day 8 CFU-S and day 12 CFU-S are shown in figure 14 and confirm earlier data (Molineux et al., 1986). Cyclophosphamide induced a rapid fall in

Table 9. Total leucocyte count (10^{-9}/l) following cyclophosphamide treatment with and without MIP-1α and/or G-CSF.

Treatment	Day 4	Day 5	Day 6	Day 7
Cyclo.	2±0.4	1.6±0.1	3.1±0.4	7.3±0.1
Cyclo/MIP	2±0.5	2.7±0.6	6.6±0.3[2]	14.2±4.0
Cyclo/G	1.5±0.2	5.5±1.1[1]	28.4±1.6[3]	26.4±2.5[2]
Cyclo/MIP/G	2.3±0.4	14.8±4.7[1]	26.2±4.9[2]	26.5±2.3[2]

Results show the means ± s.e. of 3 experiments. [1] $p < 0.05$, [2] $p < 0.01$, [3] $p < 0.001$

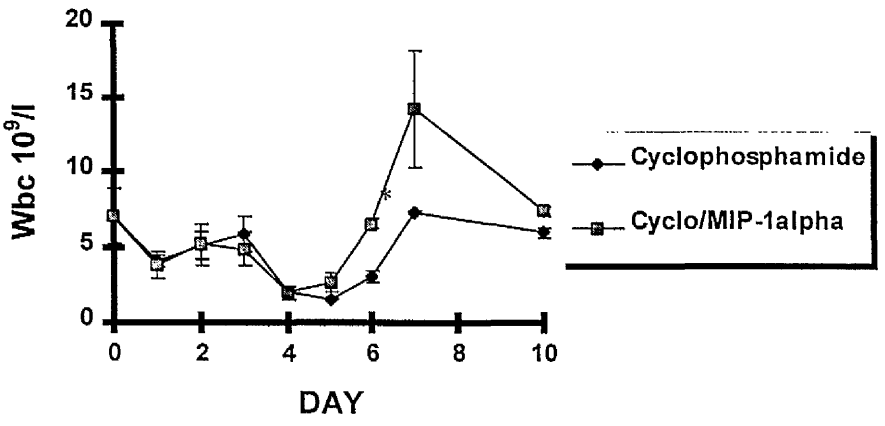


Figure 12. Leucocyte recovery following cyclophosphamide. Results are the means ± s.e. of 3 experiments. * $p < 0.01$ (at day 6 only)

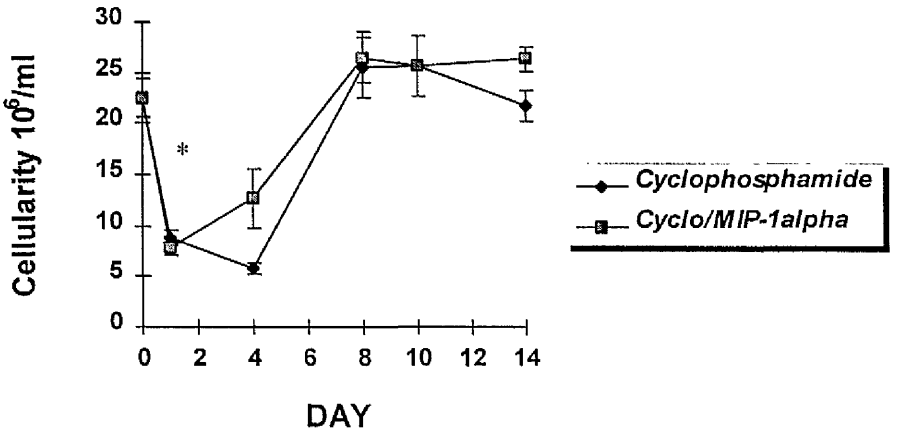


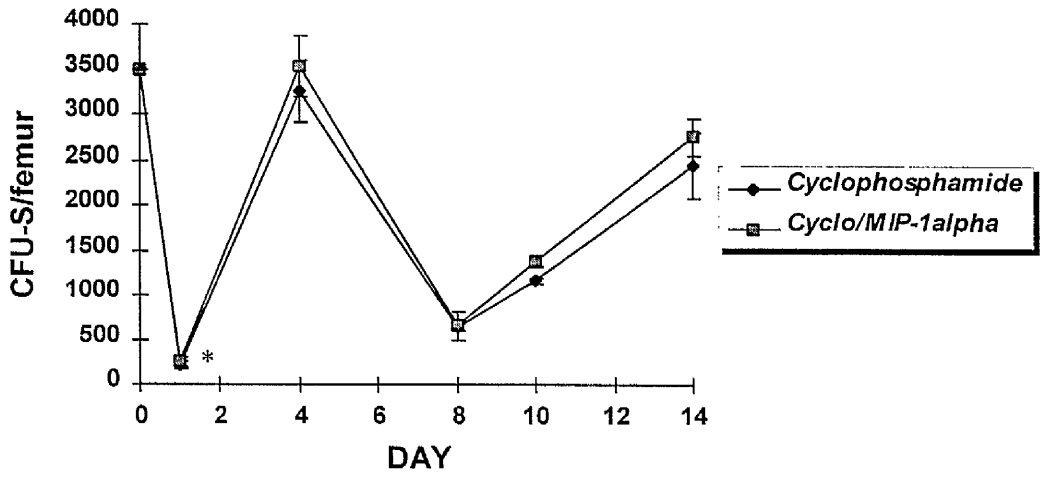
Figure 13. BM cellularity post cyclophosphamide. Results are the means ± s.e. of 4 experiments. * p = 0.05. (at day 4 only)

both populations with a nadir occurring at 24 hours. By 14 days, both CFU-S_8 and CFU-S_{12} were approaching their normal numbers (approximately 3500 per femur) having demonstrated an abortive recovery phase around day 4. The initial (1 day) cytotoxicity of cyclophosphamide was greater for 8 day CFU-S (217 ± 37/f surviving) than for 12 day CFU-S (930 ± 70/f - $p < 0.001$). Coadministration of MIP-1α neither provided any measurable protection to the cells nor enhanced their recovery over the first 8 days. During the later regeneration period, both day 8 and 12 CFU-S numbers in the MIP-1α-treated group were consistently higher at days 10 and 14. This was only a modest advantage, however, the difference in CFU-S numbers at day 14 falling within 1 standard error of the means.

3.1c Progenitor Cell Mobilisation

Haemopoietic regeneration following cyclophosphamide was accompanied by haemopoietic progenitor cell mobilisation into the peripheral blood. With cyclophosphamide alone, in vitro CFC and CFU-S numbers in blood continued to rise between days 4 and 7(Table 10), mirroring the leucocyte recovery (figure 12). This effect was augmented by G-CSF which accelerated leucocyte recovery (Table 9) and enhanced progenitor cell mobilisation (Table 10). By day 4, in vitro CFC, CFU-S_8, and CFU-S_{12} were increased 44-fold, 20-fold, and 11-fold, respectively. Mobilised progenitor cells continued to rise until at least day 7 although the greatest increase relative to cyclophosphamide occurred at day 4. Treatment with MIP-1α similarly augmented cyclophosphamide-induced progenitor cell mobilisation. Blood progenitor cell numbers continued to rise up to day 7 but again the maximal relative increase

a)



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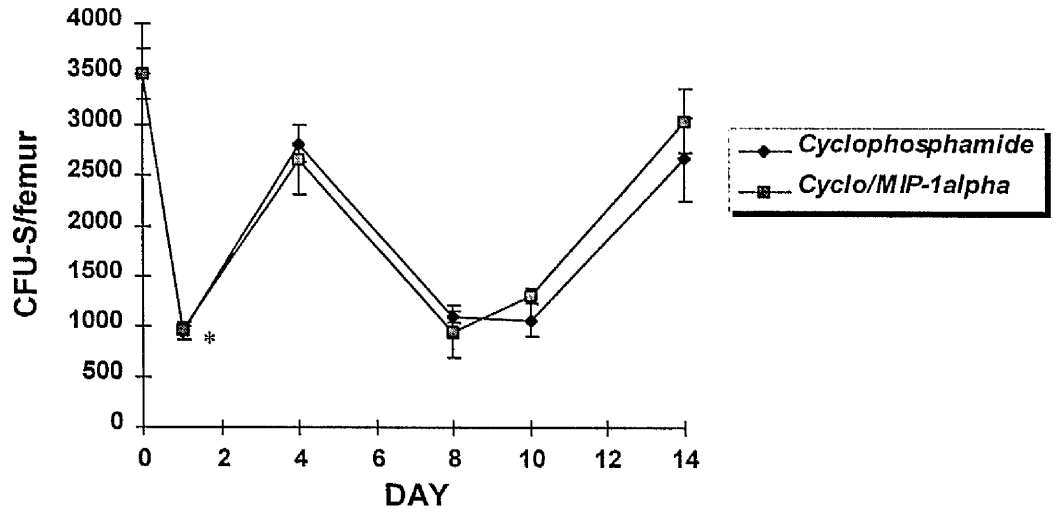

Figure 14. (a) CFU-S_8 and (b) CFU-S_{12} per femur following cyclophosphamide.

Results are the means ± s.e. of 3 experiments. * $p < 0.001$. (CFU-S_8 vs CFU-S_{12} at day 1)

occurred at day 4, when it exceeded mobilisation induced by G-CSF therapy alone. Synergy on co-administration of MIP-1α and G-CSF produced maximal mobilisation with in vitro CFC, CFU-S_8 and CFU-S_{12} increased respectively, by 58-, 46- and 25-fold compared to control on day 4. Release of in vitro CFC and CFU-S_{12} after combined MIP-1α/G-CSF appeared to plateau at about 3 x 10^4 and 2-2.5 x 10^3 /ml, respectively by day 6.

The most primitive progenitor cells that possess marrow repopulating ability (MRA) were similarly mobilised into the peripheral blood by a single administration of cyclophosphamide (Table 10c). Recovery from early damage continued at least until day 7, mirroring release of the more mature progenitor cells and leucocytes. MIP-1α and G-CSF enhanced this mobilisation to a similar degree but the combination of both factors was additive giving up to 10 fold increase over cyclophosphamide alone throughout the observation period.

3.1d Bone marrow progenitor cell numbers during mobilisation

The changes in femoral progenitor cell numbers during mobilisation are shown in table 11. As previously shown (figure 14), the number of CFU-S fell off acutely after the initial abortive recovery at day 4, to a second nadir at 7/8 days. This phase from 4-7days thus corresponded to the increasing mobilisation of progenitor cells into the peripheral blood. The enhanced mobilisation to the peripheral blood, seen with MIP-1α was reflected by a more rapid reduction in day 8 CFU-S from day 4 to day 6 when the second nadir, which was at least as low as that in the cyclophosphamide

Table 10a. GM-CFC/ml blood following cyclophosphamide treatment with and without MIP-1α and/or G-CSF.

Treatment	Day 4	Day 5	Day 6	Day 7
Cyclo.	95±76	2935±950	2935±835	4320±2010
Cyclo/MIP	4329±533	6015±215	14450±780	11850±2320
Cyclo/G	4190±1493	4350±250	20850±850	21365±705
Cyclo/MIP/G	5550±1032	12065±2990	30250±2550	17715±215

Results show the means ± s.e. of 3 experiments.

Table 10b. CFU-S/ml blood following cyclophosphamide treatment with and without MIP-1α and/or G-CSF.

Treatment	Day 4	Day 5	Day 6	Day 7
Cyclo.	14±6	217±63	462±84	612±140
Cyclo/MIP	318±58[2]	572±129	795±189	1430±542
Cyclo/G	220±111	528±108	1911±678	2256±374[3]
Cyclo/MIP/G	504±47[4]	1032±221[1]	2303±720[1]	2371±545[1]

Results show the means ± s.e. of 3 experiments. Data for CFU-S_8 and CFU-S_{12} combined. [1] $p < 0.05$, [2] $p < 0.01$, [3] $p < 0.02$, [4] $p < 0.001$ (significantly different than control)

Table 10c. MRA/ml blood following cyclophosphamide treatment with and without MIP-1α and/or G-CSF.

Treatment	Day 4	Day 5	Day 6	Day 7
Cyclo	1499±250	2749±900	3925±175	5210±1650
Cyclo/MIP	8491±1490[2]	16600±610[4]	21450±1390[4]	26500±1500[4]
Cyclo/G	6493±333[4]	11300±1340[2]	17500±3000[3]	24500±2500[2]
Cyclo/MIP/G	9540±1715[1]	28440±2870[2]	32000±3200[4]	45000±5000[2]

Results show the means ± s.e. of 3 experiments. [1] $p < 0.05$, [2] $p < 0.01$, [3] $p < 0.02$, [4] $p < 0.001$.

alone group, was reached, and 1 day earlier. At this time there was a 2.4 fold increase in mobilised CFU-S in the MIP-1α-treated cohort.

3.2 Correlation of cyclophosphamide sensitivity to CFU-S proliferation

The effect of hydroxyurea-induced stem cell recruitment into proliferation, on cyclophosphamide toxicity, is shown in figure 15. Mice pretreated with hydroxyurea, showed a greater acute reduction in both day 8 and 12 CFU-S numbers by day 1. However, as with quiescent stem cells in the normal animal the enhanced cytotoxicity of cyclophosphamide on proliferating CFU-S was not abrogated by concurrent MIP-1α therapy.

3.3 MIP-1α scheduling

The influence of varying MIP-1α schedules on cyclophosphamide-induced haemopoietic damage was investigated in a series experiments using 1-14 day continous infusion (Table 12). In agreement with previous findings (figure14), MIP-1α induced did a modest 2-fold enhancement of CFU-S recovery by day 10/11 and a 1.4-fold increase by day 14 .However, no clear dose response or schedule dependent response was evident suggesting that a short term application of MIP-1α is adequate to promote longer term recovery.

Table 11. CFU-S_8 in bone marrow of cyclophosphamide treated mice ± MIP-1α: Comparison with CFU-S_8 mobilised to peripheral blood.

Day	Cyclophosphamide		Cyclophosphamide + MIP-1α	
	CFU-S/f	CFU-S/ml	CFU-S/f	CFU-S/ml
0	3500	10±1	3500	10±1
1	217±35	-	250±50	-
4	3540±340	12±6	3540±320	357±48
5	3280±320	283±60	1800±0	680±117
6	1260±100	500±64	580±220	866±188
7	630±30	670±140	450±50	1530±470
8	640±40	-	655±155	-
10	1160±40	-	1380±60	-
14	2450±365	-	2760±208	-

Results are the means ± s.e. of at least 3 experiments.

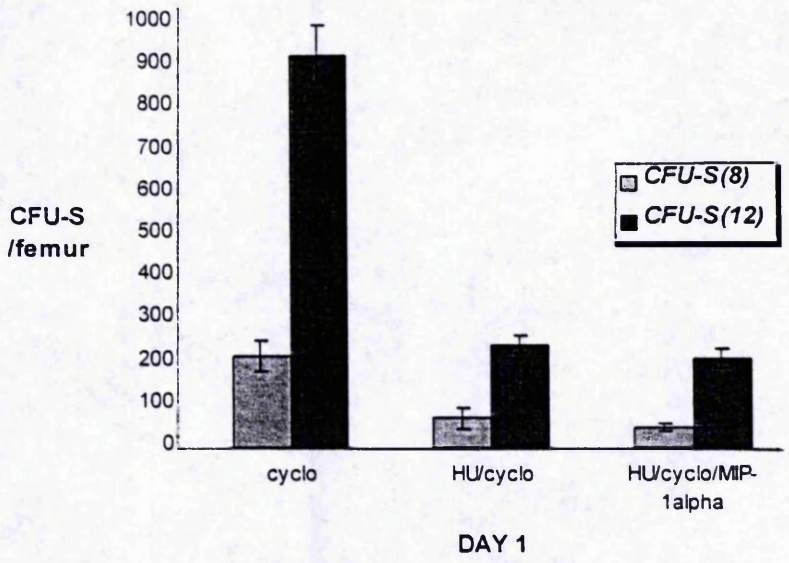

Figure 15. The number of CFU-S per femur surviving at day 1 following cyclophosphamide alone or cyclophosphamide and hydroxyurea (HU) with and without MIP-1α. Results are the means ± s.e.of 2 experiments.

Table 12. CFU-$S_{(8 \text{ and } 12)}$/femur following cyclophosphamide treatment and variable MIP-1α schedules.

Treatment	Day 10		Day 14	
	CFU-S(8)	**CFU-S(12)**	**CFU-S(8)**	**CFU-S(12)**
Cyclo.	575 ± 265	785 ± 35	2070 ± 430	1880 ± 280
1 day pump	1135 ± 265	1330 ± 70[2]	2810 ± 90	2500 ± 700
3 day pump	1070 ± 30	1400 ± 40[3]	2940 ± 940	2700 ± 500
7 day pump	1330 ± 110[1]	1180 ± 20[4]	2530 ± 350	2390 ± 290
14 day pump	1695 ± 640	1530 ± 240	3120 ± 880	2380 ± 60

MIP-1α (40μg/mouse/day) was administered via an implanted subcutaneous pump. Results show the means ± s.e. of 2 experiments. [1] $p < 0.05$, [2] $p = 0.01$, [3] $p < 0.005$, [4] $p < 0.01$

4. BCNU STUDIES

4.1 Single agent BCNU.

Preliminary experiments were carried out to determine the CFU-S recovery kinetics following a single injection of BCNU (33mg/kg). These studies revealed a CFU-S nadir around day 1 with recovery to control numbers around days 16 to 20 preceeded by a shallow (~ 60% of control) abortive recovery on day 8 (results not shown). Subsequent experiments were designed to deliver chemotherapy at a time of suboptimal BM recovery (14 days) and then repeatedly at 14 day intervals. CFU-S survival was measured on days 1 and 14 of each treatment cycle.

BM cellularities are shown in table 13. After an initial depletion of up to approximately 50% they were seen recover to control levels by day 14 during all 4 cycles of treatment irrespective of MIP1-α administration. However, there was cummulative progenitor toxicity that was most apparent with the more primitive stem cells, CFU-S_{12} and MRA (Fig. 16a-c). Some initial protection of CFU-S was apparent in most treatment cycles with MIP-1α. Control and MIP-1α-treated mice had a similar surviving fraction of CFU-S_8 by day 14 of cycle 4 (61.7% vs 66.2%). However, there was a modest enhancement of CFU-S_{12} recovery in mice receiving MIP-1α (34.2% vs 44.4%). Like the repeated irradiation experiments, this recovery advantage was most apparent during the later treatment cycles, with maximal effects observed following the fourth cycle. BCNU was toxic to the most primitive cells also (fig. 16c) with MRA numbers reduced to approximately 20% of normal after 2 cycles and to 9% after 4 cycles. A similar reduction in MRA was seen in the MIP-1α–

Table 13. BM cellularity following repeated treatments of BCNU (every 14 days) with or without MIP-1α (40μg/mouse/day for 3 days).

Day	Cellularity(10^6/femur)	
	BCNU	**BCNU /MIP1-α**
1	16	14.4
14	25.6	27
15	12.2	14.6
28	24.8	30
29	11	12.5
42	26.6	28.8
43	10	12
56	24.4	27.4

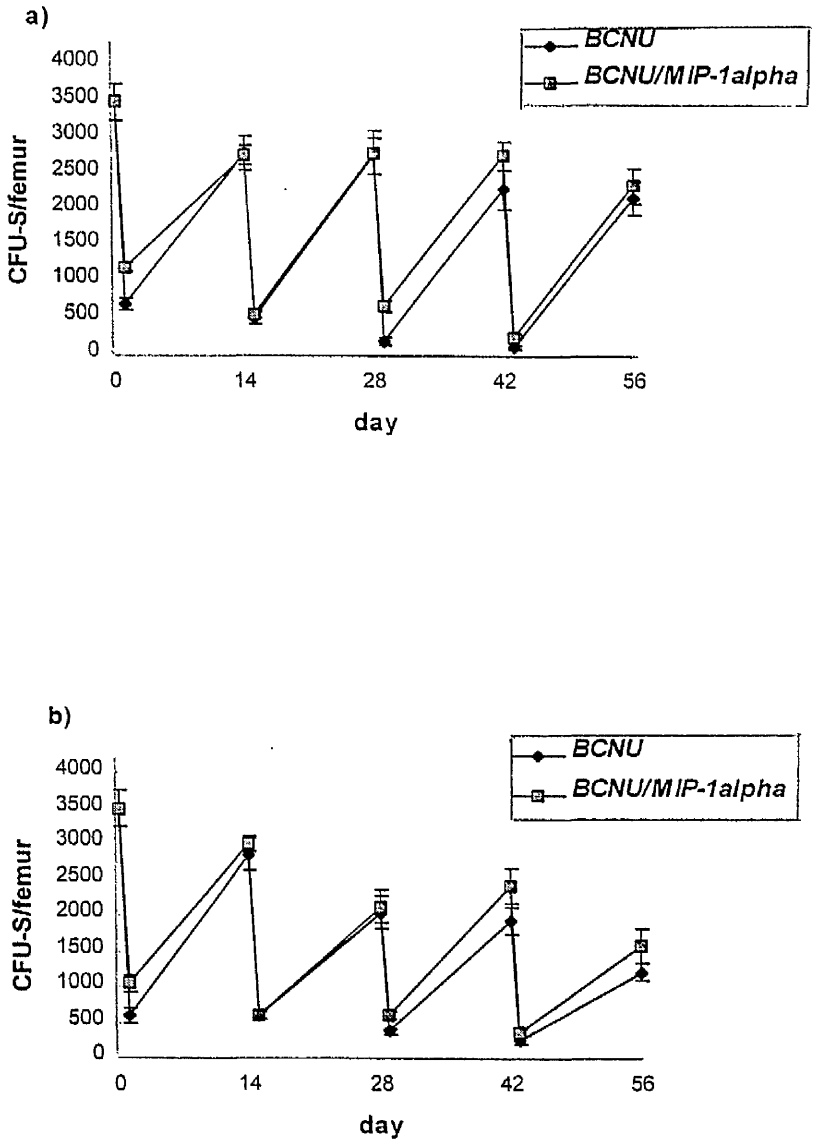

Figure 16. (a) Day 8 and (b) day 12 CFU-S recovery after repeated BCNU ± MIP-1α. Results are from 1 experiment and show the means ± s.e of 10 spleen counts from 3 mice per time point.

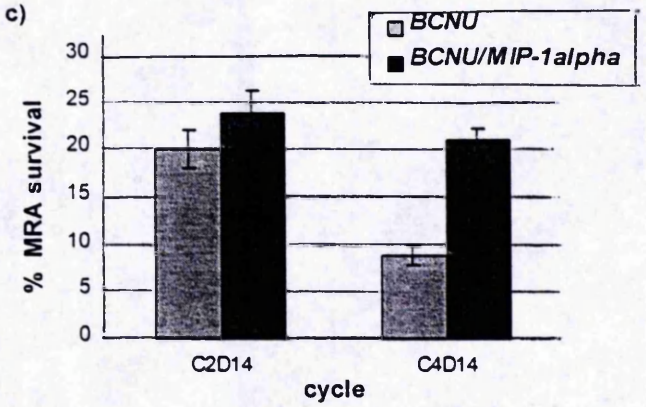

Figure 16c. Percentage MRA survival 14 days after 2 (C2D14) and 4 (C4D14) cyclesof BCNU ± MIP-1α. Results are from 1 experiment.

treated group after 2 cycles, however, the surviving fraction subsequently remained stable over at least 4 cycles.

4.2 Combined BCNU and cyclophosphamide:

Although single agent BCNU resulted in a picture of incremental BM damage the agent produced a relatively modest reduction in CFU-S numbers on day 1 of each cycle. This was in contrast to the cyclophosphamide studies in which a more marked early depletion of stem cells was observed. However, in that case there was no associated cummulative BM damage. Preliminary experiments carried out to investigate the feasibility of administering both cytotoxic agents simultaneously (results not shown) suggested an additive toxicity on BM after one cycle of treatment as compared to either agent used alone. One day after this combined treatment only 2% of normal CFU-S_8 and 5% of normal CFU-S_{12} survivied. Repeated treatments over 3 cycles resulted in a greater cumulative BM damage such that CFU-S_8 recovered to only 28% of normal and CFU-S_{12} to 17.7% of normal (figure17). In contrast, MIP-1α maintained CFU-S recovery in each cycle to a greater degree despite similar surviving numbers at day 1 of each cycle (after 3 cycles, CFU-S_8 and CFU-S_{12} were reduced to 51% and 45%, respectively). The experimental protocol was, however, associated with significant morbidity (weight loss) and mortality. Twenty percent of mice from both groups died during the study period.

In summary, therefore, the combined chemotherapy studies resulted in a greater degree of myelotoxicity and thus exposed a protective effect of MIP-1α with

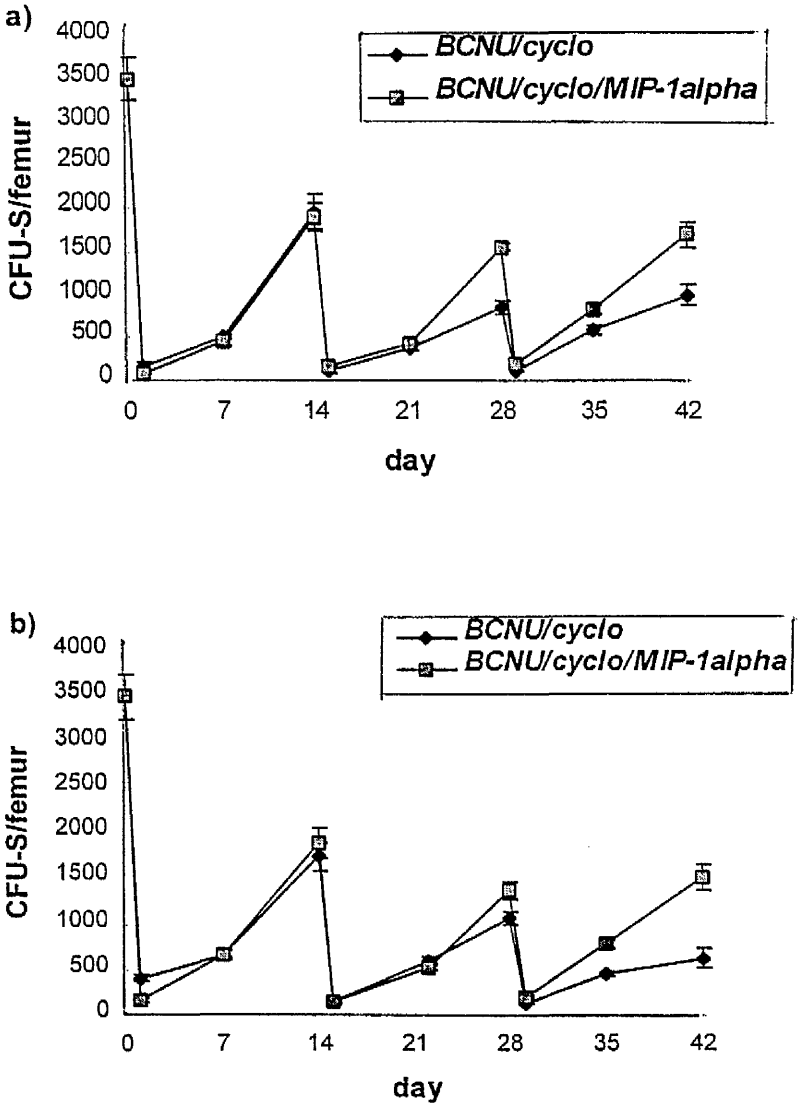

Figure 17. (a) Day 8 and (b) day 12 CFU-S survival after 3 cycles of BCNU and cyclophosphamide ± MIP-1alpha. Results are from 1 experiment.

successive cycles of treatment. However, as is also apparent in the clinical setting, the intensive chemotherapy schedule was associated with cummulative non-haemopoietic toxicity which became the dose-limiting factor. In the combined chemotherapy studies mice succumbed to toxicity typically around day 4 to day7, a time point that is highly suggestive of profound gastrointestinal damage ('gut death').

5. GUT Studies

In view of the findings in the combined chemotherapy experiments a number of pilot studies were carried out to determine the modulating role of MIP-1α on gut epithelial damage and subsequent regeneration. These studies were conducted using both irradiation and chemotherapy models of gut damage.

Irradiation resulted in a dose related reduction in crypt numbers at day 4 (Table 14). The administration of repeated bolus injections of MIP-1α had no protective effect at the 3 doses used. In mice receiving MIP-1α crypt numbers were reduced at all three dose levels at least to the same extent as with radiation alone.

During earlier dose escalation studies, BCNU (66mg/kg) resulted in a high mortality rate (80%) at day 7 associated with gut damage (emaciated and haemorrhagic intestines) observed at postmortem examination (figure 18). Mice receiving a 3 day infusion of MIP-1α had a much lower mortality rate (10%) with significant crypt regeneration at day 7 (figure 19). Repeated experiments using BCNU with or without a 7 day infusion of MIP-1α revealed a similar degree of gut damage at day 4 between control and MIP-1α-treated mice (Table 15). However, the regeneration advantage at

Table 14. Intestinal crypt survival following whole body irradiation

Treatment	Crypts/circumference Saline	Crypts/circumference MIP-1α
12 Gy	22.3 ± 2.1	17.5 ± 2.1
14 Gy	5.7 ± 1	5.0 ± 0.6
16 Gy	1.3 ± 0.2	1.0 ± 0.2
Control	99 ± 5	99 ± 5

Treatments (MIP-1α or placebo) were administered -24h, -8h, and -4 hours. Mice were killed 4 days following γ- irradiation. Each MIP-1α injection was of 10μg/mouse and data shown were size corrected.

Figure 18. Post Mortem examination of mice receiving BCNU (lower mice) or BCNU and MIP-1α (upper mice).

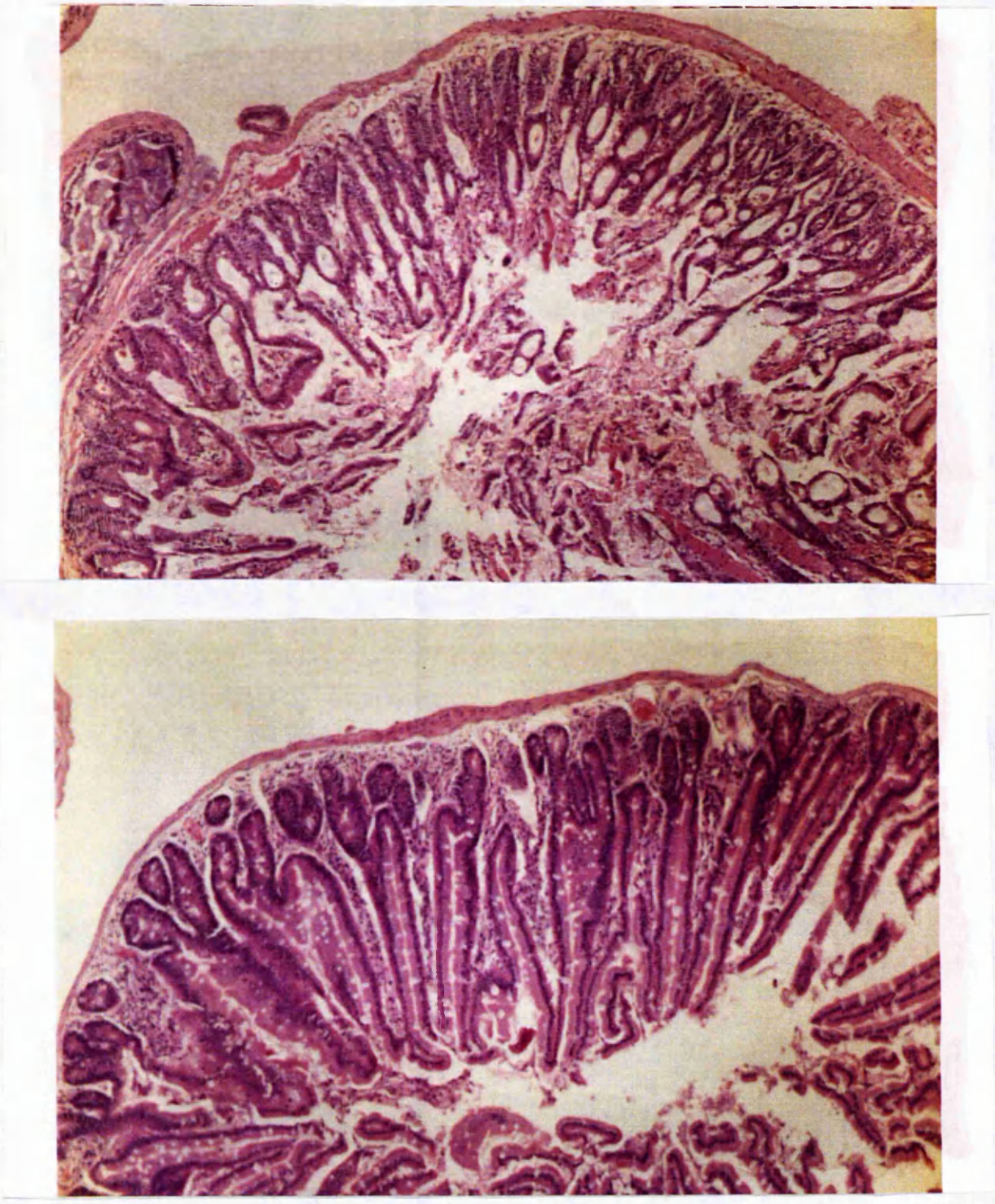

Figure 19. Histological sections of small intestine from mice receiving BCNU (upper figure) or BCNU and MIP-1α (lower figure).

day 7 seen with the 3 day infusion of MIP-1α was not reproduced (Table 15) suggesting the infusion of MIP-1α beyond 3 days continued to delay regeneration.

Administration of the S-phase specific agent, 5-FU, resulted in a dose dependent reduction in crypt numbers by day 4 with early regeneration by day 7 (table 15). In mice receiving 5-fluorouracil and a 7 day infusion of MIP-1α crypt numbers were reduced further at day 4, in an analagous fashion to that observed in the previous radiation gut model. Interestingly, however, despite this reduction, crypt regeneration at day 7 was measurably greater in the MIP-1α- treated mice at all 3 dose levels of 5-FU (Table 15).

Table 15. Surviving crypt numbers per circumference post chemotherapy with and without MIP-1alpha.

Treatment	Day 4		Day 7	
	control	MIP-1alpha	control	MIP-1alpha
5FU 7.5mg	55.4 ± 4.2	32.4 ± 5.6	42.5 ± 3.1	74.3 ± 6.6
5FU 11.25mg	17.2 ± 2.1	3.1 ± 1	64.7 ± 4.3	76.2 ± 7.4
5FU 15mg	0	0.25 ± 0.1	61.3 ± 6.2	72.2 ± 8.1
BCNU 66mg/kg	0.6 ± 0.1	0.8 ± 0.1	82.7 ± 5.3	66.6 ± 7.9

The results are from 1 experiment using 4 mice per group. MIP-1alpha was administered as a 7 day infusion, pumps inserted approximately 3 hours before chemotherapy. 5FU was injected i.p. at time 0 and 6 hours. BCNU was administered as a single i.p. injection at time 0.

Discussion

These experiments were designed as a preclinical assessment of the capacity of MIP-1α to protect haemopoiesis (and/or promote recovery) during episodes of cytotoxic treatments. Furthermore, an evaluation was made of the optimal dose schedule and duration of activity in vivo using appropriate murine models. Irradiation or cytotoxic agents were used in repeated treatment protocols with the aim of simulating a clinical chemotherapy programme and finally, some preliminary investigation was undertaken into the potential protection of gut stem cell populations.

1. Dose and Duration of Action

MIP-1α's duration of action was investigated using a model of stem cell recruitment into a proliferative phase following a single whole body exposure to 3.5Gy γ-ray irradiation (see results section 1). Earlier results had suggested that MIP-1α is active in the range of 2.5-15μg/mouse (Clements et al., 1992, Lord et al., 1992, Dunlop et al., 1992, Quesnieux et al., 1993 - see introduction) and the results of 4 separate experiments have now confirmed 10μg/mouse is a practical dose level. Similarly s.c injection was confirmed as an effective and convenient route of administration (Table 6). Measurable cell cycle inhibition was observed when MIP-1α was injected between 2 and 6 hours before BM harvesting although variable results occurred between experiments when MIP-1α was administered at the 2 hour time point. This possibly reflected the differing numbers of CFU-S in S-phase in the individual experiments and would be consistent with earlier studies that showed CFU-S were refractory to inhibition by the crude BM extract, NBME-IV, once they had proceeded beyond the

G_1/S interface and into S-phase (Lord et al, 1979). At over 8 hour, MIP-1α was no longer effective and CFU-S maintained their high proliferative activity (Table 6). Subsequent experiments requiring inhibition of progenitor cell proliferation were therefore designed, unless otherwise stated, to deliver a dose of 10μg/mouse over a 4-6 hour period by subcutaneous administration.

2. Repeated Irradiation Experiments

The concept of a dose-response curve in experimental tumour models has been fundamental to the development of chemotherapeutic protocols (Skipper 1967, Frei and Canellos, 1980) and, as a consequence, there is considerable interest in dose intensification as a means of improving tumour response rates and ultimately survival. However, repeated cycles of cytotoxic chemotherapy are associated with cumulative damage to the haemopoietic system (Testa et al., 1990) which in the clinical setting is reflected by delayed recovery of mature cells and an inevitable reduction in chemotherapeutic dose intensity. Experimentally, a model of incremental BM damage has been described using repeated sublethal (4 x 4.5Gy) irradiation (Hendry and Lajtha, 1972) that results in a 'clean' model of persistent and accumulating BM damage to haemopoietic stem cells but associated with minimal non-haemopoietic morbidity. This model was adopted to investigate the potential myeloprotective properties of MIP-1α. However the treatment interval, originally 28 days, was shortened to 14 days in order to ensure suboptimally recovered haemopoietic tissue. To maintain steady state levels over a protracted period, MIP-1α was administered as a continuous infusion using a s.c implanted mini-osmotic pump (Alzet inc.) which dispensed 40μg/mouse/day. This dosage was based on an effective active duration of

about 6 hours and is equivalent to 10μg of MIP-1α being infused every 6 hours. The pumps were inserted at least 3 hours before the mice were exposed to the irradiation. This 3 hours is required to activate release from the pumps fully. Pumps containing PBS were inserted into control mice to exclude any effect from the insertion procedure or anaesthetic. A 7 day infusion schedule of MIP-1α was devised, based largely on empirical grounds, but also to maximise any myeloprotective properties and changes in stem cell self-renewal capacity and to evaluate any modulating effect of MIP-1α on CFU-S recovery during the first 7 days. The increasing damage to the CFU-S (and MRA) populations with each cycle of radiation corroborates Hendry and Lajtha's conclusions regarding accrual of long-term damage (1972) and at the conclusion of the fourth cycle, the progenitor populations had recovered to only 10% of their normal level. The full recovery of BM cellularity by day 14 of each cycle, underpinned by a perhaps even accelerated production of peripheral blood cells in a comparable study (Dunlop et al., 1992) highlighted the lack of correlation between the degree of stem cell damage and mature cell recovery and thus emphasised the dangers of using peripheral blood measurements to reflect the quality of the bone marrow. MIP-1α resulted in little, if any, true radioprotection with similar numbers of CFU-S surviving at day 1. The infusion of MIP-1α did not inhibit the recovery of CFU-S during the first 7 days but rather produced a consistent recovery advantage that was accentuated with successive cycles of treatment. This advantage, in the absence of any clear evidence of protection, is consistent with the better maintenance of primitive cells in MIP-1α-supported LTBMC and the measured increase in stem cell self-renewal in MIP-1α-treated animals (Verfaille et al., 1994, Lord, 1995). The improved self-renewal capabilities of stem cells necessarily implies a fall in the differentiation

potential and therefore one might anticipate a detrimental reduction in the output of differentiated cells. In contrast, however, several investigators have reported an increase in the differentiated pool following exposure to MIP-1α, including an accelerated neutrophil recovery (Keller et al., 1994, Dunlop et al., 1992, Bonnet et al., 1995). At first sight these reports appear contradictory. However, as pointed out by Lord (1995), even a modest increase in self-renewal probability from say 0.65 to 0.7 will generate a sufficiently large pool of stem cells such that, within about 4 cell divisions, the output of differentiated cells will have overtaken and exceeded that generated by the lower self-renewing population, despite the reduced differentiation rate.

Whilst the radiation model provides the impetus for investigating MIP-1α with a range of cytotoxic drugs, it is not yet clear whether a 7 day exposure is necessary. Nevertheless it is now clear from assay of the blood serum that active MIP-1α is being released continuously by the minipump throughout this period (B.I.Lord personal communication) An immediate increase in the self-renewal capacity of the CFU-S surviving at day 1 may in fact be sufficient to produce the subsequent recovery advantage. Alternatively, a prolonged infusion may be required to drive and/or maintain the shift from differentiation to self-renewal. The timing and administration schedules remain the subject of further investigation but the preliminary experiments using the radiation model and variable MIP-1α schedules show recovery characteristics were improved by the presence of MIP-1α at the time of irradiation (see Table 8). Delay of exposure to MIP-1α until 1 day after irradiation ameliorated the recovery significantly and furthermore a prolonged infusion (7days > 3 > bolus) was advantageous.

3. Chemotherapy models of BM damage

The recovery advantage described in the radiation experiments provides early evidence that MIP-1α may limit progressive BM damage when used in conjunction with non-S-phase specific cytotoxins. This model, however, is not necessarily a clinically relevant one and may not be representative of the qualitative damage inflicted by commonly used chemotherapeutic drugs such as alkylating agents and anthracyclines. An attempt was therefore made to design a relevant chemotherapy model that simulated the quantitative damage seen in the radiation experiments but that was more representative of the clinical setting.

The Alkylating agents represent a major class of cytotoxic drug that have been exploited for their antineoplastic properties. Their propensity to form covalent bonds with nucleic acid ultimately results in significant cytotoxic, mutagenic and carcinogenic effects, however, the various family members produce different tissue toxicities and are non-cross resistant, despite a similar mechanism of action. All are associated with haemopoietic toxicity although there appears to be a differential sensitivity within the fine structure of the stem cell populations (Botnick et al, 1981, Down et al., 1994). Repair of alkylated DNA is possible and occurs via highly specific DNA repair enzymes such as 0^6 Alkylguanine transferase, which act rapidly to limit DNA damage (Sancar and Sancar, 1988). Cyclophosphamide is a frequently used alkylating agent that has activity against a wide range of tumours. It exerts its cytotoxic effects throughout the cell cycle but has quantitatively greater activity against rapidly dividing cells, possibly because these cells have less time to carry out DNA repair before entering DNA synthesis. Myelosuppression represents its principle

dose limiting toxicity and is well described in both cancer patients and animal models (DeWeiss et al., 1970, Molineux et al., 1986). The duration of activity of cyclophosphamide and its metabolites has not been clearly defined due to its complex metabolism. Cyclophosphamide is not cytotoxic per se but represents a prodrug that is activated by hepatic microsomal enzymes. Furthermore, the final conversion reaction to the principle active cytotoxins, phosphoramide mustard and acrolein, is an intracellular step. Any programme investigating the use of a myeloprotective agent with cyclophosphamide must therefore take into account this delayed activation and the uncertain half-life of its active metabolites.

An initial series of experiments, using an 8 hourly schedule of MIP-1α (10μg/mouse) administered as a s.c. bolus for 3 days, had no effect on CFU-S recovery (not shown). However, it was not clear whether this represented a true lack of effect or a loss of biological activity due to unfavourable pharmacokinetics of MIP-1α. In order to overcome these potential variables and ensure an adequate period of protection, MIP-1α was administered as a continuous 7 day infusion as described for the radiation experiments. The results following cyclophosphamide showed that, as with the radiation model, MIP-1α confered no measurable direct protection of CFU-S against one cycle of treatment: similar acute reductions in the CFU-S population were observed in both groups at day 1. The apparent lack of cytoprotection at day 1 is not completely unexpected as CFU-S are known to be proliferatively quiescent under steady state conditions and therefore unlikely to benefit from any potential cell cycle inhibition provided by exogenous MIP-1α. Since cyclophosphamide-induced cytotoxicity is related, at least in part, to the proliferative status of the target tissue the therapeutic benefit of MIP-1α may thus become more prominent over repeated

cycles of treatment when stem cells are recruited into cell cycle. This hypothesis was initially tested by injecting mice with cyclophosphamide 7 hours after hydroxyurea (HU). At this time, release from the HU-induced block on DNS synthesis delivers a semi-synchronised cohort of CFU-S into S-phase, resulting in a more than 2-fold and 4.5-fold increase in cylophosphamide toxicity on CFU-S_8 and CFU-S_{12}, respectively, over that seen for a quiescent population (see figure 15). Unlike the protection afforded by MIP-1α to a subsequent dose of HU (Lord et al., 1992), however, pretreatment with MIP-1α failed to ameliorate the additional toxicity induced by the administration of cylophosphamide. The conflicting results, perhaps relate to the differing routes of administration and it is possible that the s.c. route of administration (bolus injection or infusion) may not be optimal for cell cycle inhibition. It would seem more likely, however, that the contrasting results are a consequence of the differing effects of cyclophosphamide on HU-treated bone marrow compared to HU retreatment.

MIP-1α failed to attenuate or sustain the abortive recovery that characterises CFU-S kinetics over the first 8 days following treatment with cyclophosphamide (Molineux et al., 1986). A similar abortive recovery is evident following administration of a number of cytotoxic drugs but its aetiology remains poorly defined. It may represent an early regeneration wave of maturing CFU-S or alternatively a true recovery which is not maintained because of mobilisation and trafficking to the spleen (see later). This inability of exogenously added MIP-1α to attenuate the early recovery of the CFU-S population was also evident in the irradiation experiment and suggests that, in the setting of acute stem and haemopoietic cell depletion, any inhibitory signal can be overridden by an intense proliferative stimulus.

A feature of the cyclophosphamide model was a small improvement in the secondary phase CFU-S recovery in the MIP-1α-treated mice which is consistent with the observations made during the radiation experiments. A number of experiments were carried out to investigate the schedule-dependency of this response using variable dosing schedules of MIP-1α ranging from 1 day to 14 days (Table 12). A non-significant trend was observed with MIP-1α treatment producing a consistent modest recovery advantage although no clear dose response relationship was observed.

Experiments designed to evaluate repeated cycles of cyclophosphamide, injected every 10 days, did not result in a useful model of cumulative BM damage, in fact, successive injections were associated with an attenuated response and with no evidence of incremental toxicity (results not shown). Molineux et al (1986) reported similar findings using multiple cycles of cyclophosphamide administered every 14 days and suggested that this may have resulted, at least in part, from a falling half-life associated with successive injections. It may nevertheless still be that, as with the radiation model, the small effects seen after 1 cycle of cyclophosphamide/MIP-1α would be cumulative and become significant only in later cycles of treatment. However the benefits of MIP-1α in the radiation model were accentuated by falling CFU-S numbers in the control arm. It is not surprising, therefore, that repeated treatments with combined cyclophosphamide/MIP-1α did not result in a useful model of MIP-1α's cytoprotective properties against chemotherapy-induced cumulative BM damage and thus, this approach was not pursued further.

Repeated treatments with cyclophosphamide thus failed to produce a useful model of chemotherapy-induced cumulative BM damage. Since the related alkylating agents, BCNU and busulphan are known to produce a more specific stem cell defect (Morley and Blake, 1974, Botnick et al., 1981 it seemed possible that they could a more favourable model of BM damage. When comparing the myelosuppressive effects of various alkylating agents Botnick et al (1981) showed that cyclophosphamide treatment resulted in a reduction in progenitor cells that spanned both CFU-S and committed cells. In contrast, BCNU and busulphan depressed the CFU-S compartment to a much greater degree, and unlike cyclophosphamide, produced a long lasting deficit in the proliferative potential of CFU-S. Gardner et al (1993) used a competitive repopulation assay to show that repeated doses of BCNU but not cyclophosphamide was associated with a reduced potential for donor engraftment. Similarly, mice treated with busulphan developed a high degree of normal donor engraftment (~70%) as compared to cyclophosphamide-treated mice (~5% engraftment), BCNU resulting in intermediate engraftment (30%)(Down et al., 1994). The experimental evidence thus suggests that BCNU targets a similar stem cell population (with intermediate self-renewal capacity) as MIP-1α and may therefore provide a more useful model of non-S-phase cytotoxicity in which to evaluate the latter's cytoprotective properties. Preliminary experiments carried out to define the recovery kinetics of the CFU-S poulation following a single injection of BCNU (33mg/kg) established an acute reduction in the CFU-S population to approximately 20% of normal at day 1 while recovery was still incomplete at day 14. Subsequent experiments focussed solely on the CFU-S survival on day 1 and the recovery by day 14. BCNU is rapidly metabolised with a serum half-life measured in minutes (DeVita

et al., 1963), and consequently, a 3 day infusion of MIP-1α was considered sufficient to offer adequate cover for the toxic phase of BCNU. Repeated injections of BCNU, administered every 14 days, were associated with incremental damage to the CFU-S pool that was most apparent on the earlier stem cell compartment (day 12 CFU-S and MRA) and in this respect more similar to irradiation damage. Concurrent treatment with MIP-1α (40μg/day), however, did not produce any measurable direct cytoprotective effect and although it appeared to maintain recovery to a greater degree, the effects were not as marked as previously observed in the radiation model. This may have been the result of less initial BM damage (17% of CFU-S survived at day 1 compared to 0.02% following irradiation). Alternatively it may have been the consequence of a suboptimal duration of MIP-1α (3 days), as suggested by the irradiation schedule experiments (see Table 8). Attempts to increase the residual damage, by escalating the dose of BCNU, however, merely resulted in a greater incidence of non-haemopoietic toxicity with significant mortality around day 7. To attempt to overcome this, the different recovery kinetics for cyclophosphamide and BCNU were utilised. A combination of these two agents resulted in increased BM toxicity with a greater acute reduction in CFU-S and a more marked cumulative effect. In this model, the increased BM toxicity over repeated cycles accentuated the recovery advantage by MIP-1α with results more analogous to those observed in the radiation experiments. In addition, the CFU-S peak numbers were better maintained over 3 cycles compared to the initial single agent BCNU study, possibly as a consequence of a more optimal 7 day infusion of MIP-1α.

In summary therefore, the results of the cytotoxic studies suggest that the repeat cycle protection seen for radiation may also be applicable to more clinically relevant

chemotherapy. However, it appears that there may be a threshold level of bone marrow damage that is necessary before the protective properties of MIP-1α can be clearly demonstrated in an animal model. In the mouse this is not easy to achieve without introducing concomitant morbidity.

Bone marrow regeneration after cyclophosphamide, is also accompanied by peripheral blood stem cell mobilisation, an effect that is greatly enhanced by concurrent growth factor (G-CSF,GM-CSF) administration (Passos-Coelho et al., 1995). This property has been well described and exploited for transplantation purposes. Current mobilisation programmes utilise appropriate chemotherapy and G-CSF or 5 days of G-CSF used as a single agent . Lord et al (1995) have recently shown that MIP-1α also increases leucocyte numbers and progenitor cell mobilisation in mice. This effect occurred predictably around 30 minutes after injection of MIP-1α and significantly enhanced the mobilisation induced by G-CSF. In particular, MIP-1α preferentially mobilised the more primitive cells with MRA, releasing 4 times the number achieved with G-CSF alone. In the current experiments MIP-1α enhanced leucocyte recovery following cyclophosphamide and this was mirrorred by a significant increase in CFU-S and MRA mobilisation. The increase in circulating progenitor cells in the MIP-1α-treated group coincided with the reduction in the femoral CFU-S population between day 4 and day 7 (see figure 20). The femoral marrow, however, does not provide a complete picture of haemopoiesis and probably represents only 1/20 of the total haemopoietic capacity. On that basis, there appears to be a dramatic numerical reduction in the total BM CFU-S in both groups between day 4 and day 7 which is not apparently compensated by the relatively modest increase in circulating CFU-S (Table

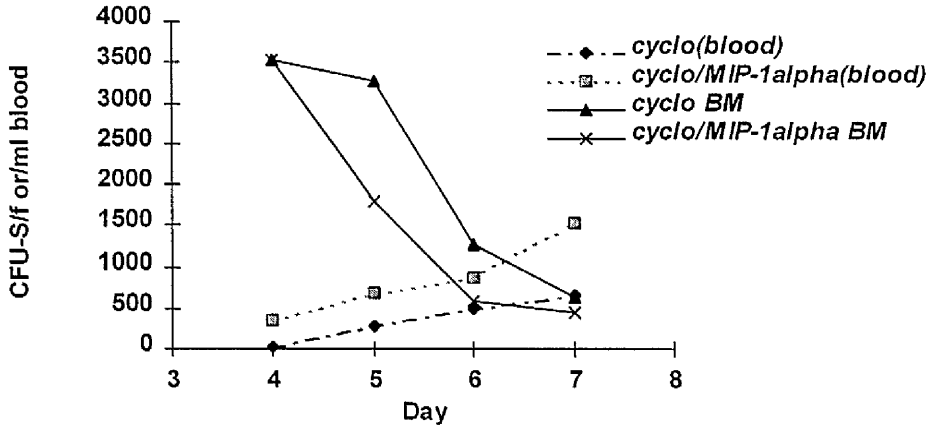

Figure 20. Comparison of femoral (BM) CFU-S_8 and blood (bl.) CFU-S_8 following cyclophosphamide. Results are the means ± s.e. of 3 experiments.

16). The ultimate deficit appears to be similar in both MIP-1α-treated and untreated mice (56-59 x 10^3 CFU-S) and almost certainly relates to CFU-S trafficking to other organs such as spleen and lungs. It is to be expected that of the mobilised CFU-S, about 10% would seed in the spleen, 10-20% would reseed the bone marrow (Testa et al., 1972) and most of the rest would be lost in the lungs, liver etc. The discrepancy between marrow loss and blood gain is not therefore surprising. It is noticable, however, that with MIP-1α, movement from the marrow is more rapid in the earlier stages and that CFU-S in the circulation are always higher than in control animals (Table 16). Irrespective of the explanation, simultaneous observation of femoral and circulating CFU-S show that BM parameters, when viewed in isolation, are not sufficient to allow full evaluation of cytoprotection by MIP-1α.

MIP-1α mobilised progenitor cells with numbers rising until at least day 7 although the greatest relative increase occurred earlier on day 4. During the earlier recovery phase, mobilisation with MIP-1α exceeded that seen with G-CSF therapy. Combined treatment with MIP-1α and G-CSF resulted in the most rapid leucocyte recovery and maximal progenitor cell mobilisation including cells with MRA. The recovery enhancement occurred despite similar numbers of precursor cells in the BM (although this may be a poor index of total CFU-S numbers as discussed earlier). MIP-1α does not appear to protect in a similar manner as described with S-phase cytotoxic drugs as comparable CFU-S kills were apparent in both treated and control mice. It is possible, however, that recovery enhancement represents a subtle increase in stem cell self-renewal that is hidden by the simultaneous mobilisation. Alternatively, MIP-1α may augment differentiation of the lineage committed progenitor cells in the absence of

Table 16. A Comparison of total marrow CFU-S and circulating CFU-S in blood.

Day	cyclophosphamide		cyclophosphamide / MIP-1α	
	BM	Blood	BM	Blood
4	70,200	20	70,200	606
5	65,600	481	36,000	1156
6	25,200	850	11,600	1472
7	12,600	1139	9000	2601
Total	57,600	1119	61,200	1995
CFU-S deficit	56,481		59,205	

The comparison shows total CFU-S assuming femoral BM represents ~ 1/20 of the total haemopoietic capacity and total blood volume of a mouse is ~ 1.7ml.

any stem cell effects as has been suggested in a previous report in which MIP-1α resulted in a 3-fold increase in granulocytes in culture and was associated with a 2-fold reduction in immature/blast cells (Keller et al., 1994). Finally, differentiation of the mobilised progenitor cells may also contribute to their apparent shortfall in the blood and also contribute to the early recovery of leucocytes.

Haemopoietic progenitor cell mobilisation is associated with the use of many chemotherapeutic agents and an increasing repertoire of cytokines. The mechanism(s) by which mobilisation occurs remains poorly understood and may vary according to the stimulus. Mobilisation following chemotherapy typically follows the recovery of the total leucocyte count and is related to the depth and duration of leucopenia (Pettengell et al., 1993). Haemopoietic growth factors such as G-CSF and GM-CSF result in a protracted mobilisation that typically peaks around day 5 of treatment (Sato et al.,1994). In contrast, the mobilisation seen following MIP-1α administration is kinetically different with an acute rise in circulating cells within 30 minutes and a preferential release of primitive cells with MRA. This rapid response, however may not be unique to MIP-1α and may well represent a common property amongst all leucocyte chemotactic factors. Jagel and Hugli (1992) for example showed that the unrelated chemotactic factors, Leucotriene B4, platelet activating factor, C5a, N-formyl-met-leu-phe (F-MLF) and Interleukin-8 all produce a rapid neutrophilia with peak responses occurring between 1 and 4 hours. This neutrophilia response was accompanied by a relative increase in non-segmented neutrophils (bands), suggesting that a major component of the leucocytosis was caused by release of BM reserves. No investigations were carried out to determine if this response was accompanied by

progenitor cell release. More recently, Laterveer et al (1995) confirmed that Interleukin-8 induced a rapid neutrophilia response and furthermore that this was associated with mobilisation of haemopoietic stem cells. The time course for mobilisation followed remarkably similar kinetics to those seen with MIP-1α with peak release of progenitor cells, including the most primitive cells with MRA, seen at around 30 minutes post IL-8 injection. The rapidity of the mobilisation response to MIP-1α suggests a different mechanism from that seen following haemopoietic growth factors or cytotoxic agents. Perhaps because of this, the mobilisation induced by the two factors together is the product of the mobilisations induced individually by the factors.

4. GUT studies

In the chemotherapy studies, dose escalation of BCNU resulted in significant gastrointestinal mucosal damage, a feature that represented the dose-limiting toxicity of this agent. In these studies it was observed that mice receiving concurrent MIP-1α had a lower mortality despite similar degrees of haemopoietic damage. Mice receiving BCNU (66mg/kg) alone had a high mortality rate with 80% dead or dying around day 7. This time scale was most consistent with profound gastrointestinal damage and subsequent postmortem examination (figures 18 and 19) confirmed severe mucosal damage in the BCNU-treated mice had been ameliorated by the 3 day infusion of MIP-1α. Investigation of mucosal damage at day 4 did not reveal any significant difference in the two groups suggesting that their better condition at 7 days with MIP-1α was the result of enhanced recovery of surviving crypts. This was not borne out in a repeat study (table 15) using a 7 day infusion of MIP-1α, perhaps as a consequence

of the more protracted schedule. In another model using escalating doses of repeated 5FU, however, initial incremental crypt damage was even higher in MIP-1α-treated mice - again suggesting growth retardation by MIP-1α - but their rate of crypt regeneration was such that by day 7 the number of crypts in the intestines of mice receiving MIP-1α had overtaken that in the control mice receiving 5-FU only (table 15). The results, whilst preliminary suggest therefore that MIP-1α has an inhibitory effect on the early regeneration of surviving stem cells and that this braking action results in an overall enhancement of stem cell self renewal which during the later recovery phase results in an amplified stem cell population.

5. Concluding Remarks

The preclinical myeloprotection studies with MIP-1α were hindered by the lack of a suitable murine model of chemotherapy-induced haemopoietic damage. The repeated sublethal irradiation model provided the strongest evidence that MIP-1α may play a role in alleviating non-S-phase cytotoxicity, probably via enhanced self-renewal of the surviving CFU-S population with a consequent amplification of the differentiation potential. However, it may not be necessarily correct to assume that chemotherapy results in a similar qualitative defect as radiation and, therefore, caution is required when extrapolating these results to the clinical setting. Despite this cautionary note, however, the radiation model did provide a platform for investigating MIP-1α's mechanism of action and for evaluating optimal dose and scheduling. The results of the chemotherapy experiments were less conclusive and the design of a suitable murine model was hampered by the non-haemopoietic effects of the cytotoxic drugs. The recognition that MIP-1α mobilises BM progenitor cells suggests that the

BM CFU-S population may not represent a complete picture of the total surviving and regenerating stem cell pool and further studies must, therefore, take into account circulating progenitor cells and trafficking to other organs. In all of the experimental models studied, MIP-1α, administered as a continous s.c. infusion, did not result in any measurable direct protective effect on CFU-S. This probably represents a true reflection of MIP-1α's inability to modulate cytotoxicity from these agents via cell cycle inhibition. It is possible, however, that the lack of protection is a consequence of unfavourable pharmokinetics and that high peak levels of MIP-1α may be required for cell cycle inhibition. Protection studies investigating MIP-1α with S-phase specific drugs have principally evaluated the i.v route of administration using an 'acute' repeated i.v. bolus schedule (Lord et al.,1992, Dunlop et al.,1992). Conversley, the protracted s.c. infusion of MIP-1α may provide optimal exposure for stem cell self-renewal.

The preliminary experiments using BCNU were consistent with the findings of the radiation model and suggest that the degree of BM damage is the limiting factor in determining the benefits of concurrent MIP-1α - the greater the degree of BM damage the greater the apparent benefit from MIP-1α. BCNU however has a 'narrow therapeutic window' for inflicting BM damage and dose escalation was associated with an unacceptable level of non-haemopoietic toxicity. The use of a combination chemotherapy model thus was attractive and of course more correctly reflects the use of chemotherapy in the clinical setting. Experimentally, BCNU and cyclophosphamide resulted in a similar picture of bone marrow damage as was seen in the irradiation studies, however, this approach was also associated with considerable non-haemopoietic toxicity. Busulphan has been shown to produce a murine model of

long term BM damage (Morley and Blake, 1974) and may provide a more useful model for investigating the myeloprotective properties of MIP-1α, however there are a number of potential drawbacks. Busulphan produces a much slower, protracted picture of BM damage compared to cyclophosphamide or BCNU and the delivery of busulphan is complicated by erratic oral absorption and the absence of a standardised parenteral formulation. The stem cell defect produced by busulphan is also very specific - a very primitive target cell which may not necessarily be sensitive to MIP-1α's stem cell effects (Quiesnaux et al., 1993, Schneider and Moore, 1991).

Further preclinical investigation of MIP-1α probably including studies with Busulphan is clearly required in order to optimise future clinical studies. This may involve an extensive search for an optimal chemotherapy model but equally experiments need to focus on the target cell population and expand upon mechanisms of action including self-renewal. MIP-1α may regulate proliferation in part via a shift from differentiation to self-renewal and so chemotherapy models should investigate the quality of surviving stem cells and not merely quantity. Despite the need for further preclinical investigation it is likely that progress will be hampered by the lack of a suitable animal model and as such it was considered reasonable to take a pragmatic approach and pursue an early clinical trial programme in parallel with ongoing laboratory studies.

The use of high dose chemotherapy and dose intensive treatment supported by haemopoietic growth factors and autologous BM transplantation has also highlighted the increasing importance of dose-limiting non-haemopoietic toxicity. Other stem cell systems, particularly gut mucosa and skin are subject to similar, if less well defined regulatory mechanisms and similar cytotoxic induced injury. Inhibitors of

haemopoietic stem cell proliferation may also regulate other stem cell systems and several reports have hinted at an inhibitory role for MIP-1α in gut, skin and germ cell proliferation (Lord et al., 1993, Parkinson et al., 1993, Hakovirta et al., 1994). Earlier experiments with MIP-1α failed to confirm an inhibitory effect on crypt stem cells using proliferation assays (Potten et al., 1995) but preliminary BCNU studies have now shown a significant advantage in the regenerative phase. Whilst this and experiments with 5-FU are consistent with an enhanced crypt stem cell self-renewal capacity further experiments are needed to confirm and develop this finding. Any conclusion on the cellular specificity and mechanism of action would be premature and collaborative studies are ongoing to evaluate any additional effects of MIP-1α on skin and germinal epithelium.

The early and predictable mobilisation of progenitor cells enriched for MRA has significant clinical potential with advantages over and above the use of G-CSF. The complimentary effects of MIP-1α and G-CSF, if confirmed in clinical studies suggest, that this may provide a realistic strategy for maximising BM recovery and peripheral blood stem cell harvesting while limiting any cumulative BM damage following repeated cytotoxic drug therapy.

The overall conclusion from the preclinical animal studies of the BB-10010 analogue was sufficient to encourage and enable its use in a number of clinical trials. The first cohort of trials have included those carried out in this study and presented in part II.

PART II: CLINICAL STUDIES

Introduction

Preclinical evaluation of BB-10010 has shown that this defined tetrameric molecule has a similar efficacy to native MIP-1α as assessed in receptor binding, calcium mobilisation and murine protection assays (Hunter et al, 1995). As summarised in part I, this MIP-1α variant ameliorated the cumulative myelosuppression associated with repeated sublethal whole body irradiation. A similar, though less marked effect, than those seen in the irradiation experiments, was observed when BB-10010 was used in combination with the alkylating agents, cyclophosphamide and BCNU, but an optimal chemotherapy model was not fully defined. During those experiments, no significant toxicity was observed and the safety of BB-10010 has since been confirmed in more detailed toxicology studies (BBL-personal communication). In none of those studies was BB-10010 associated with any pro-inflammatory effects. As a consequence of the encouraging preclinical findings, supported by a general absence of toxicity, an early clinical trials programme was developed in collaboration with British Biotech. Pharmaceuticals Limited. The preliminary results of these studies will now be presented and discussed. In all cases the term 'MIP-1α' will be substituted for BB-10010 unless the latter representation is specifically required.

The clinical evaluation of cell cycle inhibitory factors presents a number of unique problems related to the relatively low concentrations of the haemopoietic stem cells and the complexity in assessing any biological response. Assessment in conventional phase I dose finding studies is particularly limited as a consequence of several factors:

Firstly, under normal steady state conditions, and at least partly due to the influence of endogenous inhibitors including MIP-1α, the stem cell compartment is proliferatively quiescent. In order to assess the response to an exogenously added inhibitory factor some initial pertubation of the system is therefore required. Secondly, it may not be possible to define a maximum tolerated dose of inhibitor - preclinical studies evaluating these molecules have shown that they are remarkably devoid of toxicity (Guigon et al., 1980, Paukovits et al., 1991a/b, Lord et al., 1992, 1995). Furthermore in the specific case of BB-10010, detailed preclinical toxicology studies have not revealed any significant adverse effect following either chronic administration (28 day studies) or single i.v. injections up to 10mg/kg of protein.

Despite these limitations, three phase I studies have been completed using a dose range of MIP-1α from 0.1μg/kg to 300μg/kg in healthy normal volunteers and stable advanced cancer patients. The primary objectives of these studies were to determine the tolerability and pharmacokinetics of MIP-1α following bolus administration. The potential for MIP-1α to induce a neutrophil leucocytosis and progenitor cell mobilisation was also investigated although whether these endpoints accurately reflect the quantity and proliferative activity of the BM stem cell population, remains uncertain. Whilst a direct assessment of bone marrow activity may have provided a more sensitive index of response, however, this was considered both inappropriate and detrimental to patient accrual in these early studies a) for reasons outlined above and b) because previous 'in-house' experience had failed to show any consistent inhibitory effect of either MIP-1α or

a large number of its mutants with variable polymerisation potentials on colony formation by progenitor cells (BBL & CM Heyworth-personal communication).

The direct evaluation of a stem cell inhibitory factor ultimately rests on its ability to block stem cell recruitment into proliferation following the appropriate stimulus. The inhibitory effects may be quantified using flow cytometric analyses on selected BM subpopulations or alternatively by the use of in vitro suicide assays in long term bone marrow culture. The ability to measure cell cycle status in a relatively infrequent and elusive population is, however, problematical and exacerbated by a further reduction in cell numbers following chemotherapy. Measurements of stem cell viability and survival (using GM-CFC production over 6 weeks of LTBMC) offer alternative methods of assessment with BM aspirates, assayed before and after repeated cycles of chemotherapy with or without complimentary MIP-1α. A further advantage with this latter approach is that the measured outcome (stem cell survival) is a consequence of not only cell cycle inhibition but also possible effects on stem cell self renewal. In addition, the rate of mature cell recovery (neutrophils and platelets in the peripheral blood) and the ability to maintain a predefined chemotherapeutic dose intensity offer further indirect evidence of effects on stem cell populations. These parameters were investigated in two phase I/II clinical studies using combination chemotherapy with or without MIP-1α in patients with advanced breast cancer and non-small cell lung cancer.

MATERIALS AND METHODS

All procedures described below represent standard experimental haematology techniques that have been described in full in Testa N.G and Molineux G, 1993.

1. Bone marrow samples

Patients were placed in the left lateral position and after cleaning the skin with an antiseptic solution, samples were taken from the right posterior iliac spine under 1% lignocaine local anaethesia. Up to 2 mls of marrow was aspirated and at once mixed with transport medium (10mls IMDM containing 40 units of preservative free heparin and 2% fetal calf serum) in a sterile universal container. The stoppered container was inverted several times to ensure thorough mixing and then placed into a container of ice chippings until use. A bone marrow trephine was also carried out and sent for routine histological examination.

2. Preparation of aspirated bone marrow samples.

2.1 Washing and centrifugation

The suspensions of BM in transport medium were transfered to conical base, 15 ml test tubes (Falcon, New Jersey, USA) and excessive red cells were removed using a gravity sedimentation technique. A 1% solution of methylcellulose was added to give a final concentration of 0.1% methylcellulose, and 30-40 min incubation at room temperature was allowed for sedimentation of the majority of red cells. The remaining nucleated cells were then recovered and washed twice in IMDM.

2.2 Counting the cell suspension

Using a diluting pipette and white cell diluting fluid a 1 in 10 or 1 in 20 dilution of the cell suspension was made and a drop placed at the edge of a counting chamber cover slip (modified Neubauer) and allowed to flow underneath. The counting grid was inspected using the low power objective of a microscope and all the nucleated cells in five large squares were counted. If accurate counting was hampered by very high cell numbers, the count was repeated after appropriate dilution of the cell suspension with IMDM.

3. Blood progenitor cell determination.

Five ml of blood was pipetted carefully against the side wall of a sterile plastic test tube (Falcon, New jersey, USA) containing 5 ml of Ficoll (1.077g/ml density, TechGen International), taking care not to disturb the meniscus. The preparation was spun at 400g for 25 minutes at room temperature. The mononuclear cells were then harvested at the interface using a Pasteur pipette and washed twice in IMDM before counting.

4. Clonogenic assay for Mix-CFC

To a plastic universal container were added 1ml each of fetal calf serum, 5637 conditioned medium, 10% bovine serum albumin (Sigma) , recombinant erythropoietin (Amgen, California) and 5×10^4 to 5×10^5 mononuclear cells.

To this culture mixture, 0.5ml of methylcellulose was added and the combination thoroughly mixed. This final mixture was allowed to stand for approximately 5 minutes

to allow any air bubbles to disperse. From this mixture, 0.3mls was plated in triplicate into the central section of sterile 24 well tissue culture plates (Falcon, New Jersey, USA). The wells surrounding the test samples were filled with sterile distilled water to ensure adequate humidity. The culture plates were then incubated at 37°C in a humidified atmosphere of 5% carbon dioxide and 5% oxygen. After 14 days colonies were scored viewed under a dissecting microscope with a zoom lens at 30x or 40x magnfication. In this assay GM colonies have a translucent appearance and each colony consists of 50 cells or more. Erythroid colonies can be divided into the more mature CFU-E (single or paired clusters of 5-100 haemoglobinised cells) and the more primitive BFU-E (3 or greater closely arranged erythroid clusters).

5. Determination of CD34 positive cells.

Mononuclear cells were washed twice in saline supplemented with 1% BSA and incubated with a mouse anti-CD34 fluorescein isothiocyanate conjugated mAb (anti-HPCA-2, Becton-Dickenson) for 30 minutes at 4^0C. Cells were washed twice in saline supplemented with 3% BSA. Cells were stored in a fixative of 1% formaldehyde solution and were analysed on a fluorescent activated cell sorter (FACS) within 24 hours. A nonspecific isotype matched FITC conjugated mAb was used as a control (Siena et al., 1994).

6. Establishing and maintaining long term bone marrow cultures

In this in vitro system a stromal layer that is functionally and structurally similar to the in vivo haemopoietic microenvironment supports the growth and differentiation of haemopoietic cells for 8-12 weeks. The formation of the adherent layer takes place over 2-4 weeks with the development of a structured and confluent monolayer consisting primarily of macrophages, adipocytes and fibroblastoid cells. Primitive haemopoietic cells reside within this monolayer and can be visualised as compact groups of cells which have a distinct phase-dark appearance ('cobblestone areas'). Maturation of cells is associated with their release into the growth medium.

Cultures were established according to the method first published by Dexter et al (1976) and later modified by Gartner and Kaplan (1980). Briefly, between 5 x 10^5 and 2 x 10^7 nucleated BM cells in 10mls of LTBMC medium were added to 25cm^2 tissue culture flasks (falcon). Each 10mls of medium consisted of 1ml FCS, 1ml HS, 7.9ml of IMDM (350 mosmo/kg) and 0.1ml of hydrocortisone succinate (5 x 10^{-7}M) (Sigma). After inoculation, the flasks were gassed with 5% carbon dioxide in air, then capped and incubated at 33°C in the dark.

Cultures were fed routinely on a weekly basis from week 1 or 2. After removal from the incubator, flasks were agitated gently to disperse the supernatant cells. Five mls of medium were removed by pipette and replaced with the same volume of fresh cell-free medium. After regassing with 5% carbon dioxide in air the cap was replaced tightly and the flask returned to the incubator.

7. Monitoring long term bone marrow cultures

7.1. light microscopy

Before each feed the cultures were examined using a Diavertz inverted light microscope at low and high power. Flasks were immediately discarded and destroyed if there were any signs of contamination with yeast or bacteria.

Adherent layer confluence was assessed by examining multiple fields and estimating total area covered by stromal cells expressed as a fraction of the flask base area.

7.2. Count of supernatent cells and assay for GM-CFC

At each weekly feed, the 5 mls of medium removed from each LTC flask and containing the newly generated cells were retained in a conical base tube. Following centrifugation at 800g for 10 minutes the supernatant medium was discarded and the cell pellet resuspended in 1-2 mls of IMDM. A nucleated cell count was performed from which the cell count per flask was calculated. The cell concentration was adjusted by dilution with IMDM to no more than 2 x 10^6/ml and then the suspension assayed for GM-CFC. From this the total number of GM-CFC in the LTC supernatant was derived.

7.3. Assay for GM-CFC in the adherent layer.

After gentle agitation the LTC supernatant was pipetted off and 2-3 three mls of fresh IMDM were used to wash the adherent layer gently and remove any non-adherent cells.

Trypsinisation of the adherent layer was performed after the method first described by Coulombel et al (1983). The contents of one vial of trypsin (Difco) were dissolved in sufficient double distilled water to make a 5% solution which was then diluted 1:20 with phosphate buffered saline making a final concentration of 0.25%. Two or three mls of this trypsin solution was added to the culture flask which was then incubated at 37°C for 3-5 minutes. Trypsin digestion was halted by adding 7mls of IMDM and 20% fetal calf serum to the flask and a single cell suspension obtained by gentle pipetting. Following centrifugation, the supernatant was discarded. The cell pellet was resuspended and washed in IMDM to remove any traces of trypsin. A second centrifugation washing in IMDM was performed and the cell pellet resuspended for the final time in 1-2mls of IMDM. After counting, the suspension was adjusted to a maximum concentration of 2 x 10^6/ml and assayed for GM-CFC.

8. BB-10010 Pharmacokinetics and Antibody Detection

BB-10010 pharmacokinetic studies and determination of antibody generation were carried out in all clinical studies by British Biotech Pharmaceuticals ltd.

3ml blood samples collected in sodium citrate at 15 minutes to 48 hours after injection of BB-10010 were centifuged at 3000g for 10 minutes at 4^0C and the supernatent frozen at -20^0C until analysed. Samples were subsequently assayed by ELISA for BB-10010 using the Quantikine^{tm} kit (R&D Systems Inc.). The assay was validated for the determination of BB-10010 over the range 47 to 1500pg/ml. The lower limit of quantification was 93.8pg/ml.

The generation of MIP-1α antibodies was investigated using an anti BB-10010 antibody ELISA.

PHASE I STUDIES

1. A Phase I Study of MIP-1α in Advanced Cancer Patients

A dose escalation study of MIP-1α was undertaken at the Christie Hospital, Manchester in patients with stable, advanced cancer. The objectives of the study were to determine the safety, tolerability and the pharmacokinetics of MIP-1α following s.c. administration in cancer patients.

3 groups of 3 patients each received a single s.c injection of MIP-1α at doses of 1, 10 or 100μg/kg . Safety and tolerance were assessed over a 24 hour period with laboratory indices carried out at specified points during this time. 1 week later, the patients received the same total dose again, but administered s.c as a 24 hour infusion. They were observed for a 48 hour period followed by weekly assessment for 2 weeks. Laboratory monitoring consisted of routine and specialist haematology ($CD34^+$ cell and GM-CFC determination in peripheral blood), biochemistry, pharmacokinetics and antibody generation.

1.1 Eligibility Criteria

Inclusion criteria:

- Histologically proven advanced cancer
- Normal haemopoiesis as judged by a WBC >3 x 10^9/l , platelets >100,000 x 10^9/l and Hb > 10g/dl

- ≥ 18 years of age
- Bilirubin < 2 x normal and normal serum creatinine
- written, informed consent
- Concurrent endocrine therapy was acceptable if introduced and stable for at least 3 months before start of study
- Predicted survival of > 3 months

Exclusion criteria:

- History of life threatening anaphylactic reaction
- Acute illness within 2 weeks prior to the study
- Use of other investigational agents during the study
- Concomitant treatment during the study with G-CSF or other cytokines
- Concomitant treatment with steroids or chemotherapy
- Pregnant or breast feeding females
- Any further condition which, in the opinion of the investigator, would make the patient unsuitable for the study.

2. A Phase I Study of MIP-1α in Normal Healthy Volunteers

A phase I, double blind, placebo controlled, dose escalating study was carried out in healthy normal male volunteers at the Central Middlesex Hospital, London in collaboration with British Biotech Pharmaceuticals ltd and Hammersmith Medicines Research. Screening investigations and routine haematology were carried out at the

Central Middlesex Hospital and peripheral blood was analysed for $CD34^+$ cells and GM-CFC cell numbers at the Paterson Institute for Cancer Research, Manchester. The objectives of the study were to investigate the safety and tolerance of MIP-1α, the pharmacokinetics following s.c. and i.v. bolus injection and the effects of MIP-1α on numbers of circulating leucocytes and progenitor cells.

MIP-1α was administered at dose levels of 1, 10, 30, 100 and 300μg/kg s.c. and 0.1, 1, 10, 30 and 100μg/kg i.v. At each dose level 3 subjects received MIP-1α and 1 subject received placebo.

3. A Phase I Study of MIP-1α and G-CSF in Normal Healthy Volunteers

This study, carried out in collaboration with the Central Middlesex Hospital, University College Hospital, London and the Christie Hospital, Manchester was conducted to investigate the safety and possible synergistic effects of MIP-1α and G-CSF. In this study, healthy, male volunteers were recruited in groups of 4, 3 of whom received 'active' treatment and 1 received placebo. Active groups received daily injections of G-CSF (5μg/kg up to a maximum of 5 days) followed 24 hours later by a single injection of MIP-1α (fig. 22). Nine groups were studied receiving 1, 2, 3, 4, or 5 days of G-CSF followed 24 hours later by 30μg/kg MIP-1α (Groups 1-5). Groups 6-9 received 5 days of G-CSF followed by MIP-1α, (100 or 300μg/kg s.c. or 10 or 30μg/kg i.v.). Placebo groups received neither G-CSF nor MIP-1α.

Phase I Normal Volunteer Study Design

- **Grps I-V: G-CSF (5μg/kg) for 1, 2, 3, 4, or 5 days, respectively, followed 24 hours later by a single s.c. bolus injection of MIP-1α (30μg/kg)**
- **Grp VI: G-CSF (5μg/kg) for 5 days followed 24 hours later by a single s.c. bolus injection of MIP-1α (100μg/kg)**
- **Grp VII: G-CSF (5μg/kg) for 5 days followed 24 hours later by a single s.c. bolus injection of MIP-1α (300μg/kg)**
- **Grp VIII: G-CSF (5μg/kg) for 5 days followed 24 hours later by a single i.v. bolus injection of MIP-1α (10μg/kg)**
- **Grp IX: G-CSF (5μg/kg) for 5 days followed 24 hours later by a single i.v. bolus injection of MIP-1α (30μg/kg)**

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24 hour observation period (Following MIP-1α)

- Vital signs
- routine/specialist haematology
- biochemistry
- pharmacokinetics
- antibody generation

Weekly follow-up for adverse events(6 weeks)

Figure 22.

RESULTS

1. Advanced Cancer Patient Study

9 female patients with stable, stage IV breast carcinoma were enrolled between Sept. 1994 and Sept 1995 (see table 14). Median age was 52 years (range 43 to 75) and all patients had an ECOG performance status of ≤ 1. Four of the patients had received previous chemotherapy (> 6 months previously) and 2 had received a single palliative fraction of radiotherapy for painful bone metastases.

1.1 Safety and tolerability:

Mild dizziness and flushing which were considered possibly to be related to MIP-1α (table 15) were reported in 4 patients. However, no serious adverse events were observed. No inflammatory response was seen, either locally or systemically and no local pain was reported at the injection site. The development of MIP-1α antibodies was investigated using an anti-BB-10010 ELISA (carried out by BBL). Although one patient had antibodies detectable at screening (patient 1, 1μg/kg dose group), with a baseline level of 54.85 units, this did not fluctuate during the 2 week follow-up period. All other patients had undetectable (< 1.56units) antibody levels at baseline and throughout the study period.

Table 14. Phase I Cancer Study: Patient Characteristics.

Patient Number	Age	Disease Status	Current Treatment
1	57	Stage IV (pleura)	Endocrine
2	51	Stage IV (bone)	Endocrine
3	75	Stage IV (lung/soft tissue)	Endocrine
4	49	Stage IV (bone)	Endocrine (prev. CMF chemotherapy)
5	43	Stage IV (peritoneal)	none (prev. CMF chemotherapy)
6	57	Stage IV (lung)	none (prev. FAC chemotherapy)
7	52	Stage IV (lung)	none (prev. FAC chemotherapy)
8	55	Stage IV (liver/soft tissue	Endocrine
9	49	Stage IV (soft tissue)	Endocrine

Table 15. Reported Adverse Events In The Phase I Cancer Study.

Patient number	Dose group	Adverse event
1	1μg/kg	Flushing
2	1μg/kg	Headaches
4	10μg/kg	Dizziness/light headedness
8	100μg/kg	Dizziness

1.2 The activity of MIP-1α on haemopoiesis

The pharmacodynamic response to MIP-1α was determined by measuring changes in the total and differential leucocyte count and circulating numbers of colony forming cells. Neither bolus injection nor 24 hour infusion of MIP-1α produced a measurable effect on the total WBC (figures 23 and 24) or on leucocyte subsets including neutrophils, eosinophils, lymphocytes or basophils (results not shown). Although monocyte numbers fluctuated considerably during the first 4 hours this did not appear to be dose related and no placebo group was included for comparative analysis. A relative monocytosis of approximately 2-fold over baseline was, however, noted between 4 and 24 hours at the highest (100μg/kg) dose level (bolus injection and infusion- Figures 23b and 24b).

The numbers of circulating colony forming cells fluctuated considerably throughout the study period with a maximal 7-fold increase observed in the 10μg/kg dose level infusion group at 4 hours (figures 25a and 26a). Despite this increase, however, the absolute numbers of GM-CFC were low (7-140/ml) and no consistent pattern of mobilisation was evident. Furthermore, the fluctuations in GM-CFC numbers were not mirrored by changes in the $CD34^+$ cell population (figures 25b and 26b).

MIP-1α was not generally detectable in plasma after bolus or infused doses of 1 or 10μg/kg. Plasma levels following administration of 100μg/kg are shown in figure 27. At this dose level, bolus s.c. injection resulted in sustained plasma concentrations of MIP-1α over a 24 hour period with a peak concentration of 2600pg/ml at 8 hours. This peak value was not, however, completely representative of the 3 subjects and was skewed considerably as a consequence of particularly elevated levels in one individual.

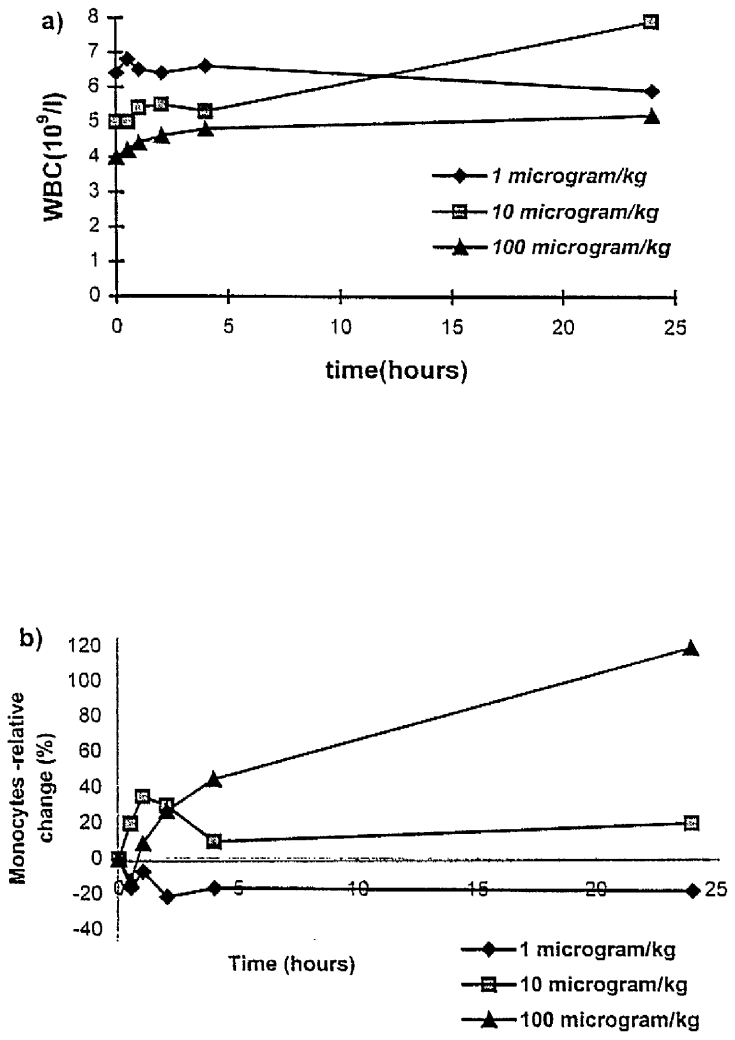

Figure 23. (a) Total WBC and (b) change in relative monocyte counts in cancer patients (3 per dose level) following bolus injection of MIP-1α.

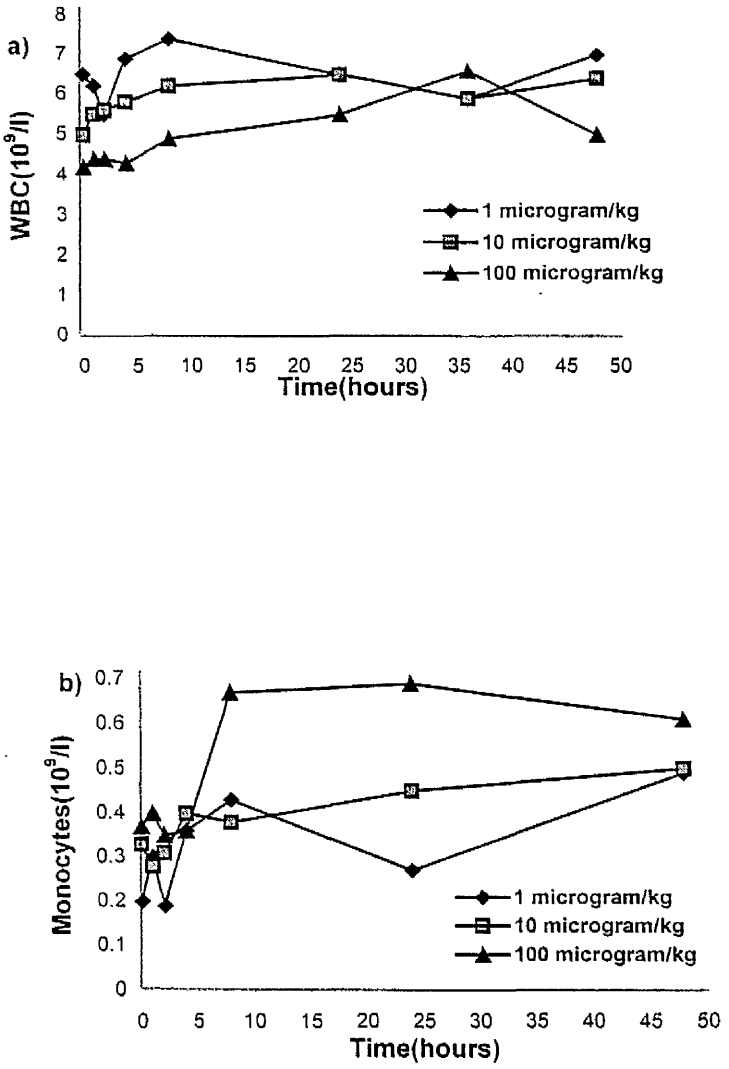

Figure 24. (a) Total WBC and (b) monocyte count in cancer patients (3 per dose level) during and following a 24 hour infusion of MIP-1α.

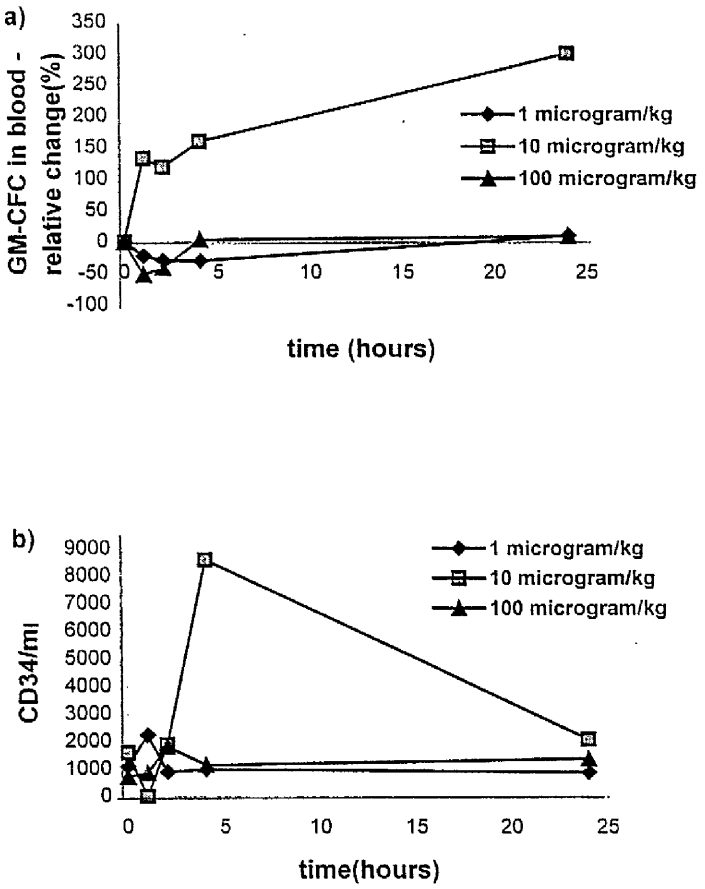

Figure 25. a) the relative change in GM-CFC/ml of blood and b) the absolute numbers of $CD34^+$ cells/ml of blood after bolus injection of MIP-1α in cancer patients (3 per dose level).

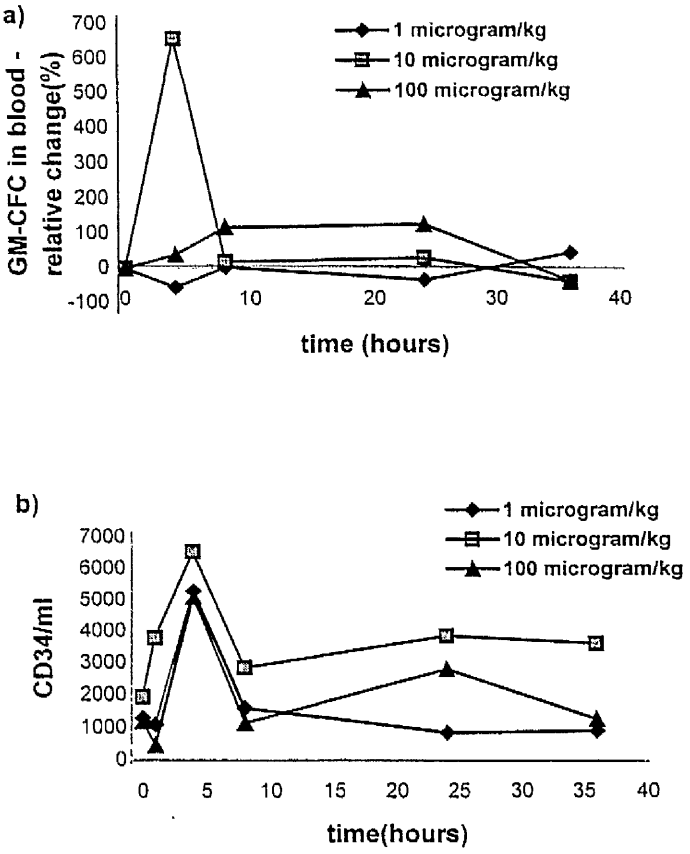

Figure 26. a) the relative change in GM-CFC/ml of blood and b) the absolute numbers of $CD34^+$ cells/ml of blood during and following a 24 hour infusion (s.c) of MIP-1α in cancer patients (3 per dose level).

Subcutaneous infusion produced a constant plasma level from 4 to 36 hours with mean plasma levels of 700pg/ml at 4 hours, 603pg/ml at 24 hours and 780pg/ml at 36 hours with levels subsequently falling to a mean of 231pg/ml at 48 hours and becoming undetectable by 1 week.

2. Normal Volunteer Studies

2.1 Safety and Tolerability

A total of 76 healthy male volunteers received MIP-1α, 36 of whom also received pretreatment with G-CSF. MIP-1α was well tolerated by all groups with no serious adverse effects seen. A number of mild adverse events (headache, 'flu-like' illness) which were considered possibly to be related to MIP-1α administration were reported. The incidence of adverse events showed no relationship to dose and a similar incidence of events occurred in subjects receiving placebo. A total of 6 volunteers reported mild redness and/or pain at the injection site. No clinically significant abnormalities were noted in routine biochemical analyses and there were no consistent changes in MIP-1α antibodies during the study period.

2.2 Effects on haematological parameters:

MIP-1α had no measurable effect on the total leucocyte count (see figure 28a) or leucocyte subsets excepting monocytes in which a modest increase of approximately 150-200% of baseline was observed with peak effects noted at 2-4 hours after i.v. injection

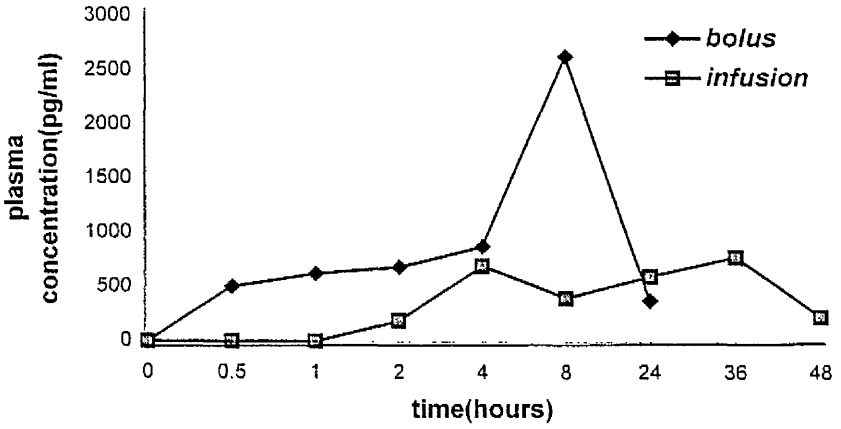

Figure 27. Plasma levels of MIP-1α following s.c. bolus and during and following s.c infusion (100μg/kg) in cancer patients (means of 3 subjects).

and at 4-6 hours following s.c. injection.. In the higher dose groups the relative monocytosis was preceeded by an initial fall in numbers (figure 28b). The change in the monocyte count mirrored drug pharmacokinetics with peak monocyte numbers corresponding to peak plasma concentrations. However, there appeared to be no real difference in circulating progenitor cell numbers between subjects receiving placebo or MIP-1α (see figure 29).

Volunteers that were pretreated with G-CSF showed a marked increase in leucocyte numbers from 5 in the placebo group to 34.5 ± 2.8 in the treated groups II to V by day 2 (figure 30a) but this was not augmented by MIP-1α over the next 24 hours. MIP-1α did result in a 2.5-fold increase in monocyte numbers in subject number 20 (group V) that peaked around the 2 hour time point, however, this followed an exaggerated response to G-CSF when compared to all other study participants. A minor increase (< 2-fold) in the relative monocyte count was noted in other groups following MIP-1α which was once again associated with an initial transient reduction (figure 30b). The monocytosis observed was, however, less marked than was seen in the previous study using MIP-1α alone.

Increasing number of days of G-CSF, pre-MIP-1α, resulted in an increase in circulating progenitor cell numbers although numbers were highly variable between individuals (figure 31). This mobilising effect of G-CSF appeared to be enhanced by subsequent MIP-1α administration except in group III. In groups II, IV and V, GM-CFC continued to rise following MIP-1α, an effect that was most marked following 2 days of G-CSF

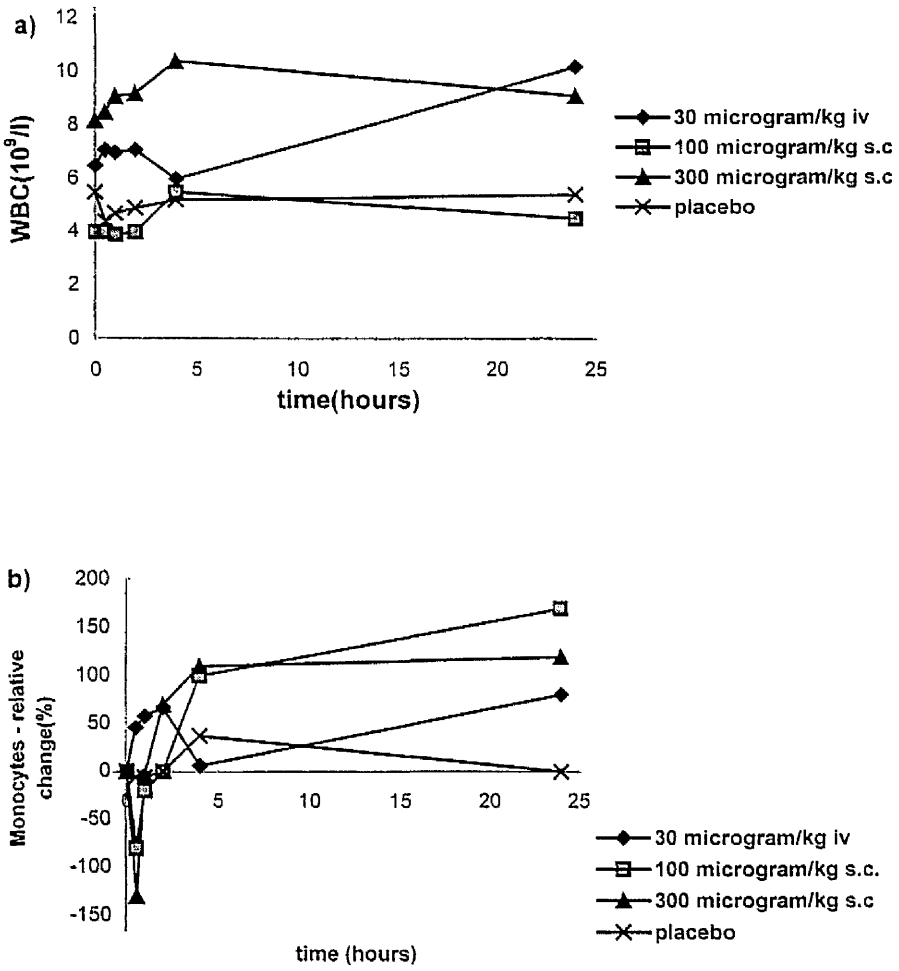

Figure 28. (a) the total WBC and (b) the relative change in monocyte counts following MIP-1α administration to healthy normal volunteers. Results are the means of 3 subjects.

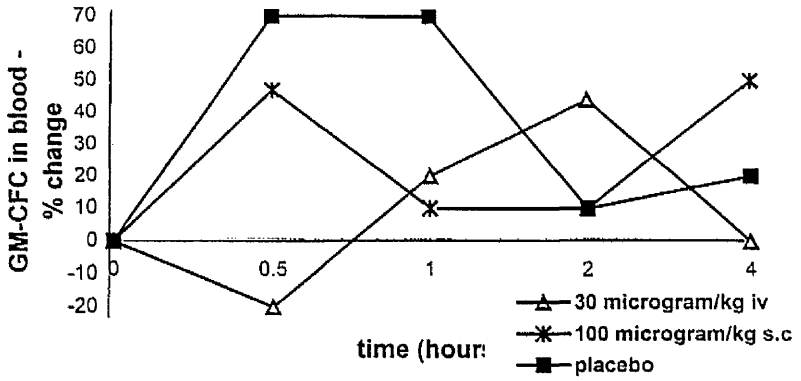

Figure 29. GM-CFC per ml of blood following MIP-1α in normal healthy volunteers. Results are the means of 3 subjects.

pretreatment (7-fold increase over baseline). MIP-1α did not increase $CD34^+$ cell numbers, except in group III, beyond that acheived with G-CSF (Figure 31b).

2.3 Pharmacokinetics

The plasma concentration of MIP-1α following i.v. and s.c. bolus administration in normal volunteers is shown in figure 32. Intravenous injection was associated with an early high peak plasma concentration followed by a rapid decline. The maximum concentrations (cmax) after 10, 30 and 100μg/kg were19700, 55700 and 787000pg/ml all occurring by 0.25 hours, the terminal half life ranging from 1.73 hours at the lower dose level to 2.78 hours at the 100μg/kg dose (Table 16). MIP-1α was not quantifiable in any subject beyond 12 hours following i.v. dosing. Area under the curve (AUC) results showed a non-linear increase with increasing dose with a mean of 3450(pg/ml)hr at 10μg/kg, 17600(pg/ml)hr at 30μg/kg and 326000(pg/ml)hr at 100μg/kg.

Subcutaneous injection resulted in a more favourable pharmacokinetic profile with a sustained plasma exposure over the 24 hour period (Table 16, figure 32b). Mean concentrations following 30, 100, and 300μg/kg MIP-1α at 24 hours were 134, 327, and 2497pg/ml, respectively. Cmax values ranged from 137pg/ml at the 10μg/kg dose to 5590pg/ml following 300μg/kg, the peaks (tmax) being acheived between 4.3 to 6.7 hours.

At the 30, 100 and 300μg/kg dose level, MIP-1α was detectable in all subjects at 24 hours after dosing which means that estimates of $AUC_{0-\infty}$ are inaccurate for s.c. doses. $AUC_{0\text{-}24\ hours}$ was determined with a mean of 4280(pg/ml)hr at 30μg/kg, 16900(pg/ml)hr at 100μg/kg and 91600(pg/ml)hr at 300μg/kg.

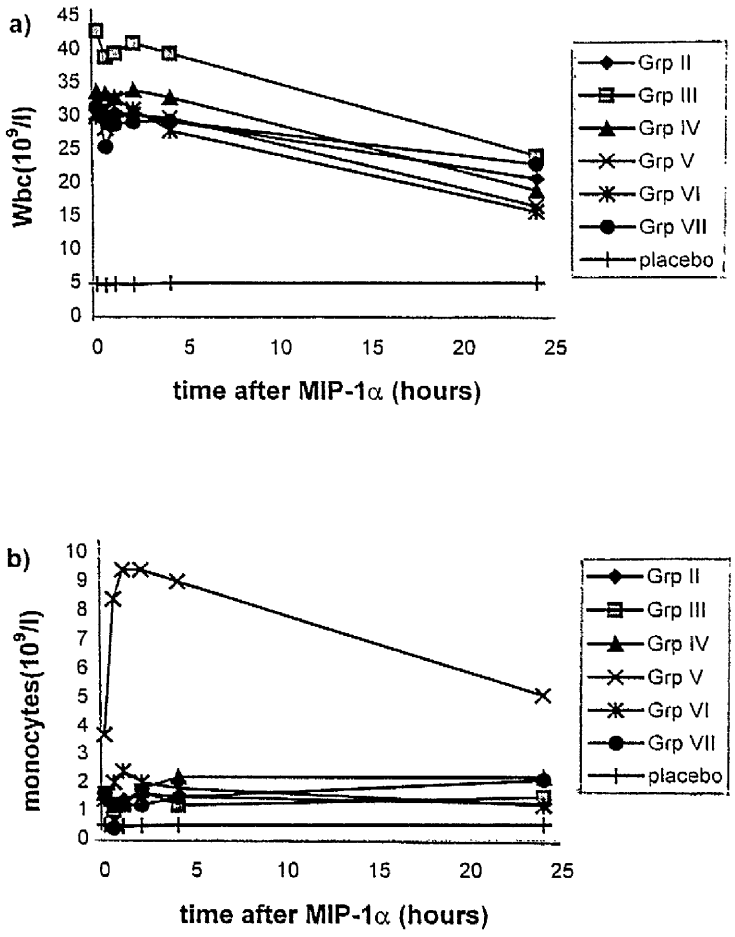

Figure 30. (a) the total WBC and (b) the absolute monocyte count in normal volunteers receiving a variable number of days of G-CSF followed 24 hours later by MIP-1α (groups II to V received 2-5 days of G-CSF, respectively followed by 30μg/kg s.c. MIP-1α, group VI and VII received 5 days of G-CSF followed by 100μg/kg s.c and 10μg/kg i.v, respectively). Results are the means of 4 subjects.

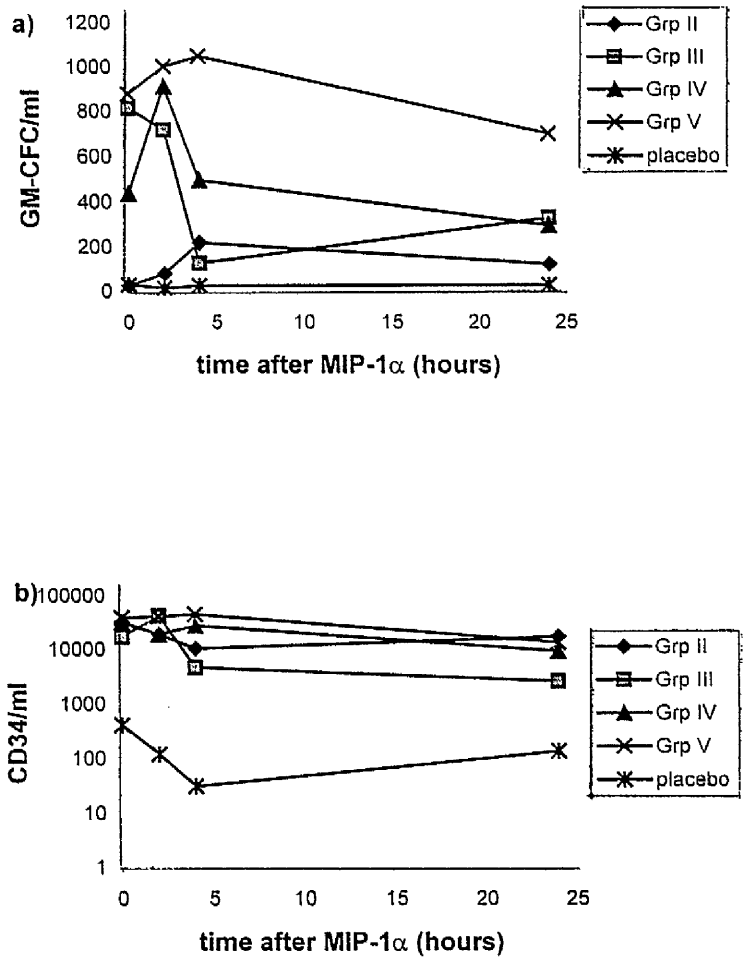

Figure 31. GM-CFC (a) and $CD34^+$ cell (b) numbers per ml of blood in normal healthy volunteers following G-CSF and MIP-1α (groups II to V received 2-5 days of G-CSF, respectively followed by 30μg/kg s.c. MIP-1α). Results are the means of 4 subjects.

Table 16. MIP-1α Pharmacokinetics Following Bolus Injection in normal volunteers.

Summary of pharmacokinetic parameters								
	intravenous MIP-1α(μg/kg)				**subcutaneous MIP-1α(μg/kg)**			
	1 (n=3)	10 (n=3)	30 (n=3)	100 (n=3)	10 (n=1)	30 (n=3)	100 (n=3)	300 (n=3)
$AUC_{0-\infty}$ (pg/ml)hr		3450	17600	326000		8630	22400	265000
$AUC_{0-24hrs}$ (pg/ml).hr					123	4280	16900	91600
Cmax (pg/ml)	563	19700	55700	787000	137	463	1170	5590
Tmax	0.25	0.25	0.25	0.25	5	4.33	6.67	6.67
Tel		1.73	2.05	2.78		12.6	9.31	44.85

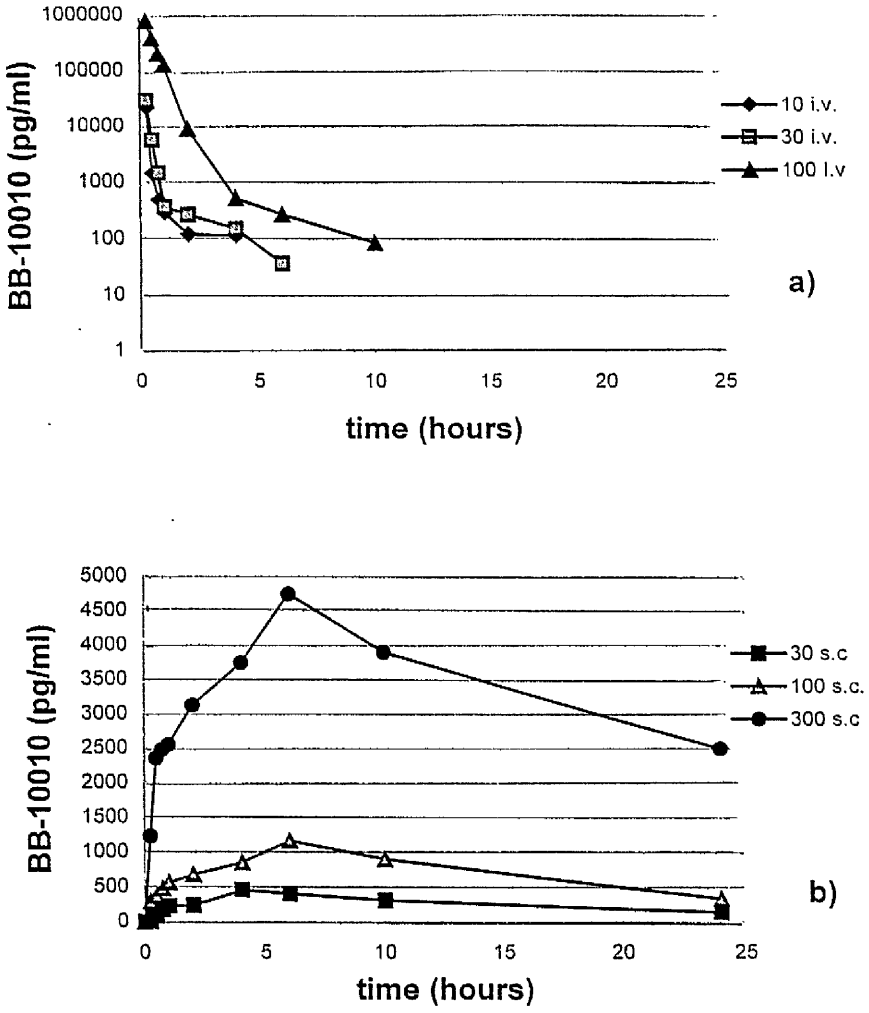

Figure 32. Serum levels of MIP-1α following a) i.v bolus and b) s.c bolus injection in normal healthy volunteers.

PHASE II STUDIES

1. A Phase II Study of MIP-1α in advanced, inoperable non-small cell lung cancer

This was an open-label, phase I/II alternate cycle randomisation study conducted in advanced or inoperable non-small cell lung cancer (NSCLC). The objectives of the study were to evaluate the safety and tolerability of repeated administrations of MIP-1α and to determine any myeloprotective properties of the molecule. The latter was assessed indirectly by measuring mature cell recovery kinetics with and without MIP-1α. In addition, febrile neutropenia, antibiotic usage, blood product transfusion and days of hospitalisation were evaluated as secondary endpoints.

1.1 Eligibility Criteria

Inclusion criteria:

- Eligible for full dose MIC chemotherapy
- Patients with histologically proven NSCLC
- Patients with normal haemopoiesis as judged by a WBC $\geq 3.5 \times 10^9$/l, platelets $\geq 150{,}000 \times 10^9$/l and Hb $\geq$ 12g/dl
- 18 years or older
- Written informed consent
- ECOG performance status ≤ 2

Exclusion Criteria

- history of life threatening anaphylaxis
- acute illness within 2 weeks prior to the start of the study
- use of other investigational agents during the study
- concomitant treatment during the study with G-CSF or other cytokines
- pregnant or breast feeding females
- Known involvement of more than 3 sites of the haemopoietic system with metastases as determined by isotope bone scan or received radiotherapy to more than one third of the skeleton
- any condition which, in the opinion of the investigator, would make the patient unsuitable for the study.

1.2 Patients and methods

Patients: 12 patients with histologically proven, inoperable NSCLC were entered into the study between July 1995 and November 1995 (table 17). Five patients had squamous cell carcinoma (SCC) and 7 patients had adenocarcinoma. None of the patients had received prior chemotherapy or radiotherapy. Seven patients had stage IV disease, 3 had stage IIIb disease, 1 had stage IIIa disease and 1 had stage II disease. The median age was 57 years (range 36 to 70) and the median ECOG performance status was 1 (range 0 to 2).

Treatment: Chemotherapy consisted of mitomycin-c ($6mg/m^2$) administered intravenously on day 0 , carboplatin ($400mg/m^2$) administered intravenously on day 0 and ifosfamide and mesna ($5g/m^2$) administered intravenously over 24 hours on day 0 (mesna $2.5g/m^2$ was given orally for an additional 12 hours). Chemotherapy cycles were repeated every 4 weeks up to a maximum of 4 cycles . If the WBC count was less than 3.5 x 10^9/l, the platelet count less than 150 x 10^9/l or the creatinine clearance was less than 50ml/min, therapy was delayed by 1 week or until recovery was adequate. No chemotherapy dose reductions were undertaken due to previous myelosuppression. If recovery had not occurred following a 2 week delay patients were withdrawn from the study and treated at the investigators discretion. Prophylactic platelet transfusions were administered if the platelet count fell to less than 20 x 10^9/l and whole blood was transfused if the Hb fell to below 9g/dl.

MIP-1α: MIP-1α was administered as a solution containing 2mg/ml. All patients received 30μg/kg of MIP-1α as a daily subcutaneous injection for 7 days, from day -1 to day 5. All doses were administered by a registered nurse although from days 2 to 5 this was carried out in the outpatient setting. Patients were randomised to receive MIP-1α either in cycles 1 and 3 (group 1) or in cycles 2 and 4 (group 2).

1.3 Results

Toxicity: A total of 46 cycles of chemotherapy was administered. Ten patients received all 4 cycles of treatment and 2 patients received 3 cycles of treatment. Patients 5 and 6

Table 17. Phase II Lung Cancer Study : Patient Characteristics.

Study number	Active cycles*	Sex	Age	Histology	ECOG status	Stage
1	1 + 3	F	66	Adeno	0	IV
2	1 + 3	M	70	SCC	2	IV
3	2 + 4	M	55	Adeno	1	IV
4	1 + 3	M	59	Adeno	2	IV
5	2 + 4	M	68	SCC	1	IV
6	2 + 4	F	52	SCC	2	IIIa
7	2 + 4	F	36	Adeno	0	IV
8	1 + 3	M	66	Adeno	2	IIIb
9	1 + 3	M	48	SCC	1	IIIa
10	2 + 4	F	63	Adeno	1	II.
11	2 + 4	M	45	SCC	1	IIIa
12	1 + 3	M	53	Adeno	1	IV

* Treatment cycles receiving additional MIP-1α treatment.

failed to complete treatment because of unacceptable chemotherapy toxicity and progressive disease, respectively. Myelosuppression represented the principle toxicity of the MIC programme (table 18) with grade IV neutropenia occurring in 69.5% of cycles and grade IV thrombocytopenia complicating 32.6% of cycles. Forty three percent of cycles were complicated by neutropenic sepsis (neutrophils of less than 0.5 $x10^9/l$ and a fever of 38.5°C on two occasions). The pattern of toxicity was similar when comparing MIP-1α-treated and -untreated cycles (table 18). A total of 17% of cycles was delayed, 75% of these in MIP-1α-treated cycles and related to prolonged infection (2 patients), suboptimal recovery of creatinine clearance (3 patients) and bleeding gastric erosions (1 patient). Two patients required a 25% dose reduction of chemotherapy for reduced creatinine clearance of less than 50ml/min (patient 2 - cycle 2, patient 10 - cycle 2). Patient 1 developed acute renal failure in cycle 4 associated with a candida septicaemia. There was one toxic death (patient 2) resulting from an episode of neutropenic sepsis in cycle 4.

MIP-1α : MIP-1α was extremely well tolerated by all patients with no significant adverse event reported in association with drug administration. There was no evidence of any local or systemic inflammatory response following MIP-1α injection and no episodes of allergy or anaphylaxis were observed. Serial measurements of MIP-1α antibodies were carried out using an anti-BB-10010 Elisa with no significant antibody levels detected throughout the study period.

Table 18. Comparison of chemotherapy toxicity between MIP-1α-treated and non-treated cycles.

Treatment Cycles And Toxicity									
Chemotherapy Cycles		**Gd IV neutopenia**		**neutropenic sepsis**		**Gd IV thrombocytopenia**		**Delays**	
MIP-1α	-	MIP-1α	-	MIP-1α	-	MIP-1α	-	MIP-1α	-
23	23	15	17	13	13	7	8	6	2
46		32		20		15		8	

Tumour Response: Whilst the evaluation of tumour response was not an objective of the study, a growth modulating effect by MIP-1α on tumour cells was considered. While patients who entered into the study did not consistently fullfill UICC criteria for response and measurable disease CXR and chest CT scanning were available for evaluation in all patients. During the study period, 2 patients progressed, there was 1 toxic death and 9 patients had stable disease.

Hospitalisation: The days of hospitalisation, intravenous antibiotic usage and blood product requirements are shown in table 19. An excess of inpatient days was recorded during the non-MIP-1α- treated cycles (145 vs 105) ($p < 0.2$) but this was largely due to patient 1 who required a prolonged hospital stay during cycle 4 with candida septicaemia and renal failure. The median inpatient stays were similar for both MIP-1α-treated and untreated cycles (8 vs 9.5). No difference was apparent for antibiotic usage or RBC transfusion between cycles. There was an excess of platelet transfusions in the non-MIP-1α-treated cycles (158 vs 86) ($p < 0.1$), however, this resulted from a high platelet requirement in a small number of patients. Episodes of neutropenic sepsis were similar in both treated and untreated cycles, however, there was considerable interpatient variation with several patients developing sepsis with each cycle (patients 1, 2, 4 and 12) as compared to others who had no episodes of sepsis (patients 3, 7 and 9).

Table 19. Days of hospitalisation, i.v. antibiotic usage and blood product transfusion with and without MIP-1α.

Pat.no	Inpatient Days		Intravenous Antibiotics		RBC Transfusions		Platelet Transfusions	
	MIP-1α	-	MIP-1α	-	MIP-1α	-	MIP-1α	-
1	19	52	12	25	4	6	16	30
2	8	12	8	14	4	4	14	30
3	0	0	0	0	0	0	0	0
4	23	17	18	15	7	0	14	8
5	8	7	8	6	4	2	6	8
6	0	17	0	16	0	4	0	22
7	0	0	0	0	0	0	0	0
8	10	13	7	7	4	6	8	28
9	0	0	0	0	0	4	0	0
10	11	6	10	6	0	4	4	16
11	5	0	5	0	3	0	8	0
12	21	21	16	12	4	3	16	16
Total	105	145	84	101	30	33	86	158
mean	8.75	12	7	8.4	2.5	2.7	7.2	13.1
median	8	9.5	7.5	6.5	3.5	3	7	12

Haematological Parameters: The neutrophil recoveries for both group 1 and group 2 over all 4 cycles of chemotherapy are shown in figure 33. The results represent the mean neutrophil counts calculated as a percentage of the average prestudy baseline value taken on day -1 of cycle 1. Administration of MIP-1α, either in cycles 1 and 3 or cycles 2 and 4, did not appear to influence the kinetics of neutrophil recovery over the 4 cycles and no intercycle variation was demonstrated. Within each group, the absolute neutrophil and platelet values at day 14 of each cycle (nadir) remained similar throughout the study, with little intercycle variation. Repeated chemotherapy resulted in a progressive reduction in the recovery value at day 28 of each cycle (peak) (figures 34 and 35). A comparison of the nadir and peak neutrophil numbers between group 1 (MIP-1α in C1 and C3) and group 2 (MIP-1α in C2 and C4) showed that group 1 had a consistently improved neutrophil recovery between days 14-28 despite deeper nadir values in each cycle (figure 36). This apparent benefit to neutrophil recovery, however, was not extended to platelet recovery.

Chemotherapy was associated with a fall in mean Hb levels of approximately 2g/dl over the 4 cycles. The fall in Hb in group 1 (11.3 ± 0.5 to 9.5 ± 1.2) was similar to that observed in group 2 (11.8 ± 0.5 to 9.3 ± 0.3) and no intercycle variation was evident following MIP-1α administration.

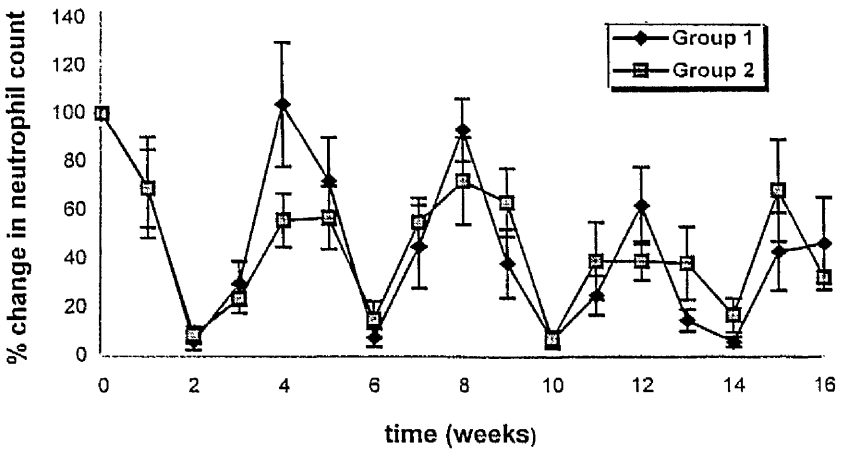

Figure 33. Neutrophil recovery over 4 cycles of MIC chemotherapy. Group 1 received MIP-1α in cycles 1 and 3 and group 2 received MIP-1α in cycles 2 and 4.

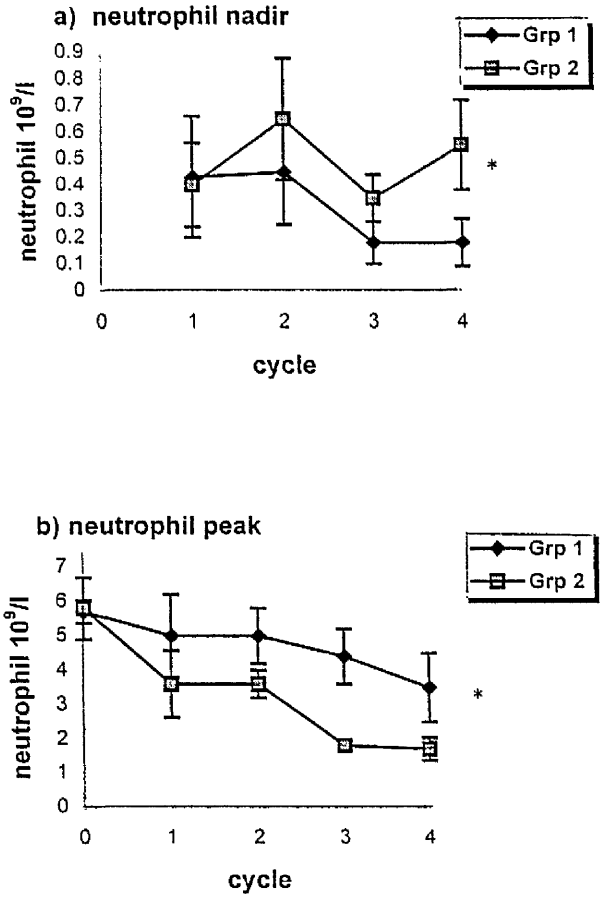

Figure 34. Results show (a) the mean neutrophil nadir numbers and (b) neutrophil peak numbers following MIC chemotherapy and MIP-1α. Group 1 received MIP-1α in cycles 1 and 3 and group 2 received MIP-1α in cycles 2 and 4. * $p > 0.1$.

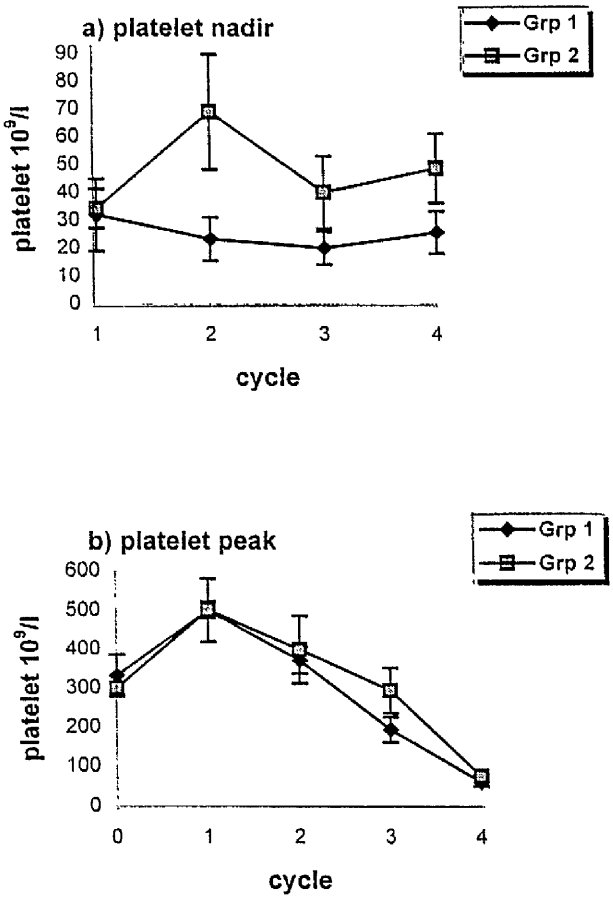

Figure 35. Results show the a) the nadir platelet counts and b) the platelet peak counts following MIC chemotherapy and MIP-1α. Group 1 received MIP-1α in cycles 1 and 3 and group 2 received MIP-1α in cycles 2 and 4.

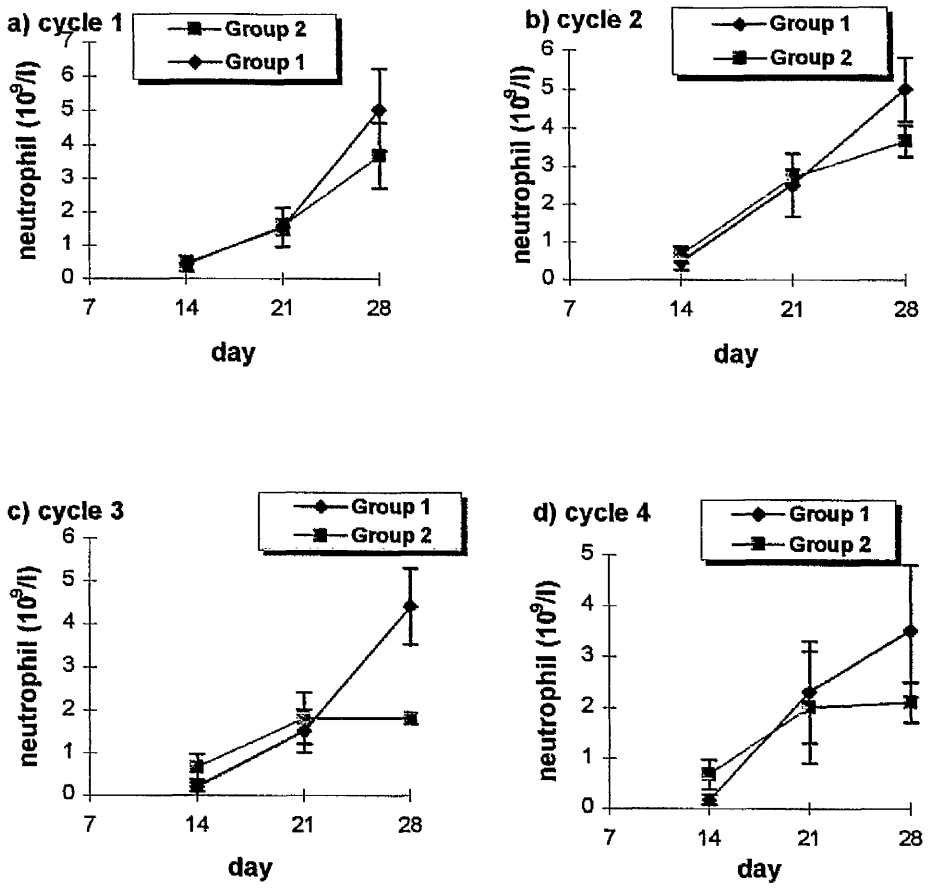

Figure 36. Results show the neutrophil recovery for each cycle of MIC chemotherapy administered with and without MIP-1α.

2. A phase II study of MIP-1α in patients with advanced breast cancer undergoing FAC chemotherapy.

A phase II randomised prospective study was carried out comparing 2 dose levels of MIP-1α (30 and 100μg/kg) in patients with locally advanced or metastatic breast cancer undergoing FAC chemotherapy. The study was designed to assess the modulating effects of MIP-1α on haemopoietic cell recovery when co-administered during all cycles of chemotherapy. The response was determined by assessing mature cell recovery (neutrophils and platelets), the mobilisation potential of the BM during chemotherapy (as determined by blood GM-CFC and $CD34^+$ cells) and BM quality, assessed by the GM-CFC production in LTBMC from pre-chemotherapy and post-chemotherapy BM. Secondary endpoints of myeloprotection were also determined, including days of hospitalisation, episodes of febrile neutropenia and blood product transfusion requirements.

2.1 Eligibility Criteria.

Inclusion criteria

- Eligible for full dose FAC
- Female patients with histologically proven breast cancer
- Patients with normal haemopoiesis as judged by a WBC $\geq 3 \times 10^9$/l , platelets $\geq 100 \times 10^9$/l and haemoglobin $\geq$ 11g/dl.
- 18 years or older

- Normal renal function (creatinine less than 1.25μmol/l) and bilirubin < 2 times the upper limit of normal.
- Written informed consent
- ECOG performance of ≤ 2

Exclusion criteria

- Previous chemotherapy except adjuvant CMF more than 12 months ago
- History of life threatening anaphylactic reaction
- Acute illness within 2 weeks prior to the start of the study
- Other investigational agents during the study or within 1 month of the start
- Concomitant treatment during the study with G-CSF or other cytokines
- Pregnancy or breast feeding
- Previous radiotherapy to more than one third of the skeleton
- Any other condition which in the opinion of the investigator would impair the subjects participation in the study

2.2 Patients and methods.

Patients: Thirty patients were recruited between August 1995 and March 1996 from 3 UK centres. Patients were randomised into 1 of 3 groups, consisting of 10 patients per group. In groups 1 and 2, patients received doses of MIP-1α of 100μg/kg or 30μg/kg for 7 days (day -1 to day 5) with each cycle of FAC chemotherapy given on day 0. Group 3 received chemotherapy alone (control). The randomisation schedule (minimisation)

balanced the potential modulating effect of previous adjuvant chemotherapy or bone metastases on haemopoietic recovery. The 3 randomisation categories included.

1. Locally advanced disease
2. Evidence of metastatic disease in less than 3 bony areas (ribs, pelvis and spine) as determined by bone scan.
3. Evidence of metastatic disease in more than 3 bony metastatic areas (ribs, pelvis and spine) as determined by bone scan.

Treatment. Chemotherapy was administered as an intravenous bolus injection repeated every 3 weeks up to a maximum of 6 cycles and consisted of 5 fluorouracil ($600mg/m^2$) day 0, doxorubicin ($50mg/m^2$) day 0, and cyclophosphamide ($600mg/m^2$) day 0. All chemotherapy was administered in an outpatient setting and was commenced 24 hours after the first MIP-1α injection. Full dose FAC was administered at 21 day intervals and was delayed by 1 week if the neutrophil count had not recovered to $\geq 1 \times 10^9/l$ or the platelet count had not recovered to $\geq$
$100 \times 10^9/l$. A 25% dose reduction in chemotherapy was considered if the patient suffered an episode of neutropenic sepsis (neutrophil $< 0.5 \times 10^9/l$ and fever ≥ 38.5 °C) or if the patient suffered any grade 3 or 4 non-haematological toxicity according to the NCI common toxicity criteria. If persistent cytopenia prevented further cycles of chemotherapy the patient was removed from study and treated at the discretion of the investigator.

Table 20. Phase II Breast Cancer Study: Patient Characteristics.

Pat. n°	Age	ECOG Score	Stage	Bone Scan	Marrow Status	Prev. Chemo	MIP-1α (μg/kg)
101	48	0	IV	-ve	normal	no	100
102	48	1	IV	-ve	normal	no	30
103	62	0	III	-ve	normal	no	30
104	42	1	IV	-ve	normal	CMF	100
105	54	1	IV	+ve	normal	CMF	no
106	55	1	IV	-ve	normal	no	30
107	64	0	IV	-ve	normal	no	100
108	51	0	IV	+ve	infiltration	no	30
109	29	1	IV	+ve	infiltration	CMF	no
110	64	1	IV	-ve	normal	no	no
111	57	0	IV	-ve	normal	no	100
112	35	1	IV	+ve	infiltration	no	30
113	60	1	III	-ve	normal	no	100
114	35	2	IV	+ve	infiltration	no	no
115	45	1	IV	+ve	normal	CMF	30
116	56	1	IV	+ve	normal	no	100
117	46	0	IV	-ve	normal	no	30
118	42	1	IV	-ve	normal	CMF	no
119	49	1	IV	-ve	normal	CMF	30
120	56	1	IV	-ve	normal	no	100

MIP-1α : MIP-1α was supplied as a sterile solution containing 2mg/ml or 10mg/ml and was administered during all cyles of chemotherapy as a daily subcutaneous injection for 7 days beginning 24 hours before chemotherapy. All injections were administered by a registered nurse either at the hospital visit or via a home visit.

1.3 RESULTS

Twenty patients were recruited and treated at a single centre (Christie Hospital, Manchester) between August 1995 and March 1996. The results presented will be restricted to these 20 patients since the author had direct access to, and involvement in these and it was for these patients only that specialist BM data was studied and analysed. A futher 10 patients were recruited from additional centres, of these 10, 8 patients completed the study and the results are compatible with those presented below. Patient characteristics (Manchester cohort) are shown in table 20. Median age was 50 years (range 29 to 64) and patients had a median ECOG performance status of 1 (range 0 to 2). Of the 20 patients, 7 had evidence of bone metastases on radioisotope bone scan. Four of these had metastatic involvement of the bone marrow on trephine examination although this was typically focal infiltration only with normal haematological indices. Seven patients were randomised to the 100μg/kg dose level, 8 to the 30μg/kg level and 5 control. All patients completed 6 cycles of chemotherapy and data were available from all 120 treatment cycles for evaluation (42 cycles for the 100μg/kg dose, 48 cycles for 30μg/kg and 30 cycles for the control group).

Toxicity

FAC chemotherapy was well tolerated with a low incidence of neutropenic sepsis in all groups. Only 6 of the 120 cycles were complicated by short-lasting sepsis (mean of 3.6 days) and included 1 patient from the control group, 2 patients at the 30μg/kg level and 3 patients at the 100μg/kg level. One patient in the control group and 2 patients in the 30μg/kg level required a 1 week delay. Only one patient (patient 20 - 100μg/kg) required two treatment delays. Non-haemopoietic toxicity was typically mild with no episodes of oral mucositis ≥ 2 NCI grade. Nausea was well controlled with standard antiemetics. MIP-1α administration was not associated with any serious adverse events. Two patients suffered a mild self-limiting cutaneous reaction characterised by erythema and swelling at the injection site (Patient 11 and 17 - see figure 37). No patient developed any systemic inflammatory response or anaphylaxis. The development of MIP-1α antibodies was investigated in all patients during the study period.

Tumour response

The study was not designed formally to assess the response of the tumour to chemotherapy and therefore not all patients necessarily met UICC criteria for measurable disease. However, they were all evaluable for response. No difference was apparent between groups or with the anticipated response based on historical data with anthracycline based chemotherapy.

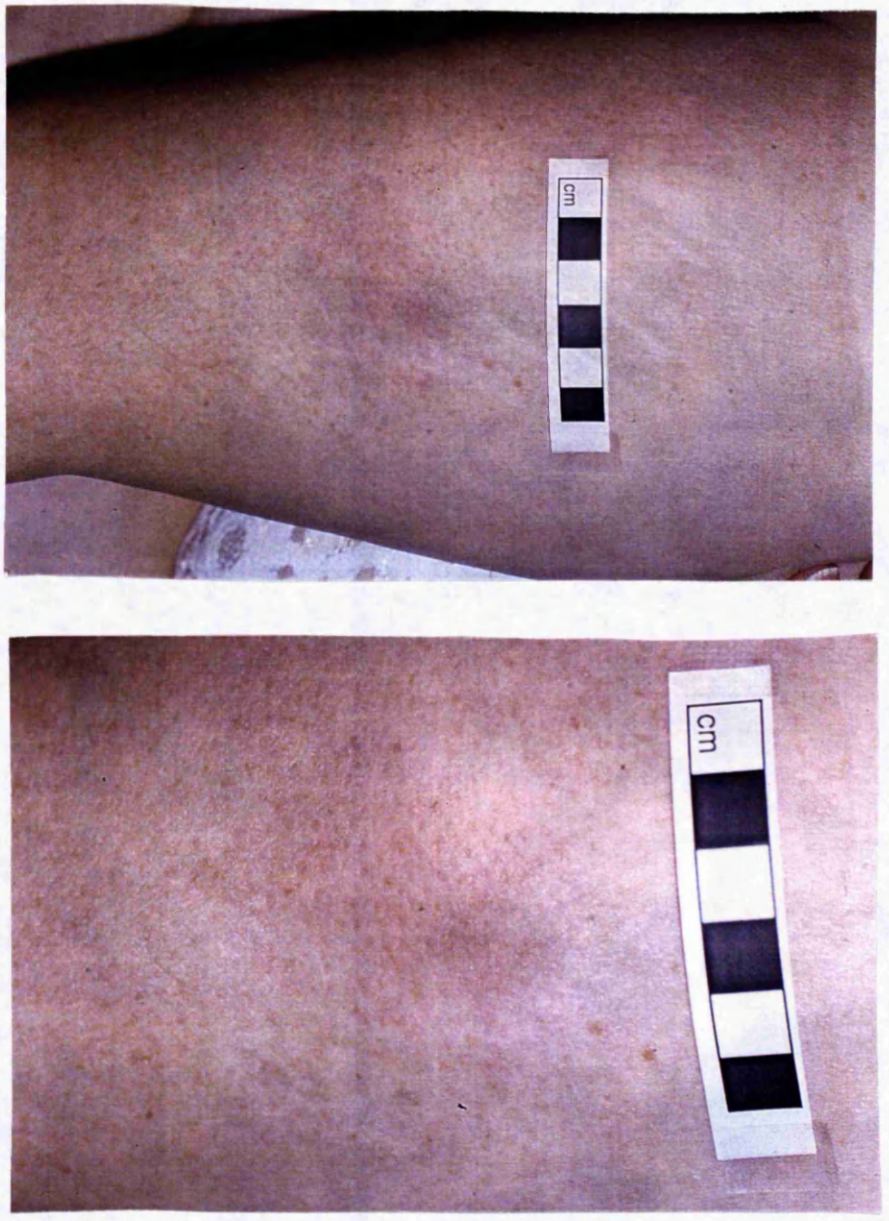

Figure 37. Cutaneous reaction following s.c. administration of MIP-1α (100μg/kg) in subject 11.

Haemopoietic recovery kinetics

Recovery of mature cells (Hb, leucocytes and platelets) was determined at day 20 of all cycles and on days 10, 14, 16 and 20 during cycles 1, 3 and 6. The recovery of neutrophil numbers in cycles 1, 3 and 6 is shown in figure 38 and Table 21. Chemotherapy treatment resulted in a neutrophil nadir at day 14 of each cycle with recovery to > 1.5 x 10^9/l by day 21. The peak recoveries of platelets, total leucocyte count (WBC) and neutrophils, as determined on day 20 of every cycle, are shown in figure 39. FAC chemotherapy was associated with a mild cummulative damage as reflected by a progressive reduction in the peak counts which was similar in all cases. Chemotherapy was not associated with any significant change in the platelet count or Hb levels which both rémained stable throughout the study period (figures 39 and 40).

Peripheral blood progenitor cell mobilisation and BM function

Blood progenitor cells (GM-CFC/ml and $CD34^+$ cells) were quantitated at days 14, 16 and 20 of cycles 1, 3 and 6. In addition, all patients underwent a pre-chemotherapy BM examination to determine the extent of BM infiltration by carcinoma cells and to assess the GM-CFC generating potential of the BM in LTBMC. A second BM examination was carried out 6 weeks following completion of chemotherapy to assess the detrimental impact of the chemotherapy on LTBMC initiation and function, and to determine any protective effect by MIP-1α. The laboratory work for this part of the trial was undertaken principally by Drs Kentaro Watanabe and Jan Duerig (Dept. Experimental Haematology

Table 21. Neutrophil recovery (10^9/l) following FAC chemotherapy and MIP-1α.

Treatment	day 0	day 14	day 20
	cycle 1		
control	4.9(3.1-7.4)	0.3(0.1-0.5)	4.1(1.9-4.9)
30μg/kg	4.4(2-6.5)	0.3(0-4.8)	2.5(0.9-7.3)
100μg/kg	4(3.4-5.8)	0.3(0.1-0.9)	2.9(0.5-5.3)
	cycle 3		
control	4.5(1.8-6.2)	0.5(0.1-0.9)	3.3(1.9-5)
30μg/kg	3(0.5-6.7)	0.4(0.1-0.6)	2.7(0.5-6)
100μg/kg	2.9(0.4-7.7)	0.5(0.1-2.8)	3.9(0.8-5)
	cycle 6		
control	2.8(1.3-4.6)	0.3(0.1-0.5)	1.2(0.4-3.2)
30μg/kg	1.8(0.6-4.6)	0.5(0.1-0.9)	1.8(0.5-4.4)
100μg/kg	2.7(0.5-3.6)	0.3(0.1-0.8)	3.2(0.5-4.2)

Results show the median(range).

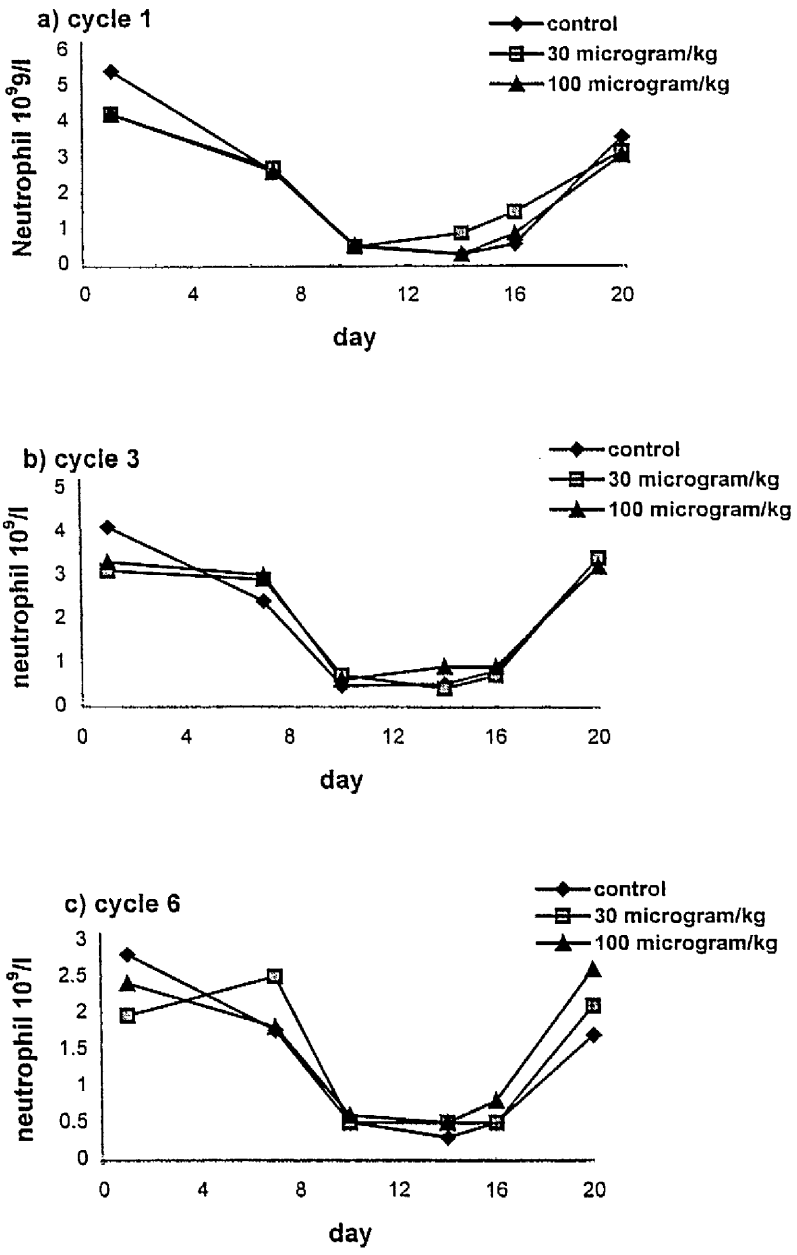

Figure 38. Results show the mean neutrophil counts for cycles 1, 3, and 6 following FAC chemotherapy ± MIP-1α

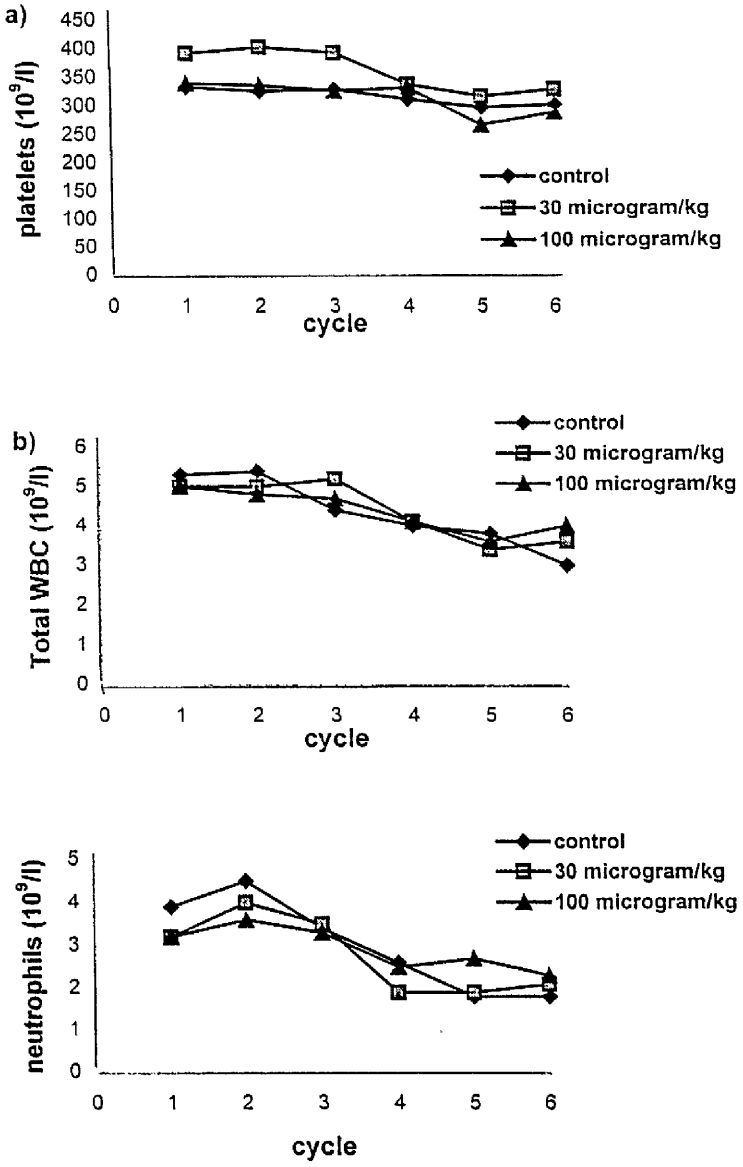

Figure 39. Results show the peak recovery counts of a) platelets, b) total WBC, and c) neutrophils for all 6 cycles of chemotherapy with and without MIP-1α.

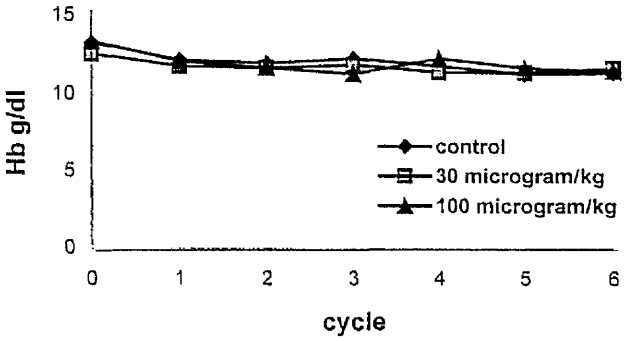

Figure 40. Mean Hb level over all 6 cycles of FAC chemotherapy.

Paterson Institute, Manchester) and will form the basis of a full report when the data are fully matured. However, the preliminary results suggest that there was a 2-3 fold increase in progenitor cell mobilisation seen in the peripheral blood of the MIP-1α-treated groups compared to control. Bone marrow analysis revealed that there was similar GM-CFC production in LTBMC in all groups. However, there was no measurable difference in the progenitor cell content (GM-CFC and $CD34^+$) post-treatment BM compared to the pre-chemotherapy examination suggesting that the cytotoxic treatment resulted in little, if any, cummulative BM damage in any group.

Discussion

The clinical studies completed to date have shown that the MIP-1α analogue, BB-10010, is extremely well tolerated, thus confirming the earlier findings in animal models. The phase I studies were designed principally to investigate the safety and tolerance of escalating doses of MIP-1α using the s.c. and i.v. routes of administration. In addition, the possibility that MIP-1α may promote antibody formation and antagonise natural MIP-1α was also considered. The results showed that neither bolus administration of up to 300μg/kg s.c and 100μg/kg i.v. nor 24 hour s.c infusion of up to 100μg/kg was associated with any significant toxicity and a maximum tolerated dose was not defined. Whilst several subjects had low levels of anti-MIP-1α antibodies at screening, no fluctuation was observed throughout the study period. MIP-1α was possibly associated with a number of mild side effects including, flushing, headache and dizziness, although the

incidence was no greater than in subjects receiving placebo. Of particular note, MIP-1α did not invoke any local or systemic inflammatory response despite previous experimental findings (see introduction) and the classification of MIP-1α as a pro-inflammatory cytokine (Wolpe et al., 1988). Six of the 85 subjects did complain of some discomfort or redness at the injection site but this was considered minor. MIP-1α was not associated with any febrile response in either the healthy volunteers or cancer patients.

The lack of proinflammatory activity of MIP-1α in these studies may relate to BB-10010's favourable properties in solution. This seems unlikely, however, given the comparable biological activity of BB-10010 and native MIP-1α in other experimental systems (Hunter et al., 1995). In early experiments using a rat model, MIP-1α was found to be pyrogenic only as part of the MIP-1 doublet (Davetalis et al., 1989) and subsequent separation of MIP-1 into its constituent parts showed that MIP-1β and not MIP-1α was the predominant partner during fever induction (Myers et al., 1993). Furthermore, those studies involved the direct injection (and therefore of a very high local concentration) of protein into the region of the hypothalamus with the ensuing response perhaps representing a non-specific pharmacological effect rather than a true physiological one. A further possibility for the relatively inert nature of MIP-1α in these studies is that the molecule has species-specific inflammatory effects. Injection of human MIP-1α into the footpad of mice was shown to produce immediate footpad swelling, characterised histologically, by a severe inflammatory response (Wolpe et al., 1988), in contrast, intradermal injection of human MIP-1α had no effect in dogs (Meurer et al., 1993). In

murine experiments the MIP-1α-induced inflammatory response was accompanied by extensive mast cell degranulation and it is possible, therefore, that the differing effects amongst species may relate, at least in part, to the variable response to MIP-1α of mast cells (Wolpe et al., 1988). This may also provide an explanation for the lack of any allergic or anaphylactic reaction in the clinical studies, despite the injection of large quantities of protein (up to 20mg at the highest dose used - 300μg/kg).

Compared to the neutrophilia induced by MIP-1α in mice (Lord et al., 1995) the effect on the total leucocyte count and neutrophil numbers in the early trials was relatively small. This, again, may represent a species-specific effect. Although MIP-1α was described as a chemotactic agent for murine neutrophils (Wolpe et al., 1988, Appelberg, 1992), the evidence for such an effect in humans is weak. McColl et al (1993) reported that human MIP-1α exerted a small dose-dependent increase in intracellular calcium in human neutrophils. However, this was weak in comparison to that induced by the c-x-c chemokines, IL-8 and GROα (MIP-1α is in the c-c structured arm of this cytokine family). Furthermore, the calcium effect was not coupled to neutrophil effector functions such as degranulation or chemotaxis. Similarly, Wang et al (1993) reported that human MIP-1α was chemotactic for human monocytes at doses that were ineffective on neutrophil chemotaxis. In these trials also, administration of MIP-1α resulted in a monocytosis. The fact that this was dose-related and mirrored the drug pharmacokinetics was also consistent with the experimental evidence that MIP-1α and related c-c chemokines act principally as chemotactic factors for mononuclear cells. The monocytosis was most apparent following s.c. injection and was frequently preceeded by

an acute, short-lived reduction in monocyte numbers. A similar phenomenon was documented following administration of colony stimulating factor when the neutrophil leucocytosis was preceeded by an initial acute fall in neutrophils numbers (Steward et al., 1989). This was explained as a possible consequence of leucocyte-endothelial interactions or pooling in the pulmonary circulation.(GM-CSF)

In the phase I clinical trials, MIP-1α did not result in a consistent release of BM progenitor cells (figures 25, 26 and 29). However, this is perhaps not unexpected given the relatively small sample size and the fact that MIP-1α on its own only resulted in a 2-fold progenitor cell increase in the peripheral blood of mice (Lord et al., 1995). In several groups receiving initial G-CSF 24 hours before MIP-1α, circulating progenitor cell numbers continued to increase for up to 4 hours post-MIP-1α injection (figure 31). This effect was most marked after 2 days of G-CSF when a 7-fold increase in circulating progenitor cells was observed and suggests that further studies should perhaps investigate an optimal dose of MIP-1α using a limited schedule of G-CSF rather than a full 5 days. Alternatively, the rising progenitor cell numbers post-MIP-1α may merely represent the peak time of mobilisation following G-CSF (Sato et al., 1995). Unfortunately, any useful comparison with control was limited by wide variation in subject response and the lack of a G-CSF treatment-only arm. The mobilising effect observed in mice occurred at a dose of 10μg/kg which translates to approximately 400μg/kg in man. The lack of a clear mobilising effect in the human studies may, therefore, merely represent a dose effect or result from differing pharmacokinetic profiles of MIP-1α in different species. Following s.c administration of 100μg/kg, the peak plasma concentration of MIP-1α observed in

man was 10-fold less than that seen in mouse, rat and marmoset (BBL-personal communication). Plasma levels of MIP-1α corresponding to those acheived using 100μg/kg in rodents were achieved in man following 300μg/kg s.c and 30μg/kg i.v. However, raising the dose to these levels did not result in any additional effect on mature cell kinetics or progenitor cell mobilisation and thus the relationship of MIP-1α effects with its pharmacokinetic properties is clearly not a simple one.

Pharmacokinetic analyses showed that plasma levels of MIP-1α were detectable despite an inability to determine a clinically significant biological effect on circulating cells and it was shown that MIP-1α, administered as a single s.c. injection of 30-300μg/kg, produced a sustained plasma concentration over a 24 hour period. This suggested that a once daily injection should be adequate for the further clinical evaluation.

These phase I studies have thus shown that MIP-1α is extremely well tolerated either as a single bolus injection or as a 24 hour infusion. No significant toxicity was observed and a maximum tolerated dose was not determined. A daily s.c. injection of 30-300μg/kg was defined as an appropriate dose range for further investigation in the phase II studies. The duration of MIP-1α activity required, however, is yet to be determined. Significant biological effects were not observed although this was not a primary objective of the phase I studies.

The design of the phase II studies posed equally challenging questions of how to determine the activity of the molecule and how to translate this into efficacy in terms of cytoprotection. The preclinical data had shown that myeloprotection was accentuated by increasing the degree of BM damage - a scenario that is not necessarily pursued in

clinical practice. Conventional dose chemotherapy protocols are primarily designed to deliver a given dose intensity in the absence of haemopoietic support. Increasing the dose intensity purely to mimic an experimental model is clearly inappropriate and for the evaluation of an unproven cytoprotective agent, unacceptable. Chemotherapy used in the management of acute leukaemia does provide an example in which protracted myelosuppression is the consequence of standard treatment and furthermore, may result in long term BM damage (Bhavnani et al., 1989, Chang et al., 1990). A major obstacle with this approach, however, is that at present there remains a degree of uncertainty surrounding the specificity of MIP-1α for normal haemopoietic progenitor cells (Ferrajoli et al., 1994).

A further problem in the design of the phase II studies is that the mode of action of MIP-1α (either via cell cycle inhibition or probably enhanced stem cell self renewal) renders the molecule particularly difficult to evaluate in the clinical setting. Measurement of cell cycle status post chemotherapy is fraught with difficulty especially given a small and elusive target cell population. Evaluation of activity would also necessitate determination of the S-phase fraction pre chemotherapy and post chemotherapy in the presence and absence of MIP-1α with the analysis compounded by considerable interpatient variability. Finally, and probably the major weakness with this approach, is the recognition that MIP-1α may be acting via additional mechanisms and not simply cell cycle inhibition, at least in the presence of non cycle active chemotherapy. For these reasons, assessment of stem cell survival and mature cell recovery may represent more realistic endpoints. However, these goals will only be achieved if there is optimal

delivery of MIP-1α to the BM microenvironment, both in terms of dose and schedule - a scenario that at present can only be guided by the phase I pharmacokinetic data.

The two phase II studies were carried out to evaluate the safety of a repeated dosing schedule of MIP-1α and investigate its myeloprotective properties, as reflected by stem cell survival and mature cell recovery when used in combination with non-cycle specific chemotherapy in patients with advanced solid tumours. In both studies a 7 day schedule of MIP-1α was used based on the murine preclinical data (irradiation model - see chapter 2) that showed maximal myeloprotective effects with the use of protracted infusions of MIP-1α. However, in contrast to the murine models and as the phase I trials had shown that this mode of delivery resulted in a similar pharmacokinetic profile over 24 hours as that seen in mice using a continuous infusion, a daily s.c injection was used.

MIC is a commonly used combination chemotherapeutic protocol used in repeated successive treatment cycles in patients with non-small cell lung cancer. It has an objective response rate of 30-40% and is associated with dose limiting myelosuppression. In a study reported by von Rohr et al (1991), 45% and 50% of cycles were complicated by grade IV leucopenia and by grade IV thrombocytopenia, respectively. The cytoprotective effect of MIP-1α was investigated using an identical dosing schedule of MIC chemotherapy to that described by von Rohr et al (1991). In this pilot study observations were limited to mature cell recovery as a clinically relevant index of efficacy. The study also provided safety data on the use of prolonged and repeated dosing of MIP-1α. Furthermore, the design using an alternate cycle randomisation potentially enabled assessment of efficacy within patients as well as between patients and avoided the need

for a much larger parallel controlled study which was considered inappropriate at this early stage of drug development.

The repeated schedule of MIP-1α was extremely well tolerated confirming our initial findings in the earlier phase I studies. No adverse events were considered directly attributable to MIP-1α administration and specifically, we did not observe a local or systemic inflammmatory response in any patient. The chemotherapy programme was associated with considerable toxicity which was comparable to that seen in the von Rohr study (table 21). Sixty nine percent of cycles were complicated by grade IV neutropenia and 32% of cycles by grade IV thrombocytopenia. Comparison between MIP-1α-treated and untreated cycles did not reveal any clinically significant differences. A modest but consistent advantage in neutrophil recovery was apparent over all 4 cycles in group 1 possibly as a result of receiving MIP-1α in the first cycle. This apparent benefit, however, was not accompanied by enhanced platelet recovery as compared to group 2.

The study confirmed the safety of MIP-1α when administered as a repeated daily subcutaneous dose of 30μg/kg but although, by the fourth cycle, the study appeared to be requiring less supportive care - blood, platelets and antibiotics - than in the von Rohr study (Table 21) it did not result in any clearly measurable effect on mature cell recovery. This, however, was not completely unexpected. Whilst the lack of effect may represent a true inability to protect against non-cycle active drugs our preclinical data, using a model of repeated sublethal irradiation, showed that MIP-1α had little protective effect in the early cycles of treatment and that significant enhancement of stem cell recovery became more prominent in the later treatment cycles. In that model, MIP-1α acted to

prevent cumulative damage to the stem cell pool, suggesting that optimal protection strategies require the administration of inhibitor with every cycle of chemotherapy. In this study, all patients received MIP-1α but none of them in every treatment cycle.

The second phase II study was designed to simulate more completely the preclinical experience in which MIP-1α was administered in all treatment cycles. Thus the effects of MIP-1α were evaluated in advanced breast cancer patients receiving 6 cycles of FAC chemotherapy, an active combination consisting of S-phase specific and non-cycle active cytotoxic drugs. The drug combination is well described in the treatment of both early (adjuvant) and advanced breast cancer patients and produces response rates of 50-60% in previously untreated patients. The use of a similar protocol, incorporating doxorubicin and cyclophosphamde only (AC), has been shown to result in a cumulative and long lasting reduction in BM progenitor cells in patients undergoing adjuvant treatment for early breast cancer (Lohrmann et al., 1978). The concurrent use of G-CSF has allowed a modest increase in dose intensity with a corresponding increase in response rates (90-100%) (van Hoef et al., 1994). The dose intensity achieved (typically 133%), however, can only be maintained during the earlier cycles of treatment and results in a greater cytotoxic insult on earlier progenitor cells (Hornung and Longo, 1992) that is reflected by progressive thrombocytopaenia and a reduction in the quality of progenitor cells mobilised by the treatment (Baumann et al., 1993). Furthermore, accelerated FAC treatment (delivered every 14 days) results in a high incidence of unacceptable mucosal toxicity (NCI grade III-IV). For these reasons, dose intensification of FAC using MIP-1α (with or without G-CSF) offers an attractive alternative strategy both in terms of limiting

Table 21. Supportive care requirements in the Phase II lung Study: A comparison of MIP-1α-treated cycles with historical data.

Treatment	Cycle Number							
	1		2		3		4	
	v.Rohr	MIP-1α	v.Rohr	MIP-1α	v.Rohr	MIP-1α	v.Rohr	MIP-1α
Blood (units)	8.4	8.3	26.8	25	45.8	50	80.9	60
platelets (units)	17.6	41.6	27.5	33	58.3	66.6	80.9	60
antibiotics (i.v)	35.2	50	44.8	41.6	50	66.6	71	60

MIP-1α study assessed 46 cycles of chemotherapy and v.Rohr (von Rohr et al., 1991) assessed 108 cycles. Results show the % episodes per cycle.

cumulative bone marrow toxicity and investigating MIP-1α's role as a mucosal protectant. In this first study it was clearly inappropriate to support intensified therapy with an unproven treatment and, therefore, MIP-1α was administered at two dose levels in combination with standard dose FAC chemotherapy. An evaluation of its efficacy was made both in terms of BM parameters and the mature cell recovery rate and the parallel controlled design of this study, with MIP-1α administered in every treatment cycle, allowed comparison of an optimal protection arm versus control (chemotherapy only). However, the study was limited by the need for a small sample size at this early point of drug development and the use of conventional dose chemotherapy of modest intensity. All patients tolerated the chemotherapy treatment extremely well with 100% of patients completing 6 cycles and only 5% of cycles delayed due to suboptimal recovery. Significant mucosal toxicity (≥ grade 3) did not occur in any patient. As with the previous studies, MIP-1α was very well tolerated with no associated serious adverse effects.

Neutrophil recovery showed a reduction in peak counts that was similar in all cases (figure 38). However, the initial neutrophil numbers in the two MIP-1α-treated groups was lower than in the controls ($4.2 \pm 0.3 \times 10^9$/l compared to $5.4 \pm 0.8 \times 10^9$/l) but was higher by the end of the sixth cycle ($1.68 \pm 0.5 \times 10^9$/l compared to $2.6 \pm 0.5 \times 10^9$/l). Normalising the cell counts to the baseline values at cycle 1 or to the level at the start of each cycle, therefore, showed a small but nevertheless, cumulative benefit of MIP-1α over the successive cycles of chemotherapy (figure 41 and 42). In the sixth cycle, FAC

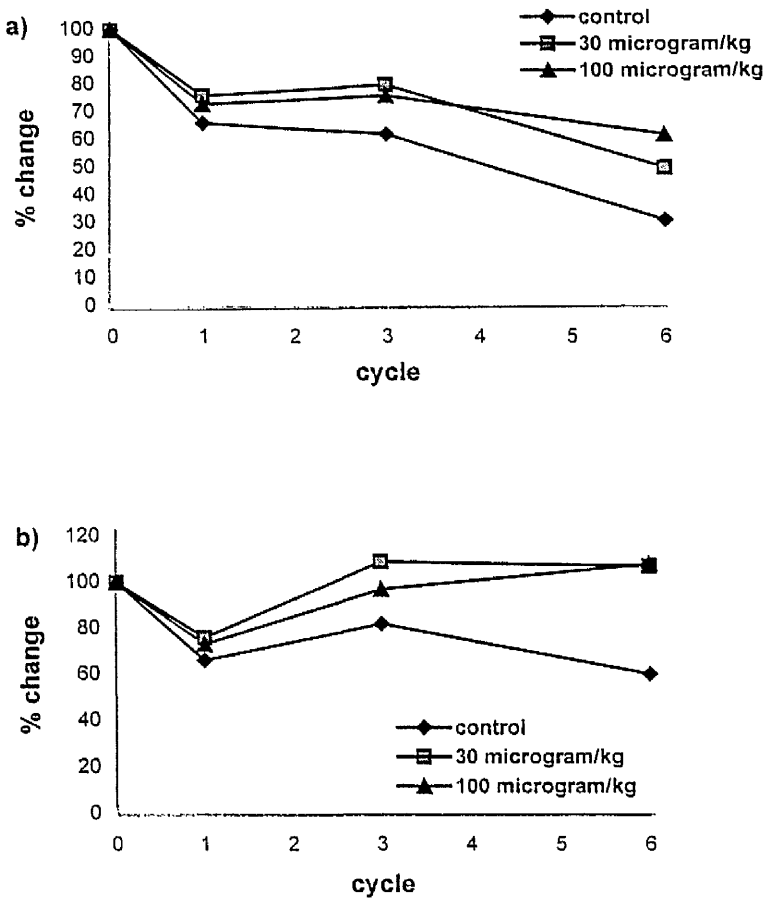

Figure 41. Results show the peak neutrophil recovery as a) a % of the baseline count at the commencement of cycle 1 and b) as a % of the neutrophil count at the initiation of each cycle.

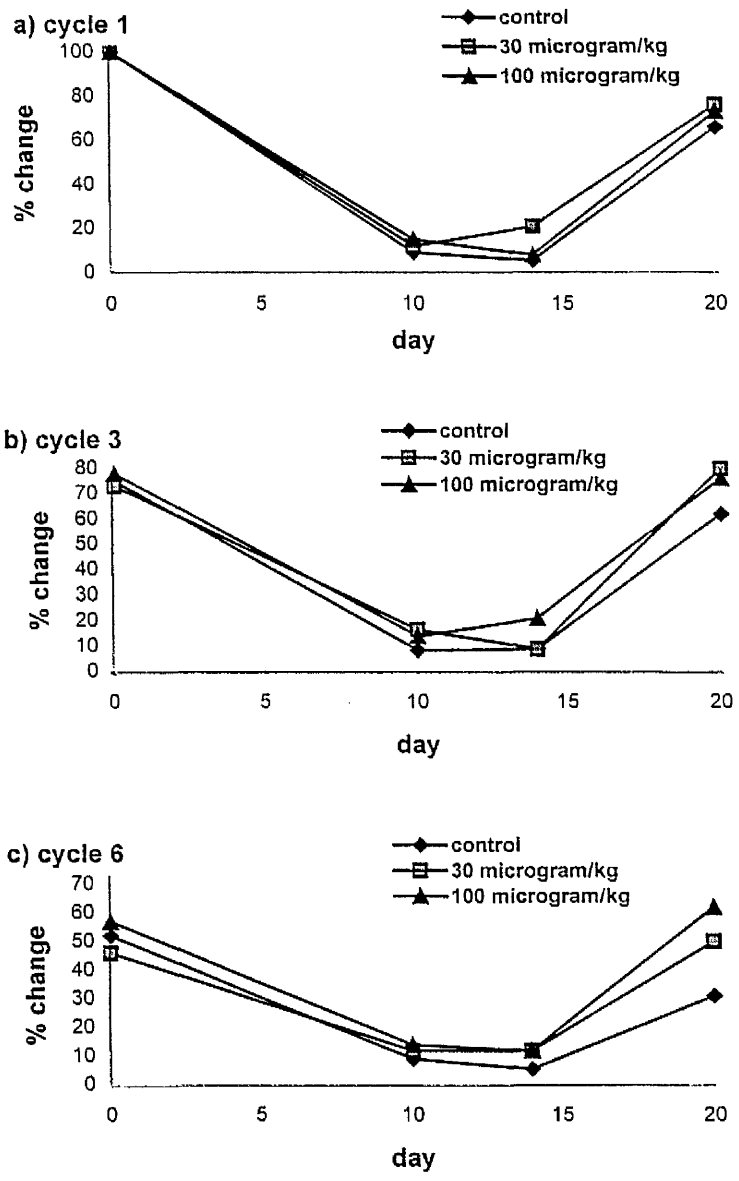

Figure 42. Neutrophil recovery during cycles 1, 3 and 6 plotted as a % of the baseline count at cycle 1.

chemotherapy alone reduced the recovery value to 60% of its starting value while MIP-1α maintained 100% recovery (Figure 41b).

The myeloprotective properties of MIP-1α were evaluated using a LTBMC technique initiated before chemotherapy and after completion of all 6 cycles of chemotherapy. This method offers a quantitative assessment for determining the degree of damage inflicted on early BM progenitors and defines a response to MIP-1α that includes protection and enhanced stem cell recovery. In addition, circulating progenitor cell numbers were determined and the degree of mobilisation used as a surrogate marker of BM damage - the anticipated advantage gained by using MIP-1α perhaps reflected by an improved maintenance of mobilisation potential over the 6 cycles of treatment. The preliminary results suggest that MIP-1α does enhance progenitor mobilisation following chemotherapy and confirm the findings shown in the murine cyclophosphamide studies. FAC chemotherapy did not appear to result in any measurable degree of cumulative BM damage with similar GM-CFC production in LTBMC before and after chemotherapy in the all groups (B.I.Lord personal communication) . This lack of BM damage was reflected clinically by a low incidence of treatment delays or dose reductions and may account for the relatively modest advantage offered by concurrent MIP-1α - In this respect it is noteworthy that in the preclinical experiments the benefit from MIP-1α was only seen following a marked degree of cumulative damage to the progenitor cell pool.

Gordon et al (1996) have recently reported their preliminary findings in a similar study of advanced breast cancer patients receiving a more myelosuppressive regimen consisting of high dose cyclophosphamide ($3g/m^2$) administered on day 1 and repeated every 21 days up to a maximum of 6 cycles. Cohorts of 3 patients received BB-10010 at doses of 5, 10, 30, and 100μg/kg s.c on days 0-2. Patients received BB-10010 alone in cycle 1 and BB-10010 with G-CSF in cycles 2-6. Their results are similar to those reported here - BB-10010 was associated with a 2-3 fold increase in circulating progenitor cells but no definitive effects on neutrophil recovery were identified. Interestingly, they reported that a 3 day programme of BB-10010 administered prechemotherapy significantly reduced the percentage of BM progenitors in S-phase from a rapidly proliferating to a slow or non-cycling state, a response that was seen at all doses. Their preliminary data would suggest that BB-10010 is active but that cyclophosphamide-induced cytotoxicity is not alleviated by cell cycle inhibition. The full results are awaited, however their findings are in marked contrast to the generally held view that MIP-1α is stem cell specific and therefore further corroborating evidence is required.

Concluding Remarks

MIP-1α has now been evaluated in 127 individuals as part of both phase I studies and phase II combined inhibitor/chemotherapy protocols. These studies have confirmed the safety and tolerability of MIP-1α using a single s.c. and i.v. bolus administration of 300μg/kg and 100μg/kg, respectively and following a 7 day repeated dosing schedule of up to 100μg/kg s.c. During these studies we have not observed any convincing evidence

to suggest a proinflammatory role for MIP-1α despite contrasting reports in the literature. Only 1 individual has developed a significant cutaneous reaction characterised by erythema and localised oedema at the MIP-1α injection site. MIP-1α did not induce a febrile response and was not associated with a measurable change in leucocyte numbers in any patient. Measurement of the acute changes in leucocyte subsets suggested a modest, dose-dependent effect on monocyte trafficking in keeping with a proposed role as a chemotactic factor for human monocytes. Mobilisation of circulating progenitor cells was variable amogst individual subjects but not stimulated to any significant extent, nor was there any specific synergy with preadministered G-CSF as had been acheived in the animal studies. Whilst this may be explained on the basis of differing pharmacokinetics between animals and humans, mobilisation of progenitor cells may have some species dependency - as revealed by the variable inflammatory response observed in humans, dogs and mice.

The preclinical data encompassed in the repeated radiation and preliminary chemotherapy models suggested that MIP-1α has an additive effect during multicyclic treatment and that its effects are accentuated with increasing marrow damage. This was also suggested by the small cummulative advantage in neutrophil recovery observed in the FAC study. Treatment with conventional dose chemotherapy may, however, not have provided an optimal model for investigation and the hypothesis might be better tested in a more dose intensive setting (subablative chemotherapy).

The potential synergistic effect between MIP-1α and G-CSF offers exciting potential and therefore deserves further investigation. Combined cytokine/chemokine protocols might

provide a further avenue to explore the role of MIP-1α in optimising peripheral blood stem cell harvesting and maintaining G-CSF driven dose intensive chemotherapy while abrogating cumulative damage on BM and epithelial mucosa. Further studies might therefore be designed to investigate the role of MIP-1α during dose intensive chemotherapy supported by colony stimulating factors. A number of phase II studies have been proposed or recently initiated to investigate the potential of MIP-1α in these settings. One study is aiming to evaluate the effect of MIP-1α (10 or 100μg/kg) on the mature cell recovery and severity of mucositis in advanced cancer patients receiving high dose etoposide and cyclophosphamide. All patients will receive one cycle of treatment with concurrent G-CSF. In a further study patients with high grade non-Hodgkins lymphoma will receive alternating high dose BEMOP (bleomycin, etoposide, methotrexate, vincristine and prednisolone) and alternating AC (adriamycin and cyclophosphamide). Patients will be randomised to receive MIP-1α or chemotherapy alone and will cross over upon experiencing grade IV neutropenia or grade III thrombocytopenia. Unfortunately, both of these trials have their limitations. The first because of the lack of a control and the latter because MIP-1α is unlikely to prevent a deep nadir (no effect on committed progenitors) but acts rather to accelerate recovery (stem cell effects).

The lack of an agreed evaluable response to MIP-1α continues to hamper progress in the assessment of its role in the clinic. Current trial protocols are designed on the empirical basis of pharmacokinetics without clear evidence that this dose and schedule provides optimal bone marrow concentrations of drug to elicit stem cell effects. Furthermore,

evaluation is confused by the application of a heterogeneous group of chemotherapeutic agents that have varying modes of cytotoxicity. It may therefore be necessary first to evaluate MIP-1α in a setting that has a more sound experimental basis. Defining an effective dose and schedule may be most straightforward employing a clinically relevant counterpart of the S-phase specific preclinical models. The treatment of CML patients may well prove to be such a model. The majority of patients with CML remain in a chronic phase characterised by a reduced stem cell self-renewal and enhanced differentiation producing a hyperproliferative state and peripheral blood leucocytosis. The management of such patients typically involves the administration of intermittent schedules of the S-phase specific cytotoxic drugs. Biologically, CML represents an ideal clinical model to test the hypothesis of stem cell protection because its progenitors carry a specific marker (the Philadelphia chromosome -t(9:22)) and, despite retaining MIP-1α receptors (Chasty et al., 1995), they are resistant to the cell cycle inhibitory properties of MIP-1α (Eaves et al., 1993a, Holyoake et al., 1993). The addition of MIP-1α in combination with an S-phase specific cytotoxic agent may enable a more specific 'targeting' of the abnormal clone and a resultant enhanced recovery of normal Ph^{-ve} haemopoiesis, which can be assessed in both peripheral blood and BM using techniques such as fluorescence in-situ hybridisation (FISH) and the polymerase chain reaction(PCR). This is now the subject of a multi centre, placebo controlled, prospective randomised clinical trial in patients with stable, chronic phase CML. In this, patients are randomised to receive MIP-1α or placebo and undergo 2 cycles of intermediate dose cytosine with BM and peripheral blood analysis for philadelphia positivity. Efficacy will

be assessed by the rate of recovery of normal haemopoiesis accompanied by a reduction in the malignant clone.

In conclusion therefore , in the clinical studies carried out to date MIP-1α therapy has not resulted in a dramatic improvement in neutrophil recovery following non-S-phase specific cytotoxic chemotherapy possibly as a result of trial strategems that have been designed primarily with limited marrow damage. Many questions remain concerning the optimal dose and duration of MIP-1α administration and the models for testing these have yet to be developed. The challenge for the future is to define a sensitive and relevant marker of activity in parallel with appropriate clinical studies.

APPENDIX.

MATERIALS.

Animal Experiments

1. Laboratory techniques: general comments

All in vitro clonogenic assays were carried out in an Envair Class II Microbiological safety cabinet thus ensuring a sterile working environment and user protection. All surfaces were sterilised with methanol before and after use. Discarded biological material and plastic items were treated with a bleach solution (virkon) in accordance with the manufacturers instructions before appropriate disposal and/or incineration.

Female B6D2F1 (C57B1 female x DBA2 male) mice aged 8-12 weeks were used throughout and all procedures were carried out, under licence, according to the provisions of the Home Office Animals (Scientific Procedures) Act, 1986.

2. Culture medium and Serum

2.1 Tellesniczky's fixative

To prepare a 500ml solution , 375ml of 70% ethanol was added to 18.75ml of glacial acetic acid and 37ml of formalin. The constituents were mixed thoroughly and stored, capped at room temperature.

2.2 Fetal calf serum

Batches of pretested sera were aliquoted into 100ml bottles and stored at -20°C until required. Bottles were thawed in turn and stored at 4°C between use.

2.3 Fischer's medium

Bottles of Fischers medium (Gibco. Paisley, Scotland) were stored at 4°C with 200mM of L-glutamine added on a fortnightly basis.

2.4 3.3% Agar

Two litres of double distilled water were added to a 3-litre round bottomed flask containing 66g of agar powder (Difco). The flask was then placed in a boiling water bath until the solution was clear and molten (around 2 to 3 hours). Once molten, 100ml aliquots were placed in sterile McCartney bottles. Agar was then tested against previous batches to ensure suitability for GM-CFC assay.

Human studies

1. Laboratory techniques: general comments

In the laboratory, all manipulations involving bone marrow, serum or medium were carried out in an Envair Class II Microbiological safety cabinet. Cabinet work surfaces were carefully swabbed with methanol before and after use. Used glass pipettes were immersed in a solution of bleach for 24 hours prior to washing and sterilisation. Plastic

items were also bleached for 24 hours before being collected for incineration. All blood samples were assayed immediately or stored at 4°C and assayed within 24 hours. All bone marrow samples were prepared and assayed immediately. Serum and culture medium were always warmed to 37°C in a water bath before use.

2. Culture media and sera

2.1 Iscoves modified Dulbecco's medium (IMDM)

Four hundred and twenty mls of double distilled water were poured into a large conical flask and 30mls of a 7.5% solution of sodium bicarbonate were added. One container (~17gms) of IMDM in powder form (Gibco, Paisley, Scotland) was emptied into the flask which was then allowed to stand at room temperature for a few minutes. The flask was agitated to complete dissolution and 500,000 units of benzyl penicillin (Glaxo, UK) and 50mg of streptomycin sulphate (Glaxo, UK) both dissolved in double distilled water, were added. The resulting solution was mixed thoroughly and finally passed through a 0.22 micron filter under suction into a sterile bottle. This 'double strength' IMDM with an osmolarity of around 610 mosmoles/kg was diluted using double distilled water to a 'single strength' concentration of 340 mosmoles/kg. In between use, the IMDM was stored in a refrigerator at 4°C and any not used after two weeks was discarded

2.2 Horse serum and fetal calf serum

Serum (Flow laboratories, UK) was aliquoted into 100ml bottles and frozen at -20°C until required. Bottles were thawed in turn and stored at 4°C between use. The sera used were

all pre-tested and shown to be suitable for clonogenic assays and the maintenance of long term bone marrow cultures.

2.3 Conditioned medium, 5637

5637 is an adherent cell line established from a human bladder carcinoma. It produces several haemopoietic growth factors and 'conditions' the supernatant culture medium making this a suitable source of growth factor for clonogenic assay (Myers et al., 1984). After 7 days incubation at 37°C, the supernatant medium is taken off the adherent cell layer, centrifuged to separate any non-adherent cells and filtered through a 0.22micron filter into a sterile bottle. The conditioned medium so obtained is stored frozen at -20°C in 150ml bottles until required. Bottled medium was thawed in turn and between use was stored at 4°C. Different batches of conditioned medium were routinely tested before experimental use to ensure optimal conditions in the GM-CFC assay.

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Minor Corrections

p 29 - interest

p 36 - not cytokine, chemokine

p 37 - principal - hermatoimmune → lymphohaemopoietic

p 38 - transcripts

p 45 - delete symbiotic

p 48 - "confirm"→ support

p 51 - drugs

p 64 - resistant

p 74 - following

p 91 - 3.5 Gy? (Table 5)

p 128 - might

p 128 - who is LW?

p 133 - ait → it

p 146 - who is BBL?

p 149 - below

p 152 - check 5 x 10^5 cells

p 178 - hospitalisation

p 208 - achieved

p 206 - which factor?

p 216 - who is J.G?

p 220 - targetting

p 231 - amongst

p 125 - check doses

p 206 - specify which factors

Fig 1 - correct arrows

Fig 12 & 13 - Explain significant point

Fig 14 - clarify legend p value

Table 10 - clarify statistics

Table 11 - +,- check

Table 19 - *not* standard errors - not p value

Table 21 - give median range