Studies on the haemotoxicity of antineoplastic agents

by

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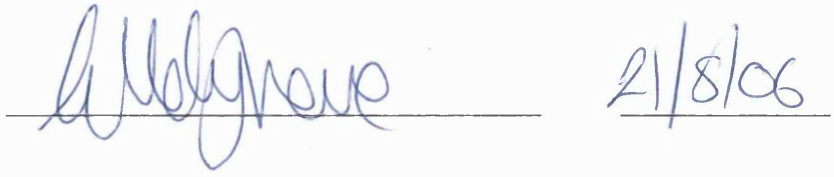
This thesis describes research conducted in the School of Pharmacy, University of London and St. George's University of London between 5th October 2001 and 4th October 2004 under the supervision of Dr. J. A. Turton and Dr. F. M. Gibson. I certify that the research described is original and that any parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication.



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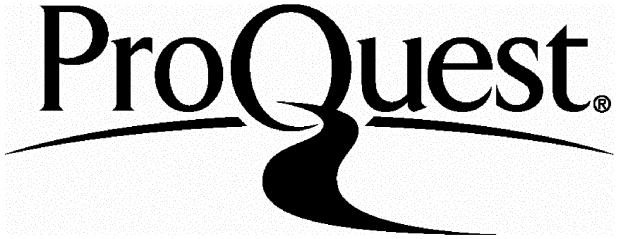

Abstract

Aplastic anaemia (AA) is a life-threatening disorder characterised by hypoplastic bone marrow and peripheral blood pancytopenia. The causes of AA are varied and include viruses, irradiation, chemicals and drugs. Recently, a new model of chronic bone marrow aplasia (CBMA) in the busulphan- (BU-) treated mouse was reported. In this model, female BALB/c mice treated with BU developed 'late-stage' (residual) CBMA on day 91 and 112 post dosing. In the mouse, CBMA shared many similarities with AA in man, however, treating mice with BU at a dose level of 10.50 mg/kg lead to high mortality. In subsequent studies, it was demonstrated that by lowering the dose of BU to 9.0 mg/kg, treated mice developed CBMA but without high levels of mortality.

Initially, in the present investigations, the model of BU-induced CBMA in the mouse was used to measure changes in the concentration of the serum cytokine fms-like tyrosine kinase 3 (FLT3) ligand (FL). During periods of bone marrow aplasia the concentration of serum FL was increased. This elevation of FL was also inversely proportional to several peripheral blood and bone marrow parameters.

The effects of administering the immunosuppressant drug cyclosporin A (CsA) to mice with BU-induce CBMA was assessed. When administered on day 57 post dosing for 30 days at 35 mg/kg/day, CsA did not protect mice from BU-induced CBMA.

Experiments were also carried out using chlorambucil (CHB), mitomycin C (MMC) and azathioprine (AZA) to investigate if these drugs were capable of inducing significant bone marrow injury and late-stage (residual) effects. It was shown that CHB, MMC and AZA caused significant bone marrow depression in the immediate post dosing period, but did not cause significant late-stage (residual) bone marrow

injury and CBMA. However, a mild residual effect on the erythroid lineage was evident in mice treated with MMC and with AZA.

It is concluded that BU-induced CBMA in the mouse is a useful model for the study of the pathophysiological aspects of drug-induced bone marrow injury, and for the assessment of therapeutic agents employed to treat such conditions. Investigations on the haemotoxicity of CHB, MMC and AZA suggested that these agents were not suitable replacements for BU in the induction of CBMA.

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Abbreviations used in this thesis

AA	Aplastic anaemia
ALG	Antilymphocyte globulin
ATG	Antithymocyte globulin
AZA	Azathioprine
Baso	Basophil
B-Cell	B-lymphocyte
BCNU	1, 3-bis (2-chloroethyl)-1-nitrosourea
BFU-E	Burst forming unit-erythroid
BFU-MK	Burst forming unit-megakaryocyte
BMT	Bone marrow transplant
BSA	Bovine serum albumin
BU	Busulphan
CAMs	Cellular adhesion molecules
CBMA	Chronic bone marrow aplasia
CD	Cluster of differentiation
CFU	Colony forming unit
CFU-C	Colony forming unit cell
CFU-E	Colony forming unit-erythroid
CFU-G	Colony forming unit-granulocyte
CFU-GEMM	Colony forming unit-granulocyte, erythroid, monocyte, megakaryocyte
CFU-GM	Colony forming unit-granulocyte monocyte
CFU-M	Colony forming unit-monocyte
CFU-MK	Colony forming unit-megakaryocyte
CFU-S	Colony forming unit-spleen
CHB	Chlorambucil
CHMF	Chronic hypoplastic marrow failure
c-kit	Stem cell factor receptor (CD117)
CsA	Cyclosporin A
DNA	Deoxyribose nucleic acid
dUTP	Deoxyuridine triphosphate
EDTA	Ethylene diaminotetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELTCIC	Extended long term culture initiating cell
Eo	Eosinophil
Epo	Erythropoietin
FAS	FAS receptor (CD95)
fasL	FAS ligand
FCS	Foetal calf serum
FD	Found dead
FL	FLT-3 Ligand
Flk2	Foetal liver kinase 2 receptor (CD135)
FLT-3	fms-like tyrosine kinase 3 receptor (CD135)
FNCC	Femoral nucleated cell count
FSC	Forward scatter height

G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte Monocyte colony stimulating factor
GTP	Guanosine triphosphate
GVHD	Graft versus host disease
Hb	Haemoglobin
HCT	Haematocrit
hEpo	Human erythropoietin
hG-CSF	Humangranulocyte colony stimulating factor
hIL-6	Human interleukin 6
HLA	Human leukocyte antigen
HPRT	Hypoxanthine phosphoribosyltransferase
HSC	Haemopoietic stem cell
HUS	Haemolytic uraemic syndrome
ICD	Intercurrent death
IFN-γ	Interferon gamma
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
ip	Intraperitoneal
IS	Immunosupression
KIE	Killed *in extremis*
KO	Knock out
Lin	Lineage marker
LNC	Lymph node cell
LTBMC	Long-term bone marrow culture
LTC-IC	Long-term culture initiating cell
Lymph	Lymphocyte
M:E ratio	Myeloid: erythroid ratio
MAHA	Microangiopathic haemolytic anaemia
MCH	Mean cell haemoglobin
MCHC	Mean cell haemoglobin concentration
M-CSF	Monocyte colony stimulating factor
MCV	Mean cell volume
MDS	Myelodysplastic syndrome
mIL-3	Murine interleukin 3
MMC	Mitomycin C
Mono	Monocyte
mRNA	Messenger ribonucleic acid
mSCF	Murine stem cell factor
MTD	Maximum tolerated dose
Neut	Neutrophil
NFAT	Nuclear factor of isolated T-cells
NF-κB	Nuclear factor of immunoglobulin K light chain in B-cells
NOD/SCID	Non-obese diabetic/severe combined immuno-deficient
NS	Not statistically significant
PALS	Periarteriolar lymphoid sheath

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Plt	Platelet
PNH	Paroxysmal nocturnal haemoglobinuria
RBC	Red blood cell
Retic	Reticulocyte
SAA	Severe aplastic anaemia
Sca-1	Stem cell antigen 1
SCF	Stem cell factor
T-Cell	T-lymphocyte
TdT	Terminal deoxynucleotidy transferase
Thy1	Cell surface antigen (CD90)
TMA	Thrombotic microangiopathy
TNF-α	Tumour necrosis factor alpha
TPMT	Thiopurine methyl-transferase
TTP	Thrombotic thrombocytopenia purpura
TUNEL	Terminal deoxynucleotidy transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labelling
WBC	White blood cell
6-MMP	6-methyl-mercaptopurine
6-MP	6-mercaptopurine
6-TGN	6-thioguanine nucleotides
7-AAD	7-Amino Actinomycin D

CHAPTER 1: Introduction

1.1. HAEMOPOIESIS

In the developing human foetus, haemopoietic stem cells develop in the aorta-gonad-mesonephros region (Gordon 2005). Principally the liver, and to a lesser extent the spleen, carry out the role of blood cell production from week 6 of gestation. In the later phases of gestation (from week 20), haemopoietic stem cells migrate from the liver to the bone marrow, which then remains the site of haemopoiesis throughout childhood and into adult life (Testa and Dexter 1997; Gunsilius *et al.* 2001).

In man, in early infancy (2 to 3 years), active bone marrow is found in all bones. In the following years active haemopoietic tissue is gradually replaced by fat and the role of haemopoiesis is carried out within the central skeleton (vertebrae, sternum, skull, and pelvis) and the proximal ends of the femurs (Testa and Dexter 1997). In times of stress, for example in severe haemolysis, expansion of haemopoietic tissue occurs down the long bones replacing the fatty deposits with active red bone marrow. In man, haemopoiesis can also take place outside of the bone marrow, in the spleen and liver; however, this extramedullary haemopoiesis is rare and usually occurs in patients with megaloblastic anaemia or myelofibrosis (Lewis 1997).

In contrast to the situation in man, haemopoiesis in mice and rats is very different. The bones of the skeleton are generally full to capacity with active bone marrow and therefore, during times of stress, it is not possible for the bone marrow to expand further within the skeleton. As a consequence, extramedullary haemopoiesis frequently occurs in the spleen (Andrews 1997).

Haemopoietic stem and progenitor cells in the bone marrow are surrounded by stroma, a supportive microenvironment consisting of collagen fibres in association with endothelial cells, fat cells and macrophages. Haemopoietic stem cells interact with the

stromal microenvironment and the extracellular matrix of the bone marrow via cellular adhesion molecules (CAMs) (Testa and Dexter 1997). As the cells mature, they leave the bone marrow microenvironment and enter the blood stream. The marrow has a rich supply of blood vessels (sinuses) into which maturing blood cells are discharged (Testa and Dexter 1997).

Blood cell production in the bone marrow is regulated by haemopoietic cytokines and growth factors (Table 1.1). Cytokines and growth factors are produced by various cells and organs including, bone marrow stromal cells, fibroblasts, lymphocytes and the kidney (Jones and Miller 1989; Hamblin 1997). Cytokines promote differentiation, proliferation and maturation of primitive haemopoietic cells. Cytokines and growth factors have varying roles in the control of blood cell production. Some act on the haemopoietic stem cell and early progenitor cells (e.g. IL-3, stem cell factor) while others act later on a particular cell lineage (e.g. Epo, G-CSF, M-CSF). Many cytokines have been identified, some have little or no effect individually in the control of haemopoiesis however, in combinations such cytokines have been shown to work in synergy to promote cell proliferation (c-kit, IL-1) (Jones and Miller 1989; Hamblin 1997; Testa and Dexter 1997).

1.2. HAEMOPOIETIC STEM CELLS

The most primitive haemopoietic stem cell (HSC) is the pluripotent stem cell from which all cells of the blood are derived (Fig. 1.1). The HSC pool constitutes a very small population within the bone marrow, being only 0.01 to 0.05% of all the cells present (Marsh and Testa 2000; Gunsilius *et al.* 2001). The HSC is characterised by a great capacity for repopulation, self-renewal and differentiation. Under normal

TABLE 1.1. Haemopoietic cells targeted by cytokines and growth factors

Cytokine	Responding cells
Erythropoietin	CFU-E
Thrombopoietin	CFU-Mk
GM-CSF	CFU-GEMM, CFU-GM, CFU-Eo, CFU-Baso, CFU-Mk, BFU-E, CFU-M, CFU-G, Dendritic cells
G-CSF	CFU-GEMM, CFU-GM, CFU-G
M-CSF	CFU-GEMM, CFU-M
IL-1	HPP-CFU
IL-2	CFU-GM, CFU-Baso, BFU-E, Dendritic cells
IL-3	CFU-GEMM, HPP-CFU, CFU-GM, CFU-Eo, CFU-Baso, BFU-E, CFU-Mk,
IL-4	CFU-GM, CFU-Baso, BFU-E, Dendritic cells
IL-5	CFU-Eo
IL-6	HPP-CFU, CFU-GM, BFU-E
IL-11	CFU-Mk, CFU-GM, BFU-E
FLT-3 ligand	LT-CIC, CFU-GEMM, CFU-GM, Dendritic cells

Abbreviations: BFU-E, Burst forming unit-erythroid; BFU-MK, Burst forming unit-megakaryocyte; CFU-E, Colony forming unit-erythroid; CFU-GEMM, Colony forming unit-granulocyte, erythroid, monocyte, megakaryocyte; CFU-G, Colony forming unit-granulocyte; CFU-GM, Colony forming unit-granulocyte monocyte; CFU-M, Colony forming unit-monocyte; CFU-MK, Colony forming unit-megakaryocyte, HPP-CFU, High proliferative potential Colony forming unit-cell, LTCIC, Long-term culture initiating cell.

Modified from Gordon (2005).

circumstances the majority of haemopoietic stem cells are quiescent or in G_0 phase of the cell cycle. This is associated with a resistance to cytotoxic chemicals (e.g. 5-fluorouracil), which in general specifically target cycling cells (Ogawa 1993; Testa and Dexter 1997).

When the pluripotent stem cell divides, it produces another pluripotent stem cell and a cell that will differentiate into one of two lineage specific stem cells: the multipotent myeloid stem cell and the lymphoid stem cell (Fig. 1.1). Through a number of cell divisions with increasing differentiation, the multipotent myeloid stem cell gives rise to myeloid progenitor cells committed to one or more lineage..

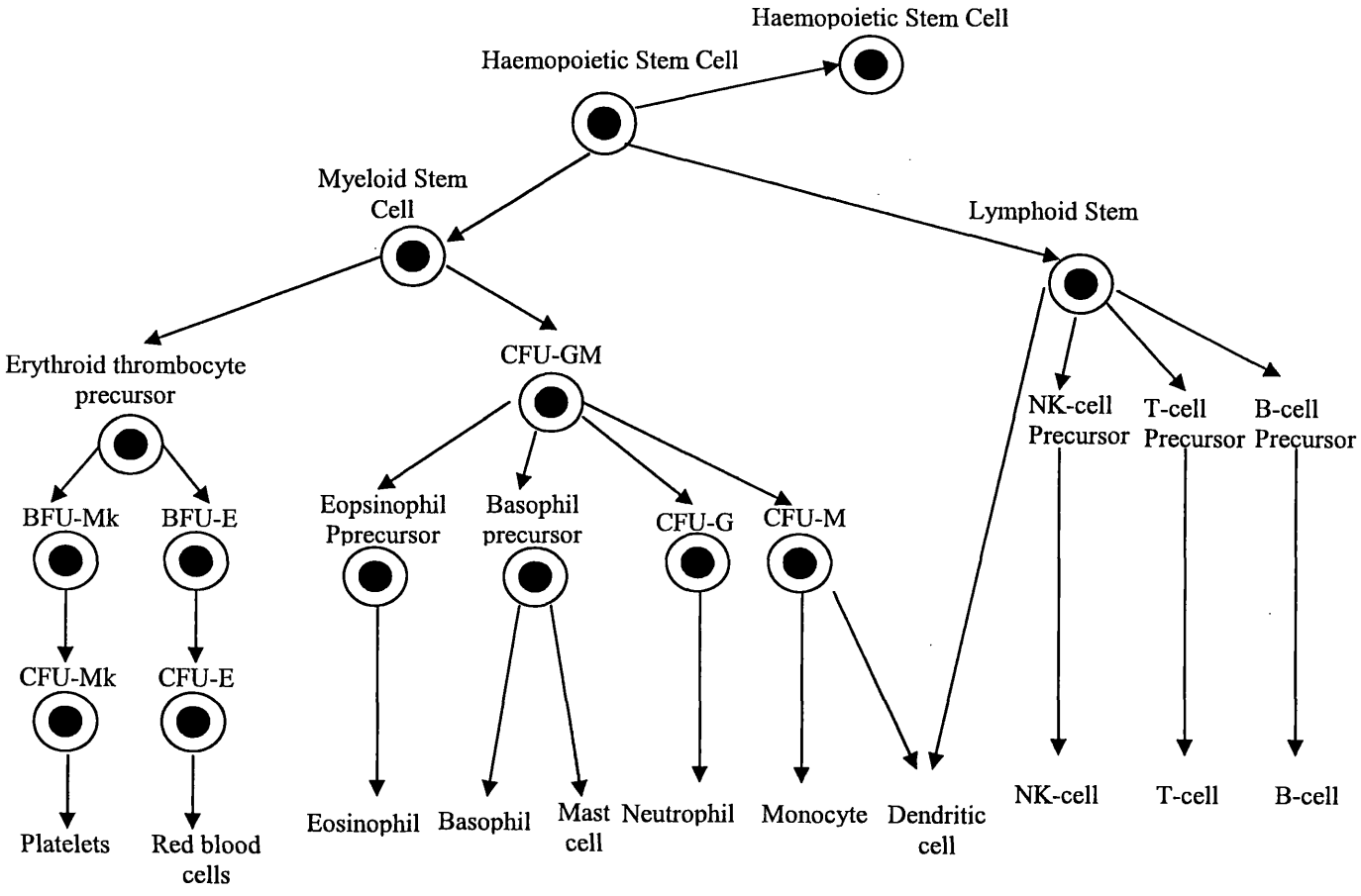


FIGURE 1.1. Haemopoiesis: A schematic diagram of blood cell production. Figure modified from Gordon (2005).
Abbreviations: BFU-E, Burst forming unit-erythroid; BFU-MK, Burst forming unit-megakaryocyte; CFU-E, Colony forming unit-erythroid; CFU-GEMM, Colony forming unit-granulocyte, erythroid, monocyte, megakaryocyte; CFU-G, Colony forming unit-granulocyte; CFU-GM, Colony forming unit-granulocyte monocyte; CFU-M, Colony forming unit-monocyte; CFU-MK, Colony forming unit-megakaryocyte.

Differentiation of the lymphoid stem cell gives rise to both B and T lymphocytes. B-cells mature within the bone marrow before travelling to B-cell rich zones within the peripheral lymphoid system (lymph-nodes or splenic follicles). Primitive T-cells however, migrate from the marrow to the thymus where they mature. In the cortex of the thymus, cells acquire CD4, CD8 and CD3 antigens. As they migrate from the cortex to the medulla of the thymus, T-cells that recognise 'self' are destroyed. At this point, the selected T-cells travel into the thymic medulla where they mature and differentiate into $CD4^+$ or $CD8^+$ cells before leaving the thymus, travelling to T-cell zones of the peripheral lymphoid system (periarteriolar sheath of the spleen and paracortical areas and medulla of the lymph nodes).

It is not possible to differentiate between stem cells and more mature progenitor cells, according to morphology. Therefore, the presence of cell surface antigens has been exploited to isolate and study stem cells (Table 1.2). A number of functional assays have also been developed to quantitate the number of stem and early progenitor cells within samples of bone marrow.

TABLE 1.2. Phenotypic markers expressed on stem and progenitor cells

Stem cells	Progenitor cells
$CD34^+$	$CD34^+$
$CD38^-$	$CD38^+$
$CD33^-$	$CD33^+$
c-Kit^+ (CD117)	c-$Kit^{-/low}$ (CD117)
$Thy1^{-/low}$ (CD90)	$Thy1^+$ (CD90)
Lin^-	Lin^+

Modified from Marsh and Testa (2000).

1.3. MEASUREMENT OF HAEMOPOIETIC STEM AND PROGENITOR CELLS IN BONE MARROW

1.3.1. Colony forming unit cell assay

The colony forming unit cell (CFU-C) assay is a short term culture system which allows the quantification of the number of committed progenitor cells in a given bone marrow sample. Individual committed progenitor cells are immobilised in a semi-solid culture medium (e.g. agar or methylcellulose). After 7 to 14 days of incubation in the presence of cytokines, progenitor cells form clones (Fig. 1.2). The clones of cells produced can be identified microscopically and scored according to morphology. The original progenitor cell determines the composition of cells within a colony (Testa *et al.* 1985; Testa and Dexter 1997; Gunsilius *et al.* 2001). Therefore, clones produced by the multipotent myeloid stem cell (CFU-GEMM) contain cells of granulocyte, erythroid, monocyte and megakaryocyte origin (Fig. 1.1). CFU-GM colonies contain both granulocyte and monocyte precursor cells. CFU-M and CFU-G colonies represent more committed progenitor cells (Fig. 1.1) whose differentiation has become restricted to a particular lineage and therefore contain cells of monocyte or granulocyte origin, respectively (Fig. 1.2 A, B). Erythroid colonies can be divided into early erythroid progenitors (BFU-E) (Fig. 1.2 C, D) and more mature progenitors (CFU-E).

1.3.2. Long term bone marrow culture

The detection of more primitive haemopoietic progenitor cells can be performed using long-term bone marrow culture (LTBMC); bone marrow cells are grown with or without a stromal feeder layer in nutrient medium for 5 to 8 weeks. At weekly intervals, half of the culture medium is removed and replaced with fresh medium. The non-

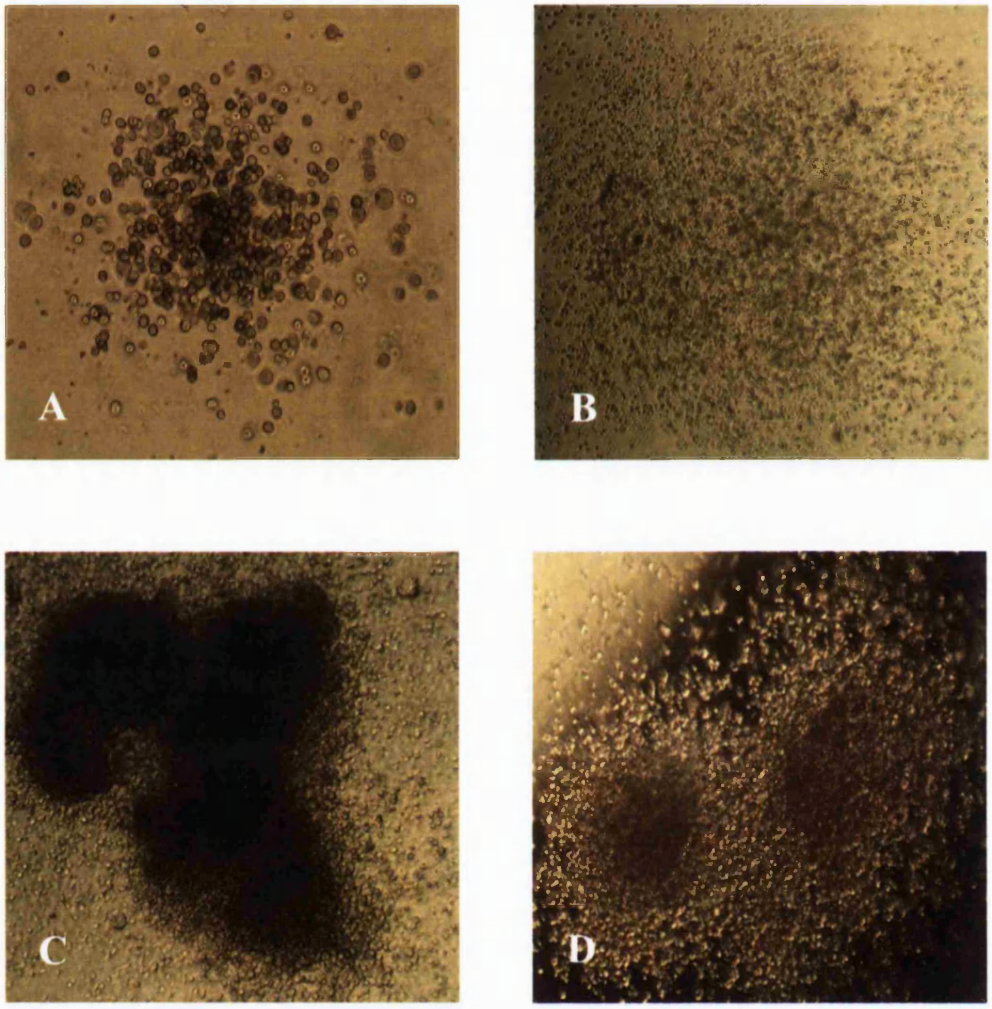


FIGURE 1.2. Committed progenitor cells from mouse bone marrow cultures. A. CFU-M, Colony forming unit-macrophage; B. CFU-G, colony forming unit-granulocyte; C and D. BFU-E, Burst forming unit-erythroid.

adherent cells, removed in the culture medium or after incubation with trypsin, are assessed for their ability to form colonies in the CFU-C assay (as described above).

The long-term culture initiating cell (LTCIC) assay allows the quantification of very primitive haemopoietic progenitors. LTCICs are the progeny of the HSC, these cells, which after 5 to 8 weeks of culture form a clone of cells in the CFU-C assay, are more primitive than the CFU-GEMM (Lapidot *et al.* 1997; Ploemacher 1997). Recently, Hao *et al.* (1996) isolated $CD34^+/CD38^-$ cells from bone marrow and cultured these cells on a stromal support. Cells within this population were able to generate colonies in the CFU-C assay after 100 days of culture and were described as extended long term culture initiating cell (ELTCIC) and are thought to be more primitive that the LTCIC.

1.3.3. Spleen colony forming units

The spleen colony forming unit (CFU-S) assay, first described by Till and McCulloch (1961), provided a way of measuring murine haemopoietic progenitor cells for their capability of multi-lineage reconstitution of murine bone marrow. Mice are firstly irradiated with a dose of radiation capable of suppressing its own haemopoietic system, followed by an intravenous injection of a bone marrow cell suspension (10^4 to 10^5 cells). After 8 to 12 days the mouse is killed and the spleen removed, fixed and examined microscopically. Nodules present on the spleen are counted and provide a value representing the number of early haemopoietic progenitor cells (CFU-S) capable of repopulating the bone marrow of an irradiated mouse (Testa *et al.* 1985; Schofield 1986). However, cells with the ability to generate CFU-S are not capable of long-term multilineage repopulation and it is therefore thought that these cells are not the most primitive haemopoietic stem cells (Eaves and Eaves 1994).

1.3.4. Non-obese diabetic/severe combined immuno-deficient mice

The identification of human haemopoietic stem cells with the ability to reconstitute the bone marrow and sustain multilineage regeneration can be studied in non-obese diabetic/severe combined immuno-deficient (NOD/SCID) mice. These mice have a homozygous mutation which leads to a defective immune system, thus, the ability of a purified population of human progenitor cells to sustain long-term multilineage reconstitution can be studied by injecting these cells into lethally irradiated NOD/SCID mice (Lapidot *et al.* 1997; Ploemacher 1997).

1.4. APOPTOSIS

Programmed cell death (apoptosis) is a method of controlling cell number and maintaining homeostasis in populations of rapidly dividing cells such as bone marrow. Apoptosis also plays a role in the removal of cells with extensive DNA damage; the removal of such a cell is important in preventing the development of malignant clones of cells (Ekert and Vaux 1997).

The survival and differentiation of cells is controlled via external stimuli in the form of growth factors. The removal of survival factors, by maintaining cells in a nutrient deficient medium, readily induces apoptosis. However, the direct binding of cytokines to cell surface receptors can trigger apoptosis, for example, the binding of FAS ligand (CD95), or tumour necrosis factor-alpha (TNF-α), to their cognate receptors (Ekert and Vaux 1997).

The receptors for both FAS and TNF-α contain 'death domains' which upon receptor activation cleave inactive pro-caspases into active caspases, which are proteolytic enzymes that specifically target and cleave intracellular proteins. Initially,

cells develop changes in the plasma membrane, which alter the permeability of the cell resulting in the loss of ions and cellular shrinkage. As cells shrink, they lose contact with neighbouring cells and become isolated. The destruction of structural proteins by caspases results in a loss of cellular architecture producing membrane irregularities and 'blebbing' (Gibson *et al.* 2000). DNA within the nucleus is digested by endonucleases producing 180 base pair fragments of DNA. Finally, the nucleus undergoes hypercondensation and collapses. The cell then fragments into apoptotic bodies containing remnants of nucleus, mitochondria and other organelles; these apoptotic bodies are phagocytosed by neighbouring cells (Campana and Cleveland 1996).

Various methods exist for detecting apoptosis using characteristic morphological changes seen in cells undergoing apoptosis; these include, light and electron microscopy and time-lapse photography. The digestion of DNA which occurs during apoptosis can be visualised by gel electrophoresis as a DNA ladder and by terminal deoxynucleotidyl transferase- (TdT-) mediated deoxyuridine triphosphate (dUTP) nick end labelling (TUNEL) staining (Gibson *et al.* 2000).

The use of flow cytometry to measure cells undergoing apoptosis is a convenient method for measuring large numbers of samples. In addition, flow cytometric analysis of cells allows the quantitative detection of small apoptotic sub-populations within a large heterogeneous population of cells.

Early populations of apoptotic cells can be identified using the DNA binding dye 7-amino actinomycin D (7-AAD) which intercalates between cystine and guanine residues of DNA. An increase in membrane permeability during the early stages of apoptosis may be responsible for the accumulation of 7-AAD by apoptotic cells; however, apoptotic cells isolated by 7-AAD fluorescence have been found to be negative for trypan blue staining which would suggest that the cell membrane is intact (Philpott *et al.* 1996).

1.5. FMS-LIKE TYROSINE KINASE 3

A member of the tyrosine kinase receptor family was discovered independently by 2 groups in 1991. Using murine placenta cells, Rosnet *et al.* (1991) cloned the FLT-3 (fms-like tyrosine kinase 3) receptor based on sequence homology to the c-fms receptor. At the same time, Matthews *et al.* (1991) discovered the Flk-2 (foetal liver kinase 2) receptor using highly enriched foetal liver progenitor cells. At the amino acid level both FLT-3 and Flk-2 receptor sequences were highly homologous signifying that both receptors are encoded by a common gene and share the same ligand (Lyman *et al.* 1993a). FLT-3 is expressed on haemopoietic progenitor cells and cells of the placenta, nervous system and gonads. In the haemopoietic system, FLT-3 is expressed on cycling stem cells and early progenitor cells particularly early B-cells (Rosnet *et al.* 1996)

The FLT-3 receptor ligand (FL) was cloned by Lyman *et al.* (1993b) and later was found to exist in both membrane bound and soluble isoforms (Lyman *et al.* 1995a), both of which are biologically active (Wodnar-Filipowicz 2003). As a consequence of FLT-3 receptor expression, the activity of FL is confined to the early stages of haemopoietic development.

FL mRNA is expressed in a variety of tissues. However, FL protein has only been found in stromal fibroblasts and T-lymphocytes (Wodnar-Filipowicz 2003). Alone, FL has been shown to have little or no stimulatory effect, however FL is able to synergise with a number of cytokines (i.e. IL-3, IL-6, and SCF) to promote proliferation and/or colony formation (Wodnar-Filipowicz 2003).

In both man and mouse, early haemopoietic progenitor cells defined by the expression of the CD34 antigen can be further divided into FLT-3^+ and FLT-3^- populations. Furthermore, human $CD34^+$/FLT-3^+ and not $CD34^+$/FLT-3^- are capable of

multilineage reconstitution of NOD/SCID mice (Sitnicka *et al.* 2003; Ebihara *et al.* 2002). This is in contrast to studies in the mouse which have shown that c-kit$^+$, lin$^-$, Sca-1$^+$ murine stem cells that do not express FLT-3 are able to support long term multilineage reconstitution in lethally irradiated hosts while, reconstitution from c-kit$^+$, lin$^-$, Sca-1$^+$ and FLT-3$^+$ cells is restricted to T and B-lymphocytes (Adolfsson *et al.* 2001; Christensen and Weissman 2001).

Studies in transgenic mice devoid of the FLT-3 receptor have illustrated the importance of the FLT-3 receptor and ligand on lymphopoiesis. Mice without the FLT-3 receptor have reduced numbers of leucocytes in the peripheral blood, bone marrow, spleen and lymph nodes, as well as a significant deficit in natural killer cell function (McKenna *et al.* 2000). Moreover, the administration of FL to mice leads to an increase in the number of circulating leucocytes in the peripheral blood, and an increase in the cellularity of the spleen and bone marrow, mainly due to a preponderance of pre-B-cells (Brasel *et al.* 1996). Administration of FL has also been shown to increase immunity to viral infection (Vollstedt *et al.* 2003) and protects against radiation-induced bone marrow failure in the rabbit following total body irradiation (Gratwohl *et al.* 1998).

A positive correlation has been identified between bone marrow injury and the plasma/serum concentration of FL. Studies in non-human primates have shown that the plasma concentration of FL increases before the onset of neutropenia following total body irradiation (Bertho *et al.* 2001). Here, a positive correlation was shown to exist between the concentration of FL in the plasma and the intensity of radiation exposure. It was therefore proposed that FL could act as a biomarker for bone marrow injury following accidental exposure to radiation, and that the concentration of FL in the plasma could be used to assess the probability of a patient developing bone marrow aplasia (Bertho *et al.* 2001).

1.6. APLASTIC ANAEMIA

Aplastic anaemia (AA) in man is a clinical syndrome resulting from the failure of the bone marrow to produce sufficient numbers of blood cells and cellular elements. The pancytopenia that ensues puts patients at risk of death from major haemorrhage or overwhelming infection. Typically, the bone marrow of aplastic patients is markedly hypocellular without evidence of a pre-existing marrow disorder. Furthermore, the marrow shows a high quantity of fat, often with only small islands of haemopoietic tissue remaining (Gordon-Smith 1989).

1.6.1. Aetiology

AA is a relatively rare disorder with an incidence in the western world of 2 to 5 cases per million of the population per year (Gordon-Smith and Lewis 1997, Young 1995). However, the incidence of AA is significantly higher in the Far East, China and Japan. This increase in incidence of AA is not due to a genetic difference between the populations but is more likely due to environmental factors such as viral infections and drug and chemical use. This interpretation is suggested by epidemiological studies, which have found the incidence of AA in immigrant populations of eastern origin residing in the West to be comparable to the indigenous population (Gordon-Smith 1989).

AA may be a rare condition however, it is associated with significant mortality. Patients with AA are categorised according to disease severity. Severe AA (SAA) is classified as marrow cellularity of less than 25% of normal and at least two of the

following (normal ranges in parenthesis):

		Normal range
Neutrophils	<0.5 x 10^9/l	(1.5-1.7 x 10^9/l)
Platelets	<20 x 10^9/l	(150-400 x 10^9/l)
Reticulocytes	<20 x 10^9/l	(29-100 x 10^9/l)

Patients with neutrophil counts of <0.2 X 10^9/l are defined as very severe AA (Camitta and Thomas 1978).

1.6.2. Causes of aplastic anaemia

AA can develop idiopathically or the disease can be acquired following exposure to viruses, radiation, or particular chemicals or drugs (Table 1.3) (Appelbaum and Fefer 1981; Young and Alter 1994; Young *et al.* 2000). Drug-induced AA occurs unpredictably in a small proportion of patients exposed to a particular toxic drug. The idiosyncratic nature of this serious drug reaction may be due to a genetic disposition (Gordon-Smith 1989). At least 400 chemicals and drugs have been suspected of causing unpredictable marrow aplasia. However, only a small number of drugs and drug families have caused this reaction to establish a significant association between exposure and AA; such drugs include chloramphenicol, penacillamine, the butazones and gold (Table 1.3).

TABLE 1.3. Causes of aplastic anaemia in man

- **Radiation**
- **Chemicals**
  - Benzene
- **Drugs**
  - Cytotoxic agents (busulphan)
  - Non-steroidal anti inflammatory drugs (butazones, indomethacin)
  - Antithyroid drugs
  - Gold
  - Penicillamine
  - Corticosteroids
  - Antibiotics (chloramphenicol, thiamphenicol)
- **Viruses**
  - Epstein-Barr (infectious mononucleosis)
  - Hepititis (non-A, non-B, non-C, non-G)
  - Human immune deficiency virus (acquired immunodeficiency disorder)

Modified from Young and Alter (1994).

1.6.3. Management of Aplastic Anaemia

Based on the pathophysiology of the disease, there are two major forms of treatment for AA: first, bone marrow transplantation where deficient stem cells are replaced, and second, immunosuppression which blocks the inhibitory action of cytotoxic lymphocytes on haemopoietic stem cells (Young and Barrett 1995).

1.6.3.1. Bone marrow transplantation

Bone marrow transplantation (BMT) initially involves a conditioning regimen involving the destruction of the patients' own stem cells and their derivatives. This is achieved by treatment with myeloablative drugs (such as busulphan) alone or in conjunction with irradiation. The elimination of the patients' immune system by these conditioning regimens is vital in preventing graft (i.e. bone marrow) rejection. Preconditioning regimens for severe aplastic patients are generally of a lower intensity

than those used in malignant disease. However, post transplant complications can occur following conditioning treatment and these may include graft versus host disease (GVHD), infections, infertility and secondary malignancies (particularly if the regimen involves irradiation). Transplants can be of three types: syngenic, from an identical twin; allogenic, from a HLA- (human lymphocyte antigen-) matched sibling, family member or unrelated donor; or autologous, from stem cells harvested from the patient's own marrow prior to conditioning. However, autologous bone marrow transplants cannot be performed in SAA patients due to the underlying deficiency in stem cells. The majority of transplants performed in SAA patients are therefore allogenic. Replacement of stem cells by transplantation from a HLA-matched sibling provides the most successful long term treatment of SAA. The probability however, of finding a HLA-matched sibling donor is low, being about 25% (Hoffbrand and Pettit 1992).

In the 1970s, allogenic BMT was first used to treat SAA. Initially, long-term survival rates were reported as less than 50%. In recent years however, five-year survival rates of transplant patients have increased significantly to 66%, with some centres reporting survival rates as high as 90% (Storb *et al.* 1994; Passweg *et al.* 1997; McCann *et al.* 2000). This increase in survival of patients has been attributed to the use of cyclosporin A to prevent GVHD and transplant rejection. However, improvements in supportive care, earlier transplantation, and improvements in transfusion practice have also contributed to the increase in survival rate.

The success of BMT is dependent on age. In patients over 20 years of age the incidence and severity of GVHD increases markedly. The survival rate in older patients has been estimated as around 50%, which is considerably lower than in younger patients (Brodsky 1998). Indeed, AA patients over 50 years of age do not qualify for bone marrow transplant because of the low survival rate and relatively high incidence of GVHD (Bacigalupo 1989).

In an attempt to increase the number of SAA patients undergoing transplants, unrelated HLA-matched donors have been used. Initial results were however disappointing. A retrospective analysis of patients undergoing BMT between 1986 and 1995 has been performed (Hows & Veim-Stone 2000). In this study, 3 year survival rates and peripheral blood recovery of transplant patients were used as end points to assess the success of BMTs from HLA-matched sibling donors, HLA-identical family donor and unrelated HLA-matched donors (Table 1.4). This study showed that transplantation of bone marrow from a HLA-matching sibling gave the best outcome. Lower survival rates were associated with other family relatives as donors, and this was also the case with unrelated donors. These lower rates could be due to a delay in transplantation, and therefore the administration of more transfusions while a match was found. The increased number of blood transfusions necessary to maintain sufficient peripheral blood cells can sensitise patients to subsequent bone marrow transplantation. However, in recent years the widespread use of white blood cell depleated blood products has lead to an improvement in successful bone marrow transplantations. Also, mismatches probably occurred in this study due to the insensitivity of serological typing. More recently, HLA compatibility, defined by high-resolution DNA typing techniques based on PCR, have produced much improved survival of leukaemia and SAA patients after unrelated donor BMT. At present, patients without a HLA-matched sibling donor are not recommended for transplant. These patients should be treated with at least 2 courses of immunosuppression (IS).

TABLE 1.4. Outcome of bone marrow transplants performed between 1986 and 1995		
	3 year survival rate	**Peripheral blood recovery**
HLA-matched sibling donor	66-71%	85-89%
HLA-matched family donor	36-54%	60-80%
HLA-matched unrelated donor	26-40%	72-85%

Modified from Hows & Veim-Stone (2000).

1.6.3.2. Immunosuppression

The use of IS to treat AA began after a study by Mathè in 1970. In this study (Mathè *et al.* 1970) anti-lymphocytic serum was administered to pancytopenic patients prior to bone marrow transplant. It was reported that the condition of patients was found to improve even when bone marrow transplants failed to graft. Therefore, treatment with anti-lymphocytic serum appeared to stimulate the host bone marrow resulting in autologous haematological recovery. Since this discovery, immune mediated destruction of the bone marrow has been considered as a probable cause of AA (Young 1996; Young and Maciejewski 1997).

Immunoglobulins, that is ATG (anti-thymocyte globulin) and ALG (anti-lymphocyte globulin), are derived from the purified serum of animals immunised with children's thymocytes in the case of ATG, and thoracic duct lymphocytes in the case of ALG (Young 1995). In the present report, from here on, both ATG and ALG will be referred to as ALG.

ALG contains antibodies that recognise multiple antigens on peripheral blood cells. The immunosuppressant action of ALG in AA patients occurs due to the

destruction of cytotoxic lymphocytes. After intra-venous administration of ALG the number of circulating lymphocytes in the patient decreases rapidly. However, the activity of these ALG preparations can vary considerably between batches. Although T-lymphocytes are the major target, other peripheral blood cells including granulocytes, platelets and bone marrow cells are also destroyed. Patients treated with ALG therefore require supportive care in the form of platelet transfusions and often steroids to combat serum sickness

Clinical trials using ALG as a therapy for AA have reported response rates of about 50% (Young 2002). However, in a study by Camitta *et al.* (1983) treatment of AA patients with ALG resulted in a 2 year survival rate of 76%.

The fungal metabolite cyclosporin A (CsA) is a potent immunosuppressive agent that primarily affects T-lymphocytes. First isolated in 1976 (Dollery 1999) CsA blocks the synthesis of IL-2 in T-helper cells by preventing the translocation of the transcription factors nuclear factor of activated T-cells (NFAT) and nuclear factor of immunoglobulin K light chain in B-cells (NF-kB) to the nucleus (Baumann *et al.* 1992). These transcription factors are essential for expression of IL-2. Inhibition of IL-2 production, and therefore release, prevents expansion of unprimed T-helper cells, cytotoxic T-cells, and T-cell dependent B-cell activation. The inhibition of T-helper cells results in a reduction of the release of interferon gamma (IFN-γ), which suppresses the proliferation of early and late haemopoietic progenitor cells (Young and Maciejewski 1997).

Patients treated with CsA for long periods of time remain in good health even with a suppressed immune system. Although a wide spectrum of side effects can develop during CsA therapy, including renal and hepatic dysfunction, hypertension, tremor, gingival hypertrophy and lymphoma, these side effects usually respond to dose adjustment (Palestine *et al.* 1984).

The discovery of the immunosuppressant properties of CsA has lead to the use of the drug in the treatment of AA. CsA alone has been shown to improve the symptoms of both AA patients (Hinterberger-Fischer *et al.* 1989; Gluckman *et al.* 1992) and patients who previously did not respond to ALG therapy (Leonard *et al.* 1989). However, the combination of ALG and CsA has been shown to be the most successful IS therapy for AA patients (Bacigalupo *et al.* 1995) and the combination is now used as the first line IS treatment in aplastic patients.

Treatment of AA with IS therapy has been shown to be successful (Young 2002). However, long term follow up studies have shown that the development of later haematological complications including paroxysmal nocturnal haemoglobinurea (PNH), myelodysplastic syndrome (MDS), and also malignant disease, during the period 5 to 10 years after treatment. Recently, an 11-year follow up study of clinical trials using ALG and CsA found that 18% of survivors developed late clonal malignant disease at 11.3 years after treatment (Frickhofen *et al.* 2003). However, the development of these secondary disorders were independent of the treatment regimen used and are just as likely to occur with ATG alone as with ATG in combination with CsA.

1.6.4. Haemopoietic stem and progenitor cells in aplastic anaemia

The stem cell defect in AA patients is both a quantitative and a qualitative deficiency. Using flow cytometry, the number of haemopoietic stem cells characterised as $CD34^{+}/CD38^{-}$ are significantly reduced in AA bone marrow (Scopes *et al.* 1994; Maciejewski *et al.* 1996). In vitro culture of AA bone marrow has also shown that there is reduced number of committed haemopoietic progenitor cells (CFU-GM, CFU-GEMM and BFU-E) and more primitive LTCICs (Marsh *et al.* 1990; 1991; Maciejewski *et al.* 1994; 1996; Rizzo *et al.* 2002). However, this deficiency in the

number of stem and progenitor cells continues to be evident in recovered AA patients who have been successfully treated with IS therapy or BMT (Maciejewski *et al.* 1996). Indeed, recovered AA patients with normal peripheral blood counts and normocellular bone marrow, continue to have a significant deficit in the number LTCIC which may last for 10 years after treatment (Podesta *et al.* 1998).

LTBMC of bone marrow from AA patients has provided evidence of a lack of proliferative capacity. LTBMCs using bone marrow samples from normal donors support haemopoietic cell production for 9 to 14 weeks. In contrast, cultures set up using bone marrow from AA patients become exhausted in 2 to 4 weeks (Gibson and Gordon-Smith 1990; Marsh *et al.* 1990; 1991). In addition, LTCICs from AA bone marrow have been shown to produce a lower number of CFU-C compared to controls (Maciejewski *et al.* 1996).

Crossover experiments to assess the function of stem cells and bone marrow stroma from AA patients have also indicated that in some cases the stromal microenvironment of the marrow of AA patients may contribute to the bone marrow defect by being less able to support haemopoiesis.

1.6.5. Apoptosis and aplastic anaemia

In the marrow of AA patients, the levels of apoptosis are increased in $CD34^+$ cells (Philpott *et al.* 1995). The changes in apoptosis levels were also shown to be associated with bone marrow damage. AA patients with low numbers of $CD34^+$ cells in the bone marrow had elevated levels of apoptotic cell death, whereas normal patients with a normal proportion of $CD34^+$ cells had a normal level of apoptosis (Philpott *et al.* 1995). This increase in the level of apoptosis in AA patients has also been demonstrated in bone marrow biopsies from AA patients (Callera and Falcao 1997).

As a result of the successful use of IS therapy in the treatment of AA, immune mediated destruction of the bone marrow has been proposed as an important cause of bone marrow destruction (Young 1996). The increase in apoptotic cell death and bone marrow damage in AA has been studied at the cellular level looking at cytokines and chemokines that are mediators of apoptotic cell death. The cytokines IFN-γ and TNF-α have been shown to exert suppressive or inhibitory effects on haemopoietic stem and progenitor cells (Zoumbos *et al.* 1985; Selleri *et al.* 1996; Dufour *et al.* 2001). An increase in the concentration of IFN-γ and TNF-α have been shown in plasma and serum samples from AA patients (Zoumbos *et al.* 1985; Schultz and Shahidi 1994). However, increases in these cytokines are not evident in all AA patients. Studies using lymphocytes isolated from the blood and bone marrow of AA patients have demonstrated an increase in the production of IFN-γ and TNF-α when stimulated in vitro, compared to normal controls (Hinterberger *et al.* 1988; Shinohara *et al.* 1991). Indeed, when mRNA levels of IFN-γ were measured in AA patients it was found that patients expressing the gene responded to immunosuppressive therapy, while patients that did not express the mRNA for IFN-γ did not respond to therapy (Nakao *et al.* 1992; Nistico and Young 1994).

More recently, the level of IFN-γ found intracellularly in mononuclear cells isolated from the peripheral blood and bone marrow of AA patients has been measured (Sloand *et al.* 2002). AA patients with cells positively stained intracellularly for IFN-γ responded to IS therapy. However, patients negative for IFN-γ staining failed to respond. It was also shown that IFN-γ positive cells were reduced in number in patients in remission who had responded to IS therapy and that the number of IFN-γ positive cells was increased in relapsing patients (Sloand *et al.* 2002). This finding is also in line with results using mRNA expression (Nakao *et al.* 1992). Therefore, AA patients expressing IFN-γ mRNA responded to IS therapy while non-responders were negative

for IFN-γ mRNA. The importance of changes in IFN-γ and TNF-α in AA patients is that these cytokines are able to modulate cell death via interactions with the FAS receptor. Indeed, expression of FAS antigen is increased in cells in vitro which are exposed to IFN-γ and TNF-α (Maciejewski *et al.* 1995a). In addition, expression of the FAS antigen has been shown to be elevated on lymphocytes isolated from the bone marrow of AA patients (Maciejewski *et al.* 1995b). The expression of FAS ligand (fasL) measured intracellularly in lymphocytes isolated from the peripheral blood or bone marrow of AA patients is increased, with levels returning towards control values in patients responding to IS therapy (Luther-Wyrsch *et al.* 2001).

1.6.6. fms-like tyrosine kinase 3 ligand and aplastic anaemia

In normal individuals, the concentration of fms-like tyrosine kinase 3 (FLT-3) ligand (FL) in plasma is low. Conversely, patients with chronic bone marrow aplasia in the form of AA or Fanconi's anaemia, have significantly elevated plasma FL levels (Lyman *et al.* 1995b). FL however, is not significantly increased in patients with anaemias not associated with a stem cell lesion i.e. pure red cell aplasia, Diamond Blackfan anaemia and α-thalassemia.

In SAA patients who undergo successful bone marrow transplant or autologous reconstitution following immunosuppressant treatment, the serum FL concentration returns to values seen in control subjects (Wodnar-Filipowicz *et al.* 1996). However, in patients who relapse, plasma FL increases once again to a similar magnitude to that seen in the pre-treatment period (Wodnar-Filipowicz *et al.* 1996).

Similarly, a study by Pfister *et al.* (2000) reported that membrane bound FL is increased on $CD4^+$ and $CD8^+$ lymphocytes of severe AA patients. In this study the level of membrane bound FL was inversely correlated with the number of $CD34^+$ cells of the

bone marrow. In addition, it was found that membrane bound FL can remain significantly elevated in AA patients for up to 20 years after autologous bone marrow reconstitution following immunosuppressant therapy.

The concentration of FL in the serum of patients undergoing radio or chemotherapy for malignant disease is at a low level in the pre-treatment period. However, immediately after radio or chemotherapy, serum levels of FL increase significantly before declining to pre-treatment levels as haematological recovery comes about (Chklovskaia *et al.* 1999; Blumenthal *et al.* 2000). Membrane bound FL is also elevated in patients receiving chemotherapy and levels follow the same pattern as soluble FL in the serum (Chklovskaia *et al.* 1999). However, increases in FL mRNA do not follow the same pattern of change. Chklovskaia *et al.* (1999) showed FL mRNA to be increased significantly 8 to 10 days after the start of chemotherapy; at this time the patients were described as having severe pancytopenia. This delay in the increase of FL mRNA expression is due to intracellular cytokine storage. FL is stored in high quantities intracellularly, and therefore protein synthesis is only increased when stores are low. Conversely, a study by Huchet *et al.* (2003) found that although plasma FL increases after radiotherapy, membrane bound FL was unchanged.

1.7. ANIMAL MODELS OF BU-INDUCED CHRONIC BONE MARROW APLASIA

In 1974, Morley and Blake (1974a) reported a series of experiments describing the development of a model of chronic hypoplastic marrow failure (CHMF) in the female Swiss and BALB/c mouse following administration of busulphan (BU). BU was administered to mice at 20, 20, 20 and 10 mg/kg by intraperitoneal injection, with each dose being administered at fortnightly intervals.

Initially, animals developed a significant bone marrow aplasia and were allowed 60 days to recover from the initial toxicity of BU before the initiation of peripheral blood and bone marrow investigations. Between 60 and 313 days post dosing, animals were killed for studies on blood and bone marrow. During this period, it was found that some mice (termed 'latent') had minor changes in the peripheral blood, such as decreased haematocrit (HCT), neutrophil, lymphocyte and monocyte counts, in addition to a reduced marrow cellularity. However, a number of animals treated with BU became pale and sick between day 60 and 313 post dosing; these mice (termed 'aplastic') were found at autopsy to have significant reductions in all circulating blood cells with a significantly reduced marrow cellularity. This peripheral blood pancytopenia and bone marrow hypoplasia was described by Morley and Blake (1974a) as CHMF, and was considered to be comparable to AA in man.

Further studies were conducted by Morley and his co-workers to characterise the changes in the bone marrow of mice with BU-induced CHMF (Morley and Blake 1974b; Morley *et al.* 1975; Morley *et al.* 1978). Indeed, it was shown that mice with CHMF had a reduction in the number of committed progenitor cells (CFU-C) and also more primitive haemopoietic progenitors as assessed by the spleen colony forming unit assay (CFU-S) (Morley and Blake 1974b; Morley *et al.* 1975; Morley *et al.* 1978).

In later publications by Morley and his colleagues (Pugsley *et al.* 1978), the changes in the haemopoietic system of mice with BU-induce CHMF were also found to involve lymphocytes, with B- and T-lymphocytes being reduced in the peripheral blood, bone marrow and spleen, as well as T-lymphocytes being significantly reduced in the thymus. It was also shown that mice with CHMF had deficient immune responses, including delayed hypersensitivity reactions and reduced antibody responses (Pugsley *et al.* 1978).

The mechanism behind the development of BU-induced CHMF in mice described by Morley and Blake (1974a) is unknown. Many ideas to explain CHMF have been suggested including drug-induced DNA damage resulting in the expression of foreign proteins on the surface of haemopoietic stem cells triggering cytotoxic T-cell killing (Young and Maciejewski 1997).

More recently the work of Morley was investigated by Andrews *et al.* (1993, 1998) and Andrews (2000). However, in these studies the development of 'late-stage' or 'residual' chronic bone marrow aplasia closely fitting the description of Morley and Blake (1974a) could not be demonstrated. Accordingly, the regimen of BU dosing was amended in order to develop a new model of chronic bone marrow aplasia (CBMA) in the BALB/c mouse (Gibson *et al.* 2003). In this study BALB/c mice were treated with 10 intraperitoneal doses of BU at 10.50 mg/kg over 22 days, and the mice were studied for 112 days after dosing. In the early post-dosing period, BU induced a 'predictable' bone marrow aplasia followed by a recovery and a return towards control. However, normality was not reached in the BU-treated animals and on 91 and 112 days post dosing BU-treated mice demonstrated a 'late-stage' CBMA. CBMA was characterised by reductions in red blood cells (RBC), haemoglobin (Hb), HCT, reticulocytes, platelets and neutrophils. Bone marrow cellularity was also reduced in addition to the number of committed progenitor cells (CFU-C). During 'late-stage' CBMA the mean cell volume (MCV) in the peripheral blood was increased as was the level of apoptosis in the bone marrow. The changes in both peripheral blood and bone marrow parameters in this mouse model mirrored the changes seen in AA patients; however, the changes were not as severe as those seen in man.

1.8. ANIMAL MODELS OF IMMUNE MEDIATED BONE MARROW FAILURE

In 1967 Barnes and Mole, reported that sporadic deaths occur in mice some time after exposure to radiation (Barnes and Mole 1967). The deaths were thought to be a result of bone marrow failure as the animals were found to have significantly aplastic bone marrow. Barnes and Mole proposed that mutations occurring as a result of irradiation resulted in the destruction of non-mutant cells of the bone marrow giving rise to bone marrow failure. The model of immune mediated bone marrow failure described by Barnes and Mole (1967) was achieved by exposing mice to a sub-lethal dose of radiation (450 to 600 rads) followed by an intravenous injection of allogenic lymph node cells (LNC).

Two strains of mice were used in the experiment of Barnes and Mole (1967). First CBA/H mice were exposed to radiation and these animals were then injected with LNC from donor C3H/H mice. These two strains of mice were chosen because they share the same histocompatibility marker, H-2. However, these antigens differ at the H-2^K locus. The injection of LNC from C_3H/H mice resulted in the destruction of the recipient bone marrow cells and resulted in death from bone marrow failure in 13 to 142 days.

The mechanism of action of the LNC in these studies is similar to GVHD which is encountered following bone marrow transplantation; however, mice in this experiment did not show the characteristic signs of GVHD such as weight loss, diarrhoea, splenomegaly, lymphoid atrophy and dermatitis.

Later, Kubota *et al.* (1978; 1979) repeated the work of Barnes and Mole. Using two strains of mice, the C_3H/He and the B10.BR, which have the same major histocompatability complex but differ at the M locus. Injection of B10.BR LNC into sublethally irradiated C_3H/He mice resulted in significant anaemia, leucopenia and

thrombocytopenia 14 to 21 days after treatment. In addition, the marrow of mice developing bone marrow failure was significantly hypocellular and had a deficiency in the number of CFU-C and CFU-S without evidence of GVHD.

Studies by Knospe *et al.* (1983) and Chiu and Knospe (1987) examined the effects of injecting LNC from different strains of mice into donor mice with the identical major histocompatability complex ($H\text{-}2^k$) but with a difference at the M locus. Further studies, by Chiu and Knospe (1989) showed that plasma from mice with immune mediated bone marrow failure was able to inhibit colony formation in the CFU-C assay. Later, studies found that the number of CFU-GM and CFU-S produced by aplastic mice were significantly reduced. Additionally, using a stromal cell culture assay, the stroma of aplastic mice was found to be disturbed, and abnormally organised (Knospe *et al.* 1994).

In a recent report by Chen *et al.* (2004) immune mediated destruction of the bone marrow has been described, again using mice with differing loci of the H-2 antigen. In this experiment mice were found to develop pancytopenia in 2 to 3 weeks. Mice with bone marrow failure were found to have increased levels of serum IFN-γ and an increase in apoptotic cells expressing the FAS antigen.

1.9. AIMS AND OBJECTIVES OF THE PRESENT PROJECT

Many experiments have been conducted in the pursuit of developing an animal model of CBMA analogous to AA in man. Models of CBMA have been described in both large and small laboratory animals; however, no model which has been developed has become widely used to investigate the pathophysiology of AA (Alter *et al.* 1978; Haak 1980; Vincent 1984).

The mouse model of drug induced CHMF described by Morley and Blake (1974a) displayed features of human AA, however, this model was time consuming with mice developing CHMF between 100 to 313 days after treatment. The changes in the BU dosing regimen used by Gibson *et al.* (2003) induced CBMA in mice in a much shorter time period. However, the CBMA induced was relatively mild in comparison to AA in man.

The study of the haemopoietic system in patients with inherited and acquired bone marrow failure syndromes is difficult as the bone marrow is sparce and therefore, bone marrow samples contain few haemopoietic stem and progenitor cells essential for investigation into the pathophysiology of these disorders. This problem is further compounded by the rarety of these disorders. It would therefore, be advantageous if an animal model of bone marrow failure could be developed to facilitate the study of haemopoietic stem and progenitor cells. As bone marrow failure can result from exposure to antineoplastic agents, we aimed to study a range of antineoplastic agents to assess their potential to induce chronic bone marrow aplasia in the rodent. A model of bone marrow failure should show features of the human condition of bone marrow failure (i.e. AA) including, a reduction in circulating peripheral blood cells and a reduction in bone marrow cellularity and the number of haemopoietic progenitor cells. In addition, the model should show further features such as an increase in the level of bone marrow apoptosis and serum cytokines (i.e. FL).

CHAPTER 2: Materials and methods

2.1. ANIMALS

Mice or rats were caged in groups of 5 to 10 or 4 to 6, respectively, with free access to diet (Rat and Mouse No.1, SDS Ltd., Witham, Essex) and mains drinking water. A temperature of 19 to 22°C was maintained, with a relative humidity of 45 to 65% and a light:dark cycle of 12:12h (lights on at 7.00h). Before the initiation of experimental procedures, animals were allowed 7 days to acclimatise. All animal procedures were conducted under local Ethical Committee guidelines and approval for Home Office Project and Personal Licences, and followed the UK Home Office (1989) "Code of Practice for the Housing and Care of Animals used in Scientific Procedures".

During periods of dosing, animals were observed daily for any signs of ill health. If animals began to lose body weight, or show overt signs of drug-induced toxicity, and it was considered that the condition of the animals would not improve, animals were autopsied. On occasions where blood and bone marrow samples could not be processed animals were killed *in extremis*. On a number of occasions animals were found dead at morning inspections. All such animals killed *in extremis* or found dead will be referred to as 'intercurrent death' animals (ICD).

Body weights of animals were determined 2 to 3 times per week during dosing periods and at approximately weekly intervals thereafter.

2.2. PREPARATION OF DRUGS

2.2.1. Chlorambucil

Chlorambucil (CHB; Sigma Chemical Co. Ltd., Poole, Dorset) was dissolved in acetone at a concentration of 4.35 mg/ml and, immediately before administration, de-ionised water was added to the CHB-acetone solution at a volume of 2 (water):1 (acetone). Control animals were treated with water:acetone (vehicle).

2.2.2. Mitomycin C

Mitomycin C (MMC; Kyowa Hakko UK Ltd., Slough, Berkshire) was dissolved in de-ionised water to a concentration of 0.25 mg/ml. Control animals were treated with de-ionised water (vehicle).

2.2.3. Azathioprine

Azathioprine (AZA; Sigma) was suspended in vegetable oil at a concentration of 5 to 15 mg/ml. Control animals were treated with vegetable oil (vehicle).

2.2.4. Busulphan

Busulphan (BU; Sigma) was dissolved in acetone at a concentration of 5 to 8 mg/ml and, immediately before administration, de-ionised water was added to the BU-acetone solution at a volume of 5 ml (water):1 ml (acetone). Control animals were treated with water:acetone (vehicle).

2.2.5. Cyclosporin A

Cyclosporin A (CsA, Sandimmune; Novartis Pharmaceuticals UK Ltd., Horsham, West Sussex) was suspended in vegetable oil at a concentration of 4.35 mg/ml. Control animals were treated with vegetable oil (vehicle).

2.3. AUTOPSY PROTOCOL

1. Animals were killed by intraperitoneal (ip) injection of pentobarbitone sodium (Sagatal, Rhône Mérieux Ltd., Harlow, Essex).

2. A sample of blood was removed from the abdominal aorta of rats, and from the right ventricle of mice following a thoracotomy incision. A 0.5 ml sample was anticoagulated with 1.5 mg/ml dipotassium ethylene diaminotetraacetic acid (EDTA) (Teklab, Sacriston, Durham), any remaining blood was collected into serum separator tubes (Microtainer; Becton Dickinson and Co., Franklin Lakes, NJ, USA).

3. The right femur was removed. The epiphyses were removed with sharp scissors and the marrow contents flushed into 3 or 5 ml of phosphate buffered saline (PBS; Sigma) or Iscove's modified Dulbecco's medium (IMDM; Life Technologies, Paisley).

4. The left femur was removed with surrounding muscle blocks intact and placed in 5 to 10 ml sterile PBS or IMDM supplemented with 10% foetal calf serum

(FCS; PAA Laboratories GmbH, Linz, Austria). Under sterile conditions the muscle and epiphyses were removed from the femur and the marrow flushed into 5 ml sterile IMDM supplemented with 10% FCS.

5. The sternum and spleen were removed from each animal at autopsy for histological examination. On occasion, the thymus, kidneys, liver and lungs were also removed for histology.

6. The contents of either left or right tibia were used to prepare a bone marrow smear. A camel hair paint brush, moistened in PBS, was used to smear bone marrow cells onto a histology slide.

2.4. ANALYSIS OF BLOOD AND MARROW SUSPENSIONS

Blood samples and bone marrow suspensions were analysed with a Bayer H*1 haematology analyser, or the ADVIA haematology analyser, using the appropriate rat- or mouse-specific software (Bayer Diagnostics UK Ltd., Newbury, Berks). The femoral marrow cell suspension was used to obtain the total nucleated cell count (femoral nucleated cell count; FNCC) using the basophil channel of the haematology analyser.

2.5. ANALYSIS OF BONE MARROW SMEARS

Tibial marrow smears were allowed to dry for 24 hours, fixed in 100% methanol for 30 minutes and stained with May-Grünwald-Geimsa. Differential counts were performed by eye on 200 cells. The proportion of cells (as %) in the myeloid, erythroid

and lymphoid lineages was calculated and converted into the absolute number of cells making up each lineage in the femoral marrow sample using the FNCC of each animal.

2.6. BONE MARROW CLONOGENIC ASSAY

The femoral marrow flush in 5ml sterile IMDM supplemented with 10% FCS was used to assess the clonogenic potential of the bone marrow. Using trypan blue exclusion, nucleated bone marrow cells were counted using a haemocytometer and cultured at 10^5 in 1 ml IMDM supplemented with 30% FCS, 1% de-ionised bovine serum albumin (BSA; Sigma), 10^{-4} M β-mercaptoethanol (Sigma), 0.05% $NaHCO_3$, 2.1 mM L-glutamine (Sigma) and 0.9% methylcellulose (Stem Cell Technologies Inc. London). Cultures were set up in duplicate in 35 mm dishes (Nunclon, Loughborough, Leicestershire) with the following growth factors added to each dish: 4 IU human erythropoietin (hEpo; Janssen-Cilag Ltd., High Wycombe, Bucks), 50 ng murine interleukin-3 (mIL-3; $1.7x10^5$ U/ml; R & D Systems Europe Ltd., Abingdon, Oxon), 50 ng murine stem cell factor (mSCF; R & D Systems), 50 ng human interleukin-6 (hIL-6; $52x10^6$ U/ml; Novartis Pharmaceuticals Ltd., Langley, Herts) and 50 ng human granulocyte colony stimulating factor (hG-CSF; 10^8 U/ml; Amgen UK Ltd., Cambridge). The cultures were incubated at 37°C in 5% CO_2 in air for 14 days. On day 14, granulocyte-macrophage colony forming units (CFU-GM), erythroid burst forming units (BFU-E) and colonies containing both granulocyte–macrophage and erythroid elements (CFU-GEM) were counted. Results are expressed as CFU-GM or total number of erythroid colonies (BFU-E + CFU-GEM) per femur. In some cases, the numbers of CFU-GM and erythroid colonies were added together to give a total number of colony forming unit cells (CFU-C) per femur.

2.7. APOPTOSIS IN BONE MARROW CELLS

A 200 µl aliquot (0.5-1.0 x 10^6cells), removed from each femoral marrow flush into IMDM supplemented with 10% FCS, was used to quantify the level of apoptosis in the bone marrow according to the method described by Philpott *et al.* (1995). Cells were washed twice by centrifuging (4°C, 400 g, 30 min) in PBS supplemented with 1% FCS and 0.05% Na azide. Excess solution was removed after centrifugation and cells resuspended in 500 µl or 450 µl of PBS for 7-amino actinomycin D (7-AAD; Calbiochem, Nottingham) unstained and 7-AAD stained cells, respectively. 7-AAD was dissolved in acetone, diluted in PBS to a concentration of 0.2 mg/ml, kept at –20°C and protected from light until use. Cells were stained with 50 µl of 7-AAD for 20 min on ice and protected from light. Cells were pelleted by centrifugation, the supernate removed, and the cells resuspended in 500 µl of 2% paraformaldehyde fixative solution (Sigma). Samples were analysed on a FACScan (Becton Dickinson, Mountain View, CA, USA) within 30 min of fixation. Data on 50000 cells were acquired and processed using Cell Quest software™ (Becton Dickinson). Scattergrams of forward scatter (FSC) vs. 7-AAD fluorescence were generated. FSC height threshold was set at 108 to exclude all RBC and debris. Regions were drawn around populations showing negative (R1), dim (R2) and bright (R3) 7-AAD fluorescence, corresponding to live, apoptotic and dead cells, respectively. A region was also drawn around remaining cell debris (R4) and RBC to exclude these data (Fig. 2.1).

2.8. ANALYSIS OF CYTOKINES

The blood deposited in serum separator tubes was allowed to stand for 75 to 90 min to allow coagulation. Tubes were then centrifuged (room temperature, 400 g, 5 min) and serum harvested. Serum samples were stored at –80°C until use.

Levels of the cytokine fms-like tyrosine kinase 3 (FLT-3) ligand (FL) in serum was detected using an enzyme-linked immunosorbant assay (ELISA; R & D Systems) with a sensitivity of typically less than 5 pg/ml measured at a wavelength of 450 nM. The concentration of FL was very high in some serum samples from drug-treated mice and to ensure measurements fell within the range of the standard curve all samples were diluted with assay diluent provided with the kit (25 µl serum, 25 µl of assay diluent). Serum samples and standards were analysed in duplicate. A standard curve was generated using a 4-parameter logistic curve fit (SoftMax® Pro Software, Molecular Devices Ltd., Wokingham, Berkshire) and the average absorbance of each sample was used to calculated the concentration of FL from the standard curve.

The presence of the cytokines interleukin-2 (IL-2), tumour necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) in serum was detected using a flourokine MAP kit (R & D Systems) with a sensitivity of typically less than 1.99, 0.42 and 5.25 pg/ml for each cytokine, respectively. Prior to analysis, each serum sample was diluted 4-fold in calibrator diluent provided with the kit. A mixture of 3 colour-coded microparticles containing immobilised antibodies specific for each cytokine (IL-2, TNF-α or IFN-γ) were incubated with samples and standards. Immobilised antibodies of interest bind to the microparticles and are detected following incubation with secondary biotinylated antibodies specific to the analytes of interest followed by incubation with streptavidin-phycoerythrin, which binds to the biotinylated antibody. Samples were analysed using a Luminex® 100™ analyser (Luminex Corp. Austin, TX, USA). This machine has two

lasers, one laser is micro-particle specific and determines which cytokine is being detected while the second laser determines the magnitude of the phycoerythrin-derived signal which corresponds to the amount of cytokine bound. Data on 10000 beads were collected per cytokine bead, per sample, and the concentration of cytokine within that sample determined from the standard curve.

2.9. TISSUES

The spleen, liver, kidneys (left and right) and thymus removed at autopsy were weighed and fixed in 10.5% phosphate buffered formalin fixative. The sternum, and on occasion the lungs (inflation fixation) were also placed in fixative. Tissues were embedded in paraffin, sectioned, and stained with haematoxylin and eosin for morphological examination by light microscopy.

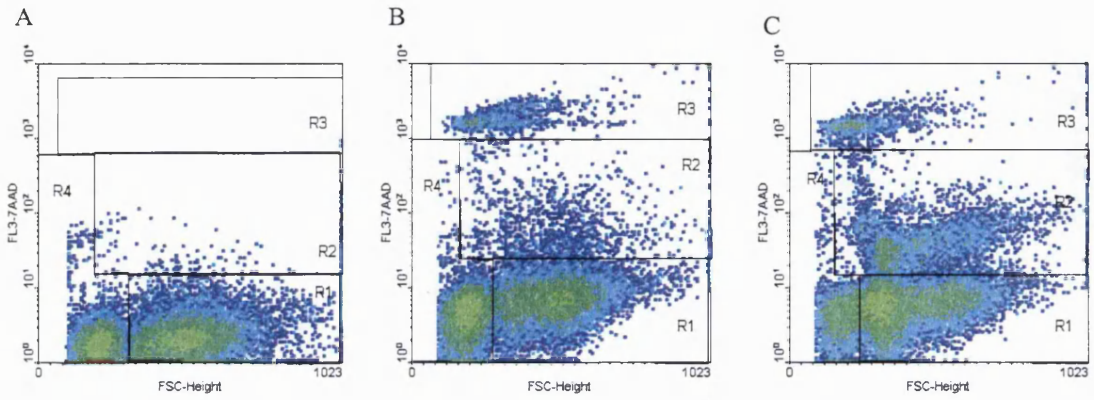


FIGURE 2.1. An example of apoptotic cell populations in mouse bone marrow identified using 7-AAD staining. Scattergrams of 7-AAD-stained mouse femoral bone marrow samples. (A), Negative control; unstained control mouse bone marrow sample. (B), control 7-AAD-stained mouse bone marrow sample showing normal levels of apoptosis; R1, live cells, 47.41%; R2, apoptotic cells, 8.22%; R3, dead cells, 6.46%; R4, cell debris, 37.72%. (C), azathioprine treated 7-AAD-stained mouse bone marrow sample with increased levels of apoptosis; R1, live cells, 55.10%; R2, apoptotic cells, 20.95%; R3, dead cells, 6.16%; R4, cell debris, 17.26%. Abbreviations: FSC-Height, forward light scatter; FL3-7-AAD, 7-AAD-fluorescence intensity.

CHAPTER 3: Studies on serum fms-like tyrosine kinase 3 (FLT-3) ligand levels and haematological changes in the busulphan-treated mouse

3.1. INTRODUCTION

The bifunctional alkylating agent busulphan (BU) is commonly used in the treatment of chronic granulocytic (myeloid) leukaemia, polycythemia vera and myelofibrosis. In addition, the myeloablative property of BU is regularly employed in conditioning regimens used prior to bone marrow transplantation in patients with malignant diseases and aplastic anaemia (AA) (Sweetman 2002).

BU is a small and highly lipophillic molecule that, once administered, undergoes extensive enzymatic metabolism; studies have found 12 metabolites of BU, however, many of these metabolites have not been fully characterised. Indeed, less than 2% of the drug is excreted unchanged (Buggia *et al.* 1994). BU interacts with nucleic acids to form DNA inter-strand crosslinks resulting in significant DNA damage. However, it is cells that are resting in the G_0 phase of the cell cycle which are more sensitive to the effects of BU (Dollery 1999).

In man, the major toxic side effect of BU treatment is bone marrow hypocellularity resulting in anaemia, leucopenia and thrombocytopenia. In some cases, long-term exposure to BU, or incidences of overdose, result in significant bone marrow toxicity leading to complete bone marrow failure, the outcome of which is pancytopenia and AA (Buggia *et al.* 1994; Bright *et al.* 2001).

Long-term treatment with BU in man can also result in damage to the lungs. 'Busulphan lung' is characterised by broncho-pulmonary dysplasia, and interstitial pulmonary fibrosis, and is associated with symptoms such as a non-productive cough and progressive dyspoenia (Buggia *et al.* 1994; Dollery 1999). If pulmonary toxicity is identified at an early stage, and drug treatment is withdrawn, the syndrome may be

reversible. However, pulmonary failure is the end-point in many cases (Dollery 1999). Further reports have described an increased risk of leukaemia, veno-occlusive disease of the liver, and cataract formation following long-term treatment with BU (Buggia *et al.* 1994; Dollery 1999).

In animals, the extent to which the bone marrow is suppressed immediately following exposure to BU is directly proportional to the dose administered. In the mouse, bone marrow depression occurring as a result of administering a single low dose of BU is readily reversible (Boggs and Boggs 1980). However, studies in both mice and rats have shown that single doses of BU at very high concentrations result in a more severe bone marrow injury. Haematological changes reported in animals treated with high doses of BU include a reduction in erythropoiesis and granulopoiesis, bone marrow hypocellularity, and a reduction in the number of committed progenitor cells (CFU-C) and spleen-colony forming units (CFU-S) (Dunn and Elson 1970; Santos and Tutschka 1974; Boggs and Boggs 1980; Anderson *et al.* 1982).

The effects on the haemopoietic system of repeat dose regimens of BU are unusual, compared to repeat dose regimens with other cytotoxic agents. In the mouse, studies have shown that following treatment with cytotoxic agents such as 5-fluorouracil, methotrexate and vinblastine the bone marrow is initially depressed before returning towards control values, and normal values are generally achieved (Trainor *et al.* 1979). This is in contrast to studies using BU which show that following an initial period of bone marrow depression and a return towards control values, normality is not achieved and 2 months post dosing mice treated with BU develop a secondary phase of bone marrow aplasia. This phenomenon, first described by Morley and Blake (1974a) as chronic hypoplastic marrow failure (CHMF), shares many similarities with the human condition of AA. For example, at a late-stage, BU-treated mice have significant

reductions in peripheral blood cell counts, bone marrow cellularity and the number of CFU-C (Morley and Blake 1974a; 1974b; Morley *et al.* 1975; 1978).

In the experiments of Morley and Blake (1974a) using female Swiss and BALB/c mice, BU was administered at fortnightly intervals at 20, 20, 20 and 10 mg/kg with CHMF occurring between 100 and 313 days after BU treatment. In order to induce 'late-stage' bone marrow aplasia in a more convenient time frame, Gibson *et al.* (2003) modified the dosing regimen of BU, treating mice with BU at 10.50 mg/kg on 10 occasions over 18 days inducing chronic bone marrow aplasia (CBMA) between 70 and 120 days post dosing.

The model of CBMA developed by Gibson *et al.* (2003) shared many similarities with the human condition of AA. However, mortality was high, with 49.3% of mice treated with BU being killed *in extremis* or found dead. To reduce the level of mortality associated with BU administration, further studies on the development of a BU-induced animal model of CBMA were conducted (Turton *et al.* 2006). BU was administered to BALB/c mice at 8.25, 9.0 and 9.75 mg/kg by intraperitoneal (ip) injection on 10 occasions over a 21 day dosing period. Immediately post dosing (days 1 to 23 post dosing) animals treated with BU developed significant bone marrow depression with many peripheral blood values being significantly reduced. A period of recovery followed and from day 50 post dosing animals displayed characteristics of CBMA as previously reported by Gibson *et al.* (2003). The severity of the 'late-stage' (i.e. post day 50) CBMA induced by BU treatment, and the level of mortality recorded, appeared to be related to the dose of BU administered. In conclusion, this study (Turton *et al.* 2006) demonstrated that BU administered at 9.0 mg/kg induced CBMA without significant mortality.

In the present experiment, BU was administered to CD-1 mice at 9.0 mg/kg on 10 occasions by ip injection over a 21 day dosing period. This experiment was devised

to use the BU-treated mouse model to examine the changes in the concentration of the serum cytokine fms-like tyrosine kinase 3 (FLT-3) ligand (FL) in relation to other haematological parameters. The CD-1 mouse was selected for use in this experiment, as this species of mouse is larger than the BALB/c mouse, and therefore would yield greater volumes of peripheral blood at autopsy for blood analysis and serum preparation. The CD-1 mouse is derived from the Swiss mouse (Charles River 2005) that was used in the initial studies of Morley and Blake (1974a) to study the development of CHMF. Therefore, it was considered that the CD-1 mouse would respond to BU treatment in a similar way as the BALB/c mouse.

3.2. MATERIALS AND METHODS

3.2.1. Experimental design

BU was prepared (as described in Chapter 2.2) and administered to female CD-1 mice (Charles River UK Ltd.; mean body weight 17.1 g). Mice were divided into two groups and treated with 10 ip injections of BU (9.0 mg/kg; n=38) or vehicle (control; n=51) over 21 days (days 1, 3, 5, 7, 9, 11, 14, 16, 18 and 21) at a dose volume of 0.1 to 0.3 ml/mouse. On days 1, 23, 72, 119 and 177 after the final dose of BU, animals (n=4 to 9) per group were killed for blood and bone marrow investigations (as described in Chapter 2.3).

3.2.2. Processing of samples

A full blood count was performed and a femoral marrow flush into 5 ml Iscove's modified Dulbecco's medium (IMDM) was prepared to assess bone marrow cellularity (femoral nucleated cell count; FNCC) (as described in Chapter 2.4). The second femur was placed in 5 ml IMDM supplemented with 10% foetal calf serum to assess the clonogenic potential of the bone marrow (as described in Chapter 2.6). Serum from control and BU-treated mice was prepared to measure the concentration of the cytokine FL (as described in Chapter 2.8). At autopsy the spleen was removed, weighed and placed in fixative, as was the sternum (as described in Chapter 2.9).

3.2.3. Statistical analysis

BU-treated and control (vehicle-treated) groups were compared using a one tailed Student's t-test (Microsoft Excel; Microsoft Ltd, Microsoft UK, Reading UK). To

identify possible relationships between the concentration of FL in the serum, and peripheral blood and bone marrow parameters, scattergrams were drawn. To produce linear relationships a $\log_{10}$ transformation was performed on serum FL data. The relationship between FL and both peripheral blood and bone marrow counts were analysed using linear regression with runs post-test performed to verify linearity (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, CA, USA).

3.3. RESULTS

3.3.1. Body weight changes

On day 1 of dosing, the mean body weight of control (vehicle-treated) animals was 15.8 g increasing to 19.1 g over the 21 day dosing period (a 20.9% increase). The mean body weight of mice treated with BU was 18.4 g on day 1 of dosing. Over the period of BU treatment the mean body weight of BU-treated mice decreased by 6.5% to a mean of 17.2 g.

After the period of BU dosing, the mean body weight of BU-treated mice increased to values comparable to controls. However, on days 90 to 125 and 160 to 175 post dosing the mean body weights of BU-treated mice were significantly reduced in comparison to control values (Fig. 3.1).

3.3.2. Haematology results

Haematological findings at day 1, 23, 72, 119 and 177 post dosing are presented in Table 3.1 and the patterns of change in time for RBC, MCV and platelets are illustrated in Fig. 3.2 A-C. On day 1 post dosing, mice treated with BU had significantly reduced peripheral blood counts (Table 3.1). The RBC (Fig. 3.2 A), Hb, HCT and the reticulocyte count were all significantly reduced to 62.1%, 65.0%, 61.5% and 19.2% of the control mean values, respectively ($p<0.001$). In addition, the MCH and MCHC were significantly increased but the MCV was normal (Fig. 3.2 B). The platelet count was also significantly reduced, to 3.3% of the control mean ($p<0.001$; Fig. 3.2 C). Profound effects of BU administration were also seen in the leucocyte parameters. The WBC, neutrophil and lymphocyte counts were all reduced significantly, as were the monocyte and eosinophil counts (reduced to 21.0% (WBC), 5.1% (neutrophils), 27.0%

(lymphocytes), 0.0% (monocytes) and 0.0% (eosinophils) of the control mean, respectively).

Significant reductions continued to be seen in the erythrocyte parameters (RBC, Hb, HCT), leucocyte counts (WBC, neutrophils, lymphocytes and monocytes) and in the platelet count on day 23 post dosing (Table 3.1). In addition to these reductions, in the peripheral blood of BU-treated animals there was an increase in MCV (Fig 3.2 B) and MCH at this time point. Although many parameters continued to be significantly reduced on day 23 post dosing there was evidence of recovery with the reticulocyte count being comparable to controls at this time point.

On day 72 post dosing, the return of erythrocyte parameters to values comparable to controls was not complete, with RBC (Fig 3.2 A), Hb and HCT continuing to be reduced significantly to 93.9%, 95.6% and 94.5% of the control mean, respectively (Table 3.1). The platelet count also continued to be significantly reduced to 68.0% of control (Fig 3.2 C). At this time point the total leucocyte count of BU-treated mice was comparable to that seen in controls however, the lymphocyte and monocyte counts remained significantly reduced.

The return of many peripheral blood counts to control values was complete on day 119 post dosing, however, significant reductions continued in the RBC, HCT and platelets. Furthermore, on day 177 post dosing, erythrocyte parameters including, RBC, Hb, HCT were significantly reduced and the MCV significantly increased. WBC, lymphocytes and eosinophils were also significantly reduced, to 67.0%, 55.4% and 63.6% of the control mean, respectively; the platelet count also remained significantly reduced at this time point.

3.3.3. Femoral nucleated cell count

On day 1 after the final BU dose, the FNCC of BU-treated mice was significantly reduced to 20.3% of the control mean (Table 3.1, Fig. 3.2 D). On days 23 and 72 post dosing, the FNCC was returning towards the control value however, the count continued to be significantly reduced to 51.2% and 70.1% of the control mean, respectively at these time points. On day 119 and 177 post dosing, the marrow cellularity of BU-treated mice was reduced, but the decreases were not statistically significant.

3.3.4. Bone marrow clonogenic assay

The number of committed progenitor cells (CFU-C) per femur within the bone marrow of BU-treated mice is shown in Fig. 3.3, expressed as a percentage of the control mean. On day 1 post dosing, the number of committed progenitors was significantly reduced in mice treated with BU to 2.0% of the control mean ($p<0.001$). On day 23 and 72 post dosing, the number of colonies per femur continued to be significantly reduced to 19.6% and 65.5% of the control mean, respectively ($p<0.001$). However, there was evidence of recovery and a return towards control values at these time points. The return of the number of progenitor cells towards control values was not complete on day 119 post dosing, and the mean number of colonies per femur of BU-treated mice was reduced to 39.0% of the control mean at this time point. However, on day 177 post dosing, the number of committed progenitor cells within the bone marrow of BU-treated mice was comparable to the control mean.

3.3.5. Serum fms-like tyrosine kinase 3 ligand

Levels of the cytokine FL in the serum of BU-treated mice were significantly increased immediately post dosing to 2370.8 pg/ml compared to 311.1 pg/ml in control animals, resulting in a more than 7-fold increase above the control mean (Fig. 3.4). The concentration of FL in the serum of BU-treated mice continued to be elevated above the control level on day 23 post dosing to 4 times the concentration seen in the vehicle-treated mice ($p<0.001$). However, there was evidence of a return towards control values at this time. On days 72, 119 and 177 the concentration of FL in the serum of BU-treated animals reached a plateau remaining significantly elevated above the control mean (Fig. 3.4).

Serum FL values measured in BU-treated mice at all 5 time points were plotted against peripheral blood and bone marrow parameters. To linearise the data the concentration of serum FL was converted logarithmically (log_{10}). Significant correlations were identified between the majority of parameters tested (Table 3.2) with the strongest relationships identified between Log_{10} FL and the platelet, RBC, neutrophil and the FNCC ($r=0.90$, $r=0.86$, $r=0.84$ and $r=0.77$, respectively; $p<0.001$) (Fig. 3.5 A-D, respectively). Relationships between serum FL values and the basophil count and the HCT were not linear and therefore linear regression analysis was not performed on these parameters.

3.3.6. Clinical observations

During the course of the experiment, some animals showed evidence of BU-induced toxicity. The condition of these mice deteriorated and some were killed *in extremis* (KIE), also, a small number of animals were found dead (FD); such mice are

categorised as inter-current death (ICD) animals. To investigate the basis of the toxicity, efforts were made to identify animals that were becoming ill. At two time points, days 43 and 55 post dosing, BU-treated animals (n=4 at each time point) showing significant clinical evidence of toxicity were autopsied. In addition, a group of control mice (n=4) were autopsied at each of the two time points for comparison and statistical analysis. A full blood count was performed and a femoral marrow flush prepared to assess marrow cellularity. The peripheral blood counts and marrow cellularity of the 8 individual BU-treated mice and the mean data for the controls are shown in Table 3.3. The BU-treated mice were found to have significant bone marrow hypocellularity and peripheral blood pancytopenia. It is therefore assumed that these BU-treated animals were losing condition as a result of bone marrow suppression.

On day 67 post dosing two BU-treated mice showed a reduced body weight and in both animals the abdomen was swollen; these 2 animals and 4 control animals were autopsied. The BU-treated mice were found to have significantly reduced RBC, Hb and HCT values with significantly increased MCV and MCH values (Table 3.4). However, the two BU-treated mice also had significantly elevated leucocyte counts (WBC, neutrophils and lymphocytes) and an increase in relative spleen weight. It is therefore considered possible that the 2 mice had developed an infection or lymphoma as a result of BU treatment.

3.3.7. Results summary

- Sustained reduction in erythropoiesis, lymphopoiesis and thrombopoiesis.

- Initial reduction in neutrophils, monocytes and eosinophils returning to normal on day 23 and 72 post dosing.

- Reduction in bone marrow cellularity and the number of CFU-Cs with increased serum FL in animals treated with BU throughout the post dosing period

3.4. Discussion

The FLT-3 receptor is expressed on haemopoietic stem cells (HSC) and early progenitor cells, particularly those of the B-lymphoid lineage (Rosnet *et al.* 1996). The FLT-3 ligand (FL) in the soluble and membrane bound forms of the ligand may therefore be involved in the control of haemopoiesis.

Studies in the mouse have shown that daily administration of FL at 10 μg/mouse for 15 days by ip injection promotes lymphopoiesis with peripheral blood and spleen WBC counts increasing significantly (Brasel *et al.* 1996). The experiments of Brasel *et al.* (1996) also showed that the administration of FL had a positive effect on the number of HSCs. Indeed, HSCs characterised as lin^-, Sca-1^+, c-Kit^+, were found in higher numbers in the bone marrow, spleen and peripheral blood of FL-treated mice.

FL administration (1μg/mouse) to neonatal mice within 24 hours of birth and daily for 6 days thereafter has also been shown to enhance immune function (Vollstedt *et al.* 2003). FL-treated mice had improved immune responses when challenged with bacteria and viruses. This improvement in immune response occurred in addition to an increase in dendritic cell number in the spleen, liver and peritoneal fluid (Vollstedt *et al.* 2003).

Further evidence for the role of FL in lymphopoiesis and the immune response has been reported using mice that have been genetically manipulated and do not express the FLT-3 receptor (McKenna *et al.* 2000). Such FLT-3 knock out (KO) mice were shown by Mckenna *et al.* (2000) to have a significant reduction in lymphopoiesis with WBC counts significantly reduced in the peripheral blood and bone marrow. Additionally, immune cells were affected with a significant reduction in the number of dendritic cells in the spleen, thymus and lymph nodes as well as evidence of a reduction in the number and function of natural killer cells.

Administration of FL by subcutaneous injection has also been shown to protect against bone marrow failure in rabbits exposed to total body irradiation (TBI). When given at 500 μg/kg for 14 days starting 2 days before TBI, FL-treated rabbits had a delayed and a less pronounced reduction in peripheral blood cells compared to untreated rabbits. FL-treated rabbits also had an increased survival rate with the best outcome achieved when administering FL in conjunction with granulocyte colony stimulating factor (G-CSF) (Gratwohl *et al.* 1998).

In the present study, CD-1 mice treated with the chemotherapeutic agent BU, an agent reported to destroy haemopoietic stem cells (Jopling and Rosendaal 2001), initially demonstrated bone marrow hypoplasia (a reduced FNCC) and peripheral blood pancytopenia. There was also a reduction in the number of haemopoietic progenitor cells (Fig. 3.3). During this initial phase of bone marrow hypoplasia, the concentration of FL in the serum of BU treated mice was increased to 2370.8 pg/ml compared to 311.1 pg/ml in vehicle-treated control mice (Fig. 3.4). The increase in FL levels was evident throughout the post dosing period with concentrations being increased significantly at all time points (days 23, 72, 119 and 177 post dosing).

Studies in man have shown that patients receiving chemotherapy for the treatment of malignant diseases or as part of a conditioning regimen to prepare for bone marrow transplant also have significantly elevated plasma FL levels (Chklovskaia *et al.* 1999). The increase in plasma FL corresponds to the period of bone marrow aplasia. Indeed, studies show the concentration of FL in the plasma to be increased immediately following chemotherapy, returning to pre-treatment levels following successful haematological recovery (Chklovskaia *et al.* 1999; Blumenthal *et al.* 2000).

An increase in plasma FL has also been identified in patients with diseases characterised by bone marrow failure such as AA and Fanconi's anaemia (Lyman *et al.* 1995b; Wodnar-Fillipowicz 1996). Serum levels of FL have been reported to be as high

as 2653 pg/ml in AA patients upon presentation and when the bone marrow is markedly aplastic. This is in comparison to a serum FL concentration of 14 pg/ml in normal subjects. The concentration of FL in the serum of AA patients then reduces following successful bone marrow reconstitution as a result of anti-lymphocyte globulin (ALG) treatment or bone marrow transplant. However, if patients relapse, FL increases in the serum of patients once again, to concentrations seen prior to treatment (Wodnar-Filipowicz *et al.* 1996).

In the present study, we measured changes in the concentration of serum FL and compared this with changes in other haematological parameters such as peripheral blood counts, bone marrow cellularity and the number of committed progenitor cells. Linear regression analysis showed significant correlations between the concentration of FL in the serum of BU-treated mice and the majority of the haematological parameters measured (Table 3.1). However, the relationship between HCT and the basophil count were not linear and linear regression analysis was not conducted; the reason for this is not clear, particularly in the case of the HCT values.

Studies describing the changes in the concentration of serum FL in mice following treatment with chemotherapeutic agents have not previously been reported. However, a recent report by Prat *et al.* (2005) has shown that levels of FL are elevated in the plasma of BALB/c and non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice following exposure to radiation. An increase in plasma FL has also been shown in non-human primates exposed to varying levels of total body irradiation (TBI) (Bertho *et al.* 2001). The study of Bertho *et al.* (2001) also showed that a positive correlation exists between the neutrophil count at its lowest point and the concentration of FL in the plasma. In addition, the concentration of plasma FL was also found to correlate with the intensity of radiation exposure.

In man, correlations have been described between the serum level of FL and both the neutrophil count and the number of committed progenitor cells (CFU-C) within the bone marrow of AA patients (Wodnar-Filipowicz *et al.* 1996). Similarly, a study by (Pfister *et al.* 2000) reported that membrane bound FL is increased on $CD4^+$ and $CD8^+$ lymphocytes of severe AA patients. In the study of Pfister *et al.* (2000) the level of membrane bound FL was correlated with the number of $CD34^+$ cells of the bone marrow.

In conclusion, in the present study in the BU-treated female CD-1 mouse, the concentration of FL in the serum correlated with bone marrow damage as measured by bone marrow cellularity, the number of committed haemopoietic progenitor cells and peripheral blood counts. The return of FL towards normal levels in BU-treated mice correlated with the time of partial haematological recovery. However, the concentration of serum FL continued to be significantly increased in BU-treated mice until day 177 post dosing during which time peripheral blood parameters were significantly reduced in comparison to vehicle-treated (control) mice.

The role FL plays however, in bone marrow injury is unclear, and more studies must be completed to further elucidate the reasons for elevated serum FL concentrations during bone marrow damage. It is possible that increases in FL occur in an attempt to increase the number of haemopoietic stem cells, and hence mature cells, after treatment with chemotherapy or radiotherapy, or indeed in AA. Cell cycle analysis of haemopoietic stem cells following BU-treatment in the mouse could provide evidence for this. FLT-3 receptor KO mice may also be used to further evaluate the role of FL in bone marrow damage.

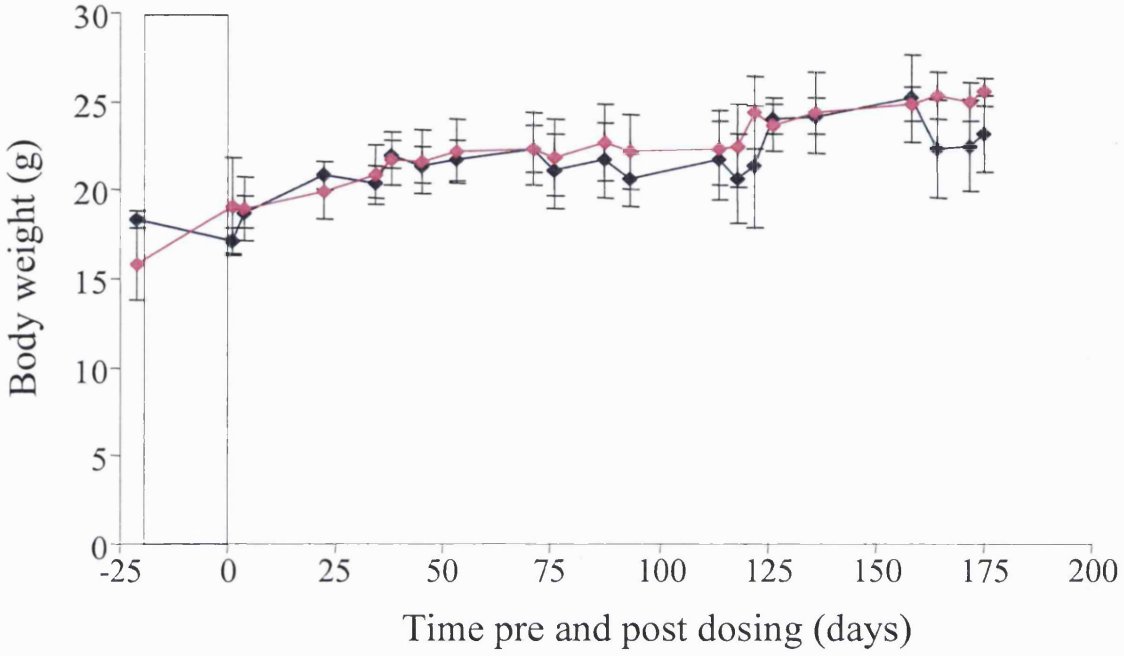
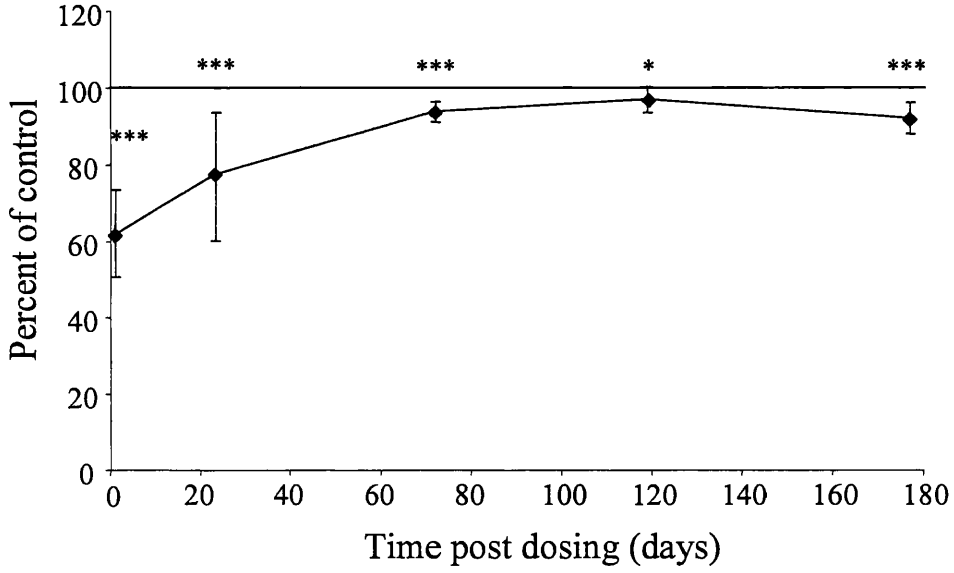
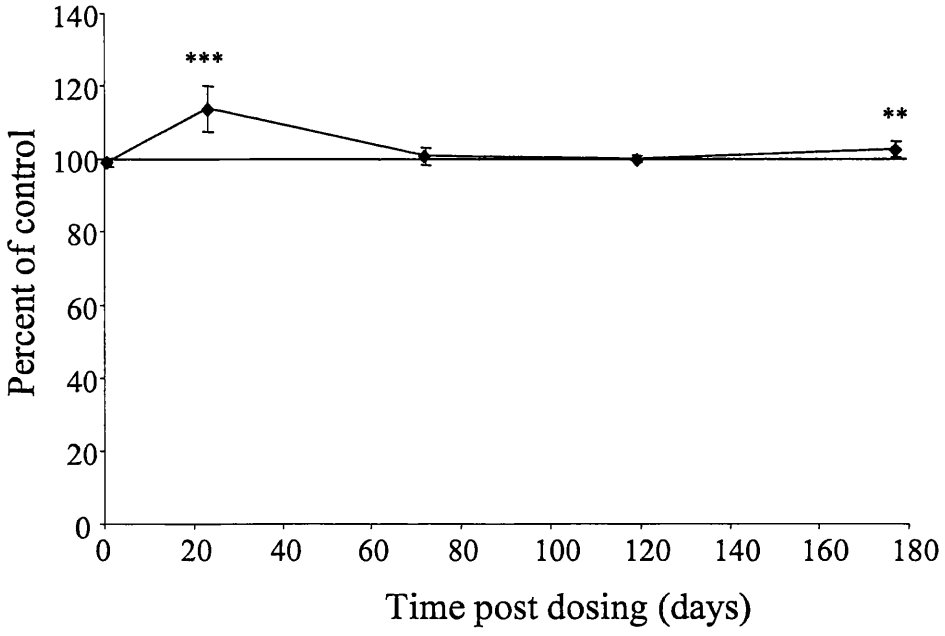


FIGURE 3.1. Body weight increases in control female CD-1 mice and mice treated with busulphan (BU) at 9.0 mg/kg. Values are means (± SD) of control (—◆—) and BU-treated (—◆—) mice. Animals were treated with 10 doses of vehicle or BU by ip injection over a 21 day dosing period (▭).

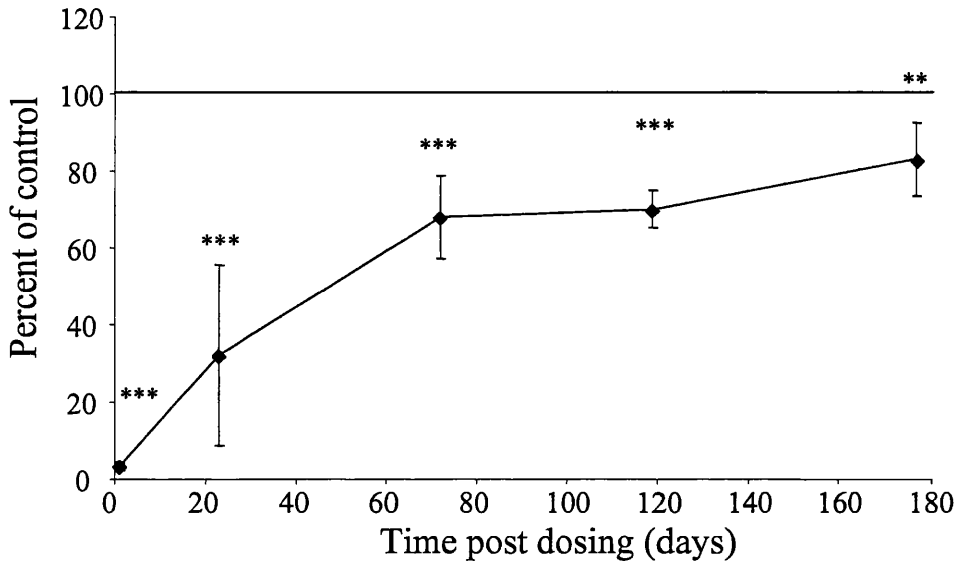
A. Red blood cells



B. Mean cell volume



C. Platelets



D. Femoral nucleated cell count

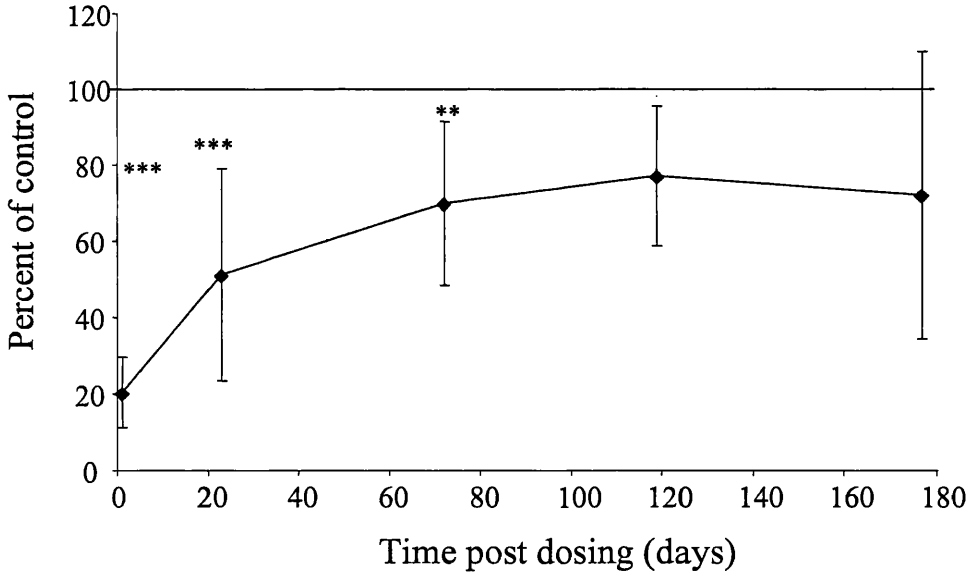


FIGURE 3.2. Results for red blood cells, mean cell volume, platelets and femoral nucleated cell counts from female CD-1 mice treated with busulphan (BU) and sampled on days 1 to 177 post dosing. Values are means (± SD), expressed as a percentage of the mean control value in animals sampled at the same time points. Animals were treated with vehicle (control) or BU (9.0 mg/kg) by ip injection on 10 occasions over 21 days. n=8 control and n=6 BU mice at each time point except on day 72 and 119 where n=7 (control), and day 177 where n=9 (control) and n=4 (BU). *Significantly different from controls, $p<0.05$; **$p<0.01$; ***$p<0.001$.

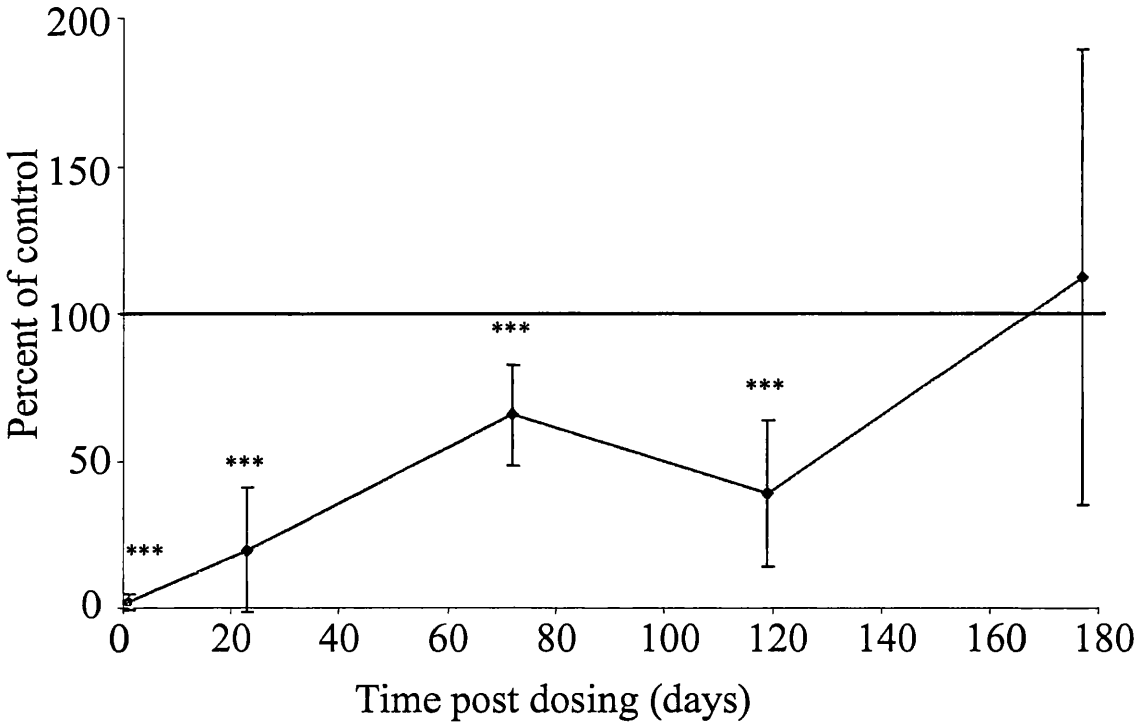


FIGURE 3.3. Committed progenitor cell (CFU-C) results from female CD-1 mice treated with busulphan and sampled on days 1 to 177 post dosing. Values are means (± SD), expressed as a percentage of the mean control value in animals sampled at the same time points. Cultures were set up in duplicate and the mean CFU-C number per femur was calculated per mouse. Number of animals per group and all other information as Fig. 3.2. ***Significantly different from controls, $p<0.001$.

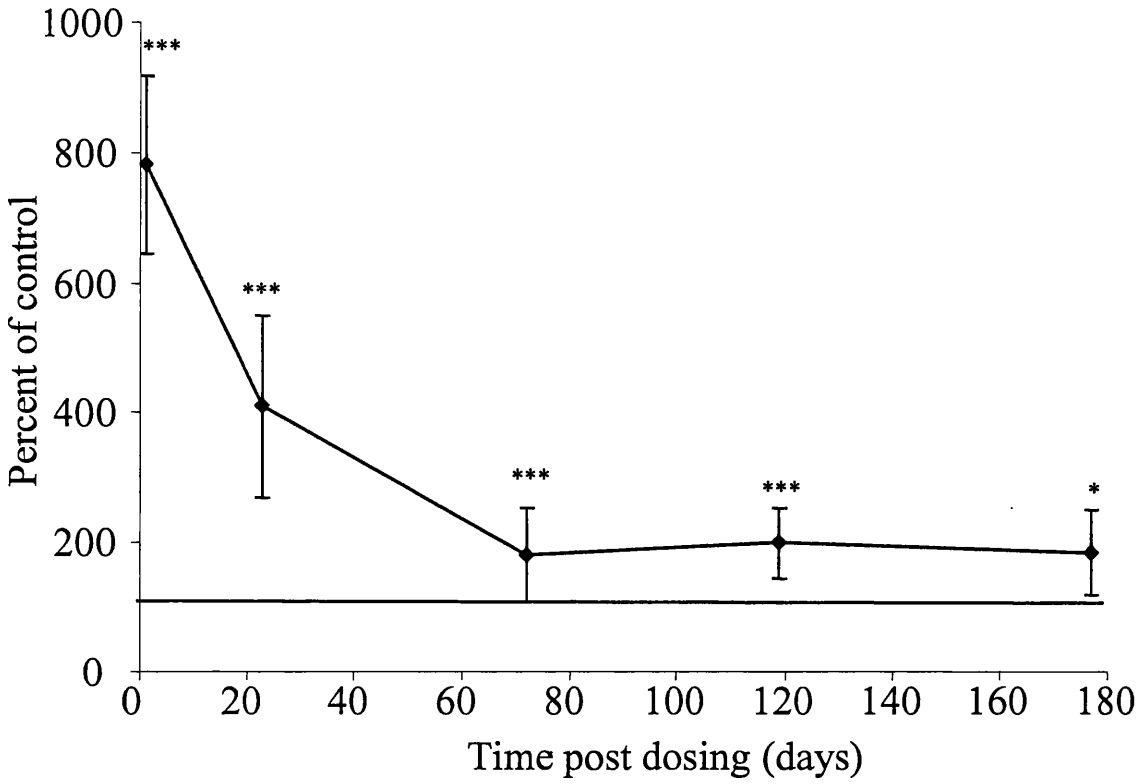
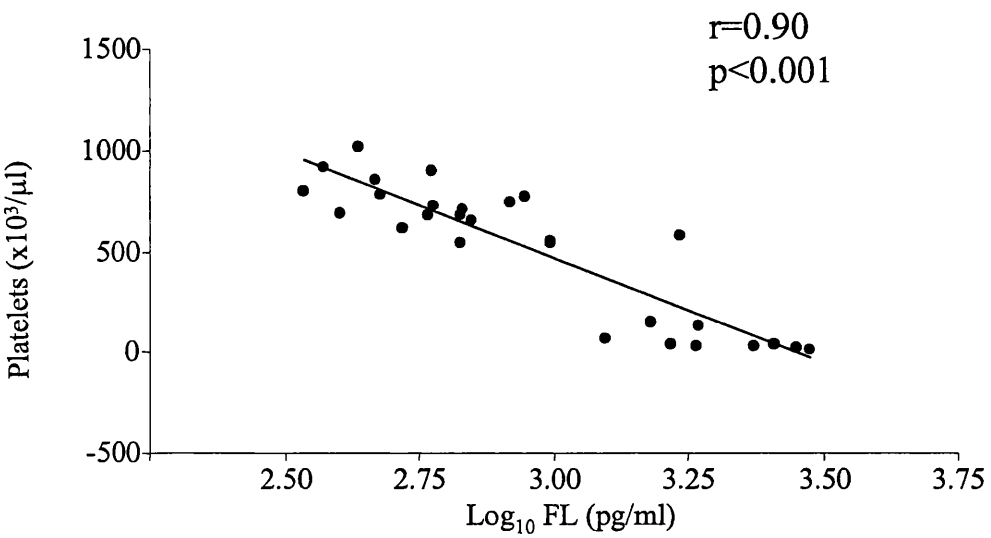
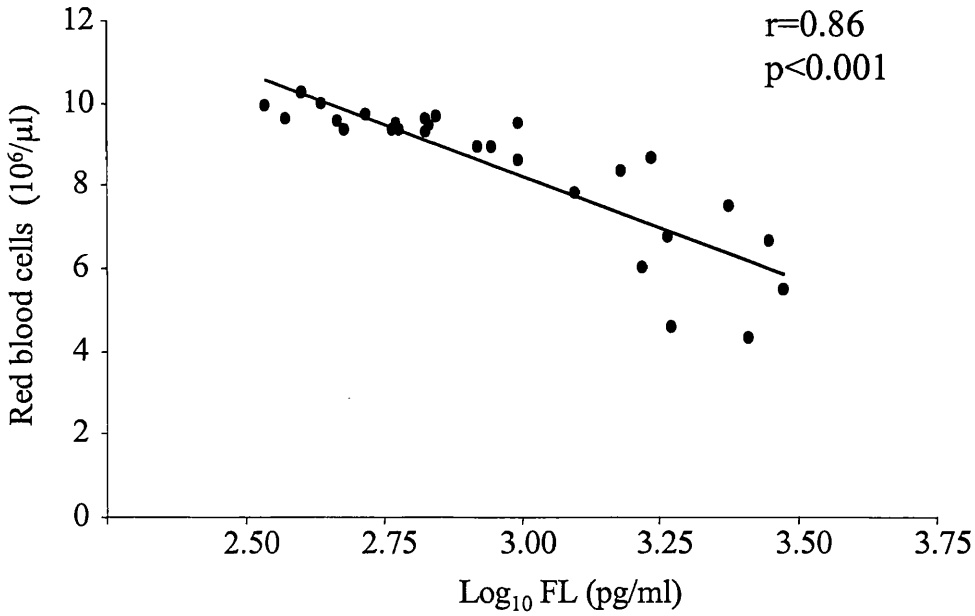


FIGURE 3.4. Serum FL results from female CD-1 mice treated with busulphan (BU) and sampled on days 1 to 177 post dosing. Values are means (± SD), expressed as a percentage of the mean control value in animals sampled at the same time points. n=6 animals per group for BU-treated mice except on day 177 where n=4. n=7 for vehicle-treated animals except on days 1, 119 and 177 where n=6, 4 and 5, respectively. *Significantly different from controls, $p<0.05$; *** $p<0.001$.

A


r=0.90
p<0.001
1500
1000
500
0
-500
Platelets (x10³/µl)
2.50
2.75
3.00
3.25
3.50
3.75
Log10 FL (pg/ml)

B


r=0.86
p<0.001
12
10
8
6
4
2
0
Red blood cells (10⁶/µl)
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2.75
3.00
3.25
3.50
3.75
Log10 FL (pg/ml)

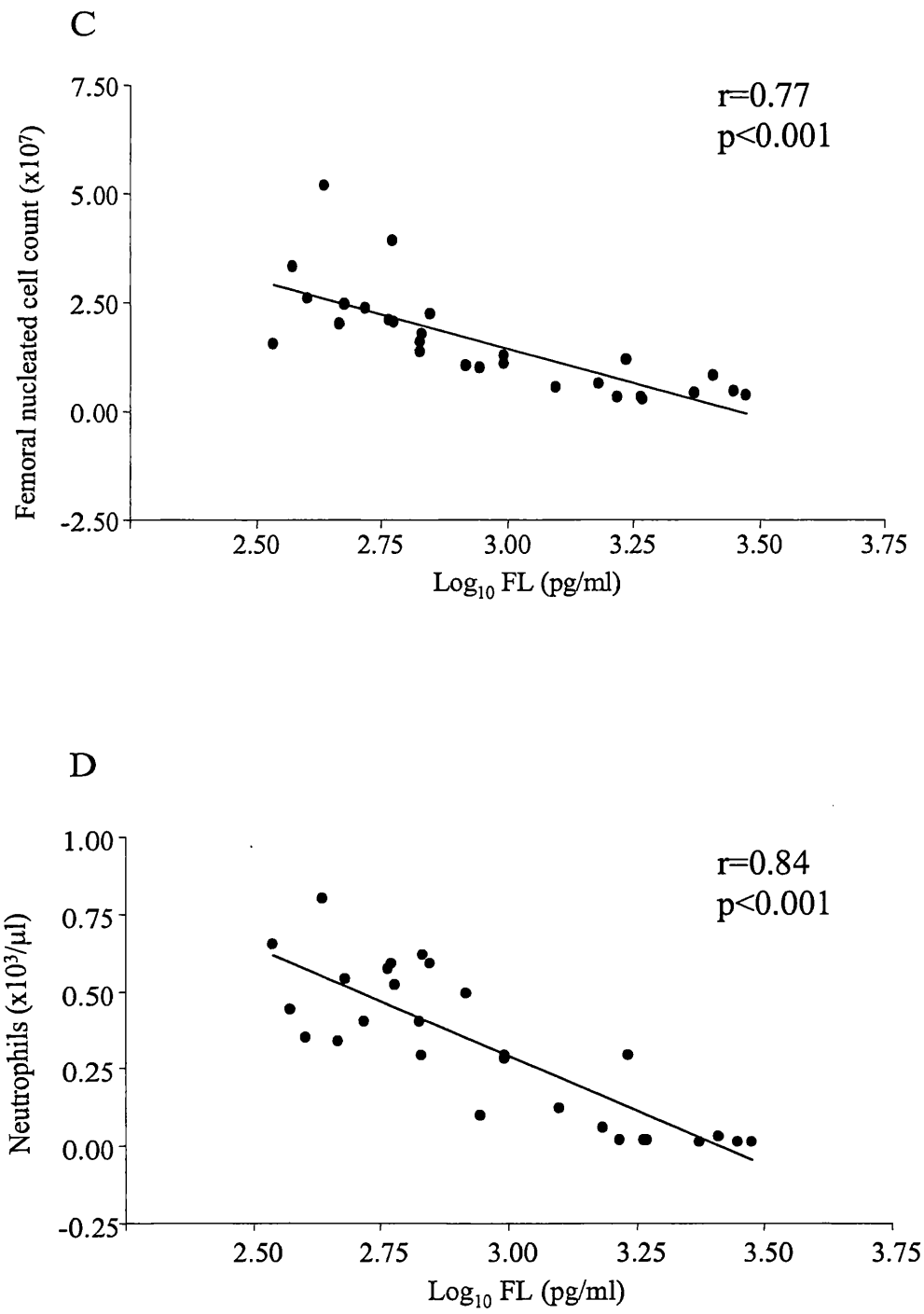


FIGURE 3.5. Scattergrams of serum FL concentration and haematological parameters. Linear regression analysis and correlation coefficients of serum FL concentration (pg/ml) and A, platelet count (10^3/μl); B, red blood cells (10^6/μl); C, femoral nucleated cell count (10^7); D, neutrophils (10^3/μl). Values are of $\log_{10}$ FL concentration and peripheral blood counts of BU-treated mice sampled on days 1, 23, 72, 119 and 177 post dosing.

TABLE 3.1. Haematological results[a] from female CD-1 mice treated with 10 doses of busulphan (BU) over a period of 21 days and sampled at 1 to 177 days after the final dose[b]

	Day of sampling									
	1		23		72		119		177	
	Control	BU	Control	BU	Control	BU	Control	BU	Control	BU
RBC	9.83 (0.36)	6.10 (1.12)***	10.15 (0.14)	7.88 (1.68)***	10.02 (0.29)	9.41 (0.27)***	9.99 (0.19)	9.70 (0.33)*	10.31 (0.22)	9.51 (0.42)***
Hb	16.0 (0.6)	10.4 (1.9)***	16.4 (0.4)	14.6 (2.6)*	16.0 (0.4)	15.3 (0.3)***	15.0 (0.7)	14.7 (0.5)	15.0 (0.5)	14.1 (0.5)**
HCT	48.1 (1.7)	29.6 (5.6)***	48.6 (1.3)	42.6 (7.6)*	47.6 (1.3)	45.0 (0.4)***	47.1 (1.1)	45.7 (1.7)*	48.1 (1.3)	45.5 (1.3)**
MCV	49.0 (1.6)	48.6 (0.5)	47.9 (1.7)	54.6 (3.0)***	47.5 (0.5)	47.9 (1.2)	47.1 (0.2)	47.1 (0.4)	46.7 (0.6)	47.9 (1.0)**
MCH	16.3 (0.5)	17.1 (0.1)***	16.2 (0.5)	18.6 (1.0)***	15.9 (0.1)	16.2 (0.3)**	15.0 (0.5)	15.1 (0.2)	14.5 (0.2)	14.8 (0.5)
MCHC	33.0 (0.4)	35.2 (0.3)***	33.7 (0.7)	34.1 (0.4)	33.6 (0.4)	33.9 (0.5)	31.8 (1.0)	32.2 (0.6)	311.3 (3.8)	309.3 (5.6)
Retic	339 (114)	65 (21)***	340 (42)	365 (125)	343 (72)	301 (39)	407 (57)	393 (39)	318 (74)	301 (80)
Plt	908 (101)	30 (10)***	1,040 (80)	335 (245)***	1,030 (86)	700 (111)***	1,010 (105)	708 (49)***	1,084 (116)	900 (103)**
WBC	1.67 (0.55)	0.35 (0.26) ***	2.44 (0.94)	0.87 (0.30)***	2.20 (0.79)	1.67 (0.39)	2.62 (1.48)	1.93 (0.49)	2.30 (0.58)	1.54 (0.96)*
Neut	0.39 (0.12)	0.02 (0.01)***	0.43 (0.18)	0.18 (0.13)**	0.51 (0.20)	0.44 (0.12)	0.45 (0.16)	0.52 (0.12)	0.45 (0.14)	0.48 (0.30)
Lymph	1.22 (0.42)	0.33 (0.25)***	1.93 (0.78)	0.65 (0.24)***	1.57 (0.59)	1.07 (0.41)*	1.98 (1.26)	1.32 (0.44)	1.68 (0.54)	0.93 (0.64)*
Mono	0.02 (0.01)	0.00 (0.00)***	0.04 (0.02)	0.01 (0.01)**	0.04 (0.03)	0.02 (0.01)**	0.04 (0.02)	0.02 (0.02)	0.07 (0.02)	0.06 (0.03)
Eo	0.02 (0.01)	0.00 (0.00)**	0.03 (0.05)	0.01 (0.03)	0.07 (0.06)	0.13 (0.01)	0.12 (0.11)	0.06 (0.07)	0.11 (0.03)	0.07 (0.02)**
Baso	0.00 (0.01)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.01 (0.01)	0.00 (0.01)
FNCC	2.17 (0.30)	0.44 (0.20)***	1.72 (0.35)	0.88 (0.48) ***	2.68 (0.56)	1.88 (0.58)**	2.50 (0.82)	1.93 (0.46)	4.64 (0.45)	3.35 (1.75)
Spleen	5,437 (541)	3,853 (403)***	5,319 (589)	5,807 (1,412)	5,583 (691)	5,337 (477)	5,275 (509)	4,666 (455)*	4,784 (493)	4,425 (328)

[a] Values are means, SD in parenthesis; n=8 for control and n=6 for BU groups at all time points, except on day 72 and 119 where n=7 (control), and day 177 where n=9 (controls) and n=4 (BU).

[b]Abbreviations and units: RBC, red blood cells, $x10^6/\mu l$; Hb, haemoglobin, g/dl; HCT, haematocrit, %; MCV, mean cell volume, fl; MCH, mean cell haemoglobin, pg; MCHC, mean cell haemoglobin concentration, g/dl; Retic, absolute reticulocyte count, $x10^3/\mu l$; Plt, platelets, $x10^3/\mu l$; WBC, white blood cells, $x10^3/\mu l$; Neut, neutrophils, $x10^3/\mu l$; Lymph, lymphocytes, $x10^3/\mu l$; Mono, monocytes, $x10^3/\mu l$; Eo, eosinophils, $x10^3/\mu l$; Baso, basophils, $x10^3/\mu l$; FNCC, femoral bone marrow nucleated cell count, $x10^7$; Spleen, relative spleen weight, mg/kg body weight. *Significantly different from controls, $p<0.05$; **$p<0.01$; *** $p<0.001$.

TABLE 3.2. Linear regression analysis and correlation coefficients of $\log_{10}$ FL and haematological parameters in busulphan-treated mice autopsied on day 1, 23, 72, 119 and 177 post dosing[a]

Parameter	Equation	r
Plt	y= -1,046.70x + 3,607.80	0.90***
RBC	y= -5.05x + 23.40	0.86***
Neut	y= -0.71x + 2.41	0.84***
FNCC	y= -3.15x + 10.90	0.77***
WBC	y= -1.99x + 7.14	0.76***
CFU-C/femur	y= -13,729x + 46,058	0.73***
MCHC	y= 3.76x + 22.30	0.71***
Hb	y= -5.60x + 30.35	0.70***
MCH	y= 3.43x + 6.34	0.66***
Mono	y= -0.05x + 0.16	0.66***
Lymph	y= -1.10x + 4.11	0.62***
Retic	y= -292.78x + 1,150.60	0.61***
Eo	y= -0.11x + 0.38	0.46***
MCV	y= 4.63x + 35.55	0.42*

[a]Abbreviations and units: as Table 3.1. FL data was converted logarithmically to linearise data for linear regression analysis. *Significant correlation, $p<0.05$; *** $p<0.001$.

TABLE 3.3. Haematological results[a] from female CD-1 mice treated with 8 doses of busulphan (BU) over a period of 21 days and killed *in extremis* on days 43 and 55 after the final dose[b]

	1	2	3	4	5	6	7	8	Mean	Control mean
RBC	4.58	2.74	4.88	5.66	1.82	2.19	1.30	5.44	3.58 (1.75)***	10.53 (0.41)
Hb	8.7	5.0	11.0	9.8	3.2	3.8	2.4	9.4	6.6 (3.4)**	16.7 (0.6)
HCT	25.6	14.9	26.1	29.0	9.3	10.6	7.5	28.5	18.9 (9.2)**	48.6 (2.2)
MCV	55.9	54.3	53.6	51.3	51.3	48.4	57.6	52.4	53.1 (2.9)***	46.2 (1.1)
MCH	19.0	18.2	22.6	17.3	17.7	17.3	18.8	17.3	18.5 (1.8)***	15.9 (0.2)
MCHC	33.9	33.5	42.2	33.8	34.5	35.7	32.7	33.1	34.9 (3.1)	34.3 (0.7)
Retic	43	54	-[c]	33	10	11	80	156	55 (50)***	307 (34)
Plt	56	20	51	24	29	31	37	55	38 (14)***	956 (110)
WBC	0.23	0.25	0.40	0.25	0.24	0.16	0.41	0.69	0.33 (0.17)***	2.21 (1.41)
Neut	0.04	0.08	0.05	0.04	0.01	0.02	0.04	0.05	0.04 (0.02)***	0.39 (0.13)
Lymph	0.19	0.16	0.31	0.21	0.22	0.13	0.35	0.61	0.27 (0.15)**	1.70 (1.30)
Mono	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00 (0.00)***	0.04 (0.02)
Eo	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.00	0.00 (0.00)	0.00 (0.00)
Baso	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00 (0.00)*	0.06 (0.07)
FNCC	0.20	0.11	0.23	0.22	0.09	0.11	0.14	0.10	0.15 (0.06)***	1.88 (0.18)
Spleen	4,412	3,667	5,500	3,294	2,611	3,056	1,778	4,333	3,581 (1,165)*	5,233 (1,201)

[a] Individual haematology results (with mean and SD) from animals treated with BU at 9.0 mg/kg and killed *in extremis* on days 43 and 55 post dosing. Animals numbered 1-4 were sampled on day 43 post dosing and animals numbered 5-8 were sampled on day 55 post dosing. n=8 for control treated animals (n=4 control mice autopsied at each time point).

[b] Abbreviations and units: as Table 3.1. *Significantly different from controls, $p<0.05$; **$p<0.01$; *** $p<0.001$.

[c] Insufficient sample volume.

TABLE 3.4. Individual haematological results[a] from 2 female CD-1 mice treated with 10 doses of busulphan (BU) over a period of 21 days and killed *in extremis* on day 67 after the final dose[b]

	1	2	Mean	Control mean
RBC	3.36	4.06	3.71 (0.50)***	9.97 (0.55)
Hb	5.9	7.1	6.5 (0.9)***	15.7 (1.1)
HCT	21.1	25.1	23.1 (2.8)***	46.0 (2.5)
MCV	62.8	61.7	62.2 (0.8)***	46.2 (0.3)
MCH	17.4	17.4	17.4 (0.0)***	15.8 (0.3)
MCHC	27.7	28.3	28.0 (0.7)***	34.2 (0.5)
Retic	586	833	710 (174)	469 (137)
Plt	341	1,674	1,008 (943)	1,015 (176)
WBC	23.26	13.05	18.16 (7.22)***	2.61 (0.34)
Neut	16.80	8.43	12.62 (5.92)**	0.73 (0.13)
Lymph	6.15	4.39	5.27 (1.25)**	1.70 (0.37)
Mono	0.18	0.07	0.13 (0.08)*	0.05 (0.01)
Eo	0.06	0.03	0.05 (0.02)	0.09 (0.05)
Baso	0.07	0.13	0.10 (0.04)**	0.00 (0.01)
FNCC	1.72	2.19	2.00 (0.30)	2.16 (0.38)
Spleen	29,389	37,857	33,623 (5,988)	5,116 (392)

[a] Individual haematology results (with mean and SD) from 2 animals treated with BU at 9.0 mg/kg and killed *in extremis* on day 67 post dosing. n=4 for vehicle-treated control animals autopsied at the same time point.

[b]Abbreviations and units: as Table 3.1. *Significantly different from controls, $p<0.05$; **$p<0.01$; *** $p<0.001$.

CHAPTER 4: The effect of administering the immunosuppressant agent cyclosporin A to mice with busulphan-induced chronic bone marrow aplasia

4.1. INTRODUCTION

The model of chronic bone marrow aplasia (CBMA) in the busulphan- (BU-) treated mouse described by Gibson *et al.* (2003), and later modified by Turton *et al.* (2006) shows many of the characteristics of aplastic anaemia (AA) in man. In this mouse model, immediately following treatment with BU, animals develop an initial phase of bone marrow hypoplasia accompanied by peripheral blood pancytopenia. The condition of the BU-treated mice then improves with many bone marrow and peripheral blood parameters returning towards control values. However, from approximately 50 days post dosing, bone marrow parameters and peripheral blood counts of BU-treated mice remain stable, but at reduced levels in comparison with vehicle-treated control animals. During this stabilised 'plateau phase', BU-treated mice were described as having chronic bone marrow aplasia (CBMA) with characteristic changes comparable to AA in man, i.e. a reduction in bone marrow cellularity, peripheral blood counts, committed haemopoietic progenitor cells, and an increase in apoptotic cell death in the bone marrow.

It is possible that the model of BU-induced CBMA described by Gibson *et al.* (2003) and Turton *et al.* (2006) would be useful in assessing new or existing therapeutic interventions, and therefore, assist in the development of improved treatment regimens for AA patients. Therefore, to investigate the possible usefulness of the model in assessing therapeutic interventions we wished to examine the effects of administering the

immunosuppressant agent cyclosporin A (CsA) to mice following treatment with BU. The drug CsA was selected due to its widespread current use in the treatment of AA. Accordingly, in the main study, female BALB/c mice were treated with 10 doses of BU at 9.0 mg/kg over a 21 day period. On day 57 post dosing (i.e. during the stabilised 'plateau phase'), animals were treated with CsA daily by gavage at 30 mg/kg for 35 days. However, prior to carrying out this experiment, a preliminary dose response study was performed in the CD-1 mouse to assess the toxicity of CsA given daily over 34 days, to identify an appropriate dose of CsA to be used in the main study.

The main aim of this study was to assess the effects of CsA treatment on the bone marrow changes occuring in response to BU treatment.

4.2. MATERIALS AND METHODS

4.2.1. Experimental design

4.2.1.1. Preliminary cyclosporin A dose response study

CsA was prepared (as described in Chapter 2.2) and administered to female CD-1 mice (mean body weight 35.0 g) at 15, 20, 25, 30, 35, 40 or 45 mg/kg (n=5 per group). Animals were treated daily by gavage for 34 days with vehicle (vegetable oil; controls) or CsA at a dose volume of 0.1 to 0.6 ml/mouse. On day 1 post dosing, all animals were autopsied for blood and bone marrow investigations (as described in Chapter 2.3).

4.2.1.2. Main busulphan-cyclosporin A study

230 female BALB/c mice (Charles River UK Ltd; mean body weight 16.0 g) were divided into 2 groups (Fig. 4.1), control (vehicle-treated; n=111; Group A) and BU-treated (n=119; Group B). Mice were treated with 10 doses of vehicle (vehicle: acetone) or BU (9.0 mg/kg) by intraperitoneal injection over a 21 day period (days 1, 3, 5, 8, 10, 12, 14, 17, 19 and 21). On day 57 post dosing, both the vehicle-treated group (Group A) and the BU-treated group (Group B) were further divided into 2 groups, to give Groups A1, A2, B1 and B2. Group A1 was treated with vehicle (vehicle-vehicle, n=51); Group A2 was treated with CsA (vehicle-CsA, n=48); Group B1 was treated with vehicle (BU-vehicle, n=50); Group B2 was treated with CsA (BU-CsA, n=54). CsA was dosed at 30 mg/kg, daily by gavage, for 35 days; mice not dosed with CsA received vehicle (vegetable oil). At weekly intervals, mice were weighed and the dose of CsA adjusted to accommodate any body weight

changes. On day 1 and 57 post BU dosing (Fig. 4.1), and on days 7, 14 and 21 of CsA treatment, and on days 1, 8, 15, 57 and 113 post CsA dosing, animals (n=4 to 9 per group) were killed for blood and bone marrow investigations (as described in Chapter 2.3).

4.2.2. Processing of samples

4.2.2.1. Preliminary cyclosporin A dose response study

In control (vehicle-treated) and CsA-treated mice (15 to 45 mg/kg CsA) a full blood count was performed and a femoral marrow flush into 5 ml phosphate buffered saline (PBS) was used to measure the femoral nucleated cell count (FNCC) (as described in Chapter 2.4). A tibial marrow smear was also prepared, stained and a differential count performed (as described in Chapter 2.5). The second femur was flushed into 5 ml Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% foetal calf serum (FCS) and used to assess apoptosis (as described in Chapter 2.7). At autopsy the spleen, liver and both kidneys were removed, weighed and placed in fixative with the sternum (as described in Chapter 2.9).

4.2.2.2. Main busulphan-cyclosporin A study

A full blood count was performed and a femoral marrow flush into 5 ml IMDM prepared to measure the bone marrow cellularity (FNCC) (as described in Chapter 2.4). The second femur was placed in 5 ml IMDM supplemented with 10% FCS and was used to measure the clonogenic potential of the bone marrow and to assess levels of apoptosis (as described in Chapter 2.6 and 2.7). Serum was prepared and used to measure the

concentration of the serum cytokine fms-like tyrosine kinase 3 (FLT-3) ligand (FL) (as described in Chapter 2.8). At each autopsy the spleen, liver, both kidneys and thymus were removed, weighed and placed in fixative with the sternum (as described in Chapter 2.9).

4.2.3. Statistical analysis

Results from the Preliminary CsA dose response study were analysed using a one-way analysis of variance (ANOVA) followed by Tukey's highest significance test for post hoc pairwise multiple comparison. In cases of violation of the assumptions for parametric testing, the Kruskal-Wallis test was used in combination with Dunn's post-test. Statistical analysis was performed using GraphPad Prism version 4.00 for windows (GraphPad Software, San Diego, California, USA).

In the Main BU-CsA study, BU-vehicle (Group B1), vehicle-CsA (Group A2) and BU-CsA (Group B2) groups were compared to mice treated with vehicle-vehicle (Group A1) using a one tailed Student's t-test (Microsoft Excel; Microsoft Ltd, Microsoft UK, Reading UK). To compare the effects of CsA administration, mice treated with BU-vehicle (Group B1) were compared to mice treated with BU-CsA (Group B2) using a one tailed Student's t-test.

4.3. RESULTS

4.3.1. Preliminary cyclosporin A dose response study

4.3.1.1. Body weight changes and clinical signs

CD-1 mice were treated by gavage with vegetable oil (vehicle control) or CsA at 15 to 45 mg/kg for 34 days. Animals in all groups remained healthy throughout the course of the experiment. Body weights were determined weekly and these showed no evidence of reductions in response to CsA treatment.

One animal dosed at 30 mg/kg CsA was found dead on day 25 of dosing; this death was thought to be the result of a dosing accident. One mouse in the 45 mg/kg group developed a swelling in the neck after 10 doses of CsA. This mass was removed at the scheduled post mortem examination on day 1 post CsA dosing and upon histological examination was identified as an abscess.

4.3.1.2. Haematology results

CsA administration appeared to have a minimal effect on the peripheral blood counts (Table 4.1). Indeed, no evidence of clear or consistent dose-related effects were seen, with very few changes being statistically significant.

4.3.1.3. Femoral nucleated cell count

The femoral nucleated cell count (FNCC) of CsA-treated animals was increased in mice treated with CsA at 20, 25, 30, 35, 40 and 45 mg/kg. These increases in bone marrow cellularity were however only statistically significant in the 45 mg/kg group ($p<0.01$) (Table 4.1).

4.3.1.4. Apoptosis

The levels of apoptosis in the bone marrow of CsA-treated mice were not significantly different from control (vehicle-treated) mice (data not shown).

4.3.1.5. Bone marrow differential cell counts

Results are shown on Table 4.2. The myeloid cell count was increased in all CsA-treated groups in comparison with the controls, however, this increase was only statistically significant in mice treated with 45mg/kg CsA. Erythroid and lymphoid lineages were generally not affected by CsA dosing.

4.3.1.6. Histopathological assessment of tissues

No treatment related changes were observed in the kidneys or liver of CsA-treated mice, apart from a slight reduction in glycogen vacuolation in the latter tissue. A slight depletion of the white pulp was seen in the spleen (in the periarteriolar lymphoid sheath) of animals treated with 35 and 45 mg/kg CsA. Some animals in the higher dose groups

appeared to have areas of reduced cellularity within the sternal bone marrow, however, this was not a general trend in the CsA-treated animals.

4.3.2. Main busulphan- cyclosporin A study

4.3.2.1. Body weight changes and clinical signs

On day 1 of the BU dosing period, the mean body weights of vehicle (n=111) and BU-treated (n=119) mice were 15.5 g and 16.4 g, respectively (Fig. 4.1). Over the 21 day dosing period, the mean body weight of mice treated with vehicle increased to 18.6 g (a 20.0% increase); however, the mean body weight of BU-treated mice increased by only 12.2%, to 18.4 g. During the BU dosing period, all animals maintained a healthy condition and there was no mortality. However, in the immediate post BU dosing period (days 1, 3 and 7), 3 BU-treated mice died unexpectedly (animals found dead (FD) or were killed *in extremis* (KIE). These will be referred to an 'inter-current' death (ICD) animals).

During the 35 day CsA dosing period, the mean body weight of vehicle-vehicle mice (Group A1) and BU-vehicle treated mice (Group B1) increased by 3.2% (from 22.0 g to 22.7 g), and 2.3% (from 21.7 g to 22.2 g), respectively. Animals treated with vehicle followed by 35 daily doses of CsA at 30 mg/kg (vehicle-CsA; Group A2) gained slightly more weight than vehicle-vehicle (Group A1) treated mice (a 4.9% increase, from 20.3 g to 21.3 g). However, the greatest increase in mean body weight was seen in BU-treated animals receiving CsA daily (BU-CsA; Group B2); in this group, the mean body weight increased from 19.9 g to 21.5 g (an 8.0% increase). In the period post CsA dosing, all groups of mice continued to gain weight at approximately the same rate as Group A1 (vehicle-vehicle) animals.

During the CsA dosing period, a total of 9 ICD mice were identified, 4 were from Group A1 (vehicle-vehicle treated mice), 1 was from Group A2 (vehicle-CsA treated mice) and 4 were from Group B2 (BU-CsA treated mice). Following CsA dosing, a further 3 ICD mice were identified, 1 from Group B1 (BU-vehicle) and 2 from Group B2 (BU-CsA). Therefore, from the 230 mice used in this experiment a total of 15 ICD animals were recorded resulting in a total mortality of 6.5%.

4.3.2.2. Haematology results

Post busulphan dosing period

Mice were sampled on day 1 and 57 post BU dosing (Fig. 4.1). On day 1 following the administration of BU at 9.0 mg/kg on 10 occasions over 21 days, mice treated with the drug displayed evidence of significant anaemia, leucopenia and thrombocytopenia (Table 4.3). The RBC, Hb and HCT were significantly reduced to 82.2%, 85.4% and 82.3% of the control mean, respectively ($p<0.001$ for each parameter) together with a significant increase in MCH and MCHC. The reticulocyte count of BU-treated mice was comparable to control values. All peripheral blood leucocytes were significantly reduced in BU-treated mice on day 1 post BU dosing with WBC, neutrophil, lymphocyte, monocyte and eosinophil counts being 23.3%, 14.0%, 25.1%, 0.0% and 30.0% of the vehicle (control) mean, respectively ($p<0.001$ for each parameter). The platelet count was reduced significantly to 25.5% of the control mean ($p<0.001$).

The majority of peripheral blood parameters in BU-treated mice were comparable to control values on day 57 post BU dosing, however, the platelet and eosinophil counts

continued to be significantly reduced to 65.6% ($p<0.01$) and 71.4% ($p<0.05$) of the control mean, respectively (Table 4.3).

Cyclosporin A dosing period

Vehicle-cyclosporin A treated mice (Group A2): On day 7 of CsA treatment, vehicle-treated mice given CsA daily at 30 mg/kg (Group A2) had significant reductions in the WBC, lymphocyte and monocyte counts, in comparison with Group A1 (vehicle-vehicle) (Table 4.4). The HCT and MCV were also significantly reduced and the reticulocyte count and MCHC were significantly increased.

After 14 days of CsA treatment, the lymphocyte count of Group A2 mice continued to be significantly reduced. At this time the reticulocyte count continued to be significantly increased, in addition to the MCV. All other peripheral blood parameters were comparable to animals in Group A1 (vehicle-vehicle) (Table 4.4).

On day 21 of CsA treatment, the WBC, lymphocyte, monocyte and basophil counts were significantly reduced in Group A2 (vehicle-CsA) animals, with all other parameters being comparable to the mice in Group A1 (vehicle-vehicle).

Busulphan-vehicle treated mice (Group B1): At autopsy on day 7 of the CsA dosing period, the RBC count, Hb, HCT and MCV of mice in Group B1 were comparable to the values of mice in Group A1 (vehicle-vehicle) (Table 4.4). However, the MCH and MCHC were significantly increased in Group B1 mice. The total leucocyte count was reduced in BU-vehicle treated mice to 54.9% of the mice in Group A1 ($p<0.01$). The reduction in total leucocyte count at this time point was due to a reduction in neutrophils, lymphocytes and monocytes (these parameters being 56.3%, 54.4% and 42.9% of the mice in Group A1

(vehicle-vehicle), respectively). The platelet count also continued to be reduced on day 7 of CsA treatment ($p<0.001$).

The peripheral blood counts of BU-vehicle treated mice (Group B1) were, in general, comparable to animals in Group A1 (vehicle-vehicle) on days 14 and 21 of CsA treatment (Table 4.4). However, the significant reduction in platelets ($p<0.001$), and monocytes ($p<0.05$) continued.

Busulphan-cyclosporin A treated mice (Group B2): The peripheral blood counts of mice treated with BU-CsA (Group B2) at autopsy on day 7 of CsA treatment were in general comparable to mice treated with BU-vehicle (Group B1) (Table 4.4). However, the reticulocyte count was significantly increased and the MCV significantly decreased in Group B2 (BU-CsA) mice in comparison with Group B1 (BU-vehicle).

On day 14 of CsA treatment, the majority of peripheral blood parameters in Group B2 (BU-CsA) animals continued to be comparable to BU-vehicle treated mice (Group B1). However, the Hb and HCT were significantly lower in BU-CsA mice (Group B2) compared to BU-vehicle (Group B1) animals (Table 4.4).

On day 21 of CsA treatment, in comparison to BU-vehicle treated mice (Group B1), the WBC and lymphocyte counts of BU-CsA treated mice (Group B2) were significantly reduced ($p<0.05$, $p<0.01$ respectively; Table 4.4).

Cyclosporin A post dosing period

Vehicle-cyclosporin A treated mice (Group A2): On day 1 after CsA dosing (Table 4.5), the significant reduction in the lymphocyte count observed in vehicle-CsA (Group A2) treated mice during CsA treatment (Table 4.4), was still evident. Additionally, at this time

point, the neutrophil and reticulocyte counts in Group A2 mice were significantly increased (Table 4.5).

In general, on days 8, 15 and 57 after the final dose of CsA, all peripheral blood parameters in vehicle-CsA treated mice (Group A2) were comparable to animals in the vehicle-vehicle (Group A1) group, except for a reduction in the monocyte count on day 8 and 15 after CsA treatment (Table 4.5, Table 4.6).

At the final autopsy, on day 113 after CsA treatment, an increase in Hb, HCT and MCV was evident in vehicle-CsA treated mice (Group A2), in comparison with vehicle-vehicle (Group A1) mice. All other peripheral blood parameters in Group A2 mice were, in general, comparable to animals in Group A1.

Busulphan-vehicle treated mice (Group B1): In comparison with Group A1 (vehicle-vehicle) animals, on day 1 following CsA treatment (Table 4.5), the RBC, platelet and monocyte counts were significantly reduced in Group B1 mice but there were also significant increases in values for MCV, MCH and reticulocytes. However, on day 8 post CsA dosing, all peripheral blood parameters in Group B1 mice (except monocytes) were comparable to the results from Group A1 animals.

On day 15 post CsA treatment, the RBC and platelet counts were significantly reduced in Group B1 mice with MCV and MCH being significantly increased (in comparison with Group A1 animals). Leucocyte parameters, namely WBC, lymphocyte and monocyte counts, were also significantly reduced in Group B1 animals. These changes in the peripheral blood of Group B1 BU-treated mice continued on day 57 post CsA dosing (Table 4.6). However, the reductions in the leucocyte parameters were more pronounced at this time point and included effects on the WBC, neutrophils, lymphocytes, monocytes, eosinophils and basophils.

Leucocyte parameters in Group B1 mice were comparable to animals in Group A1 on day 113 post CsA dosing. However, reductions continued in the erythrocyte parameters (RBC, Hb, HCT and MCHC) and also in the platelet count (Table 4.6).

Busulphan-cyclosporin A treated mice (Group B2): On day 1 after the final CsA dose, a significant reduction continued (Table 4.5) in the lymphocyte count of mice treated with BU-CsA (Group B2) in comparison to mice treated with BU-vehicle (Group B1).

On day 8 post CsA treatment, the platelet count of Group B2 mice continued to be significantly reduced in comparison with Group B1 animals (Table 4.5). At this time, leukocyte parameters (WBC, neutrophils, lymphocytes, monocytes and eosinophils) were significantly increased in BU-CsA (Group B2) animals in comparison with values seen in BU-vehicle (Group B1) animals. All other parameters were comparable to values seen in mice treated with BU-vehicle (Group B1).

On day 15 post CsA dosing, the majority of peripheral blood parameters in BU-CsA mice (Group B2) were comparable to values in BU-vehicle mice (Group B1), except for a significant increase in the reticulocyte count in Group B2 animals ($p<0.05$).

The peripheral blood counts of mice treated with BU-CsA (Group B2) continued to be comparable to values seen in BU-vehicle treated mice (Group B1) on day 57 post CsA dosing (Table 4.6). However, on day 113 post CsA dosing, the RBC, Hb and HCT were significantly increased and monocytes significantly reduced in BU-CsA treated mice (Group B2), in comparison with Group B1 animals (Table 4.6).

4.3.2.3. Femoral nucleated cell count

Post busulphan dosing period

The FNCC was significantly reduced in BU-treated mice (Group B) immediately after BU treatment (day 1 post dosing), to 45.4% of the mean value in Group A (vehicle-treated mice) ($p<0.001$; Table 4.3), before returning to values which were approximately comparable to the vehicle-treated mice (Group A) on day 57 post dosing.

Cyclosporin A dosing period

Vehicle-cyclosporin A treated mice (Group A2): On day 7 of CsA treatment, the FNCC of mice treated with vehicle followed by CsA (Group A2) was comparable to mice treated with vehicle-vehicle (Group A1). However, on days 14 and 21 of CsA treatment, the FNCC of Group A2 mice was reduced to 75.6% ($p<0.05$) and 81.6% ($p<0.05$) of the values in Group A1 (vehicle-vehicle), respectively (Table 4.4).

Busulphan-vehicle treated mice (Group B1): On day 7 and 14 of the CsA dosing period, the FNCC of mice treated with BU-vehicle (Group B1) was comparable to mice treated with vehicle-vehicle (Group A1). However, the mean FNCC of Group B1 mice was reduced on day 21 of CsA dosing to 89.6% of the mean value of Group A1 animals ($p<0.05$; Table 4.4).

Busulphan-cyclosporin A treated mice (Group B2): Throughout the CsA dosing period the mean FNCC value of mice treated with BU-CsA (Group B2) was not changed in comparison with the mean values of mice treated with BU-vehicle (Group B1) (Table 4.4).

Cyclosporin A post dosing period

Vehicle-cyclosporin A treated mice (Group A2): In the period following CsA treatment, on days 1, 8, 15, 57 and 113, the FNCC of mice treated with vehicle-CsA (Group A2) was comparable to vehicle-vehicle (Group A1) animals, except on day 8 where the FNCC of vehicle-CsA treated mice (Group A2) was significantly increased to 119.9% of the mean value in comparison with vehicle-vehicle (Group A1) animals ($p<0.05$) (Table 4.5, Table 4.6).

Busulphan-vehicle treated mice (Group B1): On day 1 post CsA treatment (Table 4.5), the FNCC in BU-vehicle (Group B1) mice was comparable to mice in Group A1 (vehicle-vehicle). However, on day 8 of the post CsA dosing period the FNCC of Group B1 mice was significantly increased to 127.3% of the mean value for Group A1 animals ($p<0.05$) before returning to a value comparable to mice treated with vehicle-vehicle (Group A1) on day 15 post dosing (Table 4.5).

On day 57 post CsA dosing, the FNCC of Group B1 animals was significantly reduced (to 73.2% of the mean value of Group A1 mice; $p<0.01$) before returning to a value comparable to Group A1 mice on day 113 post dosing (Table 4.6).

Busulphan-cyclosporin A treated mice (Group B2): During the period post CsA treatment, the FNCC of BU-CsA treated mice (Group B2) was not statistically different from mice treated with BU-vehicle (Group B1) (Table 4.5, Table 4.6).

4.3.2.4. Bone marrow clonogenic assay

The number of CFU-GM and erythroid colonies were measured in the bone marrow of vehicle-treated (Group A) and BU-treated mice (Group B) on day 1 and 57 post BU dosing, and in mice treated with vehicle-vehicle (Group A1), vehicle-CsA (Group A2), BU-vehicle (Group B1) and BU-CsA (Group B2) on days 7, 14 and 21 of CsA treatment, and days 1, 8 and 15 post CsA treatment; CFU-GM and erythroid colonies were not studied on day 57 and on day 113 post CsA administration. Results are shown in (Table 4.7.).

4.3.2.4.1. CFU-GM

Post busulphan dosing period

On day 1 after BU treatment, the number of CFU-GM colonies were significantly reduced in BU-treated mice to 19.8% of the mean value in vehicle-treated mice ($p<0.001$) (Table 4.7.). This reduction in the number of CFU-GM was also evident on day 57 post dosing ($p<0.05$). However, there was some evidence of recovery and a return towards the values of vehicle-treated animals on day 57 post dosing.

Cyclosporin A dosing period

Vehicle-cyclosporin A treated mice (Group A2): A reduction in the number of CFU-GM colonies was evident in vehicle-CsA treated mice (Group A2) at autopsy on days 7, 14 and 21 of the CsA dosing period (Table 4.7) with values being 84.6% (NS), 82.9% ($p<0.05$), and 70.9% ($p<0.05$) of the vehicle-vehicle group (Group A1), respectively.

Busulphan-vehicle treated mice (Group B1): Mice treated with BU-vehicle (Group B1) during the CsA dosing period, continued to have a reduction in the number of CFU-GM colonies at autopsy on days 7, 14 and 21 of CsA treatment, with values being 71.5% ($p<0.05$), 84.0% (NS) and 70.4% ($p<0.05$) of the mean values of Group A1 mice, respectively.

Busulphan-cyclosporin A treated mice (Group B2): During the period of CsA treatment (days 7, 14 and 21) the number of CFU-GM colonies in the bone marrow of BU-CsA mice (Group B2) was significantly reduced to 39.6% ($p<0.001$), 45.1% ($p<0.001$) and 53.8% ($p<0.05$) of the mean value seen in the BU-vehicle mice (Group B1), respectively (Table 4.7).

Cyclosporin A post dosing period

Vehicle-cyclosporin A treated mice (Group A2): Vehicle-CsA mice (Group A2) continued to have a significantly reduced number of CFU-GM colonies on day 1, post CsA dosing, in comparison with the animals in Group A1 ($p<0.01$). However, counts were comparable to the values in Group A1 mice on days 8 and 15 post CsA dosing (Table 4.7).

Busulphan-vehicle treated mice (Group B1): In the period following CsA treatment, on days 1, 8 and 15, the number of CFU-GM colonies was reduced in the bone marrow of BU-vehicle treated mice (Group B1) in comparison with the animals in Group A1; however, this reduction was only statistically significant on day 1 post CsA dosing ($p<0.05$) (Table 4.7).

Busulphan-cyclosporin A treated mice (Group B2): In comparison to mice in Group B1 (BU-vehicle), BU-CsA mice (Group B2) had a reduced number of CFU-GM colonies per femur (Table 4.7). On days 1 and 8 post CsA dosing the number of CFU-GM colonies in Group B2 mice were reduced to 48.5% ($p<0.05$) and 48.4% ($p<0.05$) of the mean value in BU-vehicle mice (Group B1), respectively. Values in the BU-CsA mice were however, not statistically reduced in the BU-vehicle mice on day 15 post CsA dosing.

4.3.2.4.2. Erythroid colonies

Post busulphan dosing period

The number of erythroid colonies per femur was significantly reduced on day 1 post dosing in BU-treated mice to 15.2% of the mean value in vehicle-treated mice ($p<0.001$) (Table 4.7). This decrease in erythroid colony formation in BU-treated animals was also evident on day 57 post dosing ($p<0.05$), although there was evidence of recovery and a return towards the values of the vehicle-treated mice.

Cyclosporin A dosing period

Vehicle-cyclosporin A treated mice (Group A2): The number of erythroid colonies in the bone marrow of the femur of vehicle-CsA treated mice (Group A2) was not significantly different from the values of animals in Group A1 (vehicle-vehicle) on days 7 and 14 of CsA treatment (Table 4.7). However, on day 21 of CsA treatment the number of

erythroid colonies in the bone marrow of vehicle-CsA mice (Group A2) was significantly reduced, to 71.4% ($p<0.05$) of the vehicle-vehicle value of animals in Group A1.

Busulphan-vehicle treated mice (Group B1): A significant reduction in the number of erythroid colonies per femur was evident in BU-vehicle treated mice (Group B1) on days 7, 14 and 21 of CsA dosing, with counts being 54.7% ($p<0.01$), 65.6% ($p<0.05$) and 54.5% ($p<0.05$) of the mean value of vehicle-vehicle treated mice (Group A1), respectively (Table 4.7).

Busulphan-cyclosporin A treated mice (Group B2): In BU-CsA mice (Group B2), a significant reduction in the number of erythroid colonies was evident, in comparison with BU-vehicle mice (Group B1), on days 7, 14 and 21 of CsA treatment. Mean values in BU-CsA (Group B2) mice were reduced to 42.2% ($p<0.01$), 49.2% ($p<0.05$) and 58.2% (NS) of the mean value of BU-vehicle mice (Group B1), respectively (Table 4.7).

Cyclosporin A post dosing period

Vehicle-cyclosporin A treated mice (Group A2): Mice in the vehicle-CsA group (Group A2) had a significant reduction in the mean number of erythroid colonies per femur on day 1 post CsA dosing ($p<0.01$) in comparison with vehicle-vehicle (Group A1) mice (Table 4.7). On days 8 and 15 post CsA dosing however, the number of erythroid colonies in Group A2 mice were comparable to the mean value in vehicle-vehicle (Group A1) animals.

Busulphan-vehicle treated mice (Group B1): The number of erythroid colonies per femur in the BU-vehicle mice (Group B1) continued to be significantly reduced on day 1 post CsA dosing, to 58.3% ($p<0.01$), in comparison with the vehicle-vehicle (Group A1) mice (Table 4.7). The number of erythroid colonies in the bone marrow of BU-vehicle mice (Group B1) continued to be reduced below the values of Group A1 mice on day 8 and 15 post CsA treatment, however these reductions were not statistically significant.

Busulphan-cyclosporin A treated mice (Group B2): The mean number of erythroid colonies per femur in the bone marrow of BU-CsA mice (Group B2) were reduced below values seen in BU-vehicle (Group B1) mice on day 1, 8 and 15 post CsA dosing (Table 4.7). However, these reductions were only statistically significant on day 8 post CsA dosing ($p<0.05$).

4.3.2.5. Serum fms-like tyrosine kinase 3 (FLT-3) ligand

The concentration of serum FL was measured in vehicle-treated and BU-treated mice on day 1 and 57 post BU dosing, and in mice treated with vehicle-vehicle (Group A1), vehicle-CsA (Group A2), BU-vehicle (Group B1) and BU-CsA (Group B2) on day 14 of CsA treatment, and also on days 1, 15, 57 and 113 post CsA treatment. Results are shown as means with standard deviations in Table 4.8.

Post busulphan dosing period

On day 1 post dosing, serum FL was significantly increased in the BU-treated mice, to 451.6% of the mean value in the vehicle-treated animals ($p<0.01$) (Table 4.8). An

elevation in the concentration of serum FL was also seen on day 57 post BU dosing when the increase was to 144.4% of the value in the vehicle-treated animals ($p<0.001$). However, at this time point (57 days post BU dosing) there was evidence of a return towards the values seen in vehicle-treated animals.

Cyclosporin A dosing period

On day 14 of the CsA dosing period, the mean concentrations of FL in the serum of mice treated with vehicle-CsA (Group A2) and BU-vehicle (Group B1) were comparable to the mean value in the vehicle-vehicle group (Group A1) (Table 4.8). The mean concentration of serum FL in mice treated with BU-CsA (Group B2) was comparable also to the mean value in the BU-vehicle treated mice (Group B1).

Cyclosporin A post dosing period

The concentrations of serum FL in vehicle-CsA treated mice (Group A2) in the post CsA dosing period (days 1, 15, 57 and 113 post dosing) were not significantly different from the vehicle-vehicle mice (Group A1) (Table 4.8). However, on days 1, 15, and 57 post CsA dosing, serum FL levels in the BU-vehicle treated animals (Group B1) were significantly increased to 141.9% ($p<0.05$), 173.3% ($p<0.01$) and 188.4% ($p<0.05$) of the values in vehicle-vehicle (Group A1) animals, respectively. However, on day 113 post CsA dosing, the mean value in BU-vehicle treated mice (Group B1) was comparable to the mean level in Group A1 animals (Table 4.8).

On days 1, 15, 57 and 113 after the final CsA dose, serum FL was increased in BU-CsA treated mice (Group B2) in comparison with BU-vehicle treated mice (Group B1); however, these increases were not statistically significant.

4.3.2.6. Organ weights

4.3.2.6.1. Spleen weights

Post busulphan dosing period

On days 1 and 57 post BU dosing the mean relative spleen weights of mice treated with BU were comparable to vehicle-treated animals (Table 4.9).

Cyclosporin A dosing period

During the CsA dosing period (day 7, 14 and 21) the mean relative spleen weights of mice treated with vehicle-CsA (Group A2) and BU-vehicle (Group B1) were comparable to values in the vehicle-vehicle treated mice (Group A1) (Table 4.9). The mean relative spleen weights of mice treated with BU-CsA (Group B2) were comparable to values seen in mice treated with BU-vehicle (Group B1) during the CsA dosing period.

Cyclosporin A post dosing period

In the post CsA dosing period, the mean relative spleen weights of mice treated with vehicle-CsA (Group A2) were comparable to the mean value in the vehicle-vehicle treated

group (Group A1) except on day 113 post CsA dosing where a reduction in the mean relative spleen weight was evident (Table 4.9).

The mean relative spleen weights of mice treated with BU-vehicle (Group B1) were comparable to the spleen weights of mice in the vehicle-vehicle group (Group A1) on days 1, 8 and 15 post CsA treatment; however, on days 57 and 113 post dosing the mean relative spleen weight were significantly reduced ($p<0.001$ at each time point) in the BU-vehicle treated group (Group B1) in comparison with group A1 (vehicle-vehicle).

The mean relative spleen weights of mice treated with BU-CsA (Group B2) were comparable to values in BU-vehicle (Group B1) mice on days 1, 8, 15, 57 and 113 post CsA dosing.

4.3.2.6.2. Thymus weights

Post busulphan dosing period

The mean relative thymus weights of BU-treated mice was significantly reduced immediately post dosing, on day 1 ($p<0.001$) before returning to values comparable to vehicle-treated mice on day 57 post BU dosing (Table 4.10).

Cyclosporin A dosing period

During the CsA dosing period (on days 7, 14 and 21) a significant reduction in mean relative thymus weight was seen in vehicle-CsA treated mice (Group A2) (Table 4.10) in comparison with vehicle-vehicle mice (Group A1). The mean relative thymus weights of mice treated with BU-vehicle (Group B1) were comparable to values in vehicle-

vehicle treated mice (Group A1) during the CsA dosing period. Mice treated with BU-CsA (Group B2) had significantly reduced mean thymus weights in comparison to BU-vehicle treated mice (Group B1) during CsA treatment on day 7, 14 and 21 ($p<0.01$ at each time point).

Cyclosporin A post dosing period

In the early CsA post dosing period (day 1) mice treated with vehicle-CsA (Group A2) continued to have a significantly reduced mean relative thymus weight before values returned to those comparable to the vehicle-vehicle mice (Group A1) at day 8, 15, 57 and 113 (Table 4.10).

The mean relative thymus weights of mice treated with BU-vehicle (Group B1) were comparable to the mean value in Group A1 mice (vehicle-vehicle) on days 1 and 8 post CsA dosing. However, a significant reduction in mean relative thymus weight was evident on days 15 and 57 post CsA dosing in BU-vehicle treated mice, before returning to values comparable to vehicle-vehicle mice (Group A1) on day 113.

The reduction in mean relative thymus weight which had been seen in mice treated with BU-CsA (Group B2) during the CsA dosing period continued into the post CsA dosing period day 1 ($p<0.001$) (Table 4.10). However, the mean relative thymus weight of mice treated with BU-CsA (Group B2) returned to values comparable to mice treated with BU-vehicle (Group B1) on days 8, 15 and 57 post dosing. However, the mean thymus weight was again reduced in BU-CsA mice on day 113 post CsA dosing ($p<0.05$).

4.3.2.7. Results summary

Group A2 (Vehicle-CsA)

- Peripheral blood counts of mice treated with vehicle-CsA were in general, comparable to mice treated with vehicle-vehicle.

- Transient reductions were evident in bone marrow cellularity, the number of erythroid colonies and CFU-GMs of mice treated with vehicle-CsA in comparison to mice treated with vehicle-vehicle.

Group B1 (BU-vehicle)

- Transient reductions in erythropoiesis and lymphopoiesis with sustained reduction in thrombopoiesis.

- Sustained reduction in erythroid colonies and CFU-GMs.

- Elevated serum F reaching statistical significance at a number of time points.

Group B2 (BU-CsA)

- In general, patterns of change in the peripheral blood of mice treated with BU-CsA were comparable to changes in mice treated with BU-vehicle.

- The number of erythroid colonies and CFU-GM were significantly reduced in mice treated with BU-CsA in comparison to mice treated with BU-vehicle.

4.4. DISCUSSION

The fungal metabolite CsA isolated by Borel *et al.* (1976) is a potent immunosuppressant agent used widely in organ transplantation and the drug is also used in the treatment of AA (BNF 2004; Young and Barrett 1995). CsA exerts its immunosuppressant effects by disrupting two transcription factors nuclear factor of activated T-cells (NFAT), and nuclear factor κ B (NFκB), both of which are essential for the expression of the cytokine interleukin-2 (IL-2). NFAT and NFκB are present within the cytoplasm of resting T-cells in an inactive form. Activation of the T-cell leads to Ca^{2+}-calmodulin dependent phosphatase activation which dephosphorylates NFAT and NFκB which the allows translocation to the nucleus and gene expression (Baumann *et al.* 1992). CsA binds intracellularly to cytoplasmic proteins (immunophillins) forming a complex which binds to, and prevents the dephosphorylation, of NFAT and NFκB thus preventing gene expression. CsA therefore blocks the synthesis of IL-2 which in turn results in a reduction in the expansion of cytotoxic lymphocytes and an inhibition in T-cell dependent B-cell activation. The immunosuppressant effects of CsA are confined to lymphocytes, CsA is not myelotoxic and therefore, it does not hinder the action of monocytes or granulocytes (Editorial 1985).

Patients diagnosed with AA are primarily treated with a bone marrow transplant if a suitable donor is available. Indeed, bone marrow transplantation in AA patients provides the best long-term survival out-come. However, if a bone marrow donor is not available AA patients are treated with anti-lymphocyte globulin (ALG) in combination with CsA. Suppression of the immune system by ALG and CsA has been shown to result in

autologous bone marrow reconstitution and remission in a large percentage of treated patients (Young and Barrett 1995).

In the preliminary dose response study, conducted to examine the effects of oral CsA treatment when administered daily for 34 days at 0, 15, 20, 25, 30, 35, 40 and 45 mg/kg, showed CsA to be well tolerated. During the period of CsA treatment, animals remained in good health with no overt signs of drug toxicity; body weight was not affected. A single incident of mortality was recorded however, but this death was believed to be as a result of a dosing accident rather than as a result of CsA treatment. At the autopsy, following the administration of 34 daily doses of CsA little evidence of drug induced toxicity was seen at any dose level. The peripheral blood counts and the bone marrow cellularity of mice treated with CsA at all dose levels were comparable to vehicle-treated controls, as were the proportion of myeloid, erythroid and lymphoid cells in the bone marrow (Table 4.1, 4.2). The only statistically significant changes were an increase in FNCC at 45 mg/kg CsA (Table 4.1), and an increase in the bone marrow myeloid cells, also at 45 mg/kg (Table 4.2). In addition, measurement of apoptosis in the bone marrow of CsA-treated mice showed no deviation from control values.

It was concluded therefore, as a result of the preliminary dose response study, that CsA could be administered orally to mice at doses of up to 45 mg/kg daily for 34 days without significant toxicity. Therefore, an appropriate dose of CsA for the main BU-CsA study was selected, namely 30 mg/kg.

The lack of myelotoxicity observed in the preliminary dose response study following CsA treatment finds a parallel with previous reports in both mice and rats. Administration of 4 doses of CsA to mice at 200 or 500 mg/kg every other day did not damage the bone marrow; indeed; only at the very high dose of 500 mg/kg was a reduced lymphocyte count observed with slight reductions also occurring in the platelet count

(Borel *et al.* 1994). In a 13 week toxicity study, rats were exposed to CsA in their diet at doses of 0, 14, 45 and 90 mg/kg (Ryffel *et al.* 1983); at 14 mg/kg a slight reduction was evident in the peripheral blood lymphocyte count. This change became more pronounced with increasing CsA dose, and in the highest CsA dose Group (90 mg/kg), distinct reductions were evident in the lymphocytes and eosinophils in the peripheral blood in conjunction with an increase in the MCH, WBC, neutrophils and monocytes (Ryffel *et al.* 1983). Rats treated with 90 mg/kg CsA also showed moderate atrophy of the thymus and severe centrilobular degeneration in the liver (Ryffel *et al.* 1983).

In the model of CBMA described by Gibson *et al.* (2003), BALB/c mice treated with BU at 10.50 mg/kg on 8 occasions over 18 days developed significant anaemia, leucopenia and thrombocytopenia in the early post dosing period (days 1 to 19). In the weeks following dosing the condition of animals began to improve. However, on days 91 and 112 post dosing animals developed CBMA characterised by significant reductions in the erythrocytes, leucocytes (WBC, neutrophils, lymphocytes, monocytes and eosinophils) and platelets, together with an increase in MCV. Bone marrow cellularity was also significantly reduced during the late-stage period (day 91 and 112), in addition to a decrease in the number of committed haemopoietic progenitor cells; bone marrow apoptosis was also increased. In this study (Gibson *et al.* 2003), mice treated with BU at 10.50 mg/kg developed significant toxicity with 49.3% of the mice catagorised as ICD. This significant mortality, associated with dosing mice at 10.50 mg/kg, led to further studies to determine if lowering the dose of BU would produce CBMA in the BALB/c mouse without significant mortality (Turton *et al.* 2005). In this more recent study, BALB/c mice were treated with BU at 8.25, 9.00 and 9.75 mg/kg on 10 occasions over 21 days. At all doses, BU induced significant anaemia, leucopenia and thrombocytopenia immediately post dosing. A period of recovery then followed, however, on days 71, 84, 106 and 127

post dosing, BU-treated mice showed evidence of CBMA with significant reductions occurring in peripheral blood counts and bone marrow cellularity. BU-induced mortality was greatly reduced in this study as a result of lowering the BU dose. It was concluded from this experiment that a dose of BU at 9.0 mg/kg administered on 10 occasions over 21 days would be sufficient to induce CBMA, but would avoid high mortality.

In BU-treated mice at day 57 post dosing (Table 4.3), and in BU-vehicle (Group B1) mice at day 7, 14 and 21 of CsA administration, and at day 1,8, 15, 57 and 113 post CsA dosing, some evidence of the characteristic features of CBMA were seen. There were reductions, in comparison with vehicle-vehicle (Group A1) mice, in RBC, Hb and HCT values, in WBC and in neutrophi and lymphocyte values; platelet counts and FNCC values showed decreases, and there were increases in MCV. CFU-GM and erythroid colonies also showed decreased counts. However, these changes were sometimes intermittent, and on occasion were not as great as in previous studies (Gibson *et al.* 2003; Turton *et al.* 2006).

A major objective of the present experiment was to investigate if the administration of CsA, to BU-treated mice showing evidence of the characteristic features of CBMA, would induce an effect where the affected parameters would return towards normal. That is, would the BU-treated/CsA-treated mice (Group B2), during and immediately after CsA treatment, show significant differences in the affected parameters to mice in Group B1 which had not received CsA? The results set out (Table 4.4; 4.5; 4.6 and 4.7) demonstrate that the administration of CsA did not induce a return towards normal in the affected parameters.

In man, the plasma concentrations of both membrane bound and soluble levels of FL have been measured in stem cell transplant recipients treated with or without CsA (Chklovskaia *et al.* 2001). This study found that plasma FL increased following the conditioning regimen of chemotherapy that is normally carried out prior to transplantation.

However, following successful stem cell transplant, the concentration of both membrane bound FL and soluble FL were reduced in patients treated with CsA, in comparison to patients not treated with CsA. This reduction in FL occurring as a result of CsA administration, described by Chklovskaia *et al.* (2001), was not observed in the present experiment (Table 4.8). The concentration of serum FL in mice treated with BU (Group B1; BU-vehicle) were raised in comparison with Group A1 (vehicle-vehicle) at all time points, but the administration of CsA did not significantly affect the level of FL at day 14 of CsA dosing, or at day 1 and 15 post CsA dosing (that is, FL levels in the BU-CsA group mice (Group B2) were not statistically significantly different to the FL levels in the BU-vehicle group (Group B1)).

Immune mediated bone marrow failure in the mouse induced by injecting lymph node cells (LNC) from donor mice into lethally irradiated hosts (the two strains having the same major histocompatability complex (H-2K) but differing at the M locus) results in significant pancytopenia (Knospe *et al.* 1983; Chiu & Knospe 1987). In this model of immune mediated bone marrow failure, however, the administration of CsA at 25 mg/kg by subcutaneous injection for 30 days (starting day 1 before irradiation and LNC injection) in a similar way to that in the present experiment did not prevent bone marrow failure (Knospe *et al.* 1984). More recently, a model of immune mediated bone marrow failure has been described by Chen *et al.* (2004) using LNC infusion. In this model, the administration of CsA (ip) at 50 μg/g daily for 10 days starting 1 hour after LNC injection resulted in a significant increase in survival (Bloom *et al.* 2004).

In the present experiment, there was some evidence to suggest that CsA was having a detrimental effect on the production of peripheral blood cells at the level of the haemopoietic progenitor cell. During the CsA dosing period vehicle-treated mice dosed daily with CsA at 30 mg/kg for 35 days (Group A2) had a reduced number of CFU-GM

colonies per femur. This reduction in CFU-GM was evident and statistically significant in comparison to vehicle-treated (Group A1) mice during the CsA dosing period (days 14 and 21 of CsA dosing) and also in the immediate post CsA dosing period (day 1). A similar reduction was evident in the number of erythroid colonies per femur in vehicle-CsA (Group A2) mice. On day 21 of the CsA dosing period, and on day 1 post CsA dosing the number of erythroid colonies per femur were significantly reduced in vehicle-CsA (Group A2) mice in comparison to vehicle-treated (Group A1) mice. Furthermore, reductions in the number of CFU-GM and erythroid colonies were greater in mice treated with BU-CsA (Group B2). In comparison to mice treated with BU-vehicle (Group B1) (Table 4.7), the number of CFU-GM colonies were reduced significantly in BU-CsA on days 7 ($p<0.001$), 14 ($p<0.001$) and 21 ($p<0.05$) of CsA dosing, and on days 1 ($p<0.05$), and day 8 ($p<0.05$) immediately post CsA dosing. Erythroid colonies were also significantly reduced in mice treated with BU-CsA (Group B2) on days 7 ($p<0.01$), 14 ($p<0.05$) and 21 (NS) of CsA dosing, and at day 8 post CsA dosing, in comparison to mice treated with BU-vehicle (Group B1). The inhibitory effect of CsA on haemopoietic progenitor cells has previously been described (Clarke *et al.* 1991). However, in the study of Clarke *et al.* (1991) the inhibitory effects of CsA were confined to the erythroid lineage with myeloid colonies being unaffected in mice treated with CsA at 125 mg/kg by gavage 4 to 5 times per week for 9 weeks. Similarly, in a study by Borel *et al.* (1977), the number of committed haemopoietic progenitor cells in the bone marrow of rats treated with CsA daily at 200 or 500 mg/kg for 14 days was, in general, comparable to control values.

The immunosuppressant effect of CsA treatment was evident in the thymus glands of mice treated with vehicle-CsA (Group A2) and BU-CsA (Group B2) (Table 4.10). This reduction in mean relative thymus weight was especially clear during CsA treatment and on day 1 post CsA treatment. In mice treated with vehicle-CsA (Group A2), and in mice

treated with BU-CsA (Group B2), this effect was not evident on days 8, 15, 57 and 113 post CsA dosing. This reduction in thymic weight has been described previously in mice treated with CsA (Kanariou *et al.* 1989; Calabrese *et al.* 2000). In the study by Kanariou *et al.* (1989), a significant reduction in the size of the thymic medulla was observed with no change in the thymic cortex of mice treated with CsA at 7 or 14 mg/kg/day for 6 weeks. The reduction in the thymic medulla was evident from day 7 of dosing and became more severe as CsA treatment progressed. However, when drug treatment was withdrawn, the thymus began to return to normal.

In the present experiment, CsA administered on day 57 post BU treatment did not protect mice from BU-induced bone marrow injury. The lack of effect of CsA administration in preventing the characteristic features evident in CBMA may be a result of poor drug absorption when administered by gavage, or could possibly be related to the dosing regimen, with either the start of treatment commencing too late, or drug treatment being withdrawn prematurely. Indeed, the actual mechanism by which BU is able to cause 'late-stage' bone marrow injury is unclear and does need further investigation.

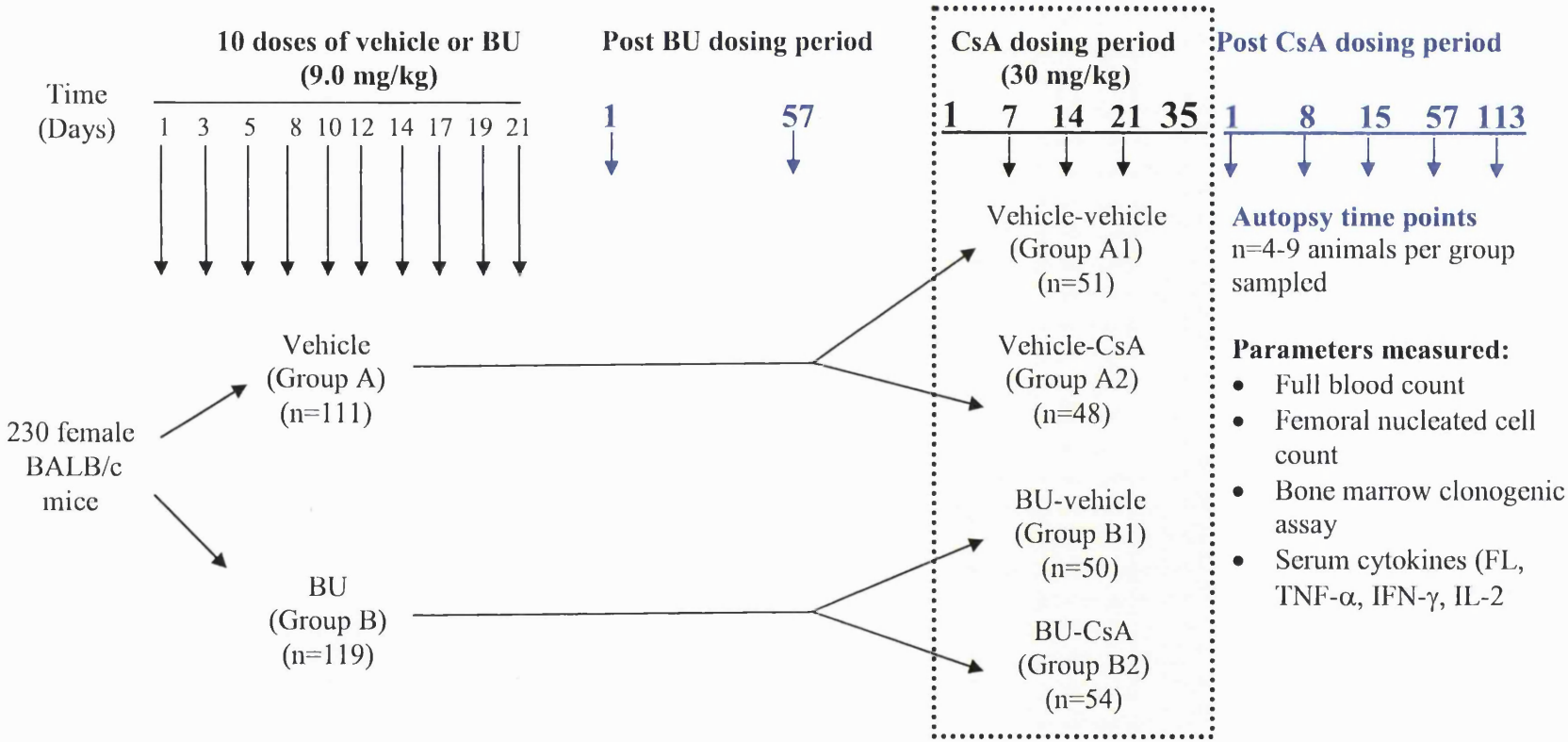


FIGURE 4.1. Experimental design of a study to investigate the effects of administering cyclosporin A (CsA) to busulphan- (BU-) treated female BALB/c mice. 230 BALB/c mice were divided into 2 groups and treated with vehicle (Group A; n=111) or BU at 9.0 mg/kg by intraperitoneal (ip) injection on 10 occasions over 21 days (Group B; n=119). On day 57 post BU dosing, both the vehicle-treated group (Group A) and the BU-treated group (Group B) were split in 2 and treated daily for 35 days with vehicle (vehicle-vehicle, Group A1; BU-vehicle, Group B1) of with CsA by gavage at 30 mg/kg (vehicle-CsA, Group A2; BU-CsA, Group B2). On days 1 and 57 post BU dosing, during the CsA dosing period (days 7, 14 and 21) and on days 1, 8, 15, 57 and 113 post CsA dosing, animals (n=4-9 per group) were autopsied for peripheral blood and bone marrow analysis.

TABLE 4.1. Haematological results[a] from female CD-1 mice treated daily by gavage with cyclosporin A (CsA) for 34 days at 0 (control), 15, 20, 25, 30, 35, 40 and 45 mg/kg and autopsied on day 1 after the final dose[b]

	CsA (mg/kg)							
	0 (control)	**15**	**20**	**25**	**30**	**35**	**40**	**45**
RBC	9.11 (0.27)	8.80 (0.63)	9.02 (0.72)	9.09 (0.75)	8.69 (0.63)	8.77 (0.37)	8.99 (0.52)	8.86 (0.60)
Hb	14.3 (0.3)	13.1 (0.6)	14.2 (0.4)	13.9 (0.7)	13.4 (0.9)	13.7 (0.5)	13.9 (0.7)	13.5 (0.6)
HCT	45.1 (1.9)	41.8 (2.4)	44.1 (2.2)	42.6 (2.9)	42.5 (1.8)	42.3 (2.0)	43.2 (1.7)	41.8 (1.8)
MCV	49.5 (0.6)	47.6 (1.1)	49.1 (1.8)	46.9 (2.3)	49.0 (2.2)	48.3 (1.1)	48.1 (1.5)	47.3 (2.1)
MCH	15.7 (0.6)	15.0 (1.0)	15.8 (0.9)	15.4 (0.7)	15.4 (0.5)	15.7 (0.5)	15.4 (0.2)	15.3 (0.7)
MCHC	31.7 (1.6)	31.5 (1.9)	32.2 (0.9)	32.8 (2.1)	31.4 (1.1)	32.5 (1.3)	32.1 (1.1)	32.3 (0.6)
Retic	360 (60)	274 (77)*	308 (75)	316 (64)	369 (116)	341 (82)	386 (115)	391 (154)
Plt	1243 (142)	1253 (136)	1448 (99)	1359 (148)	1409 (302)	1266 (337)	1095 (384)	1264 (143)
WBC	2.24 (0.26)	1.37 (0.71)	2.37 (0.48)	2.08 (0.79)	1.54 (0.20)	1.74 (0.67)	1.11 (0.26)*	2.74 (1.03)
Neut	0.30 (0.00)	0.25 (0.16)	0.43 (0.09)	0.37 (0.14)	0.30 (0.08)	0.36 (0.09)	0.30 (0.22)	0.83 (0.41)*
Lymph	1.64 (0.57)	0.98 (0.49)	1.71 (0.45)	1.53 (0.63)	1.09 (0.24)	1.14 (0.50)	0.66 (0.11)	1.60 (0.62)
Mono	0.07 (0.01)	0.06 (0.06)	0.11 (0.03)	0.09 (0.03)	0.05 (0.02)	0.12 (0.09)	0.06 (0.04)	0.18 (0.13)
Eo	0.21 (0.17)	0.06 (0.05)	0.10 (0.05)	0.08 (0.02)	0.10 (0.04)	0.10 (0.03)	0.08 (0.05)	0.12 (0.05)
Baso	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.01)	0.00 (0.01)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
FNCC	2.23 (0.33)	2.21 (0.23)	2.56 (0.36)	3.02 (0.29)	2.93 (0.50)	2.66 (0.47)	2.73 (0.36)	3.21 (0.57)**
n	5	5	5	5	4	5	5	5

[a] Values are means, SD in parentheses.

[b]Abbreviations and units: RBC, red blood cells, $x10^6/\mu l$; Hb, haemoglobin, g/dl; HCT, haematocrit, %; MCV, mean cell volume, fl; MCH, mean cell haemoglobin, pg; MCHC, mean cell haemoglobin concentration, g/dl; Retic, absolute reticulocyte count, $x10^3/\mu l$; Plt, platelets, $x10^3/\mu l$; WBC, white blood cells, $x10^3/\mu l$; Neut, neutrophils, $x10^3/\mu l$; Lymph, lymphocytes, $x10^3/\mu l$; Mono, monocytes, $x10^3/\mu l$; Eo, eosinophils, $x10^3/\mu l$; Baso, basophils, $x10^3/\mu l$; FNCC, femoral bone marrow nucleated cell count, $x10^7$. Data analysed using a one-way analysis of variance (ANOVA). *Significantly different from controls, $p<0.05$; **$p<0.01$.

TABLE 4.2. Estimated counts ($x10^7$) of myeloid, erythroid and lymphoid cells, and the myeloid:erythroid (M:E) ratio in the femoral marrow of control and cyclosporin A- (CsA-) treated female CD-1 mice[a]; animals were treated daily with CsA for 34 days at 0 (control), 15, 20, 25, 30, 35, 40 and 45 mg/kg and autopsied on day 1 after the final dose

	CsA (mg/kg)							
	0 (control)	**15**	**20**	**25**	**30**	**35**	**40**	**45**
Myeloid	1.76 (0.25)	2.00 (0.41)	2.11 (0.36)	2.18 (0.36)	2.45 (0.44)	2.35 (0.52)	2.26 (0.38)	3.59 (0.38)***
Erythroid	1.39 (0.23)	1.21 (0.20)	1.50 (0.22)	1.83 (0.45)	1.51 (0.25)	1.33 (0.27)	1.48 (0.21)	1.33 (0.69)
Lymphoid	1.29 (0.35)	1.15 (0.21)	1.36 (0.26)	1.80 (0.11)	1.52 (0.39)	1.38 (0.26)	1.46 (0.36)	1.39 (0.05)
Other	0.13 (0.02)	0.06 (0.04)	0.14 (0.06)	0.22 (0.12)	0.36 (0.13)**	0.26 (0.14)	0.25 (0.09)	0.11 (0.06)
M:E ratio	0.80 (0.17)	0.63 (0.18)	0.73 (0.16)	0.86 (0.24)	0.62 (0.06)	0.58 (0.16)	0.68 (0.20)	0.38 (0.21)*

[a] 200 cells in the tibial marrow smears were differentially counted by eye and the absolute number of cells of each lineage estimated for the nucleated marrow cell count of the femoral marrow flush. Cells categorised as 'other' include monocytes and monocyte precursors, mast cells, megakaryocyes, plasma cells and unidentifiable cells. Values are means, SD in parenthesis. Data analysed using a one-way analysis of variance (ANOVA). *Significnatly different from controls, $p<0.05$; **$p<0.01$; ***$p<0.001$. Number of animals per group and all other information as Table 4.1.

TABLE 4.3. Haematological results[a] from female BALB/c mice treated with 10 doses of busulphan (BU) at 9.0 mg/kg over a period of 21 days and sampled at day 1 and 57 after the final dose[b]

	Day of sampling			
	1		57	
	Vehicle	**BU**	**Vehicle**	**BU**
RBC	9.52 (0.11)	7.83 (1.19)***	9.53 (0.45)	9.29 (0.33)
Hb	15.1 (0.3)	12.9 (1.9)***	15.0 (0.5)	14.7 (0.5)
HCT	49.6 (1.3)	40.8 (5.8)***	48.0 (2.3)	47.4 (1.1)
MCV	52.1 (1.8)	52.2 (0.8)	50.4 (1.2)	51.0 (1.3)
MCH	15.9 (0.4)	16.5 (0.2)*	15.7 (0.5)	15.9 (0.4)
MCHC	30.5 (0.6)	31.6 (0.4)**	31.2 (0.9)	31.2 (0.7)
Retic	357 (29)	340 (102)	303 (84)	301 (38)
Plt	873 (89)	223 (127)***	1018 (93)	668 (228)**
WBC	2.58 (1.01)	0.60 (0.21)***	1.92 (0.98)	1.48 (0.43)
Neut	0.50 (0.21)	0.07 (0.03)***	0.52 (0.44)	0.38 (0.17)
Lymph	1.91 (0.83)	0.48 (0.20)***	1.29 (0.56)	1.03 (0.32)
Mono	0.05 (0.01)	0.00 (0.00)***	0.03 (0.02)	0.02 (0.01)
Eo	0.10 (0.03)	0.03 (0.01)***	0.07 (0.03)	0.05 (0.01)*
Baso	0.01 (0.01)	0.01 (0.01)	0.00 (0.01)	0.00 (0.00)
FNCC	3.24 (0.35)	1.47 (0.81)***	4.17 (0.65)	3.74 (0.29)
n	6	6	6	6

[a] Values are means, SD in parentheses.
[b] Abbreviations and units: as Table 4.1.
Data analysed using a Student's t-test. *Significantly different from controls, $p<0.05$; **$p<0.01$; ***$p<0.001$.

TABLE 4.4. Haematological results[a] from female BALB/c mice treated with 10 doses of vehicle or busulphan (BU) followed on day 57 post dosing by daily treatment with vehicle or cyclosporin A (CsA)[b] for 35 days; animals sampled on days 7, 14 and 21 of CsA treatment

	7				14				21			
	A1 Vehicle-Vehicle	**A2 Vehicle-CsA**	**B1 BU-Vehicle**	**B2 BU-CsA**	**A1 Vehicle-Vehicle**	**A2 Vehicle-CsA**	**B1 BU-Vehicle**	**B2 BU-CsA**	**A1 Vehicle-Vehicle**	**A2 Vehicle-CsA**	**B1 BU-Vehicle**	**B2 BU-CsA**
RBC	9.48 (0.15)	9.44 (0.16)	9.18 (0.57)	9.64 (0.40)	9.46 (0.24)	8.71 (7.99)	9.44 (0.12)	9.09 (0.61)	9.43 (0.42)	9.24 (0.91)	9.11 (0.19)	8.86 (0.75)
Hb	14.6 (0.4)	14.4 (0.3)	14.3 (0.7)	14.9 (0.5)	14.6 (0.4)	13.6 (3.1)	14.8 (0.1)	14.2 (0.8)+	14.6 (0.6)	14.2 (1.5)	14.4 (0.4)	13.9 (1.2)
HCT	47.6 (1.1)	46.4 (0.8)*	46.0 (2.6)	47.5 (1.8)	45.8 (1.6)	42.9 (9.4)	46.2 (0.4)	44.9 (1.6)+	46.2 (1.7)	45.1 (4.2)	46.0 (1.2)	44.6 (3.8)
MCV	50.2 (0.9)	49.2 (0.8)*	50.2 (0.6)	49.3 (0.6)+	48.4 (0.7)	49.4 (1.1)*	48.9 (0.4)	49.5 (2.3)	49.1 (1.1)	48.8 (1.2)	50.5 (0.7)**	50.3 (1.9)
MCH	15.3 (0.3)	15.3 (0.2)	15.6 (0.3)*	15.5 (0.3)	15.5 (0.2)	15.6 (0.3)	15.7 (0.2)*	15.6 (0.6)	15.5 (0.3)	15.4 (0.4)	15.8 (0.3)	15.7 (0.2)
MCHC	30.6 (0.5)	31.1 (0.5) *	31.2 (0.4)**	31.3 (0.3)	31.9 (0.5)	31.6 (0.6)	32.0 (0.3)	31.6 (0.7)	31.5 (0.3)	31.4 (0.7)	31.2 (0.4)	31.3 (1.0)
Retic	327 (51)	463 (49)***	328 (66)	409 (33)+	345 (52)	520 (168)**	321 (35)	543 (423)	328 (55)	348 (90)	296 (22)	449 (320)
Plt	1006 (100)	1031 (121)	718 (115)***	632 (238)	926 (88)	920 (95)	674 (25)***	626 (162)	916 (65)	902 (84)	681 (48)***	684 (304)
WBC	3.08 (1.00)	2.16 (0.19)*	1.69 (0.58)**	1.61 (0.69)	2.41 (0.47)	1.79 (0.93)	1.93 (0.62)	1.71 (0.79)	3.36 (1.31)	2.19 (0.68)*	2.62 (0.85)	1.69 (0.50)+
Neut	0.71 (0.31)	0.61 (0.11)	0.40 (0.19)*	0.44 (0.22)	0.60 (0.13)	0.54 (0.27)	0.44 (0.17)*	0.47 (0.17)	0.69 (0.28)	0.58 (0.18)	0.66 (0.19)	0.65 (0.24)
Lymph	2.17 (0.69)	1.41 (0.15)*	1.18 (0.40)**	1.08 (0.46)	1.62 (0.37)	1.09 (0.64)*	1.30 (0.44)	1.09 (0.55)	2.41 (0.97)	1.39 (0.47)*	1.80 (0.65)	0.87 (0.34)++
Mono	0.07 (0.03)	0.03 (0.00)**	0.03 (0.01)**	0.03 (0.01)	0.04 (0.02)	0.02 (0.02)	0.03 (0.01)*	0.03 (0.02)	0.06 (0.02)	0.04 (0.01)*	0.04 (0.01)*	0.03 (0.03)
Eo	0.11 (0.05)	0.08 (0.04)	0.08 (0.04)	0.06 (0.02)	0.14 (0.02)	0.13 (0.00)	0.14 (0.07)	0.11 (0.05)	0.17 (0.05)	0.18 (0.06)	0.10 (0.04)**	0.11 (0.05)
Baso	0.00 (0.00)	0.00 (0.01)	0.00 (0.01)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.01 (0.01)	0.00 (0.00)*	0.00 (0.00)	0.00 (0.00)
FNCC	4.24 (0.39)	3.99 (0.25)	3.87 (0.60)	3.79 (0.42)	4.31 (0.57)	3.26 (1.31)*	3.82 (0.60)	3.32 (1.18)	4.34 (0.47)	3.54 (0.80)*	3.89 (0.36)*	3.20 (1.07)
n	6	6	6	6	6	6	6	6	6	6	6	6

[a] Values are means, SD in parentheses. Abbreviations and units as Table 4.1.

[b] Animals were treated with BU (9.0 mg/kg) on 10 occasions over 21 days. On day 57 post dosing, vehicle-treated (Group A) and BU-treated mice (Group B) were each divided into 2 groups and treated daily for 35 days with vehicle (vehicle-vehicle, Group A1; BU-vehicle, Group B1) or with CsA by gavage at 30 mg/kg (vehicle-CsA, Group A2; BU-CsA, Group B2). Data analysed using Student's t-test. *Significantly different from Group A1, $p<0.05$; **$p<0.01$; ***$p<0.001$. +Significantly different from Group B1, $p<0.05$; ++$p<0.01$.

TABLE 4.5. Haematological results[a] from female BALB/c mice treated with 10 doses of vehicle or busulphan (BU) followed on day 57 post dosing by daily treatment with vehicle or cyclosporin A (CsA)[b]; animals sampled on days 1, 8 and 15 post CsA treatment

	1				8				15			
	A1 Vehicle-Vehicle	**A2 Vehicle-CsA**	**B1 BU-Vehicle**	**B2 BU-CsA**	**A1 Vehicle-Vehicle**	**A2 Vehicle-CsA**	**B1 BU-Vehicle**	**B2 BU-CsA**	**A1 Vehicle-Vehicle**	**A2 Vehicle-CsA**	**B1 BU-Vehicle**	**B2 BU-CsA**
RBC	9.75 (0.15)	8.98 (1.58)	9.42 (0.21)**	9.59 (0.43)	9.73 (0.20)	9.84 (0.34)	9.63 (0.62)	9.60 (0.21)	10.02 (0.28)	10.18 (0.18)	9.58 (0.14)**	9.57 (0.24)
Hb	14.8 (0.3)	13.6 (2.2)	14.8 (0.6)	15.2 (0.5)	15.1 (0.3)	15.1 (0.5)	15.1 (0.6)	14.8 (0.4)	15.1 (0.3)	13.3 (0.4)	14.8 (0.3)	15.0 (0.3)
HCT	47.9 (1.0)	44.0 (7.0)	47.3 (1.9)	48.4 (1.6)	49.0 (1.1)	48.9 (1.7)	48.6 (1.7)	48.1 (1.4)	47.0 (1.8)	48.2 (1.4)	46.5 (0.6)	46.9 (0.9)
MCV	49.1 (1.0)	49.2 (1.6)	50.2 (1.0)*	50.6 (1.0)	50.4 (1.1)	49.7 (1.1)	50.5 (1.5)	50.0 (1.0)	46.9 (1.2)	47.4 (1.4)	48.5 (0.8)**	49.0 (1.0)
MCH	15.2 (0.3)	15.1 (0.3)	15.8 (0.4)*	15.9 (0.5)	15.5 (0.3)	15.4 (0.1)	15.7 (0.7)	15.4 (0.4)	15.1 (0.3)	15.1 (0.3)	15.5 (0.3)*	15.7 (0.4)
MCHC	31.0 (0.4)	30.7(0.7)	31.4 (0.4)	31.3 (0.4)	30.7 (0.3)	31.0 (0.8)	31.0 (0.7)	30.7 (0.3)	32.1 (0.4)	31.8 (0.4)	31.9 (0.3)	32.0 (0.3)
Retic	302 (25)	548 (261)*	346 (47)*	341 (40)	328 (46)	329 (60)	337 (50)	399 (71)	307 (17)	316 (91)	276 (38)	349 (22)++
Plt	1003 (49)	1013 (244)	717 (61)***	628 (129)	916 (95)	950 (46)	825 (168)	644 (13)+	952 (106)	1021 (76)	762 (58)**	794 (88)
WBC	2.75 (0.73)	2.57 (0.83)	2.21 (0.78)	1.54 (0.55)	2.40 (0.87)	2.19 (0.64)	1.40 (0.38)	2.00 (0.25)++	3.22 (1.63)	2.11 (1.51)	1.74 (0.59)*	1.77 (0.62)
Neut	0.64 (0.27)	1.11 (0.30)**	0.57 (0.29)	0.43 (0.18)	0.68 (0.30)	0.61 (0.20)	0.49 (0.11)	0.66 (0.10)+	0.74 (0.27)	0.55 (0.27)	0.54 (0.17)	0.51 (.021)
Lymph	1.88 (0.43)	1.27 (0.62)*	1.50 (0.51)	0.99 (0.33)+	1.49 (0.53)	1.38 (0.39)	0.80 (0.29)	1.17 (0.28)+	2.24 (1.26)	1.39 (1.18)	1.04 (0.37)*	1.10 (0.38)
Mono	0.07 (0.03)	0.06 (0.03)	0.04 (0.01)*	0.05 (0.02)	0.07 (0.03)	0.04 (0.02)*	0.02 (0.01)*	0.04 (0.01)++	0.10 (0.05)	0.05 (0.03)*	0.04 (0.01)**	0.09 (0.04)
Eo	0.15 (0.11)	0.13 (0.11)	0.08 (0.03)	0.06 (0.05)	0.15 (0.08)	0.14 (0.05)	0.07 (0.02)	0.11 (0.04)+	0.12 (0.08)	0.12 (0.07)	0.10 (0.06)	0.00 (0.01)
Baso	0.00 (0.00)	0.00 (0.01)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.01)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.01 (0.01)	0.01 (0.01)
FNCC	4.21 (0.82)	3.15 (1.36)	4.02 (0.87)	3.11 (1.31)	2.16 (0.47)	2.59 (0.28)*	2.75 (0.39)*	2.58 (0.44)	3.33 (0.64)	3.40 (1.21)	3.30 (0.81)	3.23 (1.18)
n	6	6	6	6	6	5	6	6	5	5	6	5

[a] Values are means, SD in parentheses. Abbreviations and units as Table 4.1.

[b] Animals were treated with BU (9.0 mg/kg) on 10 occasions over 21 days. On day 57 post dosing, vehicle-treated (Group A) and BU-treated mice (Group B) were each divided into 2 groups and treated daily for 35 days with vehicle (vehicle-vehicle, Group A1; BU-vehicle, Group B1) or with CsA by gavage at 30 mg/kg (vehicle-CsA, Group A2; BU-CsA, Group B2). Data analysed using Student's t-test. *Significantly different from Group A1, $p<0.05$; **$p<0.01$; ***$p<0.001$. +Significantly different from Group B1, $p<0.05$; ++$p<0.01$.

TABLE 4.6. Haematological results[a] from female BALB/c mice treated with 10 doses of vehicle or busulphan (BU) followed on day 57 post dosing by daily treatment with vehicle or cyclosporin A (CsA)[b]; animals sampled on days 57 and 113 post CsA treatment

	57				113			
	A1	**A2**	**B1**	**B2**	**A1**	**A2**	**B1**	**B2**
	Vehicle-Vehicle	**Vehicle-CsA**	**BU-Vehicle**	**BU-CsA**	**Vehicle-Vehicle**	**Vehicle-CsA**	**BU-Vehicle**	**BU-CsA**
RBC	10.16 (0.23)	10.27 (0.17)	9.82 (0.23)**	9.88 (0.61)	9.98 (0.21)	10.08 (0.30)	9.34 (0.27)***	10.00 (0.41)++
Hb	15.1 (0.3)	15.3 (0.4)	15.1 (0.5)	15.2 (0.7)	14.9 (0.3)	15.2 (0.4)*	14.0 (0.5)***	15.2 (0.4)+++
HCT	48.5 (1.2)	47.8 (1.4)	48.7 (1.5)	48.5 (2.9)	46.7 (1.1)	48.0 (1.4)*	44.9 (1.3)**	48.1 (1.6)++
MCV	47.7 (1.4)	46.8 (1.1)	49.6 (0.1)**	49.2 (1.4)	46.8 (0.7)	47.6 (0.841)*	48.1 (1.9)	48.1 (0.6)
MCH	14.9 (0.3)	14.9 (0.2)	15.3 (0.3)**	15.4 (0.4)	14.9 (0.1)	15.1 (0.4)	15.0 (0.7)	15.2 (0.6)
MCHC	31.3 (0.6)	31.8 (0.7)	30.9 (0.3)	31.4 (0.6)	31.9 (0.4)	31.8 (0.6)	31.3 (0.4)**	31.5 (0.6)
Retic	346 (52)	397 (59)	347 (21)	346 (16)	300 (50)	284 (52)	299 (56)	303 (31)
Plt	992 (52)	897 (119)	734 (79)***	766 (94)	983 (59)	971 (40)	809 (142)**	747 (114)
WBC	3.75 (0.45)	2.92 (1.27)	1.77 (0.60)***	1.52 (0.33)	1.61 (0.44)	1.60 (0.60)	1.59 (0.54)	1.18 (0.40)
Neut	0.90 (0.21)	0.99 (0.65)	0.54 (0.16)**	0.53 (0.16)	0.52 (0.21)	0.54 (0.25)	0.62 (0.29)	0.48 (0.24)
Lymph	2.50 (0.31)	1.68 (0.55)	1.07 (0.45)***	0.84 (0.15)	0.89 (0.18)	0.82 (0.31)	0.78 (0.27)	0.56 (0.28)
Mono	0.14 (0.02)	0.09 (0.04)	0.06 (0.02)***	0.05 (0.02)	0.04 (0.02)	0.04 (0.03)	0.03 (0.01)	0.02 (0.01)+
Eo	0.19 (0.04)	0.14 (0.05)	0.10 (0.03)***	0.09 (0.04)	0.16 (0.05)	0.20 (0.08)	0.14 (0.05)	0.12 (0.03)
Baso	0.01 (0.00)	0.01 (0.01)	0.00 (0.00)**	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
FNCC	4.33 (0.57)	4.27 (0.53)	3.17 (1.02)**	3.79 (0.15)	4.45 (0.45)	4.56 (0.48)	4.10 (0.46)	3.36 (1.68)
n	6	4	6	6	6	9	7	7

[a] Values are means, SD in parentheses. Abbreviations and units as Table 4.1.

[b] Animals were treated with BU (9.0 mg/kg) on 10 occasions over 21 days. On day 57 post dosing, vehicle-treated (Group A) and BU-treated mice (Group B) were each divided into 2 groups and treated daily for 35 days with vehicle (vehicle-vehicle, Group A1; BU-vehicle, Group B1) or with CsA by gavage at 30 mg/kg (vehicle-CsA, Group A2; BU-CsA, Group B2). Data analysed using Student's t-test. *Significantly different from Group A1, $p<0.05$; **$p<0.01$; ***$p<0.001$. +Significantly different from Group B1, $p<0.05$; ++$p<0.01$.

TABLE 4.7. Clonogenic bone marrow assay results[a] from female BALB/c mice treated with 10 doses of vehicle or busulphan (BU), followed by daily treatment with vehicle or cyclosporin A (CsA)[b]

	Treatment		BU post dosing period		CsA dosing period			CsA post dosing period		
			1	57	7	14	21	1	8	15
CFU-GM	A1	Vehicle-Vehicle	3,962 (683)	10,780 (1,683)	5,316 (1,516)	6,591 (777)	5,857 (1,241)	6,152 (813)	5,339 (1,228)	4,940 (1,106)
	A2	Vehicle-CsA			4,496 (1,555)	5,465 (1,231)*	4,150 (1,345)*	4,053 (1,150)**	5,153 (1,684)	6,288 (2,671)
	B1	BU-Vehicle	785 (592)***	7,380 (1,823)*	3,802 (718)*	5,539 (1,884)	4,122 (1,585)*	4,525 (1,703)*	4,313 (2,062)	4,108 (1,163)
	B2	BU-CsA			1,506 (760)+++	2,499 (1,449)+++	2,217 (1,334)+	2,196 (1,742)+	2,089 (1,475)+	3,726 (2,136)
Erythroid colonies	A1	Vehicle-Vehicle	6,031 (955)	6,846 (1,229)	6,507 (2,462)	7,998 (1,472)	7,308 (2,716)	7,476 (1,523)	6,594 (2,462)	5,636 (2,698)
	A2	Vehicle-CsA			5,745 (2,489)	6,958 (2,527)	5,215 (2,186)*	5,040 (1,583)**	6,429 (2,360)	5,134 (1,477)
	B1	BU-Vehicle	918 (978)***	5,289 (2,341)*	3,562 (1,128)**	5,250 (2,377)*	3,980 (2,235)*	4,361 (2,160)**	4,042 (1,942)	4,512 (1,617)
	B2	BU-CsA			1,503 (990)++	2,582 (1,984)+	2,318 (1,828)	2,363 (2,310)	2,084 (1,636)+	3,094 (1,848)

[a] Values are mean and SD in parentheses. Cultures were set up in duplicate and the mean number of CFU-C per femur calculated per mouse. n=6 animals per group. Clonogenic bone marrow assays were not completed on day 57 or day 113 post CsA dosing.

[b] Animals were treated with BU (9.0 mg/kg) on 10 occasions over 21 days. On day 57 post dosing, vehicle-treated (Group A) and BU-treated mice (Group B) were each divided into 2 groups and treated daily for 35 days with vehicle (vehicle-vehicle, Group A1; BU-vehicle, Group B1) or with CsA by gavage at 30 mg/kg (vehicle-CsA, Group A2; BU-CsA, Group B2). Data analysed using Student's t-test. *Significantly different from Group A1, $p<0.05$; **$p<0.01$. +Significantly different from Group B1, $p<0.05$; ++$p<0.01$.

TABLE 4.8. Serum FL results[a] from female BALB/c mice treated with 10 doses of vehicle or busulphan (BU), followed by daily treatment with vehicle or cyclosporin A (CsA)[b]

Treatment		BU post dosing period		CsA dosing period	CsA post dosing period			
		1	57	14	1	15	57	113
A1	Vehicle-Vehicle	348.1 (42.6)	295.0 (44.7)	269.3 (73.7)	274.8 (85.9)	254.4 (19.0)	265.3 (40.5)	310.7 (94.3)
A2	Vehicle-CsA			216.1 (47.0)	279.3 (45.8)	261.9 (70.4)	276.6 (59.4)	286.3 (49.4)
B1	BU-Vehicle	1,572.0 (353.3)**	426.0 (42.3)***	327.0 (30.5)	389.9 (74.4)*	440.9 (134.0)**	499.9 (184.0)*	377.4 (48.0)
B2	BU-CsA			317.7 (61.3)	315.1 (57.9)	401.5 (177.4)	502.3 (39.2)	752.6 (473.2)

[a] Values are mean and SD in parentheses; n=4 vehicle-vehicle and vehicle-CsA groups; n=5 BU-vehicle and BU-CsA groups except on day 1 (BU post dosing period) where n=3 BU-vehicle and vehicle-vehicle groups. FL assay not completed on day 7 or 21 of CsA treatment, or day 8 post CsA treatment.

[b] Animals were treated with BU (9.0 mg/kg) on 10 occasions over 21 days. On day 57 post dosing, vehicle-treated (Group A) and BU-treated mice (Group B) were each divided into 2 groups and treated daily for 35 days with vehicle (vehicle-vehicle, Group A1; BU-vehicle, Group B1) or with CsA by gavage at 30 mg/kg (vehicle-CsA, Group A2; BU-CsA, Group B2). Data analysed using Student's t-test. *Significantly different from Group A1, $p<0.05$; **$p<0.01$; ***$p<0.001$.

TABLE 4.9. Mean relative spleen weights[a] from female BALB/c mice treated with 10 doses of vehicle or busulphan (BU) followed by daily treatment with vehicle or cyclosporin A (CsA)[b]

Treatment		BU post dosing period		CsA dosing period			CsA post dosing period				
		1	57	7	14	21	1	8	15	57	113
A1	Vehicle-vehicle	5,987 (1,140)	4,441 (854)	5,164 (585)	5,144 (293)	4,907 (1,115)	4,545 (774)	5,159 (623)	5,641 (1164)	5,419 (245)	4,754 (529)
A2	Vehicle-CsA			5,638 (408)	6,429 (2,605)	5,197 (521)	5,526 (1,887)	4,956 (993)	4,903 (323)	5,619 (653)	4,033 (410)**
B1	BU-vehicle	5,190 (999)	4,083 (526)	5,506 (1,117)	4,821 (567)	4,312 (452)	5,006 (724)	4,863 (1080)	4,698 (558)	4,069 (303)***	3,546 (426)***
B2	BU-CsA			5,196 (465)	5,805 (1,611)	5,493 (1,955)	4,603 (1,007)	4,619 (694)	5,190 (702)	3,887 (390)	3,425 (381)

[a] Values are mean and SD in parentheses; n=4 vehicle-vehicle and vehicle-CsA groups; n=5 BU-vehicle and BU-CsA groups except on day 1 (BU post dosing period) where n=3 BU-vehicle and vehicle-vehicle groups. FL assay not completed on day 7 or 21 of CsA treatment, or day 8 post CsA treatment.

[b] Animals were treated with BU (9.0 mg/kg) on 10 occasions over 21 days. On day 57 post dosing, vehicle-treated (Group A) and BU-treated mice (Group B) were each divided into 2 groups and treated daily for 35 days with vehicle (vehicle-vehicle, Group A1; BU-vehicle, Group B1) or with CsA by gavage at 30 mg/kg (vehicle-CsA, Group A2; BU-CsA, Group B2). Data analysed using Student's t-test. *Significantly different from Group A1, $p<0.05$; **$p<0.01$ ***$p<0.001$. +++Significantly different from Group B1, $p<0.001$.

TABLE 4.10. Mean relative thymus weights[a] from female BALB/c mice treated with 10 doses of vehicle or busulphan (BU) followed by daily treatment with vehicle or cyclosporin A (CsA)[b]

Treatment		BU post dosing period		CsA dosing period			CsA post dosing period				
		1	57	7	14	21	1	8	15	57	113
A1	Vehicle-Vehicle	3,507 (402)	1,713 (211)	2,265 (407)	2,203 (366)	1,964 (486)	1,833 (218)	1,897 (267)	2,250 (551)	2,103 (221)	1,538 (206)
A2	Vehicle-CsA			1,350 (176)***	1,270 (260)***	1,501 (322)*	1,301 (277)**	2,126 (294)	2,259 (159)	2,224 (345)	1,598 (269)
B1	BU-Vehicle	1,797 (349)***	1,810 (350)	1,956 (419)	2,230 (471)	1,708 (148)	1,794 (197)	1,700 (404)	1,729 (213)*	1,569 (224)***	1,516 (202)
B2	BU-CsA			1,408 (192)++	1,576 (222)++	1,281 (339)++	788 (178)+++	1,529 (363)	1,686 (346)	1,606 (351)	1,232 (428)+

[a]Relative organ weight, mg/kg body weight. Values are means and SD in parenthesis. n=6 animals per group except on day 15 post CsA dosing were n=5 for vehicle-CsA and BU-CsA, and on day 113 post CsA dosing were n=7 (BU-CsA) and n=9 (vehicle-CsA).

[b] Animals were treated with BU (9.0 mg/kg) on 10 occasions over 21 days. On day 57 post dosing, vehicle-treated (Group A) and BU-treated mice (Group B) were each divided into 2 groups and treated daily for 35 days with vehicle (vehicle-vehicle, Group A1; BU-vehicle, Group B1) or with CsA by gavage at 30 mg/kg (vehicle-CsA, Group A2; BU-CsA, Group B2). Data analysed using Student's t-test. *Significantly different from Group A1, $p<0.05$; ***$p<0.001$. +Significantly different from Group B1, $p<0.05$; ++$p<0.01$; +++$p<0.001$.

CHAPTER 5: The effect of administering two regimens of busulphan to female BALB/c mice

5.1. INTRODUCTION

Gibson *et al.* (2003) developed a mouse model of busulphan- (BU-) induced chronic bone marrow aplasia (CBMA) using the BALB/c mouse. Mice were treated with BU at 10.50 mg/kg on 10 occasions over 23 days. On days 72 and 112 post dosing BU-treated mice showed characteristic changes similar to aplastic anaemia (AA) in man. In the peripheral blood, erythrocyte, platelet and neutrophil counts were significantly reduced, as was bone marrow cellularity and the number of committed haemopoietic progenitor cells; there was also an increase in bone marrow apoptosis. However, in this study, mortality in the BU-treated mice was high (49%). This model of CBMA was later modified by Turton *et al.* (2006), in a study where BALB/c mice were treated with lower doses of BU (8.25, 9.00 and 9.75 mg/kg) to determine if CBMA could be induced in the mouse without high levels of toxicity and death.

However, in comparison with AA in man, the changes seen by Turton *et al.* (2006), in the peripheral blood and bone marrow of mice with BU-induced CBMA were relatively mild. The development of a mouse model of CBMA, with more significant BU-induced bone marrow injury and peripheral blood changes however is difficult, as increasing the dose of BU increases mortality. Therefore, in an attempt to develop a model of BU-induced CBMA, with significant changes in the peripheral blood and bone marrow, analogous to AA in man, but without significant mortality, we wished to investigate the possibility of administering two regimens of BU to the BALB/c mouse. We proposed that firstly, female BALB/c mice would be treated with 10 doses of BU at 9.0 mg/kg over a 21 day period. At

day 50 post dosing, the BU-treated mice would then be split into 6 groups and treated again with BU (i.e. a second regimen) at 1.0, 2.0, 3.0, 4.0 and 5.0 mg/kg on 10 occasions over 22 days and a sixth group of BU-treated mice would remain untreated. The objective of the experiment was to determine if a second regimen of BU dosing would induce more significant changes in the bone marrow and peripheral blood than the administration of a single BU regimen.

5.2. MATERIALS AND METHODS

5.2.1. Experimental design

231 female BALB/c mice (Charles River, UK Ltd; mean body weight 16.3 g) were divided into 2 groups (Fig. 5.1), control (n=41) and BU-treated (n=190). Mice were treated with 10 doses of BU (9.0 mg/kg) or vehicle by ip injection over a 21 day period (treated on days 1, 3, 5, 7, 9, 11, 14, 16, 18 and 21). On day 51 post dosing, BU-treated mice were divided into 6 groups and left untreated (this group is referred to as 1 x BU; n=28) or given a second regimen of BU. Animals receiving the second regimen of BU were treated with 10 doses of BU by ip injection over 22 days (day 1, 3, 6, 8, 10, 13, 15, 17, 20 and 22) at 1.0, 2.0, 3.0, 4.0 or 5.0 mg/kg (n=27 per group); these mice were therefore dosed from 51 to 72 days after the first regimen ended. On day 1 and 50 after the first regimen and at day 73, 126, 140 and 154 after the final dose of BU of the first regimen (Fig. 5.1), animals (n=4 to 8 per group) were killed for blood and bone marrow investigations (as described in Chapter 2.3); day 73, 126, 140 and 154 after the final dose of BU of the first regimen are equivalent to day 1, 54, 68 and 82 after the final dose of BU of the second regimen.

5.2.2. Processing of samples

On day 1 and 50 after the first BU regimen, and at 73, 126, 140 and 154 after the first regimen, a full blood count was performed and a femoral marrow flush into 5 ml Iscove's modified Dulbecco's medium (IMDM) was used to measure the bone marrow cellularity (femoral nucleated cell count; FNCC) (as described in Chapter 2.4). The second

femur was placed in 5 ml IMDM supplemented with 10% foetal calf serum and was used to measure the clonogenic potential of the bone marrow and to assess levels of apoptosis (as described in Chapter 2.6 and 2.7). Serum from control and BU-treated animals was prepared to measure the concentration of the cytokine fms-like tyrosine kinase 3 (FLT-3) ligand (FL) (as described in Chapter 2.8). At each autopsy the spleen, liver, both kidneys and thymus were removed, weighed and placed in fixative, and the sternum was also placed in fixative (as described in Chapter 2.9).

5.2.3. Statistical analysis

BU-treated (1 x BU) and control (vehicle-treated) groups were compared using a one tailed Student's t-test (Microsoft Excel; Microsoft Ltd, Microsoft UK, Reading UK). On days 73, 126, 140 and 154 post dosing mice treated with 1 x BU and a second regimen of BU at 1.0, 2.0, 3.0, 4.0 and 5.0 mg/kg were analysed using a one-way analysis of variance (ANOVA) followed by Tukeys highest significance test for post hoc pairwise multiple comparison. In cases of violation of the assumptions for parametric testing, the Kruskal-Wallis test was used in combination with Dunn's post-test. Statistical analysis was performed using GraphPad Prism version 4.00 for windows (GraphPad Software, San Diego, California, USA).

5.3. RESULTS

5.3.1. Body weight changes and clinical signs

On day 1 of the first BU dosing regimen (9.0 mg/kg) the mean body weight of control (vehicle-treated) mice was 15.9 g (n=41) and 16.4 g for BU-treated mice (n=190). At the end of the dosing period (day 21), the mean body weight of control (vehicle-treated) mice had increased by 17.6% to 18.7 g. However, the mean body weight of BU-treated mice increased to 17.6 g resulting in the lower percentage increase of 7.3%.

On day 51 post dosing, BU-treated mice were divided into 6 groups and left untreated (1 x BU group) or treated with a second regimen of BU at 1.0, 2.0, 3.0, 4.0 or 5.0 mg/kg. During this 22 day dosing period the mean body weight of the untreated (1 x BU) mice, and mice treated with a second regimen of BU at 4.0 and 5.0 mg/kg, did not increase. However, small increases in mean body weight were seen in animals treated with 1.0, 2.0 and 3.0 mg/kg of BU.

During the first regimen of BU dosing, all animals maintained a good state of health and there were no instances of mortality. However, between days 14 to 48 after the final dose of BU (first regimen), 13 mice out of a total of 190 were found dead or were killed *in extremis*; all such animals are referred to as inter-current death (ICD) animals. However, mortality in BU-treated mice continued into the second BU dosing regimen (day 51 to 72), a further 2 deaths occurred in the 1 x BU treatment group. In animals treated with a second regimen of BU, 2 animals died in the 1.0 mg/kg group, 3 in the 2.0 mg/kg group, 0 in the 3.0 mg/kg group, 5 in the 4.0 mg/kg group, and 8 in the 5.0 mg/kg group (a total of 20 ICD mice). The high incidence of mortality in animals treated with 5 mg/kg led to a reduced

number of animals in this group, and as a consequence, there were not sufficient animals to autopsy at the final time point (day 154).

5.3.2. Haematology results

On day 1 post dosing (after the first regimen) the effects of BU administration on the peripheral blood and bone marrow were relatively mild with significant reductions confined to the platelet, WBC, neutrophil, monocyte and eosinophil counts (Table 5.1). All other parameters showed small changes (not statistically significant; NS) or were approximately comparable to control values; however MCH and MCHC values were significantly elevated.

However, on day 50 post dosing (Table 5.1), the RBC count was significantly reduced in BU-treated mice (to 94.0% of the control mean) and Hb and HCT were also reduced (NS). In addition, the MCV and MCH were significantly increased ($p<0.01$). The platelet count was significantly reduced (to 66.0% of control) in BU-treated mice. At this time point, the WBC, neutrophil, lymphocyte and monocyte counts were also significantly reduced (the reductions were to 44.0%, 45.2%, 43.4% and 33.3% of the control mean, respectively).

On day 73 post dosing (i.e. at day 1 after the second BU dosing regimen), in the 1 x BU group mice, there were significant reductions in RBC, platelets, WBC, neutrophils, lymphocytes, and monocytes, when the data was compared with vehicle-treated control mice (Table 5.2). The MCV and MCH were also slightly elevated ($p<0.05$ and $p<0.01$, respectively) in the 1 x BU group at this time point.

On day 73 post dosing, in mice treated with a second regimen of BU at 1.0, 2.0, 3.0 or 4.0 mg/kg, in comparison with the 1 x BU group mice, the peripheral blood counts were

approximately similar; there were not statistically significant changes (Table 5.2). However, in animals treated with a second regimen of BU at 5.0 mg/kg (Table 5.2), in comparison with 1 x BU group mice, statistically significant reductions were seen in monocyte and eosinophil parameters; the platelet count was also reduced ($p<0.05$).

On day 126 post dosing (54 days after the last BU dose of the second regimen) (Table 5.3), the erythrocyte parameters (RBC, Hb and reticulocytes) were reduced in mice treated with 1 regimen of BU (1 x BU) in comparison with the vehicle (control) animals. Similarly, the platelet count was reduced, to 48.3% of the vehicle (control) mean ($p<0.01$). The WBC, lymphocyte, monocyte and eosinophil counts were also reduced in the 1 x BU group in comparison with the vehicle (control) group.

In general, at the 126 day time point, the changes in the peripheral blood counts of mice treated with a second regimen of BU were similar to mice treated with 1 regimen of BU (Table 5.3). Indeed, no statistically significant differences were identified between the 1 x BU group and mice treated with a second regimen of BU at 1.0, 2.0, 3.0, 4.0 or 5.0 mg/kg.

On day 140 post dosing (68 days after the final dose of BU of the second regimen), erythrocyte parameters including RBC, Hb, HCT and reticulocytes were reduced, and the MCV increased, in 1 x BU mice in comparison with the vehicle (control) animals (Table 5.4). At this time, the WBC, neutrophil and lymphocyte counts were comparable to the vehicle-treated controls. The platelet count was significantly reduced to 70.8% of the control mean ($p<0.001$); the MCV was increased ($p<0.05$).

The peripheral blood counts of mice treated with a second regimen of BU at 1.0, 2.0, 3.0, 4.0 or 5.0 mg/kg were reduced in line with values seen in the 1 x BU group; there were no statistcally significant differences between mice treated with a second regimen of BU and the 1 x BU group.

On day 154 post dosing (82 days after the final BU dose of the second regimen) (Table 5.5), reductions in the RBC were seen, and WBC, lymphocytes, monocytes and eosinophils were reduced, and platelets were also reduced, in mice treated with 1 regimen of BU in comparison with vehicle (control) animals.

At this time point, the peripheral blood counts of mice treated with a second regimen of BU continued to be comparable to values in the 1 x BU group.

5.3.3. Femoral nucleated cell count

Following the administration of the first BU regimen, the FNCC was significantly reduced in BU-treated mice at day 1 post dosing, to 68.8% of the control mean ($p<0.01$, Table 5.1). On day 50 post dosing, the mean FNCC value in the BU-treated animals was comparable to the controls (Table 5.1).

On day 73 post dosing (at day 1 after the last dose of the second BU regimen), the mean FNCC of the 1 x BU group mice was comparable to the vehicle dosed (control) animals (Table 5.2). The FNCC of mice treated with a second regimen of BU at 1.0, 2.0, 3.0, 4.0 and 5.0 mg/kg were comparable to the 1 x BU group. However, in animals treated with BU at 5.0 mg/kg the mean FNCC was reduced to 33.0% of the mean vehicle (control) value (NS).

On day 126 post dosing (i.e. at 54 days after the final dose of the second regimen), the FNCC was significantly reduced in the 1 x BU group mice to 65.4% of the vehicle (control) mean ($p<0.001$, Table 5.3). However, the mean FNCC in mice treated with a second regimen of BU at 1.0, 2.0, 3.0, 4.0 and 5.0 mg/kg was not different to the 1 x BU group animals.

On day 140 post dosing (i.e. at 68 days after the final dose of the second regimen), the FNCC of the 1 x BU group mice was significantly reduced ($p<0.01$, Table 5.4) in comparison with the vehicle (control) group. At this time, as at day 73 and 126 post dosing, the mean FNCC of mice treated with a second regimen of BU at 1.0, 2.0, 3.0, 4.0 or 5.0 mg/kg was comparable to the mean value in 1 x BU group mice (Table 5.4).

The mean FNCC in the 1 x BU group mice was reduced in comparison to the vehicle (control) group value on day 154 post dosing ($p<0.05$; Table 5.5) and again, as previously at day 73, 126 and 140, the mean FNCC of mice treated with a second regimen of BU (1.0, 2.0, 3.0 and 4.0 mg/kg) were not significantly different from the mean value in the 1 x BU group mice.

5.3.4. Bone marrow clonogenic assay

On day 1 post dosing (first BU regimen), the number of colonies per femur was significantly reduced in BU-treated mice to 16.5% of the control mean ($p<0.001$) (Table 5.6). Clonogenic assays were not carried out on day 50 post dosing (first BU regimen).

On day 73 post dosing (i.e. day 1 post dosing of the second BU dosing regimen), the number of colonies per femur in 1 x BU mice was comparable to the vehicle (control) group values (Table 5.6). At this time (i.e. day 1 after the second BU dosing regimen) the mean number of CFU-C per femur of mice treated with a second regimen of BU at 1.0, 2.0, 3.0 and 4.0 mg/kg were comparable to the 1 x BU group mice. However, mice treated with a second regimen of BU at 5.0 mg/kg had a significantly lower number of CFU-C per femur compared to the 1 x BU group mice ($p<0.001$).

On days 126, 140 and 154 (i.e. day 54, 68 and 82, respectively, after the second BU regimen), the number of committed progenitor cells in the bone marrow of the 1 x BU

group mice was significantly reduced to 38.7% ($p<0.01$), 50.7% ($p<0.001$) and 67.4% ($p<0.05$) of the vehicle (control) mean, respectively (Table 5.6). At these time points the number of committed progenitor cells in the bone marrow of mice treated with a second regimen of BU (1.0 to 5.0 mg/kg) was not significantly different from the 1 x BU group mice.

5.3.5. Apoptosis

On day 1 post dosing (first BU regimen), the level of apoptosis was significantly increased in BU-treated mice to 132.3% of the vehicle (control) mean ($p<0.01$) (Table 5.7). Assays for the level of apoptosis were not carried out on day 50 post dosing (first BU regimen).

On day 73 post dosing (i.e. day 1 post dosing of the second BU dosing regimen), apoptosis was significantly decreased in the bone marrow of the 1 x BU group mice ($p<0.05$). The level of apoptotic cell death was also reduced in mice treated with a second regimen of BU at 1.0, 2.0, 3.0 and 5.0 mg/kg, with values being comparable to the 1 x BU group mice. However, the level of apoptosis in mice treated with a second regimen of BU at 4.0 mg/kg was significantly lower than the mean value of the 1 x BU group mice ($p<0.05$).

On day 126 post dosing (i.e. 54 days post dosing of the second BU dosing regimen), apoptosis in the bone marrow of the 1 x BU group mice was increased significantly in comparison to values in the vehicle-treated control mice (Table 5.7). The level of apoptosis in the bone marrow of mice treated with a second dose of BU at 1.0, 2.0 and 3.0 mg/kg was not statistically different from the mean value in the 1 x BU group mice. However, a significantly lower level of bone marrow apoptosis was evident in mice treated with a

second regimen of BU at 4.0 and 5.0 mg/kg in comparison to the 1 x BU group mice ($p<0.05$).

On day 140 post dosing (i.e. at 68 days post dosing of the second BU dosing regimen), apoptosis continued to be significantly increased in the 1 x BU group mice in comparison to the vehicle-treated control values ($p<0.05$). An increased level of apoptosis was also evident in mice treated with a second dose of BU at 1.0, 2.0, 3.0, 4.0 and 5.0 mg/kg, with mean values being comparable to the values seen in the 1 x BU group mice.

On day 154 post dosing (i.e. at 82 days post dosing of the second BU dosing regimen), the level of apoptosis in the bone marrow of the 1 x BU group mice was comparable to the mean vehicle control value. At this time the level of apoptosis in the bone marrow of mice treated with a second regimen of BU were not statistically different from values seen in the 1 x BU group mice (Table 5.7).

5.3.6. Serum fms-like tyrosine kinase 3 (FLT-3) ligand FL

On day 1 post dosing of the first BU regimen, the level of FL in the serum was significantly increased in BU-treated mice to 1359.3 pg/ml compared to 376.0 pg/ml in vehicle-treated controls (361.5% of the control mean, $p<0.001$) (Table 5.8). This increase in serum FL in BU-treated mice continued, and was also seen on day 50 post dosing with the concentration of FL being 318.3% of the vehicle-treated control mean ($p<0.01$).

On day 73, 126, 140 and 154 post dosing (i.e. on day 1, 54, 68 and 82 after the second BU regimen), the concentration of FL in the serum of mice treated with 1 regimen of BU (1 x BU group) reached a plateau and remained significantly elevated, being 192.1% ($p<0.001$), 269.9% ($p<0.01$), 292.2% ($p<0.05$) and 303.7% ($p<0.01$) of the vehicle control mean, respectively.

The concentration of serum FL in mice treated with a second regimen of BU at 3.0 or 4.0 mg/kg was also increased, but the values were not statistically different from the mean concentration of serum FL in the 1 x BU group mice. Serum FL was not assayed in animals given a second regimen of BU at 1.0, 2.0 or 5.0 mg/kg.

Serum from BU-treated mice was assayed for the detection of IL-2, TNF-α and IFN-γ on days 1, 50, 126 and 154 post dosing. The concentration of these cytokines in the serum was, however, below the detectable levels of the kit (data not shown).

5.3.7. Results summary

- Sustained reduction in erythropoiesis, thrombopoiesis and lymphopoiesis with transient reductions in neutrophils.

- Reduced bone marrow cellularity and CFU-Cs with elevated apoptosis.

- Sustained increase in serum FL.

- In comparison to mice treated with 1 regimen of BU, similar haematological changes were evident in mice treated with 2 regimen of BU at 1.0, 2.0, 3.0, 4.0 and 5.0 mg/kg.

5.4. DISCUSSION

In the present experiment, the haemotoxic effects of administering 2 regimens of BU were assessed in the BALB/c mouse. The objective was to identify if 2 regimens of BU would induce a more significant effect on the bone marrow, compared with a single regimen. During the first dosing regimen, mice were treated with BU at 9.0 mg/kg on 10 occasions over 21 days.

On day 1 post BU dosing (first regimen), bone marrow toxicity was evident with significant reductions in peripheral blood parameters, bone marrow cellularity and the number of committed haemopoietic progenitor cells. In addition at this time, apoptosis and serum FL were also significantly increased. However, on day 50 post dosing, many peripheral blood and bone marrow parameters had returned toward control values. At this time, the BU-treated mice were divided into 6 groups and were left untreated (1 x BU) or were treated with a second regimen of BU at 1.0, 2.0, 3.0, 4.0 or 5.0 mg/kg.

In comparison to the vehicle-treated control mice, noteworthy changes were evident in many peripheral blood parameters of the 1 x BU group mice, including reductions in the RBC, platelets, and WBC and an increase in MCV on days 73, 126, 140 and 154 post dosing (i.e. at 1, 54, 68 and 82 days after the second BU dosing regimen) (Table 5.2 to 5.5). Bone marrow cellularity and the number of committed haemopoietic progenitor cells were reduced, and apoptosis and serum FL increased, during this time.

In general, on days 73, 126, 140 and 154 post dosing, peripheral blood counts, bone marrow cellularity and the number of CFU-C per femur were reduced in mice treated with a second regimen of BU. However, statistical analysis of the data using a one-way analysis of variance (ANOVA), demonstrated no significant difference between the 1 x BU group mice, and mice treated with a second regimen of BU at 1.0 to 5.0 mg/kg.

Haemopoietic cytokines promote the growth and differentiation of haemopoietic cells at all stages of differentiation (Jones and Miller 1989). The ability of cytokines such as stem cell factor (SCF), FL, interleukin-3 (IL-3), granulocyte colony stimulating factor (G-CSF) and thrombopoietin (TPO) to promote haemopoiesis following exposure to radiation has been studied in man, mouse and non-human primates (Herodin and Drouet 2005). Studies have shown that administration of SCF, FL, IL-3 and TPO (50μg/kg) to mice and non-human primates at 2 or 24 hours after exposure to total body irradiation (TBI) increases survival by promoting haemopoietic recovery (Herodin *et al.* 2003; Drouet *et al.* 2004).

The administration of FL alone to rabbits has been shown to have radioprotective properties (Gratwohl *et al.* 1998). In this study by Gratwohl *et al.* (1998), all rabbits exposed to TBI at 1,200 or 1,400 cGy were found dead as a result of myelotoxicity on days 5 to 16 after exposure. In contrast, when FL was administered daily for 14 days to rabbits at 500 μg/kg by subcutaneous injection, beginning 2 days prior to TBI, peripheral blood parameters (leukocytes, platelets, reticulocytes and Hb) were reduced, however, no instances of mortality were recorded. Similarly, stimulation of both the FLT-3 and G-CSF receptors using a dual-receptor agonist (ProGP), has been shown to protect mice from lethal doses of radiation (Streeter *et al.* 2003).

In the present experiment, it is unclear why a second regimen of BU administered starting at 51 days after the first regimen did not have a significant impact on peripheral blood and bone marrow parameters. At autopsy on day 50 post dosing (first regimen), the concentration of FL in the serum of BU-treated mice was significantly increased to 873.5 ± 365.9 pg/ml in comparison to 274.4 ± 72.2 pg/ml in control (vehicle-treated) mice ($p<0.01$) (Table 5.8). It is considered possible that the high concentration of FL in the serum of BU-

treated mice during administration of the second regimen (at day 51 to 72 after the first regimen) may have had a protective effect on the bone marrow thus preventing further BU-induced toxicity.

BU is reported to be especially toxic to cells that are resting and are therefore in the G_0 phase of the cell cycle (Dunn 1974). It is possible that haemopoietc stem cells (HSC) were undergoing extensive proliferation and differentiation following the first BU dosing regimen in order to repopulate the bone marrow. Therefore, when the second regimen of BU was administered on days 51 to 72 after the first BU dosing regimen, many HSCs may have been resistant to the cytotoxic effects of BU as they were not resting in the G_0 phase of the cell cycle, but actively proliferating. To investigate this hypothesis, the present experiment could be repeated and cell cycle analysis performed. If at the time of the second BU dosing regimen HSCs were proliferating rapidly, a more significant injury to the bone marrow may be induced by administering an agent toxic to rapidly dividing cells i.e. 5-fluorouracil.

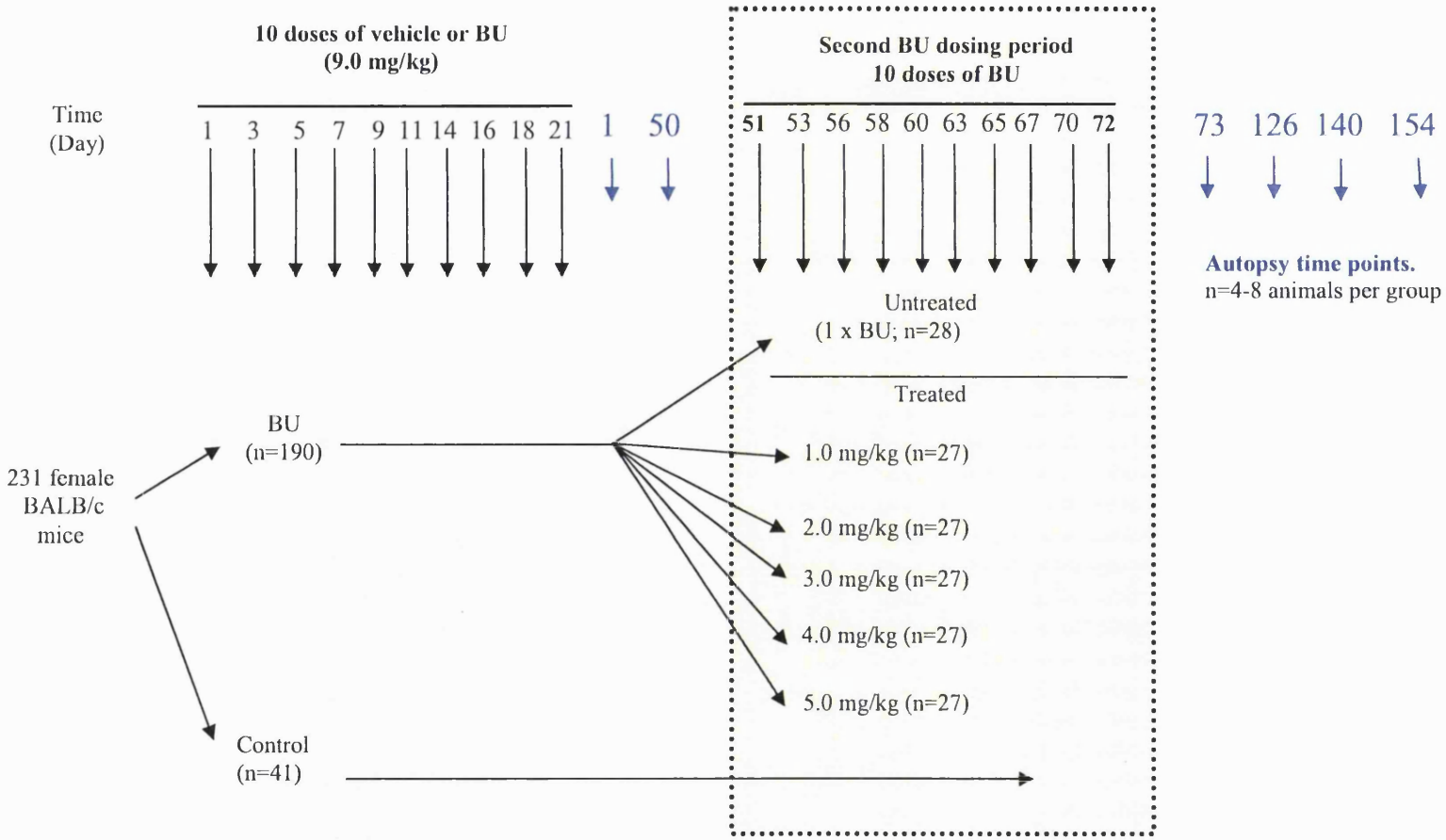


FIGURE 5.1. Experimental design of a study to investigate the effects of administering two regimens of busulphan to female BALB/c mice. 231 BALB/c mice were divided into 2 groups and treated with vehicle (n=41) or BU (n=190) at 9.0 mg/kg by intraperitoneal (ip) injection on 10 occasions over 21 days. On day 51 post BU dosing, the BU-treated group was split in 6 groups and left untreated (1 x BU; n=28) or treated with 10 doses of BU at 1.0, 2.0, 3.0, 4.0 or 5.0 mg/kg. On days 1, 50, 73, 126, 140 and 154 after the end of the first BU dosing regimen, animals (n=4-8 per group) were autopsied for peripheral blood and bone marrow analysis.

TABLE 5.1. Haematological results[a] from female BALB/c mice treated with 10 doses of busulphan (BU) at 9.0 mg/kg over a period of 21 days and sampled at day 1 and 50 after the final dose[b]

	Day of sampling			
	1		50	
	Control	**BU**	**Control**	**BU**
RBC	9.49 (0.18)	9.20 (0.41)	9.83 (0.30)	9.24 (0.16)**
Hb	15.1 (0.2)	15.0 (0.6)	15.2 (0.5)	14.7 (0.2)
HCT	48.7 (1.1)	47.3 (2.1)	48.5 (1.5)	47.0 (0.5)
MCV	51.3 (0.8)	51.5 (0.8)	49.3 (0.4)	51.0 (0.4)**
MCH	15.9 (0.2)	16.3 (0.2)*	15.4 (0.1)	15.9 (0.1)**
MCHC	309.3 (6.3)	317.8 (4.9)***	312.5 (1.5)	312.3 (2.1)
Retic	368 (59)	324 (96)	322 (52)	314 (16)
Plt	953 (111)	321 (132)**	955 (37)	630 (73)**
WBC	1.46 (0.30)	1.13 (0.94)*	3.41 (1.17)	1.50 (0.29)**
Neut	0.25 (0.06)	0.16 (0.12)*	0.62 (0.18)	0.28 (0.07)**
Lymph	1.11 (0.27)	0.94 (0.80)	2.58 (0.95)	1.12 (0.33)*
Mono	0.02 (0.01)	0.01 (0.01)*	0.06 (0.03)	0.02 (0.01)*
Eo	0.06 (0.03)	0.02 (0.02)**	0.13 (0.04)	0.08 (0.04)
Baso	0.00 (0.00)	0.00 (0.00)	0.01 (0.01)	0.00 (0.00)
FNCC	4.10 (0.62)	2.82 (0.83)**	3.86 (0.68)	3.19 (0.66)

[a]Values are means, SD in parentheses; n=6 control and BU-treated mice, except at day 50, were n=4 BU-treated mice.

[b]Abbreviations and units: RBC, red blood cells, $x10^6/\mu l$; Hb, haemoglobin, g/dl; HCT, haematocrit, %; MCV, mean cell volume, fl; MCH, mean cell haemoglobin, pg; MCHC, mean cell haemoglobin concentration, g/dl; Retic, absolute reticulocyte count, $x10^3/\mu l$; Plt, platelets, $x10^3/\mu l$; WBC, white blood cells, $x10^3/\mu l$; Neut, neutrophils, $x10^3/\mu l$; Lymph, lymphocytes, $x10^3/\mu l$; Mono, monocytes, $x10^3/\mu l$; Eo, eosinophils, $x10^3/\mu l$; Baso, basophils, $x10^3/\mu l$; FNCC, femoral bone marrow nucleated cell count, $x10^7$.

Data analysed using a Student's t-test. *Significantly different from control $p<0.05$; **$p<0.01$; ***$p<0.001$.

TABLE 5.2. Haematological results[a] from female BALB/c mice treated with 10 doses of busulphan (BU) at 9.0 mg/kg over a period of 21 days (first regimen), followed by a recovery period of 50 days; BU-treated mice were then either left untreated (1 x BU group) or treated with a second regimen of 10 doses of BU at 1.0, 2.0, 3.0, 4.0 or 5.0 mg/kg over a period of 22 days (day 51 to 72); animals were then sampled on day 73 (1 day after the last BU dose of the second regimen)[b]

	Dose of BU						
	Vehicle (control)	**1 x BU**	**1.0 mg/kg**	**2.0 mg/kg**	**3.0 mg/kg**	**4.0 mg/kg**	**5.0 mg/kg**
RBC	9.97 (1.33)	9.31 (0.49)*	9.68 (0.34)	9.60 (0.37)	9.39 (0.85)	9.71 (0.43)	8.28 (1.87)
Hb	15.1 (0.3)	14.6 (0.6)	15.2 (0.3)	15.1 (0.4)	14.8 (1.2)	15.0 (0.6)	13.0 (2.4)
HCT	47.6 (2.2)	45.5 (2.1)	47.0 (2.2)	47.7 (1.9)	46.9 (4.5)	47.7 (1.8)	41.1 (7.7)
MCV	47.7 (0.9)	48.9 (0.9)*	48.6 (1.1)	49.7 (1.7)	49.9 (1.6)	49.1 (0.6)	50.2 (3.3)
MCH	15.1 (0.1)	15.7 (0.3)**	15.7 (0.3)	15.8 (0.3)	15.8 (0.5)	15.5 (0.2)	15.8 (1.0)
MCHC	317.3 (6.5)	319.8 (1.5)	323.0 (7.6)	317.8 (6.9)	316.2 (10.3)	316.0 (4.4)	315.7 (4.8)
Retic	345 (79)	353 (95)	358 (32)	295 (22)	354 (177)	343 (55)	268 (65)
Plt	1,010 (72)	691 (151)***	785 (36)	594 (131)	650 (59)	581 (125)	182 (184)+
WBC	3.11 (1.33)	1.46 (0.71)*	1.24 (0.39)	1.80 (0.90)	1.76 (0.37)	1.28 (0.40)	0.93 (0.37)
Neut	0.67 (0.28)	0.38 (0.20)*	0.30 (0.10)	0.47 (0.19)	0.33 (0.10)	0.22 (0.09)	0.15 (0.10)
Lymph	2.21 (0.96)	0.87 (0.43)**	0.85 (0.28)	1.22 (0.73)	1.34 (0.32)	0.99 (0.32)	0.75 (0.27)
Mono	0.05 (0.03)	0.03 (0.02)*	0.02 (0.01)	0.03 (0.02)	0.02 (0.01)	0.01 (0.01)	0.00 (0.01)+
Eo	0.16 (0.08)	0.18 (0.19)	0.07 (0.03)	0.07 (0.02)	0.06 (0.01)	0.05 (0.01)	0.02 (0.01)++
Baso	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
FNCC	4.06 (0.84)	4.08 (0.52)	3.84 (0.90)	2.84 (1.82)	3.40 (1.68)	3.04 (0.66)	1.34 (0.67)

[a] Values are means, SD in parentheses; n=6 animals per group, except were n=5 (1 x BU).
[b]Abbreviations and units: as Table 5.1. 1 x BU mice were compared to vehicle (control) using a one tailed Student's t-test. *Significantly different from vehicle (control), $p<0.05$; **$p<0.01$; ***$p<0.001$. To compare the effects of the administration of a second BU dosing regimen, mice treated with 1 regimen of BU (1 x BU group), and mice treated with a second regimen of BU at 1.0, 2.0, 3.0, 4.0 or 5.0 mg/kg, were analysed using a one-way analysis of variance (ANOVA).+Significantly different from 1 x BU mice, $p<0.05$; ++$p<0.01$.

TABLE 5.3. Haematological results[a] from female BALB/c mice treated with 10 doses of busulphan (BU) at 9.0 mg/kg over a period of 21 days (first regimen), followed by a recovery period of 50 days; BU-treated mice were then either left untreated (1 x BU group) or treated with a second regimen of 10 doses of BU at 1.0, 2.0, 3.0, 4.0 or 5.0 mg/kg over a period of 22 days (day 51 to 72); animals were then sampled on day 126 (54 days after the last BU dose of the second regimen)[b]

	Dose of BU						
	Vehicle (control)	**1 x BU**	**1.0 mg/kg**	**2.0 mg/kg**	**3.0 mg/kg**	**4.0 mg/kg**	**5.0 mg/kg**
RBC	9.69 (0.15)	9.25 (0.22)**	8.89 (0.45)	9.06 (0.24)	9.08 (0.75)	9.39 (0.30)	8.12 (1.40)
Hb	14.7 (0.3)	14.0 (0.4)**	13.9 (0.6)	14.0 (0.2)	14.1 (0.6)	14.7 (0.2)	13.1 (1.4)
HCT	46.5 (1.8)	45.2 (1.2)	45.4 (1.5)	45.6 (0.9)	45.0 (1.9)	46.8 (1.0)	41.9 (4.8)
MCV	48.0 (1.1)	49.0 (0.6)	51.2 (1.0)	50.3 (1.2)	49.7 (2.3)	49.8 (0.9)	51.9 (3.1)
MCH	15.2 (0.1)	15.1 (0.2)	15.7 (0.5)	15.5 (0.3)	15.6 (0.8)	15.7 (0.5)	16.3 (1.2)
MCHC	316.8 (5.6)	308.5 (6.1)*	306.4 (9.3)	307.6 (2.5)	314.0 (4.9)	314.2 (5.5)	313.7 (4.7)
Retic	315 (27)	276 (34)*	256 (40)	264 (28)	288 (27)	271 (51)	281 (89.4)
Plt	916 (84)	442 (235)**	871 (679)	591 (56)	616 (63)	468 (264)	520 (273)
WBC	3.37 (0.90)	2.36 (0.58)*	1.66 (0.56)	2.53 (0.40)	1.59 (0.35)	2.11 (0.83)	1.48 (0.76)
Neut	0.73 (0.17)	0.62 (0.11)	0.44 (0.19)	0.83 (0.14)	0.61 (0.14)	0.47 (0.24)	0.54 (0.42)
Lymph	2.41 (0.72)	1.64 (0.48)*	1.10 (0.38)	1.51 (0.34)	0.84 (0.33)	1.53 (0.56)	0.86 (0.27)
Mono	0.09 (0.02)	0.02 (0.02)**	0.02 (0.02)	0.03 (0.02)	0.02 (0.01)	0.04 (0.02)	0.04 (0.06)
Eo	0.12 (0.05)	0.07 (0.02)*	0.09 (0.03)	0.14 (0.03)	0.12 (0.03)	0.06 (0.03)	0.03 (0.02)
Baso	0.00 (0.01)	0.00 (0.01)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
FNCC	4.22 (0.17)	2.76 (0.64)***	2.46 (1.03)	2.87 (0.42)	3.40 (0.91)	3.40 (0.50)	2.79 (0.77)

[a] Values are means, SD in parentheses; n=5 animals per group, except were n=6 (3 mg/kg), n=4 (1 x BU) and n=3 (5 mg/kg).

[b]Abbreviations and units: as Table 5.1. 1 x BU mice were compared to vehicle (control) using a one tailed Student's t-test. *Significantly different from vehicle (control), $p<0.05$; **$p<0.01$; ***$p<0.001$. To compare the effects of the administration of a second BU dosing regimen, mice treated with 1 regimen of BU (1 x BU group), and mice treated with a second regimen of BU at 1.0, 2.0, 3.0, 4.0 or 5.0 mg/kg, were analysed using a one-way analysis of variance (ANOVA).

TABLE 5.4. Haematological results[a] from female BALB/c mice treated with 10 doses of busulphan (BU) at 9.0 mg/kg over a period of 21 days (first regimen), followed by a recovery period of 50 days; BU-treated mice were then either left untreated (1 x BU group) or treated with a second regimen of 10 doses of BU at 1.0, 2.0, 3.0, 4.0 or 5.0 mg/kg over a period of 22 days (day 51 to 72); animals were then sampled on day 140 (68 days after the last BU dose of the second regimen)[b]

	Dose of BU						
	Vehicle (control)	**1 x BU**	**1.0 mg/kg**	**2.0 mg/kg**	**3.0 mg/kg**	**4.0 mg/kg**	**5.0 mg/kg**
RBC	9.59 (0.10)	9.00 (0.30)**	8.78 (0.63)	8.84 (0.82)	8.76 (0.63)	9.03 (0.45)	8.45 (0.30)
Hb	14.4 (0.3)	13.6 (0.3)**	13.6 (0.6)	13.8 (0.8)	13.8 (0.6)	13.8 (0.2)	13.1 (0.3)
HCT	46.1 (1.0)	44.5 (1.6)*	43.7 (2.0)	44.9 (2.8)	44.6 (1.7)	45.5 (0.8)	42.2 (1.2)
MCV	48.6 (1.3)	49.4 (0.8)*	49.9 (1.4)	50.9 (2.7)	51.0 (2.5)	49.4 (2.2)	50.0 (0.6)
MCH	15.0 (0.2)	15.1 (0.2)	15.6 (0.5)	15.6 (0.8)	15.7 (0.7)	15.3 (0.7)	15.6 (0.3)
MCHC	308.5 (4.9)	305.3 (4.9)	312.0 (4.3)	306.8 (4.0)	308.9 (6.2)	310.2 (1.5)	310.8 (6.4)
Retic	380 (59)	306 (27)*	305 (83)	315 (32)	285 (40)	262 (15)	279 (38)
Plt	961 (28)	680 (42)***	541 (232)	503 (262)	579 (179)	632 (130)	494 (120)
WBC	1.86 (1.29)	1.09 (0.46)	1.18 (0.99)	0.56 (0.09)	1.50 (0.51)	1.24 (0.21)	0.99 (0.46)
Neut	0.34 (0.25)	0.34 (0.15)	0.67 (0.80)	0.18 (0.06)	0.45 (0.17)	0.35 (0.09)	0.38 (0.38)
Lymph	1.36 (1.01)	0.63 (0.35)	0.45 (0.17)	0.36 (0.08)	0.93 (0.30)	0.79 (0.21)	0.55 (0.19)
Mono	0.03 (0.02)	0.01 (0.01)	0.04 (0.04)	0.01 (0.01)	0.03 (0.01)	0.02 (0.01)	0.01 (0.01)
Eo	0.09 (0.02)	0.06 (0.02)	0.05 (0.04)	0.02 (0.01)	0.08 (0.06)	0.05 (0.03)	0.03 (0.01)
Baso	0.00 (0.00)	0.01 (0.01)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
FNCC	4.10 (0.73)	2.88 (0.63)**	2.71 (1.30)	1.93 (1.03)	2.73 (1.08)	2.40 (0.61)	1.94 (0.53)

[a] Values are means, SD in parentheses; n=5 animals per group, except were n=7 (3 mg/kg), n=6 (5 mg/kg) and n=4 (1 x BU).

[b]Abbreviations and units: as Table 5.1. 1 x BU mice were compared to vehicle (control) using a one tailed Student's t-test. *Significantly different from vehicle (control), $p<0.05$; **$p<0.01$; ***$p<0.001$. To compare the effects of the administration of a second BU dosing regimen, mice treated with 1 regimen of BU (1 x BU group), and mice treated with a second regimen of BU at 1.0, 2.0, 3.0, 4.0 or 5.0 mg/kg, were analysed using a one-way analysis of variance (ANOVA).

TABLE 5.5. Haematological results[a] from female BALB/c mice treated with 10 doses of busulphan (BU) at 9.0 mg/kg over a period of 21 days (first regimen), followed by a recovery period of 50 days; BU-treated mice were then either left untreated (1 x BU group) or treated with a second regimen of 10 doses of BU at 1.0, 2.0, 3.0, 4.0 or 5.0 mg/kg over a period of 22 days (day 51 to 72); animals were then sampled on day 154 (82 days after the last BU dose of the second regimen)[b]

	Dose of BU					
	Vehicle (control)	**1 x BU**	**1.0 mg/kg**	**2.0 mg/kg**	**3.0 mg/kg**	**4.0 mg/kg**
RBC	9.89 (0.23)	9.25 (0.82)*	9.27 (0.45)	8.98 (0.60)	9.16 (0.59)	8.80 (0.69)
Hb	14.0 (0.4)	14.4 (0.9)	14.2 (0.6)	13.9 (1.1)	14.5 (0.4)	14.3 (0.5)
HCT	48.2 (1.6)	46.1 (3.2)	45.3 (2.1)	44.6 (3.8)	46.4 (1.7)	45.2 (1.6)
MCV	48.7 (1.2)	49.9 (1.7)	48.9 (1.0)	49.8 (1.5)	50.7 (0.9)	51.5 (2.7)
MCH	15.1 (0.2)	15.5 (0.6)*	15.3 (0.3)	15.5 (0.6)	15.8 (0.3)	16.3 (0.8)
MCHC	309.9 (8.5)	311.6 (6.1)	313.6 (4.7)	310.6 (4.9)	311.7 (4.1)	317.3 (1.9)
Retic	325 (28)	336 (54)	280 (27)	302 (35)	295 (29)	299 (24)
Plt	1,110 (122)	738 (244)***	733 (158)	681 (42)	771 (177)	704 (309)
WBC	2.72 (1.08)	1.22 (0.54)**	1.79 (0.96)	1.49 (0.74)	1.54 (0.48)	1.37 (0.32)
Neut	0.65 (0.34)	0.41 (0.19)	1.07 (0.67)	0.62 (0.34)	0.43 (0.94)	0.41 (0.14)
Lymph	1.86 (0.79)	0.73 (0.36)**	0.62 (0.38)	0.73 (0.32)	0.99 (0.38)	0.88 (0.17)
Mono	0.06 (0.03)	0.03 (0.02)*	0.03 (0.02)	0.03 (0.02)	0.03 (0.02)	0.03 (0.01)
Eo	0.14 (0.07)	0.05 (0.02)**	0.06 (0.03)	0.11 (0.07)	0.08 (0.02)	0.04 (0.02)
Baso	0.00 (0.01)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
FNCC	3.60 (0.84)	2.78 (1.07)*	3.19 (1.05)	3.30 (0.99)	2.84 (1.36)	2.43 (0.90)

[a]Values are means, SD in parentheses; n=7 animals per group, except were n=6 (4 mg/kg) and n=8 (control).

[b]Abbreviations and units: as Table 5.1. 1 x BU mice were compared to vehicle (control) using a one tailed Student's t-test. *Significantly different from vehicle (control), $p<0.05$; **$p<0.01$; ***$p<0.001$. To compare the effects of the administration of a second BU dosing regimen, mice treated with 1 regimen of BU (1 x BU group), and mice treated with a second regimen of BU at 1.0, 2.0, 3.0, or 4.0 mg/kg, were analysed using a one-way analysis of variance (ANOVA). As a result of significant mortality, there were not sufficient animals in the 5.0 mg/kg group to autopsy on day 154 post dosing.

TABLE 5.6. Bone marrow clonogenic assay results[a] from female BALB/c mice treated with 10 doses of busulphan (BU) at 9.0 mg/kg over a period of 21 days (first regimen), followed by a recovery period of 50 days, BU-treated mice were then either left untreated (1 x BU group) or treated with a second regimen of 10 doses of BU at 1.0, 2.0, 3.0, 4.0 or 5.0 mg/kg over a period of 22 days (day 51 to 72). Animals were sampled at day 1, 73, 126, 140 and 154 after the final dose of the first BU dosing regimen (these last 4 time points were at 1, 54, 68 and 82 days after the final dose of the second BU regimen)

	Day of sampling				
Dose of BU	**1**	**73**	**126**	**140**	**154**
Vehicle (control)	14,546 (4,680)	10,277 (3,481)	11,908 (2,965)	11,244 (1,783)	13,978 (4,041)
1 x BU	2,403 (1,451)***	7,948 (1,922)	4,609 (4,778)**	5,698 (2,469)***	9,424 (4,942)*
1.0 mg/kg		8,364 (2,594)	3,610 (2,415)	2,432 (3,082)	8,542 (4,100)
2.0 mg/kg		4,727 (1,968)	5,215 (1,688)	6,298 (2,090)	5,956 (3,809)
3.0 mg/kg		4,985 (1,534)	4,745 (2,141)	6,779 (2,659)	6,125 (4,487)
4.0 mg/kg		4,136 (2,175)	4,309 (2,011)	4,379 (3,694)	3,358 (2,315)
5.0 mg/kg		184 (286)+++	2,161 (2,569)	2,445 (1,748)	-

[a] Results are means and SD in parenthesis. Cultures were set up in duplicate and the mean number of CFU-C per femur calculated per mouse. n=6 animals per group, except on day 126, where n=5 (1.0 and 4.0 mg/kg) and n=3 (5.0 mg/kg). On day 140, n=6 animals per group, except where n=5 (1x BU and 2.0 mg/kg) and n=7 (3.0 and 4.0 mg/kg). On day 154 n=8 per group, except n=6 (4.0 mg/kg) and n=7 (2.0 mg/kg). Bone marrow clonogenic assay was not completed on day 50 after the last dose of the first BU dosing regimen. 1 x BU mice were compared to vehicle (control) animals using a one tailed Student's t-test. *Significantly different from vehicle (control), $p<0.05$; **$p<0.01$; ***$p<0.001$. To compare the effects of the administration of a second BU dosing regimen, mice treated with 1 regimen of BU (1 x BU group), and mice treated with a second regimen of BU at 1.0, 2.0, 3.0, 4.0 or 5.0 mg/kg, were analysed using a one-way analysis of variance (ANOVA).+++Significantly different from 1 x BU mice, $p<0.001$. As a result of significant mortality, there were not sufficient animals in the 5.0 mg/kg group to autopsy on day 154 post dosing.

TABLE 5.7. Apoptosis results[a] from female BALB/c mice treated with 10 doses of busulphan (BU) at 9.0 mg/kg over a period of 21 days (first regimen), followed by a recovery period of 50 days, BU-treated mice were then either left untreated (1 x BU group) or treated with a second regimen of 10 doses of BU at 1.0, 2.0, 3.0, 4.0 or 5.0 mg/kg over a period of 22 days (day 51 to 72). Animals were sampled at day 1, 73, 126, 140 and 154 after the final dose of the first BU dosing regimen (these last 4 time points were at 1, 54, 68 and 82 days after the final dose of the second BU regimen)

	Day of sampling				
Dose of BU	**1**	**73**	**126**	**140**	**154**
Vehicle (control)	9.6 (1.1)	13.2 (1.2)	10.5 (0.9)	9.0 (1.3)	11.3 (0.8)
1 x BU	12.7 (1.8)**	10.8 (2.2)*	23.0 (6.8)***	11.1 (1.7)*	12.5 (2.9)
1.0 mg/kg		12.8 (2.7)	23.6 (4.3)	11.3 (2.3)	12.3 (2.4)
2.0 mg/kg		7.6 (2.0)	24.7 (3.3)	11.5 (3.3)	12.2 (3.5)
3.0 mg/kg		7.2 (1.1)	27.6 (4.6)	10.2 (1.8)	11.3 (2.1)
4.0 mg/kg		5.9 (1.1)+	12.6 (1.9)+	10.6 (3.2)	11.9 (2.6)
5.0 mg/kg		7.9 (5.1)	12.6 (6.0)+	14.0 (3.3)	-

[a] Results are means and SD in parenthesis expressed as a percentage. n=6 animals per group, except on day 126 where n=4 (5.0 mg/kg). On day 140, n=6 animals per group, except where n=5 (3.0, 4.0, 5.0 mg/kg). On day 154, n=8 per group, except n=6 (4.0 mg/kg) and n=7 (2.0 mg/kg). Apoptosis assays were not completed on day 50 after the last dose of the first BU regimen. As a result of significant mortality, there were not sufficient animals in the 5.0 mg/kg group to autopsy on day 154 post dosing. 1 x BU mice were compared to the vehicle (control) mice using a one tailed Student's t-test. *Significantly different from vehicle (control), $p<0.05$; **$p<0.01$; ***$p<0.001$. To compare the effects of the administration of a second BU dosing regimen, mice treated with 1 regimen of BU (1 x BU group), and mice treated with a second regimen of BU at 1.0, 2.0, 3.0, 4.0 or 5.0 mg/kg, were analysed using a one-way analysis of variance (ANOVA).+Significantly different from 1 x BU mice, $p<0.05$.

TABLE 5.8. FL results[a] from female BALB/c mice treated with 10 doses of busulphan (BU) at 9.0 mg/kg over a period of 21 days (first regimen), followed by a recovery period of 50 days, BU-treated mice were then either left untreated (1 x BU group) or treated with a second regimen of 10 doses of BU at 1.0, 2.0, 3.0, 4.0 or 5.0 mg/kg over a period of 22 days (day 51 to 72). Animals were sampled at day 1, 50, 73, 126, 140 and 154 after the final dose of the first BU dosing regimen (these last 4 time points were at 1, 54, 68 and 82 days after the final dose of the second BU regimen)

	Day of sampling					
Dose of BU	**1**	**50**	**73**	**126**	**140**	**154**
Vehicle (control)	376.0 (127.5)	274.4 (72.2)	300.5 (25.6)	265.4 (45.6)	266.8 (45.9)	260.2 (15.4)
1 x BU	1,359.3 (201.5)***	873.5 (365.9)**	577.4 (72.6)***	716.3 (250.9)**	779.6 (462.8)*	790.1 (327.2)**
3.0 mg/kg			648.5 (143.9)	575.6 (127.4)	668.5 (128.9)	777.9 (247.5)
4.0 mg/kg			823.8 (195.3)	649.3 (214.4)	774.2 (257.1)	1,158.7 (338.2)

[a] Results are means and SD in parenthesis. Results expressed as the concentration of FL in serum, pg/ml. n=4 vehicle (ontrol) and n=5 (1 x BU, 3.0 and 4.0 mg/kg groups) except on days 50, 140 and 154 where n=4 (1 x BU group) and on day 126 where n=3 (1 x BU group). Serum FL analysis not completed in animals treated with a second regimen of BU at 1.0, 2.0 or 5.0 mg/kg. 1 x BU mice were compared to vehicle (control) using a one tailed Student's t-test. *Significantly different from vehicle (control), $p<0.05$; **$p<0.01$; ***$p<0.001$. To compare the effects of the administration of a second BU dosing regimen, mice treated with 1 regimen of BU (1 x BU group), and mice treated with a second regimen of BU at 3.0 or 4.0 mg/kg, were analysed using a one-way analysis of variance (ANOVA).

CHAPTER 6: Studies on the haemotoxicity of chlorambucil in the Hanover Wistar rat

6.1. INTRODUCTION

Chlorambucil (CHB) is an aromatic alanine mustard derivative. It was one of the first chemotherapeutic agents to be used in the treatment of malignant disease, and was introduced into clinical practice in 1955 (Dollery 1999). As with other alkylating agents, in man, CHB is particularly toxic to the haemopoietic system with bone marrow depression occurring in the early post dosing period. However, depression of the bone marrow by CHB has been reported to be readily reversible in comparison to other agents such as busulphan (BU) (Reynolds 1989; BNF 2004).

Following the development of a model of chronic hypoplastic marrow failure (CHMF) in the mouse using BU (Morley and Blake 1974a), Morley and his co-workers went on to examine the effects of a wide range of other anti-cancer agents on the bone marrow in the mouse (Trainor and Morley 1976; Trainor *et al.* 1979; Morley 1980). In these experiments CHB was investigated and administered at weekly intervals to mice by intraperitoneal (ip) injection at 5 and 10 mg/kg for 11 weeks. The animals were then observed for 2 to 5 months to assess the potential of CHB to cause a residual bone marrow injury resulting in 'late-stage' CHMF. These experiments demonstrated that the administration of CHB caused a reduction in marrow cellularity, the number of committed progenitor cells (CFU-C) and a reduction in spleen colony forming units (CFU-S) (Trainor and Morley 1976; Trainor *et al.* 1979; Morley 1980). It was concluded from these studies that CHB was, like BU, capable of inducing residual marrow injury and CHMF in the mouse (Trainor *et al.* 1979; Morley 1980).

With the evidence from these reports of Morley and his co-workers in mind (Trainor *et al.* 1979; Morley 1980) the present experiments were devised to assess the toxicity of CHB on rat bone marrow and the potential of the drug to induce 'late-stage' residual bone marrow injury. The objectives were also to characterise the features of bone marrow injury caused by CHB in the immediate post dosing period, to describe the patterns of change as the various haematological parameters returned towards normal, and finally, to define the changes in apoptotic marrow cells, CFU-C, and serum fms-like tyrosine kinase 3 (FLT-3) ligand (FL) in relation to the other parameters being investigated.

In addition, although apoptosis in the nucleated bone marrow cells of patients with aplastic anaemia (AA) has previously been investigated (Philpott *et al.* 1996), and similar studies have been carried out in the mouse (Gibson *et al.* 2003), comparable studies using bone marrow cells in the rat do not appear to have been reported. For this reason we decided to carry out the present study on CHB haemotoxicity in the Hanover Wistar rat, rather than in the mouse. Accordingly, a preliminary experiment was conducted to validate the use of 7-amino actinomycin D (7-AAD) staining in the assessment of apoptosis in nucleated bone marrow cells of the rat. Rat bone marrow cells were subjected to overnight incubation in culture medium with or without added nutrients. The end result of nutrient withdrawal would be an increase in apoptotic cell death which should be detectable with 7-AAD staining. Also, a preliminary dose ranging study was performed to determine an appropriate dose level of CHB to use in the main study, with the drug administered by the ip route.

A report of the present investigations on CHB has been published (Molyneux *et al.* 2004a).

6.2. MATERIALS AND METHODS

6.2.1. Measurement of apoptosis in rat bone marrow samples

A control (untreated) female Hanover Wistar rat (B & K Universal Ltd., Grimston, Aldbrough, Hull) (143 g) was autopsied and bone marrow flushed from both left and right femora into 10 ml Iscove's modified Dulbecco's medium (IMDM). Immediately (time 0 h), a 200 µl aliquot (Sample A) was removed from the bone marrow cell suspension and stained with 7-AAD (using the method described in Chapter 2.7) and the number of live, apoptotic and dead cells assessed.

The remaining bone marrow suspension was then divided into two approximately 5 ml aliquots. The cell suspensions were centrifuged and cells resuspended in IMDM supplemented with 10% foetal calf serum (FCS) (Sample B) or IMDM without FCS (Sample C), respectively. Both samples were then stored overnight (18 h) at 37°C in 5% CO_2 in air. After this 18 hour incubation, 200 µl aliquots were removed from Sample B and Sample C for the detection of apoptotic cells using 7-AAD staining.

6.2.2. Experiments on the haemotoxicity of chlorambucil

6.2.2.1. Experimental design

Experiment 1. Preliminary dose ranging study. CHB was prepared (as described in Chapter 2.2) and administered to female Hanover Wistar rats (B & K Universal Ltd.) at 5.0, 5.5, 6.0 and 7.0 mg/kg (n=3 per group). Animals were treated with 8 ip doses of vehicle (control n=4) or CHB over an 18 day period at a dose volume of approximately

0.8 to 1.2 ml/rat. On day 82 post dosing, all animals were autopsied for blood and bone marrow investigations (as described in Chapter 2.3).

Experiment 2. Main chlorambucil study. 140 female Hanover Wistar rats, mean body weight 149.6 g were divided into 2 groups, n=68 (control, vehicle-dosed) and n=72 (CHB-treated). Animals were treated with 6 ip doses of vehicle or CHB (6.3 mg/kg) over a period of 18 days (day 1, 4, 6, 11, 14, 18) at a dose volume of approximately 0.8 to 1.2 ml/rat. On days 1, 3, 9, 16, 24, 38, 45, 59 and 65 post dosing, n=6 to 8 animals per group were killed for the examination of blood, bone marrow and other organs and tissues (as described in Chapter 2.3).

6.2.2.2. Processing of samples

Experiment 1. Preliminary dose ranging study. At autopsy a full blood count was performed and a femoral marrow flush into 5 ml PBS was prepared to assess bone marrow cellularity (femoral nucleated cell count; FNCC) (as described in Chapter 2.4).

Experiment 2. Main Chlorambucil Study. At autopsy a full blood count was performed and a femoral marrow flush into 5 ml PBS was prepared to measure the FNCC (as described Chapter 2.4). A tibial marrow smear was also prepared, stained and a differential count performed (as described in Chapter 2.5). The second femur was placed in 5 ml sterile PBS and processed to assess the clonogenic potential of the bone marrow and the level of apoptosis (as described in Chapter 2.6 and 2.7). Serum was prepared from the blood to measure levels of the cytokine FL (as described in Chapter 2.8). The spleen was removed, weighed and fixed, as was the sternum, for histological examination (as described in Chapter 2.9).

6.2.3. Statistical analysis

Experiment 2. Main chlorambucil study. CHB-treated and control (vehicle-treated) groups were compared using a one tailed Student's t-test (Microsoft Excel; Microsoft Ltd, Microsoft UK, Reading UK).

6.3. RESULTS

6.3.1. Measurement of apoptosis in rat bone marrow using 7-amino actinomycin D staining

Using 7-AAD staining, the proportion of live, apoptotic and dead cells (as a percentage, excluding cell debris) were measured in rat bone marrow cells. At time 0 h, the percentage of cells identified as live, apoptotic and dead was 95.8%, 2.2% and 2.0%, respectively (Sample A) (Table 6.1; Fig. 6.1 A). Following an 18 h incubation at 37°C in culture medium (IMDM) supplemented with 10% FCS (Sample B), the percentage of cells identified as live was reduced to 86.9%. Apoptotic and dead cell populations were increased in Sample B to 4.9% and 8.2%, respectively (Table 6.1; Fig. 6.1 B). When rat bone marrow cells were maintained for 18 hours at 37°C in serum deprived medium (IMDM without 10% FCS; Sample C) the percentage of live cells identified with 7-AAD staining was reduced to 63.5%. Populations of apoptotic and dead cells were increased to 10.9% and 25.6%, respectively (Table 6.1; Fig. 6.1 C). In comparison to rat bone marrow cells maintained for 18 hours at 37°C in IMDM supplemented with 10% FCS (Sample B) the percentage of apoptotic and dead cells in Sample C were therefore increased.

6.3.2. Preliminary dose ranging study

CHB was administered at 0, 5.0, 5.5, 6.0 and 7.0 mg/kg on 8 occasions over 18 days. At all dose levels CHB was well tolerated. At autopsy on day 82 post dosing, peripheral blood and bone marrow counts of CHB-treated rats were comparable to

controls (Table 6.2). A dose level of 6.3 mg/kg was selected as an appropriate dose of CHB to use in the main study.

6.3.3. Main chlorambucil study

6.3.3.1. Clinical signs and body weight changes

During the 18 day period of CHB administration there were no clear clinical signs of drug-induced toxicity, although body weight gain was adversely affected (Fig. 6.2). However, two animals treated with CHB were unexpectedly found dead, one on the ninth day of the dosing period (after 3 CHB doses), and one at 5 days after the final CHB dose; the reasons for these mortalities could not be identified. At the beginning of the dosing period, control animals (n=68) weighed on average 155.0 g, and CHB rats (n=72) were 155.1 g. During the period of CHB dosing, the mean body weight of control and CHB treated animals increased by 25.4% to 194.4 g and 21.1% to 173.7 g, respectively. At the end of the study (day 65 post dosing); the mean body weight for CHB-treated rats was comparable to control values.

6.3.3.2. Haematology results

On days 1 and 3 post dosing, the RBC, Hb and HCT were significantly reduced in CHB-treated animals (Table 6.3); the average percentage reductions for these parameters at these times were of a similar magnitude, being 93.4% (RBC), 92.5% (Hb) and 93.0% (HCT) of the control mean values. MCV, MCH and MCHC were unaffected on days 1 and 3 post dosing, and platelet counts were slightly elevated in CHB-treated rats at these time points, however, this increase in platelets was not statistically

significant. WBC and lymphocyte counts were significantly decreased at day 1 and day 3; the reductions in lymphocytes were to 7.2% and 6.4% of the control means at these time points, respectively. Monocytes were significantly reduced at day 3 in CHB-dosed rats; neutrophils were unaffected. Reticulocyte counts of CHB-treated rats were increased on day 1 and day 3 post dosing however, this change was only statistically significant on day 1.

On day 9 post dosing, the RBC was significantly decreased in CHB-treated rats in comparison with the control mean, however, there was evidence of recovery and a return towards normal. The HCT was also returning towards normal at day 9, and the Hb level was normal. However, MCV and MCH were significantly elevated at this time point, as were the mean platelet and reticulocyte counts. Lymphocytes and WBC were still significantly reduced on day 9 to 47.2% and 20.0% of the control mean, respectively ($p < 0.001$). However, significant increases were evident in the neutrophil, monocyte and eosinophil counts.

On day 16 post dosing, mean values for the RBC, Hb and HCT were normal in CHB-treated rats, as were neutrophil, monocyte and eosinophil counts; but MCV and MCH, and platelets and reticulocytes, were still significantly raised. Lymphocytes and WBC were significantly reduced at day 16 post dosing (the lymphocyte count was 25.3% of the control). At 24, 38, 45, 59 and 65 days post dosing, many parameters in CHB-treated rats were comparable with the controls; however, lymphocytes and WBC continued to show significant reductions at 24, 59 and 65 days; the decreases in the lymphocyte counts at these 3 time points were to 44.4%, 68.6% and 60.8% of control values, respectively.

6.3.3.3. Femoral nucleated cell count

The FNCC was significantly reduced in CHB-treated rats on days 1, 3 and 9 post dosing to 53.3%, 77.6% and 25.9% of the control mean value, respectively (Table 6.3). On day 16 post dosing the FNCC was increased in CHB-treated rats however, this change was not statistically significant. This increase in bone marrow cellularity continued, being statistically significant on days 24, 45 and 59 post dosing. At 65 days the FNCC was comparable to the control value.

6.3.3.4. Bone marrow clonogenic assay

On days 1, 3, 9 and 16 post dosing the number of committed progenitor cells (CFU-C) in the femoral bone marrow of CHB-treated rats was significantly reduced to 51%, 57%, 48% and 55% of the control mean values, respectively (Fig. 6.3). On, and after, day 24, the mean number of CFU-C was at approximately normal levels and did not differ significantly from the controls. However, at day 59 the number of CFU-C was significantly reduced in CHB-treated rats before returning to a value comparable to controls on day 65.

6.3.3.5. Apoptosis

In the immediate post dosing period, days 1 and 3, the level of apoptosis in the femoral bone marrow was significantly increased to 195.3% and 202.0% of the control means, respectively (Fig. 6.4). This increase in apoptosis immediately post dosing was also evident at day 9 and 16 post dosing, however, the levels of apoptosis appeared to be returning towards control values at these time points. On day 24 post dosing, the level of apoptosis in CHB-treated rats was comparable to controls, but was significantly

increased on day 38 post dosing. On days 45 and 65 post dosing the level of apoptosis was normal in CHB-treated rats.

6.3.3.6. Serum fms-like tyrosine kinase 3 ligand

The concentration of FL was measured in the serum of rats on days 1, 3, 9, 16, 24, 45 and 65 post dosing (Fig. 6.5). On day 1 post dosing, the serum concentration of FL was significantly reduced in CHB-treated rats to 82.1% of the control mean. However, on days 3, 9, 24 and 45 post dosing, levels of FL in CHB-treated rats were increased significantly above the control levels before returning to a value comparable to controls on day 65.

6.3.3.7. Bone marrow differential counts

The overall picture in the immediate post dosing period (day 1, 3 and 9) was of reductions in myeloid, erythroid and lymphoid cells (Table 6.4). The average decreases, over these 3 time points, for the myeloid cells was to 56.2% of the controls, and for lymphoid cells, 25.1%. The data for erythroid cells at day 3 post dosing showed an increased count. However, the mean reduction in erythroid cells at day 1 and 9 was to 44.4% of the control count. By days 16 and 24 post dosing, the cell counts for each lineage, including lymphoid cells, had returned to normal, and indeed, the counts were in general, slightly higher than the control values. At 38 days, overall values for all three lineages were similar to the control results; however at day 45, the counts for myeloid, erythroid and lymphoid cells in CHB-dosed rats were increased above the controls. At 59 days post dosing the count for lymphoid cells was significantly higher than the controls. Indeed, if the counts for the lymphoid lineage at the 6 time points from 16 to 65 days are taken, the average count for the control animals is 2.265 ($x10^7$),

and for CHB-treated animals, 2.635 ($x10^7$); that is, the value for CHB-treated rats is 116.3% of the control figure.

Examination of the marrow smears of CHB-dosed animals at day 1 post dosing showed that although the overall picture was one of depletion of the cells in the myeloid, erythroid and lymphoid lineages, in some individual animals there was evidence of myeloid hyperplasia. Also, in some individual rats, the myeloid precursors showed evidence of a 'right shift' with a preponderance of mature forms, or a 'left shift' with preponderance of immature forms.

6.3.3.8. Histology of the sternum and spleen

Sections of the sternum and spleen from control rats, and from 3 animals treated with CHB and killed at days 1, 9, 24 and 65 post dosing were examined. Sternal marrow sections at day 1 post dosing showed a modest decrease in the cellularity of the marrow, in association with an increase in the number of adipocytes. These changes were also present in 1 of the 3 animals examined at day 9. No abnormalities were noted in the sternal marrow at 24 and 65 days.

The relative weight of the spleen was reduced (NS) at day 1 post dosing in CHB-treated rats (Table 6.3); at days 3, 9 and 16, the spleens from CHB-dosed animals were significantly lighter than the controls. At 24, 38 and 45 days post dosing, the spleen weight was elevated in CHB-treated rats (NS at 38 days), but the weight compared with the control animals at 59 and 65 days.

Histological sections of the spleen of CHB-dosed rats showed severe depletion of the white pulp at day 1 post dosing; all parts of the white pulp were affected, including the periarteriolar lymphoid sheath (PALS) and the marginal zone. Mild sinusoidal congestion was also present in the red pulp at this time. At day 9, depletion

of the white pulp was still prominent in CHB animals. However, by day 24 post dosing, considerable regeneration had occurred, although there was still some evidence of mild lymphocyte depletion at this time. At day 65, the white pulp in CHB-dosed animals was normal in appearance.

6.3.3.9. Results summary

- Initial reduction in erythropoiesis with compensatory reticulocytosis.

- Transient elevations in the platelet count and reduced lymphopoiesis.

- Initial reduction in bone marrow cellularity, number of CFU-Cs with increased apoptotic cell death and serum FL.

6.4. DISCUSSION

The technique of using 7-AAD staining to measure the level of apoptosis has not been performed before in our laboratory using rat bone marrow cells and there are no reports of the use of this technique being used in the rat in the literature. Therefore, a preliminary experiment was conducted to validate the use of 7-AAD staining for the analysis of apoptotic cell populations in rat bone marrow samples. 7-AAD staining of rat bone marrow from an untreated animal showed a basal level of apoptosis of 2.2%. Rat bone marrow cells were then subjected to an 18 h incubation in the presence or absence of 10% FCS. The absence of nutrients (i.e. absence of FCS) in the culture medium was a method used to induce cell death via apoptosis and therefore this procedure was used to determine if an increase in apoptosis was detectable using 7-AAD staining. Fig. 6.1 and Table 6.1 show an increase in the population of apoptotic cells in the rat bone marrow maintained in nutrient deprived medium. This result therefore supports the use of 7-AAD staining to identify and quantify apoptotic cell populations in rat bone marrow cells, as is carried out routinely using human and mouse bone marrow (Phillpot *et al.* 1995; 1996; Gibson *et al.* 2003).

CHB is a bifunctional alkylating agent (Dollery 1999) used particularly in the treatment of conditions of lymphocyte and WBC proliferation, and is the drug commonly employed at present in the management of chronic lymphocytic leukaemia, non-Hodgkin's lymphomas, Hodgkin's disease and also in ovarian cancer (BNF 2004; Sweetman 2002).

In man, CHB is administered orally, with peak plasma concentrations being found at 0.5 to 2 h after treatment (Reynolds 1989; BNF 2004). As well as being used for the indications above, CHB has also been given in the treatment of testicular tumours and advanced breast cancer. CHB has also been evaluated recently in the

treatment of non-malignant disorders including liver cirrhosis, nephrotic syndrome, and several autoimmune diseases (Sweetman 2002). The dose level of CHB used in man depends on the particular mode of use, but as a single agent a typical dosage is 0.2 mg/kg daily for 4 to 8 weeks. However, generally the drug is given as part of combination therapies; here, in intermittent dosing regimens, the dose level of CHB may be 0.4 mg/kg/day (Reynolds 1989).

The most toxic reaction of CHB in man is bone marrow depression and careful monitoring of the patient's WBC is essential. However, CHB at high dose levels may cause pancytopenia and irreversible bone marrow failure (Rudd *et al.* 1975; Reynolds 1989). Nevertheless, serious bone marrow toxicity is uncommon, and the marrow depression commonly seen is readily reversible. At therapeutic levels, CHB depresses lymphocytes, with a reversible progressive lymphocytopenia, but with lesser effects on neutrophils, platelets and Hb.

In man, CHB is only administered by the oral route (BNF 2004; Sweetman 2002). However, in the very early experimental work with CHB in the rat and mouse (Elson 1955; Elson *et al.* 1958; Alexander and Connell 1960), CHB was dissolved in arachis oil and dosed by ip injection, and the use of this route of administration has continued (Dunn and Elson 1970; Verschoyle *et al.* 1994; Van Os *et al.* 1998).

In general, in the present investigation, the pattern of the haematological response to CHB was of a mild bone marrow depression that was rapidly reversed towards normal levels. In the immediate post dosing period (day 1/3 post dosing), RBC, lymphocytes, WBC and FNCC were significantly reduced, as were marrow lymphoid cells; however, platelets and neutrophils were unaffected. At day 9 or 16, RBC were returning towards normal, as were FNCC and marrow lymphoid cells; blood lymphocytes and WBC continued to show decreased levels at these times. Also at day 9/16, MCV and MCH values showed evidence of significantly increased levels; this

finding may relate to raised reticulocyte counts at these time points and in the immediate post dosing period, or, alternatively, the raised values may be associated with dyserythropoietic changes, especially megaloblastosis. At day 24, 38, 45, 59 and 65, the overall pattern of responses was of normal values in CHB-dosed rats, but peripheral blood lymphocytes and WBC were both significantly depressed at 3 of these 5 time points. Nevertheless, cells of the lymphoid lineage in the marrow, from day 16 to 65, were increased to an average of 16.3% above the control figure. At day 1 and 9 post dosing in CHB rats, histology of the spleen showed significant lymphocyte depletion, and there was some evidence for this change at day 24 also. It is assumed that both B and T lymphocytes were affected as both B and T cell rich zones of the white pulp were ablated. The histology of the spleen was normal at day 65.

The above findings may be compared with a small number of reports in the literature. However, there are very few papers where the response to CHB has been studied for more than 20 days post dosing, and there are similarly few reports on repeat dose CHB investigations. Nevertheless, Elson (1955) and Elson *et al.* (1958) described detailed studies on the blood and marrow changes in the rat following a single dose CHB administration. The greatest depressive effect of CHB was on the lymphocyte (Elson 1955), with peripheral blood counts falling to a minimum at day 4 post dosing; normal values were regained by 18/20 days. Neutrophils were also decreased to a low point at day 4, followed by an overshoot at 8/10 days, and normal values at 15/20 days. RBC showed a slight decrease (day 4) followed by a return to normal. In a later report (Elson *et al.* 1958), marrow changes were investigated for up to 20 days following CHB administration. Erythropoietic cells in the marrow were at a minimum at 48 h post dosing, with a rapid return to normal (day 6). Megakaryocytes were resistant to CHB effects. Granulopoiesis was profoundly reduced by CHB with a maximum depression at 48 h; regeneration with a peripheral blood neutrophilia then occurred (day 10) and a

return to control levels (day 13). The volume of lymphoid tissue (spleen, thymus, lymph nodes) was also profoundly reduced after CHB dosing, with the number of lymphocytes in the marrow and blood also greatly decreased (72 h); peripheral lymphocytes fell to 10% of the control count. Regeneration of the lymphoid tissues was slow, and at 20 days (the end of the study), marrow and blood lymphocytes had not returned towards normal.

In studies on CHB in the mouse (single and repeat doses), Verschoyle *et al.* (1994) reported reductions in the weight of lymphoid tissue (thymus, spleen), and reduced blood lymphocytes and WBC; erythrocytes were unaffected, neutrophils were increased, and platelets decreased. Histological assessment of the thymus and spleen of CHB-dosed animals showed loss of lymphocytes with evidence of apoptosis. Van Os (1998) examined the effect of multiple CHB doses (6 doses administered every second week) in the mouse and investigated haemopoiesis at 20 weeks after the last dose; no significant effects were evident on WBC and marrow cell number at this time, although decreases in the long-term haemopoietic stem cell reserve were evident.

In the present study the number of CFU-C was significantly reduced during the immediate post dosing period of myelosuppression. However, the significant reduction in the number of committed progenitor cells was readily reversible, in general terms, with the mean CFU-C of CHB-treated rats being comparable to control values on day 24 post dosing.

During the early post dosing period a significant increase in the levels of apoptosis in nucleated bone marrow cells was detected in CHB-treated rats (Fig. 6.4). However, this increase in apoptotic cell death subsided as animals recovered from CHB-induced myelotoxicity, with apoptosis being in general comparable to controls from day 24 post dosing.

Changes in the serum levels of FL in CHB-treated rats were difficult to interpret (Fig. 6.5). In the immediate post dosing period (day 1), the mean level of FL was significantly reduced. This is in contrast to reports which suggest an increase in serum/plasma FL during the early stages of bone marrow suppression following chemotherapy treatment in both man and mouse (Chklovskaia *et al.* 1999; Molyneux *et al.* 2004b). However, on days 3, 9 and 24 post dosing, FL levels were significantly increased. The levels of FL were also slightly elevated on days 45 ($p<0.05$) and 65 (NS).

In conclusion therefore, the results of the present investigations with repeat dose CHB in the rat do not confirm the reports of Morley and his colleagues (Trainor and Morley 1976; Trainor *et al.* 1979; Morley 1980) who produced evidence to indicate that this drug caused residual bone marrow injury in the mouse. Our results demonstrate that CHB in the rat does not cause a residual 'late-stage' bone marrow depression, although there is some evidence of a persistent effect on peripheral blood lymphocytes.

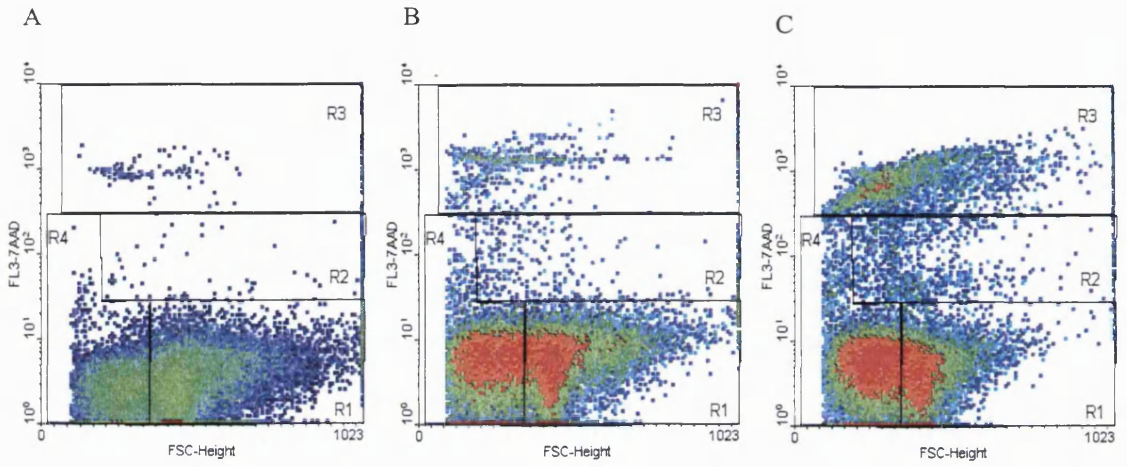


FIGURE 6.1. Measurement of apoptosis in rat bone marrow samples using 7-amino actinomycin D (7-AAD) staining. Scattergrams of 7-AAD-stained rat femoral bone marrow samples. (A), rat bone marrow sample analysed at time 0 h. (B), rat bone marrow sample maintained in IMDM supplemented with 10% FCS for 18 h at 37°C; there is a small increase in the level of apoptosis. (C), rat bone marrow maintained in serum free medium (IMDM without FCS) for 18 h at 37°C; there is an increased level of apoptosis. FSC height threshold was set at 108 to exclude all RBC and debris. Regions were drawn around populations showing negative (R1), dim (R2) and bright (R3) 7-AAD fluorescence corresponding to live, apoptotic and dead cells respectively. A region was also drawn around remaining cell debris (R4) and RBC to exclude these data. Abbreviations: FSC-Height, forward light scatter; FL3-7-AAD, 7-AAD fluorescence intensity.

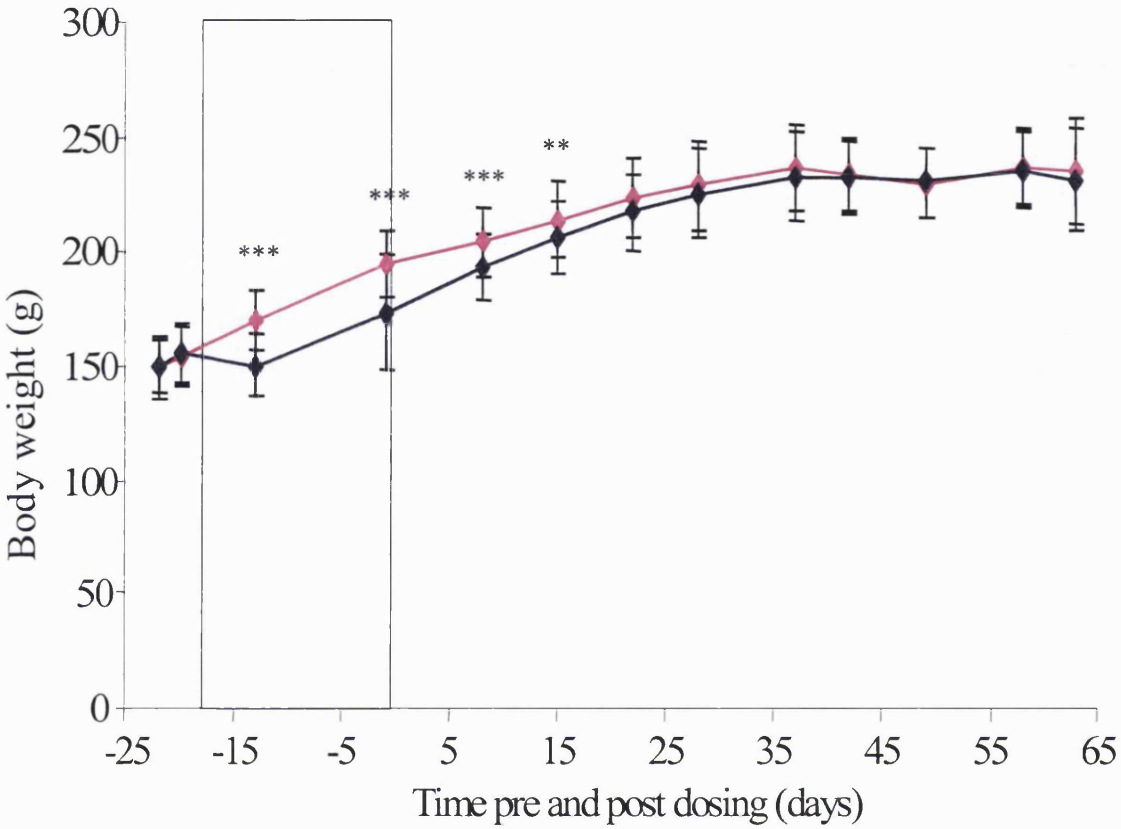


FIGURE 6.2. Body weight increases in control female Hanover Wistar rats and rats treated with chlorambucil at 6.3 mg/kg. Values are means (± SD) of control (—◆—) and chlorambucil-treated (—◆—) rats. Animals were treated with vehicle or chlorambucil on 6 occasions over an 18 day dosing period (▭). **Significantly different from controls, $p<0.01$; ***$p<0.001$.

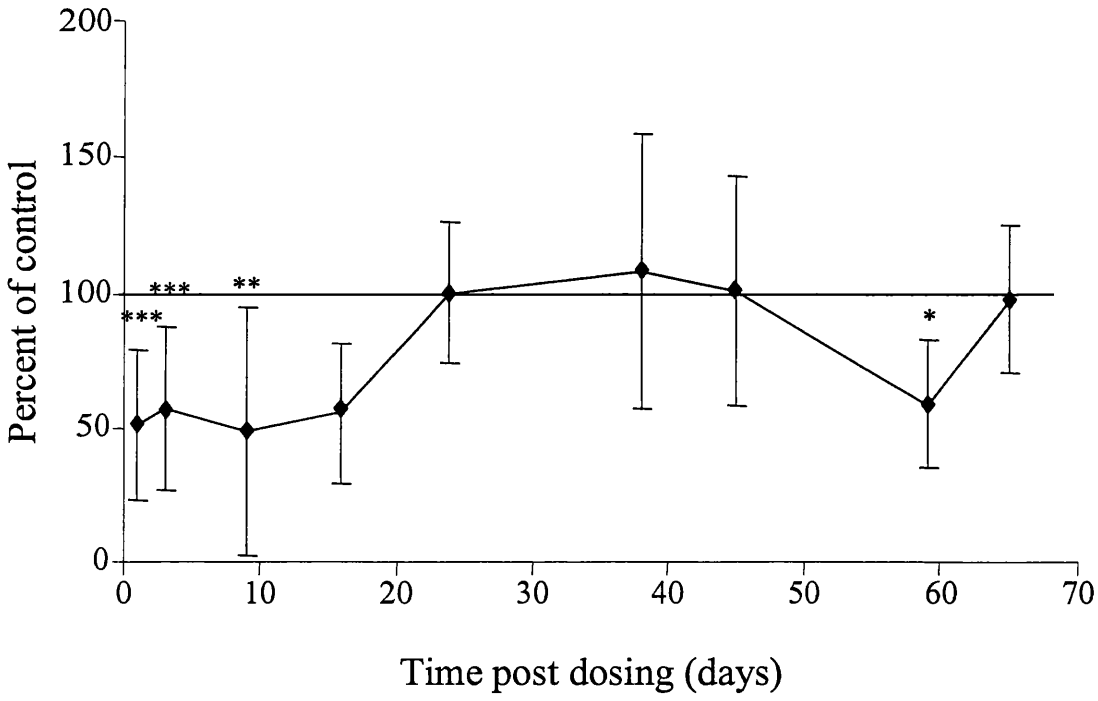


FIGURE 6.3. Colony forming unit cell (CFU-C) results from female Hanover Wistar rats treated with chlorambucil and sampled on days 1 to 65 post dosing. Values are means (±SD), expressed as a percentage of the mean control value in animals sampled at the same time points. Cultures were set up in duplicate and the mean CFU-C number per femur calculated per mouse. Numbers of animals per group and all other information as Table 6.3. *Significantly different from controls, $p<0.05$; **$p<0.01$; ***$p<0.001$.

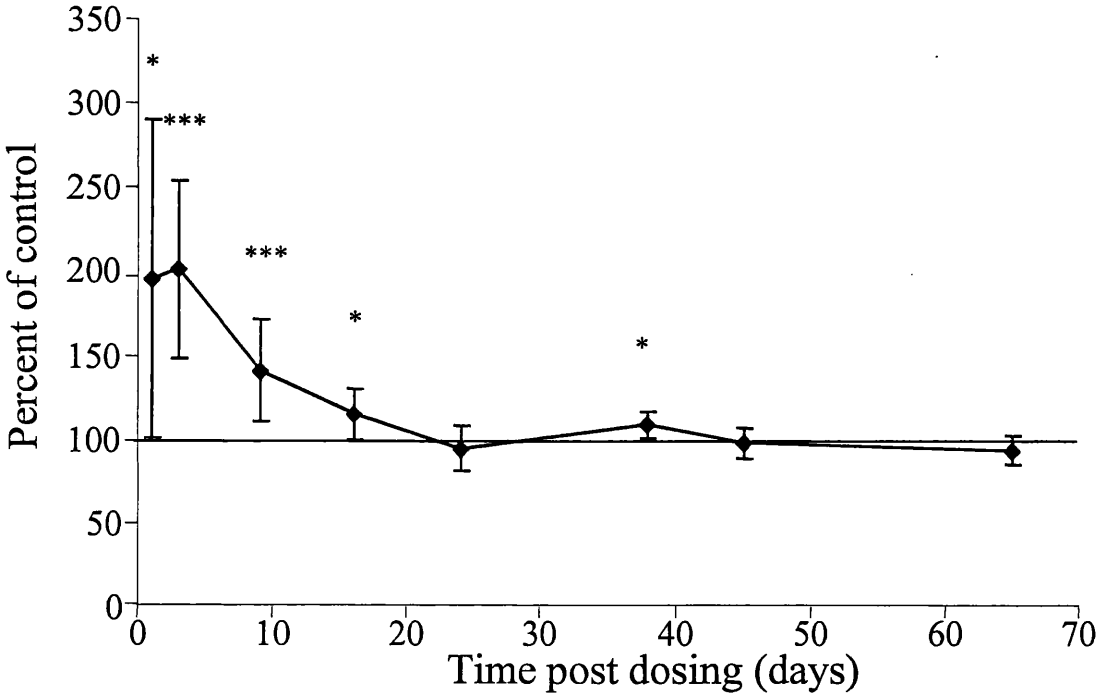


FIGURE 6.4. Apoptosis in femoral bone marrow samples; results from female Hanover Wistar rats treated with chlorambucil and sampled on days 1 to 65 post dosing. Values are means (±SD), expressed as a percentage of the mean control value in animals sampled at the same time points. Numbers of animals per group and all other information as Table 6.3. Apoptosis assay was not completed on day 59 post dosing. *Significantly different from controls, $p<0.05$; ***$p<0.001$.

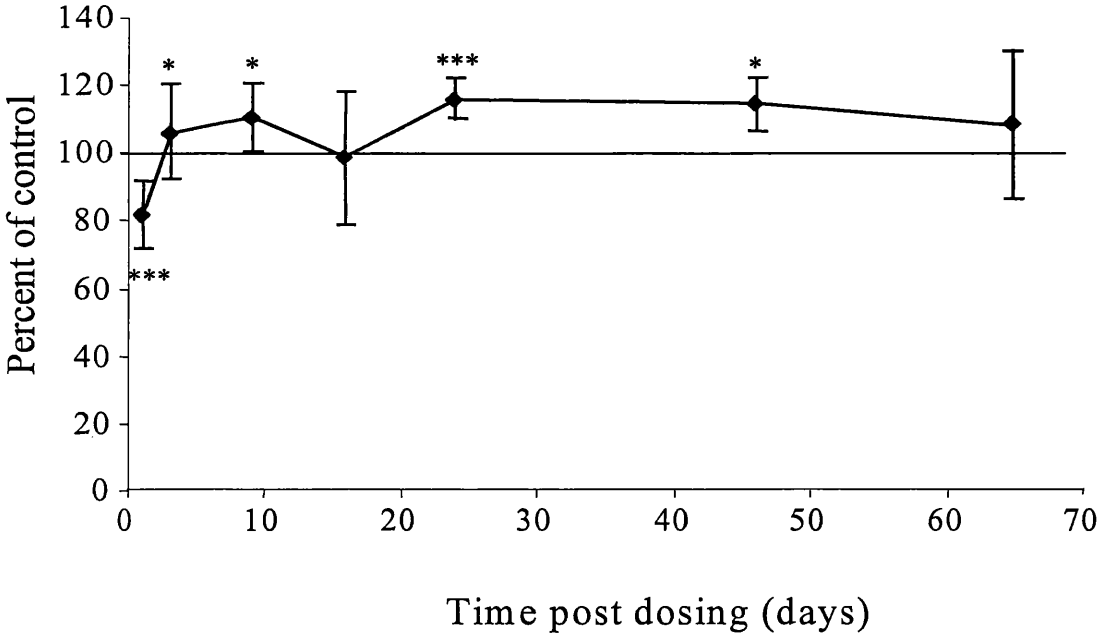


FIGURE 6.5. Serum FL results from female Hanover Wistar rats treated with chlorambucil and sampled on days 1 to 65 post dosing. Values are means (±SD), expressed as a percentage of the mean control value in animals sampled at the same time points. Numbers of animals per group and all other information as Table 6.3. FL assay was not completed on days 38 and 59 post dosing. *Significantly different from controls, $p<0.05$; *** $p<0.001$.

TABLE 6.1. Measurement of live, apoptotic and dead cells in rat femoral bone marrow samples using 7-amino actinomycin D staining[a]

Sample treatment	Live	Apoptotic	Dead
Sample A. (Time 0 h)	95.8	2.2	2.0
Sample B. (Time 18 h; IMDM with 10% FCS)	86.9	4.9	8.2
Sample B. (Time 18 h; IMDM without 10% FCS)	63.5	10.9	25.6

[a]Sample A (time 0 h); marrow was flushed from both femora into Iscove's modified Dulbecco's medium (IMDM) and assayed immediately. Sample B (time 18 h) rat bone marrow cell suspension maintained in IMDM supplemented with 10% foetal calf serum (FCS) at 37°C in 5% CO_2 in air for 18h. Sample C (time 18 h) rat bone marrow cell suspension maintained in serum-deprived medium (IMDM without FCS) at 37°C in 5% CO_2 in air for 18h. Results are expressed percentage values.

TABLE 6.2. Haematological results[a] from Hanover Wistar rats treated with 8 doses of chlorambucil (CHB) at 0 (control), 5.0, 5.5, 6.0, and 7.0 mg/kg over a period of 18 days and autopsied on day 82 after the final dose[b]

	Treatment (mg/kg)				
	0 (Control)	**5.0**	**5.5**	**6.0**	**7.0**
RBC	7.96 (0.27)	7.97 (0.20)	7.78 (0.15)	8.06 (0.33)	7.76 (0.40)
Hb	15.1 (0.3)	15.6 (0.8)	14.6 (0.2)	15.7 (0.6)	15.2 (0.3)
HCT	44.0 (2.7)	45.0 (1.2)	42.2 (0.6)	45.4 (0.2)	44.3 (2.5)
MCV	55.3 (2.2)	56.5 (0.9)	54.3 (0.6)	56.4 (0.1)	57.1 (0.3)
MCH	19.0 (0.8)	19.6 (0.7)	18.8 (0.3)	19.5 (0.2)	19.6 (0.7)
MCHC	34.3 (2.0)	34.7 (1.9)	34.6 (0.7)	34.5 (0.3)	34.3 (1.4)
Retic	237 (43)	212 (7)	207 (21)	241 (28)	240 (43)
Plt	791 (36)	757 (110)	921 (61)	772 (65)	658 (168)
WBC	2.43 (1.19)	2.07 (0.37)	1.69 (0.37)	2.15 (0.32)	2.09 (0.35)
Neut	0.54 (0.21)	0.37 (0.22)	0.26 (0.03)	0.37 (0.15)	0.30 (0.10)
Lymph	1.76 (0.99)	1.61 (0.15)	1.33 (0.41)	1.64 (0.13)	1.68 (0.45)
Mono	0.02 (0.01)	0.02 (0.01)	0.01 (0.01)	0.02 (0.01)	0.03 (0.01)
Eo	0.05 (0.02)	0.02 (0.01)	0.05 (0.02)	0.06 (0.02)	0.03 (0.01)
Baso	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.01)
FNCC	7.70 (0.86)	7.88 (1.21)	7.98 (0.74)	7.59 (1.09)	7.18 (0.51)
Spleen	2,418 (216)	2,325 (262)	2,600 (216)	2,569 (296)	2,831 (211)

[a] Values are means with SD in parenthesis. n=4 for control (vehicle-treated) rats, n=3 for all CHB-treated groups.

[b]Abbreviations and units: RBC, red blood cells, $x10^6/\mu l$; Hb, haemoglobin, g/dl; HCT, haematocrit, %; MCV, mean cell volume, fl; MCH, mean cell haemoglobin, pg; MCHC, mean cell haemoglobin concentration, g/dl; Retic, absolute reticulocyte count, $x10^3/\mu l$; Plt, platelets, $x10^3/\mu l$; WBC, white blood cells, $x10^3/\mu l$; Neut, neutrophils, $x10^3/\mu l$; Lymph, lymphocytes, $x10^3/\mu l$; Mono, monocytes, $x10^3/\mu l$; Eo, eosinophils, $x10^3/\mu l$; Baso, basophils, $x10^3/\mu l$; FNCC, femoral bone marrow nucleated cell count, $x10^7$; Spleen, relative spleen weight, mg/kg body weight.

TABLE 6.3. Haematological results[a] from female Hanover Wistar rats treated with 6 doses of chlorambucil (CHB) at 6.3 mg/kg over a period of 18 days and sampled at 1 to 65 days after the final dose[b]

	Day of sampling																	
	1		3		9		16		24		38		45		59		65	
	Control	CHB	Control	CHB	Control	CHB	Control	CHB	Control	CHB	Control	CHB	Control	CHB	Control	CHB	Control	CHB
RBC	7.24	6.76*	7.38	6.53*	7.66	7.21*	7.37	7.12	7.76	7.47	7.63	7.44	7.54	7.78	7.27	7.49	7.34	7.57
Hb	14.6	13.5*	14.7	13.1**	14.8	14.8	14.3	14.7	14.8	14.8	14.8	14.8	14.7	15.3	14.1	14.2	13.7	14.2
HCT	41.4	38.5*	41.2	37.9**	42.1	41.8	41.1	41.3	41.5	42.2	42.6	41.8	42.4	43.1	40.2	40.0	39.3	40.5
MCV	57.3	57.0	55.9	58.7	55.0	58.0***	55.7	58.1*	53.5	56.7***	55.8	56.3	56.3	55.5	55.4	53.4**	53.7	53.5
MCH	20.2	19.9	19.9	20.2	19.4	20.5**	19.4	20.7*	19.1	19.8	19.5	19.9	19.6	19.7	19.4	19.0**	18.7	18.8
MCHC	35.3	34.9	35.7	34.5	35.2	35.4	34.8	35.6	35.6	35.0	34.8	35.3	34.8	35.4	35.1	35.5	34.8	35.1
Retic	262	484***	267	410	305	429***	261	421***	281	243	247	219	234	238	169	186	276	241
Plt	972	1,098	886	1,051	842	962*	828	967**	887	827	790	821	767	897**	769	835	880	827
WBC	2.64	0.75**	2.29	0.68***	2.52	1.19***	2.83	1.05***	3.10	1.77***	2.32	2.13	2.12	2.76	2.10	1.61**	3.15	2.03**
Neut	0.35	0.53	0.34	0.51	0.39	0.73**	0.44	0.40	0.50	0.56	0.47	0.53	0.30	0.65**	0.28	0.34	0.73	0.56
Lymph	2.21	0.16***	1.89	0.12**	2.05	0.41***	2.29	0.58***	2.52	1.12***	1.77	1.48	1.76	2.02	1.75	1.20**	2.27	1.38***
Mono	0.03	0.02	0.02	0.01***	0.03	0.04**	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.03	0.02	0.02	0.04	0.02*
Eo	0.03	0.01*	0.03	0.02	0.03	0.06**	0.04	0.06	0.03	0.05	0.04	0.08	0.02	0.05*	0.03	0.04	0.06	0.03*
Baso	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
FNCC	9.63	5.13***	9.63	7.47*	8.29	2.07***	7.91	9.17	9.14	10.9*	9.05	9.09	7.28	10.17***	7.17	8.56*	8.72	8.28
Spleen	2,727	2,428	2,627	2,233*	2,501	1,962***	2,672	2,370**	2,465	2,899***	2,331	2,512	2,178	2,637***	2,379	2,668	2,535	2,402

[a] Values are means; n=8 for control and CHB groups at all time points, except n=6 for control groups at day 59 and 65, and n=7 for the control group at day 1, and CHB groups at day 1, 24 and 65.

[b]Abbreviations and units: as Table 6.2.

Data analysed using a Student's t-test. *Significantly different from controls, $p<0.05$; **$p<0.01$; ***$p<0.001$.

TABLE 6.4. Estimated counts ($x10^7$) of myeloid, erythroid and lymphoid cells, and the myeloid:erythroid (M:E) ratio in the femoral marrow of control and chlorambucil (CHB) treated rats[a]; animals were given 6 doses of CHB over a period of 18 days and sampled at 1 to 65 days after the final dose.

	Day of sampling																	
	1		3		9		16		24		38		45		59		65	
	Control	CHB	Control	CHB	Control	CHB	Control	CHB	Control	CHB	Control	CHB	Control	CHB	Control	CHB	Control	CHB
Myeloid	3.39 (1.25)	2.43 (1.32)	3.94 (1.41)	2.74 (0.98)	3.51 (0.78)	0.96 (0.38)***	2.86 (0.98)	3.30 (0.86)	3.97 (1.34)	4.48 (1.17)	3.82 (1.09)	3.51 (0.86)	3.02 (0.51)	4.31 (0.73)**	3.18 (0.40)	2.98 (0.43)	3.92 (1.31)	3.14 (0.69)
Erythroid	3.20 (1.34)	1.97 (0.71)*	2.50 (0.72)	3.61 (1.25)	2.46 (0.59)	0.67 (0.27)***	2.46 (0.86)	3.17 (0.71)	2.90 (0.53)	3.52 (0.76)*	2.44 (0.26)	2.22 (0.51)	2.06 (0.59)	3.19 (0.97)**	2.11 (0.30)	2.88 (0.35)***	2.21 (0.80)	2.83 (0.75)
Lymphoid	2.94 (0.99)	0.66 (0.16)***	3.02 (0.69)	1.02 (0.53)***	2.24 (0.68)	0.43 (0.20)***	2.43 (0.97)	2.59 (0.82)	2.20 (0.52)	2.80 (0.64)	2.68 (0.63)	3.11 (0.43)	2.01 (0.24)	2.54 (0.37)**	1.81 (0.66)	2.60 (0.65)*	2.46 (0.46)	2.17 (0.75)
Other	0.12 (0.10)	0.07 (0.07)	0.17 (0.09)	0.10 (0.05)*	0.08 (0.04)	0.01 (0.01)***	0.13 (0.09)	0.11 (0.04)	0.08 (0.06)	0.08 (0.03)	0.11 (0.04)	0.24 (0.10)***	0.19 (0.12)	0.13 (0.09)	0.08 (0.06)	0.10 (0.09)	0.12 (0.09)	0.14 (0.07)
M:E ratio	1.16 (0.48)	1.43 (0.91)	1.69 (0.90)	0.84 (0.36)**	1.46 (0.16)	1.49 (0.39)	1.23 (0.51)	1.10 (0.40)	1.35 (0.27)	1.31 (0.40)	1.56 (0.32)	1.59 (0.25)	1.54 (0.37)	1.47 (0.51)	1.53 (0.25)	1.05 (0.18)*	1.98 (0.85)	1.25 (0.74)*

[a]200 cells in the tibial marrow smears were differentially counted by eye and the absolute number of cells of each lineage estimated for the nucleated marrow cell count of the femoral marrow flush. Values are means, SD in parenthesis. Cells categorised as 'other' include monocytes and monocyte precursors, mast cells, megakaryocytes, plasma cells and unidentifiable cells. All other information as Table 6.3.

Data analysed using the Students t-test. *Significantly different from controls, $p<0.05$; **$p<0.01$; *** $p<0.001$.

CHAPTER 7: The haemotoxicity of mitomycin C in the CD-1 mouse

7.1. INTRODUCTION

Mitomycin C (MMC) is an antineoplastic cytotoxic antibiotic produced by the actinomycete *Streptomyces caespitosus* (Reynolds 1989). MMC has a wide spectrum of activity and is generally used in combinations with other cytotoxic drugs (e.g. 5-fluorouracil, vindestine, bleomycin or cisplatin). MMC is used in the palliative treatment of solid tumours particularly adenocarcinomas of the pancreas and stomach (Godfrey and Wilbur 1972), but also in the treatment of a large range of other tumours including those of the bladder, colon, rectum, lung, liver, prostate, cervix, breast, skin, oesophagus and eye (Karanes *et al.* 1986; Reynolds 1989; Veeder *et al.* 1992; Hortobagyi 1993; Sweetman 2002). However, as with other alkylating agents, MMC is toxic to the bone marrow and frequently induces profound myelosuppression (Dollery 1999).

In the mouse, few papers have been published describing the long-term effects of MMC on the haemopoietic system. However, Trainor *et al.* (1979) conducted an experiment to study the bone marrow damage caused by MMC in the BALB/c mouse. Animals were treated with 4 fortnightly doses of MMC at 2 to 4 mg/kg. At 2 to 5 months after MMC treatment, significant reductions were still evident in the bone marrow cellularity, the number of committed progenitor cells (CFU-C) and the number of spleen colony forming units (CFU-S). Trainor *et al.* (1979) thus identified MMC as an agent like busulphan (BU), chlorambucil, melphalan and 1, 3-bis (2-chloroethyl)-1-nitrosourea (BCNU) as capable of causing a residual marrow injury resulting in bone marrow suppression 2 to 5 months after treatment. However, changes in the peripheral

blood counts of mice treated with MMC in the experiment of Trainor *et al.* (1979) were not reported.

A series of experiments were therefore devised to assess the long-term 'late-stage' effects of MMC administration on the haemopoietic system of mice and to investigate the potential use of MMC in the development of a model of 'late-stage' chronic bone marrow aplasia (CBMA). Preliminary experiments were first completed to find an appropriate dose of MMC to use.

Preliminary (abstract) reports have been published (Molyneux *et al.* 2004b) and the data published as a full paper (Molyneux *et al.* 2005).

7.2. MATERIALS AND METHODS

7.2.1. Experimental design

7.2.1.1. Preliminary dose ranging studies

Preliminary Experiment 1: MMC was prepared (as described in Chapter 2.2) and a single ip dose administered to female CD-1 mice (Charles River UK Ltd.; mean body weight 26.5 g) at 6.0, 6.5, 7.0 and 7.5 mg/kg (n=3 per group). Animals were treated with a single ip injection of vehicle (water; n=3) or MMC, and studied for 96 days.

Preliminary Experiment 2: Female CD-1 mice (mean body weight 30.5 g) were treated with 4 ip injections of vehicle (water) or MMC (at 0.5 mg/kg intervals from 1.0 to 5.5 mg/kg; n=3 or 5 per group) over an 8 day period. On day 50 post dosing, animals were autopsied for blood and bone marrow investigations.

Preliminary Experiment 3: Female CD-1 mice (mean body weight 17.4 g) were treated with 8 ip injections of vehicle (water) or MMC (at 0.25 mg/kg intervals from 1.5 to 3.25 mg/kg; n=3 to 6 per group) over an 18 day period. On day 72 post dosing animals were autopsied for blood and bone marrow investigations.

7.2.1.2. Main mitomycin C study

326 female CD-1 mice (mean body weight 25.8 g) were divided into two groups, control (n=158) and MMC-treated (n=168). Animals were dosed with vehicle or MMC (2.5 mg/kg) on 8 occasions over an 18 day period (days 1, 4, 6, 8, 11, 13, 16 and 18).

On days 1, 7, 14, 28, 42 and 50 after the final MMC dose, control mice (n=8 to 12) and MMC-treated animals (n=6 to 8) were killed for the examination of blood, bone marrow and other organs and tissues (as described in Chapter 2.3). At each time point a further group of control (n=5 to 12) and MMC-treated (n=5 to 8) mice were sampled for the examination of the serum cytokine fms-like tyrosine kinase 3 (FLT-3) ligand (FL). In addition, the bone marrow cellularity (femoral marrow nucleated cell count; FNCC) was measured and organs were removed for histopathology.

7.2.2. Processing of samples

7.2.2.1. Preliminary dose ranging studies

At autopsy a full blood count was performed and the cellularity of the bone marrow assessed (FNCC) using a femoral marrow flush into 3 ml Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% foetal calf serum (FCS) (as described in Chapter 2.4).

7.2.2.2. Main mitomycin C study

At autopsy a full blood count was performed and a femoral marrow flush carried out into 3 ml IMDM supplemented with 10% foetal calf serum (FCS); this suspension was used to measure the FNCC (as described in Chapter 2.4). A bone marrow differential count was also performed using a tibial marrow smear (as described in Chapter 2.5). The second femur was placed in 5 ml sterile IMDM supplemented with 10% FCS and used to assess the clonogenic potential of the bone marrow and the level of apoptosis (as described in Chapter 2.6 and 2.7). The concentration of the cytokine FL

was measured in serum samples from control and MMC-treated mice (as described in Chapter 2.8). At each autopsy, the spleen, and on occasion the liver and both kidneys, were weighed and placed in fixative. The sternum, and on occasion the lungs (inflation fixation) were also placed in fixative for histological analysis (as described in Chapter 2.9).

7.2.3. Statistical analysis

In the main MMC study (Chapter 7.2.1.2), MMC-treated and control (vehicle-treated) groups were compared using an unpaired one tailed Student's t-test (Microsoft Excel; Microsoft Ltd, Microsoft UK, Reading UK).

7.3. RESULTS

7.3.1. Preliminary dose ranging studies

In Preliminary Experiment 1, the maximum tolerated single dose (MTD) for MMC was identified as 7.0 mg/kg (data not shown).

In Preliminary Experiment 2, with animals given 4 ip injections of MMC at 1.0 to 5.5 mg/kg, significant toxicity was seen. Many mice in the higher dose level groups (3.0 to 5.5 mg/kg) were catagorised as inter-current death (ICD) animals before the scheduled autopsy on day 50 post dosing (Table 7.1). The majority of these ICD deaths, (13 of 17 animals, 76.5%) occurred in the early post dosing period between days 1 and 8. Four animals of the 17 were, however, found dead (FD) later in the experiment on days 26, 35, 42 and 50 post dosing. At lower doses of MMC (1.0 to 2.5 mg/kg) however, there were no mortalities over the 50 day post dosing period.

At autopsy, the peripheral blood counts of mice treated with 1.0 and 1.5 mg/kg MMC appeared comparable to controls (Table 7.2), although statistical analysis was not carried out. In all other MMC-treated groups (2.0 to 3.0 mg/kg) some reductions in erythrocyte parameters (RBC, Hb, HCT and reticulocytes) and leucocyte counts appeared to be evident. The platelet counts were largely unaffected by MMC treatment in all dose level groups. Similarly, no change was seen in the FNCC; however, the FNCC was generally lower in MMC-treated mice compared to controls.

In the Preliminary Experiment 3, dose ranging study, the toxicity of MMC was assessed when given on 8 occasions over 18 days at 1.50 to 3.25 mg/kg. Mice treated with MMC at 2.75 to 3.25 mg/kg were all categorised as ICD animals before the scheduled autopsy at day 72 (Table 7.3). Deaths in these higher dose level groups tended to occur mainly at a late-stage, on days 46 to 67 after dosing. Mortality in mice

treated with MMC at 1.5 to 2.5 mg/kg was relatively low, with 1 animal being categorised as ICD in each of the 1.75, 2.25 and 2.5 mg/kg treatment groups; these mortalities were also at a late-stage, on days 70, 48 and 57, respectively.

At autopsy on day 72 post dosing, reductions were seen (NS) in the peripheral blood erythrocyte parameters (RBC, HB, HCT) and leucocytes whereas the platelet counts were largely unchanged (Table 7.4). The FNCC counts were reduced in comparison to the control mean in all MMC-treated groups, and this reached statistical significance in the 1.75 mg/kg group ($p<0.05$). The results of this experiment identified MMC at 2.5 mg/kg, when given on 8 occasions over 18 days, to be a suitable dose level to use in the main experiment as significant bone marrow injury was evident.

7.3.2. Main study

7.3.2.1. Clinical signs and body weight changes

During the 18 day dosing period, animals treated with MMC at 2.5 mg/kg remained in good health. The mean body weights of mice on the first day of dosing were 27.0 g for control (n=158) and 24.6 g for MMC-treated (n=168) animals, respectively (Fig. 7.1). At the end of the dosing period, the mean body weights were 29.1 g (control) and 27.3 g (MMC-treated), giving increases of 7.8% and 11.0% during the dosing period, respectively. In the 50 day post dosing period the MMC-treated mice failed to gain weight normally and the increase in mean body weight was to 27.8 g (a 1.8% increase) compared with an increase to 34.0 g (a 16.8% increase) in the control group.

On day 4 after the final dose of MMC, 3 mice which had been treated with the drug were found dead (Fig. 7.2); by day 7 post dosing a further 3 MMC-treated animals

were recorded as ICD. The mortality in MMC-treated animals continued, and over the 50 day post dosing period, the total number of ICD animals was 78 (46.4% mortality). This continuing mortality was entirely unexpected. Generally, the animals died suddenly, with no obvious deterioration in their condition or evidence of morbidity; it was therefore, generally, not possible to identify which animals were becoming ill. The body weights of ICD animals were not reduced in comparison with the rest of the MMC-treated group.

7.3.2.2. Autopsy observations

On days 1 and 7 post dosing, some animals treated with MMC showed evidence of an accumulation of a small amount of a 'thin' fluid (either 'milky' or clear) in the abdominal cavity. However, at the later stages of the experiment (days 14, 28, 42 and 50 post dosing), some MMC-treated mice had swollen abdomens with larger quantities of fluid. On days 28, 42 and 50 post dosing, some MMC-treated mice were found to have fluid accumulation in the thoracic cavity also. On day 14 post dosing, the fluid from the abdominal cavities of 3 MMC-treated mice was taken, and smears were prepared and stained (May-Grünwald-Geimsa). On microscopic assessment, there was a predominance of macrophages with no neutrophils or lymphocytes, and therefore it was considered that there was no clear evidence of a bacterial infection. On days 42 and 50 post dosing, the kidneys of some MMC-treated mice appeared swollen and pale in colour.

7.3.2.3. Haematology results

Haematological results have been reported (Molyneux *et al.* 2005). Changes in RBC, MCV, reticulocytes, platelets and neutrophils are displayed graphically in Fig. 7.3 A-E, respectively. On day 1 post dosing, MMC induced a profound 'predictable' bone marrow depression. Statistically significant reductions were evident in the RBC (Fig. 7.3 A), Hb, HCT and MCV (Fig. 7.3 B), reticulocytes (Fig. 7.3 C) and platelets (Fig. 7.3 D) of MMC-treated mice; the mean percentage reductions for these parameters were to 63.6% (RBC), 62.1% (Hb), 59.4% (HCT), 93.2 (MCV), 7.7% (reticulocytes) and 27.9% (platelets) of the mean control values. MCHC was significantly increased in MMC-treated mice, the MCH was however unaffected at this time point. Significant reductions were also seen in mean WBC, neutrophils (Fig. 7.3 E), lymphocytes, and monocytes (reductions to 41.0%, 15.1%, 50.3% and 2.9% of the mean control values, respectively).

On day 7 post dosing, significant reductions continued in the mean values for RBC (Fig. 7.3 A), Hb and HCT in MMC-treated mice. However, reticulocytes (Fig. 7.3 C) showed evidence of a recovery and counts were elevated above the mean control value, although this increase was not statistically significant. MCV (Fig. 7.3 B), MCH and MCHC were unaffected. Platelets were still significantly reduced (Fig. 7.3 D) but there was some indication of recovery, with the mean count being 44.0% of control. Lymphocytes in MMC mice were within normal limits on day 7 but neutrophils (Fig. 7.3 E) and monocytes remained significantly reduced, although the counts did show signs of recovery.

On day 14 post dosing, the mean values for RBC (Fig. 7.3 A), Hb and HCT continued to be significantly decreased in MMC-treated animals, however, a return towards normal was clearly evident in these parameters. A 'rebound' reticulocytosis

was also apparent (Fig. 7.3 C), with the mean reticulocyte count in MMC-treated mice increased to 262% of the control mean value ($p<0.001$); the values for MCV (Fig. 7.3 B) and MCH were also significantly raised. The platelet count was approximately normal in MMC animals (Fig. 7.3 D). Neutrophils (Fig. 7.3 E) and monocytes still remained significantly reduced in MMC-treated mice, but lymphocytes were within control ranges.

On day 28 post dosing, the mean RBC count remained significantly reduced (Fig. 7.3 A) in MMC-treated mice; however, Hb and HCT values had returned to normal. The mean reticulocyte count (Fig. 7.3 C) continued to be significantly increased in MMC-treated mice at this time (144% of control), but the platelet, neutrophil, lymphocyte and monocyte counts were normal.

By day 42 and 50 post dosing, many of the haematological parameters in MMC-treated mice had returned to control levels. However, a residual ('late-stage') effect was apparent in the mean RBC (Fig. 7.3 A), Hb, and HCT values, with all these parameters being significantly reduced both on day 42, and on day 50, in animals which had been treated with MMC.

7.3.2.4. Femoral nucleated cell count

Immediately post dosing (day 1) the mean FNCC of MMC-treated mice was very significantly reduced (to 19.6% of the mean control value) (Fig. 7.3 F). However, the FNCC in MMC-treated mice appeared to be returning towards normal on days 7, 14 and 28 being 49.1%, 58.9% and 72.0% of the control means, respectively. However, as with the mean RBC, Hb and HCT values, the mean FNCC appeared to show a residual ('late-stage') effect at day 42 and 50 post dosing (Fig. 7.3 F), with counts remaining

reduced to 65.8% and 75.3% of the mean control values at each time point ($p<0.001$ and $p<0.01$), respectively.

7.3.2.5. Bone marrow clonogenic assay

The effect of MMC on the number of colony forming unit-granulocyte monocyte (CFU-GM) and erythroid colonies per femur was highly significant and long-lasting (Fig. 7.4 A and Fig. 7.4 B). On day 1 post dosing, the number of CFU-GM colonies/femur in MMC-treated mice was reduced to 6.0% of the control mean value. The number of CFU-GM remained significantly reduced in MMC-treated mice on days 7 and 14, being 24.2% and 18.1% of the control mean, respectively. On days 28 and 42 post dosing the number of CFU-GM colonies was again reduced significantly to 46.1% and 53.1% of the control mean values, respectively. However, at day 28 and 42 post dosing there was evidence of recovery and a return towards control values. Indeed, the mean number of CFU-GM colonies/femur in MMC-treated mice was comparable to the controls on day 50 post dosing.

A similar profound effect was seen on the number of erythroid colonies/femur in MMC-treated mice (Fig. 7.4 B). On day 1 post dosing, MMC-treated mice had a significantly reduced number of erythroid colonies (5.2% of the control mean). On days 7, 14 and 28, signs of recovery were observed, with colony numbers increased to 37.3%, 20.9% and 42.2% of counts in the controls, respectively. On day 42 the number of erythroid colonies were reduced, however, not significantly. On day 50 post dosing, the number of erythroid colonies from MMC-treated mice was comparable to controls.

7.3.2.6. Apoptosis

On days 1, 7 and 14 post dosing, apoptosis in femoral marrow nucleated cells in MMC-treated mice was significantly increased to 159%, 137% and 179% of control values, respectively (control as 100%) (Fig. 7.5). On day 28 post dosing there was clear evidence of recovery and a return towards control values. However, apoptosis continued to be significantly increased at this time point. On day 42 post dosing, apoptosis in MMC-treated mice was not significantly different from the controls. However, on day 50 post dosing, apoptosis appeared to be slightly increased in mice treated with MMC.

7.3.2.7. Serum fms-like tyrosine kinase 3 (FLT-3) ligand (FL)

The concentration of FL was measured in the serum of mice treated with MMC, and in vehicle-treated controls. On days 1, 7 and 14 post dosing, the level of FL in the serum of MMC-treated mice was considerably increased to 520.3%, 584.9% and 342.0% of the mean control values (control values as 100%), respectively (Fig. 7.6). However, on day 28 post dosing, the concentration of FL in the serum of MMC-treated animals was comparable to controls. On day 42 post-dosing, FL levels were slightly reduced in MMC-treated mice but were normal on day 50.

7.3.2.8. Bone marrow differential counts

The tibial marrow smears from 6 control and 6 MMC-treated mice were randomly selected at each time point and differential counts performed (as described in Chapter 2.4.). On day 1 post dosing both myeloid and erythroid cell lineages were significantly reduced in MMC-treated animals (Table 7.5), with counts being 5.2% and

9.5% of the control values, respectively. On day 7 post dosing, the number of myeloid and erythroid cells were also significantly reduced in MMC-treated mice, however, a return towards normal was clearly evident. For cells of the myeloid lineage, in MMC mice, there was a return towards control values at day 14/28 post dosing, which in general terms remained evident for the remainder of the study. However in the case of cells of the erythroid line in MMC-treated mice, a return to control values did not occur, and counts remained significantly reduced at all time points after day 7 post dosing; the reductions were to 57.0%, 56.0%, 58.6% and 74.5% of the control counts at days 14, 28, 42 and 50 post dosing, respectively.

Cells of the lymphoid lineage, in MMC-treated mice, appeared to be 'spared' in the immediate post dosing period (day 1/7). However, at all succeeding time points the cell counts were reduced, and at day 14, 28 and 42 post dosing, the reductions were statistically significant. Cells categorised as 'other', including monocyte and monocyte precursors etc, were also, in general, reduced in MMC-treated animals at all time points.

7.3.2.9. Histopathological assessment of tissues

The sternum and spleens from MMC-treated and control mice were studied histologically at all time points; livers and kidneys were examined from selected mice at day 42 and 50 post dosing, and lungs from mice at the day 50 autopsy. The findings have been presented (Molyneux *et al.* 2005). In the sternums at day 1 post dosing there was severe depletion of the sternal bone marrow cells in all MMC-treated animals examined. At day 7 post dosing, a degree of marrow cellularity had been restored in MMC mice, but this was not to the level seen in the control animals. At 14 days post dosing, slight depletion of the sternal marrow was seen in one of four MMC-treated

animals examined. Normal levels of marrow cellularity were evident in the sternum at days 28, 42 and 50 post dosing.

The mean relative weight of the spleen was significantly reduced ($p<0.001$) in MMC-treated mice, to 50.6% of the control mean value, at day 1 post dosing. However, at day 7 post dosing, the mean relative spleen weight was increased ($p<0.001$) in MMC-treated animals to 146.1% of the mean control value (as 100%). Thereafter, in general, the mean relative weights of the spleens in MMC mice were similar to the control values. Histological study of the spleens from MMC mice, at day1 post dosing, showed a reduction in the cellularity of the red pulp (reduced haematopoiesis) in all mice examined. However, at day 7 and 14 post dosing, moderately increased haematopoiesis was evident in the red pulp of the majority of MMC mice examined. No significant abnormalities were seen in the spleens of MMC-treated mice at day 28, 42 and 50 post dosing.

At autopsy at day 50 post dosing, no significant lesions were observed in the lungs of MMC-treated mice.

The mean relative weights of livers from MMC mice were similar to the control means at day 42 and 50 post dosing; however, the relative weight of the liver of one MMC mouse autopsied on day 50 was 76,786 mg/kg body weight, compared with the mean control weight of 51,316 mg/kg body weight. On histological examination, no significant lesions were observed in the livers of MMC-treated animals sampled at day 42 and 50 post dosing. However, there was some congestion of the centrilobular sinusoids in a small number of MMC mice at these time points, but this was considered to be an agonal effect, or possibly secondary to cardiac changes (the hearts of MMC mice were not examined histologically).

The mean relative weights of the kidneys of MMC-treated mice sampled at day 42 post dosing were significantly increased to 145.4% ($p<0.05$) of the mean control

value (as 100%); in two individuals treated with MMC, the increases were 214.8% and 215.3% of the control figure (as 100%). However, at day 50 post dosing, the mean relative kidney weight of MMC animals was similar to the controls. Histological assessment of the kidney at day 42 and 50 post dosing demonstrated that lesions were present in the majority of MMC-dosed mice. The lesions, essentially comprised hydronephrosis with cortical glomerular and tubular atrophy and degeneration. The severity of the lesions varied considerably between individual animals, and also within the same individual, with unilateral changes being present in a small number of mice. In severe cases, the hydronephrotic kidneys comprised a thin rim of atrophic, basophilic and dilated cortical tubules surrounding a central cystic space.

7.3.2.10. Results summary

- Sustained reduction in erythropoiesis with compensatory reticulocytosis evident on days 14 and 28 post dosing.

- Initial reduction in platelet and leucocyte counts.

- Sustained reduction in bone marrow cellularity, CFU-Cs with increased apoptosis and serum FL.

7.4. DISCUSSION

Structurally, MMC is a quinone ring linked to an indole group; there are two side-groups, first a methoxyformamide side chain, and the second is an aziridine ring (Crooke and Bradner 1976). MMC acts as an alkylating agent after metabolic activation, mainly in the liver, and suppresses the synthesis of nucleic acids (Dollery 1999; Sweetman 2002). The drug is a cell cycle non-specific agent, but is most active in the late G_1 and early S phases of the cell cycle.

To avoid the serious and often fatal myelosuppression caused by MMC, patients are treated at 4 to 8 weekly intervals intravenously through a running saline infusion (BNF 2004). Often, however, profound leucopenia and thrombocytopenia occur at about 4 weeks after administration, with recovery at 8 to 10 weeks. However, in about 25% of patients, blood counts may not recover (Dollery 1999; Sweetman 2002). Other serious toxic effects of MMC in man include renal injury (Ravikumar *et al.* 1984; Valavaara and Nordman 1985; Verwey *et al.* 1987) and pulmonary reactions (interstitial pneumonitis) (Gunstream *et al.* 1983; McCarthy and Staats 1986; Linette *et al.* 1992; Okuno & Frytak 1997; Gagnadoux *et al.* 2002). MMC also induces the potentially fatal syndrome of thrombotic microangiopathy, which resembles the haemolytic-uraemic syndrome (HUS) (Pavy *et al.* 1982; Montes *et al.* 1993; Nishiyama *et al.* 2001; Gundappa *et al.* 2002). Here, symptoms of haemolytic anaemia and renal failure may be seen, in conjunction with hypertension and cardiovascular problems, pulmonary oedema, and neurological symptoms (Medina *et al.* 2001). The syndrome usually occurs after 6 months of MMC treatment. Hepatic veno-occlusive disease has also been described as an effect of MMC treatment (Lazarus *et al.* 1982; Craft and Pembrey 1987); the condition manifests as hepatomegaly, ascites, and liver failure. Other toxicities of MMC include gastro-intestinal effects, cardiotoxicity, dermatitis and

alopecia (Verweij *et al.* 1987). Local tissue necrosis may also be observed if drug solutions are extravasated from a vein or artery.

In the present study, with MMC administered to female CD-1 mice by ip injection on 8 occasions at 2.5 mg/kg over a period of 18 days, the drug induced a profound effect on peripheral blood (Molyneux *et al.* 2005). At day 1 post dosing, erythrocyte (Fig. 7.3 A) and reticulocyte counts (Fig. 7.3 C), and neutrophil (Fig. 7.3 E), lymphocyte and platelet counts (Fig. 7.3 D), were all significantly reduced. The marrow FNCC was also greatly decreased (Fig. 7.3 F). However, at day 7 post dosing, there was evidence of a return towards normal values; this was evident in the reticulocyte (Fig. 7.3 C), neutrophil (Fig. 7.3 E), lymphocyte and platelet counts (Fig. 7.3 D) and in the values for FNCC (Fig. 7.3 F). At 14 days post dosing, the recovery of these parameters in MMC mice continued; furthermore, RBC values also showed evidence of a return towards normal at this time (Fig. 7.3 A). At 28 days post dosing, MMC-treated mice showed a haematology picture which, for many parameters, was relatively normal, but there was evidence of a persisting effect on the RBC (Fig. 7.3 A) and FNCC values (Fig. 7.3 F), which continued to show reductions in comparison with the control animals. The effect on RBC and FNCC values continued to be seen at day 42 and 50 post dosing. This persisting and stable 'late-stage' effect at day 28, 42 and 50 in MMC-treated mice gave values for RBC of 87.8%, 89.9% and 90.4% of the mean control values; for FNCC: 79.9%, 68.8% and 76.6% of control values, respectively; a prolonged, late-stage effect was also evident in the erythroid lineage in the bone marrow smears taken from MMC-treated mice (Table 7.5).

There appear to be no haematological papers reporting repeat dose administration studies with MMC in the mouse and none where haematological observations have been made at a 'late-stage' post dosing. Benning *et al.* (1991) administered single ip doses of MMC (1.6 mg/kg) to adult CD-1 mice and studied

erythropoietic cytotoxicity up to day 7 post dosing. Kobayashi *et al.* (1981) gave single ip doses of MMC at 6.0 mg/kg to male CD2F1 mice and studied bone marrow suppression up to day 7 post dosing; there was a 90% reduction in the number of bone marrow cells on day 3 post dosing, and the lowest WBC count in the peripheral blood was seen at day 5 post dosing with recovery at day 7. Matsumoto *et al.* (1984) administered single ip doses of MMC at 2.0 mg/kg to male ddY mice and studied marrow cell counts and peripheral blood reticulocytes at a series of time points up to day 11 post dosing; at day 2 the marrow cell counts fell to 45% of control values, and at day 4 the peripheral blood reticulocytes fell to their lowest value, recovering to the control level on day 11. Bradner (1979) administered MMC at 7.5 mg/kg ip to male BDF1 mice and studied changes in peripheral blood WBC. The lowest count was seen at day 5 post dosing, with some recovery at day 7. However, Futamura and Matsumoto (1995) reported a repeat dose study of MMC in the rat. Seven daily doses of MMC were administered at 1 mg/kg ip, to male Wistar rats. Blood samples at day 1 post dosing showed significant reductions in RBC, WBC, reticulocytes and platelets.

In man, MMC may cause delayed, cumulative bone marrow suppression with a sometimes profound leucopenia and thrombocytopenia (Godfrey and Wilbur 1972; Jones *et al.* 1980; Zein *et al.* 1986; Veeder *et al.* 1992; Hortobagyi 1993; Medina *et al.* 2001). In a review of MMC toxicity, Crooke and Bradner (1976) reported that the most frequently used schedule of MMC administration was 50 μg/kg/day for 6 days, then every other day until toxicity was manifested. Using this regimen, haematological toxicity was delayed, but about 55% of patients experienced toxicity; thrombocytopenia was generally first noted in the $5^{th}/6^{th}$ week of treatment, and leucopenia in the $6^{th}/7^{th}$ week of the regimen; the duration of the leucopenia was generally 1 to 2 weeks, and the thrombocytopenia continued for 2 to 3 weeks. However, after a single MMC dose, the nadir for leucopenia was evident at 3½ weeks, and for thrombocytopenia the nadir was

at 4 weeks post dosing. Crooke and Bradner (1976), also reported that anaemia was commonly seen with all treatment regimens, but this was less severe than the effects on platelets and leucocytes; similar observations were described by Godfrey and Wilbur (1972) and Montes *et al.* (1993).

In the present investigations, preliminary dose ranging studies identified the ip single dose MTD for female CD-1 mice as 7.0 mg/kg. With 4 doses administered over 7 days the MTD was 2.5 mg/kg, and with 8 doses given over 18 days the MTD was also 2.5 mg/kg. These dose levels of MMC in the mouse, in general, find a parallel with the range of dose levels reported in other published papers. Bradner (1979) stated that the single dose ip LD_{50} in male BDF1 mice was 7.5 mg/kg; Yamada (1960) gave the ip LD_{50} as 5.2 mg/kg. Philips *et al.* (1960) reported the ip single dose LD_{50} in male Swiss mice as 8.5 mg/kg; when 5 daily doses were administered, the LD_{50} was 2.3 mg/kg. In haematological studies, Benning *et al.* (1991) used single ip doses of 1.6 mg/kg in CD-1 mice; Kobayashi *et al.* (1981) gave single doses of 6.0 mg/kg ip to male CDF1 mice, and Matsumoto *et al.* (1984) administered single ip doses of 2.0 mg/kg MMC to male ddY mice.

It is often considered that where morbidity/mortality is seen in the immediate post dosing period in mice treated with anti-cancer drugs, these effects may be related to bone marrow depression. However, although in the present MMC main study, a total of 17 mice were categorised as ICD from day 1 to 21 post dosing, there were a further 61 animals classified as ICD at a later stage, i.e. day 22 to 49 post dosing (Fig. 7.2).

The three MMC pilot dose ranging studies were carried out to identify an appropriate dose level of the drug to use in the main study which would cause no mortality. Therefore, the high level of mortality that did occur in this experiment (46.4%) was unexpected. The reasons for this mortality are unclear, but may be related to the dose of drug administered being relatively too high. The dose level of MMC used

in the main study (2.5 mg/kg) was based mainly on the results of the third pilot dose ranging study where mice were dosed on 8 occasions over 18 days at 1.50 to 3.25 mg/kg MMC. The mice in the third pilot study had an average body weight of 17.4 g, whereas mice in the first and second pilot studies weighed 26.5 g and 30.5 g, respectively. In all MMC experiments, the dose of drug administered was not changed during the period of dosing to take account of any changes in body weight. The mean body weights of mice in pilot study 3 were 17.4 g on the first day of dosing, 20.8 g on day 9, and 24.2 g on day 18 of dosing. Calculations therefore show that the level of MMC dosed on day 1 of dosing was 2.50 mg/kg, 2.09 mg/kg on day 9, and 1.79 mg/kg on day 18 of dosing. In the case of the main MMC study, mice were an average of 25.2 g on day 1 of dosing, 26.3 g on day 9, and 27.3 g on day 18 of dosing; the calculated dose levels of MMC administered on these days are therefore 2.50, 2.43 and 2.34 mg/kg, respectively. It is concluded therefore that these discrepancies in dose levels may account for high mortality in the main MMC study. Clearly, in retrospect, mice of the same body weight should have been used in the pilot and main studies.

A pronounced effect of MMC on the sternal bone marrow was evident in the histological examination of tissues from MMC mice; there was severe depletion of marrow cells in the immediate post dosing period (Molyneux *et al.* 2005). A relatively rapid return to normal cellularity was then observed at days 14 and 28 post dosing. A similar pattern of changes has been seen in earlier studies with the antineoplastic drug BU when repeatedly administered to mice (Gibson *et al.* 2003). Similarly, the histological changes evident in the spleen in the immediate post dosing period, in MMC-treated animals, compare with BU-induced spleen changes in the mouse (Gibson *et al.* 2003). Histological examination of the lungs and livers from MMC-dosed mice at day 42/50 did not reveal any significant drug-related changes. However, in the case of the kidneys from MMC-treated mice examined at late time points (day 42/50 post

dosing), hydronephrosis with cortical glomerular and tubular atrophy and degeneration was observed.

There are several reports of MMC-induced renal changes in laboratory animals. MMC caused hydronephrosis in mice repeatedly dosed with the drug (Matsuyama *et al.* 1964a, 1964b), and it was suggested that these lesions made the MMC-dosed mouse a suitable model of drug-induced hydronephrosis. Philips *et al.* (1960) reported MMC-induced renal changes (necrotising nephrosis) in the rhesus monkey, and effects on the kidney were also described by Verweij *et al.* (1988) in the rat. Bregman *et al.* (1987, 1989) studied the toxicity of MMC derivatives in the rat and reported that several compounds caused renal tubular degeneration and glomerulonephropathy. Cattell (1985) described a surgical study in the rat to determine whether MMC induced direct renal injury; the left kidney was perfused with MMC and tissues examined at a range of time points after perfusion. It was stated that lesions indistinguishable from human HUS developed, with glomerular endothelial damage, platelet accumulation and capillary wall splitting which was typical of microangiopathy; this surgical procedure in the rat was proposed as a new model of HUS. The technique of Cattell (1985) was also investigated by Blanco *et al.* (1992). These authors describe glomerular endothelial cell injury in the renal cortex with obliteration of the glomerular capillary lumen and cortical tubular necrosis.

There are many reports giving details of the nephrotoxicity of MMC in man. In a review of the literature by Crooke and Bradner (1976), cases of delayed nephrotoxicity and renal failure were described which occurred several months after the beginning of therapy. Fields and Lindley (1989) discussed the syndrome of MMC-associated thrombotic microangiopathy (TMA) where there was endothelial cell damage with resulting thrombus formation, RBC fragmentation and haemolysis. (The term TMA encompasses both thrombotic thrombocytopenia purpura (TPP) and HUS; both

syndromes involve microangiopathic haemolytic anaemia (MAHA), thrombocytopenia and renal failure; platelet thrombi in the microvasculature are seen in both TPP and HUS, but in HUS the thrombi are primarily confined to the kidney.) Similarly, several authors report cases of MMC-induced HUS/MAHA with nephrotoxic changes involving renal insufficiency, renal dysfunction, or renal failure (Pavy *et al.* 1982; Ravikumar *et al.* 1984; Valavaara and Nordman 1985; Montes *et al.* 1993; Medina *et al.* 2001; Nishiyama *et al.* 2001; Gundappa *et al.* 2002).

In conclusion, one of the main purposes in carrying out the present investigations was to determine if MMC caused significant late-stage/residual effects in the bone marrow and blood, as described by Trainor *et al.* (1979) and Morley (1980), and if the drug could therefore be considered as a replacement for BU in the development of new mouse models of late-stage bone marrow aplasia. We conclude that although there was some evidence of late-stage effects on blood erythrocytes, marrow FNCC, and cells of the erythroid lineage, the changes induced were relatively mild, and MMC is therefore not seen as a useful substitute for BU in models of chronic bone marrow aplasia in the mouse.

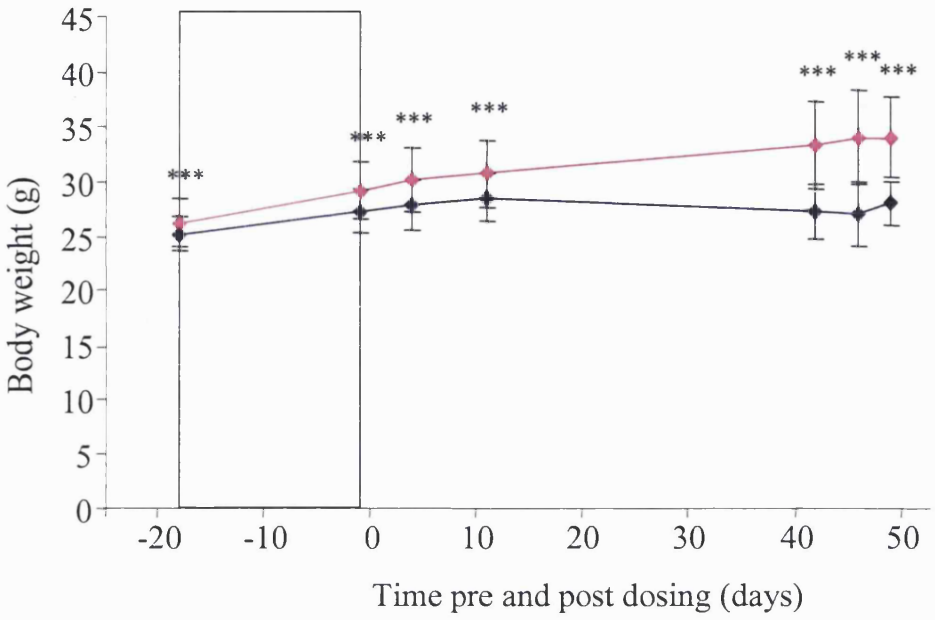


FIGURE 7.1. Body weight increases in control female CD-1 mice and mice treated with mitomycin C (MMC) at 2.5 mg/kg. Values are means (±SD) of control (—◆—) and MMC-treated (—◆—) mice. Animals were treated with vehicle or MMC on 8 occasions over an 18 day dosing period (▭). ***Significantly different from controls, $p<0.001$.

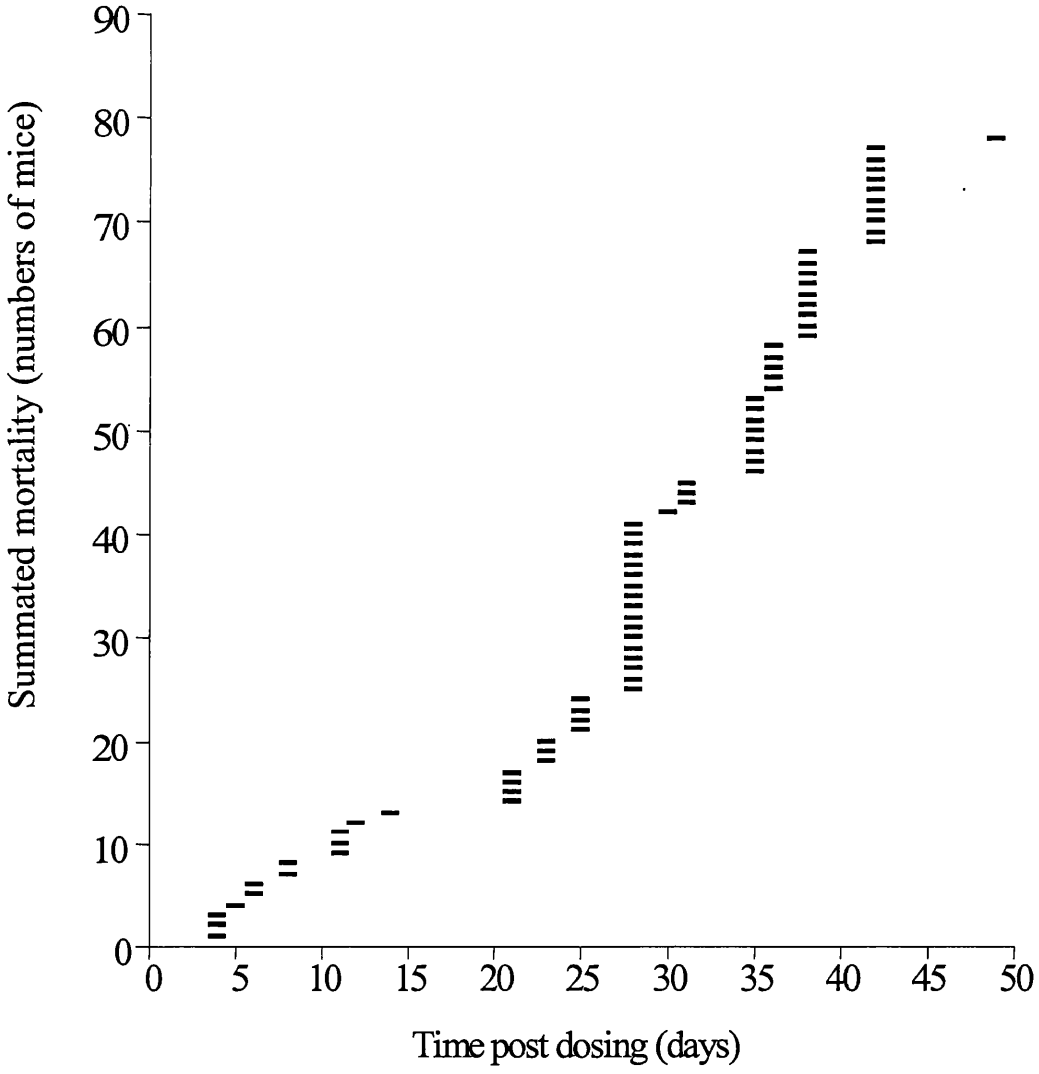
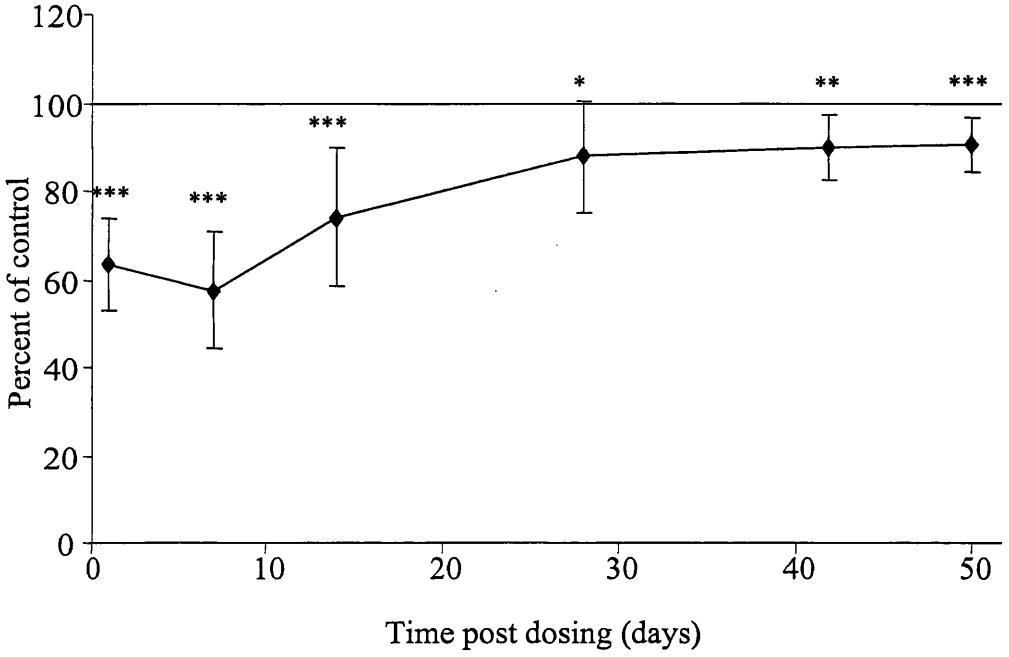
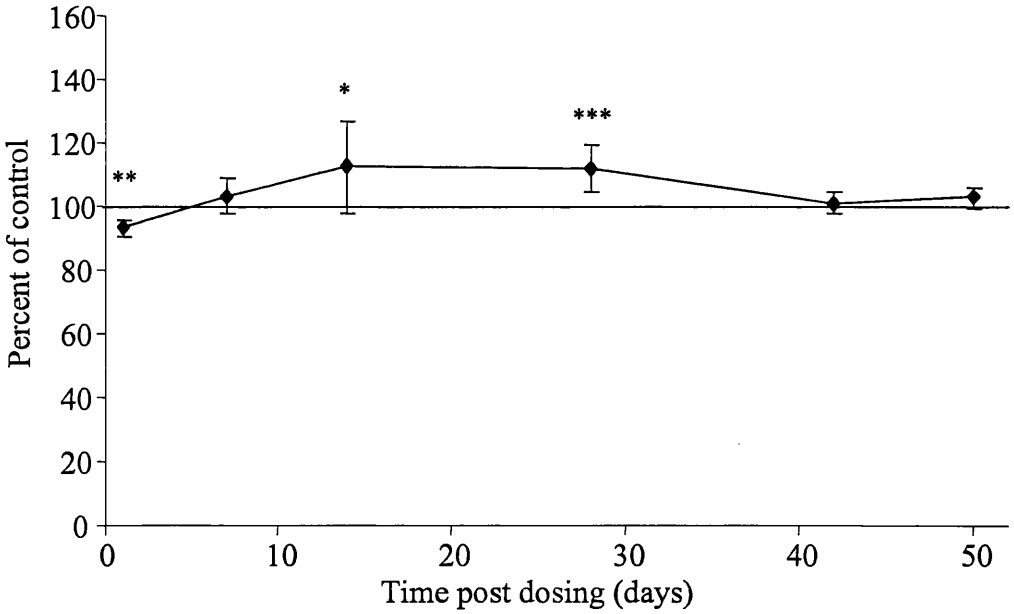


FIGURE 7.2. Mortality in female CD-1 mice treated with mitomycin C at 2.5 mg/kg on 8 occasions over an 18 day period. Each bar represents an ICD (inter-current death) animal.

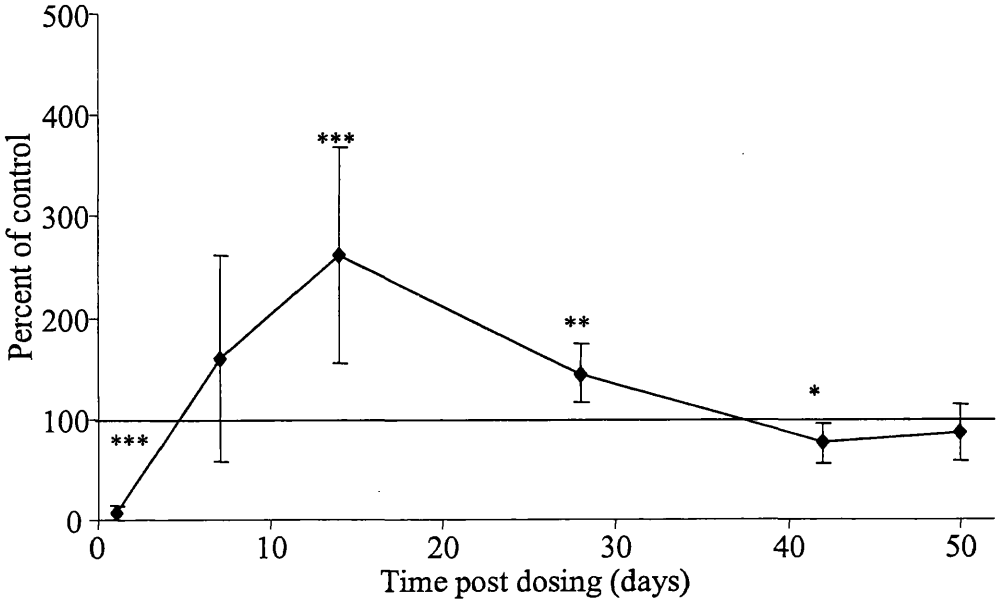
A. Red blood cells



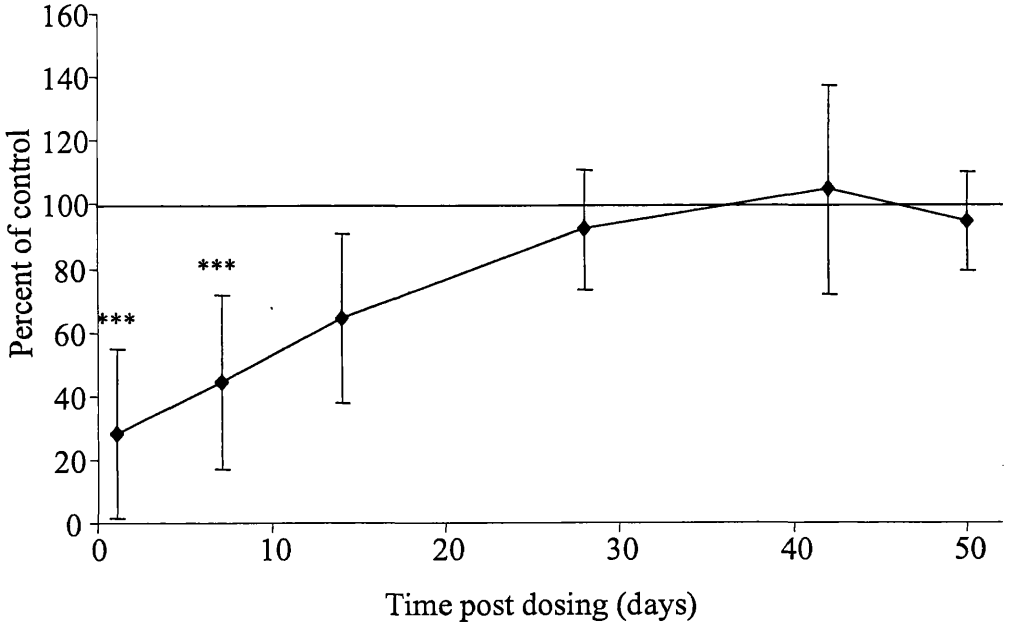
B. Mean cell volume



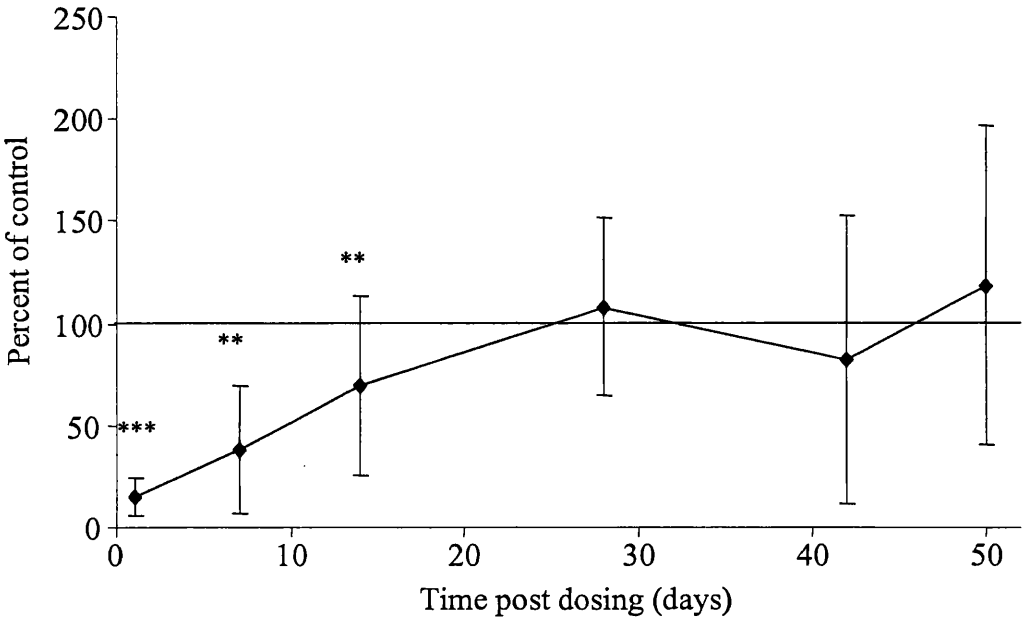
C. Reticulocytes



D. Platelets



E. Neutrophils



F. Femoral nucleated cell count

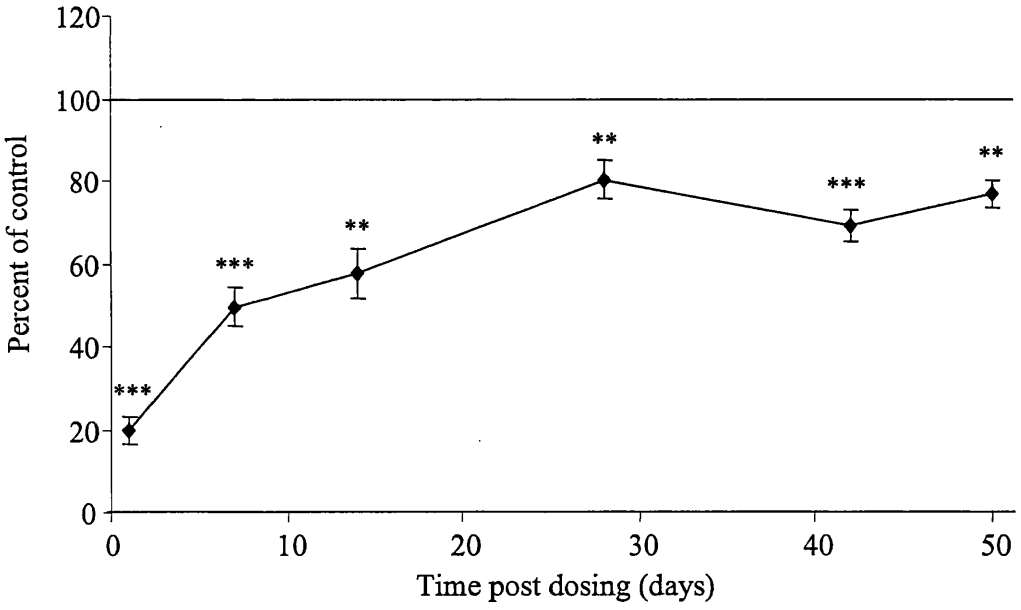
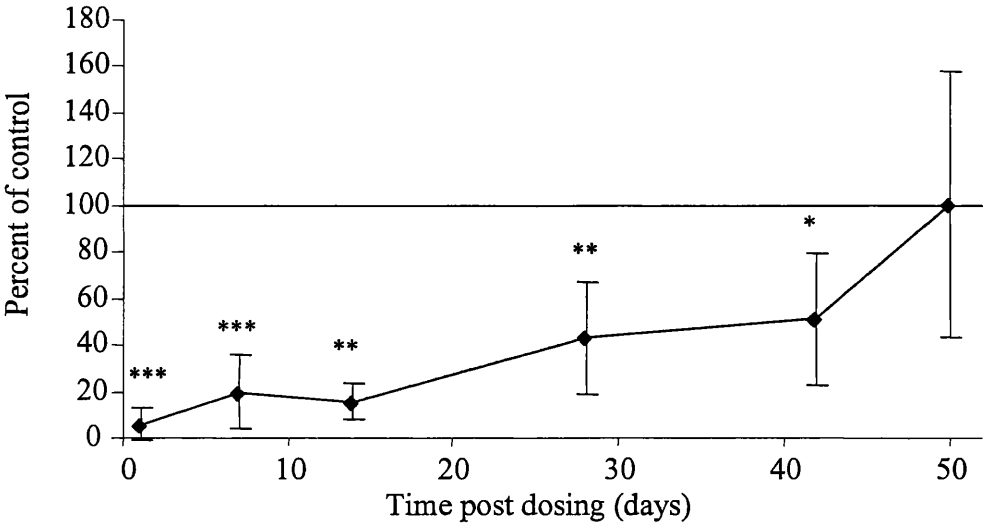


FIGURE 7.3. Results for red blood cells, mean cell volume, reticulocytes, platelets, neutrophils and femoral nucleated cell counts from female CD-1 mice treated with mitomycin C (MMC) and sampled on days 1 to 50 post dosing. Values are means (± SD), expressed as a percentage of the mean control value in animals sampled at the same time points. Animals were treated with vehicle (control) or MMC (2.5 mg/kg) on 8 occasions over 18 days and autopsied on days 1, 7, 14, 28, 42 and 50 post dosing. n=8 for control and MMC-treated mice at all time points except day 50 where n=12 (control) and n=6 (MMC). *Significantly different from controls, $p<0.05$; **$p<0.01$; ***$p<0.001$.

A. Colony forming unit-granulocyte monocyte



B. Erythroid colonies

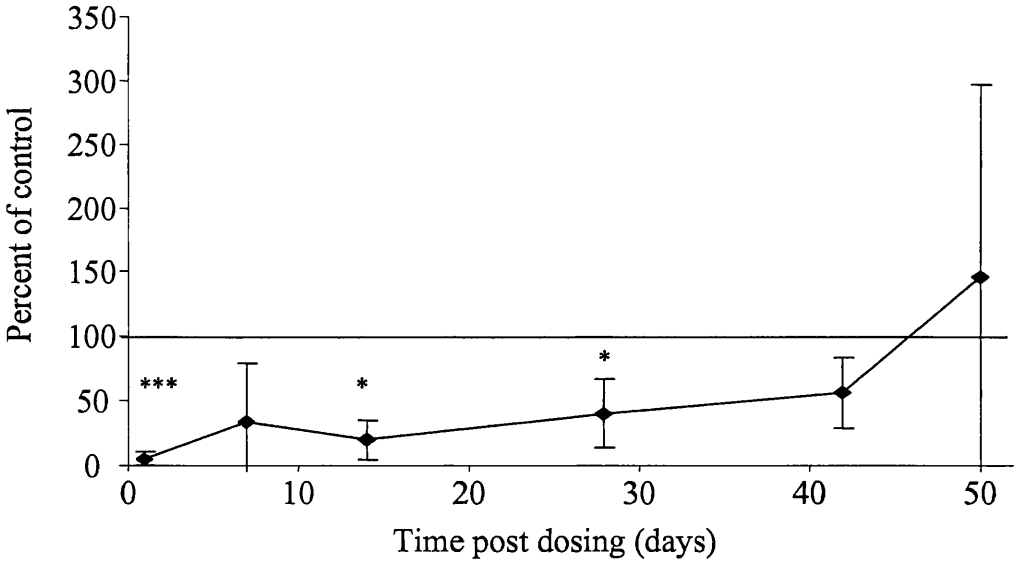


FIGURE 7.4. Results for colony forming unit-granulocyte monocyte (CFU-GM) and erythroid colonies from female CD-1 mice treated with mitomycin C and sampled on days 1 to 50 post dosing. Values are means (± SD), expressed as a percentage of the mean control value in animals sampled at the same time points. Cultures were set up in duplicate and the mean CFU-GM and erythroid colony number per femur calculated per mouse. Numbers of animals per group and all other information as Fig. 7.3. *Significantly different from controls, $p<0.05$; **$p<0.01$; ***$p<0.001$.

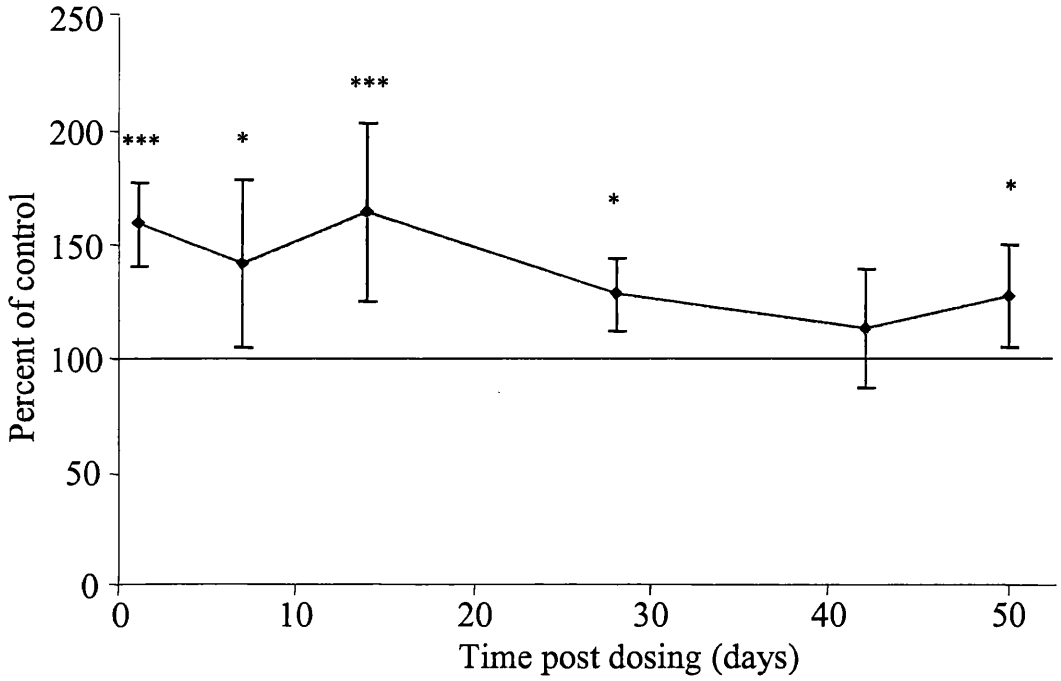


FIGURE 7.5. Apoptosis in femoral bone marrow samples; results from female CD-1 mice treated with mitomycin C and sampled on days 1 to 50 post dosing. Values are means (± SD), expressed as a percentage of the mean control value in animals sampled at the same time points. Numbers of animals per group and all other information as Fig. 7.3. *Significantly different from controls, $p<0.05$; ***$p<0.001$.

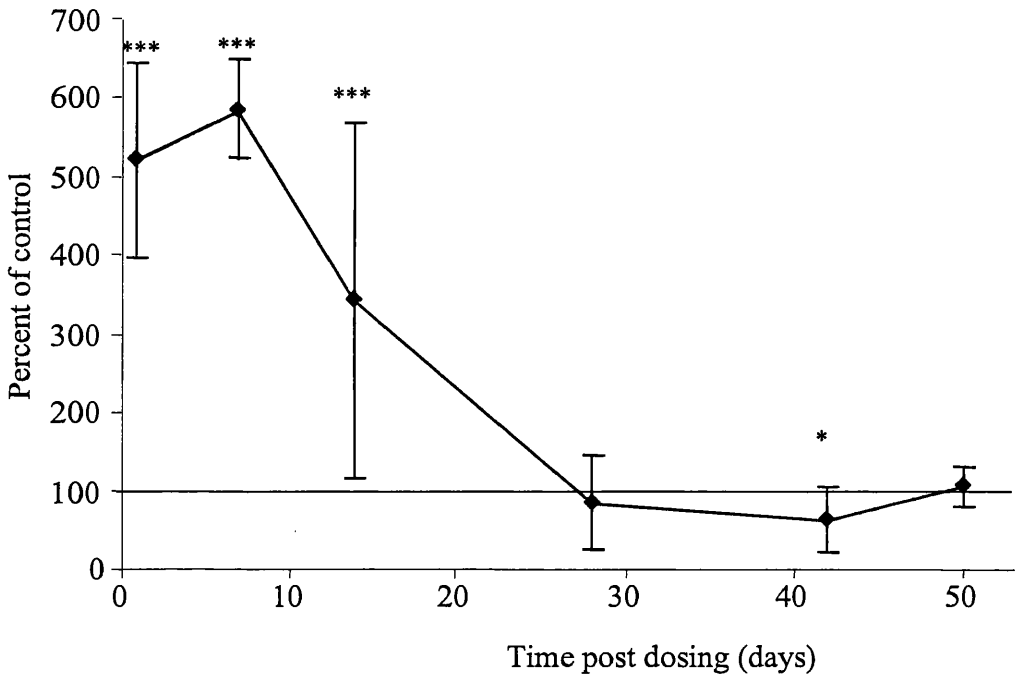


FIGURE 7.6. Serum FL results from female CD-1 mice treated with mitomycin C and sampled on days 1 to 50 post dosing. Values are means (± SD), expressed as a percentage of the mean value in control animals sampled at the same time points. n=6 for control and MMC-treated mice at all time points except day 1 where n=5 (control and MMC) and day 50 where n=12 (control) and n=5 (MMC). *Significantly different from controls, $p<0.05$; ***$p<0.001$.

TABLE 7.1. Mortality[a] in a preliminary dose ranging study in female CD-1 mice treated with mitomycin C on 4 occasions at 1.0 to 5.5 mg/kg over an 8 day period and autopsied at 50 days after the final dose

Treatment (mg/kg)	No. of mice	No. of ICD mice	ICD day (day post dosing)		
0 (control)	5	0			
1.0	3	0			
1.5	3	0			
2.0	3	0			
2.5	3	0			
3.0	3	2	26	35	
3.5	3	3	-2	1	5
4.0	3	3	4	8	50
4.5	3	3	4	8	42
5.0	3	3	3	3	3
5.5	3	3	2	2	3

[a]Animals were either killed *in extremis* or were found dead; all such animals are referred to as 'inter-current death' (ICD) animals.

TABLE 7.2. Haematological results[a] from female CD-1 mice treated with 4 doses of mitomycin C (MMC) at 0 (control), 1.0, 1.5, 2.0, 2.5 and 3.0 mg/kg over a period of 8 days and autopsied on day 50 after the final dose[b]

	Treatment (mg/kg)					
	0 (Control)	**1.0**	**1.5**	**2.0**	**2.5**	**3.0**
RBC	8.76 (0.35)	8.64 (0.17)	7.92 (0.24)	7.19 (0.16)	7.15 (0.90)	7.42
Hb	13.7 (0.38)	14.0 (1.1)	13.1 (0.5)	12.3 (0.6)	11.8 (0.7)	12.1
HCT	41.8 (1.4)	42.1 (2.5)	37.9 (0.5)	36.2 (0.7)	36.5 (5.2)	36.2
MCV	47.8 (0.9)	48.7 (2.0)	47.8 (0.9)	50.4 (1.9)	51.0 (0.9)	48.8
MCH	15.6 (0.3)	16.2 (1.0)	16.5 (0.1)	17.1 (1.1)	16.6 (1.1)	16.3
MCHC	32.6 (1.0)	33.2 (1.3)	34.5 (1.0)	33.9 (1.3)	32.7 (2.8)	33.5
Retics	377 (15)	371 (7)	341 (10)	309 (7)	307.2 (39)	319
Plt	1,484 (133)	1,501 (168)	1,524 (170)	1,684 (634)	1,365 (166)	1,261
WBC	1.67 (0.09)	1.09 (0.61)	1.02 (0.07)	0.73 (0.51)	0.91 (0.43)	0.67
Neut	0.27 (0.11)	0.21 (0.10)	0.14 (0.03)	0.20 (0.12)	0.50 (0.44)	0.24
Lymph	1.25 (0.72)	0.74 (0.42)	0.80 (0.05)	0.48 (0.38)	0.31 (0.01)	0.34
Mono	0.07 (0.04)	0.03 (0.01)	0.06 (0.04)	0.02 (0.02)	0.06 (0.01)	0.01
Eo	0.08 (0.04)	0.09 (0.09)	0.03 (0.01)	0.02 (0.01)	0.03 (0.01)	0.06
Baso	0.00 (0.00)	0.00 (0.01)	0.00 (0.00)	0.00 (0.00)	0.01 (0.01)	0.00
FNCC	2.46 (0.47)	2.38 (0.50)	2.44 (0.38)	1.90 (0.15)	2.28 (0.04)	2.70

[a]Values are means with SD in parenthesis. n=5 for control (vehicle-treated) mice, n=3 for mice treated with MMC at 1.0 and 2.0 mg/kg , n=2 for mice treated with MMC at 1.5 and 2.5 mg/kg, n=1 for mouse treated with 3.0 mg/kg. Statistical analysis not carried out.

[b]Abbreviations and units: RBC, red blood cells, $x10^6/\mu l$; Hb, haemoglobin, g/dl; HCT, haematocrit, %; MCV, mean cell volume, fl; MCH, mean cell haemoglobin, pg; MCHC, mean cell haemoglobin concentration, g/dl; Retic, absolute reticulocyte count, $x10^3/\mu l$; Plt, platelets, $x10^3/\mu l$; WBC, white blood cells, $x10^3/\mu l$; Neut, neutrophils, $x10^3/\mu l$; Lymph, lymphocytes, $x10^3/\mu l$; Mono, monocytes, $x10^3/\mu l$; Eo, eosinophils, $x10^3/\mu l$; Baso, basophils, $x10^3/\mu l$; FNCC, femoral bone marrow nucleated cell count, $x10^7$.

TABLE 7.3. Mortality[a] in a preliminary dose ranging study in female CD-1 mice treated with mitomycin C on 8 occasions at 1.50 to 3.25 mg/kg over an 18 day period

Treatment (mg/kg)	No. of mice	No. of ICD mice	ICD day (day post dosing)		
0 (control)	6	0			
1.50	3	0			
1.75	3	1	70		
2.00	3	0			
2.25	3	1	48		
2.50	3	1	57		
2.75	3	3	46	46	48
3.00	3	3	50	51	67
3.25	3	3	2	8	46

[a]Animals were either killed *in extremis* or were found dead; all such animals are referred to as 'inter-current death' (ICD) animals. This experiment was terminated on day 72 post dosing.

TABLE 7.4. Haematological results[a] from female CD-1 mice treated with 8 doses of mitomycin C at 0 (control), 1.50, 1.75, 2.00, 2.25 and 2.50 mg/kg over a period of 18 days and autopsied on day 72 after the final dose

	Treatment (mg/kg)					
	0 (Control)	**1.50**	**1.75**	**2.00**	**2.25**	**2.50**
RBC	9.03 (0.50)	8.23 (0.21)	7.43 (0.40)	7.67 (0.11)	8.18 (0.11)	7.45 (0.11)
Hb	14.6 (0.9)	13.0 (0.6)	12.8 (0.5)	12.0 (0.6)	13.0 (2.2)	12.2 (0.9)
HCT	45.3 (3.0)	40.1 (1.0)	37.9 (2.5)	36.2 (1.0)	38.3 (8.1)	37.2 (4.3)
MCV	50.2 (2.1)	48.8 (2.65)	50.9 (0.8)	47.3 (1.9)	46.7 (3.6)	49.9 (5.2)
MCH	16.2 (0.5)	15.9 (1.1.)	17.2 (0.4)	15.6 (0.7)	15.8 (0.6)	16.3 (1.0)
MCHC	32.3 (0.7)	32.5 (0.6)	33.8 (1.0)	33.0 (1.3)	33.9 (1.3)	32.9 (1.7)
Retics	288 (116)	314 (74)	276 (22)	244 (10)	235 (267)	276 (220)
Plt	1,511 (267)	1,085 (421)	1,250 (256)	1,383 (386)	1,807 (691)	1,762 (470)
WBC	2.34 (0.56)	1.02 (0.28)	0.86 (0.19)	1.85 (0.69)	1.34 (0.30)	1.06 (0.46)
Neut	0.33 (0.09)	0.19 (0.04)	0.23 (0.02)	1.02 (0.71)	0.51 (0.56)	0.39 (0.44)
Lymph	1.83 (0.46)	0.76 (0.23)	0.58 (0.17)	0.17 (0.06)	0.65 (0.24)	0.56 (0.14)
Mono	0.05 (0.01)	0.04 (0.00)	0.03 (0.01)	0.04 (0.02)	0.09 (0.09)	0.07 (0.07)
Eo	0.11 (0.06)	0.04 (0.01)	0.03 (0.00)	0.04 (0.02)	0.09 (0.13)	0.02 (0.02)
Baso	0.01 (0.01)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.01 (0.01)	0.00 (0.01)
FNCC	4.28 (0.63)	3.60 (0.35)	3.32 (0.20)*	2.96 (1.11)	3.83 (0.37)	3.27 (0.26)

[a]Values are means with SD in parenthesis. n=6 for control (vehicle-treated), n=3 for mice treated with mitomycin C at 1.50 and 2.00 mg/kg, n=2 for mice treated with mitomycin C at 1.75, 2.25 and 2.50 mg/kg. Abbreviations and units as Table 7.2. Statistical analysis conducted using a one-way analysis of variance (ANOVA).*Significantly different from controls, $p<0.05$.

TABLE 7.5. Estimated counts ($x10^7$) of myeloid, erythroid and lymphoid cells, and the myeloid:erythroid (M:E) ratio in the femoral marrow of control and mitomycin C (MMC) treated mice[a]; animals were given 8 doses of MMC over a period of 18 days and sampled at 1 to 50 days after the final dose.

	Day of sampling											
	1		7		14		28		42		50	
	Control	MMC	Control	MMC	Control	MMC	Control	MMC	Control	MMC	Control	MMC
Myeloid	1.15	0.06***	1.26	0.55***	1.20	0.88	1.56	1.44	1.40	1.08*	1.35	1.17
	(0.22)	(0.06)	(0.27)	(0.30)	(0.31)	(0.78)	(0.30)	(0.35)	(0.29)	(0.29)	(0.33)	(0.37)
Erythroid	0.95	0.09***	1.07	0.49***	1.00	0.57**	1.16	0.65*	1.16	0.68**	0.98	0.73*
	(0.12)	(0.10)	(0.18)	(0.29)	(0.22)	(0.26)	(0.37)	(0.36)	(0.42)	(0.19)	(0.14)	(0.29)
Lymphoid	0.36	0.32	0.28	0.30	0.41	0.10***	0.42	0.13**	0.39	0.16**	0.42	0.18
	(0.14)	(0.16)	(0.10)	(0.01)	(0.13)	(0.04)	(0.18)	(0.02)	(0.15)	(0.04)	(0.27)	(0.25)
Other	0.10	0.00***	0.12	0.01**	0.08	0.01**	0.05	0.02	0.07	0.01**	0.07	0.01*
	(0.06)	(0.00)	(0.09)	(0.01)	(0.05)	(0.02)	(0.04)	(0.02)	(0.04)	(0.02)	(0.06)	(0.01)
M:E ratio	1.21	0.77**	1.18	1.30	1.21	1.38	1.44	2.70*	1.26	1.66	1.37	1.88
	(0.17)	(0.30)	(0.16)	(0.91)	(0.22)	(0.63)	(0.44)	(1.15)	(0.23)	(0.58)	(0.26)	(1.03)

[a]200 cells in the tibial marrow smears were differentially counted by eye and the absolute number of cells of each lineage estimated for the nucleated marrow cell count of the femoral marrow flush. Values are means and SD in parenthesis. Cells categorised as 'other' include monocytes and monocyte precursors, mast cells, megakaryocytes, plasma cells and unidentifiable cells. n=6 for control and MMC groups at all time points.
*Significantly different from controls, $p<0.05$; **$p<0.01$; ***$p<0.001$.

CHAPTER 8: Studies on the haemotoxicity of azathioprine in the CD-1 mouse

8.1. INTRODUCTION

Purine analogues, first synthesised in the 1940s, were developed for therapeutic use in diseases of a proliferative nature such as acute leukaemia. The reason for developing these agents (e.g. 6-mercaptopurine (6-MP), 6-thioguanine) was to interfere with DNA synthesis thus hindering cell proliferation. The pro-drug azathioprine (AZA) was later developed as a slow release form of 6-MP by adding a nitroimidazole side chain to 6-MP (Elion 1989).

6-MP, and later AZA, were later found to be modulators of the immune system, capable of preventing renal graft rejection and inhibiting the immune response in rabbits injected with foreign antigens (Elion 1989). The discovery of extended renal graft survival following treatment with AZA and 6-MP was initially made in dogs (Calne 1960; 1961) and soon led to the introduction of AZA into clinical practice, and optimal results were achieved using AZA in combination with corticosteroids (Marino and Doyle 1994). The use of AZA revolutionised the practice of organ transplantation by providing essential therapy to prevent graft rejection. This led to an increase in kidney and other organ transplantation procedures and also provided the means to successfully transplant a variety of other organs and tissues e.g. liver, lung and heart (Elion 1989; Mario and Doyle 1994).

For nearly 20 years AZA in combination with corticosteriods was the principal post transplant immunosuppressant therapy in use until the discovery of cyclosporin A (CsA) (Borel *et al.* 1976) and the introduction of this drug into clinical practice in 1979 (Marino and Doyle 1994). CsA, in turn, had a dramatic effect on the field of organ

transplantation by reducing the incidence of graft rejection and increasing long-term survival, particularly when used in combination with corticosteriods (Marino and Doyle 1994).

Today, AZA is still administered to transplant recipients particularly in the prevention of renal graft rejection and the drug is commonly used as a steroid sparing agent in diseases of an autoimmune nature e.g. rheumatoid arthritis and Crohn's disease (BNF 2004).

The use of AZA is, however, not without complications. AZA induces a range of side effects that ultimately may result in the discontinuation of therapy; these changes include malaise, nausea, vomiting, diarrhoea, abdominal pain, fever and hypotension (BNF 2004). Liver toxicity is also frequently seen in the form of hepatic veno-occlusive disease and hepatitis (Satti *et al.* 1982; Lemley 1989; Dubinsky *et al.* 2003). However the most severe side effect of AZA is dose-related myelosuppression (Present *et al.* 1989; Dollery 1999).

The present studies in mice were conducted to examine the effects of AZA treatment on the haemopoietic system. AZA was chosen as this drug has previously been shown in man to cause pancytopenia and aplastic anaemia (AA) following therapeutic exposure (Bacon *et al.* 1981; Jeurissen *et al.* 1988; Burke *et al.* 1989; Sudhir *et al.* 2002). These studies would then allow us to carry out subsequent experiments aimed at evaluating AZA, administered as a second agent, to mice pre-treated with BU, as described by Gibson *et al.* (2003) and Turton *et al.* (2006). In the present investigations therefore, changes in the peripheral blood counts, the number of committed bone marrow progenitor cells, the level of apoptosis, and the concentrations of several serum cytokines (fms-like tyrosine kinase 3 (FLT-3) ligand (FL); interferon gamma (IFN-γ); tumour necrosis factor alpha (TNF-α); interleukin 2 (IL-2)) would be studied. These experiments were planned: first, a preliminary dose ranging pilot study

(experiment 1), a dose response study (experiment 2), and a time course study (experiment 3). A preliminary report in abstract form has been published (Molyneux *et al.* 2004c).

8.2. MATERIALS AND METHODS

8.2.1. Experimental design

8.2.1.1. Experiment 1; Preliminary dose ranging pilot study

Forty-eight female CD-1 mice (Charles River UK Ltd.), mean body weight 20.4 g, were randomly allocated to 12 groups (n=4 per group) and dosed by gavage with AZA (Sigma) prepared as described in Chapter 2.2, at the following dose levels: 0 (vehicle control), 25, 50, 75, 100, 125, 150, 200, 250, 300, 350 and 400 mg/kg. Animals were treated daily with AZA for 10 days and studied for 19 days post dosing. During the dosing and post-dosing periods, animals were observed for evidence of drug toxicity twice daily (or more frequently), and weighed every 2 or 3 days.

8.2.1.2. Experiment 2; Dose response study

AZA was administered to female CD-1 mice (Charles River UK Ltd.), mean body weight 25.7 g (n=60), at 0, 40, 60, 80, 100 or 120 mg/kg (n=10 per group). Animals were treated daily for 10 days with vehicle (vegetable oil) or AZA, by gavage, at a dose volume of approximately 0.20 ml/mouse. On day 1 post dosing all animals were killed for blood and bone marrow investigations (as described in Chapter 2.3).

8.2.1.3. Experiment 3; Time course study

136 female ICR (CD-1) mice (mean body weight 21.7 g) were purchased from Harlan (Harlan UK Ltd., Bicester, Oxon) and divided into 2 groups of 68 and treated

daily for 10 days with vehicle or AZA (100 mg/kg) by gavage at a dose volume of approximately 0.20 ml/mouse. On day 1, 3, 9, 22, 29, 43 and 51 post dosing, animals (n=5 to 10) were killed for blood and bone marrow investigations (as described in Chapter 2.3).

8.2.2. Processing samples

8.2.2.1. Experiment 2; Dose response study

A full blood count was performed and a femoral marrow flush into 5 ml Iscove's modified Dulbecco's medium (IMDM) was carried out to measure the cellularity of the bone marrow (FNCC) (as described in Chapter 2.4). Serum samples from both control and AZA-treated mice were prepared to measure the concentration of the cytokine FL (as described in Chapter 2.8.). A tibial marrow smear was also prepared and a differential count performed by eye on 200 cells (as described in Chapter 2.5). At each autopsy the spleen, liver and both kidneys were removed, weighed and placed in fixative with the sternum and thymus (as described in Chapter 2.9).

8.2.2.2. Experiment 3; Time course study

A full blood count was performed and a femoral marrow flush into 5 ml IMDM prepared to measure the FNCC (as described in Chapter 2.4). A tibial marrow smear was also prepared and a differential count performed by eye on 200 cells (as described in Chapter 2.5). The second femur was placed in 5 ml IMDM supplemented with 10% foetal calf serum and processed to assess the level of apoptosis and the clonogenic potential of the bone marrow (as described in Chapter 2.6 and 2.7). Serum samples from

both control and AZA-treated mice were prepared to measure the concentration of the cytokines FL, IL-2, TNF-α and IFN-γ (as described in Chapter 2.8.). At each autopsy the spleen, liver and both kidneys were removed, weighed and placed in formalin fixative, in addition to the sternum and thymus (as described in Chapter 2.9).

8.2.3. Statistical analysis

Results from Experiment 2 (Dose response study) were analysed using a one-way analysis of variance (ANOVA) followed by Tukey's highest significance test for post hoc pairwise multiple comparison. In cases of violation of the assumptions for parametric testing, the Kruskal-Wallis test was used in combination with Dunn post-test. Statistical analysis was performed using GraphPad Prism version 4.00 for windows (GraphPad software, San Diego, CA, USA).

In Experiment 3 (Time course study), AZA-treated and control (vehicle-treated) groups were compared using an unpaired one tailed Student's t-test (Microsoft Excel; Microsoft Ltd, Microsoft UK, Reading UK).

8.3. RESULTS

8.3.1. Experiment 1; Preliminary dose ranging pilot study

8.3.1.1. Clinical signs and body weight changes

At 25 and 50 mg/kg AZA, mean body weight increases during the 10 day dosing period were similar to the controls (12.7%, controls; 13.8%, 25 mg/kg; 12.5%, 50 mg/kg). At 75 and 100 mg/kg AZA, the mean body weight increases during the dosing period were slightly reduced, being 8.9% and 8.8%, respectively. At 125, 150 and 200 mg/kg AZA, the mean body weight increases dosing the dosing period showed a dose-related decrease, in comparison with the control mice, being 7.8%, -4.9% and –11.0%, respectively. At the 250, 300, 350 and 400 mg/kg dose levels, mice did not survive through the 10 day period of AZA treatment; animals were either killed *in extremis* (KIE), or on occasion were 'found dead' (FD); KIE and FD mice were grouped together and catagorised as inter-current death (ICD) animals. The mean ICD day, for mice in the 250 and 300 mg/kg AZA groups, was day 7 of dosing and for mice in the 350 and 400 mg/kg AZA groups the mean ICD was day 6 of dosing. The clinical signs of AZA toxicity were loss of condition, piloerection, stained fur in the urinary/genital region, reduced movement and closed eyes.

In the 19 day post dosing period, animals in the vehicle treated group increased in body weight by 16.9%; animals in the 25, 50, 75 and 100 mg/kg AZA groups, also increased in weight, by 28.6%, 12.2%, 24.4% and 23.0%, respectively; there was no evidence of toxicity in these groups during or after the dosing period. At 125, 150 and 200 mg/kg AZA, mice did not survive through the 19 day post dosing period; the mean ICD mice in these groups was day 10, 6 and 1 post dosing, respectively. All mice in the 125, 150 and 200 mg/kg groups showed clinical signs of toxicity, as described above.

It was decided, as a result of this dose ranging pilot study, to treat mice with AZA at 40, 60, 80, 100 and 120 mg/kg in experiment 2 (Dose response study), with autopsy at day 1 post dosing, and in Experiment 3 (Time course study) to dose with AZA at 100 mg/kg.

8.3.2. Experiment 2; Dose response study

8.3.2.1. Clinical signs and body weight changes

During the 10 day dosing period with AZA administered at 40, 60, 80, 100 and 120 mg/kg, both control and AZA-treated mice maintained a good state of health with no clinical evidence of toxic effects. The mean body weight of control (vehicle-treated) animals increased from 23.9 g to 28.0 g (a 17.2% increase). Body weight changes in mice treated with AZA were of a much lower magnitude, the mean body weight of animals treated with 40 and 60 mg/kg AZA increased by 2.1% and 2.3%, respectively. The mean body weights of animals treated with 100 and 120 mg/kg AZA increased by 4.5% and 4.1%, respectively over the 10 day dosing period. However, animals treated at 80 mg/kg, remained at approximately the same mean weight throughout the dosing period.

8.3.2.2. Haematology results

The changes in the RBC, MCV, reticulocytes, platelets and WBC are shown graphically in Fig. 8.1 A-E, respectively. At all dose levels, AZA induced a significant peripheral blood pancytopenia. Significant reductions were seen in mean erythrocyte parameters (RBC, Hb, HCT and reticulocytes), leucocyte parameters (WBC, neutrophils, lymphocytes, monocytes and eosinophils) and in the mean platelet count. A

statistically significant increase ($p<0.01$) in MCV was seen in animals treated with 80 mg/kg AZA (Fig. 8.1 B). There appeared to be general trends for dose-related effects in the changes for RBC, reticulocytes, platelets and WBC.

8.3.2.3. Femoral nucleated cell count

Administration of AZA at all dose levels induced a significant reduction in the cellularity of the bone marrow (Fig. 8.1 F). The mean FNCC of mice treated with 40 and 60 mg/kg of AZA was significantly reduced to 52.5% and 35.3% of the control mean, respectively. In mice treated with higher levels of AZA (80, 100 and 120 mg/kg), the reduction in marrow cellularity was much more pronounced being to 17.9%, 16.7% and 15.2% of the control mean, respectively. In this way there was an overall trend for a dose-related response to the level of AZA administered.

8.3.2.4. Serum fms like tyrosine kinase 3 (FLT-3) ligand (FL)

The concentration of serum FL was measured in mice treated with AZA at 0, 40, 60, 80, 100 and 120 mg/kg. A dose-related increase in serum FL was evident (Fig. 8.2). Mean levels of FL were increased to 2253.4 ± 614.3 and 2455.7 ± 541.7 pg/ml in mice treated with AZA at 100 and 120 mg/kg, respectively ($p<0.001$) compared to a mean control value of 529.4 ± 70.9 pg/ml. Animals treated with lower doses of AZA (40, 60 and 80 mg/kg) also had elevated levels of serum FL with mean values being, 828.2, 1153.4 and 1502.8 pg/ml, respectively (NS).

8.3.2.5. Bone marrow differential counts

Estimated counts of myeloid, erythroid and lymphoid cells in the femoral marrow demonstrated that the cells of the myeloid and lymphoid lineages were reduced in all groups treated with AZA, with the reductions reaching statistical significance in the 80, 100 and 120 mg/kg AZA-treated groups (Table 8.1). Cells of the erythroid lineage were also profoundly reduced at all dose levels following treatment with AZA, with the average percentage reduction being to 24.0% of the control mean ($p<0.001$ at all dose levels).

8.3.2.6. Histopathological assessment of tissues

Sections of thymus, kidney, liver, spleen and sternum were assessed from 5 control mice and mice treated with AZA at 40, 80 and 120 mg/kg (5 mice per dose level groups). Changes in the histology of the thymus were minimal with the majority of animals assessed showing signs of mild atrophy, and there was also some evidence of apoptosis occurring in animals treated with AZA at 120 mg/kg.

At autopsy on day 1 post dosing, the relative kidney weights of mice treated with AZA were comparable to control values (Table 8.2). Upon histological examination, the kidneys of mice treated with 120 mg/kg AZA were unchanged and therefore tissues from animals treated with lower doses of AZA were not assessed.

The mean relative liver weights of mice treated with AZA at 40 and 60 mg/kg were comparable to control values (Table 8.2). However, the mean relative liver weights of mice treated with higher doses of AZA (80, 100 and 120 mg/kg) were generally increased and this reached statistical significance in the 80 mg/kg group ($p<0.05$). Histological examination of the liver demonstrated centrilobular hepatocyte

hypertrophy in all mice treated with 80 and 120 mg/kg AZA, and in the majority of mice treated with 40 mg/kg AZA; livers from mice treated at 60 and 100 mg/kg were not examined.

A reduction in relative spleen weight was evident in mice treated with AZA. This reduction in mean relative spleen weight was statistically significant in all groups ($p<0.001$) with the exception of mice treated with AZA at 80 mg/kg (NS) (Table 8.2). In the spleen a significant dose-related reduction in extramedullary haemopoiesis was evident with erythropoiesis being virtually absent in the majority of animals examined. Granulopoiesis and megakaryopoiesis however did persist. In the sternal marrow, a significant reduction in cellularity was seen; all cell lines were depleted in all animals treated with 80 and 120 mg/kg AZA and in the majority of mice treated with 40 mg/kg AZA.

8.3.3. Experimet 3; Time course study

8.3.3.1. Clinical signs and body weight changes

Sixty eight control mice were dosed by gavage with vegetable oil (vehicle), and 68 with AZA at 100 mg/kg for 10 daily doses. The mean body weight of mice on day 1 of dosing was 20.7 g and 22.6 g, for control and AZA-treated mice, respectively (Fig. 8.3). At day 1 after dosing the body weight of control mice had increased by 7.2% to 22.2 g. However, at day 1 post dosing, the mean body weight of AZA-treated mice had fallen by 1.0 g to 21.6 g (a 4.4% reduction).

During the 10 day dosing period, all control and AZA-treated animals maintained a good state of health, as in the preliminary experiment, and no evidence of toxicity or mortality were recorded. In the first 10 days post dosing, control animals continued to

gain weight normally (Fig. 8.3), but there was no increase in the body weight of the AZA-treated mice, and some of these animals demonstrated a significant loss of condition. This deterioration in the condition of the AZA-treated mice during this early post dosing period (day 1 to 10) gave cause for concern as a total of 29 AZA mice were either killed *in extremis* (KIE) or were found dead (FD); these animals are categorised as inter-current death (ICD) animals. There were a further 2 ICD AZA-treated animals on day 15 post dosing, bringing the total to 31 animals. After this time point however, the condition of the AZA-treated mice began to improve and from this time (day 15), animals began to gain weight. Indeed, after day 19 post dosing, the mean body weight of AZA-treated mice was greater than controls, and this increased weight gain continued to be seen throughout the remainder of the experiment. Indeed, the increase in the body weight of the AZA-treated mice over the control animals was statistically significant at a number of time points (Fig. 8.3).

8.3.3.2. Haematology results

Changes in the RBC, MCV, reticulocytes, platelets, WBC and lymphocytes are displayed graphically in Fig. 8.4 A-F, respectively. 10 doses of 100 mg/kg AZA induced a severe anaemia (Fig. 8.4 A), leucopenia (Fig. 8.4 E, F) and thrombocytopenia (Fig. 8.4 D) immediately post dosing (day 1). RBC (Fig. 8.4 A), Hb, HCT, and MCV (Fig. 8.4 B) were all significantly reduced at this time, being 81.7%, 80.9%, 78.7% and 95.8% of control, respectively. The WBC, neutrophil, monocyte, and eosinophil counts were all significantly reduced, to 57.3%, 0.0%, 0.0% and 14.3% of control, respectively. The lymphocyte count was also reduced to 76.3% of the control mean at day 1 post dosing; however this change was not statistically significantly. Furthermore, on day 1

post dosing, reticulocytes and platelets were reduced to 0.3% and 9.2% of the control mean, respectively ($p<0.001$) at this time point.

On day 3 post dosing, the pancytopenia seen on day 1 appeared to develop further, with significant reductions in mean RBC, Hb, HCT, WBC and platelets. In addition, the reticulocytes also remained decreased, together with neutrophils, lymphocytes, monocytes, eosinophils, and basophils.

At day 9 the RBC, Hb and HCT, the MCV and all individual leucocytes remained significantly reduced compared with control values. Reticulocytes and platelets were also profoundly reduced to 0.4% and 0.6% of the mean control values, respectively ($p<0.001$).

On day 22 post dosing, a return of many haematological parameters towards control values was seen (Fig. 8.4 A-E). This return towards normal continued on day 29. However, the reductions seen in RBC, Hb and HCT, and platelets also continued to be statistically significant on either one, or both, of the day 22 and 29 time points. MCV (Fig. 8.4 B) and MCH were significantly increased in AZA-treated mice on days 22 and 29. The WBC count was comparable to control values on days 22 and 29 (Fig 8.4 E). The neutrophil count was significantly elevated on day 22 and both the neutrophil and monocyte counts were increased above the mean control value on day 29.

On day 43 post dosing, the RBC and platelet counts continued to maintain a significant reduction, while MCV (Fig. 8.4 B), MCH and MCHC continued to be increased above control values. The reticulocyte count was normal at day 43. The total leucocyte count of AZA-treated mice was significantly increased on day 43; this elevation was due to increases in neutrophil, lymphocyte (Fig. 8.4 F) and monocyte counts.

A return of all leucocytes to normal was seen on day 51 post dosing, indeed most of the haematological parameters under investigation were comparable to controls

at this time. Residual effects were seen in the RBC count (Fig. 8.4 A) which continued to be significantly reduced at this late-stage; however, Hb, HCT and MCV were comparable to control values.

8.3.3.3. Femoral nucleated cell count

The bone marrow of AZA-treated mice was significantly hypocellular on day 1 post dosing, being reduced to 15.7% of the mean control value ($p<0.001$) (Fig. 8.4 G). This statistically significant hypoplasia continued in mice treated with AZA on days 3 and 9 post dosing with the FNCC being reduced to 14.5% and 20.3% of the control mean at these time points, respectively. On day 22, the reduction in marrow cellularity was still statistically significant, however, at this time point evidence of recovery and a return towards normal was seen. On days 29 and 43, the FNCC of AZA-treated mice was reduced compared to control values, but this reduction was only significant on day 43, before returning to control ranges on day 51 (Fig. 8.4 G).

8.3.3.4. Bone marrow clonogenic assays

On day 1, 3 and 9 post dosing, the number of colony forming unit-granulocyte monocyte (CFU-GM) in the femur of AZA-treated mice was significantly reduced to 1.0%, 3.9% and 5.1% of the mean control value, respectively ($p<0.001$) (Fig. 8.5 A). On day 22, the number of CFU-GM in AZA-treated animals was showing signs of recovery; on day 29, the mean number of CFU-GM colonies per femur in the bone marrow of AZA-treated mice was comparable to control values. A significant reduction in CFU-GM colonies was evident on day 43 post dosing ($p<0.05$) before returning to values similar to the control-treated mice on day 51 post dosing.

A similar pattern of changes was seen in the number of erythroid colonies per femur after AZA treatment (Fig. 8.5 B). In the immediate post-dosing period, the number of erythroid colonies was reduced to 1.0%, 5.3% and 5.9% of the control mean values on days 1, 3 and 9, respectively. On days 22 and 29, signs of recovery were evident in the number of erythroid colonies but the numbers continued to remain significantly reduced. Indeed, at the later stages of the experiment (days 43/51), erythroid colonies remained reduced compared to the mean control value, this was however only statistically significant on day 43.

8.3.3.5. Apoptosis

Immediately after dosing (day 1), the level of apoptosis in the bone marrow cells of AZA-treated mice was significantly elevated to 245.9% of the mean control value (Fig. 8.6). On days 3 and 9 post dosing, levels of apoptosis continued to be significantly elevated compared with control mice; however, a return towards control values was clearly evident at this time. However, on days 22 and 29, the levels of apoptosis were reduced significantly, being 78.5% and 82.3% of the control mean value, respectively. At the later stages of the experiment, on days 43 and 51 post dosing, levels of apoptosis were slightly increased over the control; this effect was, however, only significant on day 51 ($p<0.01$).

8.3.3.6. Cytokine analysis

On day 1 post dosing, serum FL was significantly increased in AZA-treated mice to a mean of 1759.1 ± 447.5 pg/ml compared to the control mean value of 423.0 ± 152.2 pg/ml ($p<0.001$) (Fig. 8.7). On days 3 and 9 post dosing, FL levels continued to

be significantly elevated in AZA-treated mice to 2556.0 ± 369.2 pg/ml and 1436.1 ± 280.8 pg/ml compared to control values of 337.3 ± 45.2 pg/ml and 429.5 ± 143.0 pg/ml, respectively ($p<0.001$). On day 21 post dosing, serum FL continued to be significantly elevated in AZA-treated mice ($p<0.05$) before returning to values comparable to controls on days 29 and 43 post dosing. On day 51 post dosing FL was once again significantly increased in AZA treated mice ($p<0.05$).

Serum from control and AZA-treated mice was also assayed for IL-2, TNF-α and IFN-γ. The concentrations of these 3 cytokines in the serum of both control and AZA-treated mice were however, below detectable levels. The reasons for this are unclear.

8.3.3.7. Bone marrow differential counts

The tibial marrow smears from control and AZA-treated mice (n=6) were randomly selected at each time point and differential counts performed (as described in Chapter 2.4). On day 1 post dosing myeloid, erythroid and lymphoid cell lineages were significantly reduced in AZA-treated animals (Table 8.3), with counts being 5.7%, 31.3% and 9.8% of the control values, respectively ($p<0.001$). On day 3 post dosing, the number of myeloid, erythroid and lymphoid cells were further reduced in AZA-treated mice to 3.9%, 16.8% and 15.6% of the control mean, respectively ($p<0.001$). At days 9 and 22 post dosing, myeloid, erythroid and lymphoid cells continued to be reduced however, a return towards normal values was clearly evident at this time. From day 29 post dosing, myeloid and erythroid cell counts were comparable to control values. Lymphoid cell counts were however, slightly reduced in comparison to the controls on day 29 (NS) and 43 ($p<0.05$) post dosing. Cells categorised as 'other' (monocytes and monocyte precursors, mast cells, megakaryocytes, plasma cells and unidentifiable cells),

were reduced in AZA-treated animals on days 1, 3 and 9 post dosing before returning to values comparable to the controls from day 22 post dosing onwards. In the immediate post doing period (days 1, 3 and 9), the M:E ratio was significantly reduced ($p<0.001$) before returning to values comparable to controls on day 22 post dosing.

8.3.3.8. Histopathological assessment of tissues

Sections of liver, thymus, spleen and sternum were assessed histologically in control mice autopsied on days 1 and 51 post dosing (n=5 randomly selected mice) and in AZA-treated mice autopsied on days 1, 9, 22 and 51 post dosing (n=5 randomly selected mice).

Mild atrophy of the thymus was evident on day 1 post dosing in AZA-treated mice. This relatively mild change on day 1 became more pronounced on day 9, before returning to normal levels on day 51 post dosing.

The mean relative liver weights of mice treated with AZA were comparable to control values on day 1 post dosing (Table 8.4). However, on day 3, relative liver weights were significantly increased in AZA-treated mice ($p<0.01$). On days 9, 22 and 29 post dosing the mean relative liver weights of AZA treated mice were not statistically different from control values, however, on day 43 and 51 relative liver weights were significantly increased ($p<0.01$). Centrilobular hypertrophy was evident in the liver of AZA-treated mice autopsied on day 1 post dosing. However, this change had resolved by day 9 post dosing.

The mean relative kidney weights of mice treated with AZA were comparable to the control means on days 1 and 3 post dosing (Table 8.4). However, a significant increase in relative kidney weight in AZA mice was evident on days 9 and 22 post dosing ($p<0.001$ and $p<0.05$, respectively) before returning to values comparable to

controls on days 28, 43 and 51 post dosing. As the preliminary 2 (dose response study) AZA study did not show evidence of AZA induced kidney toxicity, the kidneys from the main AZA study were not examined histologically.

On day 1 and 3 post dosing a significant reduction in the mean relative spleen weights was evident ($p<0.001$), returning to values comparable to the controls on day 9 post dosing. An increase in mean relative spleen weight was seen on day 22 ($p<0.01$), 29 (NS) and 43 ($p<0.01$) post dosing before returning to values comparable to control on day 51 post dosing. Histological assessment of the spleen showed a marked reduction in extramedullary haemopoiesis on days 1 and 9 post dosing. This reduction in splenic haemopoiesis was no longer significant from day 9, with only mild changes evident on day 22.

The sternal sections showed a significant reduction in cellularity on days 1 and 9 post dosing. However, the cellularity of the sternum was comparable to control mice on day 22 and 51 post dosing.

8.3.3.9. Results summary

- Sustained reduction in erythropoiesis and thrombopoiesis.

- Initial reductions in neutrophils, lymphocytes, monocytes and eosinophil counts.

- Reduced bone marrow cellularity and CFU-Cs with increased apoptosis and serum FL.

8.4. DISCUSSION

The immunosuppressant drug AZA, a derivative of 6-MP, was initially developed as a slow release form of 6-MP. However, after oral administration, AZA rapidly undergoes glutathione dependant non-enzymatic cleavage, principally within erythrocytes, to yield 6-MP and an imidazole side chain (Elion 1989). 6-MP undergoes further metabolism via 3 competing pathways. Firstly, in the intestinal mucosa and liver, 6-MP is converted into 6-thiouric acid by xanthine oxidases; second, thiopurine methyl-transferase (TPMT) converts 6-MP into the inactive metabolite 6-methyl-mercaptopurine (6-MMP); and third, hypoxanthine phosphoribosyltransferase (HPRT) converts 6-MP into 6-thioguanine nucleotides (6-TGNs). The generation of 6-TGNs is essential for the therapeutic success of AZA therapy. Indeed, an increased rate of relapse is seen in children with leukaemia who, following treatment with 6-MP, produce lower than average concentrations of 6-TGNs as a result of higher TPMT activity (Lennard 1992). 6-TGNs are, however, also responsible for the toxic side effects resulting from AZA treatment (Lennard 1992). 6-TGNs disrupt purine synthesis thus reducing the availability of ribonucleotides, and in turn, reducing the synthesis of RNA and DNA (Dollery 1999). In addition, 6-TGNs become incorporated into DNA and replace guanine residues resulting in the formation of single strand breaks and kinking of chromosomes (Lennard 1992; Fairchild *et al.* 1986).

The conversion of 6-MP into 6-MMP by TPMT is of great importance, as it is the activity of this enzyme which dictates the level of toxicity expected. The activity of TPMT is subject to significant inter-individual variation owing to a genetic polymorphism. The majority of the human population (89%) are homozygous for the wild-type TPMT allele and therefore, activity is high. However, 11% of the population are heterozygous, and therefore, these individuals have one wild-type and one mutant

allele, and as a consequence have an intermediate level of TPMT activity. However, a further 0.3% of the population are homozygous for the mutant TPMT allele, and activity of TPMT is very low or negligible in these individuals (Lennard 1992; Aarbakke *et al.* 1997). Individuals with low TPMT activity have been shown to have an increased risk of myelotoxicity to AZA due to a shifting of drug metabolism from the TPMT pathway to the HPRT pathway which yields 6-TGN (Lennard 1992; Scerri 1999).

A number of papers have been published describing the association between TPMT activity and myelotoxicity in renal transplant recipients and patients with autoimmune disorders treated with AZA (Lennard *et al.* 1989; Ben Ari *et al.* 1995; Leipold *et al.* 1997; Black *et al.* 1998; Stolk *et al.* 1998; Colombel *et al.* 2000; Pandya *et al.* 2002; Schwab *et al.* 2002). Furthermore, the level of 6-TGNs in the erythrocytes of children with leukaemia is inversely proportional to the degree of neutropenia (Lennard *et al.* 1983). To reduce the risk of haematological side effects as a result of AZA treatment it has therefore been proposed that patients should first be screened to assess TPMT activity. Pre-screening would avoid treating susceptible patients with AZA, thus reducing the incidence of AZA induced myelotoxicity. Such a measure could prove cost effective in the long run when taking into account the cost of monitoring patients receiving AZA therapy and the cost of supportive care in patients developing significant toxic effects (Black *et al.* 1998).

In the present study CD-1 mice treated with AZA developed significant anaemia, leucopenia and thrombocytopenia immediately post dosing (Fig. 8.4 A, E, F and D, respectively). The peripheral blood counts of mice treated with AZA further declined on days 3 and 9 post dosing with a significant increase in apoptosis (Fig. 8.6) also occurring at these time points. This delay in the appearance of the most severe bone marrow depression (the 'nadir') may be the result of 6-TGN incorporation into DNA. It is reported that the damaging effects of 6-TGN may not be immediate, and that

following incorporation into the DNA strand, and subsequent replication of this faulty template, the production of DNA containing unilateral chromatid damage and single strand breaks may occur (Lennard 1992; Fairchild *et al.* 1986).

Profound bone marrow aplasia has been found to occur in 2% of patients treated with AZA with the majority of these cases presenting in the first 4 weeks of treatment. In man, bone marrow aplasia induced by AZA treatment is readily reversed following withdrawal of the drug (Present *et al.* 1989; Connell *et al.* 1993; Leipold *et al.* 1997). However, in the present study, mice treated with AZA continued to have significantly decreased bone marrow cellularity lasting until 28 days after dosing, with persistent reductions continuing after this time in the erythroid lineage (Fig. 8.4 A, Fig. 8.5 B).

In Fig. 8.4 A, B and Fig. 8.5 B it is shown that in the longer term, residual effects of AZA treatment were confined to the erythroid lineage. The red blood cell counts were significantly reduced at all time points following AZA treatment (Fig. 8.4 A). Similarly, bone marrow culture demonstrated a significant reduction in the number of erythroid progenitor cells within the bone marrow of AZA-treated animals at all time points (except day 51) (Fig. 8.5 B). Another effect on the erythroid line was seen in the early post dosing period, when the MCV was significantly reduced in AZA-treated mice. However, at later stages of the experiment (days 22, 29 and 43), AZA-treated mice (Fig. 8.4 B) developed significant macrocytosis. Changes in erythrocytes particularly anaemia and macrocytosis are well documented side effects of AZA treatment in man (Bottiger and Rausing 1972; McGrath *et al.* 1975; Creemers *et al.* 1993; Pruijt *et al.* 1996; Thompson and Gales 1996).

In a report by Hildner *et al.* (1998), changes in the inflammatory cytokines TNF-α and IFN-γ were measured in the serum of mice treated with AZA. In this report, mice were treated with 2 ip injections of AZA at 1, 10 and 100 mg/kg on day 0 and day 2 of the study, with serum collected on day 3. TNF-α was reduced to 42, 20 and 15% of

control in the 1, 10 and 100 mg/kg treatment groups, respectively. IFN-γ was also reduced, but not by the same magnitude, the reduction being to 90, 89 and 80% of control at 1, 10 and 100 mg/kg AZA, respectively. In man, peripheral blood mononuclear cells, separated from Crohn's disease patients undergoing AZA treatment, were shown to produce lower concentrations of IFN-γ when stimulated in culture for 48 hours (Cuffari *et al.* 2004). It was also shown in this report that the level of IFN-γ produced was related to the concentration of 6-TGN in the erythrocytes. Patients with high levels of 6-TGNs produced significantly lower levels of IFN-γ compared to Crohn's disease patients who had low levels of 6-TGN, and patients that were not treated with AZA.

In the present study (Experiment 3; Time course study) it was found that no change in the serum concentration of TNF-α, IFN-γ or IL-2 could be identified. Indeed, the concentrations of these cytokines in the serum of both the control and AZA-treated mice were below detectable limits. It is possible that assays of these cytokines carried out with mouse serum are not the optimal method of detection, and that measuring the production of the cytokines by lymphocytes in vitro may be more productive, as in the study reported by Cuffari *et al.* (2004).

The concentration of FL in the serum was found to be raised in AZA-treated mice immediately post dosing (Fig. 8.7); this increase in FL was expected, taking into account the bone marrow hypoplasia induced by AZA treatment. This increase in FL during periods of bone marrow hypoplasia has been described in primates and mice following radiotherapy (Gratwohl *et al.* 1998; Bertho *et al.* 2001; Prat *et al.* 2005). In man, an increase in the concentration of FL in plasma has been reported in patients receiving chemotherapy and radiotherapy and also, in patients with haematological disorders associated with a stem cell defect i.e. aplastic anaemia and Fanconi's anaemia

(Lyman *et al.* 1995b; Wodnar-Filipowicz *et al.* 1996; Chklovskaia *et al.* 1999; Huchet *et al.* 2003).

Significant atrophy of the thymus was observed on days 1 and 9 post dosing in AZA-treated mice. This effect has been described previously following AZA treatment in both rodents and in the rhesus monkey (De Waal *et al.* 1995; Dollery 1999). In addition to thymic atrophy, a significant reduction in the lymphocyte count of AZA-treated mice was seen in the peripheral blood on days 3 and 9 post dosing (Fig. 8.4 F). Both atrophy of the thymus, and reduced lymphocyte counts, in AZA-treated mice may be a result of an increase in apoptotic cell death. In a recent report by Tiede *et al.* (2003), apoptosis was elevated in lymphocytes treated with AZA in vitro. This increase in apoptosis was found to be a result of 6-MP interaction with the guanosine triphosphate- (GTP-) binding protein RAC-1. RAC-1 blocks the upregulation of mRNA coding for a dominant regulator of apoptosis the protein Bcl-xL which promotes cell survival by inhibiting apoptosis.

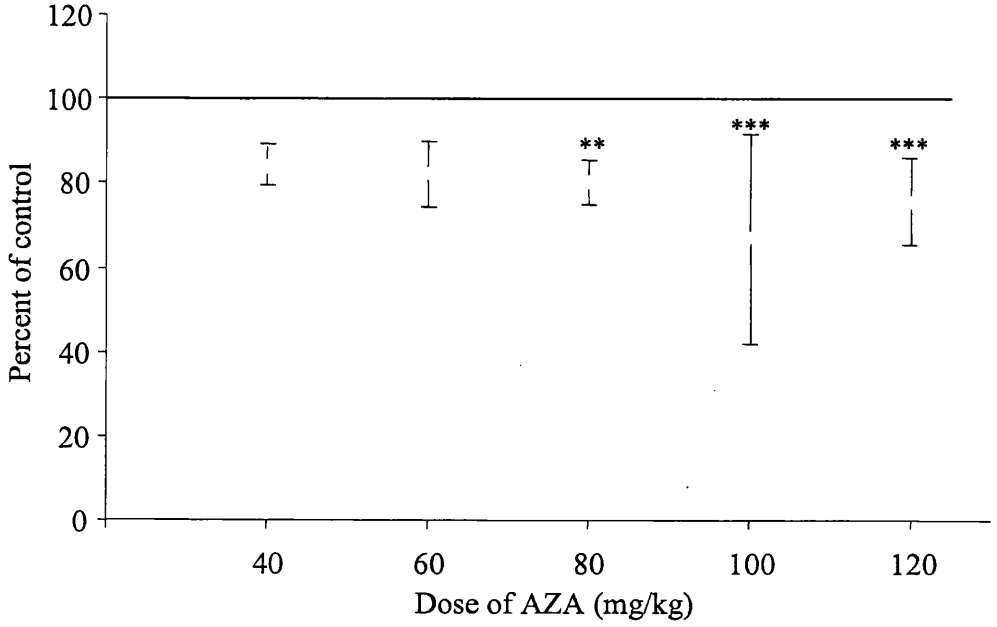
In conclusion, it has been shown in the present study that mice treated with AZA initially developed significant bone marrow depression followed by a period of recovery. However, AZA-treated mice showed evidence of a sustained (late-stage) residual injury to cells of the erythroid lineage. The effects of this disruption in erythropoiesis were seen both in the peripheral blood and in bone marrow cell cultures. The peripheral red blood cell counts of AZA-treated mice were significantly reduced at all time points with evidence of macrocytosis on days 22, 29 and 43 post dosing (Fig. 8.4 B). Similarly, bone marrow cultures showed a significant deficit in the number of erythroid colonies at all time points studied (except day 51) (Fig. 8.5 B). Therefore, the reduction in erythroid colony number corresponded to a reduction in the number of committed progenitor cells of the erythroid lineage in the bone marrow of AZA-treated mice. However, differential cell counts performed on bone marrow smears from AZA-

treated mice did not show a reduced number of erythroid cells. TPMT genetic polymorphisms which play a role on the development of bone marrow injury following AZA treatment in man also occur in some strains of mice (Hernandez *et al.* 1990). It is possible that if the present study had been conducted with a strain of mice that is known to have a genetically low TMPT activity (for example the C57BL/6J or AKR/J) a more acute bone marrow injury may have been observed involving all haemopoietic cell lineages.

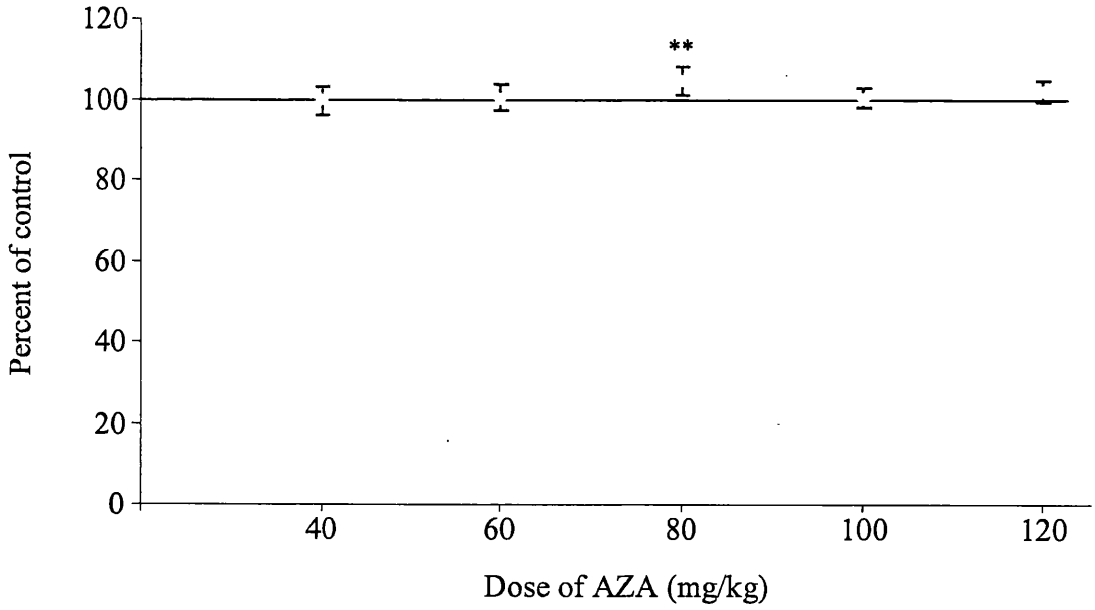
In experiment 1; preliminary dose ranging study, CD-1 mice (Charles River UK Ltd.) were treated with AZA daily by gavage at 0, 25, 75, 100, 125, 150, 200, 250, 300, 350 and 400 mg/kg. Significant toxicity was evident in mice treated with AZA at 125 mg/kg and above. All of the mice in these groups either found dead or killed *in extremis* during the dosing period or in the first 19 days post dosing. Mice treated with AZA at 25, 75 and 100 mg/kg did not however, show any evidence of AZA induced toxicity during the period of study (19 days). This is in contrast to experiment 3; time course study, here the condition of mice (ICR/CD-1; Harlan) treated with AZA daily by gavage at 100 mg/kg began to deteriorate in the immediate post dosing period. In the first 10 days post dosing, 29 AZA-treated mice were classified as ICD animals; 2 further ICD mice were identified on day 15 post dosing. From the 68 mice treated with AZA in this time course study a total of 31 ICD animals were recorded resulting in a total mortality of 45.6%.

The ICR (CD-1) mouse used in the time course study (experiment 3) was found to be more susceptible to AZA toxicity than the CD-1 mouse used in the preliminary experiments (experiment 1 and 2). Both strains of mice are derived from the Swiss mouse (Harlan 2005; Charles River 2005) and therefore, when looking for a replacement for the CD-1 mouse as a result of supply problems, it was considered that the ICR CD-1 mouse would be suitable.

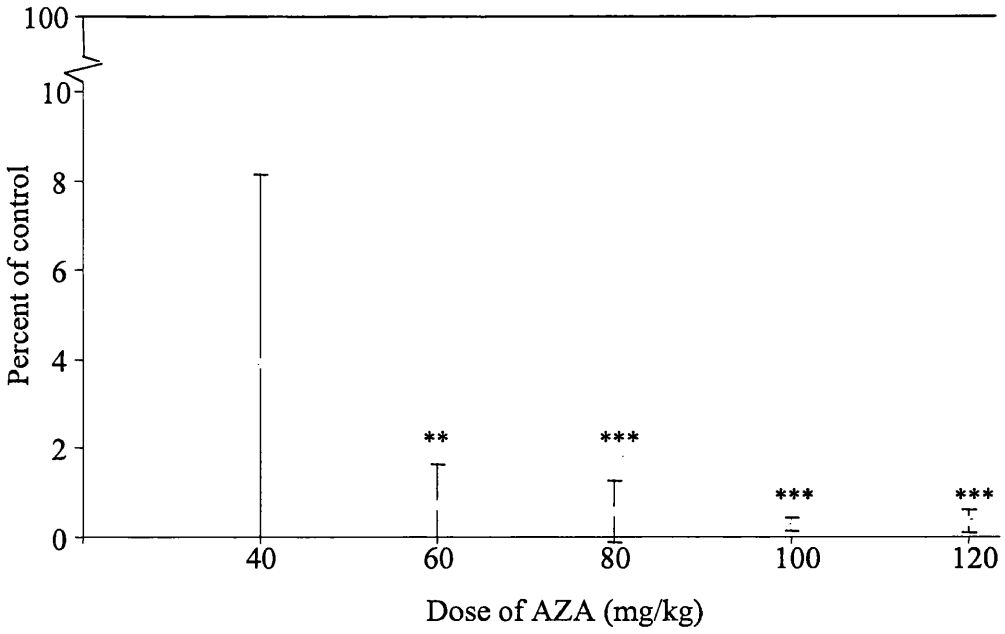
A. Red blood cells



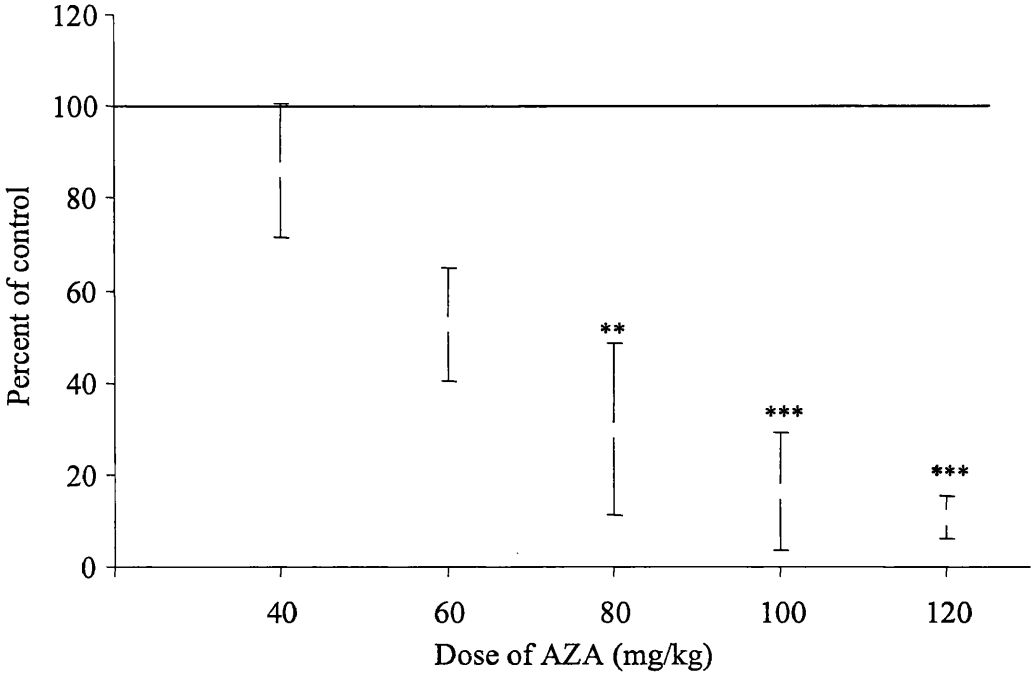
B. Mean cell volume



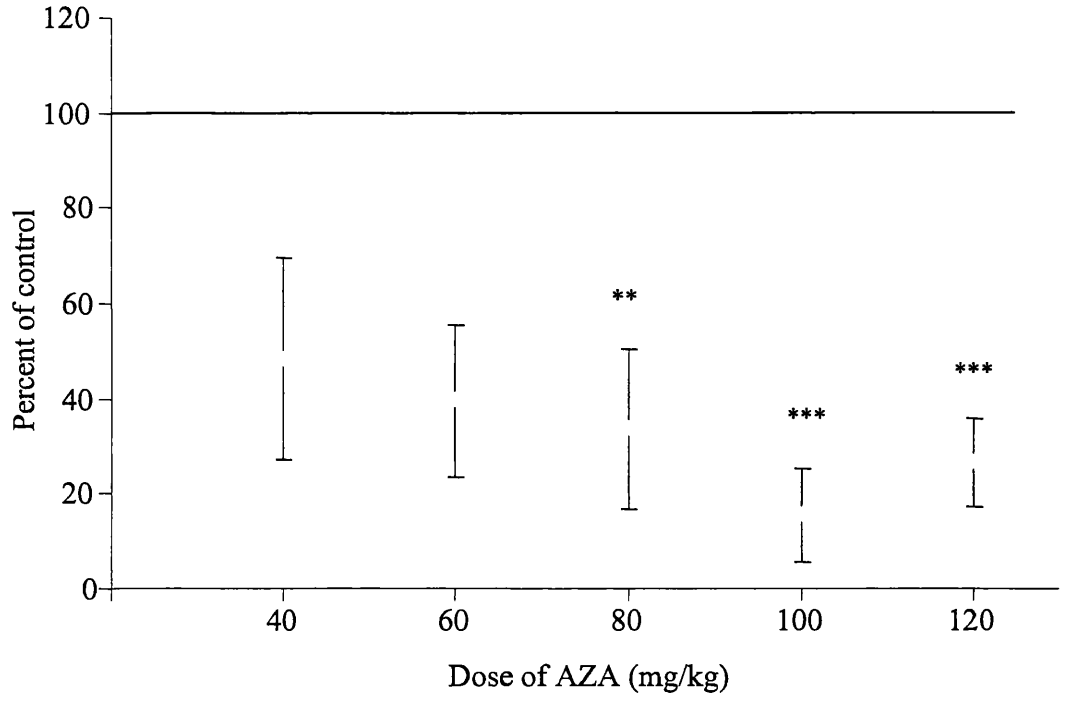
C. Reticulocytes



D. Platelets



E. White blood cells



F. Femoral nucleated cell count

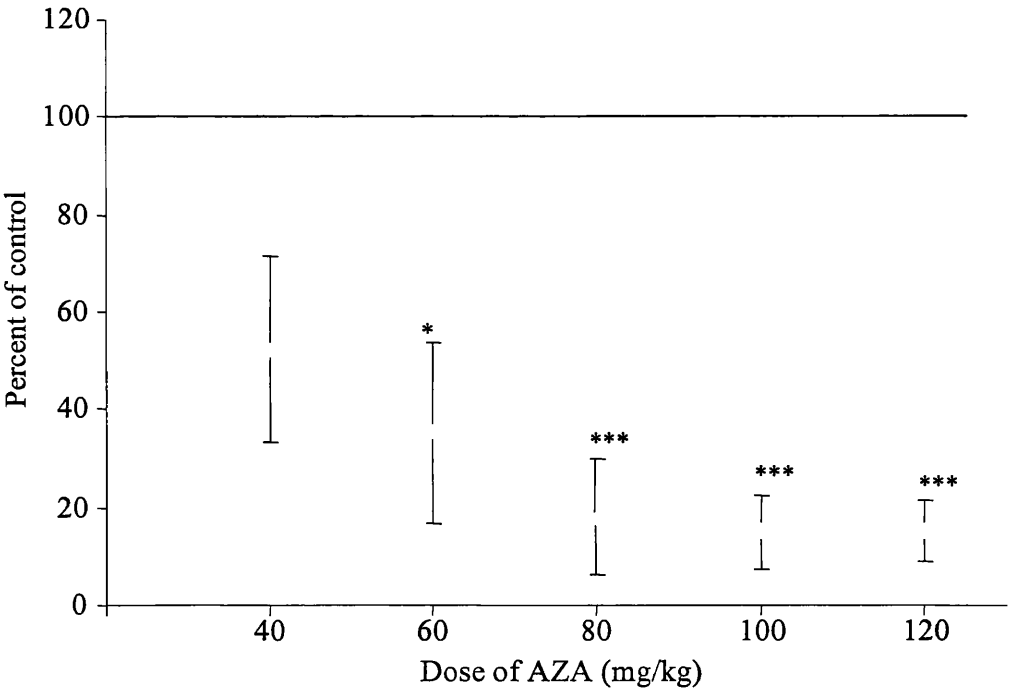


FIGURE 8.1. Results for red blood cells, mean cell volume, reticulocytes, platelets, white blood cells and femoral nucleated cell counts from female CD-1 mice treated with azathioprine (AZA) at dose levels from 40 to 120 mg/kg and sampled on day 1 post dosing. Values are means (± SD), expressed as a percentage of the mean control value. Animals were treated with vehicle (control) or AZA (40, 60, 80, 100 and 120 mg/kg) on 10 occasions daily by gavage (n=10 per group). Data analysed using a one-way analysis of variance (ANOVA). **Significantly different from controls, $p<0.01$.

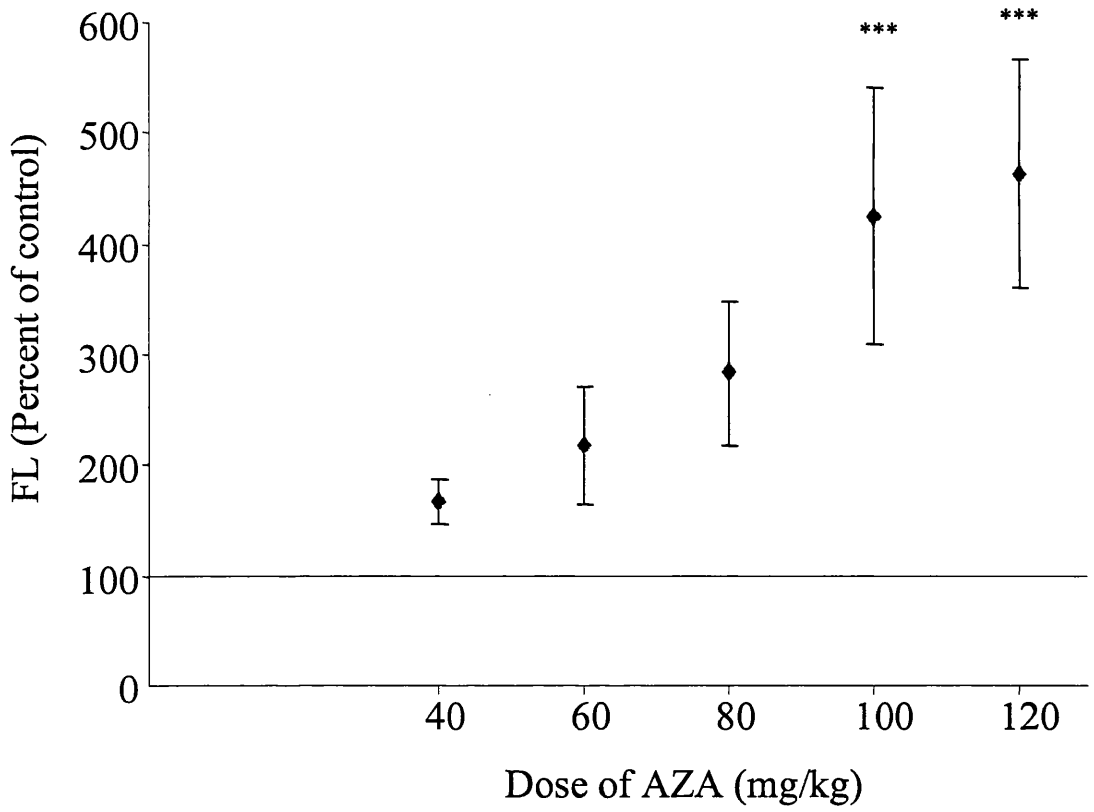


FIGURE 8.2. Serum FL results from female CD-1 mice and mice treated with azathioprine (AZA) at dose levels from 40 to 120 mg/kg and sampled on day 1 post dosing. Values are means (± SD), expressed as a percentage of the mean control value. Animals were treated with vehicle (control) or AZA (40, 60, 80, 100 and 120 mg/kg) on 10 occasions daily by gavage (n=6 per group except in the 40 and 60 mg/kg Groups where n=5). Data analysed using a one-way analysis of variance (ANOVA). ***Significantly different from controls, $p<0.001$.

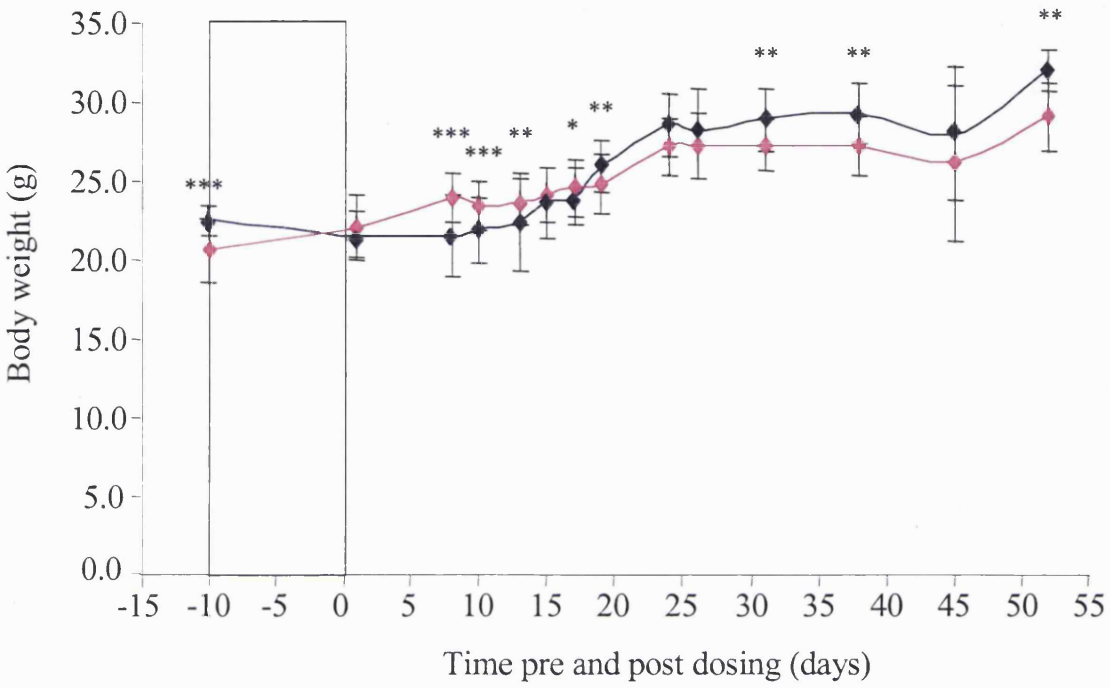
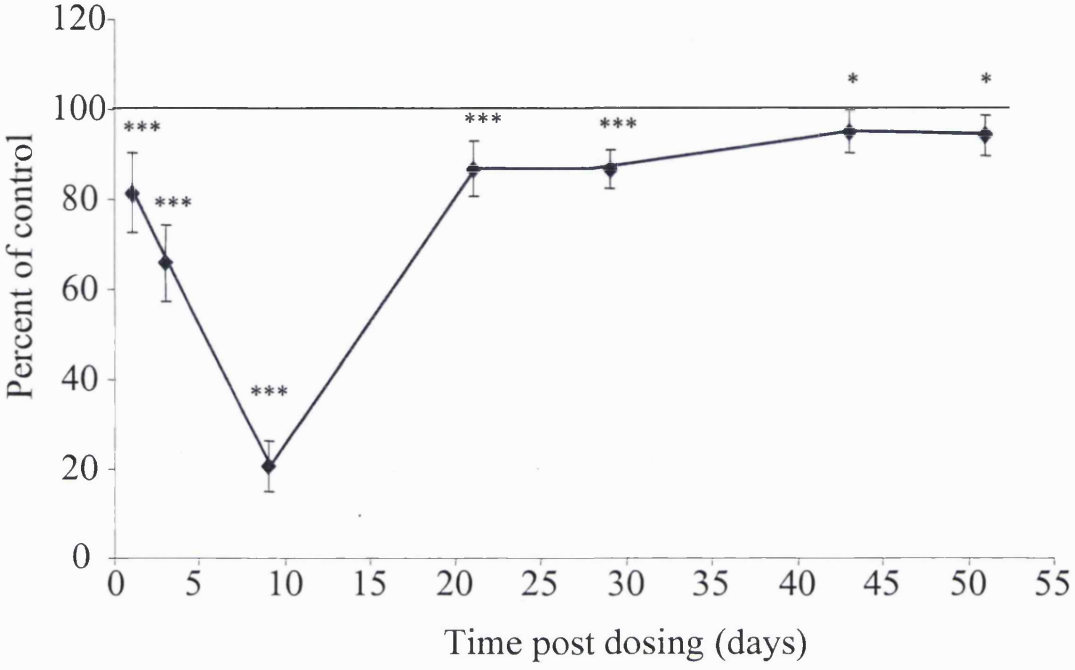
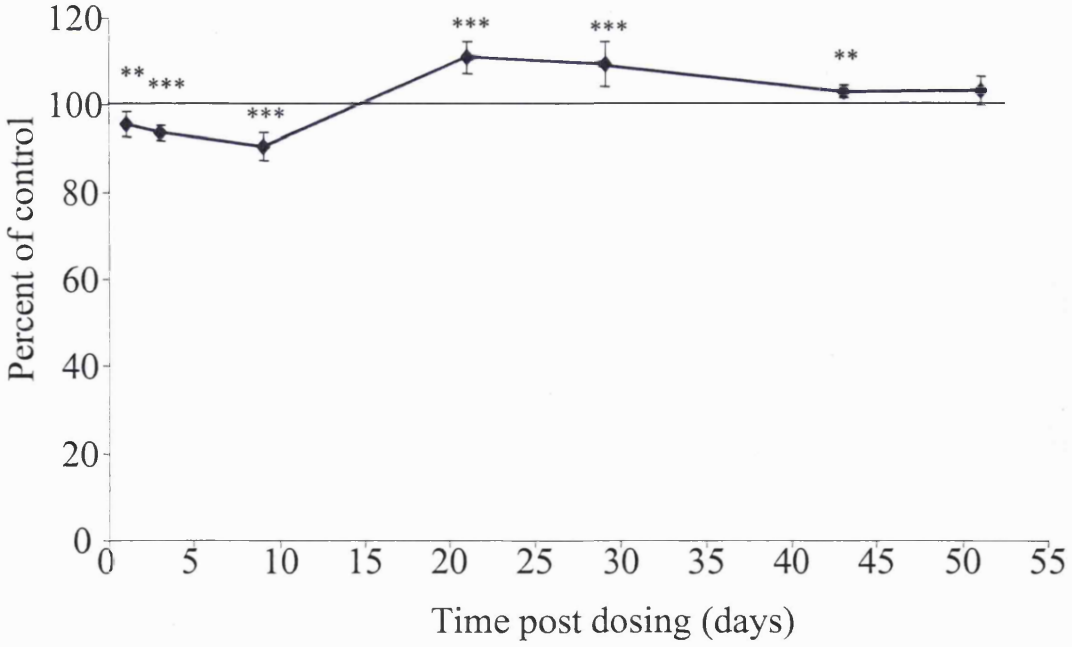


FIGURE 8.3. Body weight increases in control female ICR (CD-1) mice and mice treated with azathioprine (AZA) at 100 mg/kg. Values are means (± SD) for control (—◆—) and AZA-treated (—◆—) mice. Animals were treated daily by gavage with vehicle or AZA on 10 occasions (▭). Data analysed using a Student's t-test. *Significantly different from controls, $p<0.05$; **$p<0.01$; ***$p<0.001$.

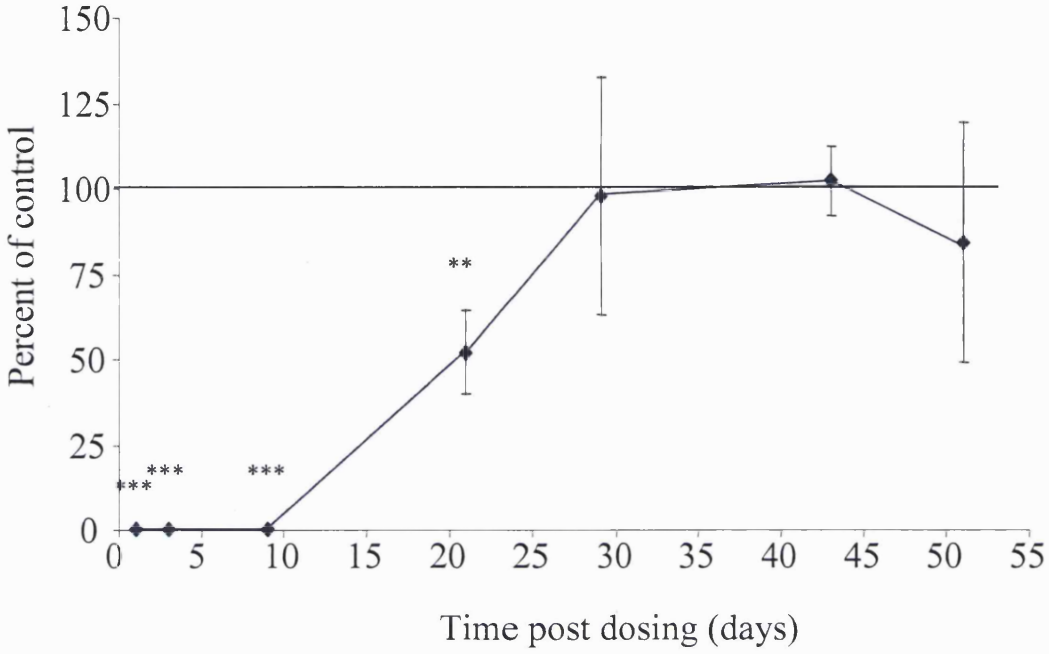
A. Red blood cells



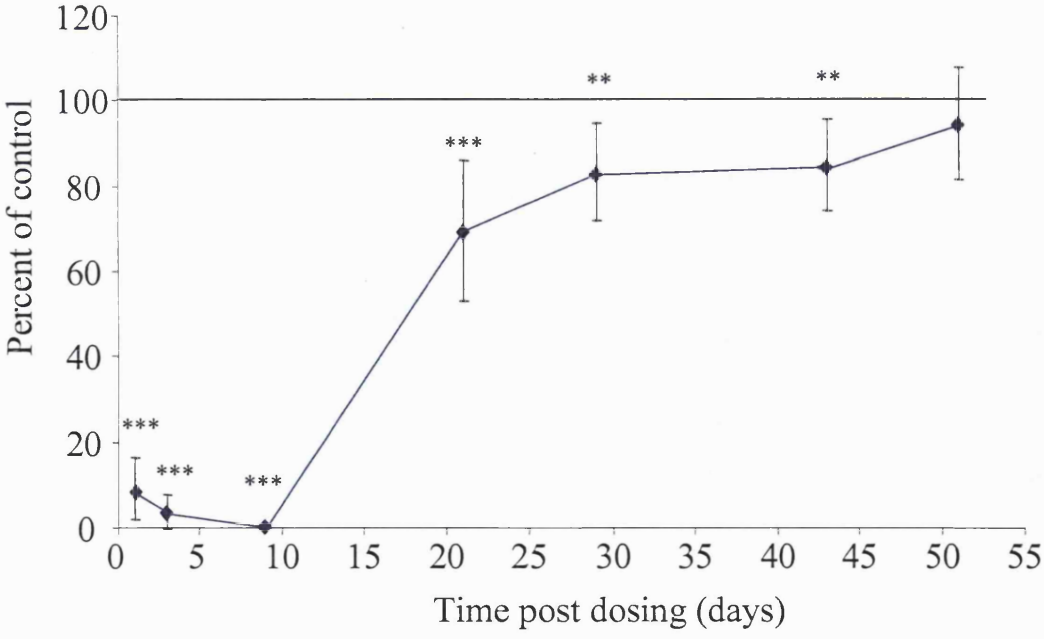
B. Mean cell volume



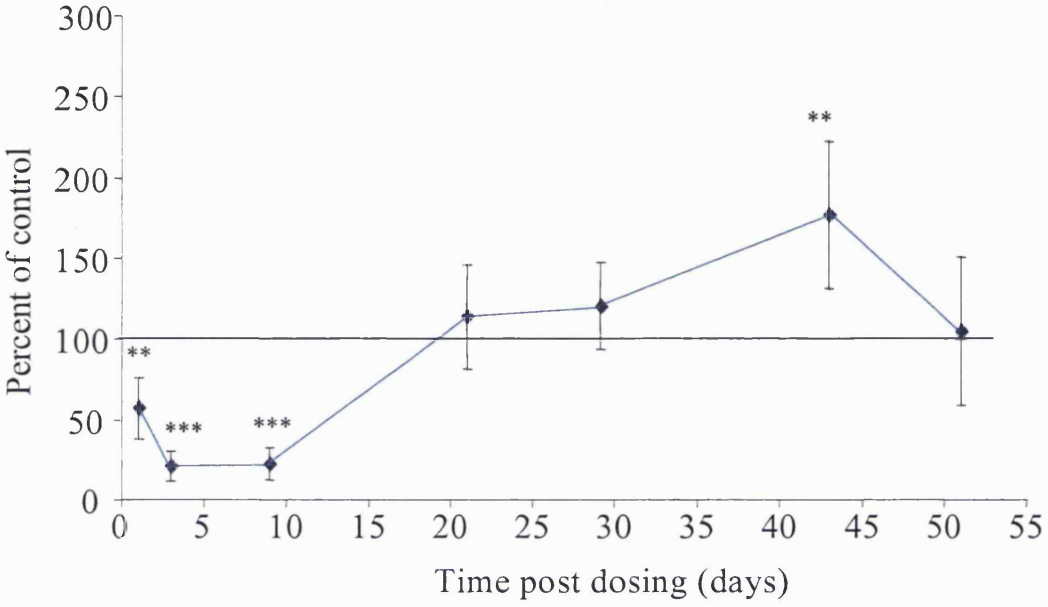
C. Reticulocytes



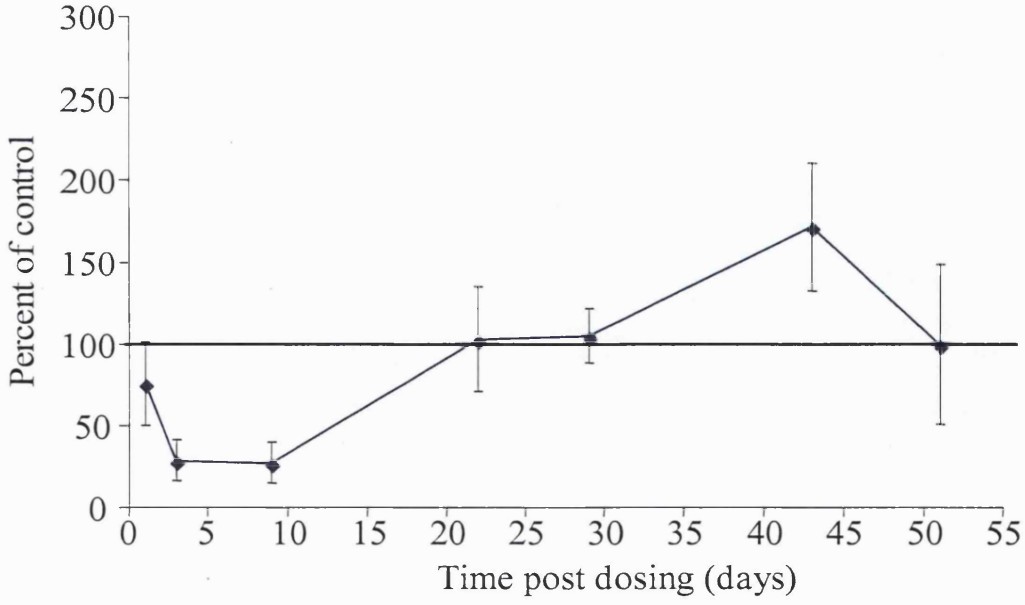
D. Platelets



E. White blood cells



F. *Lymphocytes*



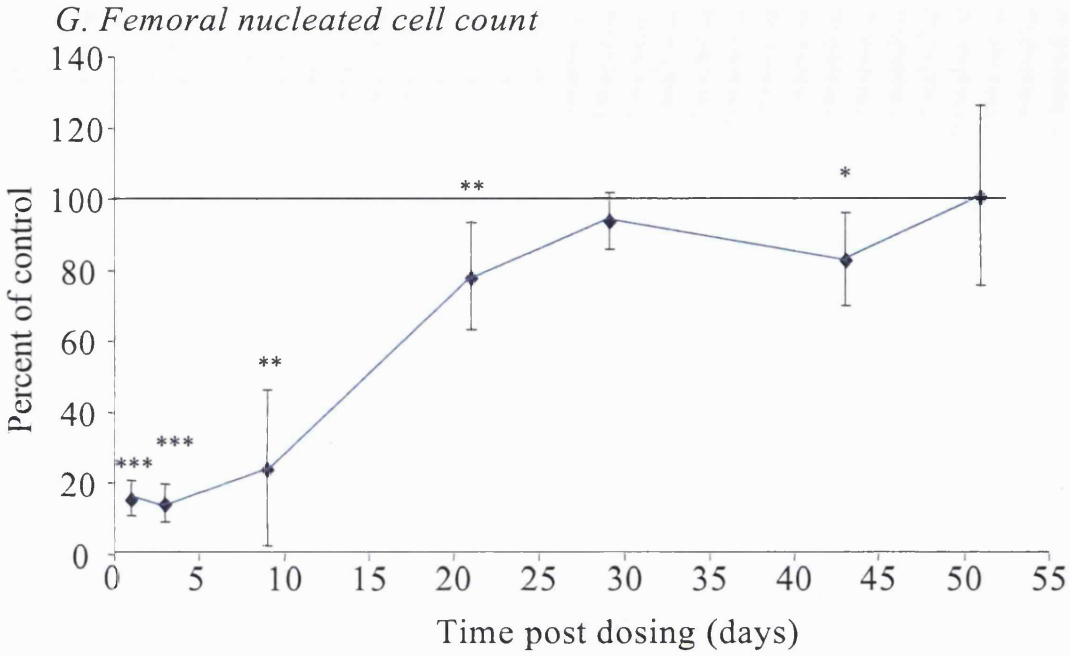
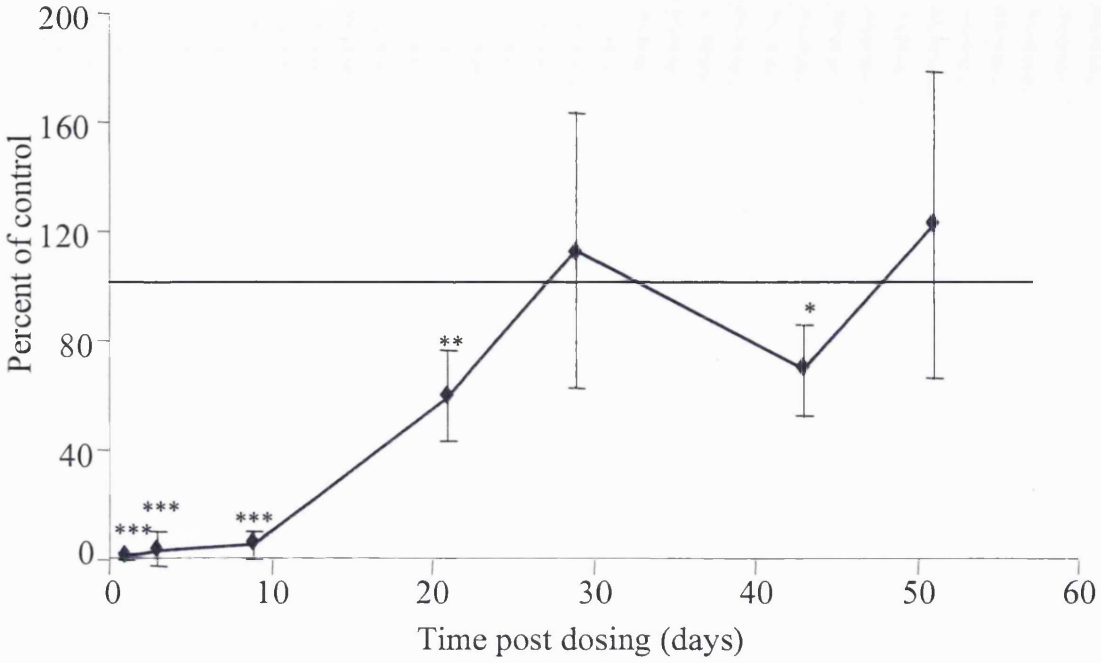


FIGURE 8.4. Results for red blood cells, mean cell volume, reticulocytes, platelets, white blood cells, lymphocytes and femoral nucleated cell counts from female ICR (CD-1) mice treated with azathioprine (AZA) and sampled on days 1 to 51 post dosing. Values are means (± SD), expressed as a percentage of the mean control values of animals sampled at the same time points. Animals were treated daily by gavage with vehicle or AZA (100 mg/kg) on 10 occasions and autopsied on days 1, 3, 9, 22, 29, 43 and 51 after the final dose. n=8 for control and AZA-treated mice on days 1 and 3 post dosing; at all other time points n=10 for control and n=5 for AZA, except at day 9, were n=8 for the controls. Data analysed using the Student's t-test. *Significantly different from controls, $p<0.05$; **$p<0.01$; ***$p<0.001$.

A. Colony forming unit cells-granulocyte monocyte



B. Erythroid colonies

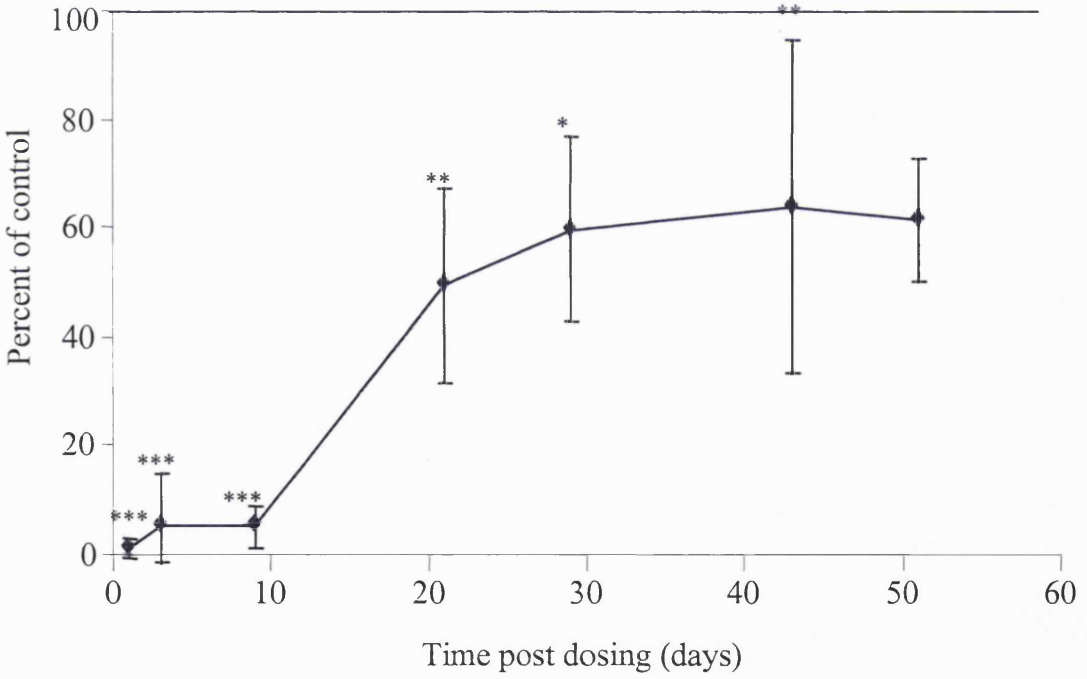


FIGURE 8.5. Results for colony forming unit-granulocyte monocyte (CFU-GM) and erythroid colony counts from female ICR (CD-1) mice treated with azathioprine and sampled on days 1 to 51 post dosing. Values are means (± SD), expressed as a percentage of control values in animals sampled at the same time points. Cultures were set up in duplicate and the mean CFU-GM and erythroid colony number per femur calculated per mouse. Number of animals per group as Fig. 8.4. *Significantly different from controls, $p<0.05$; **$p<0.01$; ***$p<0.001$.

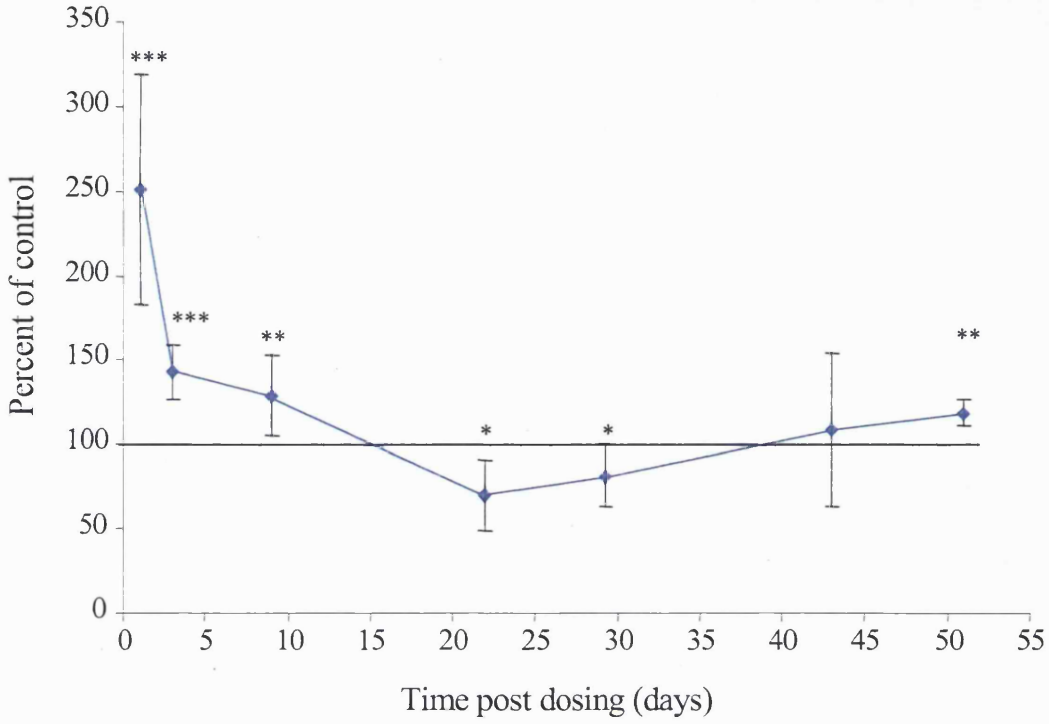


FIGURE 8.6. Apoptosis in femoral bone marrow samples; results from female ICR (CD-1) mice treated with azathioprine and sampled on days 1 to 51 post dosing. Values are means (± SD), expressed as a percentage of the mean control value in animals sampled at the same time points. Numbers of animals per group as Fig. 8.4. *Significantly different from controls, $p<0.05$; **$p<0.01$; ***$p<0.001$.

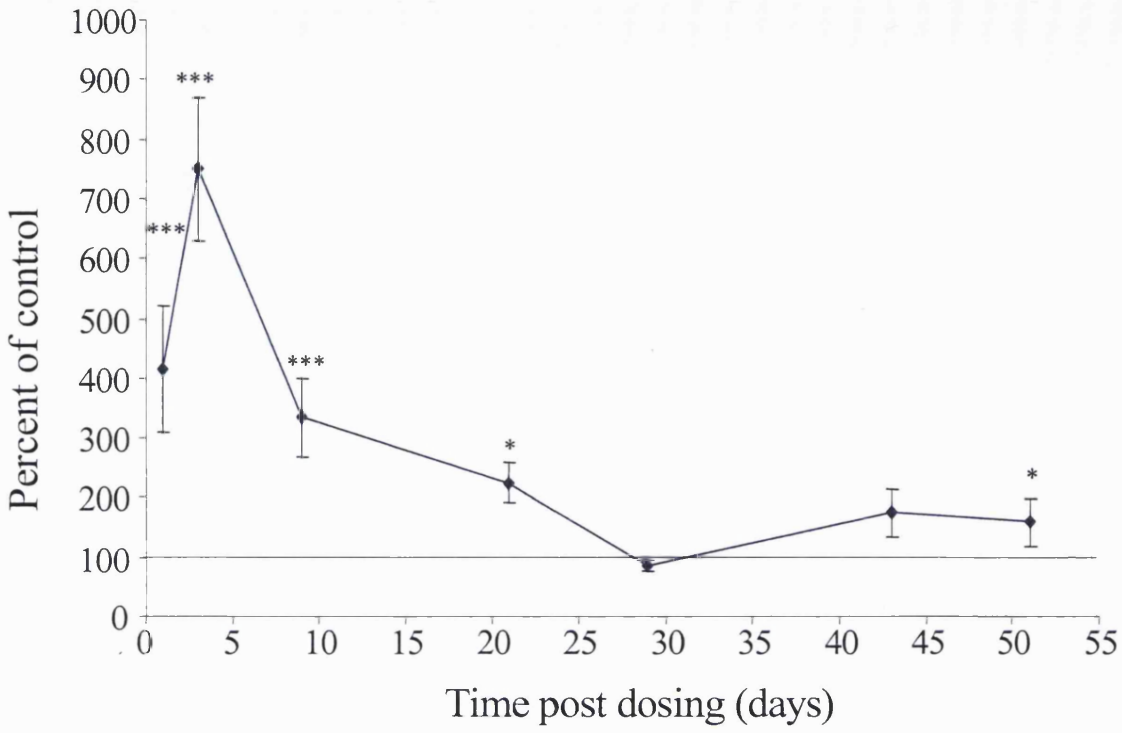


FIGURE 8.7. Serum FL results from female ICR (CD-1) mice treated with azathioprine (AZA) and sampled on days 1 to 51 post dosing. Values are means (± SD), expressed as a percentage of the mean control value in animals sampled at the same time points. n=3-7 for control and n=4-8 for AZA-treated mice. *Significantly different from controls, $p<0.05$; ***$p<0.001$.

TABLE 8.1. Estimated counts ($x10^7$) of myeloid, erythroid and lymphoid cells, and the myeloid:erythroid (M:E) ratio in the femoral marrow of control and azathioprine (AZA) treated CD-1 mice[a]; animals were given 10 daily doses of AZA at 0 (control), 40, 60, 80, 100 and 120 mg/kg and sampled on day 1 post dosing

	Treatment (mg/kg)					
	0 (Control)	**40**	**60**	**80**	**100**	**120**
Myeloid	2.63 (0.24)	1.74 (0.85)	0.82 (0.38)	0.38* (0.36)	0.13*** (0.10)	0.16** (0.18)
Erythroid	1.71 (0.15)	0.50*** (0.24)	0.30*** (0.13)	0.33*** (0.13)	0.50*** (0.20)	0.42*** (0.18)
Lymphoid	1.34 (0.27)	0.57 (0.29)	0.33 (0.16)	0.17* (0.03)	0.08*** (0.03)	0.13** (0.11)
Other	0.21 (0.05)	0.16 (0.11)	0.09 (0.03)	0.07 (0.03)	0.05** (0.03)	0.05* (0.02)
M:E ratio	1.54 (0.15)	3.88 (2.17)	2.87 (1.43)	1.24 (0.98)	0.24 (0.13)	0.42 (0.50)

[a] 200 cells in the tibial marrow smears were differentially counted by eye and the absolute number of cells of each lineage estimated for the nucleated marrow cell count of the femoral marrow flush. Values are means, SD in parenthesis; n=6 per group. Cells categorised as 'other' include monocytes and monocyte precursors, mast cells, megakaryocytes, plasma cells and unidentifiable cells. Data analysed using a one-way analysis of variance (ANOVA). *Significantly different from controls, $p<0.05$; **$p<0.01$; ***$p<0.001$.

TABLE 8.2. Relative organ weights[a] of control and azathioprine (AZA) treated CD-1 mice; animals were given 10 daily doses of AZA at 0 (control), 40, 60, 80, 100 and 120 mg/kg and sampled on day 1 post dosing

	Treatment (mg/kg)					
	0 (Control)	**40**	**60**	**80**	**100**	**120**
Kidney	6,398	6,290	6,434	6,505	6,457	6,284
	(719)	(599)	(636)	(451)	(597)	(550)
Liver	48,088	48,281	49,318	55,097*	51,933	52,903
	(3,671)	(3,583)	(5,104)	(4,704)	(4,162)	(2,925)
Spleen	4,134	2,741***	2,807***	3,395	2,784***	2,921***
	(974)	(540)	(560)	(528)	(328)	(527)

[a]Relative organ weight, mg/kg body weight. Values are means and SD in parenthesis. n=10 per group. Data analysed using a one-way analysis of variance (ANOVA). *Significantly different from controls, $p<0.05$; ***$p<0.001$.

TABLE 8.3. Estimated counts ($x10^7$) of myeloid, erythroid and lymphoid cells, and the myeloid:erythroid (M:E) ratio in the femoral marrow of control and azathioprine (AZA) treated ICR (CD-1) mice[a]; animals were given 10 doses of AZA over a period of 10 days and sampled at 1 to 51 days after the final dose

	Day of sampling													
	1		3		9		22		29		43		51	
	Control	AZA	Control	AZA	Control	AZA	Control	AZA	Control	AZA	Control	AZA	Control	AZA
Myeloid	1.59 (0.14)	0.09*** (0.06)	1.52 (0.32)	0.06*** (0.03)	1.47 (0.26)	0.31*** (0.46)	1.67 (0.30)	1.30* (0.32)	1.79 (0.29)	1.59 (0.22)	1.42 (0.29)	1.23 (0.26)	1.58 (0.43)	1.55 (0.33)
Erythroid	1.31 (0.27)	0.41*** (0.19)	0.89 (0.35)	0.15*** (0.09)	1.12 (0.25)	0.62 (0.80)	1.14 (0.18)	0.96 (0.29)	1.28 (0.36)	1.34 (0.10)	1.37 (0.14)	1.23 (0.18)	1.60 (0.37)	1.44 (0.37)
Lymphoid	1.12 (0.29)	0.11*** (0.07)	1.54 (0.37)	0.24*** (0.10)	1.36 (0.39)	0.60* (0.64)	1.20 (0.29)	0.81* (0.20)	0.90 (0.26)	0.84 (0.16)	0.77 (0.11)	0.60* (0.19)	0.79 (0.28)	1.06 (0.31)
Other	0.14 (0.04)	0.05*** (0.03)	0.13 (0.02)	0.03*** (0.01)	0.09 (0.01)	0.04* (0.05)	0.13 (0.04)	0.11 (0.03)	0.11 (0.04)	0.09 (0.02)	0.07 (0.03)	0.06 (0.02)	0.09 (0.03)	0.10 (0.05)
M:E ratio	1.27 (0.36)	0.28*** (0.22)	1.92 (0.71)	0.55*** (0.39)	1.34 (0.21)	0.33*** (0.27)	1.52 (0.45)	1.39 (0.35)	1.46 (0.33)	1.20 (0.24)	1.06 (0.29)	1.01 (0.20)	0.98 (0.13)	1.08 (0.08)

[a]200 cells in the tibial marrow smears were differentially counted by eye and the absolute number of cells of each lineage estimated for the nucleated marrow cell count of the femoral marrow flush. Values are means and SD in parenthesis. Cells categorised as 'other' include monocytes and monocyte precursors, mast cells, megakaryocytes, plasma cells and unidentifiable cells. n=6 for control and AZA groups at all time points.
Data analysed using a Student's t-test. *Significantly different from controls, $p<0.05$; **$p<0.01$; ***$p<0.001$.

TABLE 8.4. Relative organ weights[a] of control and azathioprine (AZA) treated ICR (CD-1) mice; animals were given 10 doses of AZA over a period of 10 days and sampled at 1 to 51 days after the final dose

	Day of sampling													
	1		3		9		22		29		43		51	
	Control	AZA	Control	AZA	Control	AZA	Control	AZA	Control	AZA	Control	AZA	Control	AZA
Liver	62,046	60,512	58,536	64,984**	57,444	60,687	60,199	64,786	55,369	57,158	51,824	57,688**	48,988	56,628**
	(4,393)	(5,126)	(2,946)	(3,220)	(6,139)	(6,598)	(4,248)	(6,552)	(4,754)	(5,250)	(3,945)	(3,948)	(4,397)	(4,657)
Kidney	6,989	6,749	7,084	6,714	7,016	8,030***	6,735	7,442*	6,320	6,495	5,944	6,076	6,352	6,494
	(350)	(232)	(554)	(743)	(266)	(608)	(570)	(713)	(703)	(394)	(529)	(216)	(604)	(323)
Spleen	4,529	2,692***	4,003	2,920***	4,247	4,096	4,684	5,879**	4,162	4,927	3,769	4,820**	3,882	4,114
	(484)	(150)	(602)	(505)	(963)	(737)	(602)	(1,154)	(805)	(936)	(376)	(773)	(658)	(737)

[a]Relative organ weight, mg/kg body weight. Values are means and SD in parenthesis. n=8 for control and AZA-treated mice on day 1 and 3 post dosing; at all other time points n=10 (control) and n=5 (AZA) except on day 9 where n=8 for control treated mice.
Data analysed using the Student's t-test. *Significantly different from controls, $p<0.05$; **$p<0.01$; ***$p<0.001$.

CHAPTER 9. Discussion

9.1. THE MOUSE MODEL OF BUSULPHAN-INDUCED CHRONIC BONE MARROW APLASIA

The model of BU-induced chronic bone marrow aplasia (CBMA) developed by Gibson *et al.* (2003) and modified by Turton *et al.* (2006) was used in the present studies to measure changes in the concentration of the serum cytokine fms-like tyrosine kinase 3 (FLT-3) ligand (FL) (Chapter 3). It was found that in the immediate post dosing period, peripheral blood pancytopenia and bone marrow aplasia were accompanied by a significant elevation in the concentration of serum FL. In the following 177 days after BU dosing, FL levels continued to be significantly increased in comparison to control (vehicle-treated) mice. Linear regression analysis identified positive correlations between the concentration of serum FL in BU-treated mice and many peripheral blood and bone marrow parameters, including platelets, neutrophils and the FNCC.

The changes in the concentration of serum FL in the BU-treated mouse may be compared to FL levels in the plasma of cancer patients following exposure to chemotherapy and radiotherapy (Chklovskaia *et al.* 1999; Blumenthal *et al.* 2000). In addition, it has been shown that patients with bone marrow failure, in the form of aplastic anaemia (AA) and Fanconi's anaemia, have significant increases in plasma FL (Lyman *et al.* 1995b).

The patterns of change in the concentrations of serum FL in the BU-treated mouse are another example of how this mouse model of CBMA compares with the condition of AA in man. The investigations on serum FL levels provide further support for considering the use of the BU-induced mouse model to study the pathophysiological mechanisms involved in drug-induced and other forms of bone marrow failure.

The mouse model of CBMA was used to assess the effects of administering the immunosuppressant (IS) drug cyclosporin A (CsA) to BU-treated mice (Chapter 4). On day 57 post dosing, BU-treated mice were dosed with vehicle or CsA (30 mg/kg/day) for 34 days. Administration of CsA did not however, protect mice from bone marrow aplasia, and changes in the peripheral blood and bone marrow of mice treated with BU-vehicle and BU-CsA were similar to each other and not statistically different. This result, although unexpected, would suggest that the injury to the bone marrow resulting from BU treatment is not modulated by the immune system. However, this finding needs further examination as changes to the CsA dosing schedule may result in a more positive outcome.

The extent of the bone marrow injury induced in this mouse model of BU-induced CBMA is not as severe as the condition of AA in man. With a view to inducing a more significant drug-induced injury to the bone marrow, the model of BU-induced CBMA was amended (Chapter 5). Initially, BALB/c mice were treated with BU at 9.0 mg/kg on 10 occasions over 21 days followed on day 51 post dosing with a second regimen of BU at 1.0, 2.0, 3.0, 4.0 or 5.0 mg/kg on 10 occasions over 22 days. The administration of this second BU dosing regimen beginning 51 days after the first did not induce a more significant injury to the bone marrow. More investigation is required to determine the reasons for this outcome. Studies in the rabbit have shown that when animals are treated with FL prior to total body irradiation (TBI) animals survive even though reductions are evident in periphael blood counts. Similarly, studies in the mouse and non-human primate have shown that FL administered after exposure to TBI in combination with other cytokines (i.e. stem cell factor, interleukin-3, granulocyte colony stimulating factor and thrombopoietin) prevents mortality as a result of bone marrow failure. With this evidence in mind, it is possible that endogenous FL released into the blood following the first BU dosing regimen is protecting haemopoietic

progenitor cells from further BU-induced toxicity. Secondly, haemopoietic progenitor cells may be less susceptible to the toxic effects of BU on day 50 after the first BU dosing regimen. It may be possible that the first regimen of BU destroys susceptible haemopoietic progenitor cells and that remaining progenitor cells in the bone marrow are resistant to BU. BU is particularly toxic to resting cells. If the majority of haemopoietic stem cells were cycling on day 50 after the first BU dosing regimen then cells would not be destroyed by the second regimen of BU therefore, a more significant injury would not be induced.

9.2. HAEMOTOXIC EFFECTS OF ANTI-CANCER DRUGS

The development of a more significant drug-induced bone marrow injury was investigated using the antineoplastic agents chlorambucil (CHB), mitomycin C (MMC) and azathioprine (AZA).

The administration of CHB was previously reported to cause residual 'late-stage' bone marrow injury (Trainor *et al.* 1979). However, peripheral blood and bone marrow changes (Chapter 6) as a result of CHB treatment were relatively mild and recovery following treatment was swift. However, CHB treatment did induce residual effects on the number of lymphocytes within the peripheral blood and bone marrow.

The administration of MMC to mice (Chapter 7) resulted in significant bone marrow aplasia immediately post dosing. The toxicity of MMC to the bone marrow immediately post dosing lead to incidences of mortality. On days 28 to 42 post dosing, MMC-treated mice developed significant injury to the kidney resulting in a high number of inter-current deaths. In the long-term, the effects of MMC treatment were confined to the erythroid lineage with significant reductions evident in the peripheral blood and bone marrow on days 42 and 51 post dosing.

Similarly, the administration of AZA (Chapter 8) to mice lead to an immediate reduction in peripheral blood counts and bone marrow cellularity. Unexpectedly high mortality was evident in this study as a result of bone marrow failure. Residual effects were evident following AZA treatment, however this was only to the erythroid lineage.

9.3. Future Work

To further characterise the model of drug-induced chronic bone marrow aplasia (CBMA) described by Gibson *et al.* (2003) and Turton *et al.* (2006) studies could be conducted to determine the effects of busulphan (BU) treatment on the haemopoietic stem cell (HSC) compartment. Mononuclear cells separated from bone marrow cell suspensions could be used to quantify the number of HSC according to the expression of cell surface antigens. For example, the number of HSC characterised as $CD34^+$, c-kit^+ and Sca-1^+ could be analysed in BU-treated mice immediately post dosing and during late-stage CBMA.

Experiments with cyclosporin A (CsA) have provided evidence to suggest that the immune system is not involved in the late-stage BU-induced CBMA. However, the role of the immune system in the development of CBMA following BU treatment should still be investigated to obtain further confirmation. CsA may not have protected mice from BU-induced CBMA because of inadequate drug absorption when administered via the oral route or there may have been an insufficient duration of drug treatment. Changes in lymphocyte populations following treatment with BU and the quantity of inflammatory cytokines produced (IFN-γ, TNF-α) by lymphocytes may provide answers to the role the immune system plays in the development of BU-induced CBMA.

The development of a more significant bone marrow injury in mice using cytotoxic drugs other than BU may be impossible without inducing significant toxicity to other organs resulting in high levels of mortality. Thus, the model of BU-induced CBMA (Gibson *et al.* 2003; Turton *et al.* 2006) could be modified to create a more significant injury to the bone marrow. One way of achieving this could be to use splenectomised mice. The spleen is an important haemopoietic organ with the ability to produce large numbers of blood cells particularly in times of stress. Removal of the spleen may result in the development of a more significant bone marrow injury following treatment with BU. However, it may also be possible to induce a more significant bone marrow injury by using a genetically susceptible strain of mouse or a different species.

The model of BU-induced CBMA could also be used to study the action of the cytokine FL. This cytokine is involved in early stages of haemopoiesis and is dramatically elevated in man and mouse during periods of bone marrow aplasia. This model of BU-induced CBMA could be used to elucidate the cell or tissue responsible for the production and release of FL and also to determine the effect of these elevated levels of FL on the haemopoietic stem cell population. Furthermore, FLT-3 receptor knock-out mice could be used to further study the role of FL in BU-induced bone marrow injury.

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PUBLISHED WORK RELEVANT TO THIS THESIS

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Molyneux,G., Rizzo,S., Gibson,F.M., Sulsh,S., Andrews,C.M., Pilling,A.M., Nakshbandi,T., Gordon-Smith,E.C. & Turton,J.A. (2004) Haemotoxicity of chlorambucil in the Wistar Han rat with particular reference to bone marrow culture, marrow cell apoptosis and levels of FLT3 ligand. *Comparative Clinical Pathology,* **13,** 78-81.

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ORIGINAL ARTICLE

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Haemotoxicity of chlorambucil in the Wistar Hanover rat with particular reference to bone marrow culture, marrow cell apoptosis and levels of FLT3 ligand

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Abstract We have recently developed a new model of drug-induced chronic bone marrow aplasia (CBMA) in the mouse, which shows features of aplastic anaemia (AA) in humans. Using a regimen of repeated doses of busulphan (BU) we induced late-stage (i.e. chronic) bone marrow depression. There are reports that indicate that other antineoplastic agents [e.g. chlorambucil (CHB), mitomycin, melphalan] may also cause CBMA in the mouse. Wishing to develop a model of CBMA in the rat, we investigated the potential of CHB to induce this change. Female Wistar Hanover (Wistar Han) rats were dosed with CHB (6.3 mg/kg intraperitoneally) on six occasions over 18 days. Animals ($n = 6$–8) were killed and sampled on nine occasions (at 1, 3, 9, 16, 24, 38, 45, 59 and 65 days) after the final CHB dose. A full blood count was performed, and serum was prepared for FLT3 analysis; marrow smears were produced, and the spleen and sternum were placed in histological fixative; femoral marrow suspensions were prepared for assessment of the nucleated cell count [femoral nucleated cell count (FNCC)], levels of apoptosis, and the clonogenic potential of the marrow [colony forming unit cells (CFU-Cs)]. Our results showed that at days 1 and 3 post-dosing, in general, red blood cells (RBCs), lymphocytes and FNCC were significantly reduced in CHB-treated animals; reticulocytes were increased, and platelets and neutrophils were unaffected. At 9 days, parameters in CHB rats were returning to normal, but lymphocytes were still decreased. At 16 and 24 days, many blood parameters were normal, except for reduced lymphocyte counts; this pattern generally remained until the end of the study (day 65). Levels of apoptosis in marrow cells of CHB-treated rats were increased immediately post-dosing, and this elevation persisted until day 16; thereafter, levels were generally normal. Serum FLT3 ligand (FL) levels showed some evidence of increases in CHB rats after dosing. CFU-Cs/femur were significantly reduced in CHB animals after dosing, returning to normal values at day 24. In general, marrow smears showed reductions in the myeloid, erythroid and lymphoid lineages of CHB animals at days 1, 3 and 9, returning to normal at 16 and 24 days. Histology of the spleen showed severe depletion of the white pulp immediately post-dosing in CHB-treated animals. Therefore, it is concluded from these findings that CHB does not induce late-stage (i.e. chronic) bone marrow aplasia in the female Wistar Han rat.

Keywords Apoptosis · CFU-C · Chlorambucil · FL · Haemotoxicity · Rat

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Introduction

Aplastic anaemia (AA) in humans is a condition characterised by hypocellular bone marrow, pancytopenia of the peripheral blood, and a risk of severe anaemia, infection and haemorrhage (Young and Alter 1994; Jandl 1996). The development of AA is considered to be associated with a range of aetiological agents, including viral infections, radiation, and exposure to chemicals and drugs (Heimpel and Heit 1980; Heimpel 2000; Young and Maciejewski 2000). However, the fundamental pathophysiology of AA is not well understood, and the fact that there are no convenient animal models of AA has been a contributing factor to this lack of understanding. Although the development of various models of chronic bone marrow hypoplasia/aplasia (i.e. AA) has been attempted, in both large and small laboratory animal species, none of these models has become widely used (Alter et al. 1978; Haak 1980; Vincent 1984). Nevertheless, of the various investigations that have been conducted, useful results have been achieved in studies based on the method reported by Morley and Blake (1974a). These workers developed a mouse model of drug-induced chronic hypoplastic marrow failure (CHMF) in the busulphan (BU)-treated BALB/c mouse, where 'residual' or 'late stage' bone marrow injury was described.

In both humans and laboratory animals, the response of the bone marrow to many antineoplastic agents is the development of significant marrow depression in the immediate post-dosing period (Reynolds 1989; Dollery 1999; Sweetman 2002). However, with many such anticancer agents, the marrow generally returns towards normal in the post-dosing period during the following days/weeks; this response of the marrow is dose-related and is sometimes referred to as 'predictable' bone marrow depression. The response of the bone marrow to some antineoplastic drugs (for example BU) may, however, be different in that, although the marrow may return towards normal in the post-dosing period, normality may not be achieved and the marrow may remain, to a degree, depressed. Morley and Blake (1974a, 1974b) described this phenomenon in their mouse model of CHMF as 'late-stage/chronic marrow failure', with the marrow showing evidence of 'residual injury'.

In the female BALB/c mouse model of CHMF of Morley and Blake (1974a), BU was administered on four occasions over a 6-week period, and the animals were studied over the following 300–400 days. However, after carrying out studies to follow up these findings, we (Andrews et al. 1993, 1998; Andrews 2000) have been unable to obtain results that are closely analogous to the findings of Morley and Blake (1974a) and, accordingly we have recently attempted to evaluate other regimens of BU dosing in the mouse to develop a new model of chronic bone marrow aplasia (CBMA) that shows features of the human disease (Gibson et al. 2003). Using this new mouse model as a basis, we then attempted to develop a BU-induced model of AA in the rat (J. A. Turton et al., unpublished data); however, preliminary results demonstrated that, in this species, the late-stage marrow aplasia/hypoplasia that was induced was relatively mild, in comparison with the often severe aplasia that is seen in humans.

After the initial reports by Morley and Blake (1974a, b), which described their mouse model of CHMF, Morley and his co-workers published a series of papers that investigated the pathogenesis of chronic bone marrow failure in their BU-mouse model (Morley et al. 1975; Pugsley et al. 1978; Gale and Morley 1980; Trainor et al. 1980). However, Morley and his co-workers then went on to describe a series of experiments that investigated the capacity of a range of antineoplastic agents other than BU to induce late-stage bone marrow failure; it was reported that chlorambucil (CHB), mitomycin, melphalan and carmustine had caused late-stage/chronic residual damage and marrow failure at least 2 months after the last drug treatment (Trainor and Morley 1976; Trainor et al. 1979; Morley 1980). This effect on the bone marrow was not seen with doxorubicin, cyclophosphamide, 5-fluorouracil, methotrexate or vinblastine.

In humans, a feature of the nucleated cells of the bone marrow in AA patients is that the telomeres of those cells show progressive telomere shortening (Ball et al. 1998). This finding is of considerable interest, but the mechanistic basis of the change is unclear, and the pathological processes involved are difficult to investigate in the human subject. Using techniques currently available, one cannot accurately measure telomere shortening in the bone marrow cells of the mouse, but such measurements can be made in the rat. In the longer term we wish to investigate mechanisms of telomere shortening in our rodent models of CBMA and, accordingly, have set out to develop a model of CBMA in the rat that shows characteristics that equate to AA in humans. We have therefore studied the haemotoxicity of CHB in this species and the potential of this drug to induce late-stage residual bone marrow aplasia. Our objectives were also to characterise the features of bone marrow injury caused by CHB in the immediate post-dosing period, to describe the patterns of change as the various haematological parameters returned towards normal, and to define the changes in apoptotic marrow cells, colony forming unit cells (CFU-Cs), and serum FLT3 ligand (FL; CD135) in relation to the other parameters that were being investigated.

In addition, although apoptosis in the nucleated bone marrow cells of patients with AA has previously been investigated (Philpott et al. 1996), comparable studies on bone marrow cells do not appear to have been completed with rat marrow samples. Accordingly, we therefore carried out a series of preliminary studies to confirm that our techniques for measuring apoptosis in human bone marrow samples were valid, and applicable, for similar investigations in the rat.

An initial report has been published in abstract form (Molyneux et al. 2002).

Materials and methods

Animals

Weanling female Wistar Hanover rats (B & K Universal, Grimston, Aldbrough, Hull, UK) were caged in groups of six. They were bedded on wood shavings, with food (Rat and Mouse No. 1, SDS, Witham, Essex, UK) and mains water ad libitum. A temperature of 19–22°C was maintained, with a relative humidity of 45%–65% and a light:dark cycle of 12:12 h (lights on at 7 a.m.). The animals were allowed to acclimatise for 7 days before the experiment and were observed at least three times per week for signs of ill health in the post-dosing period. Body weights were determined at approximately weekly intervals. All animal procedures were conducted under local Ethical Committee guidelines and approval for Home Office Project and Personal Licences and followed the UK Home Office (1989) *Code of Practice for the Housing and Care of Animals used in Scientific Procedures*.

Chlorambucil administration

Chlorambucil (Sigma Chemical Co., Poole, Dorset, UK) was dissolved in acetone at a concentration of 4.35 mg/ml, and, immediately before administration, deionised water was added to the CHB–acetone solution at a volume of 2:1. CHB was administered to the rats at a dose level of 6.3 mg/kg by intraperitoneal (i.p.) injection at a dose volume of 0.8–1.2 ml/rat. Control animals were treated with acetone:water (vehicle) at the same dose volume.

Tissue sampling

The rats were killed with an i.p. injection of pentobarbitone sodium (Sagatal, Rhône Mérieux, Harlow, Essex, UK). Blood was removed from the abdominal aorta, and a 0.5 ml aliquot was anticoagulated with 1.5 mg/ml dipotassium EDTA (Teklab, Sacriston, Durham, UK); the remaining blood was collected into serum separator tubes (Microtainer; Becton Dickinson, Franklin Lakes, N.J., USA). The contents of the left femur were aspirated into 5 ml phosphate buffered saline (PBS) to give a marrow cell suspension; a marrow smear was prepared from the contents of the right tibia. The right femur was removed, with surrounding muscle, and placed in 5 ml sterile PBS; under sterile conditions the muscle and epiphyses were removed from the femur, and the marrow was flushed into 5 ml sterile Iscove's modified Dulbecco's medium (IMDM; Life Technologies, Paisley, UK) supplemented with 10% foetal calf serum (FCS; PAA Laboratories, Linz, Austria). The spleen was removed, weighed, and placed in 10.5% phosphate buffered formalin fixative; the sternum was removed and placed in fixative.

Analysis of blood, marrow suspensions and tissues

Blood samples and bone marrow suspensions in PBS were analysed with a Bayer H*1 haematology analyser with rat-specific software (Bayer Diagnostics UK, Newbury, Berks., UK). Reticulocyte analysis was carried out with a Sysmex R-1000 (Sysmex UK, Milton Keynes, Bucks., UK), with voltage gain adjusted optimally for rat blood. We used the femoral marrow cell suspension in PBS to obtain the total nucleated cell count (femoral nucleated cell count; FNCC), using the basophil channel of the H*1. Tibial marrow smears were stained with May–Grünwald–Geimsa, and differential counts were performed by eye on 200 cells. Sections of spleen and sternum were prepared, stained with haematoxylin and eosin, and assessed histologically.

Apoptosis in marrow mononuclear cells

The bone marrow flush in IMDM was used to measure apoptosis according to the method described by Philpott et al. (1995, 1996). The marrow flush of 200 µl (0.5–1.0×10^6 cells) was washed twice in PBS supplemented with 1% FCS and 0.05% Na azide. Cells were then resuspended in 500 µl or 450 µl of PBS for unstained and stained cells, respectively. Cells were stained with 50 µl of 7AAD (Calbiochem, Nottingham, UK) for 20 min on ice and protected from light. Cells were fixed in 500 µl of 2% paraformaldehyde solution (Sigma) and analysed on a FACScan (Becton Dickinson, Mountain View, Calif., USA) within 30 min of fixation. Data on 50,000 cells were acquired and processed with Cell Quest software (Becton Dickinson). Scattergrams of forward light scatter (FSC) vs. 7AAD fluorescence were generated. FSC height threshold was set at 108 to exclude debris and red blood cells (RBCs). Regions were drawn around populations showing negative (R1), dim (R2) and bright (R3) 7AAD fluorescence, which corresponded to live, apoptotic and dead cells, respectively. A region (R4) was also drawn around remaining cell debris and RBCs to exclude these data (Figs. 1 and 2).

Colony forming assay (CFU-C)

Using the experiments of Lucas et al. (1999) as a starting point, we conducted preliminary experiments to establish a method of assessing bone marrow colony formation in the rat (data not shown). Accordingly, the femoral marrow cell suspension in IMDM with 10% FCS was further diluted to 25 ml with fresh IMDM. The white blood cells (WBCs), RBCs, and dead cells were counted by trypan-blue exclusion. Cells were cultured at 10^5 WBCs in 1 ml IMDM supplemented with 30% FCS, 1% deionised bovine serum albumin (BSA; Sigma), 10^{-4} M β-mercaptoethanol (Sigma), 0.05% $NaHCO_3$, 2.1 mM L-glutamine (Sigma) and 0.9% methylcellulose (Stem Cell Technologies, London, UK).

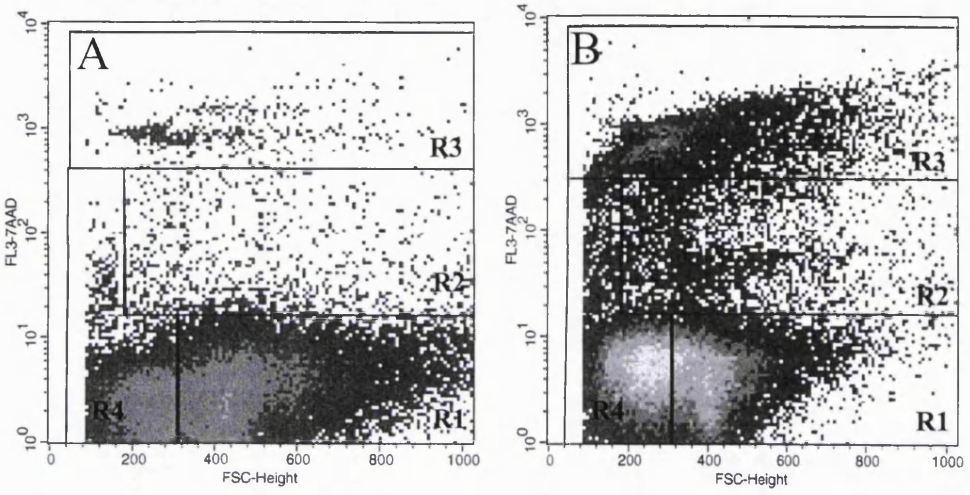

Fig. 1a, b Scattergrams of 7AAD-stained rat femoral bone marrow samples (preliminary studies). **a** Control rat bone marrow sample showing normal levels of apoptosis; *R1* live cells, 66.49%; *R2* apoptotic cells, 2.88%; *R3* dead cells, 1.21%; *R4* cell debris, 30.26%. **b** Control rat marrow sample with levels of apoptosis increased by the withholding of nutrients (maintenance in IMDM deprived of FCS for 16 h); *R1* live cells, 39.63%; *R2* apoptotic cells, 7.58%; *R3* dead cells, 13.69%; *R4* cell debris, 40.38%. *FSC-Height* forward light scatter; *FL3-7AAD* 7AAD fluorescence intensity

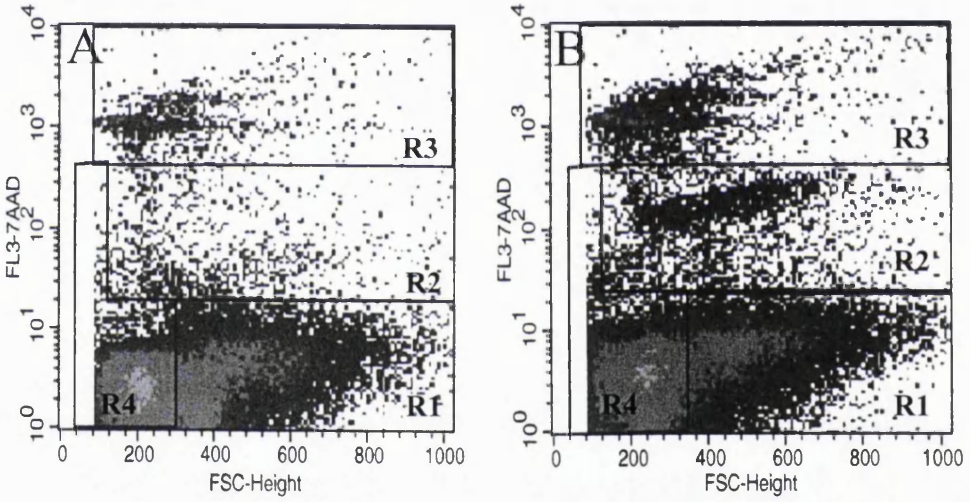

Fig. 2a, b Scattergrams of 7AAD-stained rat femoral bone marrow samples (main study). **a** Control rat bone marrow sample showing normal levels of apoptosis; *R1* live cells, 45.43%; *R2* apoptotic cells, 2.45%; *R3* dead cells, 2.68%; *R4* cell debris, 49.48%. **b** Rat marrow sample from an animal treated with CHB and autopsied 9 days after being dosed. The sample shows an increased level of apoptotic (and dead) cells; *R1* live cells, 32.18%; *R2* apoptotic cells, 6.59%; *R3* dead cells, 6.15%; *R4* cell debris, 55.12%. Abbreviations as Fig. 1

Cultures were set up in duplicate in 35 mm dishes (Nunclon, Loughborough, Leics., UK) with the following growth factor stimuli added to each dish: 4 IU human erythropoietin (hEPO; Janssen-Cilag, High Wycombe, Bucks., UK), 50 ng murine interleukin-3 (mIL-3; 1.7×10^5 U/mg; R&D Systems Europe, Abingdon, Oxon., UK), 50 ng murine stem cell factor (mSCF; R&D Systems), 50 ng human interleukin-6 (hIL-6; 2×10^6 U/mg; Novartis Pharmaceuticals, Langley, Herts., UK) and 50 ng human granulocyte colony stimulating factor (hG-CSF; 10^8 U/mg; Amgen UK, Cambridge, UK). The cultures were incubated at 37°C in 5% CO_2 in air for 14 days. Colonies of erythroid, granulocyte and monocyte origin that contained more than 50 cells were counted and combined to give a total number of CFU-Cs per culture.

FL ligand assay

The serum in separator tubes was prepared as has been previously described (Turton et al. 2002) and stored at −80°C. The presence of the cytokine FL in serum was detected with an ELISA assay with a sensitivity of typically less than 5 pg/ml (R&D Systems), in accordance with the manufacturer's instructions.

Statistical analysis

Chlorambucil-treated and control (vehicle-treated) groups were compared by a one-tailed Student's *t*-test.

Experimental design

One hundred and forty female Wistar Hanover rats, mean body weight 149.6 g, were divided into two groups: $n=68$ (control, vehicle-dosed) and $n=72$ (CHB-dosed). Animals were dosed with vehicle or CHB (6.3 mg/kg) on six occasions over a period of 18 days (day 1, 4, 6, 11, 14, 18). They were observed daily for clinical signs of toxicity during the dosing period, and at least three times each week after dosing. At 1, 3, 9, 16,

24, 38, 45, 59 and 65 days after the final CHB dose, animals from each group ($n = 6$–8) were killed and sampled for examination of blood, marrow and tissues.

Results

Clinical signs and body weight changes

During the period of CHB administration there were no clear clinical signs of drug-induced toxicity, although body weight gain was adversely affected. However, two animals treated with CHB were unexpectedly found dead, one on the ninth day of the dosing period (after three CHB doses), and one at 5 days after the final CHB dose; the reasons for these mortalities could not be identified. At the beginning of the dosing period the control animals ($n = 68$) weighed, on average, 149.7 g, and CHB rats ($n = 72$) were 149.5 g. During the period of dosing, the control animals increased in body weight by 129.8%, and by 157.3% at the end of the study (day 65 post-dosing); the results for CHB-treated rats were 117.8% ($P < 0.001$) and 154.8% [not significant (NS)], respectively. The significant reduction in body weight gain in the CHB-dosed animals, evident during the period of CHB dosing, was made good by days 8–15 post-dosing.

Haematological results

Red blood cells, haemoglobin (Hb) and haematocrit (HCT) in the CHB-treated animals were all significantly reduced on days 1 and 3 post-dosing (Table 1); the average percentage reductions for those parameters at those times were similar, being 90.9% (RBCs), 90.8% (Hb) and 92.5% (HCT) of the control values. Mean cell volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) were unaffected at days 1 and 3, and platelet counts were slightly elevated in CHB-treated rats (NS) at these times. WBCs and lymphocyte counts were significantly decreased at days 1 and 3; the reductions in lymphocytes were to 7.2% and 6.4% of those of the controls at these time points, respectively. Monocytes were significantly reduced at day 3 in CHB-dosed rats; neutrophils were unaffected. Reticulocyte counts were increased at day 1 and day 3 post-dosing in CHB-treated animals. At day 9 post-dosing, in CHB-dosed rats, the RBCs, although decreased in comparison with the controls, was returning towards normal. The HCT was also returning towards normal at day 9, and the Hb level was normal. However, MCV and MCH were significantly raised at this time point, as were neutrophils, monocytes and eosinophils; platelets and reticulocytes were also significantly elevated. Lymphocytes and WBCs were still decreased at day 9; the reduction in the lymphocyte count was to 20.0% of the control value.

At day 16, in CHB-treated rats, RBCs, Hb and HCT were normal, as were neutrophils, monocytes and eosinophils, but MCV and MCH, and platelets and reticulocytes, were still significantly raised. Lymphocytes and WBCs were significantly reduced at day 16 post-dosing (the lymphocyte count was 25.3% that of the control). In general terms, at 24, 38, 45, 59 and 65 days post-dosing, many parameters in CHB-treated rats were comparable to those in the controls; however, lymphocytes and WBCs continued to show significant reductions at 24, 59 and 65 days; the decreases in the lymphocyte counts at these three time points were to 44.4%, 68.6% and 60.8% of control values, respectively.

Femoral nucleated cell counts, marrow cell apoptosis, CFU-C and FL

The FNCC was significantly reduced on days 1, 3 and 9 post-dosing in CHB-dosed rats (Table 2), and the average percentage reduction at these time points was to 52.0% of the control counts; at 16 days the counts were raised above normal (NS), and at 24, 45 and 59 days the number of cells was also significantly increased. At 65 days the FNCC was comparable to the control value.

A series of preliminary studies was carried out to confirm that techniques previously developed to assess apoptosis in the nucleated bone marrow cells of human subjects were valid and applicable for use with the rat bone marrow samples generated in the present study. Baseline levels of apoptosis were assessed in control rat marrow samples maintained in IMDM supplemented with 10% FCS for 16 h. Marrow samples were also maintained in IMDM without FCS for 16 h; levels of apoptosis were increased as a result of the withdrawal of nutrients in those samples (Fig. 1a, b). It was, therefore, confirmed that the techniques used for assessing apoptosis in human samples were appropriate for studies in the rat.

On days 1 and 3 after CHB dosing, apoptosis in femoral marrow nucleated cells was significantly increased (Table 2). This increase in apoptosis immediately after dosing was also evident at days 9 (Fig. 2) and 16, but the levels appeared to be returning to control values. At days 24 and 45 post-dosing, levels of apoptosis in CHB-treated rats were not significantly different from the those in controls. On day 65 the level of apoptosis was normal.

The number of CFU-Cs per femur in CHB-treated rats was significantly reduced immediately after dosing. On days 1, 3, 9 and 16 the CFU-Cs of CHB-treated rats were 51%, 57%, 48%, and 55% of control values, respectively. From day 24 the number of CFU-Cs was at approximately normal levels and did not differ significantly from the controls at day 24, 38 and 45. However, at day 59 the number of CFU-Cs was again significantly reduced. On day 65 the number of CFU-Cs in CHB-treated animals was normal.

The concentration of FL was measured in the serum of the rats on days 1, 3, 9, 16, 24, 45 and 65 post-dosing. On day 1 post-dosing, the serum concentration of FL

Table 1 Haematological results from female Wistar Han rats treated with six doses of CHB over a period of 18 days and sampled at 1–65 days after the final dose. Haematological results are means; $n=8$ for control and CHB groups at all time points, except $n=6$ for control groups at days 59 and 65, and $n=7$ for control group at day 1 and CHB groups at days 1, 24 and 65. Units: *RBCs* $\times 10^6/\mu l$, *Hb* g/dl, *HCT* %, *MCV* fl, *MCH* pg, *MCHC* g/dl, *Retic* (absolute reticulocyte count) $\times 10^3/\mu l$, *Plt* (platelets) $\times 10^3/\mu l$, *WBCs* (white blood cells) $\times 10^3/\mu l$, *Neut* (neutrophils) $\times 10^3/\mu l$, *Lymph* (lymphocytes) $\times 10^3/\mu l$, *Mono* (monocytes) $\times 10^3/\mu l$, *Eo* (eosinophils) $\times 10^3/\mu l$, *Baso* (basophils) $\times 10^3/\mu l$, *Spleen* (relative spleen weight) mg/kg body weight

Parameter	Day of sampling																	
	1		3		9		16		24		38		45		59		65	
	Control	CHB	Control	CHB	Control	CHB	Control	CHB	Control	CHB	Control	CHB	Control	CHB	Control	CHB	Control	CHB
RBCs	7.24	6.76*	7.38	6.53*	7.66	7.21*	7.37	7.12	7.76	7.47	7.63	7.44	7.54	7.78	7.27	7.49	7.34	7.57
Hb	14.6	13.5*	14.7	13.1**	14.8	14.8	14.3	14.7	14.8	14.8	14.8	14.8	14.7	15.3	14.1	14.2	13.7	14.2
HCT	41.4	38.5*	41.2	37.9**	42.1	41.8	41.1	41.3	41.5	42.2	42.6	41.8	42.4	43.1	40.2	40.0	39.3	40.5
MCV	57.3	57.0	55.9	58.7	55.0	58.0***	55.7	58.1*	53.5	56.7***	55.8	56.3	56.3	55.5	55.4	53.4**	53.7	53.5
MCH	20.2	19.9	19.9	20.2	19.4	20.5**	19.4	20.7*	19.1	19.8	19.5	19.9	19.6	19.7	19.4	19.0**	18.7	18.8
MCHC	35.3	34.9	35.7	34.5	35.2	35.4	34.8	35.6	35.6	35.0	34.8	35.3	34.8	35.4	35.1	35.5	34.8	35.1
Retic	262.2	484.4***	266.8	410.1	305.3	429.1***	260.9	421.0***	280.7	243.4	246.6	219.4	233.6	237.6	168.8	185.7	276.2	241.2
Plt	972	1,098	886	1,051	842	962*	828	967**	887	827	790	821	767	897**	769	835	880	827
WBCs	2.64	0.75**	2.29	0.68***	2.52	1.19***	2.83	1.05***	3.10	1.77***	2.32	2.13	2.12	2.76	2.10	1.61**	3.15	2.03**
Neut	0.35	0.53	0.34	0.51	0.39	0.73**	0.44	0.40	0.50	0.56	0.47	0.53	0.30	0.65**	0.28	0.34	0.73	0.56
Lymph	2.21	0.16***	1.89	0.12**	2.05	0.41***	2.29	0.58***	2.52	1.12***	1.77	1.48	1.76	2.02	1.75	1.20**	2.27	1.38***
Mono	0.03	0.02	0.02	0.01***	0.03	0.04**	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.03	0.02	0.02	0.04	0.02*
Eo	0.03	0.01*	0.03	0.02	0.03	0.06**	0.04	0.06	0.03	0.05	0.04	0.08	0.02	0.05*	0.03	0.04	0.06	0.03*
Baso	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Spleen	2,727	2,428	2,627	2,233*	2,501	1,962***	2,672	2,370**	2,465	2,899***	2,331	2,512	2,178	2,637***	2,379	2,668	2,535	2,402

*$P<0.05$, **$P<0.01$, ***$P<0.001$

Table 2 Results for FNCC, marrow cell apoptosis, CFU-Cs and serum FL in female Wistar Han rats treated with six doses of CHB over a period of 18 days and sampled at 1–65 days after the final dose. Results are means (SDs); numbers of animals per group as Table 1; FL assays were not completed on days 38 and 59; apoptosis assay was not completed on day 59. Units: *FNCC* $\times 10^7$, *apoptosis* (femoral marrow nucleated cell apoptosis) %, *CFU-C* colonies/femur, *FL* pg/ml; all other information as Table 1

Parameter	Day of sampling								
	1	3	9	16	24	38	45	59	65
FNCC									
Control	9.63 (1.32)	9.63 (2.06)	8.29 (1.99)	7.91 (2.23)	9.14 (2.22)	9.05 (1.75)	7.28 (0.97)	7.17 (1.20)	8.72 (1.82)
CHB	5.13 (1.20)***	7.47 (1.92)*	2.07 (0.70)***	9.17 (1.41)	10.9 (1.42)*	9.09 (1.38)	10.17 (1.34)***	8.56 (1.22)*	8.28 (1.41)
Apoptosis									
Control	6.37 (0.68)	4.89 (1.04)	3.97 (0.56)	5.88 (0.97)	5.40 (0.45)	5.52 (0.60)	5.31 (0.28)		5.33 (0.33)
CHB	12.44 (6.04)*	9.88 (2.38)***	5.57 (1.13)***	6.77 (0.83)*	5.01 (0.71)	5.96 (0.45)*	5.28 (0.48)		5.00 (0.16)
CFU-C									
Control	16,369 (5,292)	21,481 (7,012)	22,605 (8,488)	16,161 (7,236)	16,137 (7,712)	14,705 (9,394)	17,548 (6,494)	17,700 (7,146)	16,645 (4,991)
CHB	8,295 (4,572)***	12,159 (6,498)***	10,895 (10,376)**	8,929 (4,255)*	16,146 (4,217)	18,123 (4,112)	17,648 (7,497)	10,377 (5,251)*	16,237 (4,491)
FL									
Control	610.1 (51.4)	584.4 (47.8)	516.0 (66.8)	532.3 (28.8)	510.5 (36.2)		515.4 (88.4)		536.9 (63.3)
CHB	500.7 (59.5)***	640.0 (65.0)*	571.2 (51.2)*	526.1 (104.1)	591.5 (33.1)***		590.4 (41.1)*		583.7 (116.7)

was significantly reduced in CHB-treated rats. However, on days 3, 9, 24 and 45 post-dosing, levels of FL in CHB-treated rats were increased significantly above the control levels. On day 65, levels of FL had returned to normal.

Bone marrow differential counts

Differential counts were carried out on tibial marrow smears to determine the proportion of cells (as percentages) in the myeloid, erythroid and lymphoid lineages. Taking the FNCC of each animal (see Table 2), we calculated the absolute number of cells that made up each lineage in the femoral marrow sample (Table 3). The overall picture in the immediate post-dosing period (days 1, 3 and 9) was of reductions in the numbers of myeloid, erythroid and lymphoid cells. The average decreases, over these three time points, for the myeloid cells was to 56.2% of the control values, and for lymphoid cells, 25.1%. The data for erythroid cells at day 3 post-dosing showed an increased count. However, the mean reduction in erythroid cells at days 1 and 9 was to 44.4% of the control count. By days 16 and 24 post-dosing, the cell counts for each lineage, including lymphoid cells, had returned to normal, and indeed, the counts were in general, slightly higher (NS) than the control values. At 38 days, overall values for all three lineages were similar to the control results; however, at day 45, the counts for myeloid, erythroid and lymphoid cells in CHB-dosed rats were increased above those in the controls. At 59 days post-dosing the count for lymphoid cells was significantly higher than that in the controls. Indeed, if the counts for the lymphoid lineage at the six time points from 16 to 65 days are taken, the average count for the control animals is 2.265 ($\times 10^7$), and for CHB-treated animals, 2.635 ($\times 10^7$); that is, the value for CHB-treated rats is 116.3% of the control value.

Examination of the marrow smears of CHB-dosed animals at day 1 post-dosing showed that, although the overall picture was one of depletion of the cells in the myeloid, erythroid and lymphoid lineages, in some animals there was evidence of myeloid hyperplasia. Also, in some rats, the myeloid precursors showed evidence of a 'right shift', with a preponderance of mature forms, or a 'left shift', with preponderance of immature forms (Fig. 3a–c). The morphology of the haemopoietic precursors in CHB-treated animals was comparable to that in the controls, with the exception of samples taken on day 3 post-dosing; here, there was evidence of dysmyelopoiesis with multinucleation and hypersegmentation of the neutrophil series (Fig. 3d).

Histology of the sternum and spleen

Sections of the sternum and spleen from control rats and from three animals treated with CHB and killed at days 1, 9, 24 and 65 post-dosing were examined. Sternal

Table 3 Estimated counts ($\times 10^7$) of myeloid, erythroid and lymphoid cells, and the myeloid:erythroid (*M:E*) ratio in the femoral marrow of control and CHB-treated rats; animals were given six doses of CHB over a period of 18 days and sampled at 1–65 days after the final dose. Two hundred cells in the tibial marrow smears were differentially counted by eye, and the absolute number of cells of each lineage estimated for the nucleated marrow cell count of the femoral marrow flush. Values are means (SDs). Cells categorised as 'other' include monocytes and monocyte precursors, mast cells, megakaryocyes, plasma cells and unidentifiable cells. All other information as Tables 1 and 2. $n = 8$ for control and CHB groups at all time points, except $n = 6$ for control groups at days 59 and 65, and $n = 7$ for control group at day 1 and CHB groups at days 1, 24 and 65

Cell type	Day of sampling																	
	1		3		9		16		24		38		45		59		65	
	Control	CHB	Control	CHB	Control	CHB	Control	CHB	Control	CHB	Control	CHB	Control	CHB	Control	CHB	Control	CHB
Myeloid	3.39 (1.25)	2.43 (1.32)	3.94 (1.41)	2.74 (0.98)	3.51 (0.78)	0.96 (0.38)***	2.86 (0.98)	3.30 (0.86)	3.97 (1.34)	4.48 (1.17)	3.82 (1.09)	3.51 (0.86)	3.02 (0.51)	4.31 (0.73)**	3.18 (0.40)	2.98 (0.43)	3.92 (1.31)	3.14 (0.69)
Erythroid	3.20 (1.34)	1.97 (0.71)*	2.50 (0.72)	3.61 (1.25)	2.46 (0.59)	0.67 (0.27)***	2.46 (0.86)	3.17 (0.71)	2.90 (0.53)	3.52 (0.76)*	2.44 (0.26)	2.22 (0.51)	2.06 (0.59)	3.19 (0.97)**	2.11 (0.30)	2.88 (0.35)***	2.21 (0.80)	2.83 (0.75)
Lymphoid	2.94 (0.99)	0.66 (0.16)***	3.02 (0.69)	1.02 (0.53)***	2.24 (0.68)	0.43 (0.20)***	2.43 (0.97)	2.59 (0.82)	2.20 (0.52)	2.80 (0.64)	2.68 (0.63)	3.11 (0.43)	2.01 (0.24)	2.54 (0.37)**	1.81 (0.66)	2.60 (0.65)*	2.46 (0.46)	2.17 (0.75)
Other	0.12 (0.10)	0.07 (0.07)	0.17 (0.09)	0.10 (0.05)*	0.08 (0.04)	0.01 (0.01)***	0.13 (0.09)	0.11 (0.04)	0.08 (0.03)	0.11 (0.04)	0.24 (0.10)***	0.19 (0.12)	0.13 (0.09)	0.08 (0.06)	0.10 (0.09)	0.12 (0.09)	0.14 (0.09)	0.07 (0.07)
M:E ratio	1.16 (0.48)	1.43 (0.91)	1.69 (0.90)	0.84 (0.36)**	1.46 (0.16)	1.49 (0.39)	1.23 (0.51)	1.10 (0.40)	1.35 (0.27)	1.31 (0.40)	1.56 (0.32)	1.59 (0.25)	1.54 (0.37)	1.47 (0.51)	1.53 (0.25)	1.05 (0.18)*	1.98 (0.85)	1.25 (0.74)*

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

marrow sections at day 1 post-dosing showed a modest decrease in the cellularity of the marrow (Fig. 4), in association with an increase in the number of adipocytes. Those changes were also present in one of the three animals examined at day 9. No abnormalities were noted in the sternal marrow at 24 and 65 days.

The relative weight of the spleen was reduced (NS) at day 1 post-dosing in CHB-treated rats (Table 1); at days 3, 9 and 16 the spleens from CHB-dosed animals were significantly lighter than those from the controls. At 24, 38 and 45 days post-dosing, the spleen weight was elevated in CHB-treated rats (NS at 38 days) but was comparable to that in the control animals at 59 and 65 days.

Histological sections of the spleen of CHB-dosed rats showed severe depletion of the white pulp at day 1 post-dosing (Fig. 5); all parts of the white pulp were affected, including the peri-arteriolar lymphoid sheath (PALS) and the marginal zone. Mild sinusoidal congestion was also present in the red pulp at this time. At day 9, depletion of the white pulp was still prominent in CHB animals. However, by day 24 post-dosing, considerable regeneration had occurred, although there was still some evidence of mild lymphocyte depletion at this time. At day 65, the white pulp in CHB-dosed animals was normal in appearance.

Discussion

Chlorambucil is an aromatic alanine mustard derivative. It was one of the first chemotherapeutic agents to be used in the treatment of malignant disease and was introduced into clinical practice in 1955 (Dollery 1999). CHB is used particularly in conditions of lymphocyte and WBC proliferation and is commonly employed, at present, in the management of chronic lymphocytic leukaemia, non-Hodgkin's lymphomas, Hodgkin's disease and ovarian cancer (BNF 1999; Sweetman 2002).

Chlorambucil is a bifunctional alkylating agent (Dollery 1999). The haemopoietic system is particularly susceptible to the toxicity of such agents, and CHB is an example of this susceptibility. However, with CHB, recovery of the bone marrow occurs more rapidly then with other alkylating agents such as BU (Reynolds 1989; BNF 1999; Medicines compendium 2002). CHB, in humans, is administered orally, with peak plasma concentrations being found at 0.5–2 h (Reynolds 1989; BNF 1999). As well as being used for the indications mentioned above, CHB is also given in the treatment of testicular tumours, advanced breast cancer and macroglobulinaemias. It has also been assessed recently in the treatment of non-malignant disorders including liver cirrhosis, nephrotic syndrome, and several autoimmune diseases (Sweetman 2002). The dose level of CHB used in humans depends on the particular mode of use, but, if it is used as a single agent, a typical dosage is 0.2 mg/kg daily for 4–8 weeks. However, generally, the drug is given as part of combination therapies; here, in

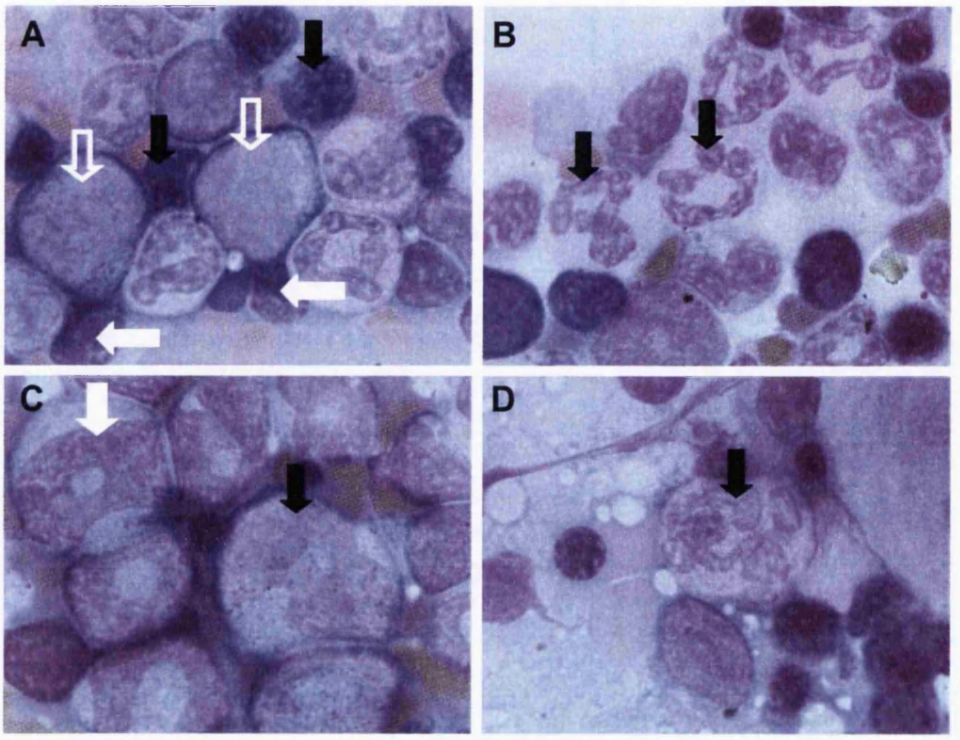

Fig. 3a–d May–Grünwald–Geimsa stained rat tibial marrow smears from control and CHB-treated animals. **a** Control rat at day 1 post-dosing, illustrating normal myeloid (*open white arrow*) and erythroid (*black arrow*) precursors and lymphoid cells (*white arrow*). The M:E ratio is 0.98:1. Original magnification (OM) ×1,000. **b** CHB-treated rat at day 1 post-dosing, with myeloid hyperplasia; the myeloid precursors show a 'right shift' with a preponderance of mature neutrophils (*black arrow*). The M:E ratio is 2.50:1. OM ×1,000. **c** CHB-treated rat at day 1 post-dosing, with myeloid precursors showing a 'left shift' (a preponderance of immature forms); promyelocytes, myelocytes (*black arrow*) and metamyelocytes (*white arrow*) predominate. The M:E ratio is 0.70:1. OM ×1,000. **d** Marrow smear from CHB-treated rat at day 3 post-dosing, showing evidence of dysmyelopoiesis with hypersegmentation and multinucleation of myeloid precursors. A developing neutrophil with hypersegmentation is shown (*black arrow*). The M:E ratio is 0.98:1. OM ×1,000

intermittent dosing regimens, the dose level may be 0.4 mg/kg (Reynolds 1989).

The most important toxic reaction of CHB in humans is bone marrow suppression, and careful monitoring of the patient's WBCs is essential. However, CHB at high dose levels may cause pancytopenia and irreversible bone marrow failure (Rudd et al. 1975; Reynolds 1989). Nevertheless, serious bone marrow toxicity is uncommon, and the marrow depression commonly seen is readily reversible. At therapeutic levels, CHB depresses lymphocytes, with a reversible progressive lymphocytopenia, with lesser effects on neutrophils, platelets and Hb levels.

Morley and Blake (1974a, 1974b) and Morley et al. (1975) and their co-workers developed a model of drug-induced CHMF in the BU-treated mouse. Those workers subsequently went on to investigate the basis of the cellular lesion underlying CHMF in their mouse model (Pugsley et al. 1978; Gale and Morley 1980; Trainor et al. 1980). However, in a series of reports, the ability of cytotoxic drugs other than BU to induce residual bone marrow damage was also investigated (Trainor and Morley 1976; Trainor et al. 1979; Morley 1980). This

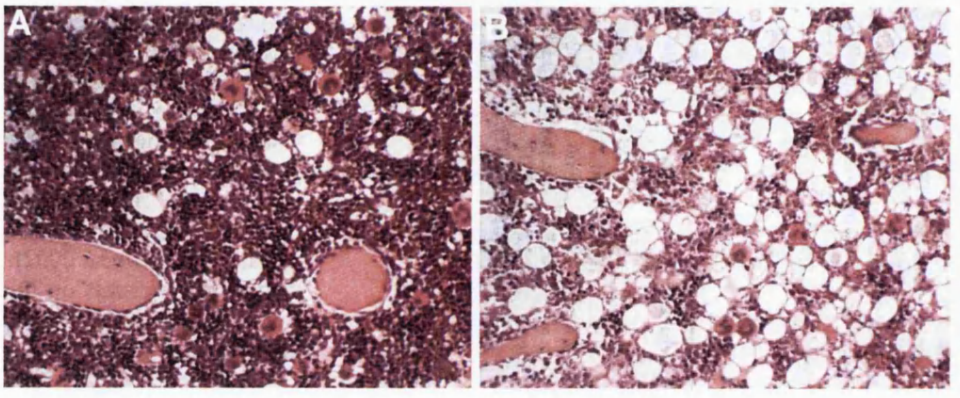

Fig. 4a, b H and E stained sections of rat sterna from control and CHB-treated animals. **a** Control rat at day 1 post-dosing, showing normal marrow cellularity. Original magnification (OM) ×200. **b** CHB-treated rat at day 1 after dosing, showing moderate depletion in the cellularity of the marrow, with increased numbers of adipocytes (OM ×200)

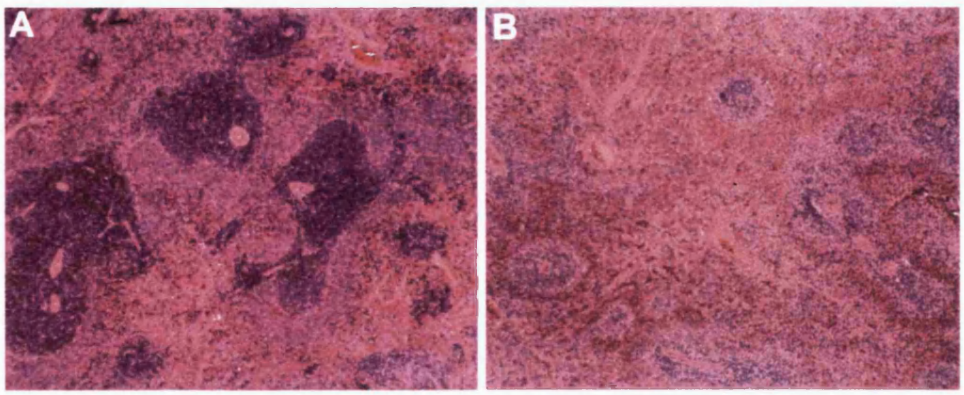

Fig. 5a, b H and E stained sections of spleen from control and CHB-treated animals. **a** Control rat at day 1 post-dosing, showing normal spleen histology with lymphoid aggregations (white pulp) and surrounding vascularised matrix (red pulp). Original magnification (OM) ×50. **b** Spleen from CHB-treated rat at 1 day post-dosing, illustrating severe depletion in the cellularity of all areas of the white pulp and mild sinusoidal congestion of the red pulp (OM 50×)

capacity of other antineoplastic agents to induce CHMF was assessed by the measuring of the depletion of pluripotent stem cells and granulocytic progenitor cells in the marrow, but with persistence for at least 8 weeks after the end of drug treatment. Evidence for residual marrow injury was identified in mice given CHB, mitomycin and melphalan, but not in animals treated with doxorubicin, cyclophosphamide, methotrexate, vinblastine and fluorouracil. Based on these findings by Morley's group, CHB was selected for study in the present investigation. The rat was chosen as there is a need to develop a convenient model of AA in this species, but previous studies with BU in the rat (J. A. Turton et al., unpublished data) had demonstrated that only a mild late-stage marrow aplasia/hypoplasia was induced with this agent. A rat model of AA would be of particular use in studies of telomere shortening and as an alternative species to the mouse in investigations on the pathophysiology of the human disease.

In humans, CHB is administered by the oral route only (BNF 1999; Medicines compendium 2002; Sweetman 2002). However, in the very early experimental work with CHB in the rat and mouse (Elson 1955; Elson et al. 1958; Alexander and Connell 1960), CHB was dissolved in arachis oil and dosed by i.p. injection, and the use of this route of administration has continued (Dunn and Elson 1970; Millar et al. 1978; Verschoyle et al. 1994; Van Os et al. 1998).

In general, the pattern of the haematological response to CHB (Table 1, 2 and 3) was of a mild bone marrow depression that was rapidly reversible towards normal. In the immediate post-dosing period (days 1–3 post-dosing), RBCs, lymphocytes, WBCs and FNCC were significantly reduced, as were marrow lymphoid cells; platelets and neutrophils were unaffected. At days 9 or 16, RBCs were returning towards normal, as were FNCC and marrow lymphoid cells; blood lymphocytes and WBCs continued to show decreased levels at these times. Also at days 9/16, MCV and MCH values showed evidence of significantly increased levels; this finding might relate to raised reticulocyte counts at these time points and in the immediate post-dosing period, or, alternatively, the raised values might be associated with dyserythropoietic changes, especially megaloblastosis. At days 24, 38, 45, 59 and 65, the overall pattern of responses was of normal values in CHB-dosed rats, but peripheral lymphocytes and WBCs were both significantly depressed at three of these five time points. Nevertheless, cells of the lymphoid lineage in the marrow, from days 16–65, were increased to an average of 116.3% over the control value. At days 1 and 9 post-dosing in CHB rats, histology of the spleen showed significant lymphocyte depletion, and there was some evidence for this change at day 24 also. It is assumed that both B and T lymphocytes were affected as both B and T cell-rich zones of the white pulp were ablated. The histology of the spleen was normal at day 65.

The above findings may be compared with a small number of reports in the literature. However, there are very few papers where the response to CHB has been studied for more than 20 days post-dosing, and there are similarly few reports on repeat-dose CHB investigations. Nevertheless, Elson (1955) and Elson et al. (1958) describe detailed studies on the blood and marrow changes in the rat following administration of a single dose of CHB. The greatest depressive effect of CHB was on the lymphocytes (Elson 1955), with peripheral blood counts falling to a minimum at day 4 post-dosing; normal values were regained by 18/20 days. Neutrophils were also decreased to a low point at day 4, followed by an overshoot at 8/10 days and normal values at 15/20 days. RBCs showed a slight decrease (day 4) followed by a return to normal. In a later report (Elson et al. 1958), marrow changes were investigated for up to 20 days following CHB administration. Erythropoietic cells in the marrow were at a minimum at 48 h post-dosing, with a rapid return to normal (day 6). Megakaryocytes were resistant to CHB effects. Granulopoiesis was profoundly reduced by CHB, with a maximum depression at 48 h; regeneration, with a peripheral blood neutro-

philia, then occurred (day 10) and a return to control levels (day 13). The volume of lymphoid tissue (spleen; thymus; lymph nodes) was also profoundly reduced after CHB dosing, with the number of lymphocytes in the marrow and blood also greatly decreased (72 h); peripheral lymphocytes fell to 10% of the control count. Regeneration of the lymphoid tissues was slow, and at 20 days (the end of the study), marrow and blood lymphocytes had not returned to normal.

In studies on CHB in the mouse (single and repeat doses), Verschoyle et al. (1994) reported reductions in the weight of lymphoid tissue (thymus; spleen) and reduced blood lymphocytes and WBCs; erythrocytes were unaffected, neutrophils were increased, and platelets decreased. Histological assessment of the thymus and spleen of CHB-dosed animals showed loss of lymphocytes, with evidence of apoptosis. Van Os (1998) examined the effect of multiple CHB doses (six doses administered every second week) in the mouse and investigated haemopoiesis at 20 weeks after the last dose; no significant effects were evident on WBC and marrow cell numbers at this time, although decreases in the long-term haemopoietic stem cell reserve were evident.

Results of the effects of CHB administration on levels of apoptosis (Table 2), as measured by 7-AAD staining, showed significant elevations on days 1, 3, 9 and 16 post-dosing, followed by a return to generally normal levels from day 24 post-dosing until the end of the study. Increases in apoptosis have previously been demonstrated in AA patients by our group, and the increases have correlated with disease severity as defined by transfusion dependence (Philpott et al. 1995, 1996). Levels of CFU-Cs (Table 2) were also affected by CHB administration, with significant decreases at days 1, 3, 9 and 16 post-dosing, followed by a return to overall normal values after day 16. Again, as many authors have shown, stem and progenitor cells at all stages of differentiation are significantly reduced in AA patients (Gibson and Gordon-Smith 1990; Marsh et al. 1990; Novitsky and Jacobs 1991; Maciejewski et al. 1996; Podesta et al. 1998). FL levels in the present study were shown to be statistically significantly high at days 3, 9, 24 and 45 post-dosing (Table 2). Values for FL are high in the serum of AA patients, and increases have been demonstrated to correlate with disease status and response to treatment (Wodnar-Filipowicz et al. 1996); levels were raised before antithymocyte globulin therapy and were reduced after response to such therapy. Furthermore, relapsing patients had increased FL levels.

In conclusion, therefore, the results of the present investigations with repeat doses of CHB in the rat do not confirm the reports of Trainor and Morley (1976), Trainor et al. (1979) and Morley (1980), who produced evidence to show that this drug caused residual bone marrow injury in the mouse that persisted at 2 months post-dosing. Our results demonstrate that CHB in the rat does not cause late-stage bone marrow depression, although there is a persistent significant effect on peripheral blood lymphocytes. However, this experiment, in which CHB induced a mild bone marrow depression in the immediate post-dosing period and a large number of sampling points was used, has defined the pattern of changes in FNCC, CFU-C, FL and apoptosis of nucleated marrow cells in relation to the responses that are seen in the more frequently employed blood parameters that are conventionally used in toxicological studies to assess haemopoietic responses to antineoplastic drugs.

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CASE REPORT

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Diabetes mellitus and fatty liver in a cow: case report

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Abstract A 5-year-old Holstein–Friesian cow was referred to the Veterinary Clinic of Shiraz University in May 2003 with a history of continuous weight and milk loss for 32 days after calving. On clinical examination the animal was moderately depressed. Pulse, respiratory rate and temperature were normal. Rumen motility was decreased in strength and rate. The faeces were dry, firm and scanty. Polyuria and polydypsia were noticed. The results of biochemical analysis revealed a significant rise in urine ketones and glucose. The blood glucose, cholesterol, triglyceride and β-hydroxybutyrate levels were considerably above the normal range. At necropsy the liver was enlarged, pale, and yellow and in the cut section the liver parenchyma had a fatty quality. The gross anatomy of the pancreas and kidneys was normal. On microscopic examination severe fatty change in the liver was observed. Fatty change in the acinar tissue of the pancreas was also seen. The number and size of islets were reduced. On the basis of characteristic staining reaction, depletion of beta cells was diagnosed. According to the history, clinical signs and laboratory findings, a combination of diabetes mellitus and fatty liver was diagnosed.

Introduction

Primary diabetes mellitus is used to describe a relatively specific disorder involving the pancreatic beta cells that results in decreased insulin levels and, therefore, insulin-sensitive hyperglycaemia (Smith 1996). It is quite a rare condition in cattle (Kaneko and Rhode 1964; Mostaghni and Ivoghli 1977). We report a rare case of combined diabetes mellitus and fatty cow syndrome.

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History

A 5-year-old Holstein–Friesian cow was referred to the Veterinary Clinic of Shiraz University in May 2003 with a history of continuous weight loss and decreased milk yield after calving. The animal was dewormed with appropriate anthelmintics. According to the owner, the animal had calved 32 days before she was admitted to the clinic. The animal had been obese during the dry period. However, she had had a poor appetite after calving and progressively lost 150 kg. Furthermore, a field veterinarian had treated the cow for retained placenta 3 days after calving. There was a history of high-level grain feeding prior to calving. Because of some economic problems the diet had been changed after parturition, and the animal had been maintained on a diet of straw, small amounts of alfalfa and concentrates. On clinical examination the animal was moderately depressed. Pulse, respiratory rate and temperature were within the normal range. Rumen motility was decreased in strength and rate. The faeces were dry, firm and scanty. Polyuria and polydypsia were noticed. On percussion of the right side of the animal, the topographic region of liver had increased. The characteristic odour of ketones was detectable on the breath. Chemical analysis of urine and blood and histopathological examination were performed by standard procedures. The serum was analysed for cholesterol by a modified Abell–Kendall/Levey–Brodie (A-K) method (Burtis and Ashwood 1994), triglyceride by the enzymatic procedure of McGowan et al. (1983), glucose by the enzymatic method of glucose oxidase (Burtis and Ashwood 1994) and β-hydroxybutyrate by the Mellanby and Williamson method (Bergemeyes 1974). The results of biochemical analysis on glucose, cholesterol, triglyceride and β-hydroxybutyrateare presented in Table 1.

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ORIGINAL ARTICLE

The haemotoxicity of mitomycin in a repeat dose study in the female CD-1 mouse

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Summary

Mitomycin (MMC), like many antineoplastic drugs, induces a predictable, dose-related, bone marrow depression in man and laboratory animals; this change is generally reversible. However, there is evidence that MMC may also cause a late-stage or residual bone marrow injury. The present study in female CD-1 mice investigated the haematological and bone marrow changes induced by MMC in a repeat dose study lasting 50 days. Control and MMC-treated mice were dosed intraperitoneally on eight occasions over 18 days with vehicle, or MMC at 2.5 mg/kg, autopsied (n = 6–12) at 1, 7, 14, 28, 42 and 50 days after the final dose and haematological changes investigated. Femoral nucleated bone marrow cell counts and levels of apoptosis were also evaluated and clonogenic assays carried out; serum levels of FLT3 ligand (FL) were assessed. At day 1 post-dosing, MMC induced significant reductions in RBC, Hb and haematocrit (HCT) values, and there were decreases in reticulocyte, platelet, and femoral nucleated cell counts (FNCC); neutrophil, lymphocyte and monocyte values were also significantly reduced. On days 7 and 14 post-dosing, all haematological parameters showed evidence of a return towards normal values, but at these times, and at day 28, values for RBC and FNCC remained significantly reduced in comparison with controls. At days 42 and 50 post-dosing, many haematological parameters in MMC-treated mice had returned to control levels; however, there remained evidence of late-stage effects on RBC, Hb and HCT values, and FNCC also continued to be significantly decreased. Results for granulocyte-macrophage colony-forming units and erythroid colonies showed a profound decrease immediately post-dosing, but a return to normal values was evident at day 50. Serum FL concentrations demonstrated very significant increases in the immediate post-dosing period, but a return to normal was seen at

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day 50 post-dosing; a relatively similar pattern was seen in the number of apoptotic femoral marrow nucleated cells. The histopathological examination of kidney tissues from MMC animals at day 42 and 50 post-dosing showed evidence of hydronephrosis with cortical glomerular/tubular atrophy and degeneration. It is therefore concluded that MMC administered on eight occasions over 18 days to female CD-1 mice at 2.5 mg/kg induced profound changes in haematological and bone marrow parameters in the immediate post-dosing period with a return to normal levels at day 50 post-dosing; however, there was evidence of mild but significant late-stage/residual effects on RBC and FNCC, and on cells of the erythroid lineage in the bone marrow.

Keywords
haemotoxicity, mitomycin, mouse, myelotoxicity

Aplastic anaemia (AA) in man has been defined as peripheral blood pancytopenia with hypocellular bone marrow (Young & Maciejewski 2000). As a result, AA is associated with severe anaemia, life-threatening infection and major haemorrhage (Heimpel & Heit 1980; Young & Alter 1994; Jandl 1996). A variety of aetiological agents have been associated with AA, and in particular, radiation, chemicals and drugs, and viral infections (Freedman 2000; Heimpel 2000). Nevertheless, the basic pathophysiology of AA is not clearly understood, and a contributing factor to this is that there are no convenient animal models of the disease. Although the development of rodent models of chronic bone marrow hypoplasia/aplasia has been attempted, none has become widely used to elucidate the pathogenesis of the human disease (Alter *et al.* 1978; Benested 1979; Haak 1980; Appelbaum & Fefer 1981; Vincent 1984, 1986; FAO/WHO 1988; Young & Maciejewski 1997). Nevertheless, of the various investigations to develop rodent models of AA, useful results have been obtained in studies based on methods described by Morley & Blake (1974a). Morley and colleagues reported a model of chronic hypoplastic marrow failure (CHMF) in the Swiss (outbred) mouse or the (inbred) BALB/c mouse treated with the anticancer drug, busulphan (BU) (Morley & Blake 1974a, b; Morley *et al.* 1975).

In both man and laboratory animals, the response of the marrow to many anticancer drugs is the development of bone marrow depression. This depression generally occurs in the immediate post-dosing period, and in many cases the effect may be profound (Reynolds 1989; Dollery 1999; Sweetman 2002; BNF 2004). With most antineoplastic drug treatments, however, the marrow then begins a recovery, with a return towards normal in the following days/weeks. This particular toxicity of many antitumour agents is dose-related, and the response is sometimes referred to as predictable bone marrow depression. However, the response of the bone marrow in man and animals to some anticancer drugs may be different to this generally observed pattern. For example, with some anticancer agents (e.g. melphalan and carmustine), the bone marrow suppression may be delayed (Reynolds 1989; BNF 2004). Alternatively, in the case of BU, although the bone marrow may be depressed and then return towards normal in the post-dosing period, normality may not be achieved, and the marrow may remain, to a degree, depressed. This phenomenon was reported by Morley and his coworkers as occurring in their mouse model of BU-induced CHMF and was described as late-stage/chronic marrow failure; the marrow was reported to show evidence of prolonged/permanent residual injury (Morley & Blake 1974a, b; Morley *et al.* 1975).

After the initial reports by Morley and his coworkers, in which the BALB/c mouse model of CHMF was described (Morley & Blake 1974a, b; Morley *et al.* 1975), the authors went on to investigate various aspects of the pathogenesis of CHMF in their BU-mouse model (Morley *et al.* 1976a, b; Pugsley *et al.* 1978a, b; Gale & Morley 1980; Trainor *et al.* 1980). However, Morley and his colleagues also carried out a related, but separate, series of investigations to elucidate whether a series of antineoplastic drugs, other than BU, also had the capacity to induce late-stage (residual) bone marrow injury in the mouse (Trainor & Morley 1976; Trainor *et al.* 1979; Morley 1980). In these studies, it was reported that mitomycin (MMC), chlorambucil, carmustine and melphalan also caused late-stage effects and residual marrow injury, and the change remained evident at least 2 months after the last dose of the drug had been administered; however, this response was not seen with several other antineoplastic

agents, e.g. cyclophosphamide, methotrexate, vinblastine, fluorouracil, mercaptopurine and adriamycin.

MMC is an antineoplastic cytotoxic antibiotic produced by the actinomycete *Streptomyces caespitosus* (Reynolds 1989). Structurally, MMC is a quinone ring linked to an indole group; there are two side-groups, first a methoxyformamide side chain, and the second is an aziridine ring (Crooke & Bradner 1976). MMC acts as an alkylating agent after metabolic activation, mainly in the liver, and suppresses the synthesis of nucleic acids (Dollery 1999; Sweetman 2002). The drug is a cell cycle non-specific agent, but is most active in the late G_1 and early S phases of the cell cycle. MMC has a wide spectrum of activity and is generally used in combinations with other cytotoxic drugs (5-fluorouracil, adriamycin, CCNU, vindesine, bleomycin, cisplatin and isosfamide). MMC is used in the palliative treatment of solid tumours particularly adenocarcinomas of the pancreas and stomach (Godfrey & Wilbur 1972) but also in the treatment of a large range of other tumours including those of the bladder, colon, rectum, lung, liver, prostate, cervix, breast, skin, oesophagus and eye (Karanes *et al.* 1986; Reynolds 1989; Veeder *et al.* 1992; Hortobagyi 1993; Sweetman 2002). The drug is used also as an intravesical instillation for superficial bladder tumours (BNF 2004). For all other indications, MMC is administered intravenously through a running saline infusion. Generally, 4- to 8-week intervals are used between drug administrations to avoid myelosuppression (BNF 2004).

The main adverse side effect of MMC in man is delayed, cumulative, possibly severe, bone marrow suppression, with a nadir at 4–8 weeks (Zein *et al.* 1986). Often, profound leucopenia and thrombocytopenia occur at about 4 weeks after administration, with recovery at 8–10 weeks. However, in about 25% of patients, blood counts may not recover (Dollery 1999; Sweetman 2002). Other serious toxic effects of MMC in man include renal injury (Ravikumar *et al.* 1984; Valavaara & Nordman 1985; Verwey *et al.* 1987) and pulmonary reactions (interstitial pneumonitis) (Gunstream *et al.* 1983; McCarthy & Staats 1986; Linette *et al.* 1992; Okuno & Frytak 1997; Gagnadoux *et al.* 2002). MMC also induces a potentially fatal syndrome of thrombotic microangiopathy (TMA), which resembles the haemolytic uraemic syndrome (HUS) (Pavy *et al.* 1982; Montes *et al.* 1993; Nishiyama *et al.* 2001; Gundappa *et al.* 2002). Here, symptoms of haemolytic anaemia and renal failure may be seen in conjunction with hypertension and cardiovascular problems, pulmonary oedema and neurological symptoms (Medina *et al.* 2001). The syndrome usually occurs after 6 months of MMC treatment. Hepatic veno-occlusive disease has also been described as an effect of MMC treatment (Lazarus *et al.* 1982; Craft & Pembrey 1987); the condition manifests as hepatomegaly, ascites and liver failure. Other toxicities of MMC include gastrointestinal effects, cardiotoxicity, dermatitis and alopecia (Verweij *et al.* 1987). Local tissue necrosis may also be observed if drug solutions are extravasated from a vein or artery.

Studies on BU-induced chronic bone marrow aplasia in the BALB/c mouse have recently been reported by Gibson *et al.* (2003) and Turton *et al.* (2006), and these authors also described a related study on the haemotoxicity of chlorambucil in the rat (Molyneux *et al.* 2004a). Bearing in mind the observations of Trainor *et al.* (1979) and Morley (1980) on the induction of residual bone marrow injury by the cytotoxic antibiotic MMC, we now wished to investigate the haemotoxicity of this drug in the outbred female CD-1 mouse (derived from the Swiss mouse), and in particular, to determine whether MMC did cause late-stage (residual) effects on the marrow and blood. The opportunity was also taken to carry out assays on serum levels of the cytokine fms-like tyrosine kinase 3 (FLT3) ligand (FL) which has been proposed as a bio-indicator in a range of bone marrow failure conditions in man (Lyman *et al.* 1995; Wodnar-Filipowicz *et al.* 1996; Chklovskaia *et al.* 1999; Blumenthal *et al.* 2000; Pfister *et al.* 2000; Huchet *et al.* 2003) and also laboratory animals (Brasel *et al.* 1996; Gratwohl *et al.* 1998; McKenna *et al.* 2000; Bertho *et al.* 2001). Preliminary (abstract) reports have been published (Molyneux *et al.* 2004b, c).

Materials and methods

Mice

Female outbred CD-1 mice (Charles River UK, Margate, Kent, UK) were caged in groups of three to 10 with free access to diet (Rat and Mouse No. 1, SDS, Witham, Essex, UK) and mains drinking water. A temperature of 19–22 °C was maintained, with a relative humidity of 45–65% and a light : dark cycle of 12 : 12 h (lights on at 07:00 hours). Animals were allowed to acclimatize for at least 7 days before the initiation of each experiment and were observed daily for signs of ill health. Body weights were determined daily, two or three times each week or at appropriate intervals. Where a mouse became ill and it was considered that it would not recover, the animal was killed. All procedures were conducted under local Ethical Committee guidelines and approval for Home Office Project and Personal Licences and followed the UK Home Office (1989) 'Code of Practice for the Housing and Care of Animals used in Scientific Procedures'.

MMC administration

Mitomycin C Kyowa (Kyowa Hakko UK, Slough, Berkshire, UK) was dissolved in de-ionized water and administered by

intraperitoneal injection in a volume of approximately 0.10–0.20 ml/mouse. Control animals were treated with water (vehicle) at the same dose volume. The dose of MMC administered was not adjusted to take account of any body weight changes during the dosing period.

Experimental design

Preliminary dose-ranging studies. Three pilot dose-ranging experiments were carried out to determine the dose level of MMC to administer in the main study. In the first experiment, mice (mean body weight 26.5 g; $n = 3$ per group) were given a single dose of MMC at 0.0, 6.0, 6.5, 7.0 and 7.5 mg/kg and were monitored for 96 days post-dosing. In the second experiment, mice (mean body weight 30.5 g; $n = 3$ per group) were given four doses of MMC at 0.5 mg/kg intervals from 0.0 to 5.5 mg/kg, administered over a period of 8 days, and the animals were studied for 50 days post-dosing. In a third investigation, mice (mean body weight 17.4 g; $n = 3$ per group) were given eight doses of MMC at 0.25 mg/kg intervals from 0.50 to 3.25 mg/kg, administered over a period of 18 days, and the animals were monitored for 72 days post-dosing.

Main study. Three hundred and twenty-six female CD-1 mice (mean body weight 25.8 g) were divided into two groups, control ($n = 158$) and MMC-treated ($n = 168$). Animals were dosed with vehicle or MMC (2.5 mg/kg) on eight occasions over an 18-day period (days 1, 4, 6, 8, 11, 13, 16 and 18). On days 1, 7, 14, 28, 42 and 50 after the final MMC dose, control mice ($n = 8$–12) and MMC-treated animals ($n = 6$–8) were killed by an intraperitoneal injection of pentobarbitone sodium (Sagatal, Rhône Mérieux, Harlow, Essex, UK) for the examination of blood, bone marrow and other organs and tissues. Control ($n = 5$–12) and MMC-treated mice ($n = 5$–8) were also sampled at the same time points to collect blood for serum preparation for the assay of the cytokine FL.

Analysis of blood and marrow suspensions

Blood was removed from the right ventricle following a thoracotomy incision, and a 0.5 ml aliquot anti-coagulated with 1.5 mg/ml dipotassium EDTA (Teklab, Sacriston, Durham, UK) or blood was collected into serum separator tubes (Microtainer; Becton Dickinson, Franklin Lakes, NJ, USA). The contents of the left femur were aspirated into 3 ml Iscove's modified Dulbecco's medium (IMDM; Life Technologies, Paisley, UK) supplemented with 10% foetal calf serum (FCS; PAA Laboratories GmbH, Linz, Austria) to give a marrow cell suspension; a marrow smear was prepared from the contents of the right tibia.

Blood samples and bone marrow suspensions in IMDM were analysed with a Bayer H*1 haematology analyser with mouse-specific software (Bayer Diagnostics UK, Newbury, Berks, UK). Reticulocyte analysis was carried out with a Sysmex R-1000 (Sysmex UK, Milton Keynes, Bucks, UK), with voltage gain adjusted optimally for mouse blood. The femoral marrow cell suspension in IMDM was used to obtain the total nucleated cell count (femoral nucleated cell count; FNCC) using the basophil channel of the H*1. Tibial marrow smears were stained with May-Grünwald–Giemsa and differential counts performed by eye on 200 cells.

Bone marrow clonogenic assays

The right femur was removed with surrounding muscle and placed in 5 ml sterile IMDM with 10% FCS (PAA Laboratories); under sterile conditions, the muscle and epiphyses were removed from the femur and the marrow flushed into 5 ml sterile IMDM supplemented with 10% FCS. Using trypan blue exclusion, the white blood cells (WBC) were counted and cultured at 10^5 WBC in 1 ml IMDM supplemented with 30% FCS, 1% de-ionized bovine serum albumin (Sigma Chemical, Poole, Dorset, UK), 10^{-4} M β-mercaptoethanol (Sigma), 0.05% $NaHCO_3$, 2.1 mM L-glutamine (Sigma) and 0.9% methylcellulose (Stem Cell Technologies, London, UK). Cultures were set up in duplicate in 35 mm dishes (Nunclon, Loughborough, Leicestershire, UK) with the following growth factor stimuli added to each dish: 4 IU human erythropoietin (Janssen-Cilag, High Wycombe, Bucks, UK), 50 ng murine interleukin-3 (1.7×10^5 U/ml; R&D Systems Europe, Abingdon, Berks, UK), 50 ng murine stem cell factor (R&D Systems), 50 ng human interleukin-6 (2×10^6 U/ml; Novartis Pharmaceuticals, Langley, Herts, UK) and 50 ng human granulocyte colony-stimulating factor (10^8 U/ml; Amgen UK, Cambridge, UK). The cultures were incubated at 37 °C in 5% CO_2 in air for 14 days. On day 14, granulocyte-macrophage colony-forming units (CFU-GM), erythroid burst-forming units (BFU-E), and colonies containing both granulocyte-macrophage and erythroid elements (CFU-GEM) were counted. Results are expressed as CFU-GM or the total number of erythroid colonies (BFU-E + CFU-GEM) per femur.

Apoptosis in femoral bone marrow mononuclear cells

Apoptosis in bone marrow cells was assessed using flow cytometry according to the method described by Philpott *et al.* (1995, 1996). Two hundred microlitres (0.5×10^6–1.0×10^6 cells) of the left femoral marrow flush was washed twice in

phosphate-buffered saline (PBS; Sigma) supplemented with 1% FCS and 0.05% Na azide by centrifuging (4 °C, 400 g, 30 min). Excess solution was removed and cells resuspended in 500 or 450 μl of PBS for unstained and stained cells, respectively. 7-amino-actinomycin D (7-AAD; Calbiochem, Nottingham, UK) was dissolved in 1 ml acetone, diluted in PBS (4 ml) to a concentration of 0.2 mg/ml, kept at −20 °C and protected from light until use. Cells were stained with 50 μl of 7-AAD (fluorescent DNA-binding agent to identify live, apoptotic and late-apoptotic/dead cells) for 20 min on ice and protected from light. Cells were pelleted by centrifugation, the supernate removed and the cells resuspended in 500 μl of 2% (w/v) paraformaldehyde solution in PBS. Samples were analysed on a FACScan (Becton Dickinson, Mountain View, CA, USA) within 30 min of fixation. Data on 50,000 cells were acquired and processed using CELL QUEST SOFTWARE™ (Becton Dickinson). Scattergrams of forward scatter (FSC) vs. 7-AAD fluorescence were generated. FSC height threshold was set at 108 to exclude debris and RBC. Regions were drawn around populations showing negative, dim and bright 7-AAD fluorescence, corresponding to live, apoptotic and dead cells, respectively, as verified previously (Molyneux *et al.* 2004a). A region was also drawn around remaining cell debris and RBC to exclude these data.

FL assay

The blood in separator tubes was allowed to stand (75–90 min), centrifuged (400 g; 5 min), the serum harvested and stored at −80 °C. The presence of the cytokine FL in the serum was detected using an ELISA assay with a sensitivity of typically less than 5 pg/ml (R&D Systems) according to the manufacturer's instructions.

Tissues

At autopsy, the spleen, and at days 42 and 50, the liver, kidneys and lungs (inflation fixation) were removed, weighed (except lungs), and placed in 10.5% phosphate-buffered formalin fixative for 21 days; the sternum was also removed and placed in fixative. Sternums were decalcified in 100% formic acid. Tissues were embedded in paraffin, sectioned and stained with haematoxylin and eosin for morphological examination by light microscopy.

Statistical analysis

MMC-treated and control (vehicle-treated) groups were compared using a one-tailed Student's t-test (Microsoft Excel; Microsoft Ltd, Microsoft UK, Reading, UK).

Results

Preliminary dose-ranging studies

Three preliminary dose-ranging pilot studies were carried out to determine a dose level of MMC to use in the main investigation. In the first experiment, lasting 96 days post-dosing, mice (n = 3 per group; mean body weight 26.5 g) were given a single dose of MMC (6.0–7.5 mg/kg); the maximum tolerated dose (MTD) was found to be 7.0 mg/kg, as assessed by body weight changes, clinical evidence of toxicity and mortality (MMC-treated animals showing significant adverse toxic effects were either killed in extremis, or, on occasion were found dead; all such animals are referred to as intercurrent death (ICD) animals). In the second experiment, mice (n = 3 per group; mean body weight 30.5 g) were given four doses of MMC (0.5–5.5 mg/kg) over a period of 8 days, and the animals were monitored for 50 days post-dosing; the MTD was found to be 2.5 mg/kg. In the third experiment, mice (n = 3 per group; mean body weight 17.4 g) were given eight doses of MMC (0.50–3.25 mg/kg) over a period of 18 days, and the animals were studied for 72 days post-dosing; the MTD at day 72 was found to be 2.5 mg/kg. As the main study was scheduled to continue for approximately 6–7 weeks post-dosing, a dose level of 2.5 mg/kg MMC was selected as an appropriate dose level to use.

Main study

Clinical signs and body weight changes. During the 18-day dosing period, animals treated with MMC remained in good health. The mean body weights of mice on the first day of dosing were 27.0 g for control (n = 158) and 24.6 g for MMC-treated (n = 168) groups, respectively. At the end of the dosing period, the mean body weights were 29.1 g (control) and 27.3 g (MMC-treated), giving increases of 7.8 and 11.0% during the dosing period, respectively. In the 50-day post-dosing period, the MMC-treated mice failed to gain weight normally, and the mean body weight at this time was 27.8 g (1.8% increase) compared with 34.0 g (16.8% increase) in the control group.

On day 4 after the final dose of MMC, three mice (out of a total of 160 MMC-treated animals at that time) were found dead; by day 7 post-dosing, a further three MMC-treated animals had been categorized as ICD. The mortality in MMC-treated animals continued, and over the 50 days of the post-dosing period, the final figures for ICD animals were 6, 7, 4, 24, 12, 24 and 1 (for weeks 1–7, respectively), involving a total of 78 animals (46.4%). This continuing mortality was entirely unexpected. Generally, the animals

died suddenly, with no obvious deterioration in their condition or evidence of morbidity; it was therefore, generally, not possible to identify which animals were becoming ill. The body weights of ICD animals were not reduced in comparison with the rest of the MMC-treated group.

Autopsy observations. On days 1 and 7 post-dosing, some animals treated with MMC showed evidence of an accumulation of a small amount of a thin fluid (either milky or clear) in the abdominal cavity. However, at the later stages of the experiment (on days 14, 28, 42 and 50 post-dosing), some MMC-treated mice had swollen abdomens with larger quantities of fluid. On days 28, 42 and 50 post-dosing, some MMC-treated mice were also found to have fluid accumulation in the thoracic cavity. On day 14 post-dosing, the fluid from the abdominal cavities of three MMC-treated mice was taken, and smears were prepared and stained (May-Grünwald–Giemsa). On microscopic assessment, there was a predominance of macrophages with no neutrophils or lymphocytes, and therefore it was considered that there was no clear evidence of a bacterial infection. On days 42 and 50 post-dosing, the kidneys of some MMC-treated mice appeared swollen and pale in colour (see *Histopathological assessment of tissues*).

Haematological results. On day 1 post-dosing (Table 1), MMC induced a profound predictable bone marrow depression. Mean values for RBC, Hb, haematocrit (HCT), reticulocytes and platelets were significantly reduced in MMC-treated mice; the mean percentage reductions for these parameters were to 63.6% (RBC), 62.1% (Hb), 59.4% (HCT), 7.7% (reticulocytes) and 27.9% (Plt) of the control values. Mean cell haemoglobin (MCH) was unaffected in MMC mice; however, MCH concentration (MCHC) was significantly increased and mean cell volume (MCV) significantly reduced. Significant reductions were also seen in mean WBC, neutrophils, lymphocytes and monocytes (reductions to 41.0, 15.1, 50.3 and 2.9% of the mean control values, respectively).

On day 7 post-dosing in MMC-treated animals, mean values for RBC, Hb and HCT remained significantly reduced. However, reticulocytes showed evidence of a recovery and counts were elevated, although not significantly, above the mean control value. MCV, MCH and MCHC were unaffected. Platelets were still reduced, but there was some indication of recovery, with the mean count being 44.0% of control. Lymphocytes in MMC mice were within normal limits on day 7 but neutrophils and monocytes remained significantly reduced, although the counts did show signs of recovery.

On day 14 post-dosing, although mean values for RBC, Hb and HCT remained significantly decreased in MMC-treated animals, a return towards normal was clearly evident in these parameters. A rebound reticulocytosis was also apparent, with the mean reticulocyte count in MMC-treated mice increased to 262% of the control mean value ($P < 0.001$); the values for MCV and MCH were also significantly raised. The platelet count was normal in MMC animals. Neutrophils and monocytes still remained significantly reduced in MMC-treated mice, but lymphocytes were within control ranges.

On day 28 post-dosing, the mean RBC count remained significantly reduced in MMC-treated mice; however, Hb and HCT values had returned to normal. The mean reticulocyte count continued to be significantly increased in MMC-treated mice at this time (144% of control), but the platelet, neutrophil, lymphocyte and monocyte counts were normal.

By days 42 and 50 post-dosing, many of the haematological parameters in MMC-treated mice had returned to control levels. However, a residual (late-stage) effect was apparent in the mean RBC, Hb and HCT values, with all these parameters being significantly reduced both on days 42 and 50 in animals which had been treated with MMC.

Immediately post-dosing (day 1), the mean FNCC of MMC-treated mice was very significantly reduced (to 19.6% of the mean control value; Table 1). However, FNCC in MMC-treated mice appeared to be returning towards normal from day 7 post-dosing onwards, being 49.1% of the mean control value at day 7, 58.9% at day 14 and 72.0% at day 28. However, as with the mean RBC, Hb and HCT values, the mean FNCC appeared to show a residual (late-stage) effect at days 42 and 50 post-dosing, with counts remaining reduced at 65.8 and 75.3% of the mean control values at each time point ($P < 0.001$ and $P < 0.01$), respectively.

Bone marrow clonogenic assay. The effect of MMC on CFU-GM and erythroid colony numbers was highly significant and long-lasting (Table 2). On day 1 post-dosing, the number of CFU-GM colonies/femur in MMC-treated mice was reduced to 6.0% of the control value. The number of CFU-GM remained significantly reduced in MMC-treated mice, being 24.2, 18.1, 46.1 and 53.1% of control values on days 7, 14, 28 and 42 post-dosing, respectively. However, on day 50 post-dosing, the number of CFU-GM had recovered to within normal (control) levels.

A similar profound effect was seen on the number of erythroid colonies/femur in MMC-treated mice. On day 1 post-dosing, the number of erythroid colonies in MMC-treated mice was decreased to 5.2% of the control values. On days 7, 14 and 28, signs of recovery were observed, with colony numbers increased to 37.3, 20.9 and 42.2% of counts in the controls, respectively. However, on days 42 and 50 post-dosing, the number of erythroid colonies from MMC-treated mice were not significantly different from the controls (Table 2).

Table 1 Haematological results from female CD-1 mice treated with eight doses of mitomycin (MMC) over a period of 18 days and sampled at 1–50 days after the final dose

	Days of sampling											
	1		7		14		28		42		50	
	Control	MMC	Control	MMC	Control	MMC	Control	MMC	Control	MMC	Control	MMC
RBC	8.73 (0.51)	5.55 (0.91)***	8.85 (0.39)	5.09 (1.18)***	8.82 (0.43)	6.53 (1.38)***	8.94 (0.57)	7.85 (1.12)*	8.72 (0.41)	7.84 (0.66)**	8.93 (0.33)	8.07 (0.56)***
Hb	14.9 (0.3)	9.3 (1.5)***	14.8 (0.5)	8.7 (2.1)***	14.8 (0.6)	11.7 (2.0)***	14.8 (0.6)	14.3 (1.6)	14.6 (0.6)	13.4 (1.0)**	14.4 (0.8)	13.2 (0.8)**
HCT	47.2 (1.3)	28.0 (4.6)***	46.7 (1.9)	28.0 (7.6)***	46.2 (1.1)	37.7 (5.5)**	45.4 (2.6)	44.3 (4.7)	44.8 (2.1)	40.7 (3.5)**	45.5 (1.5)	42.3 (2.9)**
MCV	54.2 (3.1)	50.5 (1.4)**	52.9 (2.4)	54.6 (3.0)	52.5 (2.0)	59.0 (7.7)*	50.8 (1.7)	56.8 (3.8)***	51.4 (1.8)	52.0 (1.9)	51.0 (1.8)	52.5 (1.7)
MCH	17.1 (0.9)	16.7 (0.6)	16.7 (0.8)	17.2 (0.6)	16.8 (0.8)	18.1 (1.7)**	16.6 (0.8)	18.2 (0.9)***	16.8 (0.7)	17.0 (0.7)	16.2 (0.6)	16.4 (0.7)
MCHC	31.6 (0.7)	33.1 (0.5)***	31.6 (0.7)	31.5 (1.0)	32.0 (0.7)	30.9 (1.2)***	32.7 (0.9)	32.1 (0.9)	32.6 (0.7)	32.8 (0.8)	31.7 (0.9)	31.3 (0.7)
Retic	465 (134)	36 (34)***	348 (103)	558 (357)	245 (101)	642 (260)***	323 (101)	466 (95)**	302 (80)	230 (59)*	332 (85)	287 (91)
Plt	1279 (213)	357 (342)***	1280 (154)	562 (350)***	1336 (158)	857 (357)	1279 (201)	1174 (242)	1335 (291)	1395 (440)	1377 (222)	1299 (210)
WBC	1.95 (0.44)	0.80 (0.33)***	1.60 (0.52)	1.09 (0.22)**	2.16 (0.94)	1.57 (0.59)***	1.72 (0.60)	1.43 (0.35)	2.03 (0.97)	1.53 (0.94)	1.77 (0.83)	2.25 (1.33)
Neut	0.35 (0.10)	0.05 (0.03)***	0.33 (0.14)	0.13 (0.10)**	0.44 (0.14)	0.31 (0.19)**	0.32 (0.12)	0.35 (0.14)	0.34 (0.16)	0.54 (0.75)	0.31 (0.16)	0.79 (1.00)
Lymph	1.45 (0.35)	0.73 (0.32)***	1.19 (0.44)	0.94 (0.25)	1.58 (0.76)	1.21 (0.38)	1.29 (0.50)	1.04 (0.35)	1.62 (0.84)	0.91 (0.46)*	1.29 (0.65)	1.33 (0.58)
Mono	0.04 (0.01)	0.00 (0.00)***	0.03 (0.01)	0.01 (0.01)**	0.03 (0.01)	0.02 (0.01)*	0.02 (0.01)	0.03 (0.02)	0.04 (0.02)	0.04 (0.04)	0.04 (0.02)	0.03 (0.01)
Eo	0.00 (0.00)	0.00 (0.00)	0.03 (0.04)	0.00 (0.00)*	0.08 (0.09)	0.01 (0.01)	0.08 (0.09)	0.01 (0.01)*	0.02 (0.02)	0.03 (0.04)	0.10 (0.17)	0.05 (0.06)
Baso	0.09 (0.08)	0.00 (0.00)**	0.01 (0.01)	0.00 (0.00)**	0.00 (0.00)	0.00 (0.01)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.01)
FNCC	2.49 (0.46)	0.49 (0.24)***	2.41 (0.54)	1.19 (0.54)***	2.23 (0.63)	1.28 (0.76)**	2.64 (0.37)	2.11 (0.56)**	2.72 (0.62)	1.87 (0.36)***	2.69 (0.42)	2.06 (0.30)**
Spleen	4819 (932)	2439 (496)***	4550 (633)	6646 (692)***	4168 (522)	5227 (2322)	4043 (763)	3613 (562)	4435 (904)	3201 (762)**	4197 (1070)	3460 (666)

Abbreviations and units: RBC, red blood cells ($\times 10^6$/μl); Hb, haemoglobin (g/dl); HCT, haematocrit (%); MCV, mean cell volume (fl); MCH, mean cell haemoglobin (pg); MCHC, mean cell haemoglobin concentration (g/dl); Retic, absolute reticulocyte count ($\times 10^3$/μl); Plt, platelets ($\times 10^3$/μl); WBC, white blood cells ($\times 10^3$/μl); Neut, neutrophils ($\times 10^3$/μl); Lymph, lymphocytes ($\times 10^3$/μl); Mono, monocytes ($\times 10^3$/μl); Eo, eosinophils ($\times 10^3$/μl); Baso, basophils ($\times 10^3$/μl); FNCC, femoral bone marrow nucleated cell count ($\times 10^7$); Spleen, relative spleen weight (mg per kg body weight). Values are means, SD in parentheses; $n = 8$ for control and MMC-treated mice at all time points, except at day 50, where $n = 12$ (controls) and 6 (MMC). *P*-values are significantly different from control.

*$P \leq 0.05$.

**$P < 0.01$.

***$P < 0.001$.

Table 2 Results for CFU-GM and erythroid colonies, marrow cell apoptosis and serum FL in female CD-1 mice treated with eight doses of mitomycin (MMC) over a period of 18 days and sampled at 1–50 days after the final dose

	Days of sampling					
	1	7	14	28	42	50
CFU-GM						
Control	4247 (791)	5037 (1946)	7475 (3563)	8462 (1537)	10729 (3907)	9633 (2780)
MMC	255 (303)***	1220 (964)***	1351 (743)**	3905 (2016)**	5692 (2831)*	9702 (5504)
Erythroid colonies						
Control	14485 (6112)	7267 (3362)	12271 (7101)	7903 (3403)	11353 (4279)	3271 (1521)
MMC	752 (839)***	2708 (3176)	2559 (1734)*	3333 (2021)*	6580 (2968)	4779 (4904)
Apoptosis						
Control	10.70 (1.46)	11.49 (1.88)	8.07 (1.10)	11.00 (2.18)	13.76 (4.07)	6.76 (1.70)
MMC	17.04 (1.87)***	15.67 (4.34)*	14.46 (4.48)***	13.35 (2.73)*	14.78 (2.98)	5.06 (0.55)*
FL						
Control	466.7 (50.6)	496.1 (45.0)	476.2 (56.7)	486.5 (93.5)	521.6 (70.9)	551.6 (98.9)
MMC	2428.2 (579.3)***	2902.2 (301.2)***	2065.7 (823.2)***	551.7 (155.1)	439.9 (84.3)*	488.2 (48.5)

Abbreviations and units: CFU-GM, granulocyte-macrophage colony-forming unit (colonies/femur); erythroid colonies (colonies/femur); apoptosis, femoral marrow nucleated cell apoptosis (values expressed as percentages excluding debris); FL, fms-like tyrosine kinase 3 (FLT3) ligand (pg/ml); all other information as Table 1. Values are means, SD in parentheses; numbers of animals per group as Table 1, except for FL where $n = 6$ (controls and MMC), but at day 1 $n = 5$ (controls and MMC), and at day 50 where $n = 12$ (controls) and $n = 8$ (MMC). For the bone marrow clonogenic assay, cultures were set up in duplicate and a mean CFU-GM and erythroid colony number calculated per mouse. *P*-values are significantly different from control.

*$P \leq 0.05$.

**$P < 0.01$.

***$P < 0.001$.

Apoptosis in femoral bone marrow mononuclear cells. On days 1, 7 and 14 post-dosing, apoptosis in femoral marrow nucleated cells in MMC-treated mice was significantly increased to 159, 137 and 179% of control values, respectively (Table 2). This increased level of apoptosis in MMC-treated mice was also evident at day 28 post-dosing, but at this time point the levels appeared to be returning towards control values, and on day 42, apoptosis in MMC animals was not significantly different from the controls. At day 50 post-dosing, in mice treated with MMC, apoptosis appeared to be slightly decreased.

FL assay. The concentration of FL was measured in the serum of mice treated with MMC and in vehicle controls. On days 1, 7 and 14 post-dosing, the level of FL in the serum of MMC-treated mice was considerably increased to 520.3, 584.9 and 433.8% of control values, respectively (Table 2). However, on day 28 post-dosing, the concentration of FL in MMC-treated animals was not significantly different from the controls. On day 42 post-dosing, FL levels were slightly reduced in MMC-treated mice but were normal on day 50.

Bone marrow differential counts. The tibial marrow smears from six control and MMC-treated mice were randomly selected at each time point and differential counts carried out to determine the proportion of cells (as percentage) in the myeloid, erythroid and lymphoid lineages. Taking the FNCC of each animal (Table 1), the absolute number of cells making up each lineage in the femoral marrow sample was then calculated (Table 3). On day 1 post-dosing, both myeloid and erythroid cell lineages were significantly reduced in MMC-treated animals, with counts being 5.2 and 9.5% of the control values, respectively (Table 3). On day 7 post-dosing, the number of myeloid and erythroid cells were also significantly reduced in MMC-treated mice; however, a return towards normal was clearly evident. For cells of the myeloid lineage, in MMC mice, there was a return towards control values at days 14 and 28 post-dosing, which in general terms, remained evident for the remainder of the study. However, in the case of cells of the erythroid line in MMC-treated mice, a return to control values did not occur, and counts remained significantly reduced at all time points after day 7 post-dosing; the reductions were to 57.0, 56.0, 58.6 and 74.5% of the control counts at days 14, 28, 42 and 50 post-dosing, respectively.

Cells of the lymphoid lineage, in MMC-treated mice, appeared to be spared in the immediate post-dosing period

Table 3 Estimated counts ($\times 10^7$) of myeloid, erythroid and lymphoid cells, and the myeloid : erythroid (M : E) ratio in the femoral marrow of control and mitomycin (MMC)-treated mice

	Days of sampling											
	1		7		14		28		42		50	
	Control	MMC	Control	MMC	Control	MMC	Control	MMC	Control	MMC	Control	MMC
Myeloid	1.15 (0.22)	0.06 (0.06)***	1.26 (0.27)	0.55 (0.30)***	1.20 (0.31)	0.88 (0.78)	1.56 (0.30)	1.44 (0.35)	1.40 (0.29)	1.08 (0.29)*	1.35 (0.33)	1.17 (0.37)
Erythroid	0.95 (0.12)	0.09 (0.10)***	1.07 (0.18)	0.49 (0.29)***	1.00 (0.22)	0.57 (0.26)**	1.16 (0.37)	0.65 (0.36)*	1.16 (0.42)	0.68 (0.19)**	0.98 (0.14)	0.73 (0.29)*
Lymphoid	0.36 (0.14)	0.32 (0.16)	0.28 (0.10)	0.30 (0.01)	0.41 (0.13)	0.10 (0.04)***	0.42 (0.18)	0.13 (0.02)**	0.39 (0.15)	0.16 (0.04)**	0.42 (0.27)	0.18 (0.25)
Other	0.10 (0.06)	0.00 (0.00)***	0.12 (0.09)	0.01 (0.01)**	0.08 (0.05)	0.01 (0.02)**	0.05 (0.04)	0.02 (0.02)	0.07 (0.04)	0.01 (0.02)**	0.07 (0.06)	0.01 (0.01)*
M : E ratio	1.21 (0.17)	0.77 (0.30)**	1.18 (0.16)	1.30 (0.91)	1.21 (0.22)	1.38 (0.63)	1.44 (0.44)	2.70 (1.15)*	1.26 (0.23)	1.66 (0.58)	1.37 (0.26)	1.88 (1.03)

Two hundred cells in the tibial marrow smears were differentially counted by eye and the absolute number of cells of each lineage estimated for the nucleated marrow cell count of the femoral marrow flush. Values are means and SD in parenthesis. Cells categorized as 'other' include monocytes and monocyte precursors, mast cells, megakaryocytes, plasma cells and unidentifiable cells. All other information as Tables 1 and 2, $n = 6$ for control and MMC groups at all time points. *P*-values are significantly different from control.

*$P \leq 0.05$.
**$P < 0.01$.
***$P < 0.001$.

(days 1 and 7). However, at all succeeding time points, the cell counts were reduced, and at day 14, 28 and 42 post-dosing, the reductions were statistically significant. Cells categorized as 'other', including monocyte and monocyte precursors, etc., were also, in general, reduced in MMC-treated animals at all time points.

Histopathological assessment of tissues. The sternum and spleens from MMC-treated and control mice were studied histologically at all time points; livers ($n = 12$) and kidneys ($n = 14$) were examined from selected mice at days 42 and 50 post-dosing and lungs ($n = 6$) from mice at the day 50 autopsy. In the sternums at day 1 post-dosing, there was severe depletion of the bone marrow cells in all MMC-treated animals examined (Figure 1a,b). At day 7 post-dosing, a degree of marrow cellularity had been restored in MMC mice, but this was not to the level seen in the control animals. At 14 days post-dosing, slight depletion of the marrow was seen in one of four MMC-treated animals examined. Normal levels of marrow cellularity were evident at days 28, 42 and 50 post-dosing.

The mean relative weight of the spleen (Table 1) was significantly reduced ($P < 0.001$) in MMC-treated mice to 50.6% of the control mean value at day 1 post-dosing. However, at day 7 post-dosing, the mean relative spleen weight was increased ($P < 0.001$) in MMC-treated animals to 146.1% of the mean control value (as 100%). Thereafter, in general, the mean relative weights of the spleens in MMC mice were similar to the control values. Histological study of the spleens from MMC mice, at day 1 post-dosing, showed a reduction in the cellularity of the red pulp (reduced haematopoiesis) in all mice examined. However, at days 7 and 14 post-dosing, moderately increased haematopoiesis was evident in the red pulp of the majority of MMC mice examined. No significant abnormalities were seen in the spleens of MMC-treated mice at day 28, 42 and 50 post-dosing.

At autopsy at day 50 post-dosing, no significant lesions were observed in the lungs of MMC-treated mice.

The mean relative weights of livers from MMC mice were similar to the control means at days 42 and 50 post-dosing; however, the relative weight of the liver of one MMC mouse autopsied on day 50 was 76,786 mg/kg body weight (absolute kidney weight 2.150 g; body weight 28 g), compared with the mean control kidney weight of 51,316 mg/kg body weight (mean absolute kidney weight, 1.691 g; mean body weight 30 g). On histological examination, no significant lesions were observed in the livers of MMC-treated animals sampled at days 42 and 50 post-dosing. However, there was some congestion of the centrilobular sinusoids in a small number of MMC mice at these time points, but this was considered to

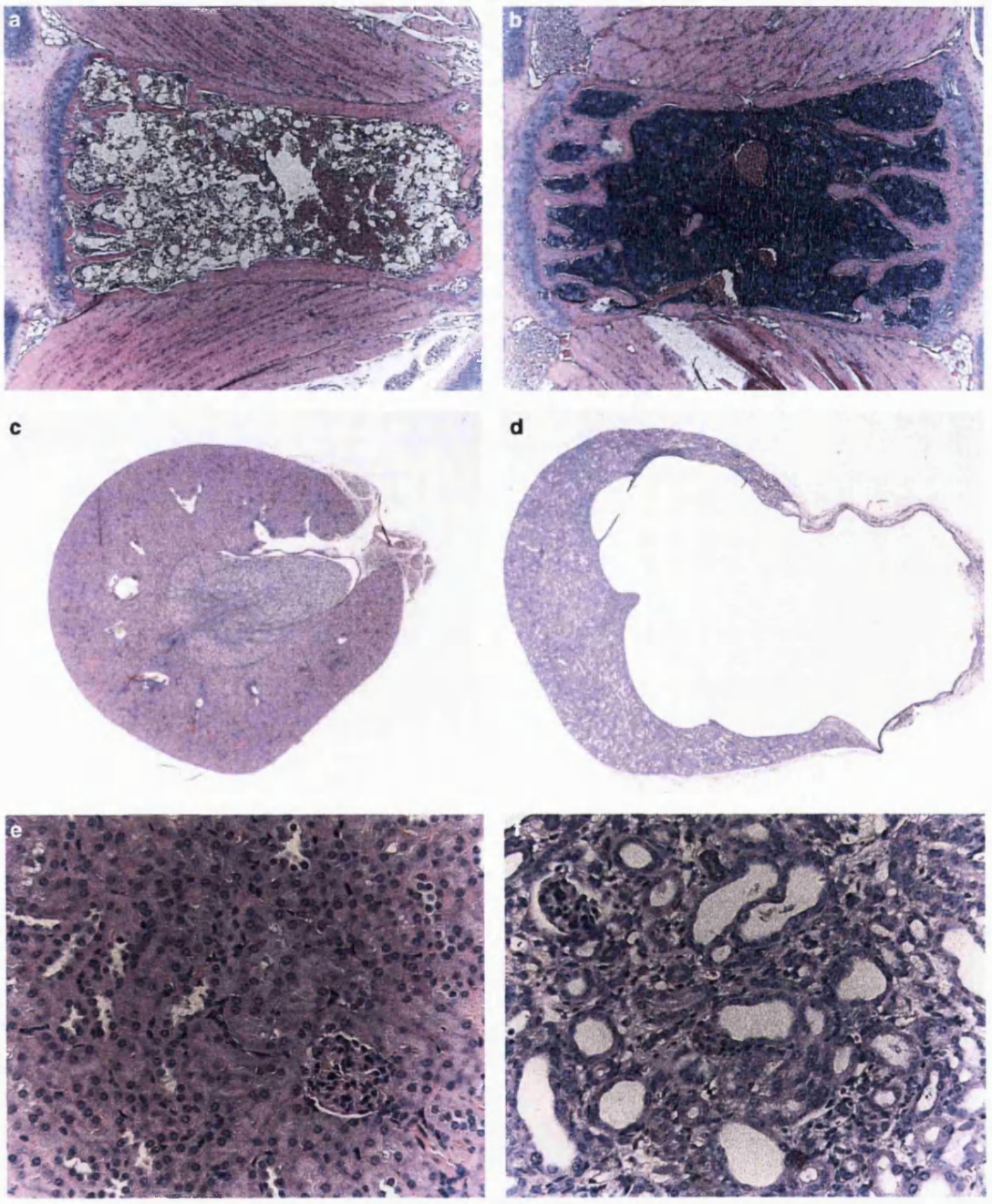

Figure 1 Haematoxylin and eosin-stained sections of the sternum and kidneys from control (vehicle-treated) and mitomycin (MMC)-treated (2.5 mg/kg) mice. (a) Sternum of a mouse, at day 1 after MMC treatment; there is severe depletion of bone marrow cellularity [original magnification (OM) ×40]. (b) Sternum of an MMC-treated mouse at day 14 post-treatment. There is complete recovery of bone marrow cellularity (OM ×40). (c) Kidney from a control (vehicle-treated) mouse, at day 50, showing the normal appearance of cortical and medullary tissues (OM ×10). (d) Kidney from a mouse treated with MMC at 50 days post-treatment; there is severe hydronephrosis with a central cystic space bordered by a thin rim of compressed renal parenchyma (OM ×10). (e) Cortex of the kidney from a control (vehicle-treated) mouse at day 50, showing the normal appearance of tissues (OM ×200). (f) Kidney cortex from a MMC-treated mouse, at day 50 post-dosing. There is cortical atrophy with dilatation of the renal tubules and interstitial fibrosis (OM ×200).

be an agonal effect, or secondary to cardiac changes (the hearts of MMC mice were not examined histologically).

The mean relative weights of the kidneys of MMC-treated mice sampled at day 42 post-dosing were significantly increased (to 145.4%; $P < 0.05$) over the mean control value (as 100%); in two individuals treated with MMC, the increases were 214.8 and 215.3% of the control figure (as 100%). However, at day 50 post-dosing, the mean relative kidney weight of MMC animals was similar to the controls. Histological assessment of the kidney at days 42 and 50 post-dosing demonstrated that lesions were present in the majority of MMC-dosed mice. The lesions (Figure 1c,d) essentially comprised hydronephrosis with cortical glomerular and tubular atrophy and degeneration (Figure 1e,f). The severity of the lesions varied considerably between individual animals and also within the same individual, with unilateral changes being present in a small number of mice. In severe cases, the hydronephrotic kidneys comprised a thin rim of atrophic, basophilic and dilated cortical tubules surrounding a central cystic space.

Discussion

In the present study, with MMC administered to mice on eight occasions at 2.5 mg/kg over 18 days, the drug induced a profound effect on peripheral blood values at day 1 post-dosing (Table 1); erythroid and reticulocyte counts, and neutrophil, lymphocyte and platelet counts, were all reduced. The marrow FNCC was greatly decreased. However, at day 7 post-dosing, there was evidence of a return towards normal values; this was evident in the reticulocyte, neutrophil, lymphocyte and platelet counts and in the FNCC. At 14 days post-dosing, the recovery of these parameters in MMC mice continued; furthermore, RBC values also showed evidence of a return towards normal. At 28 days post-dosing, MMC-treated mice showed a picture which, for many parameters, was relatively normal, but there was evidence of a persisting effect on the RBC and FNCC values, which continued to show reductions. The effect on RBC and FNCC values continued to be seen at days 42 and 50 post-dosing. This persisting and stable late-stage effect at days 28, 42 and 50 in MMC-treated mice gave values for RBC of 87.8, 89.9 and 90.4% of the mean control values and for FNCC of 79.9, 68.8 and 76.6% of control values, respectively; a prolonged, late-stage effect was also evident in the erythroid lineage in the bone marrow smears taken from MMC-treated mice (Table 3).

There appear to be no haematological papers reporting repeat dose administration studies with MMC in the mouse and none where haematological observations have been made at a late-stage post-dosing. Benning *et al.* (1991) administered single intraperitoneal doses of MMC (1.6 mg/kg) to adult CD-1 mice and studied erythropoietic cytotoxicity up to day 7 post-dosing. Kobayashi *et al.* (1981) gave single intraperitoneal doses of MMC at 6.0 mg/kg to male CD2F1 mice and studied bone marrow suppression up to day 7 post-dosing. Matsumoto *et al.* (1984) administered single intraperitoneal doses of MMC at 2.0 mg/kg to male ddY mice and studied marrow cell counts and peripheral blood reticulocytes at a series of time points up to day 11 post-dosing. Bradner (1979) administered MMC at 7.5 mg/kg intraperitoneally to male BDF1 mice and studied changes in peripheral blood WBC up to day 7 post-dosing. However, Futamura & Matsumoto (1995) reported a repeat dose study of MMC in the rat. Seven daily doses of MMC were administered at 1 mg/kg intraperitoneally to male Wistar rats. Blood samples were studied at day 1 post-dosing.

In man, MMC may cause delayed, cumulative bone marrow suppression with a sometimes profound leucopenia and thrombocytopenia (Godfrey & Wilbur 1972; Jones *et al.* 1980; Zein *et al.* 1986; Veeder *et al.* 1992; Hortobagyi 1993; Medina *et al.* 2001). In a review of MMC toxicity, Crooke & Bradner (1976) reported that the most frequently used schedule of MMC administration was 50 μg/kg/day for 6 days and then every other day until toxicity was manifested. Using this regimen, haematological toxicity was delayed, but about 55% of patients experienced toxicity; thrombocytopenia was generally first noted in the fifth or sixth week of treatment, and leucopenia in the sixth or seventh week of the regimen; the duration of the leucopenia was generally 1–2 weeks, and the thrombocytopenia continued for 2–3 weeks. However, after a single MMC dose, the nadir for leucopenia was evident at 3½ weeks, and for thrombocytopenia the nadir was at 4 weeks post-dosing. Crooke & Bradner (1976) also reported that anaemia was commonly seen with all treatment regimens, but this was less severe than the effects on platelets and leucocytes; similar observations were described by Godfrey & Wilbur (1972) and Montes *et al.* (1993).

In the present investigations, preliminary dose-ranging studies identified the intraperitoneal single-dose MTD for female CD-1 mice as 7.0 mg/kg at day 96 post-dosing. With four doses administered over 8 days, the MTD was 2.5 mg/kg at 50 days post-dosing, and with eight doses given over 18 days, the MTD was also 2.5 mg/kg at 72 days post-dosing. These dose levels of MMC in the mouse, in general, find a parallel with the range of dose levels reported in other published papers. Bradner (1979) stated that the single-dose intraperitoneal LD_{50} in male BDF1 mice was 7.5 mg/kg; Yamada (1960) gave the intraperitoneal LD_{50} as 5.2 mg/kg. Philips *et al.* (1960) reported the intraperitoneal single-dose LD_{50} in male Swiss mice as 8.5 mg/kg; when five daily doses were

administered, the LD_{50} was 2.3 mg/kg. In haematological studies, Benning *et al.* (1991) used single intraperitoneal doses of 1.6 mg/kg in CD-1 mice; Kobayashi *et al.* (1981) gave single doses of 6.0 mg/kg intraperitoneally to male CDF1 mice, and Matsumoto *et al.* (1984) administered single intraperitoneal doses of 2.0 mg/kg MMC to male ddY mice.

In carrying out the three preliminary dose-ranging studies with MMC in the present investigations, a previously unseen feature of drug toxicity was noted. In earlier experiments, in the mouse with a range of antineoplastic drugs, and with BU in particular, a pattern of morbidity and mortality, which is known to be related to bone marrow depression, was often evident in the immediate post-dosing period. For example, in a recent BU study involving 100 mice, dosed at a range of dose levels (5.0–12.5 mg/kg), 32 mice were eventually categorized as ICD, and these ICD deaths occurred from day 3 to day 23 post-dosing; the mean ICD day was day 9.8 (SD 4.9). In another recent experiment with BU, 32 mice were treated (2–16 mg/kg), and here 14 mice were eventually categorized as ICD, with the ICD deaths occurring from day 4–24 post-dosing, and the mean ICD day being day 9.2 (SD 5.4). However, in the present investigations with MMC, in the second pilot dose-ranging study, 33 mice were dosed on four occasions (0.5–5.5 mg/kg MMC), and 13 animals were categorized as ICD with mortality occurring between days 1–8 post-dosing. However, an unusual pattern of toxicity was then seen, in that there were a further four ICD animals, at a later stage, with mortality occurring on days 26, 35, 42 and 50 post-dosing. Similarly, in the first MMC dose-ranging study, with 12 mice dosed on one occasion (6.0–7.5 mg/kg), there was a total of three ICD animals, these again occurring at a later stage, on days 47, 83 and 87 post-dosing. This pattern of late-stage mortality seen with MMC had not been evident previously in other investigations with several antineoplastic drugs.

It is often considered that where morbidity/mortality is seen in the immediate post-dosing period in mice treated with anticancer drugs, these effects may be related to bone marrow depression. This is summarized in Table 4, where peripheral blood data are presented for four individual mice in the present MMC main study, which were autopsied on day 1 post-dosing. Platelet, neutrophil, reticulocyte and bone marrow counts are seen to be extremely low. However, although in the present MMC main study, a total of 17 mice were categorized as ICD from days 1–21 post-dosing, there were a further 61 animals classified as ICD at a later stage, i.e. days 22–49 post-dosing.

The three MMC pilot dose-ranging studies were carried out to identify an appropriate dose level of the drug to use in the main study, which would cause no mortality. Therefore, the high level of mortality that did occur (46.4%) was unexpected. The dose level of MMC used in the main study (2.5 mg/kg) was based mainly on the results of the third pilot dose-ranging study where mice were dosed on eight occasions over 18 days at 0.5–3.25 mg/kg MMC. The mice in the third pilot study had an average body weight of 17.4 g,

Table 4 Haematological results from four individual female CD-1 mice treated with eight doses of mitomycin (MMC) over a period of 18 days and sampled at 1 day after the final dose

	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Control
RBC	4.5	5.0	5.4	5.6	8.73 (0.51)
Hb	7.7	8.0	8.7	9.6	14.9 (0.3)
HCT	22.9	24.3	26.5	29.0	47.2 (1.3)
MCV	51.1	48.5	49.2	52.0	54.2 (3.1)
MCH	17.2	16.0	16.2	17.2	17.1 (0.9)
MCHC	33.7	32.9	32.9	33.1	31.6 (0.7)
Retic	7	9	18	13	465 (134)
Plt	86	69	82	93	1279 (213)
WBC	1.05	0.45	0.99	0.61	1.95 (0.44)
Neut	0.02	0.02	0.05	0.06	0.35 (0.10)
Lymph	1.01	0.42	0.94	0.54	1.45 (0.35)
Mono	0.00	0.00	0.00	0.00	0.04 (0.01)
FNCC	0.35	0.28	0.40	0.28	2.49 (0.46)

Abbreviations and units: RBC, red blood cells ($\times 10^6/\mu l$); Hb, haemoglobin (g/dl); HCT, haematocrit (%); MCV, mean cell volume (fl); MCH, mean cell haemoglobin (pg); MCHC, mean cell haemoglobin concentration (g/dl); Retic, absolute reticulocyte count ($\times 10^3/\mu l$); Plt, platelets ($\times 10^3/\mu l$); WBC, white blood cells ($\times 10^3/\mu l$); Neut, neutrophils ($\times 10^3/\mu l$); Lymph, lymphocytes ($\times 10^3/\mu l$); Mono, monocytes ($\times 10^3/\mu l$); FNCC, femoral bone marrow nucleated cell count ($\times 10^7$). Mean (SD) results from eight control mice sampled at day 1 post-dosing are included for comparison. A total of eight MMC-treated mice were autopsied at day 1 post-dosing. All other information as Table 1.

whereas mice in the first and second pilot studies weighed 26.5 and 30.5 g, respectively. In all MMC experiments, the dose of drug administered was not changed during the period of dosing to take the account of any changes in body weight. In the third pilot study, the mean body weights of mice were 17.4 g on day 1 of dosing, 20.8 g on day 9 and 24.2 g on day 18. Calculations therefore show that the level of MMC dosed was 2.50 mg/kg on day 1 of dosing, 2.09 mg/kg on day 9 and 1.79 mg/kg on day 18. In the case of the main MMC study, mice were an average of 25.2 g on day 1 of dosing, 26.3 g on day 9 and 27.3 g on day 18. The calculated dose levels of MMC administered on these days are therefore 2.50, 2.43 and 2.34 mg/kg, respectively. It is concluded therefore that these discrepancies in dose levels may account for high mortality in the main MMC study. Clearly, in retrospect, mice of the same body weight should have been used in the pilot and main studies.

A pronounced effect of MMC on the sternal bone marrow was evident in the histological examination of tissues from MMC mice. There was severe depletion of marrow cells in the immediate post-dosing period (Figure 1a,b). A relatively rapid return to normal cellularity was then observed at days 14 and 28 post-dosing. A similar pattern of changes has been seen in earlier studies with the antineoplastic drug BU when repeatedly administered to mice (Gibson *et al.* 2003). Similarly, the histological changes evident in the spleen in the immediate post-dosing period, in MMC-treated animals, compare with BU-induced spleen changes in the mouse (Gibson *et al.* 2003). Significant reductions in spleen size were also reported by Sokoloff *et al.* (1959) in the MMC-treated mouse, rat and chick. Histological examination of the lungs and livers from MMC-dosed mice at days 42 and 50 did not reveal any significant drug-related changes. However, in the case of the kidneys from MMC-treated mice examined at late time points (days 42 and 50 post-dosing), hydronephrosis with cortical glomerular and tubular atrophy and degeneration was observed (Figure 1c,d,e,f).

There are several reports of MMC-induced renal changes in laboratory animals. MMC caused hydronephrosis in mice repeatedly dosed with the drug (Matsuyama *et al.* 1964a, b), and it was suggested that these lesions made the MMC-dosed mouse a suitable model of drug-induced hydronephrosis. Philips *et al.* (1960) reported MMC-induced renal changes (necrotizing nephrosis) in the rhesus monkey, and effects on the kidney were also described by Verweij *et al.* (1988) in the rat. Bregman *et al.* (1987, 1989) studied the toxicity of MMC derivatives in the rat and reported that several compounds caused renal tubular degeneration and glomerulonephropathy. Cattell (1985) described a surgical study in the rat to determine whether MMC induced direct renal injury; the left kidney was perfused with MMC and tissues examined at a range of time points after perfusion. It was stated that lesions indistinguishable from human HUS developed, with glomerular endothelial damage, platelet accumulation and capillary wall splitting which was typical of microangiopathy; this surgical procedure in the rat was proposed as a new model of HUS. The technique of Cattell (1985) was also investigated by Blanco *et al.* (1992). These authors describe glomerular endothelial cell injury in the renal cortex with obliteration of the glomerular capillary lumen and cortical tubular necrosis.

There are many reports giving details of the nephrotoxicity of MMC in man. In a review of the literature by Crooke & Bradner (1976), cases of delayed nephrotoxicity and renal failure were described which occurred several months after the beginning of therapy. Fields & Lindley (1989) discussed the syndrome of MMC-associated TMA where there was endothelial cell damage with resulting thrombus formation, RBC fragmentation and haemolysis. The term TMA encompasses both thrombotic thrombocytopenia purpura (TPP) and HUS; both syndromes involve microangiopathic haemolytic anaemia (MAHA), thrombocytopenia and renal failure; platelet thrombi in the microvasculature are seen in both TPP and HUS, but in HUS, the thrombi are primarily confined to the kidney. Similarly, several authors report cases of MMC-induced HUS/MAHA with nephrotoxic changes involving renal insufficiency, renal dysfunction or renal failure (Pavy *et al.* 1982; Ravikumar *et al.* 1984; Valavaara & Nordman 1985; Montes *et al.* 1993; Medina *et al.* 2001; Nishiyama *et al.* 2001; Gundappa *et al.* 2002).

The kidney lesion induced in the present study, following the administration of repeated doses of MMC, evident at day 50 post-dosing (Figure 1d,f), and comprising hydronephrosis with cortical glomerular/tubular atrophy and degeneration, may be considered as an end-stage lesion. However, the characteristics of the earlier stages in the pathogenesis of this lesion are not known. Nevertheless, it is thought to be possible that the present end-stage lesion may have developed via pathological changes similar to those described by Cattell (1985) and Blanco *et al.* (1992), induced by MMC infusion of the rat kidney and described as being indistinguishable from human HUS. Experiments to investigate in detail the sequence of changes in the pathogenesis of MMC-induced renal disease, in the female CD-1 mouse, are at present being planned.

In conclusion, one of the main purposes in carrying out the present investigations was to determine if MMC caused late-stage/residual effects in the bone marrow and blood, as described by Trainor *et al.* (1979) and Morley (1980), and whether the drug could therefore be considered as a replacement for BU in the development of new mouse models of

late-stage bone marrow aplasia. We conclude that although there was some evidence of late-stage effects on blood erythrocytes, marrow FNCC and cells of the erythroid lineage, the changes induced were relatively mild, and MMC is therefore not seen as a useful substitute for BU in models of chronic bone marrow aplasia in the mouse.

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ORIGINAL ARTICLE

Further development of a model of chronic bone marrow aplasia in the busulphan-treated mouse

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Summary

Aplastic anaemia (AA) in man is an often fatal disease characterized by pancytopenia of the peripheral blood and aplasia of the bone marrow. AA is a toxic effect of many drugs and chemicals (e.g. chloramphenicol, azathioprine, phenylbutazone, gold salts, penicillamine and benzene). However, there are no widely used or convenient animal models of drug-induced AA. Recently, we reported a new model of chronic bone marrow aplasia (CBMA = AA) in the busulphan (BU)-treated mouse: eight doses of BU (10.50 mg/kg) were administered to female BALB/c mice over a period of 23 days; CBMA was evident at day 91/112 post-dosing with significantly reduced erythrocytes, platelets, leucocytes and nucleated bone marrow cell counts. However, mortality was high (49.3%). We have now carried out a study to modify the BU-dosing regime to induce CBMA without high mortality, and investigated the patterns of cellular responses in the blood and marrow in the post-dosing period. Mice (n = 64/65) were dosed 10 times with BU at 0 (vehicle control), 8.25, 9.0 and 9.75 mg/kg over 21 days and autopsied at day 1, 23, 42, 71, 84, 106 and 127 post-dosing (n = 7–15); blood and marrow samples were examined. BU induced a predictable bone marrow depression at day 1 post-dosing; at day 23/42 post-dosing, parameters were returning towards normal during a period of recovery. At day 71, 84, 106 and 127 post-dosing, a stabilized, late-stage, nondose-related CBMA was evident in BU-treated mice, with decreased erythrocytes, platelets and marrow cell counts, and increased MCV. At day 127 post-dosing, five BU-treated mice showed evidence of lymphoma. In this study, mortality was low, ranging from 3.1% (8.25 mg/kg BU) to 12.3% (9.75 mg/kg BU). It is concluded that BU at 9.0 mg/kg (or 9.25 mg/kg) is an appropriate dose level to

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administer (10 times over 21 days) to induce CBMA at approximately day 50–120 post-dosing.

Keywords
aplastic anaemia, busulphan, chronic bone marrow aplasia, haematology, mouse, toxicity

Aplastic anaemia (AA) is a failure of haemopoiesis resulting from injury to haemopoietic stem cells; AA involves all the bone marrow-derived cell lineages, myeloid, erythroid and platelets (Young & Alter 1994; Jandl 1996). AA is characterized by a hypocellular marrow and peripheral blood pancytopenia; the condition carries a risk of life-threatening infection, severe anaemia and major haemorrhage (Jandl 1996; Young & Maciejewski 2000). In European countries, the incidence of AA is about 1.5–2.3 per million of the population per annum (Gordon-Smith & Issaragrissil 1992; Heimpel 2000; Young & Maciejewski 2000). In nonsevere AA, the rate of recovery is about 50%; in severe AA, the chances of spontaneous recovery are approximately 10%. However, in the recovered patient, the condition may be associated with later complications of relapse and clonal evolution to myelodysplasia, acute myeloid leukaemia and paroxysmal nocturnal haemoglobinuria (Jandl 1996; Tooze *et al.* 1999). Once the patient with AA has been stabilized with blood, platelet transfusions and antibiotics, the current mainstays of treatment are bone marrow transplantation and immunosuppression with antilymphocyte (antithymocyte) globulin with or without cyclosporin (Young & Alter 1994; Young 1995).

Although AA in man may be associated with several rare congenital or inherited conditions (Alter & Young 1998; Freedman 2000), the majority of cases are acquired (Heimpel & Heit 1980; Heimpel 2000). The principal causes of acquired AA are considered to be radiation, infectious agents, and drugs and chemicals (Alter *et al.* 1978; Young 1995; Young & Maciejewski 2000). Nevertheless, in as many as 70% of patients, no identifiable causal link can be determined (idiopathic AA) (Young & Maciejewski 1997). Apart from the predictable, dose-dependent bone marrow suppression that occurs following anticancer chemotherapy (Young & Alter 1994), and which is normally reversible (Reynolds 1989; Dollery 1999; Sweetman 2002), acquired AA is idiosyncratic in that it occurs unpredictably in a small proportion of the population exposed to a particular drug, chemical or other agent. This idiosyncratic nature of acquired AA may indicate an underlying genetic predisposition (Marsh *et al.* 1999) or evidence of a pre-existing bone marrow susceptibility resulting from an earlier marrow insult or defect.

AA is also seen in some animal species (e.g. cat, dog, bovine, sheep, pig, ferret, horse and chicken), where the aetiology is generally considered to be infectious agents, drugs, toxins and radiation, or the disease may have no known cause (idiopathic AA) (Weiss 2000). However, there are examples of experimentally induced AA in several animal species. Here, the agents used include radiation (Klassen *et al.* 1972; Speck & Kissling 1973; Knospe 1988), drugs (Morley & Blake 1974a; Krishna *et al.* 1981; Den Ottolander *et al.* 1982), chemicals (Moeschilin & Speck 1967; Lock *et al.* 1996), hormones (oestrogens) (Hart 1985; Sherrill & Gorman 1985; Hart 1990), antibodies (Nettlelship 1942; Knospe *et al.* 1983, 1994; Wolk *et al.* 1998) and infective agents (Haak 1980; Camitta *et al.* 1982; Binder *et al.* 1998). The animal species involved include the mouse, rat, rabbit, ferret, dog and cow. Although the fundamental pathophysiology of AA in man is not well understood, it is generally considered that none of the above experimental models is convenient to use (Alter *et al.* 1978; Haak 1980; Vincent 1984), and none has become widely employed in attempts to elucidate the pathogenesis of the human disease (Benestad 1979; Vincent 1986; Young & Maciejewski 1997). Similarly, no experimental models are currently used in preclinical toxicity studies in the pharmaceutical industry to assess the potential of new drugs to induce AA (see Young & Keisu 1996; Macharia *et al.* 1999a).

However, Morley and colleagues (Morley & Blake 1974a, 1974b; Morley *et al.* 1975) described a mouse model of busulphan (BU)-induced chronic hypoplastic marrow failure which showed late-stage marrow aplasia/hypoplasia, and the animals demonstrated evidence of 'residual' bone marrow injury. In this model, BU was administered to Swiss or BALB/c female mice on four occasions at 14-day intervals (i.e. over a 6-week period) at 20, 20, 20 and 10 mg/kg; the animals were then studied over the following 300–400 days. We have investigated these reports (Andrews *et al.* 1993, 1997, 1998; Andrews 2000) using modifications of the original protocols but have been unable to obtain results which compare closely with those of Morley and Blake (1974a). Accordingly, various regimens of BU dosing were re-examined in an attempt to produce an easily used mouse model of

drug-induced AA, showing features of the human condition, and which developed within a relatively short period (about 120 days). These investigations, to induce chronic bone marrow aplasia (CBMA) in the mouse, have been reported (Diamanti *et al.* 1999; Macharia *et al.* 1999a, 1999b; Gibson *et al.* 2003). In essence, female BALB/c mice were treated with BU on eight occasions at 10.50 mg/kg over a 23-day period, and the animals were autopsied on five occasions over the following 112 days (days 1, 19, 41, 91 and 112 post-dosing). At 91/112 days post-dosing, mice showed evidence of CBMA with significantly reduced RBC, leucocytes, platelets and marrow-nucleated cell counts; MCV was increased. However, mortality in BU-treated mice was high (49.3%). Therefore, there was a need to modify the basic model further and define a more appropriate BU dosing regimen which would not cause high mortality. We also wished to examine the BU-induced changes in blood and marrow at a greater number of autopsy time points, as this would allow a clearer definition of the patterns of change in the various cellular responses during the post-dosing period. We now report a study to investigate these factors. A brief preliminary report has been published in abstract form (Sones *et al.* 2000).

Materials and methods

Animals

Female weanling BALB/c mice (A. Tuck and Son Ltd, Battlesbridge, Essex, UK) were caged in groups of 12–15, bedded on wood shavings, with diet (Rat and Mouse no. 1, SDS Ltd, Witham, Essex, UK) and mains drinking water provided *ad libitum*. A light : dark cycle of 12 : 12 h was maintained (lights on at 07.00 hours), with a temperature of 19–22 °C and a relative humidity of 45–65%. Animals were acclimatized for 10 days before the start of the experiment and were observed daily for evidence of ill health. Body weights were recorded twice weekly or at appropriate times. All procedures were conducted under local Ethical Committee guidelines and approval for Home Office Project and Personal licences, and followed the UK Home Office (1989) Code of Practice.

Administration of bulsulphan

Busulphan (Sigma Chemical, Poole, Dorset, UK) was dissolved in acetone at a concentration of 5–8 mg/ml following the technique of Gibson *et al.* (2003). Immediately prior to dosing, deionized water was added to the BU–acetone solution at a volume of approximately 5 ml of water to 1 ml of acetone solution. The solution was administered by intraperitoneal injection at a dose volume of 0.1–0.2 ml per mouse. Control animals were given acetone : water (vehicle) at the same dose volume and by the same route. The dose of drug administered during the 21-day dosing period was not corrected for changes in body weight.

Tissue sampling

Animals were killed by injection of pentobarbitone sodium (Sagatal, Rhône Mérieux, Harlow, Essex, UK). Blood was removed from the right ventricle following a thoracotomy incision. Blood (0.5 ml) was anticoagulated with 1.5 mg/ml dipotassium EDTA (Teklab Ltd, Sacriston, Durham, UK); the marrow contents of the right femur were aspirated into 1.0 ml of phosphate-buffered saline (PBS) to prepare a cell suspension for the measurement of the femoral marrow nucleated cell count (FNCC). A marrow smear was made from the contents of the right tibia (Smith *et al.* 1994). Here, the bone is exposed and the proximal epiphysis removed. Using a fine camel hair paint brush moistened in PBS, the brush is touched on the exposed marrow surface and the cells streaked as a series of parallel lines onto a glass slide. The preparation is left to air dry for 24 h, fixed in 100% methyl alcohol for 1 h and air dried prior to staining. For the measurement of levels of apoptosis in femoral marrow nucleated cells, the left femur was removed and the marrow contents flushed into 1 ml of Iscove's modified Dulbecco's medium (IMDM; Life Technologies, Paisley, UK) containing 10% foetal calf serum (FCS; PAA Laboratories GmbH, Linz, Austria) and supplemented with 100 IU/ml of penicillin–streptomycin; samples were stored on ice. The spleen and sternum were removed and placed in 10.5% phosphate-buffered formalin fixative (14 days fixation); the thymus gland was removed from selected animals (five mice with gross evidence of lymphoma at autopsy and three control mice at 127 days post-dosing) and processed in a similar way. In the case of two of five BU-treated mice with gross changes at autopsy (day 127) which indicated the pressure of lymphoma, blood smears were prepared from the peripheral blood sample, and spleen impressions (touch preparations; imprints) were made from a transverse cut surface of the organ.

Analysis of blood, marrow suspensions and tissue samples

Blood was analysed using a Bayer H*I haematology analyser with mouse-specific software (Bayer Diagnostics UK, Newbury, Berks, UK). Reticulocyte analysis was performed with a Sysmex R-1000 (Sysmex UK, Milton Keynes, Bucks, UK). The FNCC of the marrow cell suspension in PBS was obtained from the basophil channel of the H*I analyser.

Marrow and blood smears, and spleen impressions, were stained with May–Grünwald–Giesma. After fixation, the spleen was weighed and the weight expressed as absolute and relative weight (i.e. in relation to body weight). Sections of spleen, thymus and sternum from selected animals were prepared and stained with haematoxylin and eosin for histological assessment. The sternum was decalcified in buffered formic acid (Kristenson's solution) for 10 days at room temperature prior to staining (Bancroft & Stevens 1990).

Apoptosis in marrow mononuclear cells

Marrow samples in IMDM were analysed for apoptosis as described previously (Philpott *et al.* 1995a, 1995b). Diluted marrow was centrifuged on Ficoll-Hypaque (Amersham Pharmacia Biotech, St Albans, Herts, UK) at 400 g for 25 min at room temperature to obtain mononuclear cells (MNCs), which were washed twice in PBS supplemented with 1% foetal calf serum and 0.05% azide. Cell number and viability were assessed after trypan blue staining; bone marrow cell suspension in IMDM (10 µl) was mixed with 10 µl of trypan blue solution (0.4%; Sigma) and immediately loaded onto a haemocytometer slide for assessment of cell number and viability. MNCs (1×10^6) were incubated with 7-amino-actinomycin D (7-AAD; Philpott *et al.* 1995a, 1995b). 7-AAD (Calbiochem–Novabiochem, Nottingham, UK) was dissolved in acetone and diluted in PBS to a concentration of 200 µg/ml. This was kept at -20 °C and protected from light. 50 µl of 7-AAD solution was added to 10^6 cells, suspended in 1.0 mL PBS and mixed by vortexing. The cells were stained for 20 min at 4 °C, protected from light and pelleted by centrifugation. The supernatant was removed and cells resuspended in 500 µl of 2% paraformaldehyde solution (Sigma). Unstained cells were negative controls. Samples were analysed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) within 30 min of fixation; control unstained cells were used to adjust the FACScan settings to measure specific 7-AAD fluorescence in positively stained samples. Data on 20,000 cells was acquired and processed using Lysys II software (Becton Dickinson). Regions were drawn around clear-cut populations having negative (R1), dim (R2), and bright (R3) 7-AAD fluorescence, corresponding to live, apoptotic and dead cells, respectively. The proportion of cells within each region was calculated.

Statistical analysis

Treated and control groups were routinely compared using Student's *t*-test for unpaired samples using Microsoft Excel (Microsoft Corporation, Microsoft UK, Reading, Berks, UK). For data on mortality and the incidence of lymphoma, results were analysed using a single-tailed Fisher's exact test. Data on blood parameters from individual animals were analysed (ANOVA) using linear regression (least-squares) and tested using Pearson's correlation and t approximation (Microsoft Excel and Analyse-it Software Ltd, Leeds, Yorkshire, UK).

Experimental design

Two hundred and fifty-nine female BALB/c mice, mean body weight 15.0 g, were divided into four groups: group 1 (vehicle control), $n = 65$; group 2 (BU, 8.25 mg/kg), $n = 64$; group 3 (BU, 9.0 mg/kg), $n = 65$; group 4 (BU, 9.75 mg/kg), $n = 65$. Mice were dosed with vehicle or BU on 10 occasions over a period of 21 days (days 1, 3, 6, 8, 10, 13, 15, 17, 19 and 21). At 1, 23, 42, 71, 84, 106 and 127 days after the final BU dose, mice from each group ($n = 7$–15) were autopsied for blood and marrow examination.

Results

Clinical signs, mortality and body weight changes

In control (vehicle-dosed) and BU-treated (at all three dose levels) mice, there was no evidence of drug-induced toxicity during the 21-day dosing period. In the immediate post-dosing period (days 1–20 post-dosing), there was some evidence of a transient slight loss of condition in some BU animals (the fur becoming dull and dry), but such mice rapidly regained their normal appearance. However, over the whole post-dosing period (day 1–127), a small number of BU-treated animals showed an unexpected and rapid loss of condition. Such mice were killed when it was considered that they would not recover or, on occasion, some animals were found dead (Table 1). These mice, over a period of days, showed a rapid deterioration in the condition of the fur, a reduction in activity and responses, and an abnormal gait and hunched posture; often the ears, paws and tails of these individuals lost the normal pink colouration and became white. A total of 13 (of 194, i.e. 6.7%) mice were affected, two at 8.25 mg/kgBU, three at 9.0 mg/kg and eight at 9.75 mg/kg, giving some evidence of a dose-related effect. Mice appeared to become ill during two periods of the study, with a total of six animals affected from day 5–36 and seven animals from day 83–121 post-dosing.

One mouse treated with BU at 9.0 mg/kg was found at autopsy (day 127 post-dosing) to have a cataract in the left eye.

Table 1 Unexpected mortality[†] in female BALB/c mice treated with busulphan (BU) at 8.25, 9.0 and 9.75 mg/kg on 10 occasions over a period of 21 days[‡] and studied for 127 days after the final BU dose

BU group (mg/kg)	Number of mice in group[§]	Number of mortalities (%)[¶]
Control	65	0 (0)
8.25	64	2 (3.1)
9.0	65	3 (4.6)
9.75	65	8 (12.3)**

**$P < 0.01$ in comparison with controls; single-tailed Fisher's exact test.
[†]Mice either became ill and were killed when it was considered that they would not recover or, on occasion, animals were found dead.
[‡]There were no mortalities during the dosing period.
[§]Number of mice in each group at the beginning of the dosing period.
[¶]Days of mortalities: 8.25 mg/kg BU, days 12 and 113 post-dosing; 9.0 mg/kg, days 85, 93 and 121 post-dosing; 9.75 mg/kg, days 5, 13 and 19 (two mice), 36, 83, 93 and 98 post-dosing.

All animals were weighed on 13 occasions throughout the study (on days 2, 7, 14 and 21 of the dosing period and on days 6, 16, 22, 41, 64, 70, 83, 105 and 125 of the post-dosing period). The mean weight of all animals at the beginning of the dosing period was 15.0 g. During the 21-day dosing period, and during the period from days 1–125 post-dosing, the overall mean body weight increases were 15.3 and 37.9% for the control group; 8.5 and 30.9% for the 8.25 mg/kg BU group; 5.8 and 33.6% for the 9.0 mg/kg group; and 7.6 and 33.5% for the 9.75 mg/kg group. Therefore, mean body weight increases were not related to the dose levels of BU administered. However, from days 64–105 post-dosing (involving four weighing points), the mean body weights of all BU-treated groups were significantly reduced in comparison with the mean weight of the control animals.

Haematology findings

Results are presented in Table 2. At day 1 post-dosing, BU at all dose levels caused a predictable bone marrow depression, but there was no clear evidence that the degree of change in the parameters affected was related to the BU dose levels. In general, there were BU-induced decreases in RBC and Hb and increases in MCV and MCH. The WBCs were reduced in BU-treated mice, as were individual leucocytes; platelet counts were reduced at all BU dose levels; the FNCC and relative spleen weights were decreased.

Three animals treated with BU and sampled at day 1 post-dosing out of a total of 21 BU-treated mice autopsied at this time point had erythrocyte counts below 6.0×10^6 µl (control mean 10.77×10^6 µl). Two of these three mice with significant bone marrow depression also showed very low platelet and WBC counts and high MCV values in the peripheral blood, and the bone marrow FNCC counts were also significantly reduced; the individual results for these two mice are shown (Table 3A).

At 23 and 42 days post-dosing, the general pattern of change in BU mice was of a 'period of recovery' with a return of parameters towards normal (Table 2). However, at these time points, the values for RBC, platelets and FNCC in BU mice were still lower than in the controls, and MCV/MCH values were still raised. Nevertheless, the mean relative spleen weight was similar to the controls at day 23 post-dosing; however, in the case of two mice treated at 9.0 mg/kg BU, and 1 mouse at 9.75 mg/kg, the relative spleen weights were 194, 328 and 197% of the mean control value (control = 100%), respectively. At day 42 post-dosing, the mean relative spleen weight was reduced at all BU dose levels.

At the later stages of the study (days 71, 84, 106 and 127 post-dosing), the overall pattern of response in BU mice was of nondose-related significant reductions in RBC, platelets, FNCC and relative spleen weight, and elevated MCV/MCH values (Table 2).

To investigate the changing patterns of cell responses, in time, where parameters were affected by BU administration (i.e. RBC, platelets, FNCC, MCV/MCH and spleen weight), results from individual animals were analysed at each BU dose level and the data expressed as percentage increase/decrease, in relation to the mean control value at each of the seven autopsy time points (data not shown). However, no consistent dose-related effects of BU on these affected parameters could be identified. Therefore, data for the three BU dose levels were pooled at each autopsy time point. Results are illustrated in Figure 1a–d for RBC, MCV, platelets and FNCC, respectively. It is seen that the general pattern of response consisted of an initial significant decrease (increase for MCV) at day 1 post-dosing, followed by a period of recovery with parameters returning towards normal, and a final late, stabilized, plateau phase (days 71, 84, 106 and 127) with mean values consistently decreased (increased for MCV). The average percentage reduction for RBC over the four late-stage autopsy points was to 95.7% of the control; for platelets the figure was 67.4%; for FNCC, 73.1%, and for relative spleen weight, 90.4%. The figure was 103.3% of control for MCV. From an examination of Figure 1a–d, it is considered that values for RBC, platelets, FNCC and MCV would have entered the late-stage, stabilized plateau phase at about day 50 post-dosing.

An analysis was carried out to examine the relationships between the various parameters affected by BU dosing in

Table 2 Haematological results from female BALB/c mice treated with busulphan (BU) at 8.25, 9.0 and 9.75 mg/kg on 10 occasions over a period of 21 days and sampled at days 1, 23, 42, 71, 84, 106 and 127 after the final dose

Autopsy (days post-dosing)	BU group (mg/kg)	RBC	Hb	MCV	MCH	Retic	WBC	Neut	Lymph	Mono	Plt	FNCC	Spleen	*n*
1	Control	10.77	16.0	46.6	14.8	257.7	2.7	0.54	1.99	0.07	811	12.96	5548	9
	8.25	10.06**	15.4	48.3	15.3	237.9	2.0*	0.15***	1.76	0.02***	138***	6.20***	4766**	7
	9.0	6.97**	10.8*	51.3*	15.8*	158.9**	1.0***	0.09***	0.87**	0.01***	107***	3.57***	4798*	7
	9.75	8.22**	13.0*	50.1*	16.0***	244.1	1.4***	0.11***	1.27**	0.02***	135***	4.93***	4461*	7
23	Control	10.66	15.9	48.6	14.9	307.1	1.7	0.34	1.41	0.04	881	10.11	5044	7
	8.25	10.17*	16.6**	53.5***	16.4***	282.5	1.5	0.32	1.05*	0.04	474***	6.19***	4445	7
	9.0	8.98***	14.4*	52.8	16.1	407.1	1.6	0.59	0.95*	0.02	430**	4.94**	7293	7
	9.75	9.45**	14.9**	52.3*	15.9*	251.4	2.2	0.83*	1.25	0.04	629*	6.76*	5871	7
42	Control	10.25	15.6	49.8	15.3	224.4	1.9	0.40	1.24	0.06	797	10.79	5195	7
	8.25	9.76*	15.3	51.2*	15.8**	285.8*	1.3*	0.25**	0.97*	0.04	599***	8.83*	4484*	7
	9.0	9.44***	15.3	51.7**	16.2**	266.6	0.9***	0.22***	0.61***	0.02*	610***	8.40*	4225*	7
	9.75	10.01	15.7	50.7*	15.7**	230.1	0.9***	0.21**	0.63***	0.03	645***	9.11	4558*	7
71	Control	10.55	15.5	47.4	14.7	309.6	1.7	0.29	1.20	0.05	934	11.94	4521	7
	8.25	10.40	15.8	49.1**	15.2*	257.0*	1.5	0.31	1.08	0.04	568**	10.19*	4012*	7
	9.0	9.77***	14.8**	48.6*	15.1*	287.0	1.2	0.24	0.82	0.03	589**	8.66**	4217	7
	9.75	10.05**	15.4	48.8*	15.3**	222.1**	1.2*	0.23	0.84*	0.03	553***	9.30***	4328	7
84	Control	10.31	15.3	47.7	14.8	270.7	1.9	0.40	1.27	0.08	932	15.24	4491	8
	8.25	10.21	15.4	49.0***	15.1***	267.8	1.7	0.34	1.18	0.04**	655***	11.49*	3896***	8
	9.0	9.99*	15.2	50.2***	15.2***	282.2	1.2*	0.35	0.77*	0.03**	691***	10.49*	3977**	8
	9.75	9.73*	14.9	50.5**	15.4*	271.6	1.4	0.32	0.92	0.05	566***	11.29	4368	8
106	Control	10.39	15.7	47.9	15.1	250.7	1.4	0.29	0.93	0.05	935	13.13	4376	10
	8.25	10.28	15.8	48.5*	15.4**	271.2	2.4**	0.52**	1.59**	0.07	731***	9.26***	3614***	10
	9.0	9.31**	14.7*	50.7**	15.7*	286.5	1.0	0.26	0.62*	0.03	598***	8.45***	4250	10
	9.75	9.94**	15.5	48.9*	15.7***	238.1	1.2	0.34	0.74	0.04	643***	9.70*	3981*	10
127	Control	10.24	15.5	48.4	15.1	240.7	1.4	0.31	0.99	0.05	938	13.81	4086	15
	8.25	9.91*	15.2	49.0	15.4	272.4*	1.5	0.39	0.96	0.05	658***	10.36***	3695*	8
	9.0	9.77***	15.3	50.1***	15.6***	239.6	1.4	0.43*	0.90	0.05	699***	9.39***	3520***	15
	9.75	9.85*	15.3	49.3	15.3*	239.7	1.3	0.38	0.76	0.04*	599***	9.65***	3607**	11

*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.
Values are means; *P*-values are significantly different to control animals.
Abbreviations and units: RBC, red blood cells ($\times 10^6$/μl); Hb, haemoglobin (g/dl); MCV, mean cell volume (fl); MCH, mean cell haemoglobin (pg); Retic, absolute reticulocyte count ($\times 10^3$/μl); WBC, white blood cells ($\times 10^3$/μl); Neut, neutrophils ($\times 10^3$/μl); Lymph, lymphocytes ($\times 10^3$/μl); Mono, monocytes ($\times 10^3$/μl); Plt, platelets ($\times 10^3$/μl); FNCC, femoral nucleated cell count ($\times 10^6$); Spleen, relative spleen weight (mg/kg body weight); *n*, number of mice per group. At day 127, lymphoma-bearing mice ($n = 5$) were not included in the analysis of the 8.25 and 9.0 mg/kg BU group results; blood samples from two mice sampled at day 127 (8.25 mg/kg BU) could not be analysed due to the presence of clots.

Table 3 (A) Individual haematology results from two mice (Mouse 1 and Mouse 2) treated with 10 doses of busulphan (BU) at 9.0 mg/kg and autopsied at day 1 after the final BU dose†, and (B) from two mice (Mouse 3 and Mouse 4) treated with BU at 9.75 and 9.0 mg/kg and autopsied on days 84 and 106 post-dosing, respectively‡

	A (Day 1)				B (Day 84/106)			
	Control	BU	Mouse 1	Mouse 2	Control	BU	Mouse 3	Mouse 4
RBC	10.77	6.97*	2.15	2.83	10.39	9.31**	7.92	6.33
Hb	16.0	10.8*	3.8	4.6	15.7	14.7*	13.2	10.9
HCT	0.511	0.345*	0.133	0.150	0.497	0.469*	0.437	0.367
MCV	46.6	51.3*	62.0	52.9	47.9	50.7**	55.2	58.0
MCH	14.8	15.8*	17.7	16.3	15.1	15.7*	16.7	17.3
MCHC	31.3	30.9	28.5	30.8	31.6	31.0*	30.2	29.8
Retic	257.7	158.9**	156.0	93.7	250.7	286.5	349.4	476.5
Plt	811	107***	112	50	935	598***	101	25
WBC	2.7	1.0***	0.2	0.2	1.4	1.0	0.5	0.3
Neut	0.54	0.09***	0.02	0.01	0.29	0.26	0.04	0.04
Lymph	1.99	0.87**	0.22	0.14	0.93	0.62*	0.44	0.26
Mono	0.07	0.01***	0.00	0.00	0.05	0.03	0.01	0.00
Eo	0.06	0.00***	0.00	0.00	0.11	0.09	0.01	0.00
Baso	0.01	0.00**	0.00	0.00	0.00	0.00	0.00	0.00
FNCC	12.96	3.57***	0.7	0.9	13.13	8.45***	2.0	1.2
Spleen	5,548	4,798*	5,917	5,071	4,376	4,250	5,714	5,095

*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.
†Group mean results at day 1 post-dosing for control mice, and for animals treated with BU at 9.0 mg/kg, are included for comparison.
‡Group mean results at day 106 post-dosing for control mice, and for animals treated with BU at 9.0 mg/kg, are included for comparison.
Abbreviations and units: Baso, basophils ($\times 10^3/\mu l$); Eo, eosinophils ($\times 10^3/\mu l$); HCT, haematocrit (l/l); MCHC, mean cell haemoglobin concentration (g/dl). All other information as Table 2.

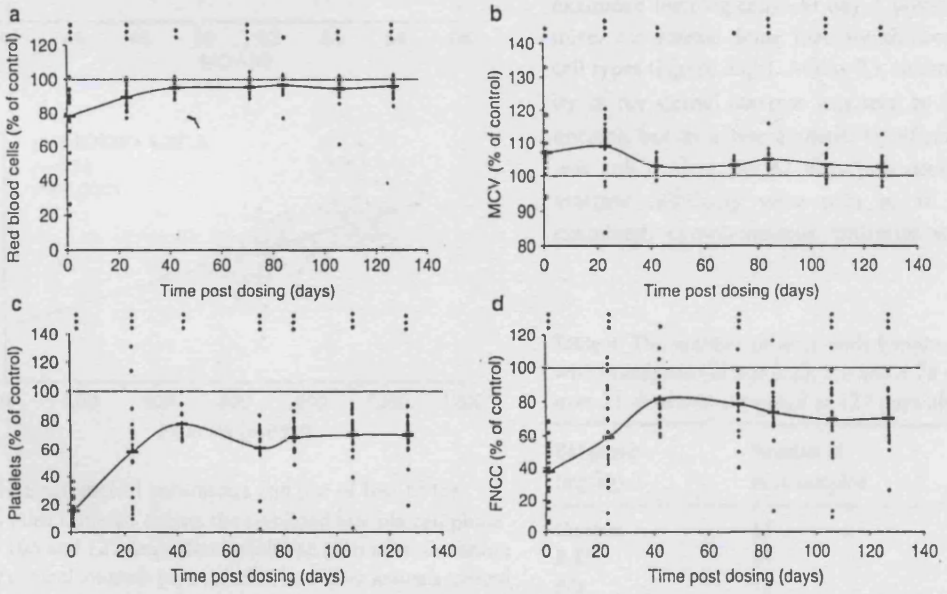

Figure 1 Haematological parameters of individual mice, expressed as a percentage of the mean control value at each time point at 1, 23, 42, 71, 84, 106 and 127 days after busulphan (BU) dosing; data from animals treated with BU at 8.25, 9.0 and 9.75 mg/kg are included. (a) Red blood cells; (b) mean cell volume; (c) platelets and (d) femoral marrow nucleated cell count. Horizontal bars indicate group mean values of pooled data; *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ are presented vertically above the data points.

individual animals at days 71, 84, 106 and 127 post-dosing. In individual BU-treated mice with reduced RBC, there was evidence of a concomitant increase in MCV and a corresponding reduction in platelets and FNCC. Furthermore, the degree of the reduction in RBC, in individual animals, was related to the magnitude of the change in the other affected parameters. Two examples of these data are given (Figure 2a,b), for RBC : MCV and platelets : FNCC.

Two mice out of a total of 109 treated with BU and examined at days 71, 84, 106 and 127, had particularly low RBC, platelet, and FNCC values, and high MCV values; the reticulocyte counts and spleen weights of these two mice were slightly increased. The individual results for these two animals are presented in Table 3B.

At the autopsy at day 127 post-dosing, five BU-treated mice were found to have enlarged spleens (weight increases of up to sixfold over the control mean spleen weight) and enlarged thymus glands. The thymus glands from these animals, and from control mice, were taken for histological examination. The haematological investigation of these mice revealed the presence of lymphoma cells in the blood and marrow (Tables 4 and 5). Although the spleens of the five BU-treated mice were enlarged, the peripheral blood picture of each animal was relatively normal, except for some variability (compared to the control animals) in the reticulocyte, platelet, WBC, neutrophil and lymphocyte counts. However, the LUC counts in the peripheral blood showed an increase in each animal due to (it is assumed) the presence of circulating lymphoma cells. It is considered that these results suggest that the development of the lymphoma in each case was at a relatively early stage. No evidence of these various changes which may be associated with lymphoma had been seen in animals before the autopsy at day 127 post-dosing. A final diagnosis of lymphoma was made for the five BU-treated mice from the above evidence, in conjunction with information from the histology of spleen, thymus and sternal marrow and the marrow smears; in the case of two of the five animals, the appearance of the spleen impressions (touch preparations; imprints) and the blood smears was also helpful.

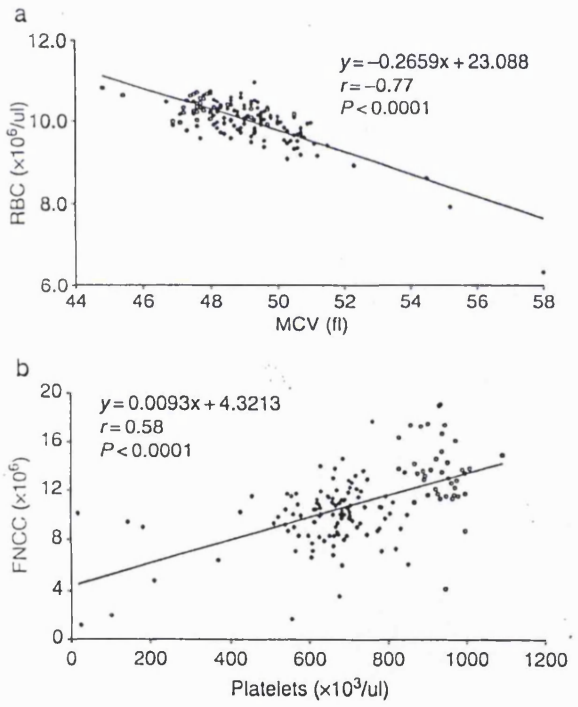

Figure 2 Haematological parameters and line of best fit for individual mice sampled during the stabilized late plateau phase at 71, 84, 106 and 127 days after busulphan (BU) administration; results for control animals (open circles) and from animals treated with BU at 8.25, 9.0 and 9.75 mg/kg (solid diamonds) are included. (a) Relationship between red blood cell count and mean cell volume; (b) relationship between femoral marrow nucleated cell count and platelet count.

Histological examination of tissues and marrow smears

Sections of sternum, spleen and thymus, and marrow smears from selected animals (control and BU-treated) were examined histologically. At day 1 post-dosing, in BU-treated mice, the sternal bone marrow showed a depletion of all cell types (Figure 3a,b). At day 23, in most cases, the cellularity of the sternal marrow was seen to be returning towards normal, but in a few animals, significant marrow depletion was still evident. At 42 days post-dosing, normal levels of marrow cellularity were seen in all BU-treated animals examined. Lymphomatous infiltrates were apparent in the

Table 4 The number of mice with lymphoma in animals treated with busulphan (BU) at 8.25, 9.0 and 9.75 mg/kg on 10 occasions over 21 days and autopsied at 127 days after the final dose†

BU group (mg/kg)	Number of mice sampled	Number of mice with lymphoma (%)
Control	15	0 (0)
8.25	14	4 (28.6)*
9.0	16	1 (6.3)
9.75	11	0 (0)

*$P < 0.05$ in comparison with controls; single-tailed Fisher's exact test.
†No mice showed evidence of lymphoma before day 127.

Table 5 Haematology results from five female BALB/c mice treated with 10 doses of busulphan (BU) over a period of 21 days and showing evidence of lymphoma at autopsy (day 127 post-dosing)*

	Control mice (SD)†	Mice with lymphoma‡				
		1	2	3	4	5
RBC	10.24 (0.25)	12.03	10.39	10.50	9.84	11.28
Hb	15.5 (0.4)	16.8	15.6	16.1	15.0	16.0
HCT	0.496 (0.013)	0.562	0.494	0.503	0.469	0.560
MCV	48.4 (1.3)	46.7	47.5	47.9	47.7	49.7
MCH	15.1 (0.4)	14.0	15.0	15.3	15.3	15.0
MCHC	31.3 (0.7)	29.9	31.7	32.0	32.0	30.2
Retic	240.7 (42.0)	436.4	82.1	158.0	–§	221.1
Plt	938 (64)	549	70	418	509	384
WBC	1.4 (0.4)	11.1	3.7	3.9	3.5	22.8
Neut	0.31 (0.09)	1.27	2.07	2.02	1.05	13.79
Lymph	0.99 (0.32)	6.15	1.24	1.70	1.87	6.62
Mono	0.05 (0.02)	0.25	0.18	0.07	0.21	0.78
Eo	0.07 (0.04)	0.06	0.00	0.01	0.17	0.08
Baso	0.00 (0.01)	–¶	0.04	0.02	0.04	–¶
LUC	0.01 (0.01)	3.38**	0.21**	0.11**	0.20**	0.95**
FNCC	13.81 (1.98)	3.2	8.9	8.1	9.4	9.0
Spleen	4,086 (426)	19,222	26,176	12,800	26,783	17,684

*The initial diagnosis of lymphoma was on the basis of the presence of an enlarged spleen and thymus, and significant numbers of lymphoma cells in the blood, marrow or spleen. No mice with lymphoma were identified before day 127 post-dosing.

†Mean (SD) results from 15 control mice autopsied at day 127 are included for comparison. All other information as Tables 2 and 3, except: LUC, large unstained cells ($\times 10^3/\mu l$).

‡A total of 15 control mice were sampled at day 127, 14 mice at 8.25 mg/kg BU, 16 mice at 9.0 mg/kg BU, and 11 mice at 9.75 mg/kg BU. Mice 1, 2, 3 and 4 were given BU at 8.25 mg/kg and mouse 5 at 9.0 mg/kg. All other information as Table 4.

§Inadequate sample volume for analysis.

¶Basophil counts are not presented due to interference from lymphoma cells.

**LUC cells in lymphoma-bearing mice include lymphoma cells and atypical lymphocytes.

sternal marrow of several BU-treated animals at day 127 post-dosing.

In the spleen, in all BU-treated animals at day 1 post-dosing, normal (i.e. minimal) levels of haemopoiesis were present. However, levels of haemopoiesis were significantly increased (i.e. to marked levels) at day 23; at day 42 post-dosing, the levels had decreased considerably (i.e. to moderate levels). At day 127, levels of splenic haemopoiesis in some BU-treated mice were normal (i.e. at a minimal level, as specified above), but in other cases haemopoiesis had not returned to the base line control levels.

Lymphoma was observed in the thymus gland and spleen of several BU-treated mice at day 127 post-dosing, and in some cases the tumour had extended into the sternal marrow and adjacent soft tissues (trachea, thoracic muscle and heart; Figure 3c,d). The tumours were of a lymphocytic type (i.e. lymphocytic lymphoma) in that they comprised lymphocytes of a well-differentiated appearance.

Examination of the tibial marrow smears from mice at day 127 post-dosing demonstrated that morphologically the lymphoma cell was a large oval cell with intense cytoplasmic basophilia, a high nuclear : cytoplasmic ratio, a relatively immature nucleus with nucleoli present and occasional cytoplastic vacuolation (Figure 3e,f).

A unilateral cataract was present in the eye of a mouse autopsied at day 127 after BU treatment.

Marrow mononuclear cell apoptosis

Studies on the levels of apoptosis in the nucleated cells of the femoral marrow were carried out in control mice and animals treated with BU at 8.25, 9.0 and 9.75 mg/kg on days 106 and 127 post-dosing. However, no significant or consistent patterns of change in the levels of apoptosis could be identified in BU-dosed animals.

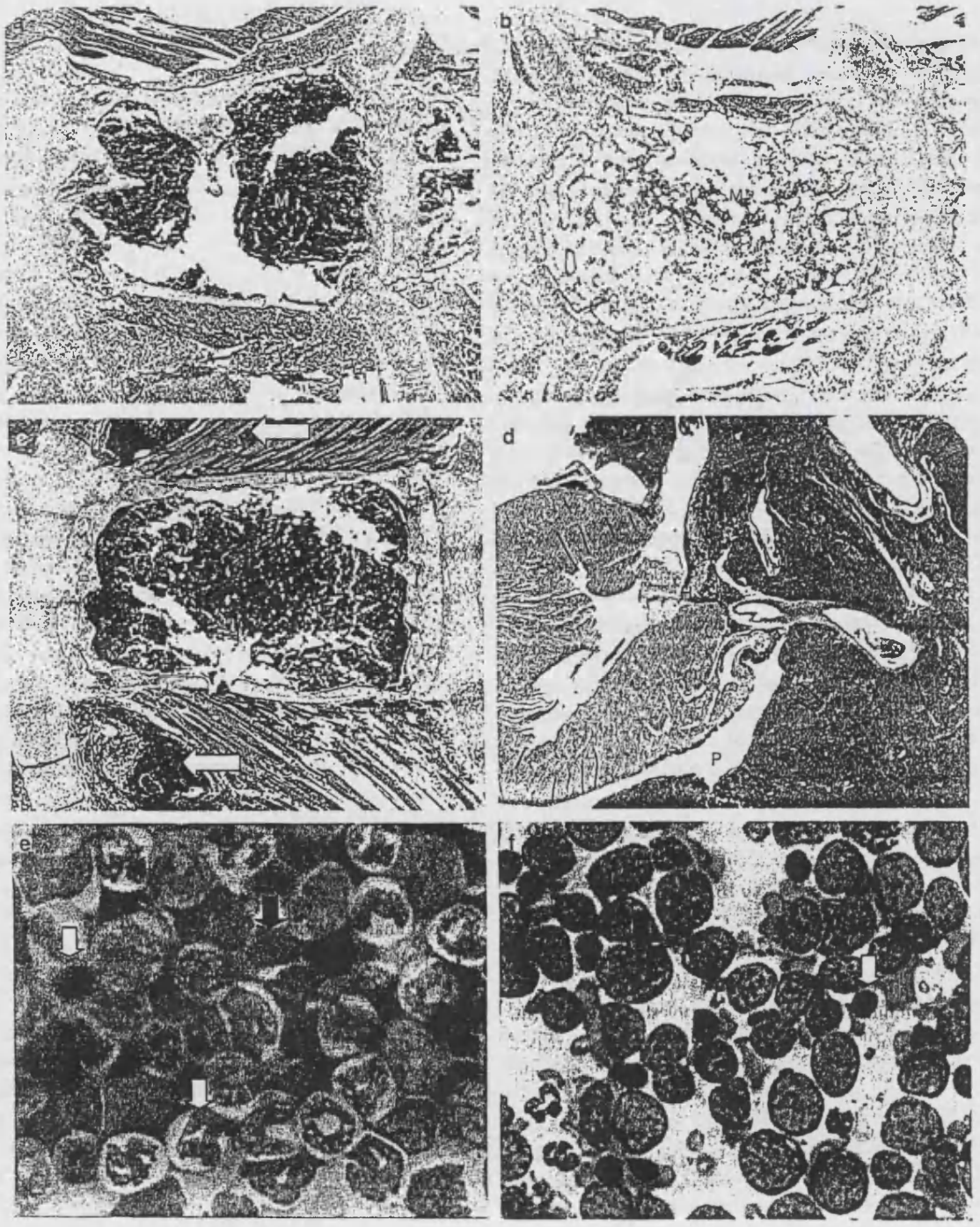

Figure 3 Haematoxylin and eosin-stained sections of mouse tissues (a–d), and May–Grünwald–Giemsa-stained tibial bone marrow smears (e, f), from control (vehicle-treated) and BU-treated animals. (a) Control mouse sternum at day 1 post-dosing showing (M) normal marrow cellularity [original magnification (OM) ×40]. (b) BU-treated (9.0 mg/kg) mouse at day 1 post-dosing illustrating (M) marked hypocellularity of the sternal bone marrow (OM ×40). (c) BU-treated (8.25 mg/kg) mouse at day 127 post-dosing; the thoracic muscle fibres are infiltrated with lymphoma cells (white arrows) (OM ×40). (d) BU-treated (8.25 mg/kg) mouse at 127 days post-dosing; lymphoma cells originating in the thymus are invading the base of the heart (H) and pericardial sac (P) (OM ×10). (e) Bone marrow smear from a control mouse at 127 days post-dosing illustrating the normal distribution of myeloid (white arrow), erythroid (yellow arrow) and lymphoid (black arrow) elements (OM ×1000). (f) Marrow smear from a BU-treated mouse (9.0 mg/kg) at day 127 post-dosing to show numerous lymphoma cells (black arrow); some normal lymphoid elements (white arrow) are also present (OM ×1000).

Discussion

In a recent report, we described a new model of CBMA in the BU-treated BALB/c mouse (Gibson *et al.* 2003). In this model, BU was administered on eight occasions over a 23-day period at 10.50 mg/kg (and 5.25 mg/kg), and the animals were autopsied at 1, 19, 41, 91 and 112 days after dosing. BU induced a predictable bone marrow depression at day 1 post-dosing, with a return of values towards normal at days 19 and 41; however, at 91 and 112 days post-dosing there was evidence of a late-stage bone marrow depression with reduced peripheral blood RBC, platelets, individual leucocytes and decreased marrow nucleated cell counts. Nevertheless, BU administration at 10.50 mg/kg caused high mortality (49.3%). The main objectives of the present investigation were therefore, first, to identify a dose level of BU that could be used to induce CBMA but which did not cause significant mortality, and second, to define clearly the patterns of response (in time) of the various parameters affected by BU administration. The results presented here demonstrate that the administration of BU on 10 occasions over a 21-day period at a dose level of 9.0 mg/kg induced only a low mortality (4.6%). Also, the patterns of response for parameters affected by BU administration (RBC, platelets and FNCC) consisted of an initial predictable depression (day 1 post-dosing), followed by a period of recovery lasting until approximately day 50 post-dosing; a late-stage, stabilized, plateau phase was then evident, with depressed RBC, platelets and FNCC counts; this 'plateau phase' period was apparent from about day 50 post-dosing until the end of the study. Such changes in RBC, platelets and marrow cell counts, in conjunction with an increased MCV, are important and characteristic features of AA in man.

In the present study, levels of mortality appeared to be related to the three dose levels of BU administered (Table 1). A comparable dose-related effect was found in the earlier investigation (Gibson *et al.* 2003), where mortality at 5.25 mg/kg BU was 0%, and at 10.50 mg/kg BU, 49.3%. Morley and Blake (1974a) also reported significant mortality in their repeat-dose BU-treated mouse study; 494 animals began BU treatment, 472 (95.5%) survived BU dosing, and at day 60 post-dosing, 325 (65.8%) were alive.

Table 2 demonstrated that BU administered at 8.25, 9.0 and 9.75 mg/kg did not cause consistent dose-related haematological changes at day 1 post-dosing, or during the late-stage plateau phase (days 71, 84, 106 and 127 post-dosing). This contrasts with the earlier investigation (Gibson *et al.* 2003) where clear dose-related effects were seen in many haematological parameters with BU administered at 5.25 and 10.50 mg/kg.

In the present study, during the late-stage plateau phase, no reductions in WBC or individual leucocytes (neutrophils, lymphocytes and monocytes) were evident. However, in the earlier investigation (Gibson *et al.* 2003), WBC, neutrophils, lymphocytes and monocytes were significantly reduced at day 91/112 post-dosing in animals treated at 10.50 mg/kg BU, but not at 5.25 mg/kg. It is considered possible therefore that the lack of an effect on leucocytes in the present study may be related to the lower dose levels of BU used (i.e. being below 10.50 mg/kg). Similarly, the lack of an effect of BU on levels of apoptosis in marrow nucleated cells at day 106/127 in the present study contrasts with the findings in the earlier investigation (Gibson *et al.* 2003), where apoptosis was significantly increased at day 91/112 post-dosing. Again, the absence of a change in levels of marrow cell apoptosis may related to the lower levels of BU used in the present study.

In the report of Morley and Blake (1974a) where a mouse model of BU-induced chronic hypoplastic marrow failure is described, animals at day 60 post-dosing were reported as entering a latent phase during which they exhibited only minor haematological changes in comparison with the control animals. However, Morley and Blake (1974a) stated that between day 60 and day 313 post-dosing, a small number of individual latent mice developed a significant bone marrow aplasia with body weight loss, pallor and loss of condition, and a marked reduction in all peripheral blood cells and marrow counts; such animals were referred to as 'aplastic' mice. Comparing the observations of Morley and Blake (1974a, 1974b) and Morley *et al.* (1975) with the results of the present study, mice sampled at days 71, 84, 106 and 127 post-dosing may equate to latent phase mice. However, two mice out of a total of 109 autopsied at these last four time points, showed more significant bone marrow depression (Table 3B). As the neutrophil and platelet counts of these individual mice were particularly low, it is possible that a more severe aplasia was developing, and these animals may therefore compare with (Morley & Blake 1974a) aplastic animals.

At the autopsy at day 127, of a total of 41 BU-treated animals sampled, five mice (12.2%) were identified with evidence of lymphoma (Table 4 and 5). These animals showed features of an enlarged thymus and splenomegaly, infiltration of the sternal marrow with lymphoma cells and the presence of lymphoma cells in the thymus gland, spleen, tibial marrow smear and peripheral blood. There was no evidence that the incidence of animals with lymphoma was related to the dose level of BU administered (Table 4).

Morley & Blake (1974a) described the presence of lymphoma in 15 of 494 (3.0%) BU-treated Swiss mice given four doses of the drug at 14-day intervals; in the investigation of Gibson *et al.* (2003), where female BALB/c mice were given

eight doses of BU, one of the seven mice autopsied at day 112 post-dosing showed evidence of lymphoma. The development of lymphoma in BU-treated female B6C3F1 mice was also described in the earlier studies of Andrews (2000); in this investigation, where mice were given four doses of BU (10–40 mg/kg), lymphoma cells were identified in 21 animals between 174 and 437 days post-dosing. Using cytochemical techniques, Andrews (2000) described a localized pattern of cytoplasmic staining for alpha-naphthyl acetate esterase and acid phosphatase in the lymphoma cells and considered this indicated that the cells were of a T-cell lineage (after descriptions of Krueger 1990). The morphological features of the lymphoma cell in the present investigation compare closely with the report of Andrews (2000) and suggest therefore that the cell identified here may also possibly be of T-cell origin. However, it is not possible to state with certainty which organ may have been the site of origin for neoplastic development.

The induction of lymphomas in BU-treated mice in the present investigation at day 127 post-dosing is consistent with the findings of other workers (Upton *et al.* 1961; Conklin *et al.* 1965; Robin *et al.* 1981; Bhoopalam *et al.* 1986). However, the identification of tumours at 127 days is considerably earlier than reported previously (Andrews 2000). Also, the presence of lymphoma in the thymus in all tumour cases in the present study is understandable, in the light of the work of Bhoopalam *et al.* (1986) who demonstrated that lymphomas induced by BU were of a T-cell origin. Nevertheless, spontaneous lymphoblastic and lymphocytic lymphoma does occur commonly in mice (Frith & Wiley 1981; Wogan 1984; Scales & Andrews 1991); in the BALB/c strain, spontaneous incidences of up to 6.7% in ageing animals are reported (Frith *et al.* 1983; Krueger 1990). In the B6C3F1 mouse, the incidence of lymphoma in ageing animals is given as 8.3 (males) and 16.8% (females) (Ward *et al.* 1979) or 8.3 (males) and 20.9% (females) (Haseman *et al.* 1998). In Eppley Swiss mice, the incidence in older animals is reported to be about 12% in males and about 27% in females (Clayson 1984).

One BU-treated mouse autopsied at day 127 was found to have a unilateral cataract, but on histological examination, no other lesions were found to be present in the eye. BU is known to be a potent cataractogenic agent (Solomon *et al.* 1955; Grimes *et al.* 1964; Grimes & Von Sallmann 1966; Gehring 1971), and there is evidence for BU-induced cataract in man (Podos & Canellos 1969; Ravindranathan *et al.* 1972; Potts 1986). The mechanism of these changes is considered to be a drug-induced reduction in the mitotic activity and proliferation of the lens epithelium with the lens eventually becoming entirely opaque (Von Sallmann 1957; Gehring 1971; Potts 1986). It is considered that the finding of a cataract in one mouse in the present study was probably related to BU treatment, as lenticular opacities have been recorded following BU administration (Conklin *et al.* 1965). However, this cannot be definitely stated as similar lesions are known to occasionally develop spontaneously in the BALB/c strain (Frith *et al.* 1983).

The present study has demonstrated that the administration of BU to female BALB/c mice on 10 occasions over 21 days induces a stabilized and long-lasting mild CBMA from about day 50 post-dosing. Animals showed significantly reduced RBC, platelets and marrow nucleated cells, and an increased MCV; these changes parallel several of the important features of AA in man. From the results of mortality at 8.25, 9.0 and 9.75 mg/kg BU (Table 1), it is suggested that administration of the drug at 9.0 (or 9.25) mg/kg would be expected to induce only a very low mortality. The mild, but statistically significant, nature of the haematological changes induced during the late-stage plateau phase make this model of CBMA particularly suitable for assessing the effect of the administration of a second agent from approximately day 50 post-dosing (Macharia *et al.* 1999a). For example, the administration of a drug with a reported potential to induce AA in man (e.g. phenylbutazone, gold salts, azathioprine, chloramphenicol, penicillamine, etc.) or an agent with therapeutic potential in the treatment of the human disease (e.g. cyclosporin and antilymphocyte globulin). However, the generally moderate nature of the late-stage changes induced in the marrow and blood is in contrast to the often severe bone marrow depression seen in the human condition. Therefore, the lack of a pronounced neutropenia, and the absence of a profound anaemia with reticulocytopenia, may be considered as relative disadvantages in investigations to elucidate the mechanisms of the pathogenesis of AA in man. Furthermore, the generally moderate nature of the late-stage changes does raise a question of terminology, and it is appreciated that there are reasons to describe the late-stage condition in the BU-treated mouse as 'chronic bone marrow hypoplasia' rather than 'chronic bone marrow aplasia'. It is of interest to note here that Morley and colleagues described the condition induced in their animals as chronic hypoplastic marrow failure (Morley *et al.* 1975; Morley *et al.* 1978; Pugsley *et al.* 1978).

Experiments are now in progress to address the generally moderate nature of the late-stage changes, and several approaches are being examined to develop a further-improved mouse model of human AA. For example, first, the use of drugs other than BU could be employed to induce marrow injury. There is, for example, evidence to suggest that chlorambucil, melphalan, mitomycin and carmustine (BCNU) may be appropriate agents to consider (Trainor & Morley 1976; Trainor *et al.* 1979; Morley 1980). Second, a re-examination of the BU-dosing regimens could be considered, with repeat doses of the drug being administered on a daily, or weekly, or fortnightly basis, and finally, other more wide-ranging possibilities could be

investigated such as the use of a more susceptible inbred mouse strain or another rodent species (e.g. rat, hamster or guinea pig), or the use of BU in conjunction with other antineoplastic agents, or BU in conjunction with radioactivity, or in association with the modulation of the immune response.

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Comparative clinical pathology, **11**, 190.

Studies on the haemotoxicity of chlorambucil in the rat with particular reference to bone marrow culture, marrow cell apoptosis and levels of the cytokine FLT-3

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Aplastic anaemia (AA) is characterised by hypocellular bone marrow, pancytopenia and a risk of severe anaemia, infection and haemorrhage. The development of AA is generally considered to be associated with viral infections, pregnancy, radiation, chemicals and drugs. However, the fundamental pathophysiology of AA is not well understood, and the fact that there are no convenient animal models of AA has been a contributing factor in this lack of understanding. Models of chronic bone marrow failure have been developed in large and small laboratory animals using infective agents, chloramphenicol, benzene, trichlorethylene, strontium 89 and irradiation. Similarly, mouse models of marrow failure induced by immunological methods have also been developed. However, none of these animal models of AA have become widely used. Nevertheless, in 1974, Morley and Blake reported a mouse model of busulphan- (BU-) induced chronic marrow failure, where 'residual' or 'late stage' bone marrow injury was described. This response of the marrow to BU contrasts with other anti-neoplastic agents, where although significant marrow depression is often seen immediately post-dosing, the marrow returns to normal over the following days or weeks. This latter response is dose related, and is sometimes referred to as cytotoxic drug-induced 'predictable' depression. In Morley and Blake's BU mouse model, BU was administered on 4 occasions over a 6-week period to BALB/c mice and animals studied over the following 300-400 days. We have been unable to obtain results (Andrews 2000), which closely parallel those of Morley and Blake. Accordingly we have evaluated other BU dosing regimes in the mouse to produce a new model of chronic bone marrow aplasia showing features of the human condition (Gibson *et al.*, 2000). We have now gone on to attempt to develop a BU-induced model of AA in the rat. However, preliminary results showed that in this species the late stage marrow aplasia induced was relatively mild compared to the severe aplasia often seen in man. Morley subsequently went on to publish a series of papers in the 1970s on his BU- mouse model. In 1979 he examined the capacity of several anticancer agents to induce late-stage marrow aplasia. He reported (Trainor *et al*) that chlorambucil, mitomycin C and melphalan caused marrow aplasia at least 2 months after drug treatment. With a view to developing a rat model of AA that equates to the severe AA in man, we have studied the effect of chlorambucil administration in the rat and its potential to cause late stage bone marrow depression.

Female Wistar-Han rats were treated with chlorambucil i.p. at 6.3mg/kg on 6 occasions over 18 days. Animals (n=6-8) were sampled at 1, 3, 9, 16, 24, 38, 45, 59 and 65 days post dosing. A full blood count was performed and serum prepared for the analysis of the cytokine FLT-3. Marrow smears were prepared and the spleen and sternum placed in fixative. Femoral marrow suspensions were prepared for the assessment of nucleated cell count, apoptosis and clonogenic potential of the marrow (CFU). The administration of

chlorambucil reduced body weight during dosing, and until 8 days post dosing. At days 1, 3 and 9 post-dosing, in general, RBC, WBC, lymphocytes and femoral nucleated cell count (FNCC) were significantly reduced in chlorambucil-treated rats; platelets and neutrophils were unaffected; reticulocytes were increased. By day 9 post dosing there was some evidence that RBC, WBC, lymphocytes, FNCC and reticulocytes were beginning to return to normal. In general, at days 16 and 24, RBC were normal and FNCC was increased; WBC and lymphocytes remained depressed. At 38/45 days, RBC, WBC, FNCC and reticulocytes were normal; at days 59/65 days all parameters of the blood were normal, except for some evidence of reduced lymphocyte counts. Levels of apoptosis in femoral marrow cells were significantly increased immediately post dosing; this elevation persisted until day 16, but thereafter returned to normal levels. Serum FLT-3 levels showed some evidence of increased values in the immediate post-dosing period. CFU/femur were significantly reduced in chlorambucil-treated rats immediately post-dosing, returning to normal levels by day 24. Spleen weights were reduced at 1, 3, 9, and 16 days, with evidence of increased weights at day 24, 38 and 45. Spleen weight returned to normal values at days 59/65.

We therefore conclude that, in the rat, chlorambucil does not induce late stage marrow depression. However, changes in levels of apoptosis, FLT-3 and CFU, together with marrow differential counts and tissue histology have provided data of considerable interest and allow us to relate detailed patterns of change in these parameters to the responses of peripheral blood values over the 9 sampling time points.

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Toxicology, **194**, 267.

Haemotoxic Effects Of Mitomycin C (MMC) In The CD-1 Mouse After Repeated Dose Administration

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Aplastic anaemia (AA) is a potentially life threatening disorder characterised by hypoplastic bone marrow and peripheral blood pancytopenia. The causes of AA are varied and include viruses, irradiation, chemicals and drugs Young and Alter (1994). Recently, Gibson *et al.* (2003) reported a new model of chronic bone marrow aplasia (CBMA) in the busulphan-treated female mouse. This model of 'late stage' bone marrow aplasia shared many similarities with the human condition (AA), however at the dose level of busulphan used, there was significant mortality. In order to develop a model of CBMA without significant toxicity we assessed the ability of mitomycin c (MMC) to induce late stage bone marrow aplasia as suggested in a report by Morley (1980).

312 female CD-1 mice were given 8 ip doses of MMC (2.5 mg/kg) or vehicle over an 18 day period. On days 1, 7, 14, 28, 42 and 50 post dosing, animals (n=13-18) were sacrificed for blood and marrow investigations. A full blood count was performed and serum prepared for analysis of the cytokine FLT3. Femoral marrow suspensions were prepared to assess the total femoral nucleated cell count (FNCC), levels of apoptosis, and number of committed progenitor cells (CFU-C).

Immediately post dosing, MMC induced a 'predictable' bone marrow depression. The FNCC and number of CFU-C were significantly reduced to below 5% control values ($p<0.001$). The percentage of marrow cells undergoing apoptosis was significantly increased ($p<0.001$). In the peripheral blood a severe anaemia was observed with reductions in erythrocyte count (RBC), Haemoglobin (Hb), Haematocrit (HCT), mean cell volume (MCV), Reticulocytes (retic), platelets (PLT) and leucocytes (WBC). The concentration of FLT3 in MMC-treated mice was greatly increased ($p<0.001$).

On day 7 post dosing the FNCC of MMC-treated mice continued to be hypocellular. RBC, Hb, HCT, PLT and WBC remained low, and there were significant reductions in CFU-C. Levels of apoptotic cells in the marrow and serum levels of FLT3 remained significantly elevated ($p<0.01$ and $p<0.001$ respectively).

On day 14 and 28, RBC, HCT and WBC remained significantly reduced in MMC-treated animals, although there were clear signs of haematological recovery and a return towards normal; a rebound reticulocytosis occurred from day 14. The FNCC and CFU-C were still significantly reduced but returning towards control values. Apoptosis and FLT3 concentrations remained greatly elevated.

On day 42-52 post dosing, many parameters had returned to normal but residual (late stage) effects of MMC treatment, were seen. However, these were confined to FNCC and RBC parameters, both of which showed significant reductions.

In conclusion, MMC does not induce late stage bone marrow aplasia in the mouse equivalent to that seen in the busulphan model of Gibson et al. (2003). Furthermore, we found evidence of significant MMC toxicity to other target organs (liver, kidneys) and resulting mortality.

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Changes in serum levels of the cytokine fms-like tyrosine kinase 3 (FLT3) ligand (FL) in the mouse following administration of mitomycin C (MMC)

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Aplastic anaemia (AA) is a rare disorder characterised by hypoplastic marrow and peripheral blood pancytopenia. In most cases AA occurs idiopathically however, a number of chemicals and drugs have been shown to induce AA in man. The cytokine FL has been shown to be indicative of bone marrow damage in man and primates after exposure to chemotherapeutic agents and irradiation. In the present experiment we have assessed the changes in serum FL levels in mice treated with MMC, an anti-neoplastic drug associated with bone marrow failure in man.
Female CD-1 mice were given 8 intraperitoneal doses of MMC (2.5 mg/kg) or vehicle over an 18 day period. After dosing, animals were autopsied at several time points over a 52 day period for blood and bone marrow investigations. A full blood count was performed, and serum prepared for FL analysis by ELISA (R & D Sysyems). Femoral marrow suspensions were prepared to assess the total femoral nucleated cell count (FNCC), levels of apoptosis, and number of committed progenitor cells (CFU-C).
Immediately post dosing (day 1), MMC induced a severe anaemia, leucopenia and thrombocytopenia with statistically significant reductions in all marrow cell lineages. FNCC and CFU-C were reduced, and apoptosis increased ($p<0.001$). At this time, levels of serum FL were greatly increased to 2428.2pg/ml in MMC animals compared to 466.7pg/ml in controls. Over the next 14 days bone marrow suppression continued and serum levels of FL remained significantly elevated ($p<0.001$). On day 28 post dosing, clear signs of recovery were seen, with many parameters, including CFU-C, apoptosis and FL returning towards control values. At day 52, all values except RBC were normal. In conclusion, serum levels of FL reflect the degree of bone marrow damage in the mouse after treatment with MMC.

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Myelotoxic Effects Of The Immunosuppressant Drug Azathioprine (AZA) In The CD-1 Mouse After Repeat Dose Administration

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Aplastic anaemia (AA) in man is characterised by peripheral blood pancytopenia and bone marrow hypoplasia. Therefore, a reduction in red blood cells (RBC), white blood cells (WBC) and platelets (PLT) is seen in the blood and reduced marrow cellularity. Causes of AA are varied but include radiation, viruses, chemicals (e.g. benzene), and drugs (e.g. penacillamine, chloramphenicol, AZA) (Young and Alter 1994). We are using busulphan (BU) to develop mouse models of AA (Gibson *et al.* 2003). In the mouse, repeat dose administration of BU causes initial bone marrow depression, followed by a return towards normal levels; however, at days 50-120 post dosing a late-stage chronic bone marrow aplasia is seen with reductions in RBC, WBC, PLT and marrow cellularity. This effect is evidence of 'residual' marrow injury with blood and marrow changes comparable to AA in man. In the present study we wished to investigate the myelotoxicity of AZA to determine if, like BU, AZA caused residual late-stage effects in all haemopoietic lineages (i.e. RBC, WBC, PLT) and reductions in marrow cellularity.

Female CD-1 mice (n=136) were divided into 2 groups of 68 and treated with AZA (100 mg/kg), or vehicle, by gavage for 10 days. On days 1, 3, 9, 22, 29, 42 and 57 post dosing, animals (n=5-11) were killed for blood and marrow investigations. A full blood count was performed (ADVIA 120) and marrow suspensions prepared to assess committed progenitor cells (CFU-C), levels of apoptosis, and the femoral nucleated cell count (FNCC).

Immediately post dosing (day 1), AZA induced marrow depression. In peripheral blood, all haemopoietic lineages were statistically significantly reduced, i.e. erythroid (RBC), myeloid (neutrophils), lymphoid (lymphocytes), and PLT. FNCC and CFU-C were significantly reduced ($p<0.001$). The percentage of marrow cells undergoing apoptosis was increased ($p<0.001$). On days 3 and 9 post dosing the bone marrow depression in AZA mice continued, with significant reductions in RBC, haemoglobin (Hb), haematocrit (HCT), PLT, reticulocyte and WBC counts. The CFU-C remained reduced and apoptosis levels in the marrow continued to be increased. On day 22 and 29, RBC, HCT, and PLT remained significantly reduced in AZA animals, although there were clear signs of haematological recovery and a return towards normal. Although CFU-C and FNCC were significantly reduced on day 22, both parameters returned to near control values at day 29. On days 42 and 57 most parameters were normal but RBC remained significantly reduced.

In conclusion, AZA induced significant depression in all haematological lineages (with an increase in apoptosis) immediately post dosing (days 1, 3, and 9). Parameters then returned towards normal at days 22, 29 and 42. On day 57 the majority of parameters were within normal limits, except RBC which remained reduced. Therefore, AZA did not induce late stage effects in all haemopoietic lineages, as in the case of BU. However, the significantly depressed in RBC at day 57, indicated a residual erythroid marrow injury.

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