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RADIOBIOLOGICAL CHARACTERIZATION OF TWO POPULATIONS OF MURINE BONE MARROW CELLS

by

M. JOAN ALLALUNIS-TURNER

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend for acceptance, a thesis entitled "Radiobiological Characterization of Two Populations of Murine Bone Marrow Cells" submitted by M. Joan Allalunis-Turner in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Medical Sciences [Radiology and Diagnostic Imaging].

Supervisor

External Examiner

Date April 11, 1985

DEDICATION

For Bob, with love.

ABSTRACT

This thesis presents studies performed to characterize cells obtained from the central marrow cavity (Fraction 1) and the compact bone (Fraction 3) of murine femurs.

When irradiated *in vivo*, [illegible]FU-GM were relatively radioresistant as compared [illegible]U-GM. Treatment with misonidazole (MISO) before irradiation abrogated the radioresistance of Fraction 3 and did not effect the sensitivity of Fraction 1. When removed from their microenvironment and irradiated *in vitro*, the aerobic sensitivities of Fraction 1 and Fraction 3 CFU-GM were similar; the radioresistance which had been demonstrated for Fraction 3 *in vivo* was no longer evident. When irradiated under hypoxic conditions, a lower oxygen enhancement ratio (OER) was observed for Fraction 3 CFU-GM compared with Fraction 1 CFU-GM. These results suggest that the *in vivo* microenvironment of Fraction 3 may be relatively hypoxic.

Fraction 3 cells were twice as sensitive as Fraction 1 cells to the cytotoxic effects of MISO under hypoxic conditions *in vitro*. This increased sensitivity to MISO cytotoxicity and the decreased radiobiological OER observed for Fraction 3 may be explained in part by the lower levels of intracellular glutathione found in Fraction 3 cells.

In vitro cultures of Fraction 1 and Fraction 3 in reduced oxygen tensions demonstrated that only a small increase in plating

efficiency, could be attributable to the reduction in oxygen concentration. However, significant numbers of CFU-GM survived incubation under extremely hypoxic conditions (0.6% oxygen).

Under hypoxic conditions, bone marrow cells bound appreciable amounts of MISO. Little binding was observed for well oxygenated marrow cells. Autoradiographic analysis of *in vivo* binding patterns in normal marrow demonstrated that more MISO was bound to Fraction 3 equivalent anatomic areas than was bound to Fraction 1 equivalent areas. Uniformly elevated binding was observed for both Fraction 1 and Fraction 3 areas in regenerating marrow.

The repopulation kinetics of Fraction 3 CFU-GM were [redacted] to those of Fraction 1 following a sub-lethal dose of total body irradiation. For both populations, pre-treatment with MISO enhanced cell kill one to two days after irradiation and may have impaired recovery at latter times.

Fraction 1 and Fraction 3 cell populations were shown to contain cells with self-renewal potential as measured by *in vitro* re-plating assays. Both cell fractions could efficiently restore hematopoietic function to lethally irradiated mice with Fraction 3 cells being more efficient.

Finally, an evaluation of the radioprotective abilities of an experimental agent, diethyldithiocarbamate (DDC), suggested that this compound is a non-specific stimulant of the hematopoietic system in general and will be of limited clinical use.

In conclusion, these studies have demonstrated that populations of murine hematopoietic cells can be separated on the basis of their

spatial distribution within the femur. The finding that compact bone associated cells were relatively radioresistant, sensitive to MISO, associated with elevated MISO binding in vivo and proliferated at low oxygen tensions in vitro suggests that the normal microenvironment of these cells may be relatively hypoxic. The finding that Fraction 3 cells restored hematopoietic function more efficiently than Fraction 1 cells suggests that this microenvironment may serve as a stem cell reserve.

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Mr. Rick Crilly designed the irradiation jig, and with the assistance of Miss Glenna Stirret, performed the X-ray dosimetry.

Mr. Zbigniew Krezolek cared for the animals, and Miss Zina Mosleh assisted with media preparation.

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Ms. Viki Bjerklund and Miss Karen Brown provided secretarial assistance.

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ABBREVIATIONS

BCNU = 1-3-bis(2-chloroethyl)-1-nitrosourea

CER = colony enhancement ratio

CFU-GM = colony forming unit-granulocyte/macrophage

CFU-S = colony forming unit-spleen

DDC = diethyldithiocarbamate

DMF = dose modifying factor

DTNB = 5-5'-dithiobis-(2-nitrobenzoic acid)

EDTA = ethylenediaminetetraacetic acid

GSH = glutathione

Gy = Gray

HEPES = N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HPP-CFU = high proliferative potential colony forming unit

$LD_{50/30}$ = dose resulting in 50% survival over 30 days

$LD_{50/28}$ = dose resulting in 50% survival over 28 days

LET = linear energy transfer

MISO = misonidazole

NADPH = nicotinomide-adenine dinucleotide phosphate

OER = oxygen enhancement ratio

PBS = phosphate buffered saline

PHA = phytohemmagglutinin

PLD = potentially lethal damage

RBE = relative biological effectiveness

RTOG = Radiation Therapy Oncology Group

TCA = trichloroacetic acid

THSC = totipotent hematopoietic stem cell

TLD = thermoluminescence dosimetry

WR-2721 = S-2-(3-aminopropylamino)-ethylphosphorothioic acid

CHAPTER ONE

Introduction

The discovery of X-rays by Roentgen in 1895 caught the imagination of scientists, physicians and hobbyists alike. By 1896, X-rays were being used in medicine to locate foreign bodies, treat tuberculosis and infectious diseases, detect tooth decay, and in industry to check the integrity of iron castings and distinguish genuine from fake diamonds (Brecher and Brecher 1969). Initially, X-rays and other radiations were considered harmless. Early determinations of X-ray output were sometimes based on estimates of the relative amounts of erythema produced on the operator's hands and forearms (Mould 1983). The severe radiation burns and skin ulcerations which were later reported were thought by some to have arisen from the static discharge of the electric coils or from the generation of an ozone layer near the skin (Brecher and Brecher 1969). However, more astute observers blamed the rays themselves for the skin damage and were quick to recognize that diseased tissues would be better targets for the deleterious effects of the radiation. 1897 marked the first documented case of the use of X-rays in the treatment of inoperable cancer (Mould 1983). This was soon followed by other attempts to control or cure tumors. However, ten years elapsed before shielding of X-ray tubes became standard practice. As a result, the risk of normal tissue damage was consiserable for both the patient and the physician.

In the years to follow, radiation was used to treat a variety of acute and chronic disorders. As late as 1942, X-rays were reported as being effective therapy for such relatively benign conditions as warts, persistent nosebleeds, hayfever, sinusitis, conjunctivitis and psoriasis (Brecher and Brecher 1969). The epidemiological studies of the 1950's and 1960's which described many late effects of irradiation including sterility and induction of malignancies soon halted the indiscriminate use of radiation in benign disease.

Radiotherapy, as practiced today, combines a respect for normal tissue damage with the realization that tumor cure may also be achieved. Indeed, the major goal of radiotherapy is to optimize the therapeutic ratio by delivering the maximum dose to the tumor while not exceeding the tolerance of the normal tissues.

Despite advances in computerized treatment planning, the increasing use of high energy irradiation, and the use of intricate shielding techniques, radiosensitive normal tissues are often unavoidably exposed during standard radiotherapy. One of the goals of radiobiology has been to establish the response of these tissues to ionizing radiation. The effects of radiation on rapidly self-renewing tissues such as the bone marrow and the gastrointestinal system were among the first to be studied.

Many of the early experiments with bone marrow attempted to define the tolerance of this organ to acute, whole body irradiation. Failure of the hematopoietic system was recognized as the probable cause of death in animals dying eight to thirty days after moderate

doses of irradiation (Quastler 1945). Consequently, many of the early studies used comparisons of survival rates and $LD_{50/30}$ doses to study the influence of various factors on bone marrow related lethality. Kohn and Kallman (1956) were among the first to observe that sensitivity to whole body irradiation was under genetic control. Their studies noted differences in $LD_{50/28}$ doses which ranged from 5.44 Gy to 6.65 Gy for five strains of mice tested. Djerassi et al. (1960), using four different strains of mice, also observed differences in $LD_{50/28}$ doses.

Age and gender differences were other factors identified as influencing radiation tolerance. Lindop and Rotblat (1962) studied the acute radiation sensitivity of mice which were irradiated at ages ranging from one day to 108 weeks. Their studies demonstrated an early peak in radiosensitivity at four weeks of age and a decline to maximum radioresistance at 40 weeks followed by a steadily increasing sensitivity in older animals. They also noted that after 10 weeks of age, female mice were less radiosensitive than were male mice, a finding noted earlier by Kohn and Kallman (1956).

Acute lethality studies were also used in investigations which explored the ability of exogeneous agents to modify radiosensitivity. The sensitizing effects of molecular oxygen, and the protective effects of hypoxia, were first described for bacteria by Alper and Howard-Flanders (1956). Attempts were made to alter the radiosensitivity of mice by allowing them to breathe nitrogen, 5% oxygen, or pure oxygen at one atmosphere shortly before irradiation. Significant increases in $LD_{50/30}$ values were observed in the hypoxic

animals (Dowdey et al. 1950, Wright 1960, Phillips and Ainsworth 1969). However, attempts to sensitize animals with one atmosphere of oxygen were not successful (Lindop and Rotblat 1962).

In vivo studies such as those described above continue to be used to assess the effects of radiation and radiation modifiers on the hematopoietic system. These assays offer the advantage of allowing the naturally occuring interactions among hematopoietic cells, long and short range growth factors and the hematopoietic environment, to be preserved relatively unperturbed after irradiation. For this reason, in vivo studies have the potential to provide information which may be more clinically relevant than in vitro assays of single colony types.

A complementary approach has been to evaluate the effects of radiation on the individual components of the hematopoietic system. These studies attempt to provide an insight into the mechanisms which dictate the host's response to radiation. Although understanding the radiation effects on the production of and response to growth factors and on the hematopoietic microenvironment are of fundamental importance, these areas have received relatively less attention than the effects of radiation on the hematopoietic stem cells and their committed progeny.

In 1961, clonal assays were first used to directly assess the effects of irradiation on hematopoietic cells. Till and McCulloch (1961) were the first to develop a system to enumerate the number of pluripotential stem cells in mouse bone marrow. Lethally irradiated mice were injected with syngeneic marrow cells, and their spleens

were removed eight to ten days later. The number of visible nodules, or "colonies", was linearly related to the number of the cells injected and was shown to correspond to the number of stem cells contained in the innoculum.

In vitro colony assays were developed shortly thereafter, and allowed for the quantification of marrow colonies consisting of myeloid cells (Bradley and Metcalf 1966), erythroid cells (McLeod et al. 1974, Iscove et al. 1974), megakaryocytes (Metcalf et al. 1975a), T-cells (Roszenszajn et al. 1975), and B-cells (Metcalf et al. 1975b).

Using these in vivo and in vitro clonal asays, radiobiologists were able to directly assess the effects of radiation on the hematopoietic cells. The single hit multi-target theory was frequently used to construct radiation survival curves. In this model, survival equals $1-(1-e^{-KD})^n$, where n equals target number, D_0 equals the dose, measured on the exponential portion of the survival curve, which reduces survival from 1 to 1/e, where e equals the natural logarithm, and K equals $1/D_0$. The sensitivity of CFU-S (McCulloch and Till 1962, Till and McCulloch 1963 and 1964) and CFU-GM (Senn and McCulloch 1970) were soon determined using estimates of D_0 values. Determinations of the oxygen enhancement ratio (OER) (Phillips 1968, Hendry and Howard 1972, Hendry et al. 1975), repair capacity (Till and McCulloch 1963 and 1964), and relative biological effectiveness (RBE) of neutrons to gamma radiations (Hendry 1972, Feola et al. 1974, Phillips et al. 1974, Maruyama et al. 1983) were also made. In all cases, the survival curves were

described to have small initial shoulders followed by exponential slopes. The relatively straight curves observed over two to three decades of cell kill suggested that the bone marrow had a uniform response to irradiation.

Recent studies, however, have suggested that sub-populations of hematopoietic cells may express different radiosensitivities. Santos Mello and co-workers (1974) have studied the induction of chromosome aberrations in PHA-responsive human T-cells following acute or chronic irradiation. Their results have demonstrated a biphasic response to irradiation consistent with the existence of two sub-populations of T-cells differing in their radiosensitivity. These results have been supported by the work of Ekstrand et al. (1982) who described differences in radiosensitivity among T_μ and T_γ sub-populations.

Similar variations in radiosensitivity have been noted for large human monocytes. Kwan and Norman (1978) identified monocytes by their ability to phagocytize latex particles. They irradiated monocytes with varying doses of gamma rays and assessed their survival after four days in culture. Their results indicated that although most of the cells were radioresistant (D_0 = 6.5 Gy), 25% were relatively radiosensitive (D_0 = 0.5 Gy).

These differences in radiosensitivity are not limited to the mature blood cells. Shvets (1983) reported the results of a histological survey of spleens, femurs, and illiac crests obtained from mice after whole body irradiation. The mice were rescued with bone marrow that had been previously irradiated with various doses of

gamma irradiation. When regenerating foci (colonies) of erythroid, myeloid, and megakaryocytoid cells were scored and plotted as surviving fractions, biphasic curves were obtained for all of the colony types. The D_0 values for all of the radiosensitive populations ranged from 0.1 to 0.5 Gy. In bone marrow and spleen, radioresistant populations with D_0 values ranging from 1.15 to 2.1 Gy and 1.15 to 1.25 Gy, respectively, were noted.

Biphasic survival curves obtained after in vivo irradiation of mice were interpreted by Millard and Blackett (1981) as evidence of a radioresistant sub-population of marrow CFU-GM. These investigators theorized that the observed radioresistance may have resulted from the cells residing in a spatially distinct microenvironment which was relatively hypoxic.

Studies by Allalunis et al. (1983) directly tested the in vivo radiosensitivity of murine CFU-GM which were harvested as three populations according to their spatial distribution within the mouse femur. The CFU-GM of the central marrow cavity (Fraction 1) and the endosteal region (Fraction 2) expressed radiosensitivities similar to that described by other workers for unseparated marrow. CFU-GM isolated from the compact bone region of the femur (Fraction 3) were relatively radioresistant. Fraction 3 cells were also sensitive, in vivo, to the cytotoxic effects of the hypoxic cell sensitizer, misonidazole (MISO).

The studies described in this thesis have extended the initial report by Allalunis et al. (1983) and have attempted to provide a biological profile of these radiosensitive and radioresistant

populations of bone marrow cells. Chapter Three describes the results of experiments which used basic radiobiology techniques to investigate the sensitivities of these cells. Included among the investigations were determinations of the response to *in vitro* irradiation under controlled oxygenation conditions and the *in vitro* sensitivity to MISO.

Chapter Four presents the results of a series of experiments which used radiobiological techniques to address a question of current clinical interest. The ability of a thio phosphate compound, WR-2721, to protect normal tissues during radiotherapy is currently being investigated in Phase I clinical trials sponsored by the RTOG. Several additional compounds, including diethyldithiocarbamate (DDC), have been proposed as being radioprotectors of normal tissues in radiotherapy procedures. Laboratory studies which evaluated the effects of DDC as a marrow radioprotector are reported in this section.

Chapters Five and Six address questions of a more theoretical nature, specifically, do radioresistant Fraction 3 sub-populations play a role in the maintainance of hematopoiesis. Data is presented which was obtained from studies of the role of Fraction 3 cells in marrow regeneration after sub-lethal doses of irradiation. The effects of low oxygen tensions on *in vitro* growth are considered. The binding of radiolabelled MISO to marrow cells is discussed. The ability of Fraction 1 and Fraction 3 cells to produce *in vitro* colonies with self-renewal potential is also compared. Finally, evidence which suggests that Fraction 3 cells may serve as a hematopoietic reserve is considered.

CHAPTER TWO

Materials and Methods

Animals

The C57Bl/10J mice used in these studies were obtained from two sources. The Small Animal Breeding Unit of the University of Alberta supplied male and female mice which were used for most experiments. However, studies of the effect of animal age on *in vitro* colony growth required large numbers of age-matched mice. These experiments necessitated the purchase of additional mice from Jackson Laboratories. All animals were housed five per cage with Purina Lab Chow and water available *ad libitum*. A 12 hour light/dark cycle was maintained. Unless otherwise noted, mice were six to twelve weeks old at the time of experimentation. Animals of the same gender were used in each experiment.

Three month old female C3H/HEJ mice used in the bone marrow radioprotector studies were obtained from the Small Animal Breeding Unit of the University of Alberta.

Media

MEM Alpha medium (Flow Laboratories) was reconstituted from powder with distilled water and supplemented with sodium bicarbonate according to manufacturer's instructions. No antibiotics or antimycotics were added. All media were filtered through 0.45μ filter units (Nalge Corporation) and stored at 4°C for periods not

exceeding three weeks. Alpha medium (1 x concentration) with 10% fetal calf serum (Flow Laboratories) was used for all routine cell handling procedures. Alpha medium at 2x concentration was used to prepare the methylcellulose solution required for in vitro cultures. Boiling distilled water (250 mL) was added to 9 gm of previously autoclaved methylcellulose (Fluka). The mixture was stirred until room temperature was achieved. Following the addition of 250 mL alpha medium (2x concentration), the mixture was stirred at 4° C until the medium was clear (usually 24 hours). The methylcellulose was poured into sterile 17 x 100 mm culture tubes (Falcon) and stored at -20°C until further use.

Mouse lung conditioned medium was used to stimulate the in vitro proliferation of CFU-GM (Sheridan and Metcalf 1973). Lungs were excised, rinsed free of clots with sterile saline and finely minced using surgical scissors and forceps. One mL alpha medium was added to each 40 mg lung tissue contained in 17 x 100 mm culture tubes. The tissue was incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 48 hours. After incubation, the supernatant was removed, heat inactivated (56° C for 30 min.), then centrifuged to remove debris (3000 rpm x 20 min.). The medium was then dialyzed against distilled water at 4°C for 24 hours, centrifuged (3000 rpm x 20 min.), filter sterilized through a 0.45μ filter, poured into 17 x 100 mm culture tubes and stored at -20°C until required.

Mouse spleen cell conditioned medium (Metcalf and Johnson 1978) was used to stimulate the in vitro growth of high proliferative potential colony forming units (HPP-CFU). Mouse spleens were gently

pressed through a sterile metal screen in order to obtain a single cell suspension. Cells were then electronically counted (Model ZBI, Coulter Electronics) and diluted to 10^7 cells per ml with alpha medium containing 10% fetal calf serum and 0.25% pokeweed mitogen (GIBCO). These cultures were incubated in tissue culture flasks (Corning) at 37°C in a humidified atmosphere of 5% CO_2 in air for five days. Following incubation, the medium was decanted, centrifuged (3000 rpm x 20 min.), filter sterilized and stored frozen.

Cell Harvest Techniques

Bone marrow cells were removed from different anatomic regions of mouse femurs using modifications of previously described techniques (Kilgore et al. 1979; Allalunis et al. 1983). Cells from the central marrow cavity (previously designated Fraction 1) and from the endosteal region (previously designated Fraction 2) were isolated together by flushing the excised femur with 4.5 mL collagenase (1 mg/mL) (GIBCO) in alpha medium for eight minutes. The action of collagenase was inhibited by the addition of 0.5 mL fetal calf serum. The resultant cell suspension, designated as Fraction 1, was transferred to a sterile tube and held on ice. The femurs from which Fraction 1 cells had been removed were finely minced with surgical scissors and incubated in the presence of 2.5 mL fresh collagenase solution in alpha medium for 15 minutes. The bone fragments were vigorously stirred at five minute intervals. This incubation procedure was terminated by the addition of 2.5 mL fetal calf serum

and the resulting cell suspension (Fraction 3) was transferred to a sterile dilution tube and held on ice.

Drugs and Biologicals

MISO, (1-(2-nitro-1-imidazole)-3-methoxy-2-propanol) was a gift of Hoffman-La Roche (Etobicoke, Ont.). ^{14}C-MISO, labelled in the 2 position of the imidazole ring, was generously provided by Dr. W. E. Scott of Hoffman-La Roche (Nutley N.J.) at a specific activity of 230 μCi/mg. The sodium salt of DDC was obtained from Sigma Co. Ltd. and was used without further purification. WR-2721 (S-2-(3-Aminopropylamino)-ethylphosphorothioic acid) (lot AJ-682) was a gift of the National Cancer Institute (USA). All drug solutions were prepared immediately before use with sterile distilled water or sterile pyrogen free saline as required.

Hematopoietic Cell Assays

CFU-GM were assayed using modifications of techniques originally described by Bradley and Metcalf (1966). Bone marrow or spleen cells were cultured in a mixture of alpha medium containing 1.2% methylcellulose, 20% fetal calf serum and 10% lung conditioned medium. Four to eight replicate tissue culture plates (10 x 35 mm) (Falcon), each containing one mL media, were used per experimental group. All plates were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. After seven days, aggregates of 50 cells or more were scored as colonies with the aid of an inverted microscope.

HPP-CFU colonies were assayed using a modification of the

technique originally described by Johnson (1983). Bone marrow cells were cultured in alpha medium containing 1.2% methylcellulose, 20% fetal calf serum, 10% spleen cell conditioned medium, 2% HEPES buffer (1M) and 7.5×10^{-5}M 2-mercaptoethanol (Kodak). Four to eight replicate plates were incubated as described above. Colonies of 50 or more cells were counted seven and fourteen days after plating.

For experiments in which individual colonies were re-cultured, a micropipet equipped with sterile tips was used to remove colonies from the original culture and to resuspend them in 0.4 mL fresh media contained in each well of a 24 well microtiter plate (Falcon). After each well was microscopically examined to ensure that the colonies were resuspended and free of clumps, the plates were incubated as described above for an additional seven to ten days. The number of wells containing secondary colonies was then scored.

Individual colonies were also scored according to morphology by removing each colony with a micropipet equipped with disposable tips. The colonies were smeared onto glass microscope slides and examined after staining with panoptic (Wright's, hematoxylin and eosin) or histochemical specific stains (leukocyte peroxidase, α-napthyl acetate esterase, napthol AS-D chloracetate esterase).

CFU-S were assayed according to techniques described by Till and McCulloch (1961). Lethally irradiated (see below) three month old mice were intravenously injected with bone marrow cells suspended in 0.5 mL alpha medium. Spleens were removed eight days later and preserved in Bouin's fixative. Individual spleen colonies were enumerated.

Irradiation Procedures

A ^{137}Cs unit (Picker) was used for all *in vitro* irradiation procedures. The dose rate delivered to a flask placed in the middle of the irradiation field was 1.86 Gy/min as determined by a Victoreen ionization chamber (model 500). Suspensions (6 mL) of cells (2 x 10^6/mL) were slowly stirred in glass flasks specially designed for radiobiological studies (Chapman *et al*. 1977). Chambers were gassed with a mixture of 5% CO_2 in nitrogen (<5 ppm O_2) or 5% CO_2 in air at a flow rate of one liter per minute for 60 minutes before irradiation commenced. Gas flow was maintained at this rate throughout the irradiation exposure. This procedure was more than adequate to produce conditions of extreme hypoxia or aerobic conditions as measured radiobiologically (Chapman *et al*. 1977). Cells were sampled through side ports and held on ice until cultured. Cell survival data was fit to the linear quadratic model with a computer program (Gillespie *et al*. 1975).

For assays of CFU-S, mice were irradiated with an X-ray unit (Siemens) operated at 300 kV and 12 ma (half value layer equal to 3.4 mm copper). Unanesthetized mice were placed in a circular plexiglass jig designed by Mr. Rick Crilly of the Cross Cancer Institute, who also performed the dosimetry. Space filling wax phantoms were used to enhance the effects of irradiation scatter. The surface dose received by the mice was 0.443 Gy/min as measured by TLD capsules. Dose variation across the jig had a total deviation of 1% as measured by film density. The percent dose drop across the abdomen was 6% as

measured by TLD capsules.

A ^{137}Cs unit was used to irradiate mice for studies of DDC radioprotection and for studies which measured the regeneration of CFU-GM after irradiation. Unanesthetized mice were restrained in ventilated polypropylene tubes and irradiated with gamma rays at a dose rate of 1.42 Gy/min as determined by a Victoreen ionization chamber.

Animal Survival Studies

In experiments which compared the ability of Fraction 1 and Fraction 3 cells to rescue lethally irradiated animals, three month old male mice were irradiated with 9 Gy X-rays as described above. Fraction 1 and Fraction 3 cells were isolated, washed free of collagenase, resuspended in alpha medium and injected into a pre-warmed lateral tail vein of recipient mice. Animals were monitored for survival for 30 days.

Evaluation of DDC as a Radioprotector

In studies which evaluated the effect of a single dose of DDC, mice received 15 or 30 mg DDC intraperitoneally 15 min before exposure to 2, 4, 6, or 8 Gy gamma radiation. In studies of the effects of multiple doses of DDC, mice received 15 mg DDC at 48 hr, 24 hr and 15 min before irradiation. Control animals received equivalent volumes of pyrogen free saline or DDC without irradiation. CFU-GM or CFU-S survival was assayed immediately after irradiation. In a parallel series of experiments, mice received 600 mg/kg WR-2721

30 minutes before irradiation. CFU-GM survival was assayed as described above.

In these experiments, the surviving fractions were calculated as follows. The colony enhancement ratio (CER) was determined by dividing the number of colonies obtained from unirradiated control animals treated with DDC by the number of colonies obtained from unirradiated control mice treated with saline. DDC survival values were obtained by comparing the number of colonies obtained from DDC treated irradiated mice with the number of colonies obtained from saline treated irradiated mice. These values were then normalized to account for the CER observed in DDC treated, unirradiated mice (Allalunis-Turner and Chapman 1984). Dose modifying factors (DMF) were determined by dividing the irradiation dose required to produce 37% survival in DDC treated animals by the dose required to produce 37% survival in saline treated animals.

In studies which measured the kinetics of the increase in CFU-GM following treatment with DDC or WR-2721, animals received a single dose of DDC (5, 15, or 30 mg) or WR-2721 (200, 400, or 600 mg/kg) at 0 hr. Bone marrow and spleen cells or bone marrow cells only were obtained from C57B1/10J and C3H/HEJ mice respectively and were assayed for CFU-GM survival at various times following treatment. Animals injected with equivalent volumes of saline served as controls in these experiments.

In Vitro Cytotoxicity of MISO .

Experiments were designed to determine the effects of MISO on

the survival of Fraction 1 and Fraction 3 cells maintained in aerobic or hypoxic conditions. Bone marrow cells were isolated as previously described, placed in glass spinner flasks at 37°C and de-gassed using procedures identical to those described above for irradiating cells in vitro under aerobic or hypoxic conditions. High concentrations of MISO were added after one hour of de-gassing, resulting in final concentrations which ranged from 120 to 1200 µg/mL. Cell samples were obtained from the flasks at various times after the addition of MISO and the survival of CFU-GM was determined.

Effects of Low Oxygen on Bone Marrow CFU-GM

Studies were designed which compared the survival of CFU-GM incubated in a conventional manner with the survival obtained following incubation under conditions in which the concentration of oxygen in the incubation chambers was precisely controlled at various levels. Fraction 1 and Fraction 3 marrow cells were isolated as described above. Aliquots of each population were cultured in standard growth media and incubated at 37°C for seven days in a humidified atmosphere of 5% CO_2 in air. These cultures served as controls. Other Fraction 1 and Fraction 3 cells were cultured under defined oxygen conditions in the following manner. Four mL cells in modified growth media were added to 50 mm glass dishes. (A modification of the medium consisting of the addition of 20 mM HEPES buffer and the reduction of sodium bicarbonate to 20% of normal levels was necessary to maintain normal pH balance in the cultures in

the absence of CO_2 in the gas mixtures used in these chambers.) The culture dishes, along with a similar glass dish filled with distilled water to provide humidity, were placed in specially designed, air-tight aluminum chambers (Koch et al. 1979). The chambers were sealed and the air was evacuated and replaced with four changes of nitrogen gas containing precisely determined amounts of oxygen which ranged from 0.2 to 18%. Following the gassing procedure, the chambers were placed in a 37°C incubator for seven days. At the end of the incubation, the oxygen levels inside the chamber were determined using a polarographic oxygen electrode (Koch 1984). Colonies were scored and the plating efficiency was compared to that of control cultures.

^{14}C-MISO Binding Studies

The binding of ^{14}C labelled MISO to bone marrow cells was determined by several methods. In studies which determined the rate of binding in vitro, Fraction 1 bone marrow cells were incubated in glass spinner flasks or aluminum chambers with ^{14}C-MISO (10 to 100 μM) at 37°C under aerobic or hypoxic conditions identical to those described earlier. After the addition of MISO, the cell suspensions were sampled over a three hour period. The method of Koch et al. (1984), described below, was used to determine the binding rate. Duplicate samples which had been labelled for various times were washed four times with ice cold phosphate buffered saline (PBS). Following the fourth wash, the cell pellet was resuspended in 100 μL PBS to which was added 100 μL 10% trichloroacetic acid. The cell

lysate was held on ice for 20 min, transferred to small centrifuge tubes and spun at 5000 rpm for two minutes. The resultant supernatant (acid soluble fraction) was decanted, added to a plastic scintillation vial (Fisher) containing 10 mL Scintiverse I (Fisher). The acid insoluble pellet was disolved in 100 µL 1N NaOH for 30 minutes, neutralized with 100 µL 1N HCl, and added to 10 mL scintillation fluid as above. All sample vials were thoroughly mixed, wiped clean and counted in a Beckman liquid scintillation counter (Model LS7000) programmed to record disintegrations over an energy range of zero to 156 kV. Following the subtraction of background counts, the binding rate was calculated and expressed in terms of moles of ^{14}C-MISO bound per million cells per hour.

The in vivo binding of radiolabelled MISO to normal and regenerating bone marrow cells was also determined. A three month old male mouse which had received 9 Gy irradiation plus 10^6 Fraction 1 bone marrow cells nine days before served as a source of regenerating bone marrow. An untreated three month old male mouse served as control. ^{14}C-MISO was injected according to the following schedule: 50 µM at -180 minutes; 25 µM at -135, -90, and -45 minutes. Animals were sacrificed at 0 min. The femurs, humerii and the spleen were removed, fixed in 10% phosphate buffered formalin, decalcified (femur and humerus) and imbedded in paraffin blocks. Four micron serial sections were obtained from each block for histological examination and autoradiography.

De-waxed, dehydrayted histological sections were prepared for autoradiography using techniques described by Ulberg et al. (1982). Liquid nuclear emulsion (NBT-2 Kodak) was melted at 43° C, diluted with an equal volume of distilled water and poured into a plastic dipping pot and held at 43°C. Glass slides were dipped vertically into the emulsion, gently removed and allowed to gel on chilled metal trays. Slides were then dried for two to three hours in a light-tight dessicator box, wrapped in foil, placed into a light-tight container and stored at 4°C for 7 to 38 days. Slides were developed with D19 (Kodak) for three minutes, rinsed in an acetic acid stop bath, fixed for six minutes in commercial fixative (Kodak), then rinsed in running water for five minutes. After air drying, slides were stained with hematoxylin and eosin. The distribution of labelled MISO in the tissue sections was determined microscopically. For quantitative grain counts, the number of grains per nuclei in a 100 μm^2 area of interest was determined.

Age Survey

One hundred C57Bl/10J male weanling mice were used to determine the effects of age on the growth of Fraction 1 and Fraction 3 bone marrow. Bone marrow cells were sampled at intervals of two to four weeks as the mice aged to 12 months. The relative CFU-GM plating efficiency was determined, as was the number of secondary colonies generated from HPP-CFU. Femurs were also removed, decalcified and sectioned for histological examination.

Glutathione (GSH) Measurement

Intracellular GSH was measured using modifications of a technique described by Tietze (1969). Marrow cells were washed in ice-cold phosphate buffered saline, pelleted, then re-suspended in one mL tricholoracetic acid (TCA) (5% TCA in 0.01N HCl with 0.5 mM EDTA). The cell lysates were centrifuged to remove debris, aspirated, then frozen in glass tubes. Immediately before assay, cell lysates were extracted five times with equal volumes of ether. To prepare cells for spectrographic analysis, a 200 µL aliquot of the lysate was added to a two mL cuvette containing NADPH (200 µM), DTNB (100 µM), and GSH reductase (1.57 units/mL) in buffer (0.1M KH_2PO_4). The absorbance at 412 nm was measured in a Beckman spectrophotometer (Model DU-7). For each sample, GSH content was determined by comparison to standards containing known amounts of GSH.

Statistics

A Student's t-test was used to evaluate the differences observed between experimental groups (Zar 1974).

CHAPTER THREE

Results

Effects of MISO on CFU-GM Survival After Irradiation In Vivo

Mice were injected with a single dose of MISO (1 mg/g) or pyrogen free saline (0.01 mL/g) 30 min before irradiation with a ^{137}Cs source. The survival of Fraction 1 and Fraction 3 CFU-GM was determined immediately after irradiation or within one hour. Table 1 presents the pooled results of eight separate experimental trials. All of the data were expressed as a pecentage of the unirradiated control values. MISO had no radiosensitizing effect on Fraction 1 CFU-GM in vivo, as the survival of Fraction 1 cells obtained from MISO treated animals was comparable to that observed with Fraction 1 cells from saline treated animals. However, Fraction 3 cells were sensitized to MISO. At all radiation doses tested, CFU-GM survival following MISO treatment was reduced as compared to that of saline treated controls.

CFU-GM Survival After In Vitro Irradiation

Survival data for Fraction 1 and Fraction 3 CFU-GM after irradiation in vitro are shown in Figures 1 and 2, respectively. The aerobic radiosensitivities of Fraction 1 and Fraction 3 cells were similar. However, under hypoxic conditions, Fraction 3 cells were significantly more radiosensitive than were Fraction 1 cells. The oxygen enhancement ratios (OER) (determined at the 10% survival

Table 1

Effects of Misonidazole on CFU-GM Survival
Following In Vivo Irradiation

	Dose (Gy)	CFU-GM Survival as % Control	
		Saline	Misonidazole
Fraction 1	0.75	53 ± 5	46 ± 2
	1.50	32 ± 4	30 ± 5
	3.00	23 ± 4	19 ± 3
	4.50	11 ± 4	9 ± 3
Fraction 3	0.75	83 ± 18	24 ± 11 *
	1.50	42 ± 8	16 ± 9 *
	3.00	54 ± 5	15 ± 3 *
	4.50	27 ± 4	3 ± 1 *

* Differences between mean values are significant at $p < .001$

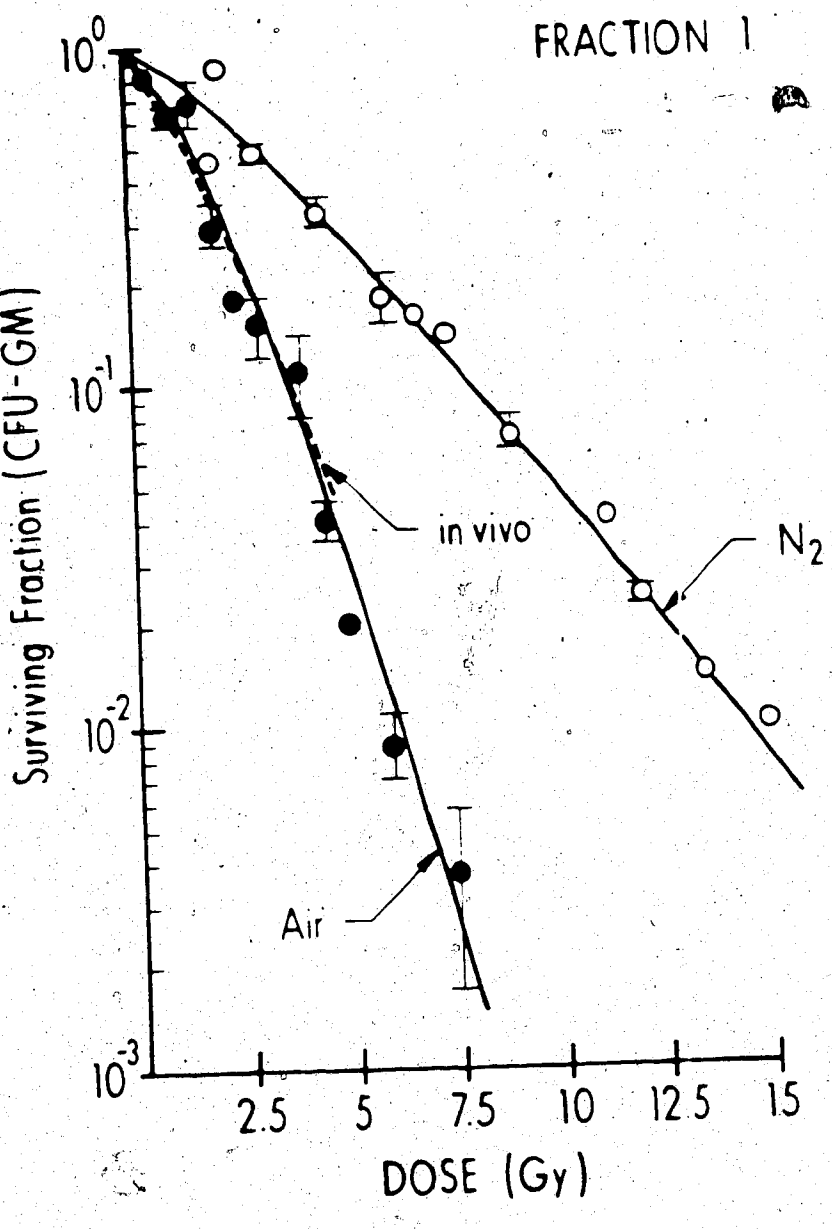

Figure 1 : The in vitro radiation sensitivity of Fraction 1 CFU-GM in air or in nitrogen. The in vivo sensitivity of Fraction 1 CFU-GM is depicted by the dashed line. Standard errors are provided for points at which three or more determinations were made.

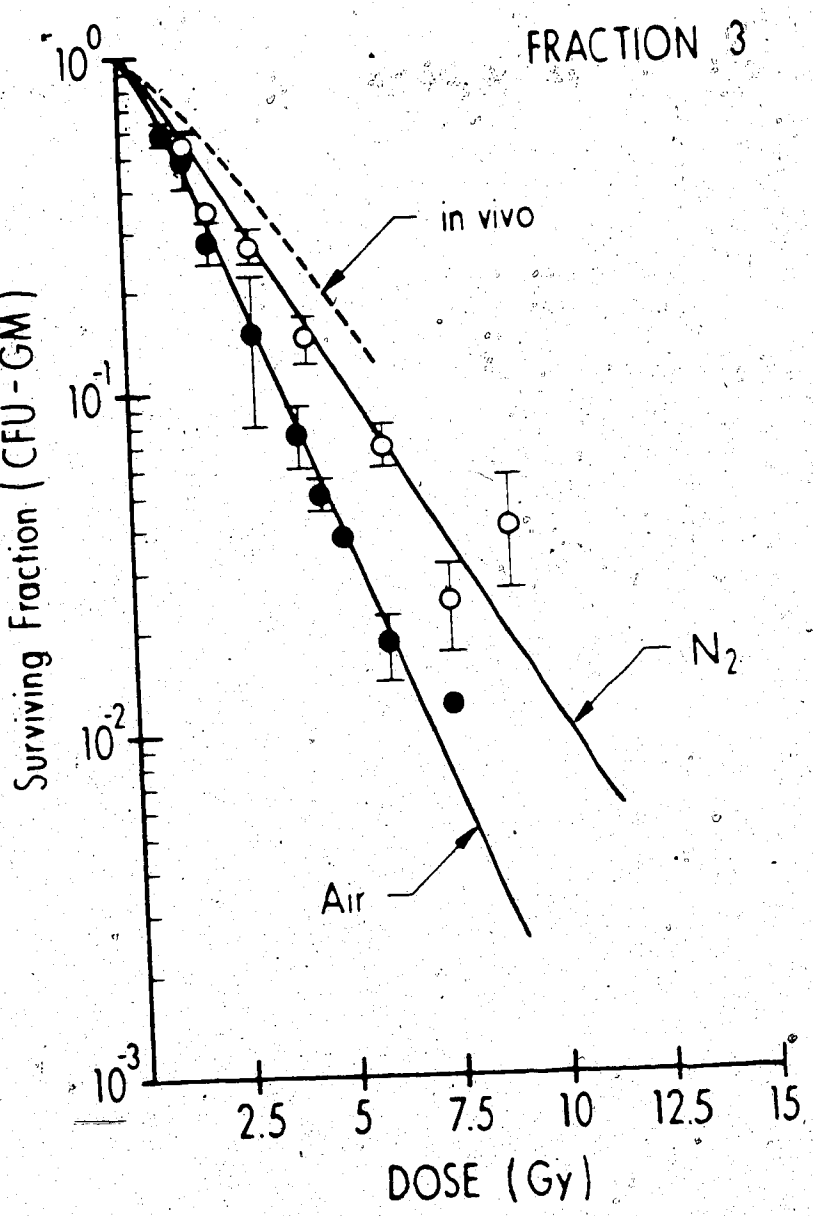

Figure 2 : The in vitro radiation sensitivity of Fraction 3 CFU-GM in air or in nitrogen. The in vivo sensitivity of Fraction 3 CFU-GM is depicted by the dashed line. Standard errors are provided for points at which three or more separate determinations were made.

level) for Fraction 1 and Fraction 3 were 2.2 and 1.5, respectively. For comparison, the irradiation inactivation data which was previously determined for Fraction 1 and Fraction 3 CFU-GM obtained from animals irradiated in vivo are indicated in each figure by the dashed line. Best fit values of α and β derived from the linear quadratic model of radiaton inactivation are shown in Table 2 for Fraction 1 and Fraction 3 cells. In this model, survival equals $\alpha D + \beta D^2$, where α defines the probability of radiation inactivation being produced by a single track lethal event and β defines the probability of the induction of a two track lethal event.

Cytotoxic Effects of MISO In Vitro

The survival of Fraction 1 and Fraction 3 CFU-GM after incubation in hypoxia with MISO is shown in Figures 3 and 4, respectively. No decrease in CFU-GM survival was observed with control populations of these cells incubated with various concentrations of MISO in air, nor in nitrogen without added MISO, for the exosure times investigated. Surviving fractions were computed and CFU-GM survival was found to decrease both as hypoxic exposure time and the concentration of MISO was increased. The relative cytotoxicity of different hypoxic cell sensitizers has been previously compared by plotting the reciprocal of the time required to kill the same proportion of cells by various concentrations of each drug (Chapman et al. 1979). In Figure 5, the reciprocal of time required to kill 50% of Fraction 1 and Fraction 3 CFU-GM is plotted versus the concentrations of MISO investigated. These data indicate

Table 2

α and β Values from the Linear Quadratic Model of Radiation Inactivation of Fraction 1 and Fraction 3 CFU-GM *In Vitro*

	α (Gy^{-1})	β (Gy^{-2})
Fraction 1		
Air	0.35 ± 0.14	0.072 ± 0.041
N_2	0.24 ± 0.045	0.0059 ± 0.0057
Fraction 3		
Air	0.54 ± 0.067	0.019 ± 0.016
N_2	0.46 ± 0.071	---

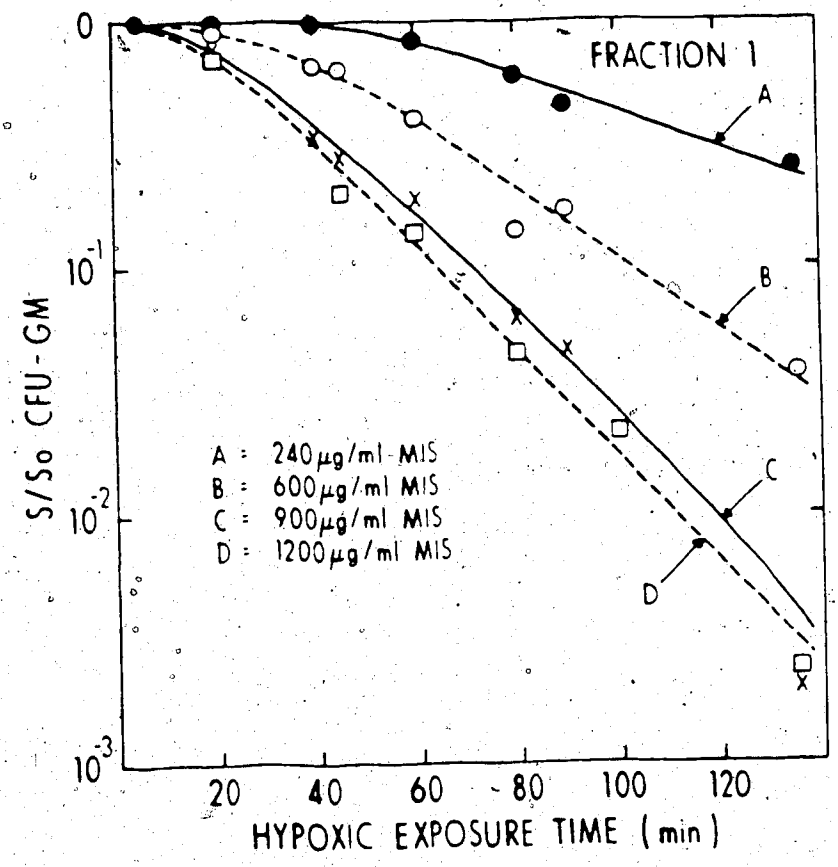

Figure 3 : The survival of Fraction 1 CFU-GM incubated in nitrogen with various concentrations of misonidazole. Pooled results from three separate experimental trials.

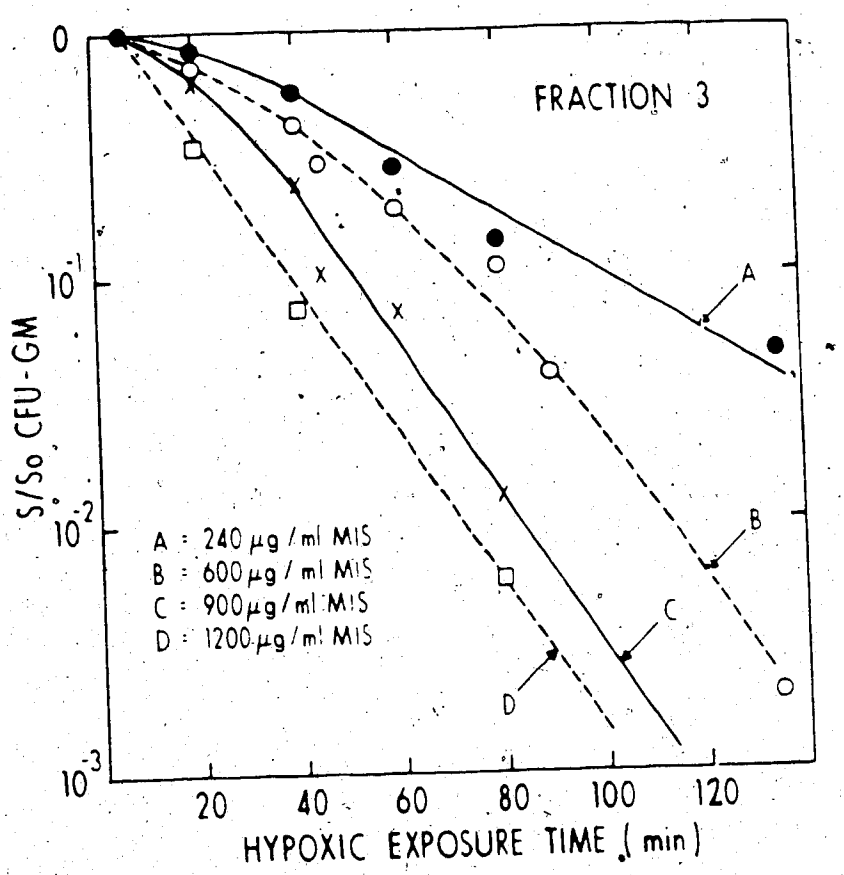

Figure 4 : The survival of Fraction 3 CFU-GM incubated in nitrogen with various concentrations of misonidazole. Pooled results from three separate experimental trials.

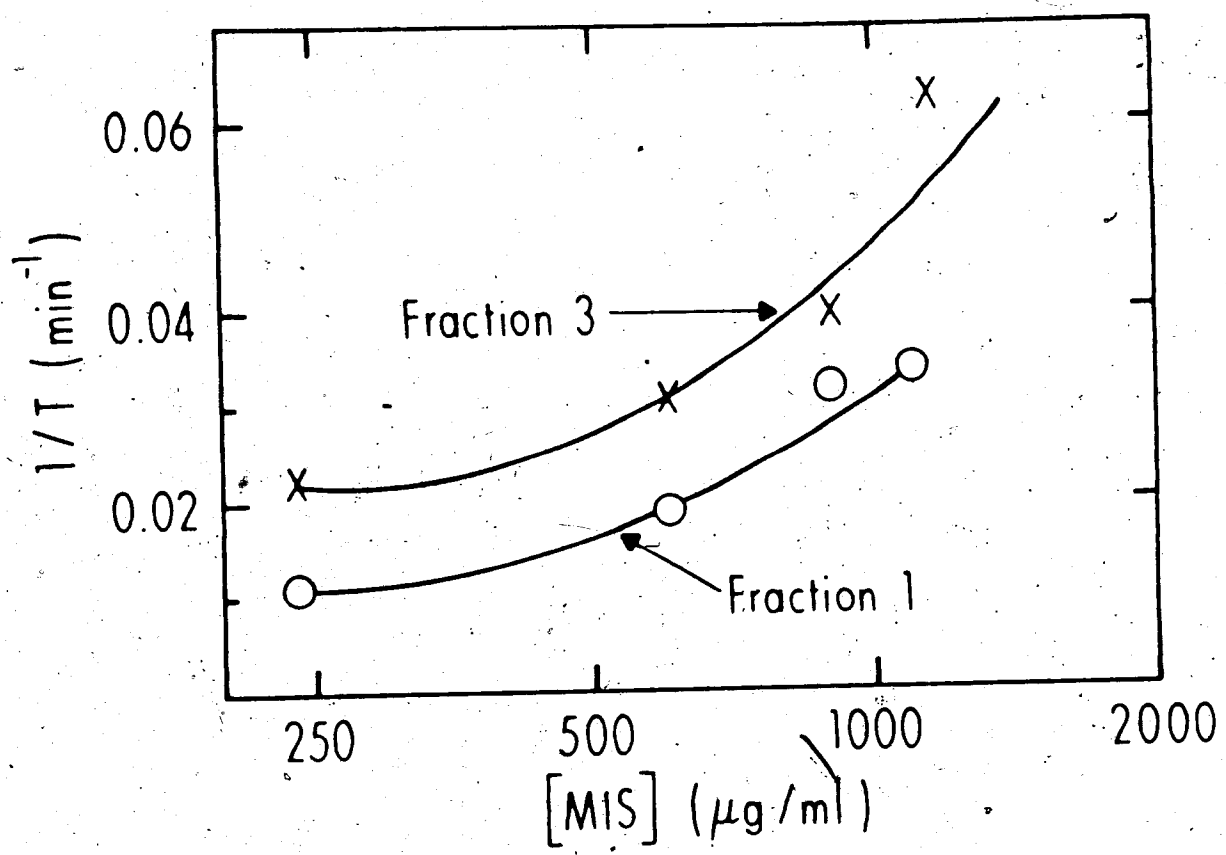

Figure 5. Plot of the reciprocol of the time required to kill 50% of Fraction 1 or Fraction 3 CFU-GM versus increasing concentrations of misonidazole. The data were derived from the results presented in Figures 3 and 4.

that Fraction 3 cells were approximately two times more sensitive to the cytotoxic action of MISO in vitro than were Fraction 1 cells.

Measurement of Intracellular GSH Levels

\Intracellular GSH levels were determined for duplicate samples of both Fraction 1 and Fraction 3 cells. The GSH content of Fraction 3 cells was approximately 42% of that measured in Fraction 1 cells. The results are presented in table 3.

Table 3

Intracellular Glutathione Levels in Fraction 1 and Fraction 3 Cells

	Glutathione (mol/10^6 cells)	
	Fraction 1	Fraction 3
Sample 1	4.0×10^{-10}	1.7×10^{-10}
Sample 2	2.8×10^{-10}	1.2×10^{-10}

Discussion

Investigators in Great Britian and Canada were among the first to promote the use of oxygen mimetic drugs in an attempt to radiosensitize hypoxic tumor cells (Adams 1973, Chapman et al 1974, Begg et al. 1974, Denekamp et al. 1974). Many compounds with varying electron affinities and partition coefficients have been synthesized and tested. The chemistry of these compounds, along with their mechanism of action, pharmacokinetics, effects on normal and tumor tissues, and interactions with radiation and chemotherapy have been the subject of several recent research symposia (Hypoxic Cell Sensitizers in Radiobiology and Radiotherapy 1977, Radiosensitizers : Their Use in the Clinical Management of Cancer 1979, Conference on Chemical Modifiers : Radiation and Cytotoxic Drugs 1981, Chemical Modifiers of Cancer Treatment 1983). In addition, the National Cancer Institute (USA) currently funds an active synthesis, pre-clinical screening program and clinical trials. One of the most promising agents to emerge from the basic and clinical investigations has been the 2-nitroimidazole compound, MISO. MISO has been shown as an effective hypoxic cells sensitizer in in vivo and in vitro tumor models although early clinical trials have reported no significant therapeutic gains (Fowler et al. 1976, Denekamp and Fowler 1978, Chapman et al. 1979, Chapman et al. 1981, Phillips et al. 1982). Because of the wealth of laboratory and clinical data generated with this drug, it has become the benchmark

to which the effectiveness of other sensitizing agents must be compared.

Because the bone marrow has been generally assumed to be a well-oxygenated tissue, there has been relatively little concern that MISO will have any myelotoxic effects. However, contradictory results were obtained when the effects of MISO on the hematopoietic system were investigated. Turner et al. (1980), in their survey of the effects of MISO on hematopoiesis in normal and tumor-bearing mice, found no evidence of radiosensitization or cytotoxicity. Similar results were obtained by Clement et al. (1980) and by Rose et al. (1980) using the CFU-S assay in normal mice. However, in the only study to date on the effects of MISO on human bone marrow, Allalunis et al. (1980) noted cytotoxic effects on CFU-GM following in vivo administration. When MISO was combined with chemotherapeutic agents, enhanced marrow damage was noted by Tannock (1980), Allalunis et al. (1982) and Pedersen et al. (1982). This finding led these workers to conclude that , because of incresed marrow damage, a therapeutic benefit may not be obtained with MISO.

The above studies were all performed with unseparated populations of marrow cells. The most common procedure for isolating these cells is to flush several passages of media through a mouse femur. This is particularly easy in the mouse as the central marrow cavity of the femur is relatively free of trabecular bone extensions. Most of the axial cells can be removed using this technique. Longer or more vigorous washing of the femur will also remove the cells which are located in the irregularly shaped niches of the endosteal

area. However, considerable numbers of cells are left behind, confined to discrete islands within the compact bone (Chan and Metcalf 1972, Meck and Mel 1973). A collagenase wash technique described by Kilgore et al. (1979) and modified by Allalunis et al. (1983) facilitates the recovery of hematopoietic cells from spatially distinct microenvironments within the mouse femur. Preliminary studies suggested that the sensitivities of the compact bone associated cells to irradiation and the effects of the hypoxic cell sensitizer, MISO, were significantly different from the sensitivities observed for pooled central cavity and endosteal region cells. A more complete radiobiological profile is provided in this report.

In Vivo Effects of MISO

When the effects of MISO on compact bone associated marrow cells were determined in vivo, a dose related cytotoxicity was observed (Allalunis et al. 1983). The results presented in this thesis have extended that original report to include an estimate of MISO's effect on marrow radiosensitivity. All animals were irradiated 30 min after injection with MISO, a time which corresponds to the peak plasma levels of the drug (Conroy et al. 1980, Workman 1980).

For Fraction 1, MISO had no radiosensitizing effect, as surviving fractions determined from saline and MISO treated mice were comparable. These results are similar to those reported in the first surveys of MISO's effect on the bone marrow and suggest that Fraction 1 cells are relatively well oxygenated.

In contrast, Fraction 3 cells were radioresistant in vivo. This radioresistance has been previously described (Allalunis et al. 1983) and is in agreement with the reports of Millard and Blackett (1981) and Greenberger et al. (1980) who noted a radioresistant sub-population of CFU-GM in murine marrow. Pre-treatment with MISO was able to abrogate this radioresistance. A dose modifying factor (DMF) of 1.3 was estimated at the 37% survival level. This value is somewhat greater than that observed by other workers who studied the radiosensitizing effects of MISO in murine tumor models (Denekamp 1974, Brown 1975). However, the DMF observed for Fraction 3 may reflect both the radiosensitizing and the previously described cytotoxic effects of MISO for this population of cells.

Misonidazole has also been reported to sensitize other normal rodent tissues including skin (Brown 1975, Stewart et al. 1982), spermatogenic stem cells (Suzuki et al. 1977) and spinal cord (Yuhas 1979). These findings suggest that a range of oxygen tensions may exist within normal tissues, and that mildly hypoxic populations of normal cells may be at risk when hypoxic cell sensitizers such as MISO are employed.

In Vitro Radiosensitivity

The aerobic response of Fraction 1 and Fraction 3 cells to irradiation was similar to that described by other workers. Senn and McCulloch (1970) and Testa et al. (1973) have reported D_0 values which range from 1.6 to 1.8 Gy. The aerobic sensitivities reported here fall within that range. For comparison, the radiation

inactivation of Fraction 1 and Fraction 3 cells obtained from air breathing animals irradiated *in vivo* was provided. For Fraction 1, *in vivo* and *in vitro* radiation sensitivities were not significantly different. In contrast, the sensitivity of Fraction 3 cells irradiated *in vivo* was considerably less than that observed for Fraction 1 cells (Allalunis *et al.* 1983). This result has suggested that part of the radioresistance observed for Fraction 3 cells *in vivo* may be attributable to the relative hypoxia of their microenvironment. Misrahy *et al.* (1962) and Brookes (1968) have described a reduced pO_2 and blood flow for this region of the femur, factors which could combine to produce a mildly hypoxic environment for these cells.

The irradiation of Fraction 1 cells *in vitro* under conditions of extreme hypoxia produced radioresistance, yielding an average OER value of 2.2. Earlier studies using unseparated murine bone marrow cells have reported OERs ranging from 2 to 2.6 (Till 1963, Blackett *et al.* 1974). In contrast, when Fraction 3 cells were irradiated *in vitro* under conditions of extreme hypoxia, an average OER of 1.5 was obtained. A difference between Fraction 1 and Fraction 3 cells irradiated in hypoxia suggests an difference in inherent radiosensitivity. A similar pattern of radiation response has been observed for GSH deficient cells. Comparable sensitivities were observed when normal fibroblasts and fibroblasts obtained from patients with 5-oxyprolinuria (a genetic disease characterized by decreased levels of intracellular GSH) were irradiated under aerobic conditions. However, when irradiated under hypoxic conditions, the

GSH deficient cells were relatively more sensitive and therefore exhibited a lower OER (Deschavanne et al. 1981, Malaise 1983, Astor 1984). Increased sensitivity to irradiation under hypoxic conditions has also been described for normal cell which had been artificially depleted of GSH with diethylmaleate, an agent which binds GSH (Bump et al. 1982). These results support the competition theory of radiation damage (Chapman et al. 1973). In this model, electron affinic species which oxidize free radicals compete with hydrogen donating species which reduce free radicals. The oxidation of free radicals results in damage fixation, whereas radical reduction results in damage repair. In this case, a reduced concentration of intracellular GSH suggests that fewer hydrogen donating species would be available to repair radiation induced radicals in critical targets (Midander et al. 1982). These unrepaired lesions would contribute t the enhanced cell kill which is manifest as a lower OER. The aerobic sensitivity is not as affected since thiols do not compete as effectively as oxygen for target radicals (Biaglow and Varnes 1983). The in vitro results obtained in this study are compatible with the competition model of radiation damage. Fraction 3 cells were found to contain approximately one half the concentration of intracellular GSH as was found in Fraction 1 cells. It is therefore likely that the increased sensitivity of cells irradiated under hypoxic conditions may, in part, be due to their reduced GSH content.

Misonidazole Cytotoxocity

The results obtained when the cytotoxic effects of MISO were

assessed *in vitro* suggest that an inherent difference in sensitivity may also contribute to the response of Fraction 3 to MISO. When Fraction 1 and Fraction 3 cells were exposed to MISO *in vitro* under aerobic conditions, no decrease in CFU-GM survival was observed. When cells were incubated with MISO under hypoxic conditions, CFU-GM survival decreased both as the drug concentration and the exposure time increased. When the relative sensitivities of each populaion were evaluated by comparing the time required to kill an equivalent proportion of cells, it was observed that Fraction 3 cells were approximately twice as sensitive to MISO hypoxic toxicity as were Fraction 1 cells.

The factors which contributed to the differences in *in vitro* and *in vivo* radiosensitivity and cytotoxicity observed for Fraction 1 and Fraction 3 cells have yet to be elucidated. It is unlikely that differences in cell cycle parameters have contributed to these effects. Chapman *et al*. (1971), in studies of V79 cells, established that the OER is constant for all phases of the cell cycle with the exception of mitotic cells (Chapman *et al*. 1979). As regards the difference in sensitivity to MISO's cytotoxic effects, one may postulate that differences in the quantity or composition of nitroreductase enzymes could contribute to the observed differences. However, the more probable explanation for the difference in both radiosensitivity and cytotoxicity observed between Fraction 1 and Fraction 3 cells is the difference in their respective intracellular GSH contents. Astor *et al*. (1983) have shown that human cell lines which are deficient in GSH are more sensitive to MISO's cytotoxic

effects. Similarly, normal cells which have been depleted of GSH with diethyl maleate also express an enhanced sensitivity to MISO (Bump et al. 1983).

These data have led us to postulate that some bone marrow cells which reside in a relatively hypoxic microenvironment within compact bone may acquire a level of endogenous thiols which is significantly lower than is found in marrow cells which reside in the central regions of the femur. Further studies may identify additional factors which contribute to the difference in inherent radiosensitivity and drug sensitivity of Fraction 1 and Fraction 3 cells.

CHAPTER FOUR

Results

Radioprotective Effects of (DDC)

The effects of a single dose of DDC (15 mg) on the radiation survival of Fraction 1 CFU-S and CFU-GM are presented in Figures 6 and 7, respectively. Dose modifying factors (DMFs), measured at the 37% survival level, ranged from 1.4 to 2.9 when DDC and saline treated animals were compared. However, when the DMFs were calculated using normalized values obtained from DDC treated, unirradiated animals, a range of 0.9 to 1.6 was observed. Similar results were obtained when a single dose of 30 mg DDC was used, with the dose modifying factors calculated from normalized data ranging from 0.9 to 1.1. In a companion experiment which calculated the effects of WR-2721 on CFU-GM survival, calculations of dose modifying factors based on normalized data yielded range of 1.9 to 3.4. These results are depicted in Figure 8.

Figure 9 presents the increase observed in Fraction 1 CFU-GM following treatment with WR-2721 or DDC. A single dose of DDC (15 mg) resulted in an increase of CFU-GM obtained from C57Bl/10J bone marrow (CER =2.5) and spleen (CER = 3.9), and from C3H/HeJ bone marrow (CER = 1.9). A single dose of WR-2721 (400 mg/kg) resulted in a CER of 1.3 for C57Bl/10J marrow cells.

The radioprotective effects afforded by multiple doses of DDC are shown in Figure 10. Following multiple doses of DDC, mice were refractory to its stimulatory effects on CFU-GM proliferation (CER =

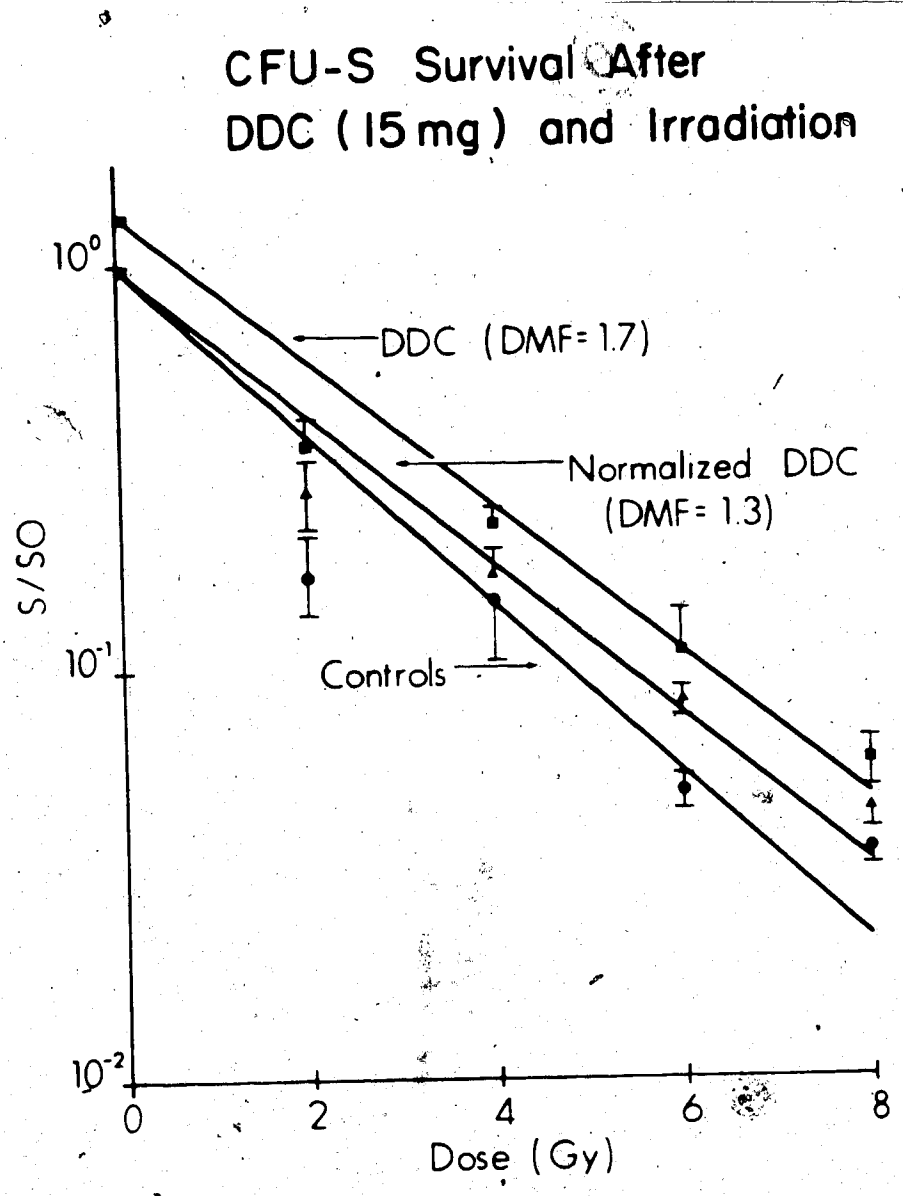

Figure 6 : The survival of bone marrow CFU-S after in vivo treatment with a single dose of DDC and irradiation. A representative experiment from three separate trials is shown. Reprinted with permission [Int. J. Radiat. Oncol. Biol. Phys. 10:1569, M.J.Allalunis-Turner, J.D. Chapman, Evaluation of DDC as a radioprotector of bone marrow.] Copyright 1984 Pergamon Press, Ltd.

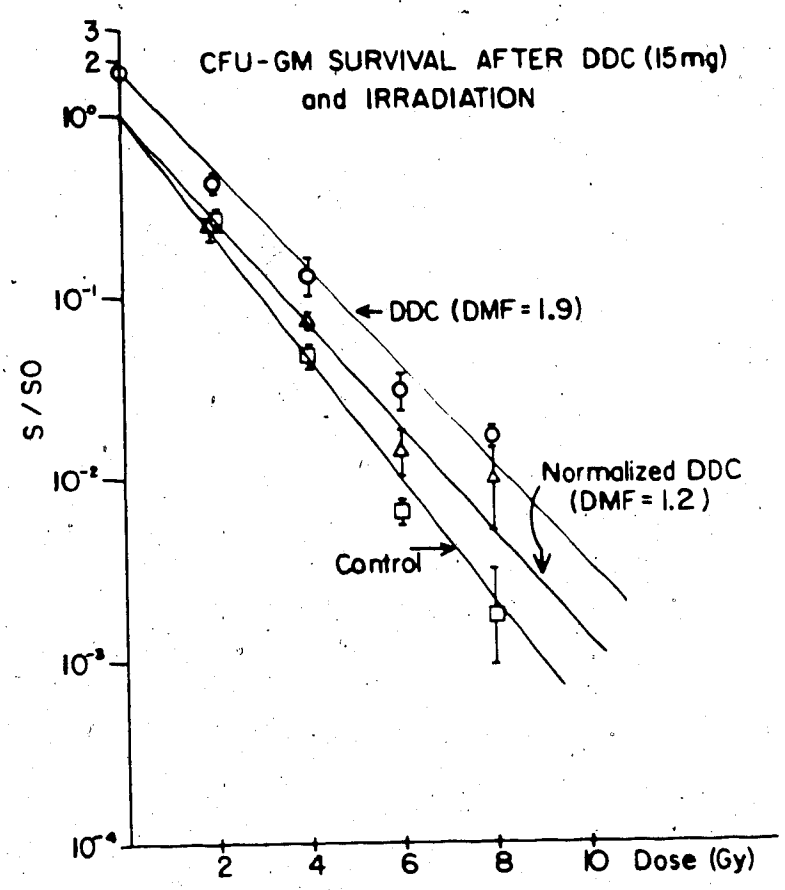

Figure 7 : The survival of bone marrow CFU-GM after in vivo treatment with a single dose of DDC and irradiation. A representative experiment from six separate trials is shown. Reprinted with permission from [Int. J. Radiat. Oncol. Biol. Phys. 10:1569, M.J.Allalunis-Turner, J.D. Chapman, Evaluation of DDC as a radioprotector of bone marrow] Copyright 1984 Pergamon Press, Ltd.

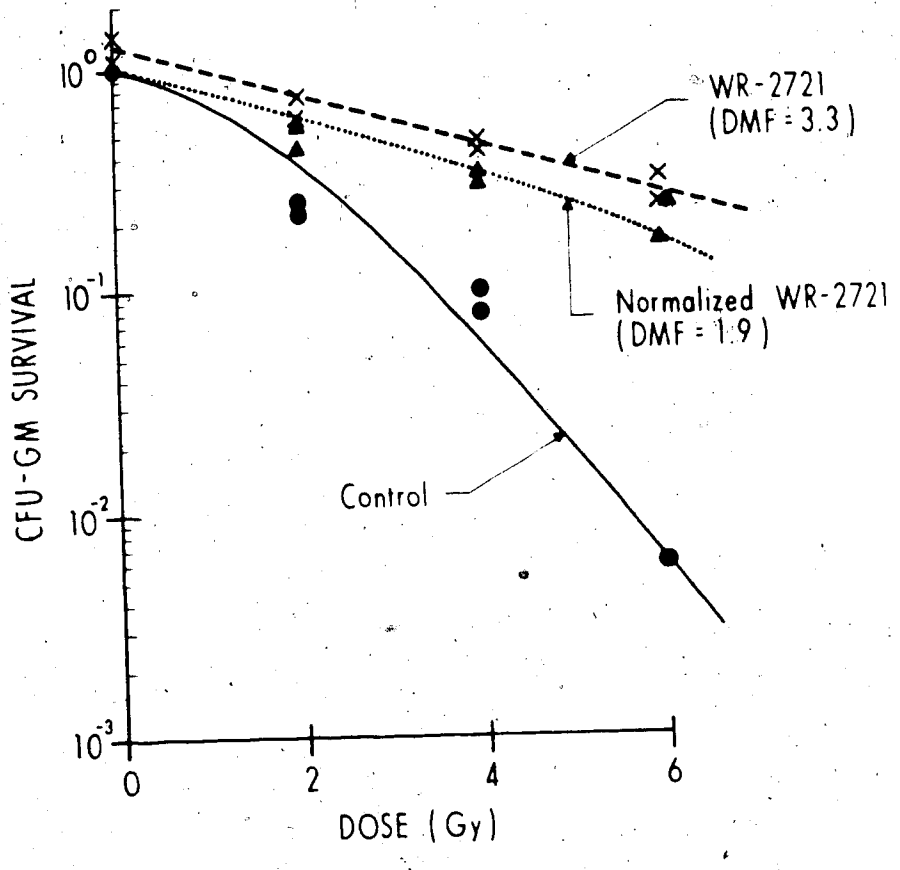

Figure 8 : The survival of bone marrow CFU-GM after in vivo treatment with a single dose of WR-2721 and irradiation. The pooled data from two separate trials are shown.

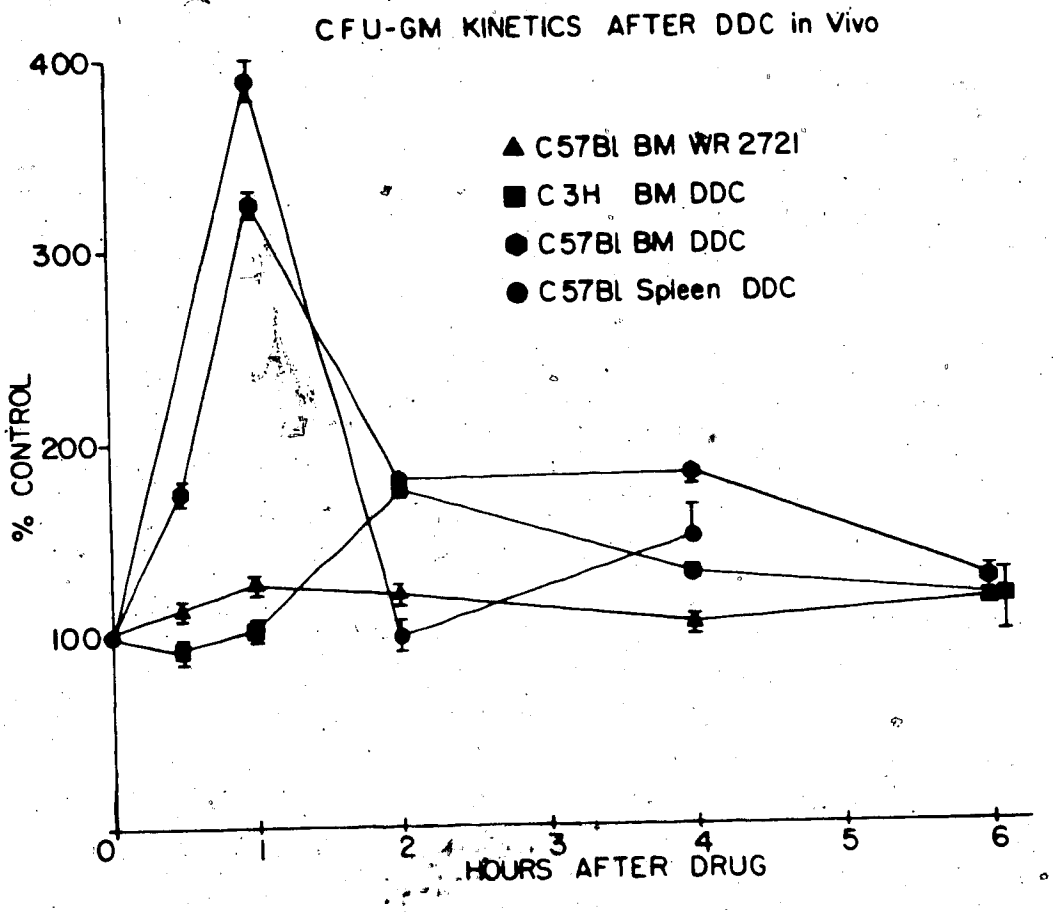

Figure 9 : The survival of bone marrow and spleen CFU-GM after in vivo treatment with a single dose of DDC or WR-2721. The pooled data from four separate trials are shown. Reprinted with permission from [Int. J. Radiat. Oncol. Biol. Phys. 10:1569, M.J.Allalunis-Turner, J.D.Chapman, Evaluation of DDC as a radioprotector of bone marrow] Copyright 1984 Pergamon Press, Ltd.

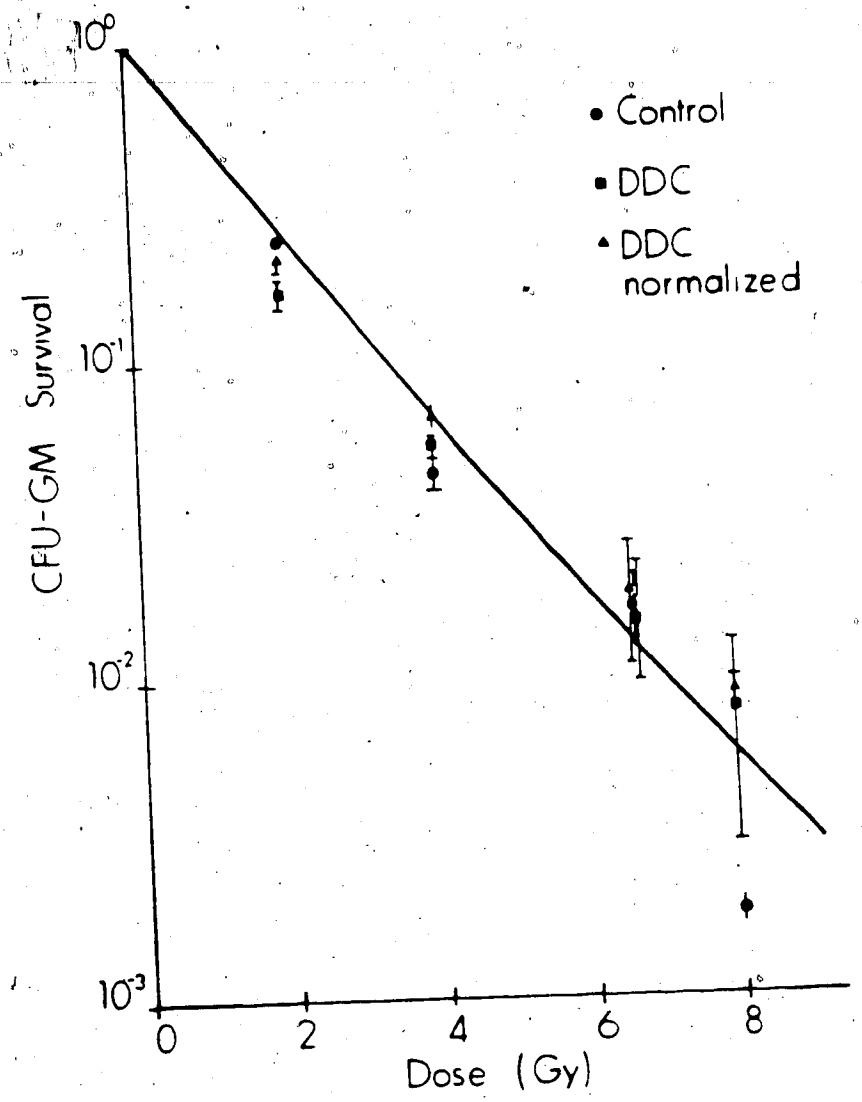

Figure 10 : The survival of bone marrow CFU-GM after in vivo treatment with multiple doses of DDC followed by irradiation. The pooled data from two separate trials are shown. Reprinted with permission [Int. J. Radiat. Oncol. Biol. Phys. 10:1569, M.J. Allalunis-Tuner, J.D.Chapman, Evaluation of DDC as radioprotector of bone marrow]. Copyright 1984 Pergamon Press, Ltd.

1.1 to 1.3). In addition, little or no radioprotection was observed (DMFs ≐ 1.0 to 1.2).

Discussion

In 1949, Patt and colleagues reported that treatment with cysteine before irradiation protected rats from bone marrow related death. Since that time, there has been an active search for other agents which would be selectively radioprotective for normal tissues with little protection for tumors. Because the bone marrow is often the dose limiting organ when large fields of irradiation are required, many studies have employed hematopoietic damage as the biological endpoint.

Search for marrow radioprotectors have evolved along two distinct paths. The first approach has been to modulate the post-irradiation recovery and proliferation of hematopoietic cells. Treatments as diverse as post-irradiation bleeding (Boggs et al. 1972) or pre-irradiation injection with endotoxin (Hanks and Ainsworth 1964, Smith et al. 1966a and 1966b, Boggs et al. 1968, Stevenson et al. 1981), colchicine (Brecher et al. 1967), carbon particles (Mori and Nakamura 1970, Nakamura 1972, Fujita et al. 1983), cytosine arabinoside (Millar et al. 1978 and 1982), methotrexate (Millar et al. 1978) and cyclophosphamide (Blackett and Aguado 1979) have been reported to protect the hematopoietic system from irradiation. Among the indicators of enhanced recovery noted by the above workers were post-irradiaton increases in marrow CFU-S, CFU-GM, endogenous CFU, marrow cellularity, $LD_{50/30}$ survival rates, serum CSF and spleen iron uptake.

An alternative approach to the use of biological modifiers of hematopoietic response has been the attempt to protect normal tissues at the molecular level by promoting the repair of radiation induced free radicals. Compounds containing a hydrogen-donating species, such as sulfhydryl amines, can effectively reduce free radicals. Cysteamine (Devik and Lothe 1955, Nelson et al. 1963, Smith et al. 1966c), cysteine (Devik and Lothe 1955) and AET (aminoethylisothiurea dihydrobromide) (Vittorio et al. 1969) were effective in chemical systems and in some animal models. However, the large doses which were often required to produce effective radioprotection in vivo were also associated with unacceptable systemic toxicities. In the early 1960's, the Anti-Radiation Drug Development Program of the United States Army began a systematic search for compounds which would be effective radioprotectors at physiologically tolerable concentrations. WR-2721 was among the most promising compounds developed. Early screening in mice carrying a transplantable mammary carcinoma demonstrated dose modifying factors of 2.4 for skin and 2.7 for bone marrow in the absence of appreciable tumor protection (Yuhas and Storer 1969). This initial report was soon followed by many others describing the effectiveness of WR-2721 both as a radioprotector and as a chemoprotector (reviews by Phillips 1980, Yuhas et al. 1980, Yuhas 1982).

While experimental and clinical trials with WR-2721 continue, the screening of other protective compounds is proceeding. Early studies by Van Bekkum (1956) suggested that analogues of dithiocarbamic acid were effective marrow radioprotectors. Based on

this observation, several investigators have proposed that DDC could offer selective normal tissue protection (Evans et al. 1983, Milas et al. 1984). The studies in this thesis have evaluated the ability of DDC to protect normal murine marrow and have compared its effects to those of WR-2721.

Radioprotective Effects of DDC

The radioprotective effects of single or multiple doses of DDC are modest as compared to those observed for WR-2721 (Allalunis-Turner and Chapman 1984). Average DMFs of 1.3 and 1.2 were observed for CFU-S and CFU-GM, respectively. These values were lower than those observed by other investigators who used estimates of $LD_{50/30}$ dose or CFU-S survival as endpoints (Evans et al. 1983, Milas et al. 1984). These values were also markedly lower than those reported by Phillips et al. (1973), Yuhas et al. (1980) and Wasserman et al. (1981) in their studies of WR-2721. This finding suggests that WR-2721 will afford greater marrow protection in radiotherapy protocols in which the bone marrow is the dose limiting organ.

The differences in DMF observed in this study and the large values reported by other workers was likely due to the resolution of two separate effects of DDC on the bone marrow. A single dose of DDC caused a marked increase in the number of bone marrow and spleen CFU-S and CFU-GM. The post-irradiation surviving fraction must be normalized to account for this non-specific stimulation of stem cells. Consequently, the resultant estimates of the radioprotective

effects of DDC were significantly lower than those observed by other investigators. $LD_{50/30}$ assays or endogenous colony assays used by others cannot distinguish this stimulatory effect, as un-irradiated, DDC treated control animals cannot be incorporated into the design of these experiments. For this reason, the direct radioprotective effects of DDC on bone marrow stem cells were possibly overestimated.

The non-specific stimulation of hematopoietic stem cells by DDC was similar to that described for marrow cells stimulated by non-thiol agents such as endotoxin and carbon particles. The mechanism by which DDC stimulated hematopoietic cells has yet to be determined. One possible explanation could be its ability to trigger cells into cycle and thus into a more radioresistant phase. This effect has been described for endotoxin (Smith et al. 1966b, Quesenberry et al. 1972 and 1973) but remains to be established for DDC. Similarly, an increase in growth factor production could enhance the post-irradiation repopulation of stem cells (Fujita et al. 1983), a phenomenon which also awaits investigation.

It is unlikely that the effects of DDC are due to endotoxin contamination. When C3H/HeJ mice were challenged with DDC and assayed for CFU-GM survival, a small increase was observed. Because C3H mice are genetically incapable of responding to endotoxin (Sultzer 1968), these results suggest that endotoxin contamination was not responsible for the effects observed with DDC.

An additional factor which would limit the usefulness of DCC in fractionated radiotherapy protocols was its lack of effectiveness in a multi-dose treatment schedule. Little radioprotection or

hematopoietic cell stimulation was seen in mice which had three consecutive daily treatments with DDC. In contrast, WR-2721 is able to offer equivalent or only slightly reduced protection when used in a ten dose schedule (Utley et al. 1976).

The small radioprotective activity which can be observed with DDC is likely due to the presence of thiol groups. Stromme and Eldjarn (1966) have identified metabolites of DDC, including free thiol groups, bound to liver and spleen nuclei, mitochondria and microsomes, and have suggested that a concentration of hydrogen donating species in the sub-cellular fraction may offer protection to target molecules.

In conclusion, these studies suggest that DDC will be of limited use as a clinical radioprotector. An enhanced proliferation of stem cells similar to that observed for hematopoietic cells may not be observed for other normal tissues whose stem cell renewal systems possess a different hierarchical structure. In addition, Milas et al. (1984) have reported that DDC has a small protective effect on the FSa tumor and its micrometastases. Because the normalized values for DDC marrow protection are also relatively small, it is unlikely that a significant therapeutic gain could be acheived. Finally, the reports that DDC has radiosensitizing effects on neutrophils (Rigas et al. 1978), T-cells (Rigas et al. 1980) and erythrocytes (Stone et al. 1978) add a cautionary note to the contemplation of the clinical use of DDC.

CHAPTER FIVE

Results

Bone Marrow Regeneration After Total Body Irradiation

This series of experiments compared the kinetics of the regeneration of Fraction 1 and Fraction 3 CFU-GM following total body irradiation with 3 Gy X-rays. The effects of treatment with MISO before irradiation were also evaluated. The [illegible]ed results of three experimental trials are presented in Figures 11, 12, and 13. Fraction 1 and Fraction 3 CFU-GM were decreased to comparable levels one to two days following irradiation. An abortive rise in colony number was observed at days three to four, followed by a further decline in colony number to less than 10% of control values. Except for a single peak at day 17 observed with Fraction 1 cells, both Fraction 1 and Fraction 3 CFU-GM remained below control levels for the 65 day span of the experiment. The effects of pre-irradiation treatment with MISO on CFU-GM survival is depicted in Figures 12 and 13. In the initial period after irradiation (days 1 to 3), an enhanced cell kill was observed for both Fraction 1 and Fraction 3 CFU-GM obtained from animals which had been treated with MISO. At latter recovery times (days 4 to 28), no significant differences were noted between saline and MISO treated groups. However, the data obtained on days 43 to 65 suggests that long term recovery may have been compromised by MISO treatment.

The ability of Fraction 1 and Fraction 3 bone marrow cells to repair potentially lethal damage in vivo is presented in Figure 14. The surviving fractions of CFU-GM were obtained at 30 minutes, six

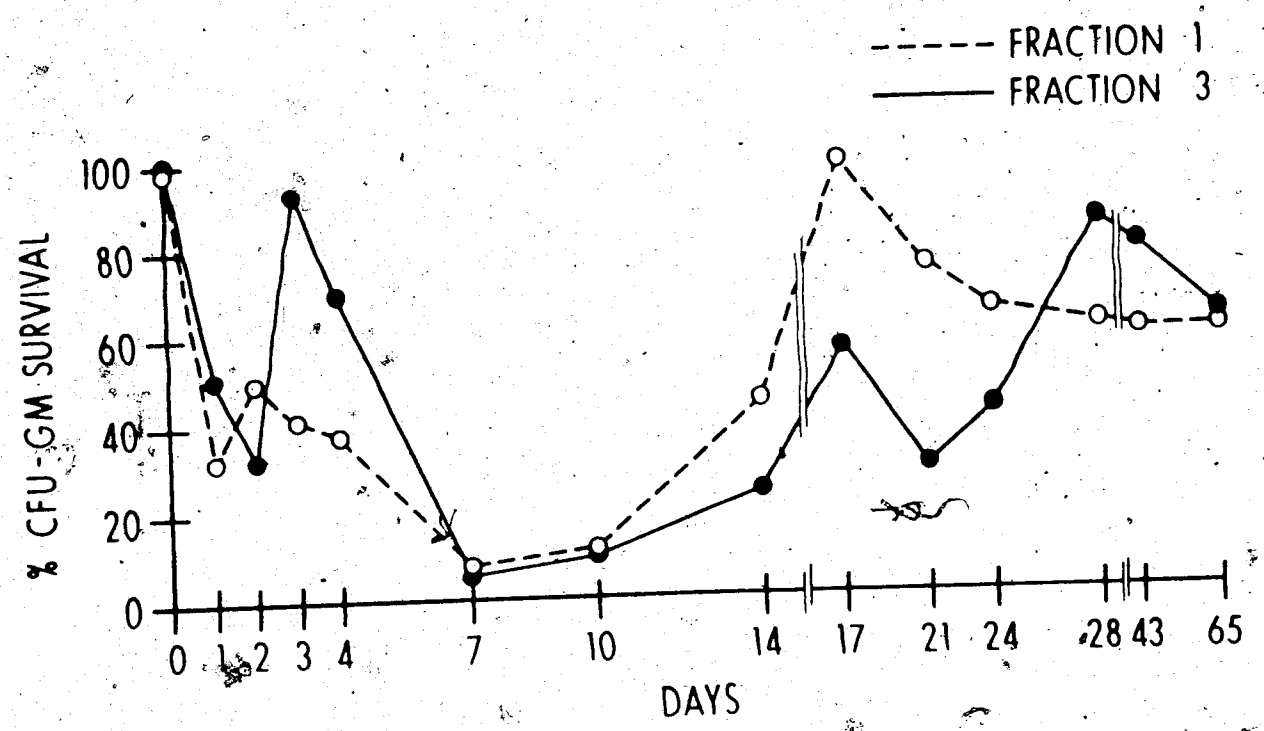

Figure 11 : The survival of Fraction 1 and Fraction 3 CFU-GM after total body irradiation (3 Gy). The pooled results from three separate trials are shown. The surviving fractions are expressed as a percentage of the number of colonies obtained from unirradiated control mice.

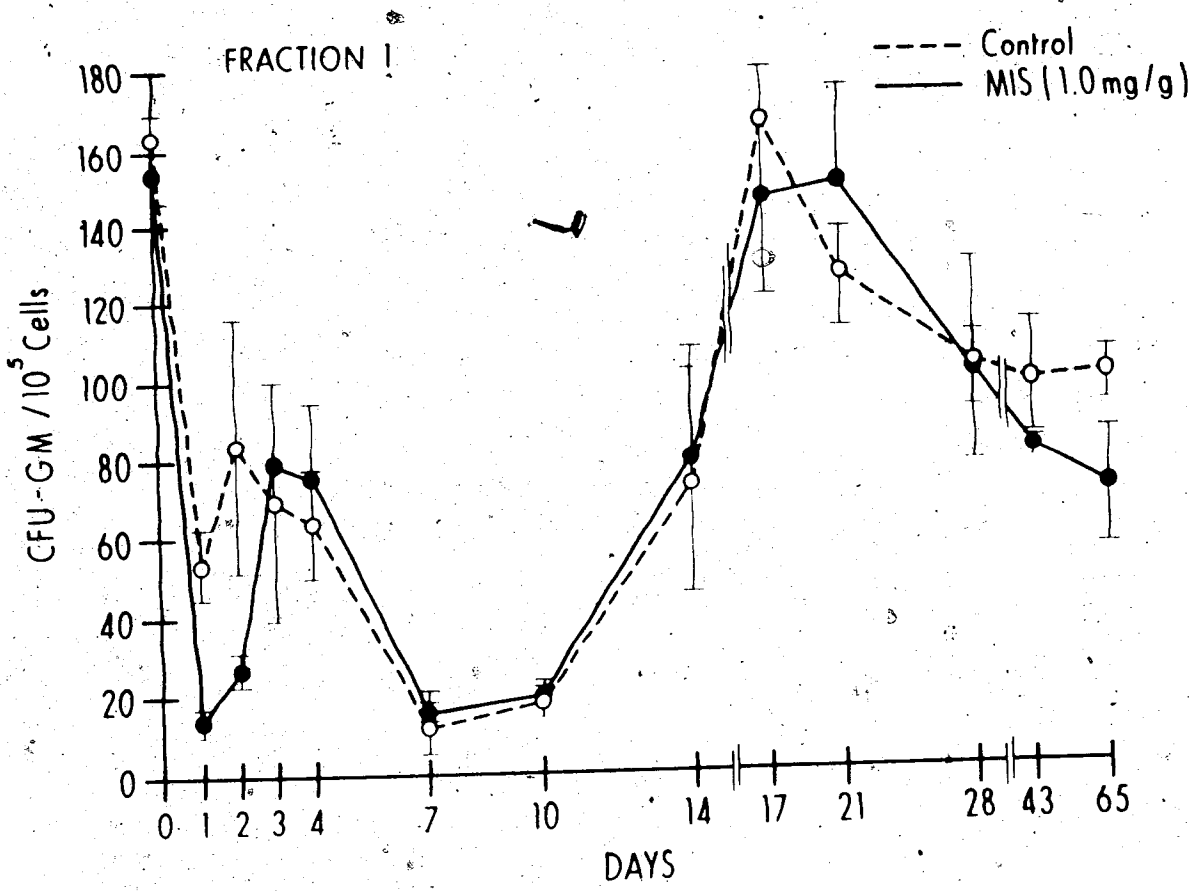

Figure 12 : The effect of in vivo treatment with misonidazole on the survival of Fraction I CFU-GM after total body irradiation (3 Gy). The pooled results from three separate trials are shown.

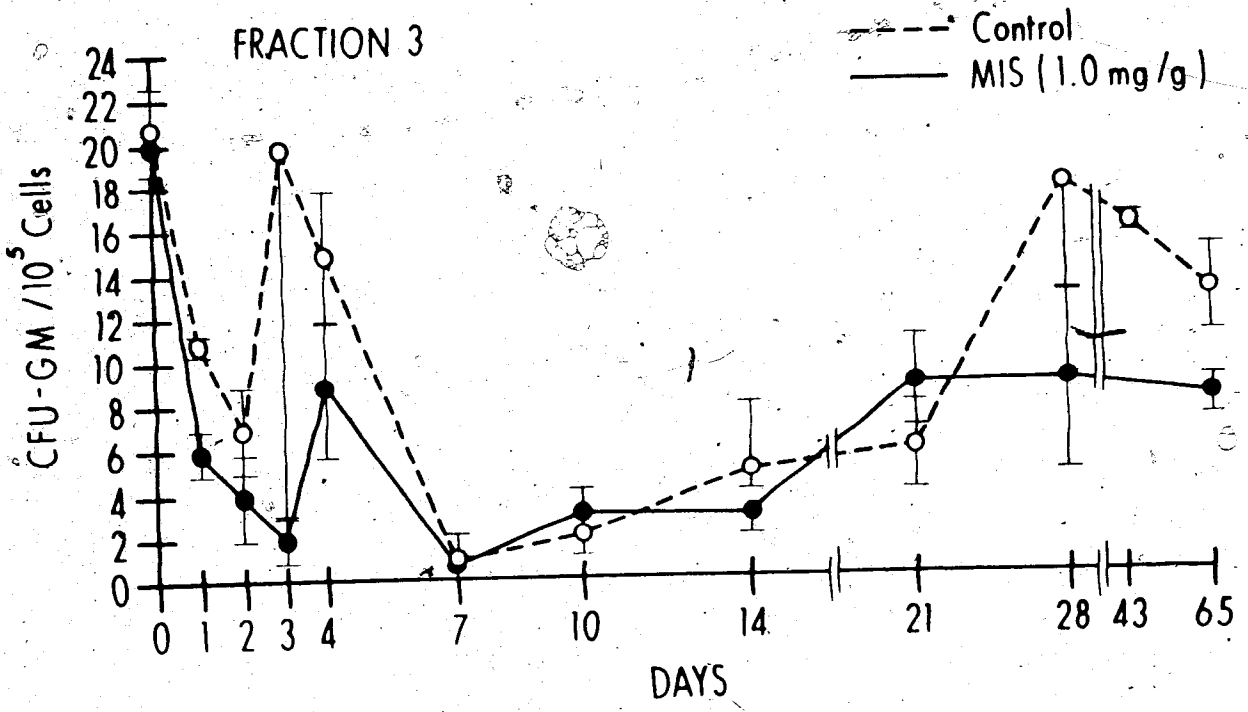

Figure 13 : The effect of in vivo treatment with misonidazole on the survival of Fraction 3 CFU-GM after total body irradiation (3 Gy). The pooled results from three separate trials are shown.

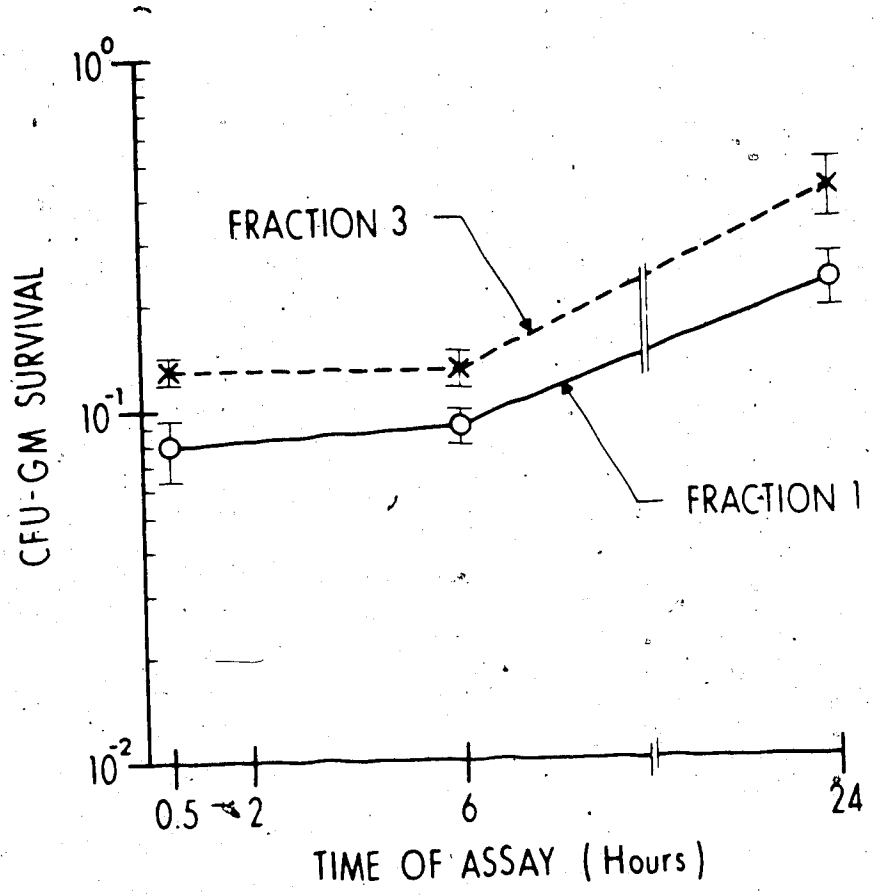

Figure 14 : The survival of Fraction 1 and Fraction 3 CFU-GM measured at various times after total body irradiation (3 Gy). The pooled results from two separate trials are shown.

hours and 24 hours after 3 Gy irradiation. No evidence of repair was noted in the first six hours following irradiation, an amount of time which has been described as sufficient for potentially lethal damage repair to occur (Little et al. 1973). Some evidence of recovery or repopulation was noted at 24 hours for Fraction 1 and Fraction 3 cells as is indicated by the increased survival.

Discussion

Marrow Regeneration After Total Body Irradiation

The effects of irradiation on the histological appearance of the bone marrow have been well described (Casarett 1980a, Harris 1981). The early effects, which occur within minutes or hours, include a G_2 block of proliferating cells, disruption of the microvasculature with hemorrhage and clot formation, reproductive death of radiosensitive cells following recovery from G_2 and mitotic block, cellular and chromosomal aberrations, and lysis of damaged cells. The intermediate phase, occurring a few days later, is characterized by the removal of cellular debris and clotted material and results in a marrow of hypoplastic appearance. Radiosensitive cells continue to undergo mitotic death, mature blood elements become depleted, and small, abortive foci of regenerating colonies appear. The final recovery phase, following a dose dependent period of hypoplasia, is usually accompanied by an "overshoot" of regenerating cells, with a gradual return of differentiated cells to normal limits within a few weeks.

A similar pattern of damage and regeneration has been described for both pluripotential and committed hematopoietic cells following single doses of whole body irradiation. The results presented in this chapter compare the regeneration kinetics of Fraction 1 and Fraction 3 CFU-GM following a sub-lethal dose of whole body irradiation.

The regeneration pattern of Fraction 1 CFU-GM was similar to

that described by other workers (Hall 1969, Chen and Schooley 1970, Chan and Metcalf 1973, Sugavara and Shikita 1982). Specifically noted was a rapid initial decline in CFU-GM, followed by an abortive rise which was one half the value of the subsequent recovery peak (Harris 1981). The recovery to control levels was followed by a gradual decline to a characteristic sub-normal values (Covelli and Metalli 1973, Ainsworth et al. 1974).

A somewhat different pattern was observed for Fraction 3 CFU-GM. The initial nadir was delayed for 24 hours. Similarly, the final recovery peak was delayed relative to that observed for Fraction 1 cells. The differences observed between Fraction 1 and Fraction 3 cells suggest that Fraction 3 cells may have a lower mitotic frequency. Casarett (1980b) has postulated that although mitotic linked cell necrosis is dose dependent, the relative frequency of cell division (or length of interphase) is also reflected in the estimate of cell death following irradiation. If Fraction 3 cells have a lower mitotic frequency, they would require more time for the initial damage to become expressed. Similarly, a longer period of time would be required for cells to regenerate to pre-irradiation levels. Both of these phenomena have been observed with Fraction 3 cells, as initial damage was manifest 24 hr later and the recovery peak was not acheived until 11 days after that of Fraction 1 cells.

The diminished levels after recovery observed for Fraction 1 and Fraction 3 cells at later times (four to nine weeks post irradiation) are consistent with the results of Ainsworth et al. (1974), who noted persistant, late CFU-S depression (approximately 50% of

control values) following fractionated or single doses of neutron or gamma irradiation. These workers have concluded that the direct effects of stem cell killing were largely responsible for the reduced number of CFU-S observed as late as one year after irradiaton. Their concommitant studies of the effects of irradiation on the microvasculature were inconclusive. However, other studies support the postulate that damage to the stroma and microvasculature of marrow contribute to many of the late effects observed after irradiation. (Chamberlin et al. 1974, Nelson et al. 1974, Bentley 1982). Reduced blood flow (Pitkanen and Hopewell 1983), progressive arteriolo-capillary fibrosis (Casarett 1980a), venous and arterial tortuosities (Ainsworth et al. 1974) and telangectasia (Rubin 1984) have been described as late consequences of irradiation. In addition, stromal damage measured as failure to support ectopic marrow (Chertov and Gurevitch 1979) or deletion of stromal cells (Werts et al. 1980) has been observed. Because maintainance of hematopoiesis requires an intact stroma and microvasculature (Trentin 1971, Bentley 1982) the above factors may contribute to the diminished survival observed for Fraction 1 and Fraction 3 cells in the final weeks after irradiation.

The effects of MISO on marrow regeneration have also been evaluated for Fraction 1 and Fraction 3 cells. No radiosensitizing effects have been previously noted for unseparated marrow (Turner et al. 1981) or for Fraction 1 cells (Allalunis et al. 1983) assayed immediately after irradiation. In these studies, when CFU-GM survival was assayed 24 to 48 hours after irradiation, enhanced

damage as measured by a decreased survival was noted for both cell fractions. Because the survival level measured at 24 hours is a function of dose (El-Nagar et al. 1984), the effects of MISO was equivalent to having administered a larger initial dose of irradiation (ratio of colony surviving fractions equal to 2.3 and 1.8 for Fraction 1 and Fraction 3 respectively measured at 24 hours). A similar enhancement of damage by MISO has been described for marrow recovering following treatment with cytotoxic agents. In these studies, mice treated with MISO in addition to cyclophosphamide or BCNU had an impaired recovery as measured by white blood cell count (Tannock 1980), CFU-GM (Allalunis et al. 1982), or $LD_{50/30}$ survival (Pedersen et al. 1982, Allalunis et al. 1983). In the chemotherapy studies, an alteration in drug pharmacokinetics may have contributed to the enhanced marrow damage. In the case of irradiation, MISO's cytotoxicity and radiosensitizing properties must be considered. The enhanced damage of MISO to Fraction 3 CFU-GM is consistent with earlier descriptions of their sensitivity to MISO. The decreased survival of Fraction 1 is less readily explained. Previous studies have described MISO's ability to inhibit repair of potentially lethal damage (PLD) (Sakamoto and Aritake 1981, Brown et al. 1983). The question of whether normal bone marrow cells have the capacity to repair PLD remains controversial. However, the observation that misonidzole diminished Fraction 1 CFU-GM recovery at 24 hours is consistent with the interpretation that some form of marrow recovery or repair was inhibited. In the intermediate phase of post-irradiation recovery, there was no difference between MISO and

saline treated animals. Recovery peak values were also comparable for each group. However, in the latter phase of the observation period, a decreased CFU-GM survival was observed in MISO treated animals, suggesting that a greater primary damage to the stem cell compartment was incurred and was manifest as a diminished repopulation capacity. Alternatively, it may be postulated that MISO enhanced damage to the stroma and microvasculature and resulted in a diminished capacity to support hematopoiesis. However, to date, there have been no reports of MISO enhancing either the acute or late effects of low doses of irradiation on the supporting stroma or microvasculature of bone marrow.

Another possibility which must be considered is that MISO has diminished the production of growth factors. In their studies of marrow regeneration after irradiation, Morley et al. (1971) and Chan and Metcalf (1973) have noted a dose dependent increase in serum colony stimulating factor (CSF) which preceded the regeneration of CFU-GM. In addition, Chan and Metcalf (1972) have demonstrated the production of CSF by bone associated cells after the marrow had been removed from the central cavity of the femur. The production of this CSF was not significantly impaired by moderate doses of radiation. The nature of this radioresistance was not explored, but it is possible that the same factors which confer radioresistance on Fraction 3 cells may also have contributed to the radioresistance of the CSF producing cells. As such, these may have been a target for the sensitizing effects of MISO. Although investigators have demonstrated that MISO does not interfere with the aerobic production

of CSF by human mononuclear cells (Allalunis et al. 1980). The in vivo effects have yet to be investigated.

CHAPTER SIX

Results

Growth of CFU-GM Under Controlled Oxygen Conditions

Fraction 1 and Fraction 3 CFU-GM were incubated in a conventional manner and under experimental conditions in which the oxygen concentration in the gas phase was precisely regulated. The oxygen concentration of the air mixture used to gas the reference laboratory incubator was 18%. The range of oxygen concentrations which were initially established in the experimental chambers are shown in table 4. The oxygen concentrations which were measured in the experimental chambers at the termination of the experiment are also provided for comparison. A slight leak was detected in chamber 2 as was indicated by the small increase in oxygen at the end of the experiment.

Table 5 shows the plating efficiencies of CFU-GM following incubation under normal or reduced oxygen tensions. Two experimental trials were performed. For both Fraction 1 and Fraction 3 cells, incubation in 18% oxygen (balance nitrogen) under experimental conditions improved the plating efficiency over that observed with standard incubation conditions. This improvement was greater for Fraction 3 cells, which were increased to more than three times the standard control values. The plating efficiency of Fraction 3 cells was slightly improved by culture at reduced oxygen tensions, with approximately three to four times more colonies proliferating in oxygen tensions of 2.0 to 0.6% than were obtained under standard

Table 4

Initial and Final Oxygen Concentrations in Experimental Chambers

	Initial	Final
Chamber 1	18.0 %	18.3 %
2	6.6	8.6
3	2.1	2.1
4	0.66	0.65
5	0.21	0.19

Table 5

Effect of Various Oxygen Concentrations on CFU-GM Growth

Oxygen Concentration	Plating Efficiency *
A. Fraction 1 Cells	
Air	.0028 ± .0002
18.0 %	.0045 ± .0003
6.0	.0040 ± .0004
2.0	.0038 ± .0005
0.6	.0039 ± .0004
0.2	.0024 ± .0003
B. Fraction 3 Cells	
Air	.00024 ± .00005
18.0 %	.00079 ± .00009
6.0	.00079 ± .00009
2.0	.00110 ± .00010
0.6	.00094 ± .00010
0.2	.00065 ± .00008

* Estimates of error = $\sqrt{n}/n$; n = total colonies counted

incubation conditions. A similar magnitude of increase at low oxygen tensions was not observed for Fraction 1 cells. Also of interest is the observation that conditions of severe hypoxia (0.2% oxygen for seven days) still permitted the proliferation of significant numbers of CFU-GM (51% and 71% of control values for Fractions 1 and 3, respectively).

The increased plating efficiency observed for bone marrow cultured in modified media in an atmosphere of 18% oxygen in nitrogen prompted an investigation of whether marrow culture under conventional incubation conditions could be improved by similar media changes. The modified media did not support the growth of CFU-GM when the cultures were incubated under standard conditions (data not shown). However, when the standard formulation of MEM alpha medium was supplemented with HEPES buffer (1 molar solution, pH = 7.3) used in experimental conditions, an increase in plating efficiency was observed for both Fraction 1 and Fraction 3 cells (Table 6). Subsequent to this observation, all CFU-GM cultures were supplemented with 20 µL HEPES buffer per plate in order to ensure optimum growth conditions.

Binding of MISO to Hematopoietic Tissues In Vitro

The rate of binding of radiolabelled MISO to bone marrow cells was determined following incubation under aerobic or hypoxic conditions in vitro. Table 7 presents the binding rates which were obtained using two different systems. The binding rates presented in Part A were calculated from data obtained using glass flasks which

Table 6

Effect of HEPES Supplemented Media on CFU-GM Growth

	HEPES (µl/plate)	% Control
Fraction 1	0	100
	10	159
	15	179
	20	189
	40	200
Fraction 3	0	100
	10	252
	20	305
	40	323

Table 7

In Vitro Binding of ^{14}C-Misonidazole of Bone Marrow Cells

	Misonidazole (μm)	Oxygenation	Average Binding Rates (10^{-13}M/ 10^6 Cells/ hr)	
			Acid Soluble	Acid Insoluble
A.	10	Air	0.4	*
	10	N_2	11.9	5.5
B.	10	N_2	4.2	2.3
	100	N_2	22.7	16.7
	200	N_2	44.6	17.6
C.	10	Air	0.9	0.5
	10	4.5% O_2	1.0	0.5
	10	1.0	1.2	0.6
	10	0.25	1.6	0.7
	10	N_2	7.3	3.2

* Counts not above background

were continuously gassed under conditions which were identical to those described for the MISO *in vitro* cytotoxicity studies. The binding rates presented in Part B were calculated from data obtained using aluminum chambers identical to those used for the low oxygen plating efficiency studies. These chambers were also used to establish atmospheres containing small, controlled amounts of oxygen, in which the binding rates presented in Part C were determined. All of the rates are expressed as moles of MISO bound to one million cells per one hour of drug exposure time.

Little binding to aerobic cells was observed in these studies. Under anoxic conditions, significantly greater binding was achieved. The rate of binding was independent of the meth[illegible] to establish severe hypoxia, as the values obtained in [illegible] and B are comparable. The results pr[illegible]n Part B also indicate that the binding rate was increase[illegible] amount of drug available to the cells was increased. The sl[illegible]f this increase was consistent with half order kinetics.

The binding rates presented in Part C were obtained from experiments designed to determine the effects of small amounts of oxygen on the binding of MISO to bone marrow cells. Oxygen concentrations of 4.5%, 1%, and 0.25% inhibited MISO binding, as the binding rate was increased only 1.6 times that observed for cells exposed under aerobic conditions. The removal of all oxygen increased the rate of binding to approximately eight times that observed for aerobic cells.

Autoradiographic Analysis of MISO Binding In Vivo

Histological sections of hematopoietic tissues were examined using autoradiographic techniques to determine the pattern of radiolabelled MISO binding following in vivo exposure to the drug. Other studies which have examined the binding patters of ^{14}C-MISO in V79 or EMT6 spheriods have established that binding occurs as a function of the oxygen tension of the tissue, with the greatest amount of label being concentrated in the hypoxic regions (Franko and Chapman 1982, Franko et al. 1982). In the studies in this thesis, quantitative grain counts were obtained for histological sections of normal or regenerating femurs, humerii, and spleens. When bones were examined, counts were made in anatomic areas which corresponded to the presumed location of Fraction 1 (central marrow cavity) or Fraction 3 (compact bone associated) cells. The counts were calculated both as the number of grains in a 100 μm^2 area or as the number of grains per nuclei contained in a 100 μm^2 area.

Table 8 shows the binding patterns for both normal and regenerating tissues. Approximately two to three times the number of grains were found per cell in the Fraction 3 equivalent areas as compared to the number of grains counted in the Fraction 1 equivalent areas of normal bone. The normal spleen was uniformly labelled.

In regenerating tissues, greater amounts of label were found in regenerating Fraction 1 equivalent areas when compared to Fraction 1 equivalent areas of normal tissues. Fraction 3 equivalent areas were also heavily labelled.

In tissue sections obtained from regenerating spleens, the

Table 8

In Vivo Binding of ^{14}C-Misonidazole to Hematopoietic Tissues

Tissue	Grain Counts (grains/$100^2\mu$)	
	Fraction 1	Fraction 3
Normal Femur	443 ± 62	711 ± 59
Regenerating Femur	903 ± 54	826 ± 50
Normal Spleen	299 ± 42 *	390 ± 31 **
Regenerating Spleen	337 ± 17 *	904 ± 171 **
	Average Grains / Cell	
Normal Femur	0.19 ± 0.03	0.64 ± 0.03
Regenerating Femur	3.4 ± 0.5	1.99 ± 0.10

---

* Red pulp areas of the spleen
** Areas of the spleen associated with hematopoietic cells

amount of label confined to the red pulp areas was similar to that observed in the normal spleen. However, approximately three times more label was found associated with foci of regenerating hematopoietic cells.

In Vitro Estimates of Self-Renewal Potential

The effect of animal age on the ability of Fraction 1 and Fraction 3 cells to generate CFU-GM and HPP colonies was investigated. Mice ranging in age from six weeks to 12 months were studied. The number of colonies obtained from these animals is shown in table 9. A small variation in the absolute number of CFU-GM was observed over the course of the experiment. This is presumed to reflect inter-animal biological differences. No significant decreases in CFU-GM were noted for either Fraction 1 or Fraction 3 cells as the age of the mice increased.

HPP colonies were recognized by the presence of erythroid, myeloid and megakaryocytoid cells within the same colony and by their ability to generate secondary colonies in replating assays, as is presented in Table 9. These results suggest that the relative proportions of both Fraction 1 and Fraction 3 HPP-CFU decline with age.

Table 9

Number of Fraction 1 and Fraction 3 Primary and Secondary Colonies from Mice of Various Ages

	Age (months)	Primary Colonies (mean and range)		% Secondary Colonies
A. Fraction 1	1.5	140	(122 - 166)	17
	2.0	167	(127 - 231)	--
	3.0	171	(162 - 178)	36
	4.0	168	(128 - 218)	16
	5.0	166	(131 - 200)	18
	6.0	166	(153 - 186)	13
	9.0	157	(141 - 186)	6
	12.0	157	(128 - 204)	5
B. Fraction 3	1.5	24	(16 - 29)	25
	2.0	37	(21 - 43)	--
	3.0	21	(9 - 33)	29
	4.0	18	(15 - 26)	27
	5.0	29	(13 - 45)	23
	6.0	19	(13 - 31)	19
	9.0	20	(17 - 30)	10
	12.0	12	(10 - 17)	15

Demonstration of Day 7 and Day 14 CFU-GM

Two sub-sets of CFU-GM colonies can be recognized by their different rates of growth in vitro. Representative plate maps which depict the relative location of Fraction 1 and Fraction 3 colonies following 7 and 14 days of culture are shown in Figures 15 and 16. In these maps, the colonies present at day 7 are indicated by the "X" symbol. The colonies present at day 14 are indicated by the "O" symbol. In the instance in which a colony was observed at the same location on both day 7 and 14, the symbols are superimposed. The "X" and "O" symbols which stand alone represent colonies which were present at day 14 but not at day 7 ("O" symbols), or conversely, colonies which were present at day 7 and not at day 14 ("X" symbols). In these studies, the colonies on seven different plates of Fraction 1 and Fraction 3 CFU-GM were mapped. For these pooled data, an average of 28 and 29% of the colonies present in cul[redacted] Fraction 1 and Fraction 3 CFU-GM could be identified as day 14 colonies.

Rescue of Lethally Irradiated Mice by Fraction 1 and Fraction 3 Cells

A comparison of the ability of Fraction 1 and Fraction 3 cells to rescue lethally irradiated (9 Gy) mice is shown in Table 10. Between 10 and 30 animals were used in each group. Animals alive 30 days after irradiation and marrow transplant were considered to have received a successful marrow transplant. Control animals which received irradiation without marrow rescue did not survive to 30 days. The results in these experiments suggest that Fraction 3 cells were more efficient than Fraction 1 cells in restoring hematopoiesis.

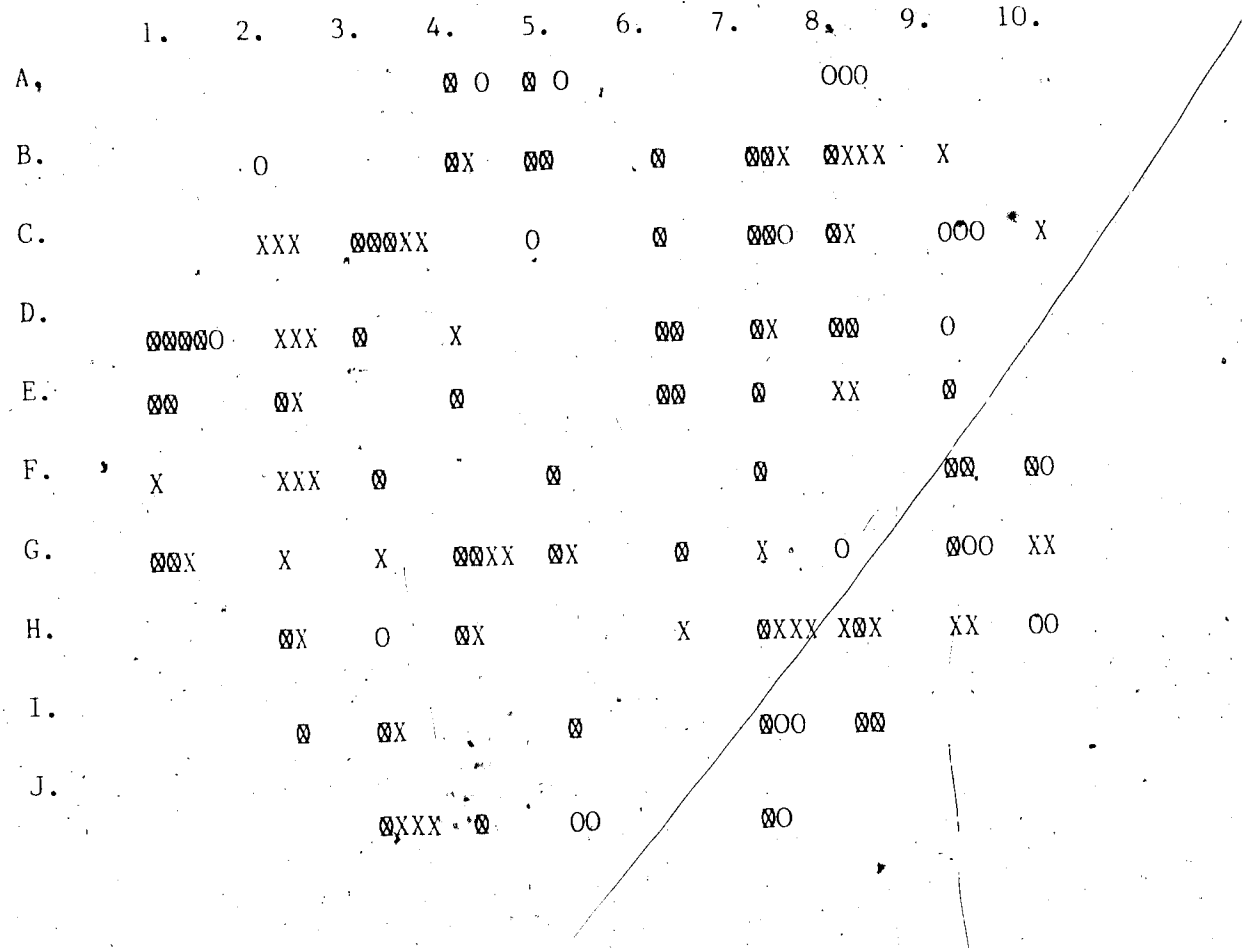

Figure 15. A plate map which represents the relative location of Fraction 1 CFU-GM is illustrated. The "X" symbols and "O" symbols represent the locations at which a colony was observed at day 7 or day 14 respectively. Where symbols are superimposed, a colony was observed at both day 7 and 14. In this plate, there were 108 colonies on day 7 and 83 colonies at day 14. Of this number, 25 colonies (=29%) could be identified as day 14 colonies.

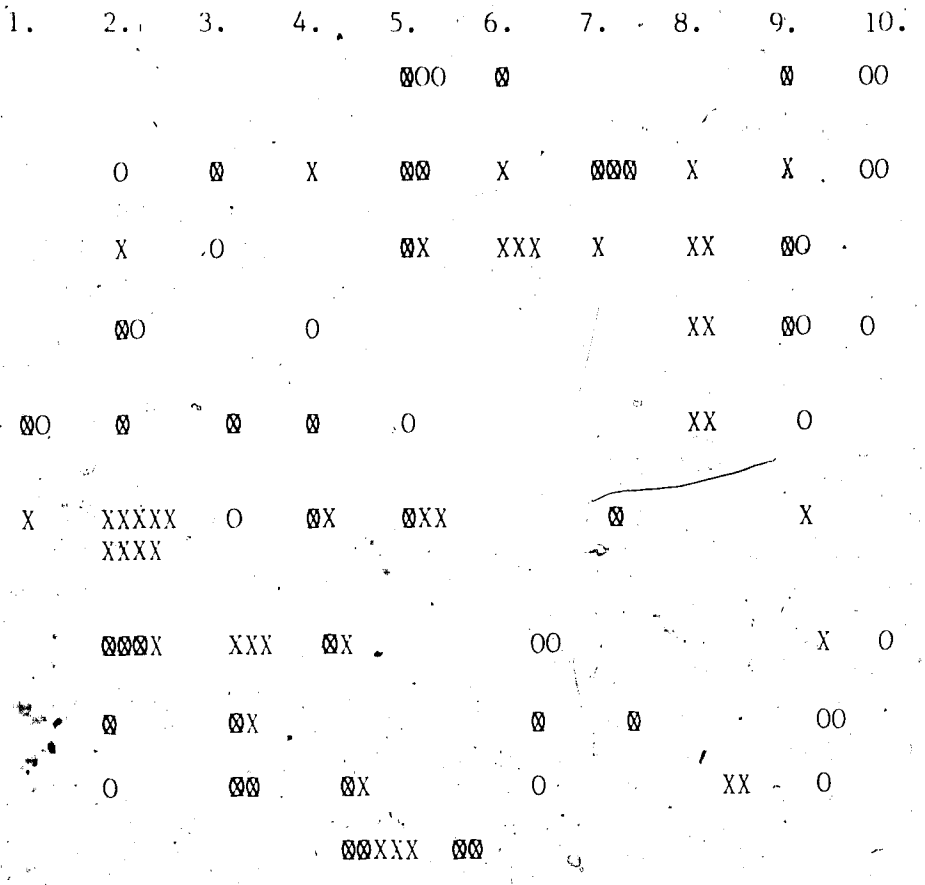

Figure 16. A plate map which represents the relative location of Fraction 3 CFU-GM is illustrated. The "X" symbols and "0" symbols represent the locations at which a colony was observed at day 7 or day 14 respectively. Where symbols are superimposed, a colony was observed at both day 7 and 14. In this plate, there were 78 colonies at day 7 and 60 colonies at day 14. Of this number, 25 colonies (=41%) could be identified as day 14 colonies.

Table 10

Comparison of Animal Survival Rates Following Whole Body Irradiation and Rescue With Fraction 1 or Fraction 3 Cells

Number of Cells	Survival Rates	
	Fraction 1	Fraction 3
1.0×10^6	100 %	100 %
9.5×10^5	100	---
9.0×10^5	70	---
8.5×10^5	69	---
8.0×10^5	54	---
7.5×10^5	21	100
6.5×10^5	---	80
6.0×10^5	---	76
5.0×10^5	0	0

Discussion

Effect of Low Oxygen on CFU-GM In Vitro

It has been suggested that the in vitro proliferaion of both normal and tumor cells may be improved by culture at lowered oxygen tensions. When studies were conducted which compared standard (18% O_2) and reduced (1-5%) oxygen levels, increased plating efficiencies were observed at the lower oxygen concentrations for human fibroblasts (Taylor et al. 1974, Balin et al. 1976), murine fibroblasts (Richter et al. 1972, Bradley et al. 1978), CFU-GM (Bradley et al. 1978), CFU-E (Rich and Kubanek 1982), rat fetal lung cells (Tanswell et al. 1984) and chick embryonic myocardial cells (Hollenberg 1971). This phenomenon has also been observed with cultures of Erlich ascites tumor cells (Gupta and Eberle 1984), B16 melanoma cells (Courtney 1976), human melanoma cells (Gupta and Krishan 1982, Joyce and Vincent 1983) and other freshly excised human tumor samples (Sridhar et al. 1983).

The results reported in this thesis identify a factor other than oxygen which may influence in vitro plating efficiencies. For both Fraction 1 and Fraction 3 CFU-GM, a minor component of the increased plating efficiency was attributable to reduced oxygen tensions. The largest component of the observed increase in proliferation was the result of an overall improvement in plating efficiency which accompanied a change in culture media.

In the studies cited above, pH balance was maintained by an atmosphere of 5-10% CO_2 with sodium bicarbonate in the culture media. In the studies in this thesis, CO_2 was not used, the concentration of sodium bicarbonate was reduced and HEPES buffer was added to maintain pH balance. When Fraction 1 and Fraction 3 CFU-GM were cultured in modified media in an atmosphere of 18% oxygen in N_2, two to three times more colonies were observed as compared to the number of colonies obtained from cultures grown in standard media in an atmosphere of 18% oxygen with 5% CO_2. These results suggest that the improvement in plating efficiency was largely attributable to changes in methods used to regulate pH balance. A reduction in the oxygen tension in the experimental chambers resulted in no ehancing effect for Fraction 1 CFU-GM and a small enhancing effect for Fraction 3 CFU-GM. These results were unexpected since the premise behind these experiments and those cited above predicted that a reduced oxygen tension would provide an environment which more closely resembles that encountered in vivo where the venous pO_2 is in the range of 5-6% O_2 (Wintrobe et al. 1974). However, the results of these studies indicate that this simple hypothesis is inadequate, and that factors other than atmospheric oxygen tension can exert a controlling effect on CFU-GM proliferation.

The increased plating efficiencies observed both in standard cultures with added HEPES buffer and in cultures maintained in experimental chambers in HEPES modified media suggest that the addition of HEPES per se was responsible for the increased cell proliferation. The underlying mechanism which controls this

increased proliferation remains to be determined. HEPES is a hydrogen ion buffer which has been shown to offer equivalent or improved buffering capacity to low and high density cultures of mammalian cells or SV-40 transformed cells (Eagle 1971, Itagaki and Kimura 1974). It has been assumed that the addition of HEPES buffer in these cultures has provided adequate, if not superior, pH regulation. An increase in intracellular pH is known to accompany DNA synthesis and mitosis, although a causative relationship has yet to be established (Nuccitelli and Heiple 1982). The possibilty therefore exists that the addition of HEPES to the media resulted in a slightly elevated pH and a subsequent increase in mitosis. This hypothesis remains to be tested.

It is unclear why the use of a different buffer system would obliterate the stimulatory effects on cell proliferation which have been previously reported for low oxygen tensions. What is clear, however, is the observation that other variables in addition to atmospheric oxygen tension may exert a controlling effect on cell growth in vitro.

MISO Binding Studies

The presence of hypoxic cells in experimental tumors has been well established (Tannock 1976, Chapman et al. 1983b, Suit 1984). Analysis of survival curve data, recently reviwed by Moulder and Rockwell (1984), provides indirect evidence that significant proportions of many experimental tumors are hypoxic. The ability of hypoxic cell sensitizers such as MISO to modify tumor cell kill and

influence cure rates provides additional evidence that the presence of hypoxic cells may modify animal tumor response to therapy (Sheldon et al. 1976, Denekamp 1980, Law et al. 1981, Siemann 1982). The development of radiolabelled hypoxic cell compounds has allowed a direct visualization of hypoxic regions in EMT-6 tumors labelled in vivo (Chapman et al. 1981a, Garrecht and Chapman 1983), V79 and EMT-6 spheriods labelled in vitro (Franko et al. 1982, Franko and Koch 1984, Franko et al. 1984) and human tumor explants labelled in vitro (Franko and Koch 1984, Franko et al. 1984). In each case, the binding of radiolabelled MISO was confined to areas of the tumor which were presumed to be hypoxic based on calculations of the oxygen diffusion distance or the absence of any histologically identifiable venous structures. These above studies have confirmed in tissue samples observations originally made using single suspensions of EMT-6 (Miller et al. 1983, Chapman et al. 1983) or V79 cells (Chapman et al. 1983) incubated with MISO in vitro under aerobic or hypoxic conditions.

A feature common to all of the histological studies noted above was the demonstration that normal tissues generally bind MISO at a much lower rate. In their study which included a survey of 13 normal tissues, Garrecht and Chapman (1983) noted two to six times less binding in most normal tissues when compared to EMT-6 tumors labelled in the same animal. The liver, which retained significant amounts of MISO, was an exception. Franko et al. (1984) also reported that normal stromal tissues associated with tumors had significantly reduced uptake of MISO. These results were consistent with the

assumption that most normal tissues are relatively well oxygenated and would therefore not be expected to bind MISO. However, the studies reported in this thesis have suggested that some bone marrow cells may be relatively hypoxic. The following studies have used the preferential binding of MISO to hypoxic tissues as a tool to investigate the presumed hypoxia of Fraction 3 cells.

The pattern of metabolic binding of ^{14}C-MISO to murine bone marrow cells was similar to that described for other types of cells (Chapman et al. 1983a, Miller et al. 1983, Koch et al. 1984). Little drug was bound to marrow cells incubated in air (average binding rate at 10 μm = 0.6 x 10^{-13}M/10^6 cells/hr), as the presence of oxygen is thought to block the metabolic reduction of the MISO nitro group which is a necessary step in the binding process (Varghese et al. 1976). When marrow cells were incubated with MISO under extremely hypoxic conditions, the binding rates were significantly increased (average binding rate at 10 μM = 7.4 x 10^{-13}M/10^6 cells/hr). However, the rate of binding which was observed for marrow cells was approximately 30 to 50 fold less than that observed when EMT-6 cells (Chapman et al. 1983) or hepatoma cells (Chapman 1984, unpublished data) were incubated with equivalent amounts of the drug under hypoxic conditions. These results suggest that a qualitative and/or quantitative difference in nitroreductase enzymes may exist between normal marrow and tumor cells. Alternatively, inherent differences in cellular energy levels or redox state may have contributed to the observed difference in results (Olive 1984). Because binding rates have yet to be

determined for other normal tissues, there is no information as to whether a similarly reduced rate of binding relative to that of tumors will also be observed.

Despite the relatively smaller amounts of MISO which were bound, the kinetics of the reaction were similar to those described earlier by Chapman et al. (1983) and later confirmed by Koch et al. (1984) for EMT-6 and V79-WNRE cells. When oxygen is absent, MISO binds to cells with half order kinetics. A logarithmic plot of marrow binding rates versus MISO concentration was consistent with half order kinetics. The studies by Koch et al. have indicated that the kinetics of this reaction is highly dependent upon oxygen concentration. A change to a first order reaction was noted when cells plus MISO were incubated in the presnce of 2 μM oxygen. A second as yet uncharacterized change in kinetics was observed at oxygen concentrations which approached those of air. A similar oxygen dependence of MISO binding to marrow cells has yet to be investigated.

The in vivo binding pattern of ^{14}C-MISO to hematopoietic tissues was assessed by autoradiographic techniques. In normal femurs and humerii, approximately two to three times more label was associated with the hematopoietic cells localized in the spongiosa region of the distal ends of the long bones or in the compact bone. These areas are presumed to be the sites from which Fraction 3 cells are harvested. This finding provides indirect evidence that the microenvironment of these cells may be relatively hypoxic as compared to that of the central marrow cavity. The magnitude of the

difference in the amount of MISO bound to Fraction 1 and Fraction 3 cells is consistent with the assumption that the difference in pO_2 of these two populations of cells may be less than 2%.

When the binding to regenerating tissues was determined, both Fraction 1 and Fraction 3 equivalent areas were heavily labelled. This suggests that relative marrow hypoxia may exist during the regeneration process. Other investigators have noted disruptions in the marrow microvasculature following [illegible] which would support this hypothesis.

Areas of increased label density were also noted in sections of regenerating spleen. In this case, more label was associated with foci of hematopoietic cells. This observation, coupled with the uniformly increased labelling pattern observed in regenerating marrow has suggested that the process of marrow self-renewal and differentiation which occurs during regeneration may be facillitated by an environment of a relatively reduced pO_2. An analagous period of intense marrow proliferation which occurs during fetal development is known to proceed at a pO_2 which is lower than that of the post-natal or adult condition. The proposed miniaturization of highly sensitive oxygen electrodes (Koch 1984) would allow this hypothesis to be directly tested.

In Vitro Self Renewal Potential

The hematopoietic system has been extensively studied at the tissue, cellular and molecular level, and has been the subject of several recent comprehensive reviews (Till and McCulloch 1980, Till 1982, Broxmeyer 1983). What emerges is a picutre of exceeding complexity and heterogeneity. For example, within the class of CFU-S, differences in bouyant density and cell size (Worton et al. 1969), self renewal potential (Monette and Stockel 1982) and expression of certain antigenic determinants have been described (van den Engh and Golub 1974, Monette and Stockel 1981). A similar range of physical and biological differences has been descri[illegible] hematopoietic cells which are assayed in vitro (Broxmeyer 1983).

In addition to differences in biological properties, several investigators have described differences in the physical distribution of hematopoietic cells. Hendry and Lord (1972) have described a spatial distribution of CFU-S within the mouse femur. In their model, CFU-S are concentrated along the endosteal surface of the bone and decrease in number with the square of the distance away from the bone. A similar pattern has been described by Gong (1978) for rat bone marrow. These results complement those of Shackney et al. (1975) who described a kinetic gradient across mouse femurs in which the highest proliferation rate was observed in the sub-endosteal region. These observations have suggested that anatomic zones within the femur differ in their stem cell content and in their proliferative activity, and that Fraction 1 cells may differ from Fraction 3 cells in other biological parameters as a result of their

physically distinct location within the femur. A full biological comparison of Fraction 1 and Fraction 3 cells, complete with estimates of cell size, density and cell cyle characteristics remains to be investigated. Instead, two small windows of comparison were chosen. The first of these determined if Fraction 3 cells contain any cells capable of giving rise to HPP-CFU, and compared the self-renewal potential of these colonies with that observed for Fraction 1 cells. The second comparison between Fraction 1 and Fraction 3 cells estimated the frequency of day 14 CFU-GM in each fraction.

HPP colonies have been described by several investigators and are thought to be the in vitro equivalent of CFU-S (Till and McCulloch 1980). HPP in vitro colonies share a similar range of size, cell cycle parameters (Baines et al. 1982 and 1984), sensitivity to rabbit anti-mouse brain serum (Monette and Stockel 1981) and sensitivity to hydroxyurea and 5-fluorouracil as do CFU-S (Keller et al. 1984). In addition, they have been shown to exhibit a considerable range in self renewal probability, as measured by the ability of primary colonies to generate secondary colonies in re-plating assays (Johnson et al. 1982). Experiments in this thesis have attempted to detemine whether HPP colonies can be identified among Fraction 3 cells and whether the self renewal probability of these colonies is equivalent to that of Fraction 1 cells. In these experiment, the proportion of primary colonies forming secondary colonies was approximately 20 to 24% for both Fraction 1 and Fraction 3 cells derived from young adult mice. For both populations of

cells, the number of HPP colonies declined with age, a phenomenon which had not been previously reported. These studies therefore suggest that pluripotential cells can be found among Fraction 3 cells and that their numbers are approximately equivalent to those of Fraction 1 cells.

The second aspect of these studies sought to determine whether two known sub-populations of CFU-GM exist in both Fraction 1 and Fraction 3 cells and to determine their relative frequency. CFU-GM which are usually assayed in in vitro cultures at day seven are thought to arise from somewhat less mature populations of cells which do not appear in cultures until day 12 or later (Jacobsen et al. 1979, Aglietto et al. 1984). In these experiments, plate maps were constructed after 7 and 14 days incubation for both Fraction 1 and Fraction 3 CFU-GM. In the representative maps provided, it can be observed that some colonies are present only at day 7 and others only at day 14. In some locations, colonies were counted at both day 7 and 14. These results indicate that 28 and 29% of the total number of colonies of Fraction 1 and Fraction 3 cells counted at day 14 were not present at day seven. These results are consistent with the interpretation that both sub-populations of CFU-GM are present in both fractions of cells. These observations and those obtained from the HPP-CFU assay suggest that hematopoietic cells located within the compact bone do not differ significantly from those found in the central cavity with regard to these two estimates of hematopoietic stem and progenitor cells. Although a CFU-S and CFU-GM gradient may exist for hematopoietic cells confined within the central core of the

femur, these results suggest that compact bone cells do not differ significantly when estimates of HPP-CFU and day 14 CFU-GM colonies are made.

Survival of Lethally Irradiated Mice

The ability of transplanted bone marrow cells to rescue lethally irradiated recipients was first demonstrated over 30 years ago (Lorenz et al. 1952; Congdon et al. 1952). Much information has been subsequently obtained concerning the number of cells which are required to ensure survival (Kurnick and Nokay 1965; Doherty 1969, Doherty and Smith 1969), the repopulating efficiencies of various hematopoietic tissues (Rabotti 1964), the influence of lymphocytes on engrafment (Sharkis et al. 1978) and the patterns of repopulations within the marrow itself (Salner et al. 1982). It is generally accepted that following lethal doses of irradiation, survival is a function of the number of donor cells which have been transplanted. It has also been suggested that the marrow transplantation assay is the only valid assay to establish the presence of totipotent hematopoietic stem cells (THSC) (Boggs et al. 1982, Phillips 1984). This rescue assay was therefore chosen to investigate whether fraction 3 cells contain THSC, and if so, were their numbers comparable to those found among Fraction 1 cells.

These studies have established the fact that Fraction 3 cells do contain THSC as measured by the ability of Fraction 3 cells to ensure animal survival for a period of at least 30 days (Bond et al. 1965). The comparative survival rates, based on a comparison of the

absolute number of cells required to rescue animals, or based on the relative number of clonogens in each fraction, suggest that Fraction 3 cells were more efficient that Fraction 1 cells in their ability to restore marrow function. However, this latter observation must be cautiously interpreted. At least two other factors should be considered as having the potential to modify the results of these studies. The first concerns the observation that factors other than the number of THSC may influence marrow engrafment. These include the presence of accessory cells such as thymocytes (Lord and Schofield 1973) and anti-theta sensitive cells (Sharkis et al. 1978) in the donor transplant material. Therefore, these experiments cannot exclude the possibility that Fraction 3 contains proportionately more accessory cells than does Fraction 1 cells, and that the enhanced animal survival rate observed for these cells is a function of cells other than THSC.

Finally, it must be recognized that the harvest procedure differs between Fraction 1 and Fraction 3 cells. Both populations were exposed to equivalent concentrations of collagenase for equivalent periods of time. However, Fraction 3 cells were removed from the femur by the repeated grinding of small pieces of bone. This mechanical disruption was not controlled for as Fraction 1 cells were not subject to the same type of agitation. The possibility exists that the relative numbers of THSC or accessory cells could have been artificially enhanced in Fraction 3 cells.

In spite of these cautionary notes, the preliminary indication that Fraction 1 and Fraction 3 cells may differ in their ability to

reconstitute hematopoiesis is an intriguing one worthy of further consideration.

CHAPTER SEVEN

Summary

The studies reported in this thesis have provided a radiobiological characterization of two populations of marrow CFU-GM. Compact bone associated cells differed from central marrow cavity cells in their radiosensitivity, sensitivity to MISO, intracellular glutathione content, uptake of ^{14}C-MISO, and their ability to rescue lethally irradiated mice. Some of these differences, such as the radiation and MISO sensitivities, can be explained by the hypothesis that the microenvironment of Fraction 3 cells is relatively hypoxic. However, differences in physiological oxygen tension do not readily explain relative differences in GSH content. It is not known whether chronically hypoxic cells become depleted of GSH. However, studies with cells made acutely hypoxic for several hours have demonstrated no reduction in intracellular GSH levels (Koch, personal communication 1985). Therefore, mechanisms in addition to the relative intracellular concentrations of GSH may contribute to these observed results.

The presumed hypoxia of Fraction 3 cells may also have little direct influence on the relative ability of Fraction 1 and Fraction 3 cells to rescue lethally irradiated animals. In both experimental studies and clinical practice, a direct correlation can be made between marrow engraftment and the number of donor cells administered. These studies suggest that Fraction 3 cells may

contain proportionately more THSC. However, other factors such as the number of accessory cells in each population may modify this interpretation.

In addition to comparing some of the biological properties of Fraction 1 and Fraction 3 cells, the studies in this thesis have examined two topics of current interest in the field of experimental oncology. The first of these concerned the proposed use of DDC as a marrow radioprotector. Experiments in this thesis were able to distinguish between the stimulatory and protective effects of DDC on hematopoietic stem cells. This resolution of the mode of action of this drug has prompted the conclusion that DDC will be of limited clinical value as a marrow radioprotective agent. This observation is in contrast to the conclusions reached by other investigators who have not resolved the stimulating effects of DDC on the hematopoietic system.

The second topic has questioned the role of oxygen and pH balance in the culture of normal and neoplastic cells. The addition of HEPES buffer to standard tissue culutre media was shown to result in an increased plating efficiency for Fraction 1 and Fraction 3 CFU-GM. A subsequent reduction in oxygen tension did not enhance the number of Fraction 1 colonies, and resulted in a relatively small increase of Fraction 3 colonies. These results are in contrast to the observations made by several other investigators using a variety of normal and neoplastic cells, and suggest that other factors in addition to relative oxygen concentration may control cell proliferation in vitro.

This thesis has described several biological differences which exist between two populations of murine CFU-GM. However, the most striking difference between these two populations is the fact that they reside in physically distinct locations within the femur. In other stem cell renewal systems, such as the intestine and the skin, the stem cells are confined to a single anatomic location. In the gut, the stem cells occupy the intestinal crypts, while in the skin, the stem cells are located in the center of a circle of 10-11 basal nucleated cells (Potten 1974). The marrow is unique in that its stem cells can exist in more than one physically distinct microenvironment.

The influence of the marrow environment on hematopoiesis has yet to be conclusively established. Several studies have suggested that short range promoters and inhibitors of hematopoietic cell proliferation are locally produced in the marrow and that the relative proportions of each may vary significantly over distances of a few tens of microns (Wolf 1978, Lord and Wright 1984). According to this model, one might expect that the cells in Fraction 3 are subject to slightly different proliferative controls than are the cells of Fraction 1.

In contrast to the above model in which locally resident cells exert a regulatory influence on hematopoiesis, the possibility exists that the microenvironment is relatively passive. If this were the case, marrow cells might lodge in any of several available niches within the bone. This model sees the marrow as a highly adaptable organ with self-regulatory properties independent of its

physical support system.

The influence of growth factors on the maintainance of hematopoiesis has been well characterized on both the cellular and molecular level. The possible modulating effects of different physical marrow environments on the interactions of hematopoietic stem cells with their regulating factors remains to be determined.

From an anatomic point of view, the physical location of Fraction 3 marrow cells may more closely resemble that of human marrow cells, as human marrow in the adult is largely confined to areas of trabecular bone. It remains to be seen whether some of the biological properties which have been attributed to Fraction 3 cells would also be observed with populations of human marrow cells. Other studies have shown that human marrow CFU-GM express a sensitivity to MISO cytotoxicty which was similar to that described for Fraction 3 cells (Allalunis et al. 1980). A comparison between the response of human marrow CFU-GM and murine Fraction 1 and Fraction 3 CFU-GM to other cytotoxic drugs with a known specificity for hypoxic cells would be of interest.

In conclusion, the studies described in this thesis have raised several interesting questions about the radiobiology of the hematopoietic system. While many of these questions remain unanswered, these experiments have provided a framework upon which future studies of sub-populations of hematopoietic cell may be based.

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