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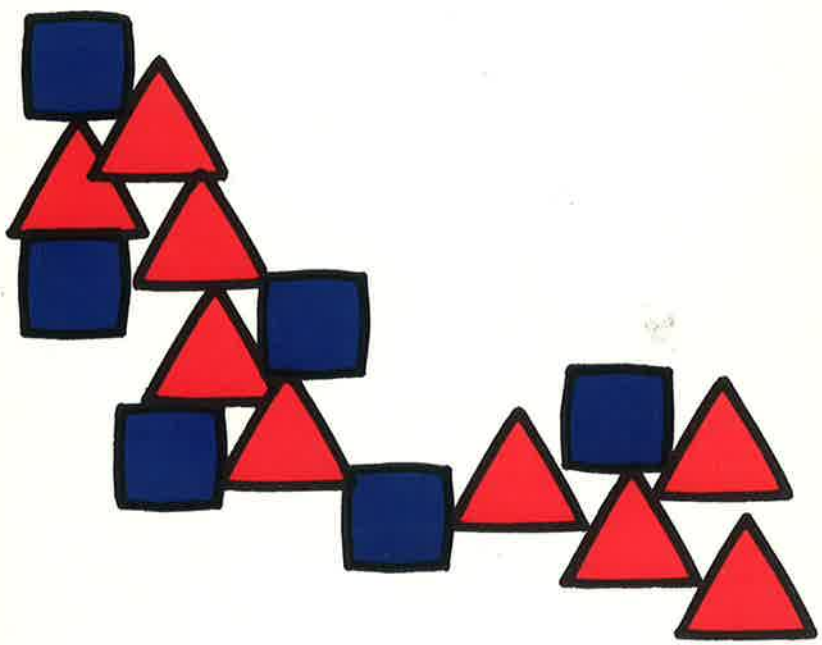
PROTECTION OF HAEMOPOIETIC STEM CELLS DURING CYTOTOXIC TREATMENT



H.J.Keizer

PROTECTION OF HAEMOPOIETIC STEM CELLS DURING CYTOTOXIC TREATMENT

The work described in this thesis has been performed at the Radiobiological Institute TNO, Rijswijk, in a joint appointment with the Interuniversity J. A. Cohen Institute for Radiopathology and Radiation Protection, Leiden.

This thesis is available as a publication of the Radiobiological Institute TNO,
151 Lange Kleiweg, Rijswijk, The Netherlands, 1976

ISBN 90 211 4015 2

STELLINGEN

I

Voor het bedrijven van kanker therapie in de kliniek zal meer en meer zowel een klinische als research opleiding gewenst zijn.

II

Terwijl kanker vooral voorkomt bij de rijken, is lepra een ziekte van de straatarmen van deze wereld.

Zeegers, In: "Telex", een uitgave van de Stichting Carosi, 1976

III

De beenmerg stamcel van de muis kan gemakkelijk zodanig gemanipuleerd worden dat hij minder gevoelig is voor chemische anti-kanker stoffen.

IV

Wanneer proefdieren tijdens totale-lichaamsbestraling genarkotiseerd of vastgebonden zijn, kan dit leiden tot verminderde stralingsgevoeligheid van de te onderzoeken weefsels.

V

Het stralingsbeschermend effekt van pentobarbital voor de beenmerg stamcel van de muis hangt af van de kinetische toestand van de stamcel populatie.

VI

In afwachting van het uiteindelijke anti-kanker middel, moeten we niet ophouden nieuwe wegen te zoeken waarop de nu beschikbare therapeutische mogelijkheden optimaal kunnen worden gebruikt.

VII

Het onderzoek naar stoffen die beenmerg minder gevoelig maken voor intensieve kanker chemotherapie dient gestimuleerd te worden.

VIII

Research is de kunst van iets oplossen; daarom kan het voordeliger zijn een basis probleem te bestuderen in micro-organismen dan in zoogdiercellen die zoveel gecompliceerder zijn.

Perutz, Nature *262*, 449, 1976

IX

Beter begrip van het pleiotrope fenotype van cytostatica-resistente mutante cellijnen en in het bijzonder van hun collaterale gevoeligheid zou van grote waarde zijn bij de ontwikkeling van optimale combinaties van cytostatica voor de behandeling van resistente tumoren.

Bech-Hansen et al., J. Cell. Physiol. *88*, 23, 1976

X

De snelheid waarmee de L1210 leukemie resistent wordt voor therapie met combinaties van cytostatica, is afhankelijk van de manier van combineren en het behandelingsschema.

Schmid et al., Cancer Treatment Rep. *60*, 23, 1976

XI

Er bestaat mogelijk een relatie tussen de langdurige blootstelling van het personeel van operatiekamers aan lage concentraties gasvormige narkosemiddelen en de verhoogde frekwentie van abortus, congenitale misvormingen in het nageslacht, nierziekten, leveraandoeningen en kanker in deze groep mensen. Het aanbrengen van relatief goedkope afvangsystemen is gewenst.

Van Stee, Ann. Rev. of Pharmacol. and Toxicol., *16*, 67, 1976.

27 oktober 1976 H. J. Keizer

PROTECTION OF HAEMOPOIETIC STEM CELLS DURING CYTOTOXIC TREATMENT

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN
DOCTOR IN DE GENEESKUNDE
AAN DE RIJKSUNIVERSITEIT TE LEIDEN, OP GEZAG
VAN DE RECTOR MAGNIFICUS DR. D. J. KUENEN,
HOOGLERAAR IN DE FACULTEIT DER WISKUNDE
EN NATUURWETENSCHAPPEN
VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN
TE VERDEDIGEN OP
WOENSDAG 27 OKTOBER 1976 TE KLOKKE 16.15 UUR

DOOR

HENDRIK JAN KEIZER

geboren te Haarlem in 1947

W. D. MEINEMA B.V. – DELFT – 1976

Promotor:
Prof. Dr. L. M. van Putten

Co-referenten:
Prof. Dr. E. L. Noach
Prof. Dr. P. Thomas

Voor Roel en Jos

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Wat we meemaken is een explosie van weten;
elke dag zwellen onze hoofden verder op en de
rest van het lichaam tobt maar goeig door...

Godfried Bomans, in: In de kou

CHAPTER 1

INTRODUCTION

A. Tumour chemotherapy

Cancer chemotherapy has been applied with increasing success during the last two decades. Cures can be obtained for eleven types of cancer, where a cure is defined in the cancer patient who survives several years after treatment and has a subsequent survival experience indistinguishable from the survival experience of the whole population of the same age and sex (Zubrod, 1972). Despite these successes, major problems remain. Chemotherapy of microbial diseases was greatly favoured by the fact that microorganisms have a cell wall instead of a cell membrane and this essential difference could be exploited to selectively kill microorganisms with drugs. Unfortunately, a similar basic difference has not yet been shown to exist between normal and malignant cells. The lack of such a difference is a major obstacle to adequate drug control of human malignancies.

As a consequence, the administration of anti-cancer drugs to cancer patients or experimental tumour-bearing animals results not only in tumour cell kill but also in the elimination of a proportion of normal cells. Since the currently used drug screening models select against drugs which are effective in killing resting cells, the majority of anti-cancer agents currently used are most effective against cells which are actively traversing the cell cycle (Valeriote and Van Putten, 1975). The obvious conclusion is that the normal tissues most sensitive to anti-cancer drugs are those with a rapid turnover rate; these tissues include the haemopoietic system, the epithelial lining of the gastrointestinal tract and the skin. Such tissues are therefore called "critical normal tissues".

Elimination of haemopoietic cells may lead to pancytopenia, placing the host at risk for infection, haemorrhage and anaemia. Loss of epithelial cells in the gastrointestinal tract results in stomatitis, nausea, vomiting and diarrhea. Damage of skin cells may lead to alopecia, erythema and abnormal pigmentation. A much smaller number of drugs exert their toxic effect primarily on tissues with a slow turnover rate, e.g. Bleomycine can cause lung fibrosis and the vinca alkaloid

spindle poisons (Vincaleucoblastine, VLB; Vincristine, VCR) are toxic to the nervous tissue.

To reduce or avoid damage to normal tissue during cancer chemotherapy, several measures could be considered from a theoretical point of view.

1. Selection of drugs. New drugs or analogs of already existing drugs with a high specificity for certain types of malignant cells could be developed.
2. Drug scheduling. Based on cell kinetics, pharmacological principles or empirical evidence, drugs would be given at specific intervals to obtain a maximum degree of tumour cell kill with a minimum degree of cell kill in critical normal tissues.
3. Combined modality chemotherapy. Combination of chemotherapy, at nontoxic levels, with other treatment modalities such as surgery, radiotherapy and immunotherapy could lead to dose reduction and thus to less damage to normal tissues.
4. Development of measures which preferentially protect critical normal cells against cytotoxic agents. This is a poorly investigated area in cancer research, primarily due to insufficient knowledge concerning the essential differences between normal and malignant cells. Moreover, incomplete knowledge about the physiological mechanisms regulating cell proliferation and differentiation makes it difficult to manipulate the cell cycle of critical normal tissue cells, despite the fact that the therapeutic ratio might be greatly improved if proliferation in critical normal tissues could be blocked.

One method by which preferential protection of a normal tissue can be obtained is through the use of folinic acid (citrovorum factor) given simultaneously or shortly after Methotrexate (MTX; amethopterin). This treatment has been demonstrated to preferentially rescue normal cells from the toxic effects of the drug using an *in vivo* mouse model (Chabner and Young, 1973). In man, this treatment was shown to improve the therapeutic index for osteogenic sarcoma treated with MTX in high doses (Jaffe, 1972; Jaffe et al., 1974). To explain the preferential rescue of normal cells by folinic acid, three hypotheses have been advanced (see Tattersall et al., 1975). These are: 1. a deficiency in cellular uptake of MTX in neoplastic cells. If a similar difference also exists for folinic acid no neoplastic cell rescue will occur, resulting in an improved therapeutic gain; 2. normal bone marrow

stem cells are recruited into cycle more rapidly than are tumour cells and therefore also rescued more quickly; this early rescue will be more important for normal cells. 3. higher concentrations of folinic acid are required to rescue neoplastic cells than to rescue normal cells.

As noted above, rapidly growing tissues are more sensitive to presently used cytostatics than are nonproliferating tissues. Therefore, it might be reasoned that, if the proliferation rate of normal critical cell systems, e.g., haemopoietic tissue and gastrointestinal tract, could be slowed temporarily, the selectivity of cell kill by cytostatic compounds might be improved. In other words, fewer normal cells would be killed without reduction of the tumour cell kill. Since most anti-cancer drugs kill cells with a differing effectiveness dependent on the cell cycle phases (for a review see Madoc-Jones and Mauro, 1975; Hill and Baserga, 1975), the normal cell should be blocked in a phase of cell cycle which is resistant to the particular drug in question.

Obviously, one has to avoid the occurrence of the same effect in malignant cells treated simultaneously. Unfortunately, this requires a detailed insight into the mechanisms which control proliferative and differentiative events in normal cells and into the differences between normal and neoplastic cells in regard to these mechanisms. Nonetheless evidence has been presented by Bruce, Lin and Bruce (1970) which suggests that several compounds result in a preferential protection of normal critical cells during cytostatic treatment. They described a system whereby mice were exposed to the general anaesthetics halothane and nitrous oxide for a 24-hour period. Halothane was administered at a dosage of 0.37 per cent, a dose which does not cause narcosis but rather a state of sedation. Under this condition, the mice respond to stimuli such as noise and pain. This exposure of mice to low dosages of general anaesthetics decreases the sensitivity of haemopoietic stem cells to Arabinosylcytosine (Ara-C) and Vincaleukoblastine (VLB) as shown by an increased survival of femoral haemopoietic stem cells after fractionated chemotherapy. In contrast, they found that the sensitivity of leukaemic stem cells to chemotherapy was unchanged by the low dosages of the anaesthetics. Since the haemopoietic stem cell kill in the air-exposed cytostatic-treated animals indicated that recruitment of resting (G_0) stem cells into cycle must have occurred, they postulated that both general anaesthetics delay the entry of resting normal cells into cycle. Keizer et al. (1973) demonstrated that administration of injectable anaesthetics to mice during chemotherapy, also protected a

proportion of the stem cells. By analogy, data were presented recentyl, which showed that 24-hour halothane exposure *in vivo* decreased DNA synthesis (as measured by ^{125}IUdR incorporation into DNA) preferentially in mouse femoral and splenic haemopoietic cells. This was also found occasionally in the cells of the small intestine. In contrast, neither experimental tumours, e.g. an osteosarcoma, L1210 leukaemia and AKR lymphoma, nor other normal tissues such as skin and muscle showed a similar effect (Evenwel, Keizer and Van Putten, 1976). These data suggest that the decreased toxicity of anti-cancer agents for haemopoietic stem cells during exposure to halothane or nitrous oxide may be associated with a decreased DNA synthesis rate in these cells.

Outline of the study

If the principle of reversible depression of bone marrow growth can be applied clinically, this may greatly improve the therapeutic gain of cytostatic treatment of malignancies. There are two reasons for investigating CNS depressants other than the volatile anaesthetic halothane and the gaseous anaesthetic nitrous oxide.

1. The use of both halothane and nitrous oxide has a number of disadvantages, such as the technical facilities which are needed to expose patients continuously, the intensive patient care that is necessary and the severe liver toxicity occasionally seen after halothane exposure.
2. More insight may be obtained into the mechanism(s) which render mouse haemopoietic stem cells less sensitive to cytostatic treatment when CNS depressants are given concomitantly

In section B of this chapter, data will be presented showing that the clinical use of a number of drugs known to depress the function of the central nervous system* may ultimately lead to clinically evident bone marrow depression. Analysis of the data may reveal drugs which are well suited to protect normal cells during cytostatic treatment.

Chapter 2 gives a description of the experimental procedures and techniques which have been used during this study.

* The central nervous system (CNS) includes the cerebrum, cerebellum and the spinal cord. Compounds which exert an inhibitory effect on this system are referred to as CNS depressants.

A series of experiments using a number of injectable CNS depressants to investigate the effect of this type of drug on haemopoietic stem cell survival during fractionated cytostatic treatment was started. These will be described in chapter 3.

CNS depressants are also described as occasionally modifying the effect of ionizing radiation, as shown by the animal survival after whole-body radiation exposure. The results obtained with a number of compounds which are frequently used to anaesthetize experimental animals are described in chapter 4.

Chapter 5 deals with attempts to clarify the mechanism of the radioprotective effect of one of these drugs, pentobarbital.

In chapters 3, 4 and 5, the survival of the haemopoietic stem cell will be used as the main parameter to quantify the effect of CNS depressants during cytotoxic treatments with both chemotherapy and ionizing radiation. In order to facilitate interpretation of the experimental data presented in these chapters, section C of this introduction deals with the description of a number of characteristics of the haemopoietic system of the mouse under normal conditions and after cytotoxic therapy.

B. Clinical evidence of drug-induced reversible bone marrow depression

An ideal drug, given to protect critical normal cells during cytostatic treatment, would have the following features: it should inhibit cellular proliferation; it should not kill the cells; it should not exert any other effects after continuous exposure; the effect should be reversible; and it should discriminate between normal and malignant cells. In the next paragraphs, data on a number of drugs which are used in man and are known to ultimately lead to bone marrow depression will be presented and discussed on the basis of the above-mentioned criteria.

In 1956, Lassen and co-workers introduced nitrous oxide anaesthesia as a part of the treatment of tetanus to avoid weeks of psychological stress resulting from being totally paralysed but fully conscious during curare treatment (Lassen et al., 1956). In most cases, a mixture of 50 per cent oxygen and 50 per cent nitrous oxide was used. A number of drugs, such as antibiotics, chloralhydrate, pentobarbitone and d-tubocurarine were given simultaneously. When treatment was continued for more than two weeks, signs of aplastic anaemia developed.

Despite repeated blood transfusions, pronounced granulocytopenia and thrombocytopenia with severe haemorrhages developed, followed by septicaemia. Bone marrow biopsy revealed severe damage to the blood forming cells, "presumably by some drug or other". A few days after cessation of anaesthesia, signs of repopulation were seen in the bone marrow.

Analysis of the data suggested that nitrous oxide was the probable cause of the haematological problems. Two new cases of tetanus were again treated according to the above-mentioned drug regimens. Since nitrous oxide exposure was started a few days after the rest of the treatment had been initiated, the effect of nitrous oxide on the bone marrow and the peripheral blood cell counts could be carefully studied.

It was shown that within four days after the beginning of exposure, leukocyte and thrombocyte counts in the peripheral blood were sharply decreased, indicating that nitrous oxide has a direct toxic effect on these blood cells. The bone marrow picture showed depressed erythropoiesis of the megaloblastic type, pointing to a maturation block in the cells. A few days after cessation of the nitrous oxide exposure, peripheral cell counts were again normal, whereas the bone marrow showed regeneration similar to that following a toxic infectious episode. Similarly, Lassen and Kristensen (1959) demonstrated a considerable decrease in the total number of myeloid cells and thrombocytes in the peripheral blood of patients suffering from chronic myeloid leukaemia who had been exposed to nitrous oxide (25 per cent) for a period ranging from 5–15 days, suggesting that nitrous oxide exerts a direct toxic effect on mature haemopoietic cells. The marrow in the nitrous-oxide-treated leukaemic patients resembled that seen in megaloblastic anaemia. Thus, these data support those obtained in the tetanus studies and suggest that continuous nitrous oxide exposure in man may have a two-fold effect, namely:

1. a direct toxic effect whereby haemopoietic cells are killed; and
2. a maturation arrest.

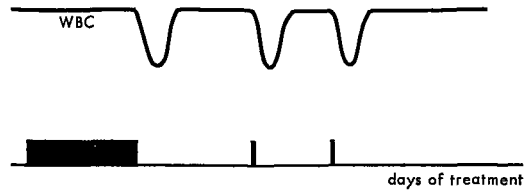
Green, Douglas and Eastwood (1963) described the effect of exposure of rats to an eighty per cent nitrous oxide mixture for a period of 2–6 days. Peripheral blood studies revealed that the absolute numbers of both polymorphonuclear leukocytes and of lymphocytes decreased; however, the relative number of lymphocytes increased.

A progressive hypoplasia was seen in the marrow. Reproduction of

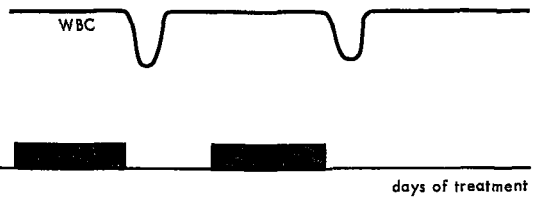
cells apparently ceased and mitoses disappeared. No particular cell form seemed to have escaped, except for the megakaryocytes. A bone marrow picture resembling that of megaloblastic anaemia was not reported, in contrast to the situation in man; this suggests a difference in response to nitrous oxide between haemopoietic tissue in the rat and in man. The mechanism of this nitrous oxide effect is still largely unclear, although it was recently shown that nitrous oxide blocks cultured HeLa cells in mitosis (Brinkley and Rao, 1973). Bruce and Koepke (1966) exposed rats to 0.45 per cent halothane for periods of 24 to 115 hour. They reported a peripheral granulocytopenia, an increase in the ratio of dividing to maturing cells in the marrow granulocytes, a normal uptake of labelled thymidine in the DNA of dividing marrow cells, but a failure of labelled DNA to progress into more mature cell types with the passage of time. A toxic effect, whereby cells are killed, has not been reported for halothane. In this respect, halothane and nitrous oxide have different effects.

In the foregoing, the effect of continuous exposure to the general anaesthetics halothane and nitrous oxide on haemopoietic cells has been described. It may be interesting to determine which other drugs ultimately lead to bone marrow depression after continuous exposure, and, moreover, what are the mechanism(s) by which they exert their effects. One approach to this subject, is to select drugs that can reversibly inhibit cell growth in the haemopoietic system, and thus to determine which drugs give rise to clinical signs of depression of growth in the haemopoietic system, i.e., in patients with agranulocytosis or aplastic anaemia. A variety of drugs are already known to result in these clinical conditions after varying periods of administration (see, among others, the publications of the Swedish Adverse Drug Reaction Committee, e.g. Böttiger et al., 1973; Böttiger et al., 1974). At regular intervals new descriptions of drugs causing agranulocytosis or aplastic anaemia appear in the literature, e.g. propranolol (Nawabi et al., 1973).

Two main categories are often considered in the causation of agranulocytosis and aplastic anaemia (see, for instance, Pisciotta, 1973). One type produces a disease characterized by a sensitization period followed by a sudden onset of agranulocytosis with violent chills, fever and collapse. The rapid fall in peripheral leukocytes suggests peripheral destruction. Readministration of very small amounts of the drug brings about precipitous granulocytopenia (see



A. Type I agranulocytosis; peripheral leukocyte destruction



B. Type II agranulocytosis; block of proliferation

Figure 1.1. Schematic representation of the course of two types of drug induced agranulocytosis. WBC, white blood cells.
Part A: agranulocytosis associated with peripheral leukocyte destruction; after sensitization, readministration of only a small amount of the drug brings about precipitous granulocytopenia.
Part B: agranulocytosis associated with a block of proliferation; no sensitization occurs.

figure 1.1a). Nieweg (1973) described this syndrome as being a type of allergy. Obviously, drugs belonging to type 1 do not meet the above mentioned criteria for an ideal compound. In type 2 disease, there is also a latent period followed by sudden leukopenia, which may be asymptomatic until the onset of an infection becomes evident. It may be possible to readminister the drug during a certain period before the next decline in the number of granulocytes occurs (see figure 1.1b). Type 2 toxicity is generally associated with an interference with production rather than peripheral destruction. The bone marrow picture in this type is hypoplastic or aplastic and is devoid of granulocyte precursors and normoblasts and there are numerous empty fat spaces and few lymphocytes.

Recovery starts with lymphocyte repopulation followed by granulocyte regrowth. A major group of drugs possessing type 2 toxicity is the class of phenothiazine derivatives. For one member of this class of drugs, chlorpromazine, the mechanism by which marrow toxicity is

produced has been investigated in detail (see review Pisciotta, 1973). Since the incidence of chlorpromazine-induced agranulocytosis is low, a cellular defect in chlorpromazine-sensitive patients has been proposed. This defect was shown to be a deficient incorporation of thymidine triphosphate (TTP) into DNA, even in the absence of chlorpromazine. Functionally, the marrow of these chlorpromazine-sensitive subjects has fewer precursor cells in cell cycle and a smaller number of *in vitro* colony forming cells. Under normal conditions, they have a sufficient number of proliferating cells to maintain a normal leukocyte count. Drug exposure eliminates the limited number of cycling cells and a shortage of cells develops soon thereafter. Although such detailed information is not available for other drugs, this example suggests that, generally speaking, drugs giving type 2 toxicity do not result in agranulocytosis in normal individuals, despite long periods of administration.

The conclusion here is that clinical data on agranulocytosis and aplasia do not reveal drugs which may be extremely well suited to protect normal cells by depressing their DNA synthesis rate during chemotherapy.

C. The haemopoietic system of the mouse

Haemopoietic tissue is one example of the so-called self renewal tissues, in which a sufficient number of highly differentiated cells of the erythroid, granuloid, lymphoid and megakaryocytic lines is maintained throughout the lifespan of an individual. Microscopic investigations of haemopoietic tissue and haemopoietic cells in the blood (and other tissues) have permitted the morphological characterization of the different cell lines each being a spectrum of cell stages between immature and fully differentiated. The maintenance of a constant number of mature cells requires the existence of stem cells which give rise to both new stem cells and differentiating cells. The great similarity in the morphology and staining properties of the cells at an early stage of development of the cell lines suggested a common "stem cell" for all haemopoietic cell lines. Twenty years ago, however, the view that each cell line consisted of the progeny of its own stem cell also found support. The suggestion that the lymphoid system had its stem cell and the granuloid, erythroid and megakaryocytic systems had a common stem cell, was also proposed at that time.

A wealth of information on the morphology of the haemopoietic tissue under normal conditions and during conditions of disease and a wide variety of other types of stress was collected. However, insight into the mechanisms involved in self maintenance of populations and the production of mature cells and the mechanisms by which their production is controlled remained unavailable.

This was primarily due to the lack of a functional and sensitive quantitative assay for cell survival in the haemopoietic tissue at the stem cell level. In 1956, it was shown in mice that the efficacy of bone marrow transplantation in the prevention of radiation-induced bone marrow death was cell mediated (Vos, Davids, Weijzen and Van Bekkum, 1956; Ford, Hamerton, Barnes and Loutit, 1956). A next step forward was made when it was demonstrated that the number of mice which survived lethal irradiation after bone marrow transplantation correlated with the number of viable cells in the inoculum of isologous bone marrow (Van Bekkum and Vos, 1957; and others). However, this technique of quantitative bone marrow assay was time- and mouse-consuming. The eventual method for a quick and sensitive quantitative estimation of the regenerative capacity of mouse bone marrow, i.e. the number of haemopoietic stem cells, came in 1961. Till and McCulloch (1961) showed then that mouse bone marrow cells injected into lethally irradiated syngeneic recipients were able to form colonies which were visible on the surface of the spleen and that the number of spleen colonies was directly proportional to the number of injected cells. This technique is known as the "exogenous spleen colony assay".

A colony forming unit (CFU) is defined as the cell (or group of cells) in a bone marrow or spleen cell suspension which upon injection into a lethally irradiated recipient gives rise to a macroscopically visible colony containing cells of haemopoietic origin on the surface of the spleen. Haemopoietic proliferation can also be observed in the femoral bone marrow of the recipient mice following transplantation of haemopoietic cells (Trentin et al., 1968; Curry et al., 1967; Wolf et al., 1968). The use of radioactively labelled haemopoietic cell inocula in the rat showed that a number of cells lodge in organs other than spleen and bone marrow, primarily the lung and liver (Gregusová and Hupka, 1961), where they probably die. This indicates that only a fraction of the injected cells lodge in the spleen and form colonies. Thus, the number of CFU in a haemopoietic cell suspension as determined by

the spleen colony assay represents only a fraction of the real number of potentially colony forming cells. It is now widely accepted that the fraction of stem cells which lodges in the spleen and forms a macroscopic and recognizable colony on the splenic surface after i.v. injection, is approximately 0.05 (Lahiri, Keizer and Van Putten, 1970) rather than 0.2 as proposed by Siminovitch et al. (1963).

What is the nature of the colony forming cells? A series of investigations by Becker et al. (1963), Fowler et al. (1967), and Wu et al. (1967), using mouse haemopoietic cells bearing a chromosomal marker, provided evidence for the view that the majority of the colonies originated from a single cell. The descendants within a single colony showed the same chromosomal marker. These investigations also revealed that the CFU are able to differentiate into haemopoietic cells of all cell lines. The self-renewal property of the CFU was demonstrated by Trentin and Fahlberg (1963) by retransplantation of single colonies. Although no splenic colonies of a lymphoid nature were found, it was established that CFU can also give rise to progeny of the lymphoid line (Micklem and Loutit, 1966; Wu et al., 1968). The spleen colony forming cell (CFU) is now usually considered as the pluripotent haemopoietic stem cell; at any rate, it is the most primitive haemopoietic cell which can be identified by a functional assay. Its morphology has been described (van Bekkum et al., 1971).

Models to define the sequence of the events that take place between the CFU and the mature cells have been postulated (Lajtha, 1970; Trentin, 1970; Hellman et al., 1970; McCulloch and Till, 1971). Briefly, these models are based on the existence of two categories of progenitor cells:

1. pluripotent stem cells (speen colony forming cell = CFU) capable of differentiating into erythropoietic, granulocytic and megakaryocytic lines as well as possessing self-renewal capacity;

2. early committed cells, progeny of pluripotent parents but restricted in ways in which they can undergo differentiation and with limited capacity for self-renewal to maintain independent populations of progeny. Two types have been recognized and generally accepted up to now: a. the CFU-c, which is a progenitor cell committed to granulopoiesis and which requires a Colony Stimulating Factor (CSF) to form colonies in culture (Bradley and Metcalf, 1966; Pluznik and Sachs, 1966; Van den Engh, 1976); b. the CFU-e, the progenitor cell

committed to erythropoiesis and which is erythropoietin-dependent (Stephenson et al., 1971).

The kinetic state of mouse haemopoietic stem cells

As in all dividing mammalian cell systems, the mouse haemopoietic cells traverse the cell cycle stages G_1 (pre-synthetic), S (DNA synthesis), G_2 (premitotic) and M (mitosis), according to the concept proposed by Howard and Pelc (1951, 1953). A G_0-phase has been proposed to explain how some cell populations such as liver and bone marrow stem cells alter their rate of cell production in response to a loss of cells (Lajtha, 1963; Quastler, 1963). The G_0 cells were conceived to act as a reservoir from which cells could be randomly triggered in order to supply cells for division when required (for reviews on G_0 phase, see Epifanova and Terskikh, 1969; Burns and Tannock, 1970; Van Putten, 1974). These cells are referred to as G_0-phase cells, G_0 cells, resting cells or noncycling cells (fig. 1.2).

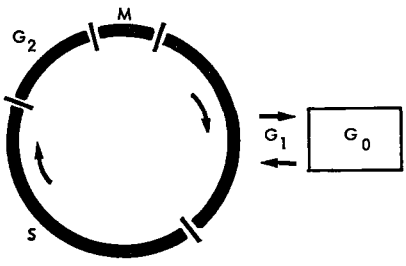


Figure 1.2. Diagrammatic representation of the cell cycle of mammalian cells. Neither the exact location of the G_0-phase cells has been indicated, nor has it been tried to visualize the pool of differentiating cells. M, mitosis; G_1, pre-synthetic phase; S, DNA synthesis (duplication of genetic material); G_2, pre-mitotic phase; G_0, population of non-proliferating cells.

There are several available techniques for measuring the proportion of mouse haemopoietic stem cells which are "actively" traversing the cell cycle stages, i.e. cycling cells; the *in vitro* or *in vivo* tritiated thymidine (^{3}H-TdR) suicide technique and comparable techniques using other compounds such as ^{125}Iododeoxyuridine (^{125}IUdR) or hydroxyurea (HU) are most often used. Both ^{3}H-TdR and ^{125}IUdR are specifically incorporated into DNA and cause cell death due to beta-

irradiation of the genetic material (see, for a recent discussion of these data, Hofer et al., 1975). Thus, only cells in S-phase are killed, allowing one to estimate the number of stem cells in S-phase by calculating the number of surviving spleen colony forming cells in a suspension treated with ^{3}H-TdR compared to an untreated control bone marrow suspension. Hydroxyurea prevents entry of cells into S and also kills cells already in S-phase. Using one or another of the above-mentioned techniques, several groups of investigators determined the kinetic state of normal mouse bone marrow, i.e. the fraction of stem cells in S-phase. The *in vitro* ^{3}H-TdR suicide technique in which the cells are incubated in a ^{3}H-TdR medium with a high specific activity (about 20 Ci/mmol) showed that less than 10 per cent of the mouse haemopoietic stem cells are in S-phase (Becker et al., 1965; Iscove et al., 1970; Moore and Metcalf, 1972; Metcalf, 1972; Lahiri and van Putten, 1972; Blackett et al., 1974). An exception was found in studies of Croizat, Frindel and Tubiana (1970), in which figures of roughly 20 per cent were reported when using the *in vitro* incubation technique. These higher figures were attributed, however, to infection in the animals. The *in vivo* ^{3}H-TdR suicide technique, in which a much higher dose of the compound is injected into the mice, shows that a larger proportion of the stem cells are in S-phase, with 20 per cent as the mean figure (Blackett et al., 1974; Dicke and Van Bekkum 1972). It might be argued that recruitment of CFU into cycle is responsible for the considerably larger number of CFU in S-phase when using the *in vivo* ^{3}H-TdR suicide technique. This effect is also seen after hydroxyurea (Vassort et al., 1973), irradiation (Croizat, Frindel and Tubiana, 1970) and treatment with other cytostatic agents such as Ara-C (Millard and Okell, 1975).

At present, a majority of the investigators in this field accept a model for the normal mouse in which less than 10 per cent of haemopoietic stem cells are in S-phase. Higher values in an otherwise "normal" animal are believed to indicate that the animal is either anaemic or infected (Lajtha, 1975). Assuming that, at the most, 10 per cent of the stem cells are in S-phase and also assuming that under steady state conditions those cells not in S-phase have an age distribution with equally large frequencies in the other phases of the cell cycle, it can be calculated that 20 per cent (2 times 10 per cent, since the duration of S-phase (Ts) is about $\frac{1}{2}$ of the duration of one cell cycle (Tc) (Frindel et al., 1967)) of the mouse haemopoietic stem cells

are actively traversing the cell cycle. This indicates that 80 per cent of the stem cells are in a resting state (G_0-phase cells). There is evidence that not only stem cells but also more mature cells may be either resting or cycling in response to differing demands for cell production or differentiation (Becker et al., 1965; Iscove et al., 1970). For comparison, it should be noted that 20–50 per cent of the haemopoietic colony forming cells in tissue culture were found to be in S-phase (Blackett et al., 1974; Lajtha et al., 1969), indicating that this cell population contains a much smaller proportion of resting (G_0-phase) cells.

Differentiation of mouse haemopoietic colony forming cells

Evidence in favour of the existence of a pluripotent haemopoietic stem cell from which all different cell lines arise has been mentioned in earlier paragraphs. The study of differentiation pathways of haemopoietic colony forming cells is hampered by the lack of methods by which stem cells can be distinguished from the rest of the cells in a haemopoietic cell population. The spleen colony technique gives some insight into the processes of stem cell differentiation. However, this method is biased by a great variety of largely unknown factors which may be involved in the process of proliferation and differentiation. An important portion of the data concerning *in vivo* differentiation of haemopoietic stem cells has come from histological examination of colonies produced from treated or untreated haemopoietic cells in normal and treated recipients. These date have been reviewed by Dunn (1971a).

Spleen colonies may be of one cell line, e.g. purely erythroid or purely myeloid, or mixed, consisting of two or three cell lines. In the spleen, the majority of granulocytic colonies grows in the pulp, whereas the erythroid colonies tend to grow under the splenic capsule (Lewis, O'Grady and Trobaugh, 1968). Seven or eight days after bone marrow transplantation, 50–60 per cent of the colonies are erythroid, 15–20 per cent granulocytic, 4–5 per cent megakaryocytic, 10 per cent mixed and the remainder undifferentiated (Jenkins, Upton and Odell, 1969; Trentin et al., 1968; Silini et al. 1968; Gidali and Fehér, 1967; Curry and Trentin, 1967). This gives a ratio of erythroid colonies to granulocytic colonies (E:G) of 3:1 or 4:1. An increase in time interval to more than ten days changes this spleen colony distribution as follows: a number of pure colonies give rise to other

cell lines; consequently the number of mixed colonies increases. The ratio E:G, however, remains at 3:1 or 4:1. In mixed colonies, the second line of differentiation occurs among the immature cells in the periphery of the initially pure colony (Wolf and Trentin, 1968). Trentin (1970; 1971) suggested that this was because the colonies encroach upon another type of microenvironment. The colonies which develop in the femoral marrow are randomly distributed (Trentin et al., 1968). Seven or eight days after transplantation, the majority of the colonies in the bone marrow are pure (Trentin et al., 1968; Wolf and Trentin, 1968). Moreover, the E: G colony ratio is 0.5:1 or 1:1 (Trentin et al., 1968). Whole spleens transplanted subcutaneously support haemopoiesis and give rise to spleen colonies with the same E:G colony ratio as do normal spleens, i.e. 3 to 4:1 (Wolf and Trentin, 1968). Pieces of marrow stroma transplanted into the spleen support haemopoiesis with an E:G colony ratio similar to that of bone marrow *in situ*, 05:1 or 1:1. Colonies growing across the junction of marrow and spleen show abrupt transition of haemopoietic type, i.e. erythropoiesis in spleen stroma and granulopoiesis in marrow stroma (Wolf and Trentin, 1968). The above-mentioned data, among others, indicate the importance of the micro-environment for the differentiation of haemopoietic colony forming cells and strongly suggest that it is no longer correct to say that the decision process by which a differentiation pathway is chosen by the pluripotent stem cell is stochastic, random or uncontrolled. (Tavassoli, 1975).

A large number of agents, including radiation, endotoxin, cytostatics and hormones, have been used in attempts to modify the number and histological type of the spleen colonies (see review by Dunn, 1971a). For example, irradiation of donor mice with doses up to 500 rad decreases the E:G colony ratio at 7 or 8 days after transplantaton from 3:1 or 4:1 to 1:1 (Wolf and Trentin, 1970). A similar ratio has been found for endogenous spleen colonies treated in the same way (Jenkins, Upton and Odell, 1969). Marrow treated with Methotrexate 24 hours before transplantation into irradiated recipients results in an increase in the E:G colony ratio from 4:1 to 15:1 at day 8 (Dunn, 1971b). Changes in spleen colony histology may reflect direct changes in differentiation pathways of the colony forming cells. These changes, however, can also be induced via other mechanisms (O'Grady, Lewis and Trobaugh, 1968), e.g. an increased proliferation rate of erythroid cell lines induced by erythropoietin that leads to very large erythroid

colonies which overgrow the smaller colonies of other cell lines. Thus, a change in spleen colony histology does not necessarily reflect changes in differentiation pathways. Similarly, changes in spleen colony histology do not always reflect changes in bone marrow morphology, e.g. oestradiol depresses erythropoiesis in the donor but granulopoiesis in the colonies which arise after transplantation of the treated marrow. These data illustrate the difficulty one faces when studying the influence of radiation or drugs on the differentiation of haemopoietic stem cells. Furthermore, the time course of the number of CFU revovered from spleens of sublethally irradiated mice or from spleens of supralethally irradiated and bone marrow transplanted mice, shows a decrease during the first days. This aspect will be discussed in the following paragraph.

The loss of stem cells following irradiation

A reduction in the number of colony forming cells which survive in the spleen after a sublethal dose of radiation or in the number of colony forming cells early after transplantation of haemopoietic cells in lethally irradiated recipients has been shown (Lahiri and Van Putten, 1969; Kubanek et al., 1969; Kretchmar and Connover, 1969; and others). This early decrease in the number of CFU is usually referred to as "the dip" (fig. 1.3). This dip can be abolished by pretreatment of the donor animal with Vinblastine (Smith, Wilson and Fred, 1968) or pretreatment of either the donor or the recipient with a sufficient dose of ionizing radiation (Lahiri, Keizer and Van Putten, 1970). The latter authors suggested that the loss of CFU is due to differentiation of multipotential stem cells, with subsequent loss of colony forming ability. Similarly, a roughly threefold increase in the percentage of colony forming cells in S-phase (DNA synthesis) has been shown to occur in both the spleen and the femur in the first days after sublethal irradiation (Lajtha et al., 1969; Croizat, Frindel and Tubiana, 1970; Guzman and Lajtha, 1970; Gidali and Lajtha, 1972). Lahiri and Van Putten (1972) found an increase in the number of femoral stem cells in S-phase from 7 to 25 per cent within minutes following the irradiation.

The dip in the time course of CFU growth in the spleen after sublethal irradiation or transplantation of haemopoietic cells indicates that the loss of stem cells is larger than the increase by repopulation.

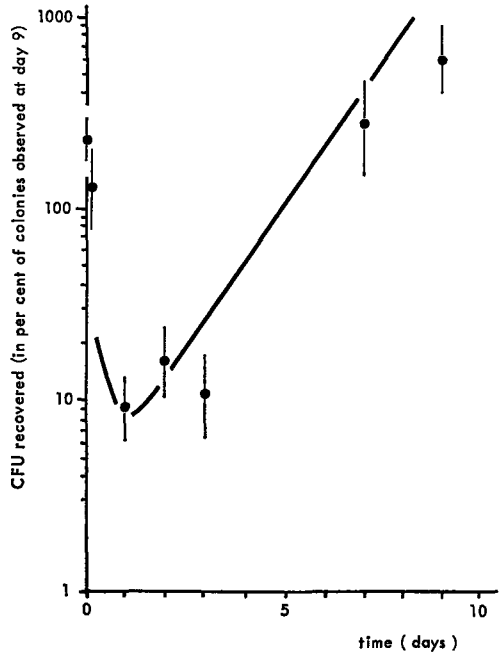


Figure 1.3. Illustration of the decrease in number of haemopoietic stem cells (the "dip") in the spleens of mice which received a whole-body radiation dose of 450 rad X-rays (represented by the points). A similar decrease in the number of haemopoietic stem cells is also observed when they are transplanted into mice whose own bone marrow has been eradicated after 750 rad whole-body X-irradiation (solid line). Note that immediately after sublethal irradiation (dots) the spleen contains 2.5 CFU for every colony observed at 9 days in similar animals. From Lahiri, Keizer and Van Putten (1970).

Thus, in the first few days after irradiation, the differentiation stimulus is larger than the proliferation stimulus. Radiation induced differentiation has also been observed in other tissues, such as mouse-yolk-sac-derived erythroid cells (Fantoni et al., 1972) and *in vitro* mouse neuroblastoma cells (Prasad, 1974; Yang et al., 1974). The latter authors observed that radiation resulted in a differentiation stimulus on neuroblastoma cells which was similar to that of cyclic AMP.

The haemopoietic system of the mouse and chemotherapy

Since the size of the spleen colony forming cell (CFU) population is considered to be a reflection of the repopulating capacity of the haemopoietic system, the spleen colony technique has frequently been used, and continues to be used, to determine the effect of anti-cancer drugs on this tissue. Based on a comparison with mouse lymphoma cells, W. R. Bruce and coworkers (1966, 1968) were able to subdivide chemotherapeutic drugs into three classes according to the type of dose-survival curve to which they gave rise. These were:

1. *nonspecific* agents: these give exponential dose-effect curves which are linear when survival fractions are plotted on a logarithmic scale against the dose on a linear scale; there is no difference between the dose-survival curves for either normal colony forming cells or lymphoma cells. Nitrogen mustard and X- and gamma radiation belong to this group.

2. *phase-specific* agents give dose-effect curves which plateau at the higher drug dosages; lymphoma cells are much more sensitive. ^{3}H-thymidine, Vinblastine, Methotrexate, Azaserine and Arabinosylcytosine are drugs in this group. Since ^{3}H-TdR only kills cells which are synthesizing DNA, it was concluded that the agents belonging to this group only kill cells while they are traversing a particular proportion of the cell cycle. The cells which are not passing through the phase in which they are sensitive to the drug are unaffected by even very high doses of the agent as indicated by the survival plateau in the curves.

3. *cycle-specific* agents lead to exponential survival curves with different slopes for both types of cells; the difference found in slopes is in contrast to the type 1 agents. Agents which belong to this group do not kill cells only in a a specific cell cycle phase but are generally effective against proliferating tissues; 5-Fluorouracil, Actinomycin-D, Cyclophosphamide and Bis-chloroethylnitrosourea (BCNU) are members of this group. The difference in slope of resting (normal bone marrow) and rapidly proliferating (lymphoma) cell survival might be explained by differences in time available for repair of the lesions before the next S-phase fixes the damage (Van Putten and Lelieveld, 1970a).

This classification of anti-cancer drugs into the three groups proposed by Bruce et al. has been confirmed and extended by Van Putten and co-workers (1971; 1972) (fig. 1.4).

They arrived at the same classification, using normal resting colony forming cells and rapidly proliferating colony forming cells. In contrast to the colony forming cell population in a normal mouse, a minority of the cells in a rapidly proliferating colony forming cell population is in a resting state (see chapter 2 for the technique of the production of such a rapidly proliferating cell population). However, the differences between the two models emphasized an important principle in cancer chemotherapy. Nitrogen mustard which, according to Bruce et al., falls into the type 1 drug classification (nonspecific), was found to be cycle-specific by Van Putten and Lelieveld (1971).

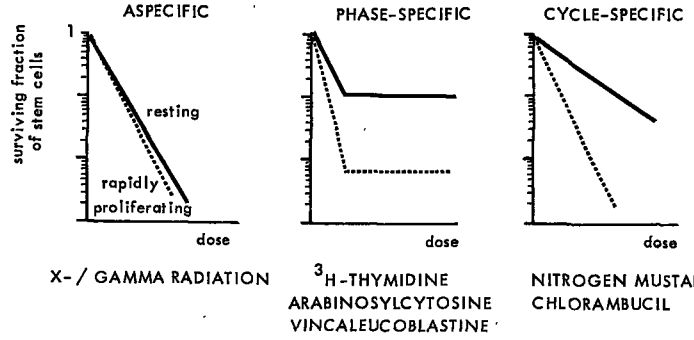


Figure 1.4. Schematic representation of the dose-effect relationships for chemotherapeutic agents in killing resting and rapidly proliferating normal haemopoietic spleen colony forming cells (after Van Putten, 1972).

In addition, drugs which had been classified as cycle-specific according to the lymphoma model did not show this cycle specificity for rapidly proliferating normal cells (Van Putten, Lelieveld and Kram-Idsenga, 1972). These data emphasized the importance of intrinsic differences in sensitivity to anti-cancer drugs among cell types. This was confirmed by the studies of Van Putten et al. (1972) in which it was shown that cycle-specific drugs (type 3) are not necessarily effective in all experimental tumours with a high growth fraction (GF) and a relatively short cycle time (Tc), where the growth fraction is defined as the fraction of cells which actively traverse the proliferation cycle and the cycle

time as the time it takes for a cell to complete one cell cycle (G_1, S, G_2 and M).

Recruitment

It was discussed earlier that the percentage of mouse haemopoietic stem cells which is in an active cell cycle could be estimated to be less than 20 percent. Circumstantial evidence suggests that a similar situation may exist in man (Frei et al., 1969). This does not imply that, for instance, phase-specific drugs can "safely" be given during a course of chemotherapy. It has been shown that treatment of these resting tissues with cytostatics can recruit resting cells into cycle (W. R. Bruce et al., 1969; Bhuyan et al., 1973; Van Putten, 1973, Van Putten et al., 1973). These cells may be subsequently killed by the circulating drug. It is thought that this recruitment of resting cells into cycle is mediated by corrective regulatory mechanisms switched on due to either loss of stem cells or loss of more differentiated or maturing cells.

Synchronization

Cells participating in proliferation under steady state conditions can be described by an age distribution function in which cells differing in age have equally large frequencies. Synchronization procedures per definition wil change this frequency distribution and cause a temporarily increase or decrease in the frequency of cells of a given age. There are various methods for synchronizing cells both *in vitro* and *in vivo* (see for reviews, Nias and Fox, 1971; Frindel and Tubiana, 1971).

A variety of drugs cause an accumulation in or just before a certain phase, e.g. Ara-C kills cells in S and induces a G_1/S progression delay (synchronization in S, when the block is released); VLB kills cells in M and also causes cells to accumulate in M (for a review of the site of action of anti-cancer drugs, see Madoc-Jones and Mauro, 1975; Klein, 1974; Hill and Baserga, 1975. Many successful attempts to synchronize both normal (haemopoietic and gastrointestinal cells) and malignant cells *in vivo* have been made in both experimental animals and man (for a review of *in vivo* experimental and clinical treatment protocols, see Van Putten, Keizer and Mulder, 1976).

The radiation syndromes

During the first 3 or 4 weeks following whole-body irradiation of mammals, fatalities may occur due to injury to the haemopoietic system, the gastrointestinal system and the central nervous system. As can be seen in fig. 1.5, the animals die within an interval of a few hours to a few days after exposure to dose ranges exceeding 12,000 rad (cerebral syndrome). At a lower dose level, between 1200 and 12,000 rad, death occurs between 4–7 days due to denudation of the epithelium of the gastrointestinal tract. Anorexia and watery diarrhea are the main features leading to a shock syndrome. Radiation dosages lower than roughly 1200 rad are insufficient to cause lethal damage in the aforementioned organ. The animal will die after dosages between about 700 and 1200 rad from a loss of function of the haemopoietic system (bone marrow syndrome). The haemopoietic tissues are depleted of the various cell lines, leading to granulocytopenia after a few days, thrombocytopenia between 8–12 days and anaemia during the second and the third week. Sepsis and haemorrhages are the main clinical symptoms occuring during the bone marrow syndrome. Treatment with isologous bone marrow or spleen cells protects the irradiated mice from haemopoietic death up to the irradiation dose level at which the intestinal syndrome will occur and kill animals from 4–7 days after irradiation.

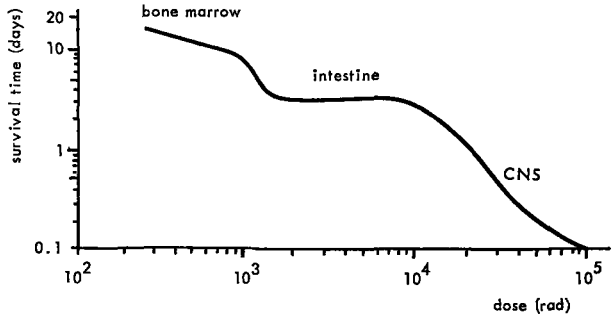


Figure 1.5. Survival time after lethal whole-body radiation exposure in mice, showing the three radiation syndromes. From Van Bekkum and De Vries, 1967.
1. Region of bone marrow syndrome
2. Plateau of intestinal syndrome
3. Region of cerebral syndrome

Radiosensitivity of mouse haemopoietic stem cells

As previously mentioned, the work of several groups led to the development of the spleen colony technique which enabled the investigator to measure the radiation sensitivity of mouse haemopoietic cells (Till and McCulloch, 1961). With the aid of this technique, dose survival curves can be made for mouse spleen colony forming cells (CFU). A typical dose survival curve is shown in fig. 1.6. An explanation of the different parts of the curve and of the symbols used is given in the legend.

Terminology by which the radiosensitivity of the mouse colony forming cells or mammalian cells in general can be expressed when the

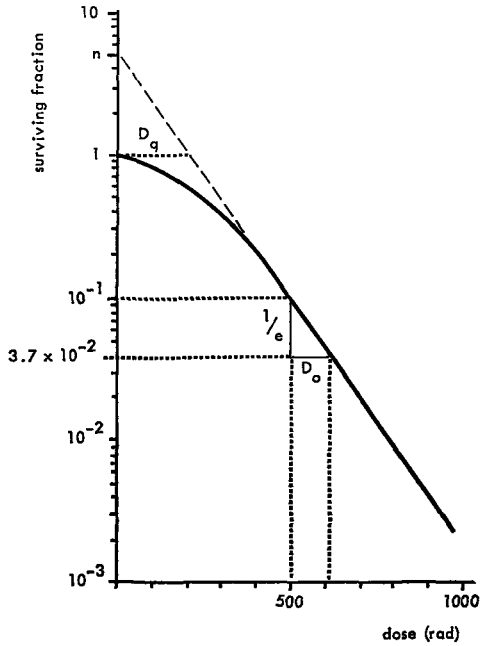


Figure 1.6. Dose-survival curve for mammalian cells (including spleen colony forming cells) after X- or gamma radiation exposure. On the vertical axis the surviving fraction is plotted on a logarithmic scale against the dose plotted on a linear scale on the horizontal axis. The curve has an initial shoulder followed by a straight portion. The width of the shoulder is characterized by the extrapolation number n, or by the dose D_q, the dose at which the extrapolated straight portion of the curve cuts the dose axis trough 1. The straight portion of the curve is characterized by the slope D_0 (D_{37}), i.e. the dose needed to reduce survival to 37 (1/e) per cent.

radiosensitivity of a cell population is determined via a quantitative survival assay, has been developed. Dose survival curves obtained in such a way are characterized by the following two parameters. 1. The slope of the final straight portion expressed as the dose required to reduce a population to 37 per cent of its initial value, known as the D_0. 2. The width of the shoulder, indirectly indicated by the n-value, was for a long time held to reflect only the ability of the cell population to repair sublethal damage to the DNA. Thus, the n-value should express only the degree of accumulation of sublethal damage. Several authors, however, have come to the conclusion that this is no longer tenable.

Another important factor that can significantly modify the n-value of the dose-survival curve of a spleen colony forming stem cell population is the time between administration of the cytotoxic treatment and assay of the surviving stem cells, indicating the complexity of the shoulder phenomenon (for a discussion of this subject, see Van Putten et al., 1970b; and, in the broader scope of experimental radiotherapy and chemotherapy, Valeriote and Van Putten, 1975).

In vivo versus in vitro irradiation of haemopoietic stem cells

The D_0 values and extrapolation numbers that have been obtained after X- or gamma-irradiation of mouse haemopoietic stem cells *in vivo* do not show significant differences from the *in vitro* data (irradiation of freshly prepared suspensions under euoxic conditions). This can be illustrated by data from Van Putten et al. (1970). These authors reported D_0 values 64 and 73 rad for the *in vivo* and *in vitro* situation respectively, and n-values 2.6 and 3.2 respectively, which are essentially the same as those obtained by other investigators (see review Lajtha, 1965). Comparison of the *in vivo* and *in vitro* radiosensitivity of normal resting stem cell population with a rapidly proliferating stem cell population did not reveal significantly different D_0 values. However, the extrapolation numbers appear to be lower in the case of the rapidly proliferating stem cells: $n = 1.1$ and $n = 1.2$ for *in vivo* and *in vitro* irradiation respectively, compared to before mentioned extrapolation numbers $n = 2.6$ and $n = 3.2$.

The age-response of haemopoietic stem cells

The age responses of cultured mammalian cells to ionizing irradiation are, in general, very similar. Some differences do exist; these depend on the cell line, the method of synchronization and the radiation dose. The data indicate that late G_2 and M cells are most sensitive and late S cells are the least sensitive. For G_1 cells, there is a difference between cells with a short or long cycle time. In cells with short cell cycle times (mouse L cells), G_1 cells are rather resistant. In cells with long cell cycle times (HeLa cells, Chinese hamster cells), early G_1 cells, are quite resistant, whereas late G_1 cells are sensitive (see Fabrikant 1969).

After synchronization *in vivo* using hydroxyurea, mouse spleen colony forming cells were found to be most sensitive in M and in-

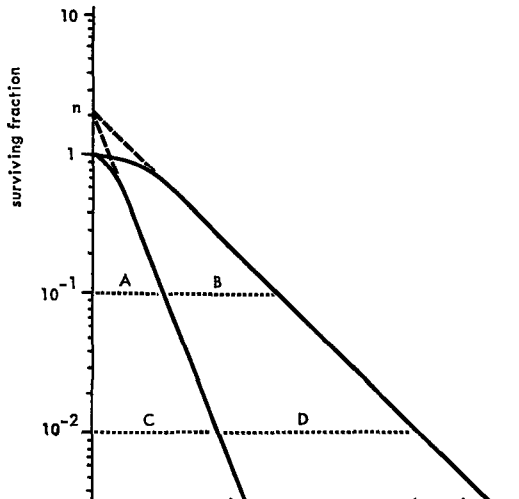


Figure 1.7. Schematic representation of a typical dose-survival curve for mammalian cells exposed to X- or gamma rays under aerated and hypoxic conditions. The ratio (A+B)/A=2.5, is identical to the ratio (C+D)/C=2.5. Thus, at all levels of survival the dose required under hypoxic conditions is 2.5 times greater than under aerated conditions, i.e. the O.E.R. is 2.5. The extrapolation number n needs not to be the same for the two dose survival curves.

creasingly resistant through S-phase (Chaffey and Hellman, 1971). A logical consequence of the finding that S-phase cells are more radioresistant than cells in other cell cycle phases would be that a cell population with a high proportion of S-phase cells (the hemopoietic stem cells of lethally irradiated and bone marrow transplanted mice or of sublethally irradiated mice) would be predicted to be more radioresistant than a normal haemopoietic cell population *in vivo*, which is largely in a resting state (G_0-phase). However, this is not the case, as shown by the essentially similar D_0 values (Van Putten et al., 1970b). These data suggest that radiation effects other than merely cell kill, such as differentiation, may also be involved in the determination of the radiosensitivity of the haemopoietic stem cell population.

The oxygen effect

In the absence of oxygen, a dramatic decrease in the radiosensitivity of many biological entities to X- and gamma radiation is observed. Typical dose-survival curves under aerated and hypoxic conditions are presented in fig. 1.7.

There is one major difference between the two curves, namely, that the radiation dose required to produce a given degree of biological damage is higher in the case of hypoxia. The ratio of hypoxic to aerated doses giving a similar response is often assumed to be similar at all survival levels. This ratio is defined as the oxygen enhancement ratio (OER). For ionizing irradiation, such as X- or gamma rays, the OER has a value of between 2.5 and 3.

CHAPTER 2

EXPERIMENTAL PROCEDURES

Irradiation

Irradiation was accomplished with either a Philips Mueller machine at a dose rate of 60 rad/min (300 kV; 10 mA; HVL of the beam, 3 mm Cu) or with a ^{137}Cs source of about 600 Ci (model Gammacel 20) providing gamma rays at a dose rate of 122 rad/min. In an earlier stage of the study, recipient mice for spleen colony assay received a radiation dose of 750 rad whole-body X-irradiation. When a ^{137}Cs source became available, C57BL/Ka recipient mice received two separated fractions of 600 rad whole-body gamma-irradiation at a 16-hr interval. This was done in an attempt to reduce the occasional high mortality occurring in these mice after 750 rad whole-body X-irradiation followed by bone marrow transplantation. This alternative treatment was effective in suppressing the formation of endogenous spleen colonies. Recipient mice of other mouse strains received 950 rad whole-body gamma-irradiation given in a single session.

Mice

All chemotherapy experiments, including those with halothane, employed 8 to 15-week-old (C57BL × CBA)F1 hybrid male mice and 9 to 15-week-old C57BL/Ka male mice. Nine to 15-week-old (DBA2 × BALB/c)F1 hybrid male mice and 9 to 15-week-old C57BL/Ka female mice were used in the nitrous oxide and ^{3}H-TdR suicide experiments, respectively. For all experiments in which animals were immobilized, either mice chosen randomly among 20 to 40-week-old (C57BL × CBA)F1 hybrid female mice (data given in figures 4.1–4.6), 9 to 15-week-old C57BL/Ka male mice (immobilization plus Ro-07-0582), or 8 to 15-week-old (DBA2 × BALB/c)F1 hybrid male or female were used.

Immobilization of mice during whole-body irradiation

Three types of immobilization were used:

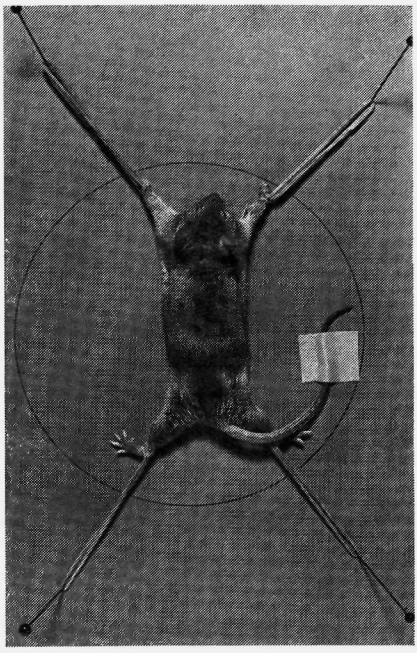


Figure 2.1. Physical restraint for mice during radiation exposure. The mouse has been restrained by means of 4 rubber bands on each of the four legs of the mouse. This method leads to stretching of the mouse and constriction of the lower legs.

a. Anaesthesia. Each anaesthetic compound was injected 10–15 minutes before radiation exposure.
b. Physical restraint by means of rubber bands on each of the four legs of the mouse (fig. 2.1) starting 10 to 15 minutes before radiation exposure. This method leads to stretching of the mouse and constriction of the lower legs.
c. Physical restraint by means of adhesive tape over each of the four legs of the mouse (fig. 2.2) starting 10–15 minutes before radiation exposure. This method presumably causes less stretching of the mouse and no constriction of the legs.

Spleen colony technique for assay of haemopoietic stem cells

The theoretical considerations of this technique have been described previously (see Chapter I). The practical aspects of it are as follows:

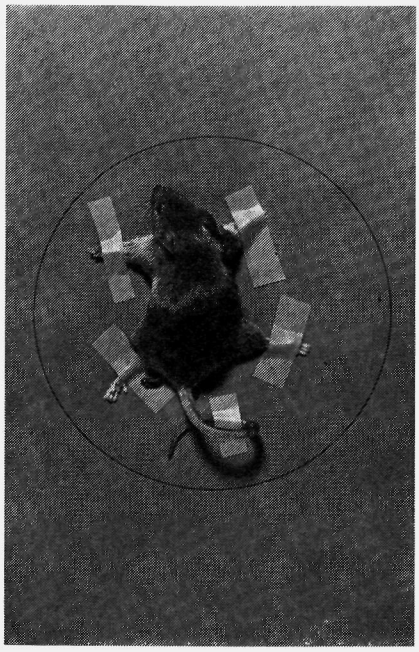
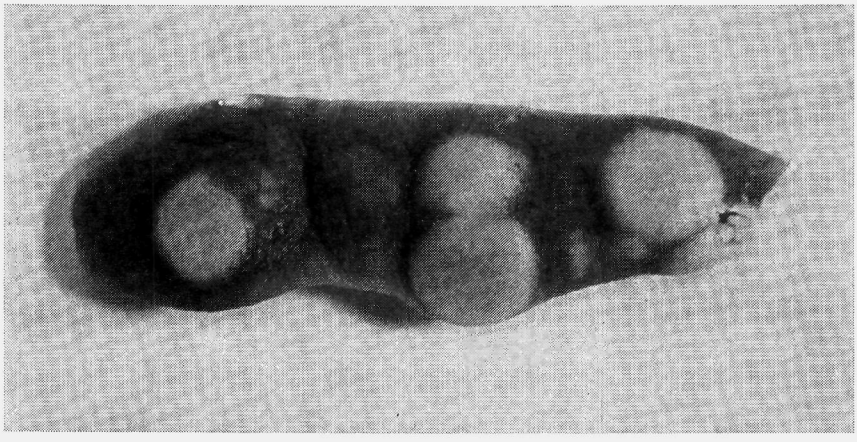


Figure 2.2. Physical restraint for mice during radiation exposure. The mouse has been restrained by means of adhesive tape over each of the four legs of the mouse. This method causes less stretching of the mouse and no constriction of the legs.

Figure 2.3. Photograph of mouse spleen with superficial colonies containing haemopoietic cells. The colonies developed after 950 rad whole-body gamma-irradiation followed by i.v. transplantation of 10^5 isologous haemopoietic cells.



Cell suspensions were prepared in Hanks' solution either by quantitatively washing femur shafts with a syringe and 20 gauge needle and passing the resulting suspension through fine-mesh nylon gauze or by mincing the spleens using a pair of scissors and filtering the suspension through fine-mesh nylon gauze. Sufficient aliquots of the suspensions, giving 5–15 superficial spleen colonies, were injected into the tailvein of heavily irradiated* recipient mice. These recipients were killed 9 days after injection and the spleens were fixed in Telleyesniczky's solution. The colonies visible on the surface of the spleens were then counted** (fig. 2.3). Since each colony represents the progeny of a single haemopoietic stem cell, the number of spleen colony forming cells in the original suspension can be calculated. The effect of several sorts of treatments, e.g. radiation and chemotherapy, can be easily assayed by use of this technique.

Fractionated chemotherapy of haemopoietic stem cells

The basic treatment schedule was as follows: the cytostatic drugs were injected 3 times, at 4-hr intervals, either into normal control animals or into animals which had been treated with CNS depressants. Twelve hours after the start of the cytostatic treatment, the animals were killed by dislocation of the neck and the femurs and/or spleens were collected to estimate the CFU survival. The time points at which the CNS depressant had been administered depended on the experiment and the type of the compound, varying, however, from 3 hours before the start of the experiment to a few hours before the end of it. Injections of chemotherapeutic agents were always given between 2 a.m. and 2 p.m. Modifications of treatment schedules for cytostatic drugs and for CNS depressants are indicated in the results section. The dosages of the CNS depressants were chosen around one dose which was found to induce sedation or anaesthesia.

* See under irradiation.

** Sources of error are found in the biological variation (number of stem cells per femur or spleen), preparation of the suspension and random distribution of the cells after i.v. injection. The last aspect has been discussed by Blackett (1974).

Production of rapidly proliferating haemopoietic stem cells

Less than 10 per cent of the haemopoietic stem cells of a normal mouse which is neither infected nor anaemic are in S-phase, i.e. the majority of the CFU are in a resting state (G_0-phase cells). There are two experimental possibilities for obtaining a haemopoietic cell population in which the majority of the stem cells is actively traversing the cell cycle:

1. Mice are exposed to a dose of ionizing radiation which kills the majority of the haemopoietic cells but which is not sufficiently high to cause the death of the animal. Five to seven days later, most of the stem cells are in cell cycle.
2. Mice are irradiated with a dose which is sufficient to kill 100 per cent of the animals due to the bone marrow syndrome. Isologous bone marrow transplantation i.v. is then performed; this protects the mice from being killed. Ninety-eight to 99 per cent of the transplanted CFU are actively traversing the cell cycle (rapidly proliferating) five to seven days later.

The advantage of the second method is then one can be sure that the repopulating haemopoietic cells have not been affected by sublethal radiation damage. Therefore, to produce the repopulating marrow, one quarter of the content of a femur, containing $2.5-5 \times 10^6$ cells, was injected into the tailvein of recipient mice which received 950 rad gamma rays a few hours earlier. Seven days later, these transplanted animals served as donor animals to be subjected to further treatment followed by assay of surviving CFU in their spleens or femurs.

In vitro estimation of number of femoral marrow haemopoietic stem cells in S-phase

When looking at an haemopoietic cell suspension, one cannot discriminate between the potential spleen colony forming stem cells and the non-stem-cell pool. Therefore, the number of DNA synthesizing (S-phase) stem cells cannot be estimated by means of autoradiography. To solve this problem, the haemopoietic cell suspension is incubated with a DNA precursor which is radioactively labelled. The specific activity of the label must be high enough to kill the cell when the label

is incorporated into DNA. By means of the spleen colony technique, the decrease in spleen colonies, i.e. the loss of stem cells, is determined as a measure of the number of spleen colony forming stem cells in S-phase.

The nucleoside thymidine (TdR) is preferentially incorporated into the DNA of cells in S-phase. When tritiated (^{3}H-TdR), this compound kills the cells in S by beta-irradiation of the nuclear material.

Femoral bone marrow cell populations investigated were prepared using Hanks' Balanced Salt Solution to which 50 ml of calf serum, 10^5 I.U. of penicillin and 100 mg of streptomycin per liter had been added. Aliquots (0.4 ml) of the cell suspensions, containing approximately 10^7 cells/ml, were incubated with either 0.1 ml of ^{3}H-TdR (final activity 100 μCi/ml; specific activity, 18.5–21 Ci/mmol) or with 0.1 ml of physiological saline for 20 min at 37 °C. The reaction was stopped by using ice-cold Hanks' solution and, after appropriate dilution, the number of surviving CFU was assayed in lethally irradiated isogeneic recipient mice. The number of CFU specifically killed by the ^{3}H-TdR was assumed to reflect the number of CFU in S-phase.

Estimation of DNA synthesis rate in femoral marrow and splenic cells

As with ^{3}H-TdR, ^{125}IUdR is exclusively incorporated into DNA. This labelled nucleoside was used to measure the *in vivo* DNA synthesis rate in haemopoietic cells (stem cells and non-stem-cells) of mice which had been exposed to either nitrous oxide or normal air for one or more days. The advantage of ^{125}IUdR is the ease of counting gamma rays from ^{125}I which abolishes the need for preparing clear scintillation counting samples.

One hour before the end of anaesthesia, 5 μCi of the ^{125}IUdR (specific activity 2–6 Ci/mmol) in 0.2–0.3 ml was injected i.p. The animals were killed by cervical dislocation one hour later and tissues of interest collected and weighed immediately. In the case of femoral bone marrow, a suspension was prepared by quantitative removal of the contents of 2 femurs. According to a modified Schmidt-Tannhauser method, tissues were dissolved in 2 ml 1 N NaOH over a 24-hr period at 37 °C. The tubes were then cooled to 0 °C and 10 ml of an ice-cold 15 per cent trichloroacetic acid (TCA) solution was added to precip-

itate the DNA. The precipitate was washed twice at 0 °C using a 5 per cent TCA solution to achieve separation of DNA and RNA and the washed DNA was finally counted in a well-type scintillation NaI(Tl) gamma counter. The number of counts obtained from each tissue sample was expressed as a percentage of the number of counts per μCi of injected activity.

Production of hypoxic bone marrow cells

To investigate the radiosensitizing properties of Ro-07-0582 on completely hypoxic mouse haemopoietic cells, mice were killed by cervical dislocation and exposed to whole-body-irradiation 10 minutes thereafter. One hour before the irradiation (50 minutes before killing), these mice received either 0.25 ml physiological saline or Ro-07-0582 at a dosage of 1 g/kg i.p. The CFU assay was performed on the femoral marrow of all groups immediately after irradiation.

Identification of hypoxic cells in the femoral marrow

The compound Ro-07-0582* was used to identify hypoxic haemopoietic stem cells in the femoral marrow of the mouse. The suspension of the drug was freshly prepared for each experiment and suspended in a 2 per cent carboxymethylcellulose solution. It was injected i.p. one hour before radiation exposure at a dosage of 1 g/kg body weight. This dose is close to the maximum tolerated dose (1.2 g/kg). The decrease in the femoral CFU survival (as compared to a like treated control) in the Roche-compound-treated animals was considered to reflect the fraction of hypoxic cells. To distinguish between a sensitizing and toxic effect of the compound for stem cells, a well-oxygenated *in vivo* bone marrow cell population was also irradiated in the presence of the sensitizer.

Halothane and Nitrous Oxide

Air containing 0.4 per cent halothane was produced with a Fluotec Vaporizer® at a flow rate of 2 l/min and led into a perspex cage of about 11 liters volume, which was adapted to serve as an anaesthetic

* Thanks are due to Hoffmann-La Roche of Basel for providing Ro-07-0582.

chamber. This dose of halothane is sedative. Thus, the mice respond to stimuli during the entire exposure time and ate and drank during a major portion of a 12-hr period. A control group of mice was exposed to normal air under exactly the same conditions.

Proper mixing of nitrous oxide and air resulted in a gas mixture containing 50 percent N_2O, 30 percent N_2 and 20 percent O_2, which was led into an anaesthetic chamber with a volume of 6 liters. A similar vessel was used to expose another group of mice to normal air under similar controlled conditions. Exposure of mice to this gas mixture has almost no visible effects on the mice and they can ambulate, eat and drink during the total period of exposure, which could easily be continued for 48–72 hours.

In vitro irradiation of mouse haemopoietic cells

Femoral bone marrow cell suspensions were incubated for 20 minutes in Hanks' medium containing different concentrations of pentobarbital at 37 °C. This was done in small plastic tubes containing 2 ml of the haemopoietic cell suspension (approximately $10—15 \times 10^6$ cells/ml). Immediately after the incubation period, the suspensions (in the original tubes) were exposed to gamma-irradiation at different dosages. The irradiated suspensions and, simultaneously, the incubated but nonirradiated control suspensions were assayed for the number of CFU using the spleen colony technique. Alternatively, femoral bone marrow cell suspensions of normal mice and of animals treated with pentobarbital 20 min earlier were incubated in the normal Hanks' medium at 37 °C for 30 min. These suspensions were also subsequently exposed to gamma radiation and the number of surviving CFU was assayed using the spleen colony technique.

Drugs

Cytostatics

1-β-D-arabinofuranosylcytosine	Cytosar® (cytarabine), Ara-C; Upjohn Company;
Vincaleucoblastine	Velbe®, (vinblastine sulphate), VLB; Lilly;
Nitrogen mustard	Mustine Hydrochloride®; HN_2; Boots.
Chlorambucil	Amino Chlorambucil, ACA; Wellcome.

CNS Depressants

Pentobarbital sodium	Nembutal®; S. A. Abbot; 60 mg/ml pentobarbital sodium;
Chloralhydrate	Each solution was prepared freshly before use;
Fluanison-Fentanyl	Hypnorm®; Janssen Pharmaceutics and Philips-Duphar; butyrophenon neuroleptic; per ml 10 mg Fluanison and 0.2 mg Fentanyl (base).
Dehydrobenzperidol®	Janssen Pharmaceutics; butyrophenon neuroleptic; 2.5 mg/ml.
Phencyclidine	Sernylan®; Parke, Davis and Company; Phencyclidine hydrochloride, 20 mg/ml;
Diazepam	Valium®; Hoffmann-La Roche; suspension 10 mg/2 ml.
*Flupenthixol**	Fluanxol depot®, H. Lundbeck and Co.; flupenthixol decanoas 20 mg/ml.
Halothane	Fluothane®, ICI Limited.
Nitrous Oxide	N_2O

Miscellaneous

Prednisolone	Di-adreson-F-aquosum ®; Organon; Prednisolone sodium succinate.
Triton WR 1339	TWR; formaldehyde polymer of polyoxiethylene ether of octylphenol; nonionic detergent. Suspensions prepared in 0.2% carboxymethyl cellulose (CMC) solution.
Ro-07-0582	Hoffmann-La Roche. Prepared in a 2% carboxymethylcellulose (CMC); 1 g Ro-07-0582 per 10 ml CMC.
Propranolol	Inderal ®; ICI; 1 mg/ml.

* Thanks are due to Lundbeck and Co of Amsterdam for providing Flupenthixol.

CHAPTER 3

CNS DEPRESSANTS DURING FRACTIONATED CHEMOTHERAPY

The rationale for the experiments presented in this chapter can be briefly summarized as follows.

Exposure of mice to the general anaesthetics halothane and nitrous oxide during fractionated chemotherapy using Ara-C and VLB reduces the toxicity of the drugs for haemopoietic stem cells. This result is in contrast to the situation in leukaemic stem cells, where anaesthetics provided no protection. A lower number of the normal stem cells induced into cycle was proposed as an explanation for these results (Bruce, Lin and Bruce, 1970). To investigate whether this effect is shared by other members of the group of CNS depressants, a system was developed to test a number of these compounds. The reasons to investigate this effect in more detail are two-fold: 1. to clarify the mechanism; and 2. to investigate the clinical usefulness.

The scientific literature concerning the numerous cellular effects of CNS depressants is still growing. Therefore, it is worthwhile to summarize the main concepts which will serve as background knowledge for interpreting the data to be presented. Although a number of theories have been advanced, the exact mechanism by which anaesthetics exert their anaesthetic effect is still not precisely known. Inhibition of synaptic transmission in the central nervous system seems the most likely explanation at present. The question of how this block of transmission is obtained is still a matter of debate. At the cellular level, it has been shown that membrane (lipids, proteins), cytoplasmic (mitochondria, microtubules, microfilaments, and other constituents), and nuclear material can be involved. Thus, these drugs may lead to changes in membrane properties, cytoplasmic transport, motility, energy production and protein synthesis in many *in vivo* and *in vitro* cellular systems (for a thorough review, see Halsey, Millar and Sutton, 1974). While many investigators are still attempting to solve the problem of anaesthesia, others are concerned with the action of anaesthetic agents on cells other than those of neural origin.

Although anaesthetics act upon a variety of biological systems,

there are many which are relatively or entirely unaffected. Therefore, anaesthetics do not produce an overall and uniform depression of all biological systems. In fact, anaesthetics show quite a remarkable target selectivity (Nunn, 1974).

Inhibition of cell division by CNS depressants

One aspect of the cellular effects of anaesthetics which may be of importance in explaining the results presented subsequently is the finding that anaesthetics have an inhibitory effect on cell growth and cell division (Henderson, 1930). Oestergren et al. showed that many anaesthetic compounds induce a typical arrest of plant cells in metaphase, which is usually called c-mitosis* (Oestergren, 1944).

It may be questioned if it is justified to assume that these compounds also cause c-mitosis in animal tissue. A major obstacle in answering this question adequately is the difficulty in demonstrating c-mitosis *in vivo* in animals. Circumstantial evidence, however, suggests that this type of inhibition of cellular division might also be obtained in animal tissue by using many of the same anaesthetic agents which produce c-mitotic effects in plants (Hall, 1962). The antimitotic effects were found to be not limited to anaesthetics but shared by certain narcotics (levallorphan, nalorphane) and tranquillizers such as meperidine, promethazine and promazine (Simon, 1964); a thorough discussion of the cellular effects of these narcotics and of several hallucinogenic drugs is found in a review of Zimmerman and McClean, 1973. The type of inhibition of cellular division could not be exactly characterized in all studies. In 1966, on reviewing the whole set of data on this subject, Andersen concluded that the available data suggested that most anaesthetic agents, in addition to some narcotics and tranquillizers, interfere with normal cell division by producing c-mitosis. Inhibition of DNA synthesis with an apparent delay in S-phase was observed in rat intestinal cells *in vivo* after prolonged halothane exposure (Bruce and Traurig, 1969). Using halothane, S-phase was shown to be affected in *in vitro* systems employing human lymphocytes (Cullen, Sample and Chrétien, 1972), mouse lymphocytes (D. L. Bruce, 1972a, b; 1974), mammalian hepatoma cells (Jackson, 1973),

* c-mitosis: Arrest of cell division in metaphase first seen after colchicine by inactivation of the mitotic spindle; chromosomes may be found in characteristic configurations (Levan, 1938).

Chinese hamster fibroblasts (Sturrock and Nunn, 1974), *Vicia faba* (Grant, Powell and Radford, 1974) and mouse haemopoietic cells *in vivo* (Evenwel, Keizer and Van Putten, 1976). Inhibition of growth of mammalian hepatoma cells was reported by Jackson and Epstein (1971) and Jackson (1972) and of mouse bone marrow cells by Nunn, Sturrock and Howell (1976).

Grant et al. (1974) did not exclude the possibility of an action of halothane in G_1-phase. Sturrock and Nunn (1975), using Chinese hamster fibroblasts, demonstrated a dose-dependent inhibition of cell multiplication with a prolonged interphase, a delayed entry of the cells into M, prolonged M-phase and c-mitosis after exposure to anaesthetics. Multipolar and multinucleated cells were also observed by these authors (Sturrock and Nunn, 1976). These data have been interpreted to mean that anaesthetics act on every phase of the cell cycle (Sturrock and Nunn, 1975). It is not clear whether any common mechanism explains all of the observed effects. There is still no generally accepted answer to the question as to whether the mechanism by which anaesthetics inhibit cell multiplication in plants such as *Vicia faba* and *Pisum sativum* differs from the manner in which they are inhibitory in mammalian cells. In plants, it has been demonstrated that interference with the function of the microtubules of the mitotic spindle is involved in the production of c-mitosis (Nunn et al., 1971, 1974). To further clarify the effects of CNS depressants on cellular multiplication, research is required on those factors which have been suggested to play a role in the control of cellular proliferation. In this respect, a number of recent developments dealing with the cellular effects of some local anaesthetics and tranquillizers may lead to further answers. This aspect will be discussed in another section of this chapter.

Experimental procedure

Most of the experiments described in this chapter were performed by using the following experimental system. Normal mice were treated with 3 injections of either vincaleucoblastine (VLB) or 1-β-D-arabinosylcytosine (Ara-C) at 4-hour intervals. In addition to this treatment, a second group received an injection of a CNS depressant, either an hypnotic or a tranquillizer, always 15–30 minutes before the first cytostatic treatment was given. The treatment with the CNS depressants before the second and the third injection of the anticancer-

drug was not done in all cases. Four hours after the last challenge with the cytostatic drug, the mice were killed and the number of haemopoietic stem cells in the femoral marrow was determined in all groups using the spleen colony technique (see fig. 3.1).

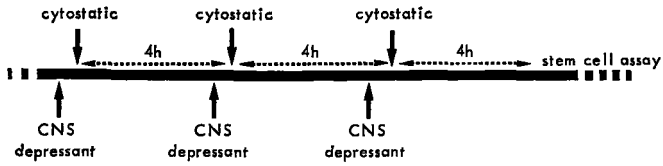


Figure 3.1. Schematic representation of the basic treatment protocol, in which chemotherapy is applied to mice with the concomitant use of CNS depressants. Unless indicated otherwise in the text, the CNS depressants were injected always 15–30 minutes before cytotoxic treatment.

Experimental data

Fluanisone-Fentanyl

The data given in Table 3.1 indicate that the CFU survival after fractionated VLB therapy is always higher in the fluanison-fentanyl treated animals, with the exception of experiment I. The mean fraction of protected stem cells is 37 per cent, a figure which is statistically significant ($p < 0.01$).

Dehydrobenzperidol (DHBP)

The mean increase in CFU survival after chemotherapy when DHBP was given during the cytostatic treatments was 46.6 percent ($p < 0.001$) (Table 3.2). Two other conclusions can be drawn from these data; one is that the fraction of CFU spared during chemotherapy seemed to be highest at the higher dosages of the CNS depressant (exp. I, II and III). Moreover, the effect of DHBP is seen not only during treatment with VLB, but also when using Ara-C (exp. 1).
To calculate the mean per cent increase, data obtained for VLB and Ara-C have been pooled since both drugs belong to the same class of anti-cancer agents, the phase-specific drugs, i.e. they kill only the cycling cells and no resting cells. Moreover the dosages used of both drugs are equipotent, i.e., they result in percentages of surviving CFU which are at the plateau region of the dose-survival curves (see introduction). Finally, the data obtained with VLB and Ara-C in our system do not show essential differences.

Table 3.1. Effect of Fluanisone–Fentanyl on stem cell survival after chemotherapy.

Treatment		Number of surviving CFU in the femoral marrow						
CNS depressant (mg/kg)	Cytostatic (mg/kg)	Exp. I	Exp. II	Exp. III	Exp. IV	Exp. V	Exp. VI	Mean per cent protection*
None	VLB 3×10	2440	2320	2186	2100	3020	2540	
Fluanisone – Fentanyl								
16–0–0	,,	2300 (−5.3)						
16–8–8	,,		2862 (23.4)					
16–0–16	,,			3292 (50.6)				
16–0–16	,,				4215 (100.7)			37 ($p<0.01$)
8–8–8	,,		3173 (36.8)					
8–0–8	,,					3800 (25.8)		
4–4–4	,,						3147 (23.9)	
4–4–4	,,		3257 (40.4)					

Effect of a mixture of Fluanisone and Fentanyl during fractionated treatment with Vincaleucoblastine (VLB) on the survival of femoral haemopoietic stem cells. The figures in parentheses represent the difference (in percentages) with the group which did not receive the CNS depressant. Fluanisone and VLB were injected at 4 hour intervals, i.p. and s.c., respectively. Depending on the strain of mice, the number of CFU in the femur of an untreated control mouse ranges from 5000–9000 and the number of CFU in the spleen of an untreated control mouse ranges from 3000–5000.

* Per cent protection $= \dfrac{\text{stem cells after cytostatic with CNS depressant} - \text{stem cells after cytostatic}}{\text{stem cells after cytostatic}} \times 100$

Phencyclidine

This drug, an arylcycloalkylamine, resulted in a 42.5 per cent ($p < 0.001$) higher CFU survival (Table 3.3). It must be admitted that only one dosage, 40 mg/kg body weight, was tested in two experiments. This dosage combined with the dose schedule is highly sedative in comparison with the other treatments; mice treated with the protocols given are under deep narcosis for many hours.

Diazepam (valium)

The data obtained with this drug again show the wide variations in the percentages of CFU which are protected from the cytotoxic effect of the anticancer drug (Table 3.3.). The mean increase in survival is 18.6 per cent, a figure which is not statistically significant.

Pentobarbital sodium

No significant increase in CFU survival was observed after treatment with this barbiturate at the dosages used (Table 3.3.). Pentobarbital seems to be ineffective in protecting CFU from the cytotoxic effects of vinblastine. In summarizing the data presented up to this point, it appears that both fluanisone-fentanyl and dehydrobenzperidol result in higher survival percentages, 34 and 38 per cent, respectively. The figure of 42.5 per cent which was obtained with phencyclidine may be an overestimation, for reasons mentioned earlier, i.e., the prolonged and deep narcosis induced by the drug at the dosage used. Diazepam gave rise to a statistically insignificant increase of 18.6 per cent and pentobarbital sodium also had no significant effect (11.3 per cent increase).

Since it is unknown what blood or tissue levels of the CNS depressants are needed to modify the process of cell kill by anti-cancer agents, we injected the "anaesthetics" at regular time intervals during cytostatic treatment. It is also unknown whether the time of termination of the state of narcosis may be a factor of importance in the protection phenomenon. However, it should be remembered that, in the case of the phenothiazines, the drugs remain in the plasma for exceedingly long times. For these reasons, it seemed logical to initiate experiments with CNS depressants which could be given in a depot form. This would presumably give more constant mean blood and tissue concentrations. The two most active drugs, dehydrobenzperidol and fluanisone

Table 3.2. Effect of Dehydrobenzperidol on stem cell survival after fractionated chemotherapy.

Treatment		Number of surviving CFU in the femoral marrow							
CNS depressant (mg/kg)	Cytostatic (mg/kg)	Exp. I	Exp. II	Exp. III	Exp. IV	Exp. V	Exp. VI	Exp. VII	Mean per cent protection
None	Ara-C 3×150	2100							
None	VLB 3×10		1920	2186	1754	2700	3020	2300	
Dehydrobenzperiodol 48–0–48	Ara-C 3×150	3253 (55)							
48–0–0	VLB 3×10		4092 (113)						
48–0–0	,,			3440 (57)					
32–0–0	,,		2960 (54)						
24–0–0	Ara-C 3×150	3093 (47)							
24–0–0	VLB 3×10			3800 (83)					
20–20–20	,,				2480 (41)				46.6 ($p < 0.001$)
16–0–0	,,		3627 (89)						
12–12–12	,,				2314 (32)				
10–10–5	,,					3493 (29)			
8–8–8	,,						3323 (11.1)		
4–4–4	,,						3385 (12.9)		
4–4–4	,,				2347 (35)				
2–2–2	,,							2187 (−5)	

Effect of Dehydrobenzperidol during fractionated cytostatic treatment using VLB and Ara-C on the survival of femoral haempoietic stem cells. All drugs were given at 4 hr intervals. Dehydrobenzperidol and Ara-C were injected i.p. and VLB s.c.

(+ fentanyl), both belong to a subclass of the neuroleptics, the butyrophenones. Therefore, another neuroleptic in oil solution, flupenthixol, a thioxanthene derivative, was chosen to be tested in our chemotherapy system; the oil solution of this compound served as a depot preparation.

Flupenthixol

The data obtained with this long-acting neuroleptic have been collected in Table 3.4. The mean number of surviving CFU was raised from 3087 to 4759, an increase of 52.3 percent ($p < 0.001$). The effect was seen when using both VLB and Ara-C. It is noteworthy that a remarkable protective effect of flupenthixol was seen after one very high dose of VLB (20 mg/kg s.c.) followed by CFU assay 16 hours later (exp. V, Table 3.4).

A single i.p. injection of chlorpromazine (10 mg/kg), a drug which is structurally closely related to flupenthixol, gave an identical response.

Comparison of neuroleptics with halothane

In the original system described by Bruce and co-workers (1970), mice were exposed to 0.37 per cent halothane or 50 per cent nitrous oxide for a 24-hour period. During this period, the mice were challenged with 6 injections of 1 mg/mouse Ara-C at 4-hr intervals. The authors reported increased survival percentages for normal femoral CFU in the halothane and nitrous oxide exposed mice after Ara-C treatment, which were 120 per cent (range 90-200) and 101 per cent (range 77–145) higher than the survival in the air exposed and Ara-C treated mice. A comparable, although lower (61 per cent) increase was obtained when using VLB during halothane exposure. With our "best compound", flupenthixol, a mean per cent increase in surviving CFU of 52.2 (range 9–131) was obtained (see Table 3.4). This figure is definitely lower than those obtained by Bruce et al.; however, these authors treated their mice for a 24-hour period and a 12-hour period was used in this study. This prompted us to compare our neuroleptic drugs more directly with the Bruce-system.

In the first experiment (Table 3.5), a 12-hour period of halothane exposure did not change the CFU survival (4213 against 4000) after 3×200 mg/kg Ara-C. In contrast, the neuroleptic compound dehydrobenzperidol improved the CFU survival, increasing the CFU

Table 3.3. Effect of Phencyclidine, Diazepam and Pentobarbital on stem cell survival after fractionated chemotherapy.

Treatment		Number of surviving CFU in the femoral marrow							
CNS depressant (mg/kg)	Cytostatic (mg/kg)	Exp. I	Exp. II	Exp. III	Exp. IV	Exp. V	Exp. VI	Exp. VII	Mean per cent protection
Phencyclidine	VLB 3×10	2186	2100	1754	4600	3369	2900	2700	
40–40–40	,,	3093 (41.5)							42.5 ($p < 0.001$)
40–40–0	,,		3013 (43.5)						
Diazepam									
16–16–8	,,	2400 (9.8)							
8–8–8	,,			2775 (58)					
8–8–8	,,		2587 (23.2)						18.6 (n.s.)
8–8–0	,,	2615 (18.9)							
8–8–0	,,				5120 (11.3)				
4–4–4	,,		1893 (−9.9)						
Pentobarbital									
60–30–15	,,					3714 (10)			
60–30–15	,,							3320 (22)	
60–30–15	,,						3240 (12)		
60–0–0	,,						2587 (−11)		
30–30–30	,,					3814 (13)			11.3 (n.s.)
30–30–0	,,						2990 (3)		
15–15–15	,,						3356 (16)		
15–0–0	,,						3446 (26)		

Effect of CNS depressants on the survival of femoral haemopoietic stem cells during fractionated cytostatic treatment using VLB. All drugs were given at 4-hour intervals. Phencyclidine and Pentobarbital were injected i.p., Diazepam i.m. and VLB s.c.; n.s., statistically nonsignificant.

Table 3.5. Comparison of the effect of halothane and various neuroleptics on the stem cell survival after fractionated chemotherapy.

Treatment		Number of surviving CFU in the femoral marrow			
CNS depressant	Cytostatic (mg/kg)	Exp. I	Exp. II	Exp. III	Exp. IV
None	None	9467	7800	9467	2640*
None	Ara-C	4000			867*
	3×200				
None	6×50		1500	2224	
Halothane		4213			1493*
0.4% – 12 hr	3×200	(5)			(72)
Dehydrobenzperidol		5920			1166*
3×8 mg/kg	3×200	(48)			(34)
Halothane			2733	3900	
0.4% – 24 hr	6×50		(82)	(75)	
Flupenthixol				3107	
8 mg/kg i.m.	6×50			(40)	

Effect of CNS depressants during fractionated cytostatic treatment of mouse femoral haemopoietic stem cells. All injections were given of 4-hour intervals. Halothane exposure and treatment with neuroleptics started about one half hour before the first cytostatic treatment was given.
* stem cell survival in the spleen

number from 4000 to 5920 (48 per cent). In experiment II, the protocol of Bruce was repeated, i.e., six injections of Ara-C at 4-hour intervals; survival increased from 1500 to 2733 (82 per cent) in the halothane group. A survival figure of 75 per cent was found in experiment III and three injections of flupenthixol improved CFU survival by 40 per cent. This gives the impression that the neuroleptics might be less potent than halothane. The last experiment given in table 3.5 is again an illustration of this; exposure to halothane for 12 hour improves CFU survival in the spleen after 3×200 mg/kg Ara-C at 4-hr intervals by 72 per cent, whereas only a 34 per cent increase was obtained with dehydrobenzperidol.

Amino-Chlorambucil (ACA) and Nitrogen-Mustard (HN_2)

The original idea leading to these studies was that halothane and nitrous oxide rendered normal mouse haemopoietic stem cells less sensitive to phase-specific cytostatic drugs, presumably by inhibition of DNA synthesis or other metabolic events necessary for the cells to

Table 3.4. Effect of Flupenthixol on stem cell survival after fractionated chemotherapy.

Treatment		Number of surviving CFU in the femoral marrow									Mean per cent protection
Flupenthixol (mg/kg)	Cytostatic (mg/kg)	Exp. I	Exp. II	Exp. III	Exp. IV	Exp. V	Exp. VI	Exp. VII	Exp. VIII	Exp. IX	
None	VLB 3×10	*3320*	*3813*	*2700*	*4107*						
40	,,	4160 (25)	4160 (9)	6240 (131)							
16	,,				6680 (63)						
8	,,				5320 (27)						
None	1×20					1813*					
8	,,					3350* (85)					
4	,,				5160 (26)						
2	,,				4680 (14)						52.2 ($p < 0.001$)
None	Ara-C 3×150						2427	2650	3020		
4	,,						3086 (27)				
4	,,							4480 (69)			
4	,,							3733 (41)			
8	,,								5760 (91)		
8	,,								5890 (95)		
None	3×300									3940	
24	,,									5086 (29)	
10 (CPZ)**	VLB 1×10					3600* (98)					

Effect of Flupenthixol during fractionated cytostatic treatment using Ara-C and VLB on the survival of femoral haemopoietic stem cells. VLB injected s.c., Ara-C i.p. One single injection of VLB was followed by CFU assay 16 hour later. Flupenthixol was always injected i.m.

* stem cell survival in the spleen

** CPZ = chlorpromazine, 10 mg/kg i.p.

proceed through the cell cycle. Based on this idea, a logical approach seemed to be to use Ara-C and VLB, the best known representatives of the phase-specific anti-cancer drugs. The results obtained with these drugs were presented earlier in this chapter.

It seemed of interest, however, to determine the effects of our "best neuroleptics" on CFU survival during cytostatic treatment with HN_2 and ACA. These compounds are cycle-specific but not phase-specific. Both HN_2 and ACA were administered in one single injection and CFU assays were done 16 hours thereafter. When ACA was administered, a notable protective effect on the haemopoietic stem cells was seen with phencyclidine, dehydrobenzperidol and flupenthixol (Table 3.6; exp. I, II and III). The increased CFU survival ranges from 87 to 293 (mean 210) per cent of the survival of CFU in treated animals which did not receive a CNS depressant. No protection was seen when HN_2 was used (Table 3.6; exp. IV and V). In summary, the protective effect on haemopoietic stem cells found when using CNS depressants during treatment with anti-cancer agents seems not to be restricted to phase-specific cytostatic drugs alone; the fact that this response was definitely observed during treatment with ACA but not with HN_2 might suggest that differences in the site of action at the cellular level play a role.

Prednisolone and Triton WR 1339

Significantly higher survival percentages of haemopoietic CFU resulted from the use of several anaesthetics and tranquillizers concomitantly with cytostatic treatment. Let us assume, as a working hypothesis, that CNS depressants reversibly depress DNA synthesis in haemopoietic stem cells and therefore render these cells less sensitive to cytostatic treatment. In an attempt to further characterize this effect of CNS depressants, another rather nonspecific inhibitor of growth and cell division, a glucocorticosteroid, was tested in the CFU chemotherapy model. This class of compounds has been shown to reduce choline uptake and its incorporation into cellular lipids in a number of *in vitro* growing cells (Makman et al., 1971; Story et al., 1973a, 1973b). This phenomenon is accompanied by inhibition of thymidine incorporation into nucleic acids. Inhibition of cell division and DNA synthesis following prednisolone administration has been observed in thymocytes (Dougherty and White, 1945), fibroblasts (Pratt and Aronow, 1966) and liver cells (Howard, 1964). Other investigators demonstrated that

Table 3.6. Effect on stem cell survival when CNS depressants are given during chemotherapy with cycle-specific drugs.

Treatment		Number of surviving CFU in the femoral marrow					
CNS depressant (mg/kg)	Cytostatic (mg/kg)	Exp. I	Exp. II	Exp. III	Exp. IV	Exp. V	Mean per cent protection
None	Amino-Chlorambucil 30 mg/kg i.p.	165	86	68			
Phencyclidine 4×20	,,	545 (230)					
Dehydrobenzperidol 4×10	,,	308 (87)					
Flupenthixol 1×10	,,		277 (222)				210 ($p < 0.001$)
1×16	,,			224 (229)			
1×20	,,		317 (268)				
1×40	,,		338 (293)	173 (140)			
None	Nitrogen-Mustard 5 mg/kg s.c.				6		
Flupenthixol 1×40	,,				5 (−17)		−17 (n.s.)
1×16	,,				5 (−17)		
None	10 mg/kg i.p.					101	
1×40	,,					83 (−16)	
1×10	,,					138 (36)	10 (n.s.)

Effect of CNS depressants during cytostatic treatment of femoral haemopoietic stem cells using Amino-Chlorambucil (ACA) and Nitrogen-Mustard (NH_2).

Cytostatics were injected once, CFU assay was done 16 hours later. Phencyclidine and Dehydrobenzperidol were injected i.p. at 4-hour intervals. Flupenthixol was injected once, i.m.

* injected three hours before ACA

glucocorticosteroids also delay cell division and, conversely, accelerate cell maturation (see, for instance, Carson et al, 1973; Adamson and Klass, 1976). The mechanism of these inhibitory effects is unknown. It should also be realized that glucocorticosteroids may give rise to a wide variety of other effects *in vivo*. Surprisingly, we found that prednisolone (1 or 3×0.4 mg/kg, i.p.) gives rise to significantly increased survival percentages, after treatment with both Ara-C and VLB. The mean per cent increase was 37.2 ($p < 0.01$) (Table 3.7, upper part).

Prednisone, another synthetic glucocorticosteroid, is presently part of several anti-cancer drug combination protocols and is particularly used against certain types of leukaemias and lymphomas. One explanation advanced to explain its effectiveness was that the compound possessed lympholytic activity. The data presented here concerning the protective effect of prednisolone for normal CFU during fractionated chemotherapy of haemopoietic stem cells may also play a role in the therapeutic effect of prednisone.

To investigate the role of membrane alterations in the protective effect of CNS depressants for normal CFU, it was decided to test the non-ionic detergent Triton WR 1339 in the mouse CFU model. This preparation has been shown to have considerable chemotherapeutic effects *in vivo* and to decrease cancer dissemination and metastases in some experimental models, (Franchi et al., 1971) but not in others (Hellmann, 1973). In certain tumour models, membrane changes have been suggested to explain some of the effects. The lower part of Table 3.7 illustrates that the concomitant use of this compound also improves CFU survival after fractionated chemotherapy (mean increase in survival 29.5 per cent; $p < 0.01$). The dosages and dose schedules used do not indicate a dose-dependency.

In summary, two compounds with a broad spectrum of poorly characterized cellular effects resulted in significantly increased survival percentages of haemopoietic stem cells during fractionated cytostatic treatment of 37 and 30 per cent, respectively.

Halothane versus Nitrous Oxide

As mentioned earlier, these two compounds rendered normal haemopoietic spleen colony forming cells less sensitive to a phase-specific cytostatic treatment. Since fractionated administration of cytostatic agents triggers resting cells into cycle (recruitment) and

Table 3.7. Effect of prednisolone and Triton WR 1339 after fractionated chemotherapy on stem cell survival.

Treatment		Number of surviving CFU in the femoral marrow						
Protective agent (mg/kg)	Cytostatic (mg/kg)	Exp. I	Exp. II	Exp. III	Exp. IV	Exp. V	Exp. VI	Mean per cent protection
None	VLB 3×10	2700	2540	1867	3940	3020	2427 2507*	
Prednisolone 1×0.4	,,	3200 (19)	3947 (56)					
3×0.4	,,			2429 (30)				
3×0.4	,,			2600 (39)				37.1 ($p < 0.01$)
3×0.4	Ara-C 3×300				5600 (42)			
Triton WR 1339 1×275	VLB 3×10		3330 (31)					
3×275	,,			2514 (35)				
1×550	,,					3692 (22)		29.5 ($p < 0.01$)
3×550	Ara-C 3×150						3214 (30)	

Effect of Prednisolone and Triton WR 1339 on femoral haemopoietic stem cell survival after fractionated cytostatic treatment using Ara-C and VLB.
All injections were given at 4-hour intervals, VLB s.c. and Ara-C i.p. The two independently determinated cytostatic treated control values in experiment VI illustrate the small variations which can be obtained within one experiment.
* duplicate assays

makes the stem cells sensitive to the cytostatic drug in repeated schedules, it was suggested that exposure to these gases might prevent this recruitment and thereby prevent the normal CFU from being killed.

Recently, evidence was presented which revealed that the halothane treatment reversibly decreased DNA synthesis in femoral and splenic haemopoietic cells (Evenwel, Keizer and Van Putten, 1976), suggesting a relationship between the reduction in DNA synthesis rate and the decreased sensitivity to phase-specific cytostatic treatment. This inhibition of DNA synthesis rate was also seen in a rapidly proliferating stem cell population, where the majority of these cells are actively traversing the cell cycle. This leads to the interpretation that inhibition of recruitment of haemopoietic stem cells may not be the unique cause of the protective effect seen during cytostatic treatment but may rather be secondary to other inhibitory effects.

Since nitrous oxide exerts protective effects on femoral CFU during cytostatic treatment similar to those produced by halothane, it seemed interesting to determine whether *in vivo* exposure to nitrous oxide also depressed the DNA synthesis rate in haemopoietic cells. This was done by means of the ^{125}IUdR uptake into DNA as the test parameter. Considering an exposure time of 24 hour it can be seen that only 1 out of 4 experiments resulted in a significantly depressed DNA synthesis rate in the spleen cells (Table 3.8). In the second experiment of this series concerning 24 hour exposure, the DNA synthesis rate is even significantly higher in the spleen cells. In neither of these 4 experiments was a decreased DNA synthesis found in femoral haemopoietic cells and in only 1 experiment was there a significantly lower value in small intestinal cells. Increasing the exposure time from 24 hour to 48 and 72 hour did not further decrease the DNA synthesis rate in any of these tissues (exp. 5, 6 and 7, Table 3.8) in contrast however, surprisingly, significantly higher values were found in the spleen, suggesting that synchronization (accumulation of cells) in S-phase occurs. The bone marrow and intestine data in the air and nitrous oxide exposed animals were not significantly different.

The first seven experiments given in Table 3.8 were done using normal untreated mice, whose haemopoietic stem cell population is mainly in a resting state. The last experiment in Table 3.8 shows that a rapidly proliferating stem cell population did not respond to 24 hour nitrous oxide exposure, again in contrast to the effect of halothane (compare with Evenwel, Keizer and Van Putten, 1976). By analogy,

Table 3.8. Continuous Nitrous Oxide exposure: the effect on different *in vivo* mouse tissues.

Treatment	Exposure time (hr)	Mean ^{125}IUdR uptake ±S.E. (per cent of activity per μCi injected) Spleen*		Bone marrow**	Small intestine*	
Air	24	3.7±0.44		0.29±0.07	8.9±0.6	
N_2O		4 ±0.71		0.30±0.02	10.2±0.7	
Air	24	1.8±0.09		0.22±0.05	7.9±1	
N_2O		2.81±0.7	$p<0.05$	0.3±0.06	4.6±0.35	$p<0.02$
Air	24	7.9 ±1.7		0.34±0.05	17.7±1.8	
N_2O		7.8 ±1.4		0.42±0.02	13.0±1.9	
Air	24	17 ±3.3		0.90±0.1	10 ±0.4	
N_2O		5.4±0.8	$p<0.01$	0.90±0.1	8.9±0.3	
Air	48	13 ±2		0.14±0.04	11.4±0.3	
N_2O		18.9±2.5	$p<0.05$	0.19±0.06	10.9±0.6	
Air	72	6 ±0.8		0.09±0.014	9.9±0.9	
N_2O		11.9±2.25	$p<0.05$	0.12±0.025	9.5±0.4	
Air	72	3.4±0.5		0.04±0.01	6 ±0.3	
N_2O		7 ±0.9	$p<0.01$	0.06±0.01	6.2±0.45	
Air***	24	24.3±1.6		0.13±0.01	5.7±0.3	
N_2O***		25.6±1.6		0.16±0.01	6 ±0.5	

Effect of continuous Nitrous Oxide exposure on DNA synthesis in various *in vivo* mouse tissues; N_2O 50 percent, O_2 20 percent and N_2 30 percent. One hour before the gas exposure ended the mice were injected with 5 μCi ^{125}IUdR i.p. One hour later the mice were killed and tissues of interest were collected.

* per gram of tissue

** per two femurs

*** donors received 950 rad gamma rays plus 3×10^6 bone marrow cells i.v. seven days earlier to produce a rapidly proliferating stem cell population.

it has been demonstrated that halothane inhibits transformation of lymphocytes by phytohaemagglutinin, whereas nitrous oxide fails to do so (D. L. Bruce, 1974, 1976). These data suggest that the decreased sensitivity of haemopoietic stem cells during fractionated chemotherapy does not necessarily correlate with a decrease in DNA synthesis in these cells. Interference of the compounds with other cellular processes, e.g., uptake into the cell or transport through the cell, may be involved.

Table 3.9. Extent of protection seen with various CNS depressants.

Protective agent	Increase of survival (per cent)	Class
Pentobarbital	11.3 (n.s.)**	barbiturate
Diazepam	18.6 (n.s.)**	benzodiazepine
Fluanisone*	37 ($p < 0.01$)	butyrophenone
Phencyclidine	42.5 ($p < 0.001$)	arylcycloalkylamine
Dehydrobenzperidol	46.6 ($p < 0.001$)	butyrophenone
Flupenthixol	52.2 ($p < 0.001$)	thioxanthene

Collection of data from Tables 3.1–3.4.
* mixture with fentanyl
** n.s., not statistically different

Discussion

The results obtained with the CNS depressants used are summarized in Table 3.9. Before discussing these results, the following remarks should be made: 1. Although several dosages of the CNS depressants have been tested, the optimal dosage is not known. This is mainly because of the neuroleptic and anaesthetic effects of the used CNS depressants. Further, the effect of CNS depressants during chemotherapy of haemopoietic stem cells shows rather wide variation between one experiment and the other. Therefore, when considering these results main emphasis should be given to the quality of the protective effect of CNS depressants rather than to the absolute magnitude of this effect.

It can be concluded that pentobarbital does not protect haemopoietic stem cells during cytostatic treatment. Theoretically, there may be two explanations: 1. the plasma and tissue pentobarbital levels were too low; 2. the drug does not prevent haemopoietic stem cells from being killed by a phase-specific anti-cancer drug.

After injection of an anaesthetic dose of pentobarbital, anaesthesia starts very quickly thereafter and lasts for about one hour. The plasma half-life of this drug in the mouse is likely to be approximately 40–80 minutes. The fact that DNA in bone marrow and spleen cells were depressed to a major extent for a period of at least 3 hour after a single pentobarbital injection *in vivo* (Baserga and Weiss, 1967; Rambach et al., 1952) makes it reasonable to assume that pentobarbital was present in the femoral marrow and spleen in a significant concentration for the whole period during which chemotherapy was applied. This

strongly suggests that pentobarbital does not prevent haemopoietic stem cells from being killed by a phase-specific anti-cancer drug. Thus, it seems that inhibition of DNA synthesis as such does not indicate a decreased sensitivity to VLB. Since the reverse, that rapid cell growth renders cells more sensitive to anti-cancer agents, is one of the dogmas of modern cancer chemotherapy, this might suggest that depression of other cellular processes is needed in order to decrease the sensitivity of the stem cells to cytostatic treatment.

The mean increase in stem cell survival which resulted from the use of diazepam was 18.6 per cent. Thus, diazepam is only moderately effective in protecting haemopoietic stem cells against cytostatic drugs.

Fluanisone (in a mixture with fentanyl) and dehydrobenzperidol, which are both neuroleptic agents (butyrophenones), resulted in almost similar protection, 34 and 38 per cent increase, respectively. Similar to the other experiments, the variations between the experiments are rather large. Nevertheless, the increases in survival are statistically significant, $p < 0.01$ and $p < 0.001$, respectively.
Flupenthixol, injected once i.m., resulted in the highest percentage of protected stem cells (52.2 percent).

Nitrogen-Mustard and Amino-Chlorambucil

The toxicity of ACA to mouse haemopoietic stem cells could be decreased, as shown by a two- or three-fold increase in survival after the cytostatic treatment. Two conclusions can be drawn:

1. the protection phenomenon is not restricted to phase-specific drugs such as VLB and Ara-C. HN_2 and ACA are considered as cycle-specific compounds in the mouse stem cell model, i.e., they are more toxic to rapidly proliferating stem cells.
2. the protection phenomenon is also seen at bone marrow stem cell survival fractions of about 1 or 2 per cent (one femur contains approximately 8000 spleen colony forming cells). Concomitant administration of CNS depressants increases survival to about 4–5 per cent.

In the mouse, it is known that many fewer than one per cent of the haemopoietic stem cells can give rise to a complete bone marrow restoration. These data are not available for man; if it may be postulated that the same is true for man, an increase in stem cell survival from 1 to 5 per cent, although involving small numbers of surviving stem cells, would be relevant.

Comparison between neuroleptic compounds and halothane

It was observed that halothane does not significantly alter the survival of femoral haemopoietic stem cells during a 12-hour period with 3 injections of Ara-C. In contrast, during 24 hour exposure with 6 injections of Ara-C there was a considerable increase (80 per cent) in stem cell survival. Assuming that recruitment of resting stem cells into cycle and differentiation of stem cells (with loss of colony forming ability) are more pronounced during a treatment period of 24 hour than during a 12-hour period, these data can mean that 1. halothane excerts its protective effect by inhibition of the recruitment of resting cells (G_0-phase) into cycle; 2. a considerable loss of CFU by differentiation will occur during a 24-hour period of cytostatic treatment; halothane may give its protective effect by prevention of this cell loss; 3. halothane causes a metaphase block. It may be reasoned that it takes approximately 12 hours to collect a significant number of stem cells in M-phase and thereby render them insensitive to drugs interfering with DNA synthesis. A further observation is that the neuroleptics dehydrobenzperidol and flupenthixol again resulted in an increase in survival in the order of 40–50 per cent, which confirms the percentages found in the first series of experiments. Finally, the protective effect of halothane is higher (70–80 per cent) than these neuroleptics. The question as to whether this is due to specific properties of this compound or because of different or more constant plasma and tissue levels remains unanswered.

For pentobarbital, it has already been suggested that depression of DNA synthesis need not necessarily be related with a decreased sensitivity of the cells to phase-specific cytostatic drugs. Two additional sets of data may also point into this direction. Firstly, prednisolone in surprisingly low concentrations had a remarkable protective effect for haemopoietic stem cells during chemotherapy. The mechanism by wich prednisolone inhibits cell division is largely unknown. There is evidence to suggest that cortisone inhibits the activity of DNA polymerase in rat liver (Henderson and Loeb, 1970), and this might inhibit cell division. The protective effect of Triton WR 1339 in our system is completely unclear, although a membrane stabilizing effect of this drug has been proposed. Twenty-four hour nitrous oxide exposure does not depress DNA synthesis in femoral marrow cells and did so in only 1 out of 4 experiments in splenic cells. Continued exposure

(48–72 hour) to nitrous oxide even stimulates DNA synthesis in our system. These data together indicate that one has to be careful in pinpointing the mechanism of protection by CNS depressants during cytostatic treatment; a decreased DNA synthesis rate is neither a sufficient nor a constant finding. Other possibilities to explain the protective effect for haemopoietic stem cells of a variety of CNS depressants during fractionated chemotherapy have to be considered. One such a possibility is the interference of CNS depressants with control systems that play an important role in the regulation of major cellular processes:

1. A system which is controlled by a membrane adenyl cyclase;
2. A system of microtubules and microfilaments.

In a number of cells the two systems have been shown to be closely associated. The effect of CNS depressants on these two systems will be discussed in the following paragraphs.

The adenylate cyclase system has been shown to be an important component in the regulatory control of cellular functions such as proliferation and differentiation. The importance of cyclic AMP, calcium and cyclic GMP has been recognized for the control of cell proliferation (see, for instance, the reviews of Whitfield et al., 1973, and Berridge, 1975), and differentiation (Hsie et al., 1971a, b; Kram et al., 1973). In particular, the importance of the adenyl cyclase system for the control of proliferation in the haemopoietic tissue has been shown by Whitfield and co-workers (see review, 1973). The work of Byron (1972, 1974, 1975) supports this view. Using a model in which haemopoietic cells were incubated *in vitro*, it was shown that neurotransmitters, either beta-adrenergic or cholinergic, induced DNA synthesis in previously resting cells. Subsequently, antagonists of these drugs, such as the beta-adrenergic-receptor blocker propranolol, selectively blocked this induction of DNA synthesis. In addition, a number of other agents such as steroids (Byron, 1971), prostaglandins (Fehér and Gidali, 1974) and parathormone (Gallien-Lartigue and Carrez, 1974) were found to give similar results. Evidence suggesting that cyclic AMP, calcium and cyclic GMP are involved in this triggering of resting haemopoietic stem cells in cycle was presented. Consequently, the presence of a neurotransmitter-sensitive membrane receptor system, which can initiate intracellular mechanisms leading to haemopoietic cell proliferation, has been proposed. Nerve fibers pass throughout the bone marrow and terminate in close proximity to

bone marrow cells (Calvo, 1968). It has been speculated that neurotransmitters from these nerve endings regulate stem cell proliferation (Byron, 1974). It may be hypothesized that halothane and other CNS depressants act on these nerve endings in the haemopoietic tissue or later on in the chain of events controlling the cell cycle. The specificity of the halothane effect for haemopoietic cells can be explained by the extremely rapid response, as evident from proliferation and differentiation, in the haemopoietic stem cell pool after cytotoxic challenge. No other tissue in man is known to react in the same way.

A system of microtubules (MT) and microfilaments (MF) has been shown to exist in both the cytoplasm of interphase cells and the mitotic apparatus. It seems likely that the material of the cytoplasmic structure is reutilized in the assembly of spindle microtubules. Reciprocally, the reappearance of cytoplasmic microtubules in the late telophase/early G_1-phase suggests that spindle tubule subunits may then, in turn, be recycled into cytoplasmic microtubules (Brinkley et al., 1975). These cytoplasmic structures seem to be involved in the maintenance of cytoplasmic rigidity, cellular motility, the distribution of membrane receptors, cytoplasmic transport and other major cellular functions. Evidence has been presented showing that calcium and the cyclic mononucleotides cAMP and cGMP are involved in the control of these cellular structures (see, among others, Weisenberg, 1972; Borman et al., 1975; Oliver et al., 1975). This may suggest that cellular control mechanisms in which calcium and the cyclic nucleotides are involved act via the MT and MF systems (a review discussing recent evidence is that of Marx, 1976). Apparently, the disruption of the MT and MF system can lead to disturbances in a variety of cellular functions.

Recent observations have indicated that a number of tranquillizers inhibit adenylate cyclase systems in cells obtained from nervous tissues and peripheral tissues. This was shown for dopamine (brain cells), noradrenaline (fat cells) and glucagon (liver plasma membranes) stimulated adenylate cyclases, using neuroleptics such as phenothiazines, thioxanthenes and benzodiazepines (Free et al., 1974; Miller et al., 1976). Wolleman (1974) reported the inhibitory effect of chlorpromazine on phosphodiesterase activity in rabbit brain cells. (Phosphodiesterase degredates cAMP to adenosine-5′-monophosphate). Two differences between the effects of the neuroleptics on nervous and peripheral cells have been established: a. There was no correlation

between the neuroleptic effect of the drug and the peripheral inhibitory effects. b. Effects on peripheral adenylate cyclase systems were obtained only after drug dosages which were usually 100 times higher than in the case of brain adenylate cyclase systems (Miller, 1976).

In addition, several local anaesthetics and tranquillizers have been shown to cause a redistribution of membrane receptors, a process which is probably controlled by the cytoplasmic MT and MF systems (Ryan et al., 1974; Poste et al., 1975; Nicolson et al., 1976).

As mentioned above, MT and MF structures are presumably involved in a number of cellular events. Thus, these data may suggest that local anaesthetics and tranquillizers exert major inhibiting effects on a number of cellular processes, the importance of which cannot be wholy appreciated at the moment. The recent work of Lipski (1976) has demonstrated that the injection of isoproterenol, a beta-adrenergic agent, elevates the level of cAMP in mouse bone marrow cells *in vivo*. Moreover, propranolol, which is a selective blocker of beta-adrenergic-receptors, prevented this effect. This is interesting, in view of the fact that propranolol decreases the toxicity of fractionated courses of both VLB and Ara-C towards mouse haemopoietic stem cells (Table 3.10). This strongly suggests that the sensitivity of stem cells to cytostatic drugs might be influenced by manipulation of the systems which are involved in the control of cellular proliferation. That these processes may be associated with the adenylate cyclase system and the cyclic nucleotides, on one hand, and the MT and MF systems, on the other, allows one to speculate that the mechanism of action of anaesthetics and tranquillizers during cytostatic treatment of mouse haemopoietic stem cells may also be related to changes in these subcellular compounds. This speculation is strengthened by the finding that both of these classes of compounds have marked inhibitory effects on MT and MF systems, on several adenylate cyclase systems and on the phosphodiesterase activity, as was pointed out earlier.

In summary, these data indicate that the mechanisms of protection by CNS depressants during cytostatic treatment cannot yet be attributed to a single cellular event. The evidence presented suggests that CNS depressants may interfere at one point or another with the control system which regulates major cellular processes such as proliferation and differentiation.

Do CNS depressants discriminate between normal and malignant cells?

If one wishes to decrease the toxicity of anti-cancer agents preferentially so that non-malignant cells alone are protected by manipulation of principal cellular events, it is necessary that the malignant cells are not affected by the manipulative measures. The anaesthetics halotane and nitrous oxide have been shown to discriminate between normal and malignant haemopoietic stem cells (Bruce, Lin and Bruce, 1970). In addition, *in vivo* exposure to halothane has been found to depress IUdR incorporation into DNA in normal splenic and femoral haemopoietic cells and occasionally in normal intestinal cells, but not in a variety of malignant cells of both haemopoietic and non-haemopoietic origin (Evenwel, Keizer and Van Putten, 1976).

In contrast, halothane exerted disruptive effects on the MT and MF systems in neuroblastoma cells *in vitro* (Hinkley and Telser, 1974) and nitrous oxide was shown to block cultured HeLa cells in mitosis. It is unknown, however, whether the effects of these two drugs would have resulted in a change of the sensitivity of these types of malignant cells to anti-cancer agents. Recent evidence has shown that several malignant cells of different origin have significantly decreased numbers or are totally devoid of cytoplasmatic MT an MF structures (Fonte et al., 1974; Brinkley et al., 1975). The disruption of the microtubule (MT) and microfilament (MF) systems has been shown to be involved in the effect of several general anaesthetics, local anaesthetics and tranquillizers. Insight in the functions of the MT and MF systems shows that they play an important role in the control of a number of major cellular processes. Using the Skipper-method*, no effect of dehydrobenzperidol on the survival of leukaemic mice during cytostatic treatment was observed (Keizer, 1974). Since this technique can only discriminate between surviving fractions of leukaemic cells which differ by at least a factor of 10, a cell survival assay with a higher accuracy, e.g., the leukaemic stem cell assay, might be of help in answering this question. Analogous to the spleen colony forming cell assay of a normal haemopoietic cell suspension, this technique is used

* Skipper method: With this technique, varying number of leukaemic cells are injected into mice and the time of death of the animals is scored. Alterations in the time of death after cytostatic treatment allows an estimation of the number of leukaemic cells which survived.

Table 3.10. Effect of Propranolol during fractionated chemotherapy.

Treatment		Number of surviving CFU in the femoral marrow				
Protective agent (mg/kg)	Cytostatic (mg/kg)	Exp. I	Exp. II	Exp. III	Exp. IV	Mean per cent protection
None	VLB 3×10	1920	2300			
None	Ara-C 3×150			3020	693*	
Propranolol 3×5	VLB 3×10	3280 (71)	2853 (24)			
3×2.5	,,	2720 (41)	2859 (23)			48 ($p<0.01$)
3×5	Ara-C 3×150			5650 (87)	983* (42)	

Effect of the beta-adrenergic receptor blocker, propranolol, during fractionated chemotherapy of mouse femoral haemopoeitic stem cells. All injections were given at 4-hour intervals, propranolol and Ara-C i.p., VLB s.c.
* CFU survival in the spleen

to estimate the number of leukaemic stem cells in a leukaemic cell suspension. In contrast, the recipient mice do not have to be irradiated to induce leukaemic stem cell growth. Preliminary experiments suggest that flupenthixol does not protect leukaemic stem cells during chemotherapy. This finding shows that it cannot be excluded that normal and malignant cells may respond differently to treatment with certain CNS depressants during chemotherapy.

Manipulation of normal critical cells: speculations on future developments

There are a number of reasons for assuming that research in the area of normal cell manipulation will be intensified in the near future (for a discussion of this subject, see Van Putten, Keizer and Evenwel, 1976). Briefly, these points can be summarized as follows:

1. the lack of a difference between normal and malignant cells which can be exploited to kill tumour cells selectively;
2. resting normal cells in different patients are more similar than resting tumour cells, thus permitting a more uniform manipulation schedule;
3. during the course of a fractionated cytostatic treatment, resting normal cells are recruited into cycle more rapidly than are resting tumour cells.

Moreover, the results to date strongly suggest that manipulation of the cell cycle of normal critical cells can be achieved, so that they are less responsive to cytostatic treatment.

The choice of CNS depressants seems to be justified for two additional reasons:

1. their effects are considered to be reversible;
2. they have no uniform effect on any type of cell, i.e., they probably differ greatly in their effect on in the CNS and in their effect on peripheral tissues. This supports the idea that a drug with a preferential effect on critical normal cells may be selected. An ideal drug, in this respect, should be devoid of neuroleptic or anaesthetic effects, but should strongly decrease toxicity of ani-cancer agents to normal critical cells. The finding that both α- and β-flupenthixol were able to inhibit a number of stimulated adenylate cyclase systems in both nervous and peripheral cells, whereas β-flupenthixol is almost devoid of neuroleptic activity (Miller, 1976), illustrates that the idea of

developing drugs with the capacity to "anaesthetize" peripheral normal cells should not be rejected out of hand. Another approach to investigate the possibilities of normal cell manipulation in order to investigate this phenomenon in more detail, may be found in the use of drugs such as cyclic AMP, cyclic GMP and drugs which specially inhibit the adenyl cyclase system, such as the beta-adrenergic-receptor-blocker propranolol, and the phosphodiesterase blocker Ro-20-1724 (a compound of the Roche company). A model as presented in this chapter might be suitable to test these drugs. Finally the effect of our protective compounds (CNS depressants and others) directly on the adenyl cyclase system and on the MT and MF systems should be investigated in particular on haemopioetic cells.

CHAPTER 4

IMMOBILIZATION OF EXPERIMENTAL ANIMALS DURING RADIATION EXPOSURE

A radiobiologist is frequently faced with the necessity of immobilizing experimental animals for a limited period of time. For this purpose, a wide variety of anaesthetic drugs is available. These include ether, chloralhydrate, pentobarbital, fluanisone, tribromoethanol and ketamine. A second type of immobilization is achieved by physical restraint of the animals.

Paterson and Matthews (1951) reported that anaesthesia using two courses of an orally administered 10 per cent ethyl alcohol solution (1 ml) at 80 and 20 minutes before whole-body X-irradiation of mice increased the LD_{50} value by 70 rad and permitted the non-survivors to live longer. Similarly, a 5 percent ethyl alcohol solution, which rarely rendered the mice unconscious, provided the same protection. Since two known anaesthetic compounds, thiopental and ethylcarbonate, did not affect LD_{50} values at clearly anaesthetic dosages, the authors concluded that anaesthesia *per se* played no part in the radioprotective effect of ethyl alcohol. The protective effect of ethyl alcohol was confirmed by Cole and Ellis (1952) in mice but not in rats. Thiopental gave rise to a moderate radioprotective effect in rats (Williams and De Long, 1953). Detailed studies of Langendorff and Koch (1954) showed that hexobarbital sodium and ethyl alcohol given shortly before a 500 Röntgen whole-body X ray exposure resulted in a significantly increased survival after 30 days. In contrast, ether and urethane were found to potentiate radiation damage. These authors also suggested that not narcosis, but some property of the drug itself played a part in the protection phenomenon. Van Bekkum (1969) observed in mice that both types of immobilization, anaesthesia and restraint without anaesthesia, caused a considerable increase in the LD_{50}. Moreover, a mortality pattern suggestive of the intestinal syndrome instead of the bone marrow syndrome was observed in a proportion of the animals. As a consequence, animal survival could no longer serve as a measure of bone marrow cell survival.

A variety of drugs which are used to anaesthetize laboratory animals

can be shown to have a radioprotective effect specifically on the haemopoietic system and, less consistently, on the gastrointestinal system. For pentobarbital sodium, this effect has been related to an increased haemopoietic stem cell survival after whole-body radiation exposure (Keizer and Van Bekkum, 1971; Keizer and Van Putten, 1974, 1976). Riches and co-workers (1973) demonstrated that tribromoethanol anaesthesia during radiation exposure resulted in a higher femoral bone marrow cellularity and higher splenic weight at day 10 after irradiation.

In this chapter, data on the effects of a number of CNS depressants, both hypnotics and tranquillizers, during whole-body irradiation are reported. In addition, the effect of immobilization of mice by means of restraint without anaesthesia will be presented. For this purpose, total-body X-irradiation was given to both non-anaesthetized mice and to mice which were under narcosis. The effect of these CNS depressants during irradiation was determined by using the following two parameters:

1. the number of mice dying between 10 and 30 days after a total body radiation dose, less than approximately 1100 rad X rays (bone marrow syndrome).
2. the number of mice dying within 5 days after a total body radiation dose, more than approximately 1100 rad X rays (gastrointestinal syndrome). In addition, data were collected at the cellular level on the effect of immobilization of mice during whole-body radiation exposure. For this purpose, the spleen colony technique was used to determine the haemopoietic stem cell survival. Attempts to further characterize the effect of CNS depressants during whole-body radiation exposure will also be described.

Experimental data

An increase of 0 to 100 per cent mortality from damage to the haemopoietic tissues occurred within a dose range of approximately 200 rad X rays (from 700 to 900 rad) (fig. 4.1; dotted line with open circles). Both pentobarbital and a mixture of fluanisone and fentanyl (when injected shortly before radiation exposure), caused a shift of the dose-survival curves to the right. This means that higher radiation doses were needed to obtain the same mortality as in the non-anaesthetized group. For comparison, a calculated dose-survival curve for

control mice, based on pooled data from the Radiobiological Institute, has been added (fig. 4.1; dotted line with crosses). A significantly higher number of pentobarbital anaesthetized mice survived longer than 30 days, even after a radiation dose known to be sufficient to kill non-anaesthetized mice within 5 days after irradiation. After calculation of the best fit on a probability scale, the lines were redrawn on a linear scale. This plot (fig. 4.2) shows two things:

1. the protective effect of anaesthesia is seen only after radiation dosages which usually kill more than 50 per cent of the animals; and
2. the difference between the control lines and the two anaesthetisia lines is statistically significant in this region ($p < 0.05$), since there is no overlap of the 95 percent confidence limits. The dose mortality curves obtained after scoring the mortality at day 5 after total-body X-irradiation also show a shift to the higher dose levels when the mice were treated with the CNS depressants (fig. 4.3). This shift was about 150 rad X rays over the total high or low dose range. After probit analysis, the data were redrawn on a linear scale. The statistical significance of the difference between the lines is shown by the lack of an overlap of the 95 per cent confidence limits (fig. 4.4). Comparison of fig. 4.1 and fig. 4.3 indicates that, at day 30 after irradiation, a few mice are still living in the anaesthetized group, whereas mice in the control group had already died from gastrointestinal damage. This indicates that the dose difference between the two radiation syndromes is becoming smaller.

Other compounds which are also frequently used to anaesthetize experimental animals, such as chloralhydrate and ether, have the same effect as pentobarbital and fluanisone-fentanyl on the 30-day survival (figure 4.5). Moreover, the number of 5-day survivors after 1300 rad X rays indicates that these anaesthetics also alter the radiation effect on the gastrointestinal system (fig. 4.6). It is interesting to note that halothane anaesthesia* did not alter 30 day survival. Also, in this series of experiments, the administration of pentobarbital resulted in the most marked change in mortality. These data (fig. 4.5 and 4.6) do not show the overlap in mortality from bone marrow death and gastrointestinal death (compare with figs. 4.1 and 4.3). The fact that

* The halothane concentration used in these experiments was 1.5 per cent, which is just sufficient to immobilize the mice. This is in contrast to the concentration used in the experiments described in chapter 3, which was 0.4 percent allowing the mice to ambulate.

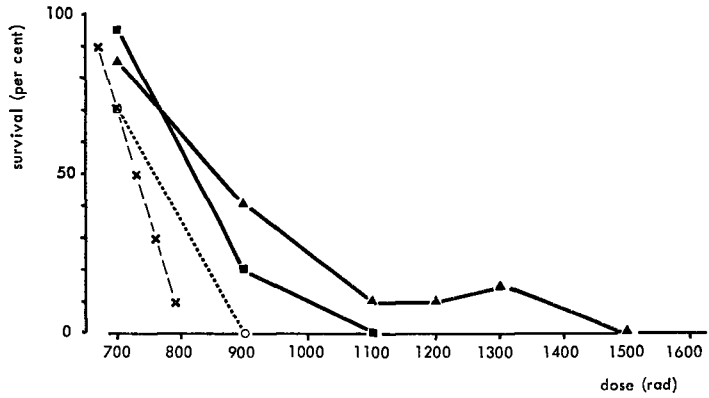


Figure 4.1. Dose-survival curves of mice after whole-body X-irradiation. Survival scored at day 30. Each point reflects survival in 20 mice exposed O----O, control mice; ■■, mice anaesthetized with Fluanisone-Fentanyl (15 mg/kg body-weight); ▲▲, mice anaesthetized with Pentobarbital (60 mg/kg body-weight); x-----x, this dotted line represents a calculated curve, based on pooled data for control mice obtained in the Radiobiological Institute TNO over the last 10 years.

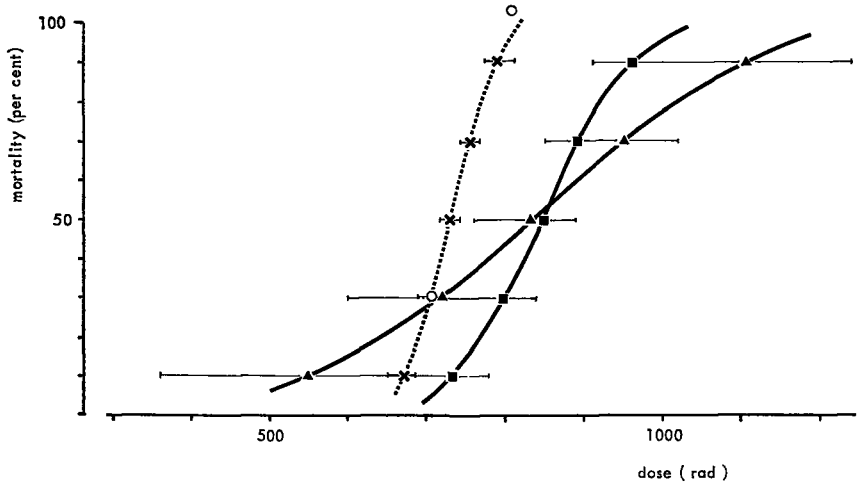


Figure 4.2. Dose-mortality curve after whole-body X-irradiation of mice. After probit analysis of the data from fig. 4.1, the calculated best fit has been redrawn on a linear scale.
O O, experimental points for control mice; ▲▲ Pentobarbital; ■■ Fluanisone-Fentanyl; x-----x, calculated curve based on pooled data for control mice obtained in the Radiobiological Institute over the last 10 years.

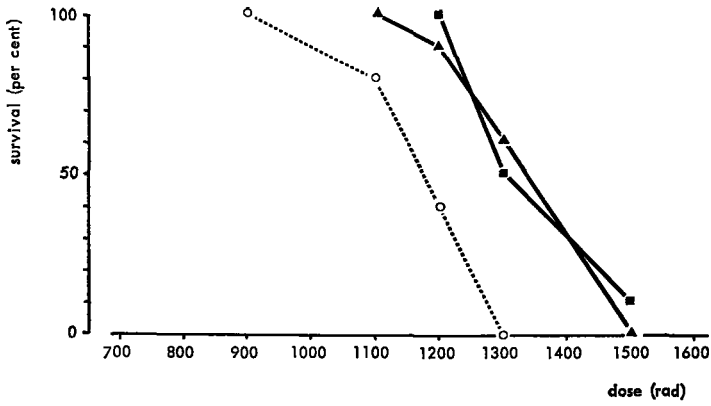


Figure 4.3. Dose-survival curves of mice after whole-body X-irradiation. Survival scored at day 5. Each point reflects survival in 20 mice exposed.
○ ○, control mice; ■■, mice anaesthetized with Fluanisone-Fentanyl (15 mg/kg. i.p.); ▲▲, mice anaesthetized with Pentobarbital (60 mg/kg i.p.).

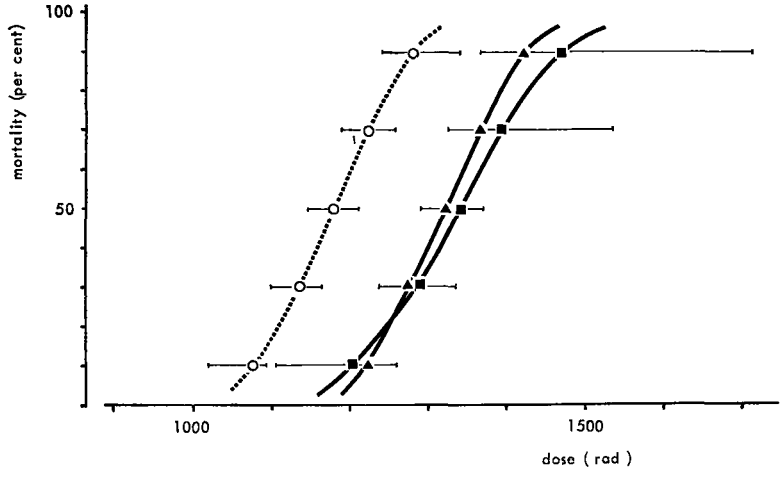


Figure 4.4. Dose-mortality curves of mice after whole-body X-irradiation. After probit analysis of the data from fig. 4.3, the calculated best fit has been redrawn on a linear scale.
○ ○, control mice; ▲▲ Pentobarbital anaesthetized mice; ■■ Fluanisone-Fentanyl anaesthetized mice.

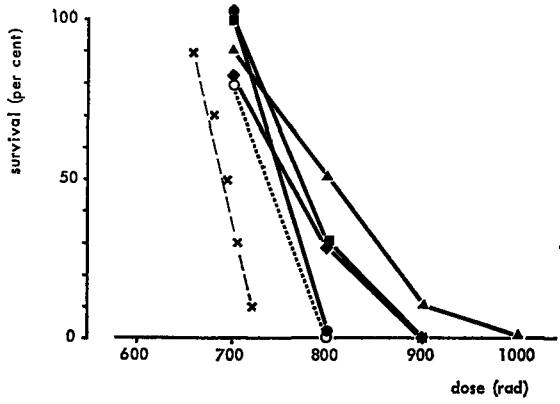


Figure 4.5. Dose-survival curves of mice after whole-body X-irradiation. Survival scored at day 30. Each point reflects survival in 10 mice exposed.
○ ○, control mice; ●●, Halothane (1.5 per cent) exposed mice;
■■, Chloralhydrate (400 mg/kg i.p.) anaesthetized mice;
◆◆, Ether anaesthetized mice
▲▲, Pentobarbital (60 mg/kg) anaesthetized mice.
++, the dotted line represents a calculated curve, based on pooled data for control mice obtained in the Radiobiological Institute TNO. Each point reflects survival in 30–50 mice exposed.

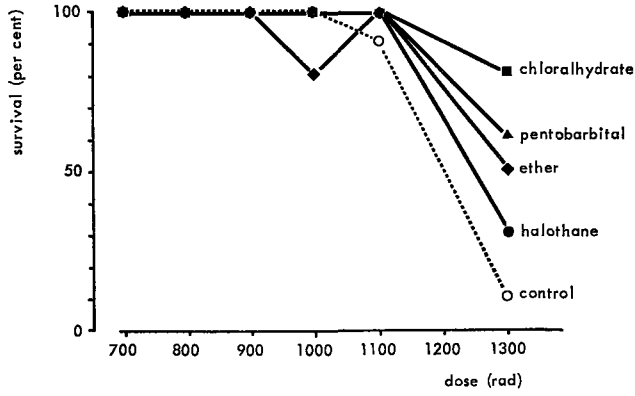


Figure 4.6. Dose-survival curves of mice after whole-body X-irradiation. Survival scored at day 5. Each point reflects survival in 10 mice exposed.
○ ○, control mice; ●●, Halothane (1.5 per cent) exposed mice;
■■, Chloralhydrate (400 mg/kg i.p.) anaesthetized mice;
◆◆, Ether anaesthetized mice; ▲▲, Pentobarbital (60 mg/kg i.p.) anaesthetized mice.

mice of different strains were used for the experiments illustrated in figs. 4.1 and 4.2 and the experiments given in fig. 4.5, and fig. 4.6 might play a part.

To investigate the dose-dependency of the effect of pentobarbital during whole-body irradiation, a dose of 15 mg/kg body weight instead of the usual dose of 60 mg/kg body weight, was investigated. No effect of this dose of pentobarbital (15 mg/kg) on the 30-day or 5-day survival following radiation exposure was observed (fig. 4.7). Apparently this dose is below the threshold for the protective effect of pentobarbital.

In an attempt to further characterize the effect of CNS depressants at the cellular level during whole body irradiation, dose survival curves were made for femoral stem cells from control and pentobarbital treated mice (fig. 4.8). The D_0 values were 74 and 90 rad X rays with extrapolation numbers $n = 1.6$ and $n = 1.4$, for the control and pentobarbital groups, respectively. Statistical analysis showed that the difference between the two D_0 values is not significant. However, the lines are not similar ($p < 0.005$)*. In a similar way, the effect of pentobarbital anaesthesia during radiation exposure on the survival of splenic CFU was determined. In the case of splenic CFU, however, no effect of pentobarbital was observed (Table 4.1). When mice were

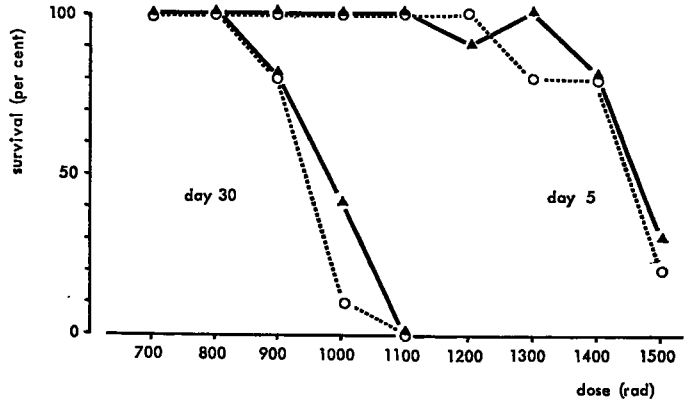


Figure 4.7. Effect of a non-anaesthetic dose of Pentobarbital (15 mg/kg i.p.). Dose survival curves of mice after whole-body gamma-irradiation. Survival scored at day 5 and day 30. Each point reflects survival in 10 mice exposed. ○ ○, control mice; ▲▲, Pentobarbital (15 mg/kg i.p.).

* see appendix to this chapter for data on statistical analysis.

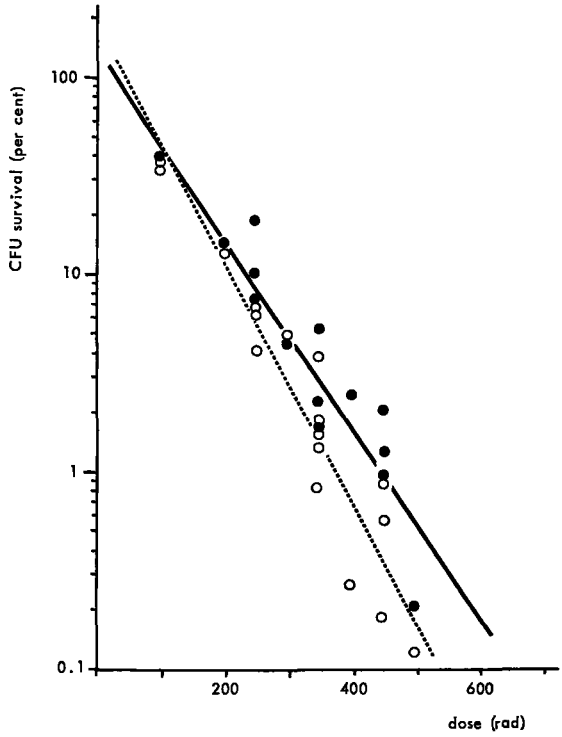


Figure 4.8. Effect of Pentobarbital anaesthesia on stem cell survival in the femoral bone marrow after whole-body X-irradiation. Each experiment was done with 4–5 donor mice. Each point represents the survival based on the number of colonies in 10 spleens.
○ ○, control mice, $D_0 = 74$ rad, $n = 1.6$; ●●, Pentobarbital (60 mg/kg i.p.) anaesthetized mice, $D_0 = 90$ rad, $n = 1.4$. Lines are statistically different, $p < 0.005$.

immobilized by means of rubber bands during whole-body irradiation (see fig. 2.1), a surprising increase in the survival of haemopoietic stem cells in the femoral bone marrow of mice was observed (fig. 4.9). The D_0 value of the physical restraint curve was 95 rad with an extrapolation number $n = 1.3$. The slope of this line is also not statistically different from the control line but again the lines were not similar ($p < 0.001$)*. This restraint technique clearly causes 1. stretching of the animals; and 2. constriction of the legs of the animals

* see appendix to this chapter for data on statistical analysis

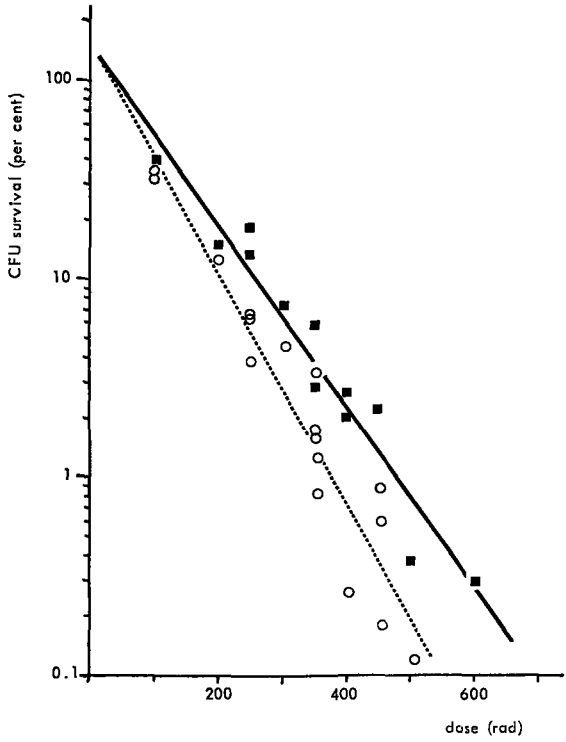


Figure 4.9. Effect of physical restraint without anaesthesia on stem cell survival in the femoral bone marrow after whole-body X-irradiation. Each experiment was done with 4–5 donor mice. Each point represents the mean survival based on the number of colonies in 10 spleens.
○ ○, control mice, $D_0 = 74$ rad, $n = 1.6$ (from figure 4.8);
■■, physical restraint mice, $D_0 = 95$ rad, $n = 1.3$. Lines are statistically different, $p < 0.001$.

by the rubber bands. The protective effect of this type of restraint disappeared immediately after the mice were released (Table 4.2).

In an effort to avoid these effects of physical restraint, the four legs of the animals were fixed by means of adhesive tape (which causes almost no mechanical pressure) in such a way that the animals were not stretched (see fig. 2.2). The data obtained in this way indicate that now a higher radiation dose was needed to obtain the same mortality after 30 days as compared to the control irradiated mice (fig. 4.10), that is, 1100 rad instead of 800 rad X rays. No effect on gastrointestinal damage (5-day mortality) could be observed.

Table 4.1. Survival of haemopoietic stem cells in the spleen of normal and Pentobarbital anaesthetized mice after 300 rad whole-body gamma-irradiation

	CFU survival (per cent)					
	Exp. I	Exp. II	Exp. III	Exp. IV	Mean±E.S.	
Resting						
Saline	0.96	2.3	3.4	3.8	2.6±0.64	n.s.**
Pentobarbital*	0.90	3.2	2.8	3.2	2.5±0.55	

Each experiment was done with 4–5 donor mice; stem cell survival was based on the number of colonies in 10 spleens.
* 60 mg/kg
** n.s., not significantly different

Table 4.2. Effect of 450 rad whole-body X-irradiation on CFU survival in restrained and released mice

	Percent CFU survival (mean ±S.E.)*
Irradiation of control	0.64±0.08
Restraint during irradiation	1.75±0.54
Pentobarbital during irradiation	1.03±0.14
Restraint before irradiation	0.69±0.05

Restraint and Pentobarbital anaesthesia (60 mg/kg i.p.) started 10–15 minutes before radiation exposure. One group of mice was irradiated immediately after they had been released.
* mean of 3 experiments.

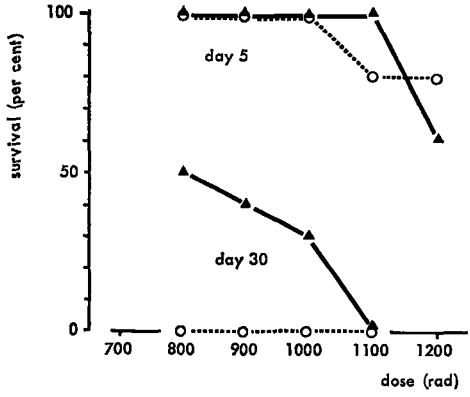


Figure 4.10. Effect of immobilization by means of adhesive tape on the survival of mice during whole-body X-irradiation.
Survival scored at day 5 and day 30 after radiation exposure.
Each point reflects survival in 10 mice exposed. ○ ○, control mice; ▲▲, restrained (adhesive tape) mice.

Conclusions and discussion

1. Three techniques were employed to immobilize mice during whole-body-exposure to ionizing iradation. These were anaesthesia and physical restraint using either rubber bands or adhesive tape. The test parameters for the effect of the irradiation were 1. whole animal mortality after whole-body irradiation, and 2. CFU survival in the femur and spleen.

All three immobilization methods affected one or more of the test parameters in the same direction, that is, they afforded radioprotection; a higher radiation dose was needed to achieve an identical effect as was seen in controls.

2. Our data show that all but one drug, halothane (1.5 per cent), increase the 30-day survival percentage or the lifespan after doses of irradiation high enough to cause gastrointestinal damage. This suggests that the effect is a feature which a variety of anaesthetics have in common. In some experiments, the effect was seen only after radiation dosages that cause more than 50 per cent kill (LD_{50}). This may explain why other authors concluded that particular anaesthetic drugs had no radiation modifying effect, since this conclusion was based on changes in the LD_{50} (see introduction to this chapter). The radiation damage potentiating effect of ether, as reported by Langendorff and Koch (1954), could not be confirmed in this study.

3. The failure of a non-anaesthetic dose of pentobarbital to act as a radioprotective agent might suggest that: 1. anaesthesia *per se* plays a role in changing the radiation sensitivity; 2. anaesthesia does not play a role, but the effect is dose-dependent. The work of other investigators had already suggested that the effect is not secondary to the production of anaesthesia but that other cellular effects of the particular anaesthetic drug gave rise to the changed radiosensitivity of certain cells, thus supporting the seond hypothesis: the effect is dose-dependent.

4. Pentobarbital and restraint improved haemopoietic stem cell survival in the femoral bone marrow after whole-body irradiation. This suggests that these immobilization techniques exert their radiation modifying effect directly at the cellular level.

5. The number of isologous haemopoietic cells needed to protect 50 per cent of a lethally irradiated mouse population is approximately 5×10^4 cells (Van Bekkum and Vos, 1957; Van Putten, 1964). This

number of cells, approximately 0.5 per cent of the content of one femur, contains at most 1000 haemopoietic stem cells. This calculation serves to illustrate that a small number of protected cells can give rise to changes in the 30-day survival after radiation exposure.

6. Splenic CFU were not protected. Assuming that tribromoethanol has a similar effect as pentobarbital in this study, this observation does not support the suggestion of Riches et al., that tribromoethanol anaesthesia exerts its protective effect on both the femoral and the splenic haemopoietic cells. These authors observed a greater spleen weight and a higher femoral cellularity in anaesthetized mice 10 day after irradiation. The greater spleen weight might be explained by an increased migration of haemopoietic cells from the femur due to an increased stem cell survival in the femur. A less likely explanation may be that tribromoethanol increases the radioresistance of the splenic CFU. This then might be a specific finding for tribromoethanol.

7. In conclusion, one has to be careful in interpreting radiation effects on normal haemopoietic and intestinal cells when CNS depressants are given shortly before radiation exposure to immobilize the experimental animals. It is unknown whether other normal tissue cells are also affected. In addition, it cannot be excluded that malignant cells are also affected. These data suggest that radiobiological studies, either with normal or malignant cells, should be considered very critically when they involve either physical or pharmacological restraint.

Further investigation into how CNS depressants modify radiation effects of normal cells is required. Similarly, the effect of anaesthetics on tumour cell survival after radiation exposure should be determined. The following chapter examines some of these problems.

Statistical appendix

Conditions under which two dose-survival curves are statistically different.

The shape and significance of the various parts of a dose-survival curve obtained after radiation exposure of mammalian cells, in particular haemopoietic stem cells, have been discussed in chapter 1 ("Radiosensitivity of mouse haemopoietic stem cells" and" The age-response of haemopoietic stem cells").

Considering two X ray dose-survival curves obtained for mouse haemopoietic stem cells, the following combinations of D_0 and n-values can theoretically exist.

1. Both D_0 and n-values are statistically different.
2. D_0 but not n-values are statistically different.
3. Neither D_0 nor n-values are statistically different.
4. n-values but not D_0 are statistically different.

Obviously, the fourth combination results in two regression lines which are parallel but not similar. It is generally assumed that the third combination where neither the D_0 nor the n-values differ significantly, characterize two lines which are identical. This is not necessarily true. This situation can be illustrated by considering three radiation dose-survival curves for femoral marrow stem cells. Each curve was generated using whole-body irradiation, the variable was the presence and method of production of restraint. For mice neither restrained nor anaesthetized, a curve with $D_0 = 74$ rad and $n = 1.6$ was obtained. For anaesthetized mice, $D_0 = 90$ rad and $n = 1.4$. A curve for physically restrained mice yielded a $D_0 = 95$ rad and $n = 1.3$. Statistical analysis (Diem and Lentner, Documenta Geigy, Wissenschaftliche Tabellen) of the data showed that both the anaesthesia curve and the restraint curve differ significantly from the control,

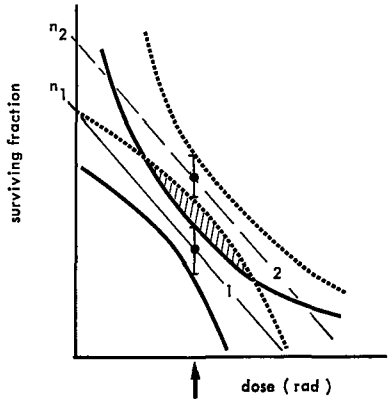


Figure 4.11. Schematic representation of two calculated dose survival curves for haemopoietic stem cells; 1. solid line with extrapolation number n_1; and 2. dotted line with extrapolation number n_2. The 95 per cent confidence limits of the two regression lines have also been given. The shaded area reflects the area with no overlap of the 95 per cent confidence limits.

$p < 0.005$ and $p < 0.001$ respectively. Moreover, the D_0 values and the n-values were shown not to differ significantly.

This illustrates that dose-survival curves, with similar D_0 and n-values can, however, be statistically different lines. A graphical illustration is shown in fig. 4.11, giving two dose-survival curves with their 95 percent confidence limits based on *mean* survival levels at the indicated dose (see fig. 4.11). The dotted area between the two lines indicates the area of no overlap of the 95 percent confidence limits, indicating that the difference between the two curves is statistically significant.

CHAPTER 5

THE MECHANISM OF RADIOPROTECTION BY PENTOBARBITAL

In the previous chapter we have shown by use of several test parameters that a variety of CNS depressants exert a slight, but significant, radioprotective effect on the haemopoietic and the gastrointestinal systems. In this chapter, experiments which were designed to elucidate the radioprotective effect of one of the CNS depressants, pentobarbital sodium, on the haemopoietic stem cell of the mouse will be described. Based on some known systemic and cellular effects, a number of hypotheses have been advanced to explain the mechanism of the radioprotective effect of pentobarbital. These systemic and cellular effects of pentobarbital are summarized briefly in the following.

Effects of barbiturates on the central nervous system and other cell systems

The barbiturates reversibly depress the activity of all excitable tissues. Not all tissues are affected at the same dosage and concentration; the CNS is exquisitely sensitive, so that when barbiturates are given in sedative or hypnotic doses, very little effect on skeletal, cardiac, or smooth muscle occurs. Even in anaesthetic concentrations direct effects on peripheral excitable tissues are mild (Goodman and Gilman, 1975). The drugs have been shown to inhibit bioluminescence in bacteria, to reduce oxygen consumption in various mammalian tissues and to inhibit respiration in cell-free preparations of liver and brain mitochondria. The barbiturates have also been shown to inhibit cell division and the synthesis of nucleic acids and proteins in a variety of systems (Table 5.1). Morover, the drug has been shown to block mitosis (Caratzali et al., 1969; Whyatt et al., 1973). In some of these systems, the inhibitory effects have been shown to be reversible, even after more than 3 weeks of incubation (Baserga and Weiss, 1967; Fink and Kenny, 1970).

The precise mechanism by which barbiturates inhibit DNA synthesis is still a matter of debate. In the case of pentobarbital,

Table 5.1. *In vivo* and *in vitro* growing, normal and malignant cells in which pentobarbital has been shown to inhibit DNA- and protein synthesis.

Cell type	Author
In vivo	
Bone marrow	Rambach et al. (1952)
Spleen	
Dejunum	Baserga et al. (1967)
Ehrlich ascites	
In vitro	
Ehrlich ascites	Beck et al. (1975)
HeLa	Baserga et al. (1967)
Mouse heteroploid cells	Fink et al. (1971)
Rat hepatoma	Jackson et al. (1971)
Murine mastocytoma	Whyatt et al. (1973)
Murine lymphoblasts	

Baserga and Weiss (1967) presented evidence supporting a direct effect at the DNA level. Whyatt and Cramer (1973) found that not only DNA synthesis but also RNA and protein synthesis were inhibited, suggesting that the inhibited DNA synthesis was secondary to the other inhibited processes. Beck et al. (1975) presented data suggesting that the observed inhibition of DNA, RNA and protein synthesis might be secondary to the depressed metabolism of the cells. The effects of barbiturates on the various tissues need not necesarily be based on a common mechanism involving identical receptors.

Effect of pentobarbital anaesthesia on the cardiovascular system

The effect of pentobarbital anaesthesia on the cardiovascular system of the mouse can be divided into two main categories:

1. A decrease in the cardiac output to 50 per cent of resting value and, subsequently, a decrease in the absolute perfusion of all tissues (Zanelli et al., 1975, 1976; Johnson, Fowler and Zanelli, 1976). A drop of 5 °C in body temperature during pentobarbital anaesthesia was observed by the latter authors.
2. A redistribution of cardiac output among the normal body organs: kidneys and intestines receive relatively more blood while lungs and muscle receive less (Aardal et al., 1973; Zanelli et al., 1975).

It is open to spectulation as to whether, under treatment with pentobarbital, these changes affect the oxygen supply of the organs concerned and thus may explain the radioprotective effect of pentobarbital anaesthesia.

Distribution of pentobarbital after intraperitoneal injection

Because of their high perfusion rates, the brain and certain visceral organs (liver, kidney, heart, etc.), which together receive 70 per cent of the total cardiac output, exhibit maximal concentration of the barbiturates very soon after i.v. or i.p. administration. On the other hand, minutes are required for muscle and skin to equilibrate with plasma and even more time is required to redistribute to fat. There is no impenetrable barrier to the diffusion of barbiturates in the body; consequently, if the drug remains in the plasma for a sufficiently long time, it will be distributed to all tissues and fluids. As muscle and fat take up the barbiturate, the plasma concentration falls and the drug diffuses out of the brain along a concentration gradient. As much as 90 per cent of the initial peak concentration may be given up by the brain and viscera to the other tissues.

In the next hours, most barbiturates are converted to inactive metabolites. The principal site of biotransformation is the liver. A number of barbiturates, such as barbital, phenobarbital and aprobarbital, are also excreted partially unchanged by the kidneys. The plasma half-life of pentobarbital sodium in the mouse falls within the range of one hour.

Hypotheses to explain the radioprotective effect of pentobarbital

1. The drastic effect of pentobarbital anaesthesia on the cardiovascular system may suggest that a decreased oxygenation of the femoral bone marrow occurs, leading to a decreased radiosensitivity of the haemopoietic cells. To test this hypothesis, the hypoxic cell radiosensitizer Ro-07-0582 was used.
2. Direct interference with the chain of events leading to radiation damage of biological material is considered as a possible mechanism by which several compounds exert their radioprotective effect. Pentobarbital is distributed to all tissues after injection into mice; therefore

this compound might result in a decreased radiation sensitivity in all haemopoietic cells. This implies that femoral and splenic haemopoietic cells should be equally affected. This aspect was investigated. The effect of pentobarbital during radiation exposure of haemopoietic cells *in vitro* might give additional information.

3. Pentobarbital has an inhibitory effect on cellular processes such as DNA, RNA and protein synthesis. This suggests that modifications in cellular growth rate may be involved in the radioprotective effect of pentobarbital. To test this, the effect of pentobarbital on recruitment of resting cells into cycle was examined. The difference in the radiation response of a rapidly proliferating cell population in the femur and spleen during radiation exposure was also investigated, since this system should not be affected by pentobarbital if interference with recruitment of resting stem cells is the main mechanism of protection.

Experimental data

To test the hypoxia hypothesis, experiments were performed which were designed to investigate whether any hypoxic cells could be detected in the femoral bone marrow during pentobarbital anaesthesia or during immobilization by physical restraint. Use was made of a sensitizer of hypoxic cells *in vivo*, Ro-07-0582, a 2-nitroimidazole derivative.

First, we verified the sensitizing effect of Ro-07-0582 on completely hypoxic femoral CFU. The CFU were made hypoxic by killing the mice about 10 min before radiation exposure. In fig. 5.1, the dose-survival curves are shown for hypoxic CFU in mice treated either with physiological saline or with Ro-07-0582, 60 minutes before irradiation. It can be seen that Ro-07-0582 considerably increases the radiosensitivity of hypoxic CFU, as evidenced by a decrease in the D_0 value from 281 rad gamma rays to 161 rad gamma rays. The effect of Ro-07-0582 on well-oxygenated femoral CFU is indicated in fig. 5.2, showing a slight increase in the D_0 from 96 to 108 rad gamma rays.

The decrease in D_0 from 281 to 161 rad also indicates that the Roche compound is not able to render the hypoxic CFU as radiosensitive as the CFU in a well-oxygenated haemopoietic cell pupulation (normal CFU, $D_0 = 96$), i.e., the fraction of hypoxic CFU is larger than the proportion sensitized by Ro-07-0582. From these data (figs. 5.1 and 5.2), it may be concluded that Ro-07-0582 has a considerable radio-

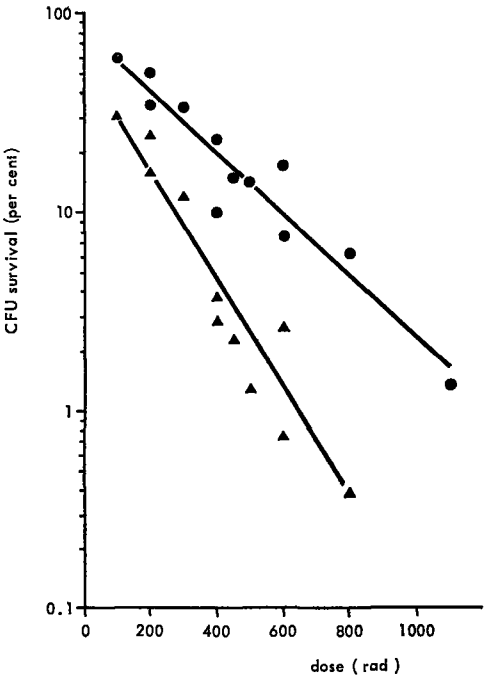


Figure 5.1. Effect of Ro-07-0582 on survival of hypoxic stem cells after whole-body gamma-irradiation. Dose survival curves for femoral bone marrow CFU. ●●, Dead animals (10 minutes before irradiation exposure) plus physiological saline, $D_0 = 281$ rad, $n = 0.8$; ▲▲, Dead animals (10 minutes before radiation exposure) plus Ro-07-0582 (1 g/kg i.p.), $D_0 = 161$ rad, $n = 0.6$. Each donor group consisted of 4–5 mice. Each point reflects the mean survival based on the number of colonies in 10 spleens. Ro-07-0582 and physiological saline were injected one hour before radiation exposure. D_0 values are statistically significant $p < 0.001$.

sensitizing effect on hypoxic femoral CFU but not on well-oxygenated CFU. Consequently, this drug seemed suitable to detect the presence of hypoxic femoral CFU, either in restrained mice or in pentobarbital anaesthetized mice. The results are shown in table 5.2. The drug did not affect the CFU survival after irradiation of unrestrained control mice. As expected, physical restraint during irradiation increased CFU survival from 0.48 to 1.83 per cent. When Ro-07-0582 was given to restrained mice, the CFU survival decreased to 0.94 per cent. This might indicate that a major part of the radioprotection seen during restraint is caused by hypoxia of the bone marrow cells; the 50 per cent reduction seen with the Roche compound is similar to that obtained

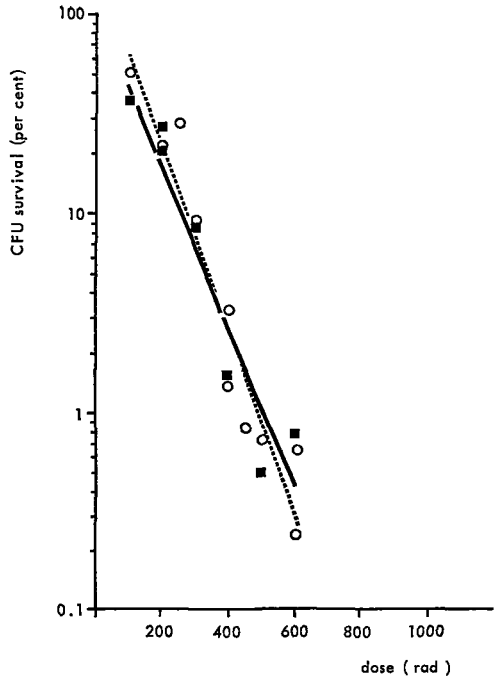


Figure 5.2. Effect of Ro-07-0582 on survival of well-oxygenated stem cells after whole-body gamma-irradiation. Dose survival curves for femoral bone marrow CFU. ○ ○, Living animals plus physiological saline, $D_0 = 96$ rad, $n = 1.8$. ■■, Living animals plus Ro-07-0582 (1 g/kg i.p.), $D_0 = 108$ rad, $n = 1.1$. Each donor group consisted of 4–5 mice. Each point reflects the mean survival based on the number of colonies in 10 spleens. Lines are not statistically different.

with this compound on completely hypoxic bone marrow cells (see fig. 5.1). By analogy, the proportion of hypoxic CFU in the femoral marrow during restraint may be larger than the proportion sensitized by the Roche compound. Since the radiosensitivity of the femoral CFU during pentobarbital anaesthesia does not change in the presence of Ro-07-0582 (Table 5.2), this suggests that no significant proportion of hypoxic CFU are present in the femoral marrow during pentobarbital anaesthesia.

The experimental data described next are the results of attempts to further elucidate the mechanism by which pentobarbital exerted its radioprotective effect on the femoral bone marrow. A first step was to determine whether pentobarbital affected the radiosensitivity of mouse femoral CFU when irradiated *in vitro*. The concentrations of pento-

Table 5.2. Test for hypoxia as a factor in radiation exposure by restraint or anaesthesia. The effect of Ro-07-0582 on mouse femoral stem cell survival after radiation exposure.

		Per cent CFU survival (Mean* ± S.E.)	
no restraint	+ 450 rad X	0.48 ± 0.12	n.s.****
no restraint + Ro-07-0582**	+ 450 rad X	0.63 ± 0.17	
restraint	+ 450 rad X	1.83 ± 0.48	$p < 0.05$
restraint + Ro-07-0582	+ 450 rad X	0.94 ± 0.13	
no anaesthesia	+ 450 rad gamma	1.24 ± 0.08	n.s.
no anaesthesia + Ro-07-0582	+ 450 rad gamma	1.44 ± 0.11	
pentobarbital***	+ 450 rad gamma	1.86 ± 0.09	n.s.
pentobarbital + Ro-07-0582	+ 450 rad gamma	1.81 ± 0.08	

The mice were anaesthetized or restrained, 10–15 minutes before radiation exposure. Ro-07-0582 was injected one hour before irradiation.

* the figures represent the mean of six (upper part) or four (lower part) experiments.

** 1 g/kg i.p.

*** 60 mg/kg i.p.

**** n.s., not significantly different

barbital which were used, 0.3 and 0.6 mg/ml suspension, are on the average 10 times higher than the *in vivo* dose*. Incubation alone does not kill bone marrow CFU (Table 5.3, last vertical column). The data given in fig. 5.3 and in table 5.3 indicate that neither of the two pentobarbital concentrations results in a radioprotective effect on the femoral bone marrow CFU *in vitro*. Thus, the *in vivo* situation is needed for pentobarbital to exert its radioprotective effect. Therefore, pentobarbital was given to mice at a dose of 60 mg/kg body weight i.p. and the femoral bone marrow was collected and irradiated *in vitro* 15–20 minutes later. After irradiation, the suspensions were assayed for the number of surviving CFU. The dose survival curve prepared for femoral marrow of non-anaesthetized control mice had a D_0 of 102.1

* It is assumed that the expression of the dose as mg/kg body weight is in an order of magnitude comparable to mg/l, which is 10^{-3} mg/ml. Thus, 60 mg/kg *in vivo* is similar to 0.06 mg/ml *in vitro*. Since *in vitro* pentobarbital concentrations of 0.3 mg/ml and 0.6 mg/ml were used, these concentrations are 5 and 10 times higher, respectively, than those used *in vivo*.

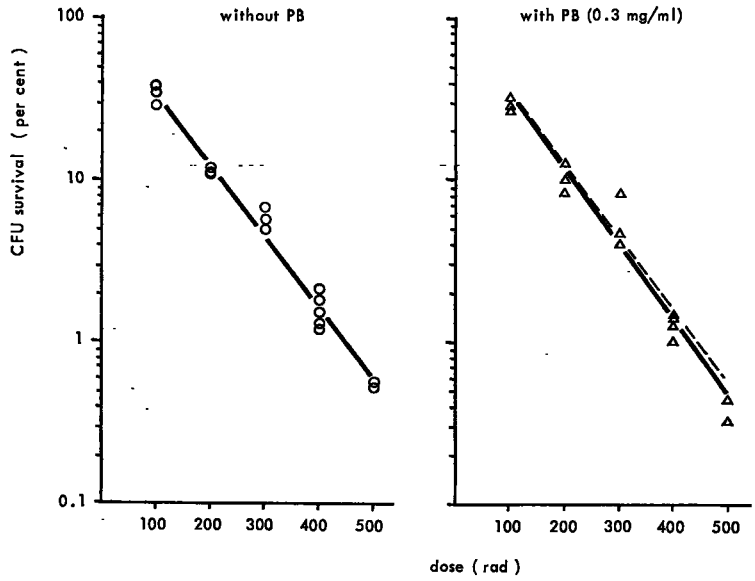


Figure 5.3. Dose survival curves for femoral marrow stem cells irradiated *in vitro*. Femoral bone marrow cell suspensions (2 ml, containing $10–15 \times 10^6$ cells/ml) were incubated for 30 minutes at 37 °C. Left side, ○ ○, irradiation after incubation without Pentobarbital, $D_0 = 98$ rad, $n = 0.94$. Right side, △ △, irradiation after incubation in Pentobarbital medium, $D_0 = 94$, $n = 0.94$. For comparison the control curve has been added as a dotted line. Each point reflects the mean survival based on the number of colonies in 10 spleens. Lines are not statistically different.

Table 5.3. Effect of pentobarbital during *in vitro* irradiation of bone marrow cell suspensions.

Incubated in	Per cent CFU survival (mean ± S.E.*)		
	200 rad gamma rays	400 rad gamma rays	unirradiated control incubation (CFU per femur)
Hanks' medium	14.5 ± 1.1	2.2 ± 0.09	6500
Hanks' medium + 0.3 mg/ml pentobarbital	15.1 ± 1.1	2.1 ± 0.09	6307
Hanks' medium + 0.6 mg/ml pentobarbital	15.2 ± 1.1	1.5 ± 0.3	7043

Normal femoral bone marrow cell suspension ($10–15 \times 10^6$ cells/ml) were incubated at 37 °C for a 20-min period. Immediately after incubation, the cell suspensions were irradiated and CFU assay was done.

* each figure is the mean of 4 or 5 experiments.

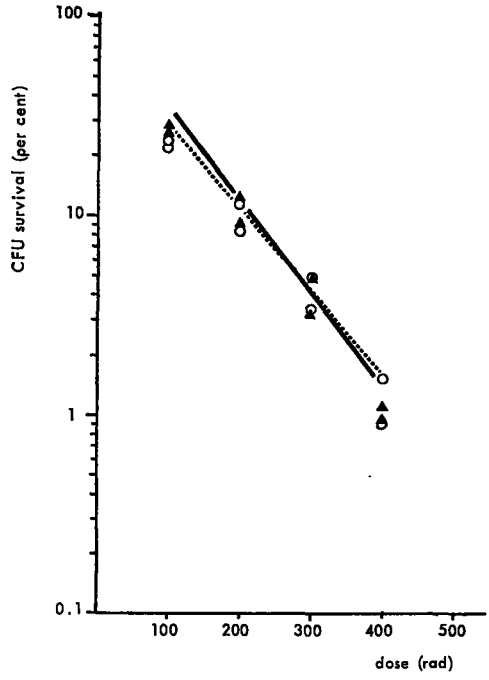


Figure 5.4. Effect of Pentobarbital anaesthesia followed by *in vitro* gamma-irradiation of the femoral bone marrow cells (2 ml, containing $10–15 \times 10^6$ cells/ml). Dose-survival curves for femoral bone marrow stem cells. ○ ○, control mice, $D_0 = 102.2$ rad, $n = 0.65$. ▲▲, Pentobarbital (60 mg/kg i.p.) anaesthetized mice. $D_0 = 92.5$ rad, $n = 0.87$. Each point reflects the mean survival based on the number of colonies in 10 spleens. Pentobarbital was injected 15–30 minutes before radiation exposure. Lines are not statistically different.

rad gamma rays with an extrapolation number $n = 0.65$, whereas the D_0 value of the anaesthetized marrow was 92.5 rad gamma rays ($n = 0.87$). The difference between these curves is not statistically significant (fig. 5.4). This again indicates that pentobarbital did not have a radioprotective effect on the marrow CFU when irradiation was performed *in vitro*.

As mentioned in the introduction to this chapter, the inhibiting effect of pentobarbital on DNA synthesis *in vivo* and *in vitro* has been described in normal and malignant cells (Baserga and Weiss, 1967; Whyatt and Cramer, 1973; Beck et al., 1975). Consequently, the hypothesis was advanced that pentobarbital might exert its radioprotective effect by inhibiting DNA synthesis. This hypothesis is as

Table 5.4. Effect of pentobarbital on the recruitment of bone marrow CFU into DNA synthesis after 175 rad total-body gamma-irradiation.

	Percentage of CFU in S-phase	
controls	24.7±3.2*	
pentobarbital**	11.7±2.1	53 per cent inhibition ($p < 0.05$)

Mice were irradiated, either unanaesthetized or anaesthetized by means of Pentobarbital. Ten minutes after radiation exposure the femoral bone marrow cells were incubated with ^{3}H-TdR (final activity 100 μCi/ml, specific activity 18.5–21 Ci/mmol), at 37 °C for 20 minutes. After incubation the number of surviving spleen colony forming cells was determined. Each donor group consisted of 4–5 mice.

* mean±S.E. of 9 experiments

** 60 mg/kg i.p. (15 minutes before radiation exposure)

follows: During and immediately after radiation exposure of mouse haemopoietic stem cells (CFU) *in vivo*, the fraction of CFU which is synthesizing DNA (S-phase) increases from less than 10 per cent to about 30 per cent (see chapter 1). If pentobarbital, by its inhibitory effect on DNA synthesis, prevents this recruitment and, if these non-cycling cells (G_0-phase cells) are less radiosensitive than S-phase cells, this might explain the decreased radiosensitivity of the pentobarbital treated haemopoietic cell system in the femoral marrow.

Table 5.5. Survival of resting and rapidly proliferating stem cells (after 300 rad total-body gamma-irradiation) in the spleen of normal and Pentobarbital anaesthetized mice.

	CFU survival (per cent)					
	Exp. I	Exp. II	Exp. III	Exp. IV	Mean±S.E.	
Resting						
Saline	0.96	2.3	3.4	3.8	2.6±0.64	n.s.**
Pentobarbital*	0.90	3.2	2.8	3.2	2.5±0.55	
Radpidly proliferating						
Saline	2.7	4.0	5.2	5.1	4.25±0.29	n.s.
Pentobarbital*	2.1	4.3	6.6	8.4	5.35±0.69	

Mice were anaesthetized or injected with physiological saline, 10–15 minutes before radiation exposure. Each donor group consisted of 4–5 mice. Data on normal spleen stem cell survival were taken from Table 4.1.

* 60 mg/kg

** n.s., not significantly different

Table 5.4 shows that, indeed, pentobarbital *in vivo* prevents the CFU from entering S-phase (12 per cent CFU kill after incubation with the S-phase specific ^{3}H-TdR) after a sublethal dose of whole-body irradiation compared with the non-anaesthetized group (25 per cent loss). Since the phenomenon of recruitment of CFU into S-phase after irradiation is assumed not to occur in a cell population which is already rapidly proliferating, we investigated the effect of pentobarbital during irradiation of rapidly proliferating femoral CFU in the spleens of mice 7 days after lethal irradiation followed by bone marrow transplantation. It has been shown that 40–50 per cent of the CFU in these spleens are in S-phase (Lahiri and Van Putten, 1972).

Table 5.5 shows that pentobarbital did not cause a significant in-

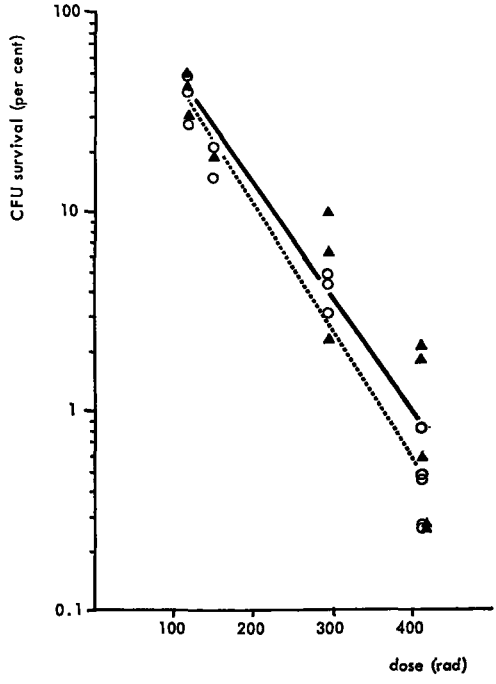


Figure 5.5. Dose-survival curves for rapidly proliferation haemopoietic stem cells in the femoral marrow after *in vivo* whole-body gamma-irradiation. Each donor group consisted of 5–10 mice. ○ ○, control mice $D_0 = 67.5$ rad, $n = 2.13$. ▲▲, Pentobarbital (60 mg/kg i.p.) anaesthetized mice, $D_0 = 75.1$ rad, $n = 1.9$. Each point reflects the mean survival based on the number of colonies in 10 spleens. Lines are not statistically different.

crease in CFU survival after irradiation of the chimaeric spleens. Similarly, pentobarbital did not change the radiosensitivity of normal, resting splenic CFU, in contrast to femoral CFU. This points to some difference between the mouse spleen and femur in their response to pentobarbital.

For this reason, we investigated the effect of pentobarbital during irradiation of rapidly proliferating CFU growing in the femur. Fig. 5.5 gives the non-anaesthetized curve, $D_0 = 67.5$ rad gamma rays with extrapolation number $n = 2.13$, and the curve of anaesthetized mice, $D_0 = 75.1$ with $n = 1.9$. The difference between these curves is not statistically significant. This means that the rapidly proliferating CFU population in the femoral marrow does not respond to pentobarbital during irradiation. Thus, these data support the hypothesis that prevention of recruitment of the CFU may play a role in the pentobarbital effect.

Discussion

Hypoxia

To test the hypoxia hypothesis, use was made of the hypoxic cell radiosensitizer Ro-07-0582. This compound was found to radiosensitize a major percentage of a completely hypoxic femoral bone marrow CFU population. This was demonstrated by a reduction in the D_0 from 281 rad gamma rays to 161 rad, whereas the well-oxygenated population had an essentially unchanged D_0 of about 100 rad gamma rays. The decrease in D_0 from 281 to 161 rad also indicates that the Roche compound does not render the hypoxic CFU as radiosensitive as CFU in a well-oxygenated hemopoietic cell population, i.e. the fraction of hypoxic CFU is larger than estimated by Ro-07-0582. By analogy, the proportion of hypoxic CFU in the femoral marrow during restraint may be larger than estimated by the Roche compound.

Since the radiosensitivity of the femoral CFU during pentobarbital did not change in the presence of Ro-07-0582, this suggests that there is not a significant proportion of hypoxic CFU present in the femoral marrow during pentobarbital anaesthesia. It might be hypothesized that 20–25 minutes of pentobarbital anaesthesia, by its effects on cardiac output and organ perfusion, decreased the Ro-07-0582 levels in the femoral bone marrow. We showed, however, that the Roche compound had exactly the same sensitizing effect on hypoxic femoral CFU

after injection of one quarter of the usual dose* (Keizer, 1975). From the above-mentioned considerations, a valid conclusion seems to be that pentobarbital anaesthesia probably does not exert its radioprotective effect on the femoral bone marrow CFU by causing hypoxia in a proportion of these cells.

Pentobarbital as "chemical radioprotector"

How ionizing radiation produces biological damage is still not completely understood. Direct (by events in biological macromolecules) and indirect (by ionization of water) effects of ionizing radiation are usually considered. During the ionization of water, "free radicals" among many other transformations are produced. Free radicals do not carry an electric charge, but do have excess energy and are therefore highly reactive. In the absence of oxygen, all biologic systems are very much less sensitive to X- and gamma-irradiation. It is thought that the highly reactive products which are formed after reaction of oxygen and free radicals play a major role in the production of biological damage. Theoretically, "chemical radioprotection" can be obtained in the the following three ways: 1. interaction with oxygen; 2. inactivation of free radicals; and 3. interaction with cellular components, so that a given component is less susceptible to the damaging radiation products.

Concerning the interaction with cellular components, it has been suggested that the protector may combine with a receptor group, e.g. the prosthetic group of an enzyme. The inhibition of enzymes leading, to a decreased radiosensitivity of the cell has been described as a "biochemical shock" (Filippovich et al., 1973). The authors were able to correlate the maximal enzyme inhibition with the maximal radioprotective effect. Thus, these data provide some circumstantial evidence to support the hypothesis that pentobarbital may act like a "chemical radioprotector" during radiation exposure. We have already seen that, after injection, pentobarbital is distributed to all tissues (including the haemopoietic tissues) and fluids of the body. Moreover, the drug inhibits a variety of cellular constituents and processes. But our data also show that pentobarbital did not change the

* A comparable plateau of the radiosensitizing effect of Ro-07-0582 between dosages of 0.25 and 1 g/kg body weight, has been reported for normal mouse skin and an WHT intradermal squamous carcinoma 9 (Adams, Denekamp and Fowler, 1976).

radiosensitivity of splenic CFU during whole-body exposure, nor the sensitivity of cells *in vitro*. This suggests that another mechanism may be involved in the radiation effect modifying properties of pentobarbital.

Pentobarbital and the inhibition of DNA synthesis

Exposure of mouse CFU *in vivo* to whole-body irradiation induces an influx of resting CFU (G_1- or G_0-phase cells) to go into cycle. This recruitment of resting cells into S-phase starts very quickly after (or during) irradiation. The data presented in this chapter show that pentobarbital anaesthesia during irradiation decreases the number of these early recruited cells.

This can mean three things:

1. The cells in which pentobarbital depresses DNA synthesis are to be considered as S-phase cells prevented from continuing DNA synthesis;
2. They are cells which could not initiate DNA synthesis, and remain as resting cells (G_0-phase cells);
3. Not only is DNA synthesis inhibited, but the differentiation of stem cells is simultaneously blocked. When stem cells differentiate, they will not give rise to spleen colonies after injection into lethally irradiated animals. Thus, prevention of differentiation means that more cells will give rise to spleen colonies, i.e., a greater stem cell survival will be measured.

As pointed out in chapter 1, CFU become increasingly radioresistant during S-phase. This does not support the hypothesis (see under point 1) that the inhibition of DNA synthesis in S-phase cells leads to a decreased radiosensitivity of the cells.

The radiosensitivity of resting CFU cannot be determined directly, since resting cells as such cannot be synchronized. For this reason, indirect means have to be used to estimate the radiosensitivity of resting cells. In a bone marrow CFU population from germfree mice, the fraction of cells in S-phase is "zero", as determined by the ^{3}H-TdR suicide technique (Croizat, Frindel and Tubiana, 1970). Comparing the radiosensitivity of this noncycling CFU population with a rapidly proliferating CFU population, the authors found that noncycling CFU might be less sensitive to ionizing radiation (Croizat, Mary, Frindel, and Tubiana, to be published). Another attempt to determine the radiosensitivity of resting CFU has been made by Duplan and Feinendegen (1970). They observed that the radiosensitivity of an

in vivo CFU population was significantly decreased after elimination of the CFU in S-phase. The authors interpreted this to mean that haemopoietic CFU in G_1 (G_0) and G_2 phase were definitely more radioresistant than CFU in S-phase. Using liver cells, Coggle (1968) came to a similar conclusion. Thus, the presence of fewer bone marrow CFU in the more radiosensitive cell cycle phase (early S-phase) might explain the decreased radiosensitivity of mouse femoral CFU when pentobarbital is given during exposure to ionizing radiation. One limiting factor is that the recruitment phenomenon must already have occurred during the radiation exposure. Thus, an extremely rapid response of the CFU to ionizing radiation is necessary. The rapidity of radiation induced changes was examined by Catravas and McHale (1974). These authors demonstrated significant changes in the levels of several enzymes in the rat brain as early a 4 minutes after radiation exposure. These data show that the inhibited recruitment hypothesis is not necessarily invalidated because of the extremely short time interval. The fact that normal splenic stem cells showed no change in radiosensitivity when pentobarbital was administered, might be explained by a number of differences in the proliferative and differentiative state between femoral and splenic CFU under steady state conditions and after transplantation or radiation exposure (Guzman and Lajtha, 1969; Lahiri and van Putten, 1970). It can be hypothesized that these differences may lead to differences in the response of these two types of CFU to pentobarbital during ionizing radiation.

Cellular depletion of haemopoietic tissue by radiation exposure induces an influx of noncycling cells into S-phase at the stem cell level. This can be measured by applying the ^{3}H-TdR-suicide technique (see chapter 2) to the CFU in a haemopoietic cell suspension. The effect of radiation depletion on the differentiation of stem cells cannot be quantified by such an easy functional test as the spleen colony assay. Differentiation will always have to be followed by CFU division in order to maintain a constant level of CFU; if this association is indeed coupled with a simultaneous activation mechanism, early cell doubling might indicate the presence of an early loss from differentiation. Thus, the proportion of DNA synthetizing haemopoietic stem cells would give an indirect estimate of the differentiation of these stem cells. Inhibition of this influx of stem cells into S (by pentobarbital) might imply the inhibition of differentiation of the stem cells. Stem cells that differentiate can probably not complete a sufficient

number of doublings to produce a visible spleen colony. Therefore, inhibition of differentiation after radiation exposure would increase CFU survival. This hypothesis may serve as an explanation as to how pentobarbital increases haemopoietic stem cell (CFU) survival during irradiation.

A variant of the "differentiation prevention" hypothesis might be found in the concept that differentiation requires a prior round of cell division (Holtzer, 1963; Ebert and Kaighn, 1966). This phenomenon has been observed in embryonic tissue and in a number of adult tissues. These include, among others, the haemopoietic tissue in man (Lajtha, 1959; Paul et al., 1968; Gross et al., 1970), mouse mammary epithelium (Lockwood et al., 1967; Topper and Vonderhaar, 1974; Vonderhaar and Topper, 1974), and plant cells (Fosket, 1968). Using *in vitro* embryonic kidney mesenchyme, it has been demonstrated that suppression of mitosis by X rays does not interfere with differentiation while inhibition of DNA synthesis with 5-fluorodeoxyuridine inhibited differentiation (Sobel, 1966). A similar phenomenon might be obtained in the haemopoietic stem cell population using pentobarbital or other CNS depressants during radiation exposure.

Most of the evidence presented favours the conclusion that pentobarbital exerts its protective effect by an inhibitory effect on certain cellular events, ultimately involved in cellular DNA synthesis and cellular differentiation. As reported in chapter 3, pentobarbital does not exert protection to spleen colony forming cells during cytostatic treatment. Thus, this finding does not support the hypothesis that all CNS depressants exert their protective effect to haemopoietic stem cells during cytostatic treatment by interference with the progression of the cells through the cell cycle or by prevention of differentiation of stem cells. One possible explanation that can be put forward is the continued presence of the chemotherapeutic drug in intracellular pools, thus providing drugs which can kill cells even after transplantation into the recipient animal. Pentobarbital, because of its high lipid solubility, is presumably washed out of cells during the preparation of the suspension, and thus is no longer available to protect the cells.

The presented data, however, demonstrate that the radioprotective effect of pentobarbital is associated with one particular kinetic state of the haemopoietic stem cells. For the protective effect of pentobarbital is only seen in a normal resting haemopoietic stem cell population and

not in the spleen and not in rapidly proliferating stem cell populations, neither in the femoral marrow nor in the spleen.

In conclusion, the majority of the presented evidence suggests that pentobarbital exerts its protective effect by an inhibitory effect on cellular events ultimately involved in cellular DNA synthesis and cellular differentiation.

CHAPTER 6

GENERAL DISCUSSION

The question as to how a given treatment X gives rise to response Y in an *in vivo* or *in vitro* situation is complicated by the following considerations:

1. The response is usually not restricted to one single tissue or organ.
2. Usually, more than one cellular constituent responds to the treatment, not allowing one to pinpoint the effect to a a single event.
3. Knowledge on the control of many cellular processes underlying any given response is far from complete.

This reasoning is also valid when anaesthetics or tranquillizers are administered to man or an experimental animal. Although the effect is known, the mechanism can not be attributed to a single cellular event; several candidadate explanations are available. The effect of CNS depressants on the central nervous system results in sedation, sleep, or narcosis and all other effects on peripheral tissues are considered as side effects. These side effects are neglected as long as they remain within acceptable limits. Obviously, those drugs with almost no or only moderate side effects are selected. In the work described here two "side effects" of CNS depressants are reported:

1. They decrease the cell loss of murine spleen colony forming cells produced by anti-cancer agents.
2. They decrease the cell loss of mouse murine spleen colony forming cells and less consistently of gastrointestinal cells produced by ionizing radiation.

This raises two questions. 1. What is the relevance of these findings for clinical cancer treatment and 2. What is the mechanism by which these effects are produced.

Relevance of the protection against cytostatic agents for clinical application

One of the major problems in cancer chemotherapy is that anti-cancer agents do not discriminate between normal and malignant cells. Nor is it likely that a breakthrough from this perspective can be

expected in the near future. Therefore, any approach aimed at the improvement of the specificity of anti-cancer agents has to be investigated with scrupulous care. Preferential protection of normal cells during cytostatic treatment is one such an approach. The general anaesthetics halothane and nitrous oxide have been shown to be able to preferentially protect a major proportion of normal mouse haemopoietic stem cells from damage during fractionated chemotherapy. Malignant lymphoma cells were not found to respond in the same way. The studies presented in this thesis have shown that several other compounds of the group of the CNS depressants, such as injectable anaesthetics and tranquillizers, give rise to a similar, although less significant effect on normal haemopoietic stem cells. Obviously, the use of these injectable compounds is easier than the exposure to volatile or gaseous anaesthetics. Moreover, the occasional severe liver toxicity seen after halothane can be avoided.

It may be assumed that difficulties with bone marrow regeneration after fractionated chemotherapy occur only when haemopoietic stem cell survival is reduced below 5 per cent. After fractionated administration of phase-specific drugs, as in our model, at least 30–50 percent of the stem cells will survive treatment; protective measures are thus not essential. In the clinical situation, e.g. when Ara-C is given at 4-hour intervals over a period of 72 hour, much lower survival percentages of stem cells are likely to be observed. The data presented for chlorambucil show that the protection phenomenon also occurs at stem cell survival levels of 1 or 2 percent, levels closer to those critical in the clinical situation.

For halothane it has been shown that only normal haemopoietic cells and, occasionally, intestinal cells respond. Several types of normal and malignant cells did not respond to halothane exposure. In the case of the injectable CNS depressants (using the L 1210 leukemia stem cell assay) preliminary experiments suggest that these malignant cells also do not respond to these compounds. The specificity of the haemopoietic cells in this phenomenon may be explained by the extremely rapid response of this tissue compared to other normal tissues to growth regulating factors. Considered thus far, each aspect supports the idea of a clinical application of protection against anti-cancer compounds by CNS active agents. Another illustration of the importance of the protection phenomenon can be found in the following. To cure a tumour, the cell number has to be reduced by a factor of at least 10^8.

In contrast, a reduction of normal haemopoietic stem cell survival by a factor of 10^4 is considered to be critical. Obviously a modification of cell survival of 40–50 percent is of more importance at the haemopoietic stem cell survival level than at the tumour cell survival level.

Corticosteroids have shown their value in a number of chemotherapy treatment protocols for haematological malignancies. Since CNS depressants in our system result in a percentage protection which is approximately a factor 1.5 higher than that obtained after prednisolone, it might be expected that CNS depressants would produce benefits of approximately the same magnitude as steroids. However, the toxicity of the CNS depressants at the high doses probably required, must not be overlooked.

Relevance of the protection against radiotherapy

The presented data show that immobilization of mice, either by anaesthesia or by physical restraint without anaesthesia (two different types), during whole body irradiation exposure, decreases the radiosensitivity of the haemopoietic cells and the intestinal cells. Thus, the mortality from the bone marrow syndrome is decreased and the duration of life of the mice dying from the intestinal syndrome is increased. No data have been presented on the effect on experimental tumours of immobilization during radiotherapy. A direct protective effect on tumour cells by pentobarbital and other CNS depressants cannot be excluded. Thus, application of CNS depressants to decrease normal cell kill might not produce a therapeutic advantage for irradiation.

A major obstacle in obtaining adequate control of ilio-pelvic tumours by radiotherapy is the severe gastro-intestinal damage occurring during local irradiation. A number of miscellaneous drugs have been tested to decrease this gastrointestinal damage during local radiotherapy, but results thus far have been disappointing. Therefore, an approach in which CNS depressants are utilized deserves further attention.

Mechanism by which CNS depressants protect haemopoietic stem cells during cytotoxic treatment. A unifying hypothesis.

The haemopoietic stem cell population in the mouse is unique in the sense that it responds extremely rapidly after stimulation to such

perturbation as caused by radiotherapy and chemotherapy. Two aspects of this response can be demonstrated using the spleen colony forming cell assay. One is the recruitment of resting (G_0-phase) stem cells into cycle (S-phase) and the other is differentiation of stem cells to cells which do not possess the capacity to form a spleen colony. These two features of the haemopoietic stem cell population form the core of the hypothesis concerning the mechanism whereby CNS depressants protect when applied during cytotoxic treatment. CNS depressants may block the progress of stem cells in the cell cycle in a phase in which cells are insensitive to a particular cytostatic drug. In addition, by inhibiting unknown cellular processes, CNS depressants may prevent differentiation of stem cells. Thus, the number of spleen colony forming cells is increased. However, inhibition of other cellular events which result in a decrease of the toxicity of the cytostatic, must also be considered. It was demonstrated that normal splenic and rapidly proliferating splenic and femoral marrow stem cells did not respond to pentobarbital. Moreover, no response to pentobarbital was observed when haemopoietic cells were irradiated *in vitro*, indicating that the *in vivo* situation is needed for pentobarbital to exert its radioprotective effect. It may be assumed that in a rapidly proliferating stem cell population, recruitment of resting stem cells into cycle and induction of differentiation of stem cells is of minor importance. Moreover, splenic and femoral marrow stem cells have been shown to respond differently to ionizing radiation and transplantation into lethally irradiated recipients. Splenic stem cells were found to have a greater tendency for differentiation and to be more radiosensitive than stem cells in the femoral marrow (Guzman and Lajtha, 1970; Lahiri and Van Putten, 1969). Thus, our observations strongly suggest that interference of pentobarbital with proliferation and differentiation of stem cells can explain its radioprotective effect.

The level at which CNS active drugs interact with processes involved in proliferation and differentiation appears to be the chain of events leading from a membrane receptor-adenylate cyclase system through cyclic AMP, Calcium and cyclic GMP. An alternative locus of interaction is at the level of cytoplasmic structures such as microtubules (MT) and microfilaments (MF) (see also the discussion of chapter 3). Moreover, the first system involving calcium and cyclic nucleotides is associated with the functioning of MT and MF. Anaesthetics, local

anaesthetics and tranquillizers have pronounced effects on both systems.

The protective effect of CNS depressants to haemopoietic stem cells during radiotherapy can be associated with alterations in the cell cycle of the cells. The known effect of CNS depressants on both adenyl cyclase controlled systems and on MT system suggests that a common mechanism can give rise to both the protective effect observed during chemotherapy and during radiotherapy. Recent evidence has shown that the cellular level of cyclic nucleotides may influence the radiosensitivity of these cells and affect the radioprotective effect of cysteamine, a known radioprotective drug. Briefly, it was demonstrated by Langendorff and Langendorff (1971) that an increase in the cellular level of cyclic AMP, obtained after *in vivo* administration of either ATP, 3′-AMP, 5′-AMP and 3′+5′-AMP, significantly improved the 30-day survival of mice after whole-body radiation exposure. They also showed that when the system which controls the cellular levels of cyclic AMP was blocked by a *in vivo* β-adrenergic receptor blocker, the protective effect of a number of radioprotective agents appeared to be abolished. Prasad (1972) showed that prostaglandine E1 and Ro 20-1724, both known to increase cyclic AMP levels, markedly decreased the radiosensitivity of Chinese-hamster ovary cells. Mitznegg (1973) provided strong evidence that the radioprotective effect of cysteamine is enhanced by the cellular levels of cyclic AMP. Finally Pazdernik et al. (1974) showed, using *in vitro* haemopoietic colony forming cells, that the survival of these cells after radiation exposure can be improved by compounds that increase cellular cyclic AMP levels, and also that these compounds markedly enhance the radioprotective effect of cysteamine for these cells. Although data on this subject are far from complete, they provide indirect evidence for the hypothesis that interference with cyclic nucleotide systems can decrease the radiosensitivity of cells. In this respect it may also be noted that pentobarbital anaesthesia in rats has been shown to either decrease or increase the levels of cyclic AMP and cyclic GMP in a number of tissues, such as heart, liver, testis, muscle, and kidney (Kimura et al., 1974). Thus, a unifying hypothesis can be proposed in which the protective effects of CNS depressants for haemopoietic stem cells during cytotoxic treatment are explained by the effect of these drugs on the cyclic nucleotide system and the system of MT and MF, secondarily controlling cellular proliferation and differentiation.

In summary, the possibility exists for the manipulation of normal critical cells during cancer treatment, in order to preferentially render these normal cells less sensitive to cytotoxic treatment. Moreover, it can be speculated that common mechanisms may explain the protective effect for both chemotherapy and radiotherapy.

SUMMARY

The results that can presently be obtained in cancer chemotherapy have an empirical rather than pharmacological basis. Nevertheless, several principles have been formulated to explain the responses seen, among which is the dogma that fast growing cells are more sensitive than resting cells. The majority of currently used anti-cancer agents have been selected according to this principle. Consequently, normal tissues which turn over rapidly, such as the bone marrow, the intestinal epithelium, and the skin, form the main obstacle to adequate drug control of human malignancies, and are defined as the "critical normal tissues". Fortunately, the effect of anti-cancer agents on the haemopoietic stem cells can easily be studied in the experimental mouse model. The haemopoietic stem cell, identified by colony formation in the spleen of the mouse can be used to estimate the regenerative capacity of the haemopoietic tissue after cytotoxic treatment.

In man, one usually tries to decrease the toxicity of chemotherapeutic drugs by dose scheduling and combined modality treatment protocols. Another approach that has been explored only minimally thus far is the inhibition of proliferation preferentially in rapidly dividing normal tissues. In this regard, general anaesthetics were shown to protect a major proportion of spleen colony forming cells during fractionated application of anti-cancer agents. Similarly, anaesthetics have been shown to decrease the radiosensitivity of normal haemopoietic cells and occasionally of intestinal cells. These observations were the stimulus to the research presented in this thesis.

In Chapter 3, a number of injectable anaesthetics and tranquillizers were investigated from the perspective of protection of hematopoietic stem cells from the effects of anti-cancer agents. Injectable drugs were chosen because of their ease of application. Using the phase-specific anti-tumour drugs Ara-C and VLB, it was shown that, at the dosages used, diazepam and pentobarbital did not give rise to a significant protection of stem cells. In contrast, fluanisone (in a mixture with fentanyl), phencyclidine, dehydrobenzperidol and flupenthixol result in 37, 42, 47 and 52 per cent protection, respectively, when results are expressed as the increase in stem cell survival taken as a percentage of the cytostatic treated controls. Comparison of dehydrobenzperidol and flupenthixol with halothane, suggested that the latter compound was the more potent. When amino-chlorambucil, a cycle-specific cytostatic

drug was given, the protection phenomenon was also obtained. In contrast, when nitrogen mustard was used, no protection was obtained when CNS depressants were applied concomitantly. Prednisolone resulted in a 37 per cent protection. Similarly, Triton WR 1339, a non-ionic detergent that is associated with membrane stabilizing properties, also gave rise to a 30 per cent protection. These data suggested that haemopoietic stem cells could be manipulated easily, and in a way such that they are less sensitive to cytostatic treatment. Pentobarbital inhibits DNA synthesis *in vivo*, but does not protect stem cells during cytostatic treatment. The reverse is true for nitrous oxide, indicating that inhibition of DNA synthesis is neither a sufficient, nor a constant finding and can therefore not explain the protective effect of CNS depressants. Anaesthetics and tranquillizers have been reported to exert inhibitory effects on two closely related control systems: the adenyl-cyclase-cyclic-nucleotide-system and on the cytoplasmic microtubule-microfilament system. Consequently, the hypothesis was advanced that CNS depressants may protect spleen colony forming cells during cytostatic treatment by interference with these control systems, It was however not possible to identify the exact mechanism of action.

For the study of the protection of bone marrow and intestinal cells by anaesthesia and physical restraint during whole-body radiation exposure, three factors were investigated: 1. the number of surviving animals at day 5; 2. the number of surviving animals at day 30; and 3. the number of surviving stem cells in the femoral marrow immediately after radiation exposure using the spleen colony technique. It was shown that the number of 30-day-survivors increases significantly when the mice are anaesthetized or restrained during radiation exposure. Moreover, the duration of life of the 5-day-survivors was extended by some of the immobilization techniques. A non-anaesthetic dose of one of the anaesthetic compounds, pentobarbital, did not affect 30- and 5-day survival. Using the spleen colony assay it was shown that the effect on 30-day survival is associated with an increase of haemopoietic stem cell survival in the femoral marrow. A similar radioprotective effect was not found for the splenic stem cell population during pentobarbital anaesthesia. The data indicate that one must be careful in interpreting radiation effects in animals which have been immobilized during radiation exposure (Chapter 4).

In chapter 5 studies are described which characterize in more detail the radioprotective effect of pentobarbital. The effect was not found to be

caused by hypoxia of a fraction of the stem cells through the use of the hypoxic cell radiosensitizer Ro-07-0582. This finding was in contrast to the radioprotective effect of physical restraint, which could be abolished using Ro-07-0582. Further, pentobarbital did not exert its protective effect under *in vitro* conditions. Comparison of haemopoietic stem cells with different kinetic characteristics demonstrated that rapidly proliferating stem cells, either in the spleen or in the femoral marrow, did not respond to pentobarbital during radiation exposure. Thus, the results indicated that the radioprotective effect of pentobarbital was associated with a normal resting femoral stem cell population only. The kinetic state of the resting stem cell population is known to change very rapidly in response to cytotoxic treatment. Based on the inhibitory effects of pentobarbital in particular, and CNS depressants in general, the hypothesis was put forward that pentobarbital exerts its radioprotective effect on cells by interfering with the cell kinetic response of the haemopoietic stem cell population.

A unifying hypothesis was put forward to explain the protective effect of CNS depressants during both chemotherapy and radiotherapy. In it, the inhibitory effects of these compounds were identified to lie in control mechanisms which are ultimately involved in the regulation of the cellular processes of proliferation and differentiation. These control mechanisms were located in the membrane receptor adenyl cyclase-cyclic-nucleotides-chain and in the system of microtubules and microfilaments, which may be linked. The limitations of the hypothesis and experimental data were also discussed.

Finally, it was concluded that further exploration of the protection phenomenon is worthwhile from two perspectives, the first being possible clinical application, and the second that manipulation of the cell cycle by CNS depressants might provide insight in the control of cellular proliferation and differentiation.

SAMENVATTING

Chemische stoffen die gebruikt worden bij de behandeling van tumoren (cytostatica), maken in hun werking geen onderscheid tussen tumorcellen en normale cellen. Tijdens de chemische behandeling van een tumor worden dan ook heel wat normale cellen gedood. Delende cellen zijn gevoeliger voor de meerderheid van de tegenwoordig gebruikte cytostatica dan rustende cellen. Daarom zullen vooral die weefsels met veel delende cellen tijdens therapie beschadigd worden, het beenmerg, het darmslijmvlies en de huid. Deze weefsels worden de „kritieke normale weefsels" genoemd.

Het zou een grote winst zijn als de snel delende normale weefsels tijdelijk stil gelegd konden worden. Deze benadering is nog weinig onderzocht in de chemische behandeling van kanker. De inhaleerbare narcosemiddelen, lachgas en halothaan, zijn in staat een aanzienlijk deel van de beenmerg stamcellen in de muis te beschermen tegen herhaalde injecties van de anti-kanker stoffen. Dit werd niet gevonden voor leukemische stamcellen. Als verklaring werd gesuggereerd, dat de gebruikt narcosemiddelen de beenmerg stamcellen in hun delingscyclus ophielden op een plaats die minder gevoelig is voor de circulerende anti-kanker stof.

In dit proefschrift werd deze waarneming verder onderzocht in muizen, voornamelijk aan de hand van de bloedvormende stamcel, die d.m.v. de miltkolonie techniek kan worden aangetoond. Hierbij werd gebruik gemaakt van inspuitbare narcosemiddelen of tranquillizers om twee redenen:

1. Ze zijn gemakkelijker toe te dienen dan gassen en de klinische toepassing is daardoor aantrekkelijker.
2. Om inzicht te krijgen in het mechanisme van de stamcel bescherming door het uitbreiden van het aantal gebruikte stoffen.

Hoofdstuk 3 beschrijft het effect van anaesthetica en tranquillizers op de beenmerg stamcel na gefractioneerde toediening van Arabinosylcytosine en Vincaleucoblastine. Het bleek dat, met de gebruikte doseringen, diazepam en pentobarbital geen statistisch significante bescherming gaven. Wel echter, fluanisone (in een mengsel met fentanyl), phencyclidine, dehydrobenzperidol en flupenthixol die een bescherming gaven van respectievelijk 37, 42, 47 en 52 procent. Hierbij is de bescherming gedefinieerd als het verschil in stamceloverleving tussen de met cytostatica en anaesthetica behandelde groepen en de alleen

met cytostatica behandelde groep, in procenten van de overleving in de alleen met cytostatica behandelde groep. Vergelijking van dehydrobenzperidol en flupenthixol met halothaan, liet zien dat de laatste het meest effectief was in het beschermen van de stamcellen. Het beschermingseffect werd ook bereikt wanneer de behandeling gebeurde met het cytostaticum Amino-chlorambucil. Echter niet wanneer Stikstofmosterd werd gebruikt. Het gebruikt van prednisolone (synthetisch bijnierschorshormoon) en Triton WR 1339 (membraan stabilizerend detergent) resulteerde in een bescherming van 37 en 30 procent respectievelijk.

Deze gegevens laten zien dat de beenmerg stamcel blijkbaar makkelijk te manipuleren was, zodanig dat hij minder gevoelig werd voor cytostatica. Pentobarbital remde de DNA synthese *in vivo*, maar beschermde de beenmergstamcellen niet tegen cytostatica. Het omgekeerde werd gevonden voor lachgas. Dit suggereert dat remming van DNA synthese alleen niet voldoende was om beenmerg stamcellen te beschermen tegen gefractioneerde toediening van cytostatica. Een andere verklaring werd gezocht in de remmende werking die anaesthetica en tranquillizers hebben op een systeem waarbij adenyl cyclase en cyclische nucleotiden betrokken zijn en op een systeem waarbij cytoplasmatische microtubuli en microfilamenten een rol spelen.
Deze systemen, die volgens recente onderzoekingen blijkbaar nauw met elkaar verbonden zijn, spelen en centrale rol bij de regulering van belangrijke cellulaire processen zoals celdeling en cel-differentiatie.

In hoofdstuk 4 werd onderzocht wat het effect is van narcotiseren of vastbinden van muizen tijdens totale lichaamsbestraling, maatregels die vaak onvermijdelijk zijn in de radiobiologie. Als criteria werden gebruikt de overleving 5 dagen na de bestraling (welke een maat geeft voor de darmschade) en de overleving 30 dagen na bestraling (welke een maat geeft voor de beenmergschade). Het bleek dat, zowel narcose als vastbinden tijdens bestraling, het aantal 30 dagen overlevenden vermeerderde en de levensduur van de 5 dagen overlevenden verlengde. Een niet narcotische dosis van pentobarbital had geen effect op bovengenoemde criteria. Door middel van de miltkolonie techniek werd aangetoond dat narcose en vastbinden tijdens bestraling een grotere stamceloverleving in het dijbeenmerg tot gevolg had. Dit werd niet gevonden in de milt stamcel populatie.

Een gedetailleerder onderzoek naar het effect van pentobarbital tijdens totale lichaamsbestraling werd beschreven in hoofdstuk 5.

Allereerst werd met behulp van een 2-nitroimidazol derivaat, Ro-07-0582, aangetoond dat het stralingsbeschermende effect van pentobarbital niet berustte op een zuurstofgebrek in de cellen. Het stralingsbeschermende effect van het vastbinden van muizen kon d.m.v. Ro-07-0582 ongedaan worden gemaakt, wat suggereerde dat hier wel van een zuurstofgebrek sprake was. Verder bleek dat pentobarbital geen stralingsbeschermend effect had wanneer de beenmerg cellen *in vitro* werden bestraald. Dit was noch het geval wanneer pentobarbital *in vitro* werd toegediend, noch wanneer het reeds *in vivo* toegediend was geworden. Blijkbaar zijn de *in vivo* omstandigheden onmisbaar voor pentobarbital om zijn stralingsbeschermend effect te veroorzaken. Bij verder onderzoek van deze *in vivo* omstandigheden bleek dat de stamcellen in de milt tijdens bestraling niet werden beschermd. In een normale stamcel populatie bevindt ongeveer 80 procent van de stamcellen zich in een rustfase. Er kon worden vastgesteld dat pentobarbital geen stralingsbeschermend effect uitoefende op een stamcel populatie die slechts één of twee procent van deze rustende stamcellen bevatten. Opmerkelijk was dat dit ontbreken van het pentobarbital effect vastgesteld werd zowel in de milt als in het beenmerg.

Het is bekend dat de rustende toestand van een normale beenmerg stamcelpopulatie snel verandert na behandeling met cytostatica of straling. Gefundeerd op de kennis omtrent de cellulaire effecten van pentobarbital in het bijzonder, en remmers van de functie van het centrale zenuwstelsel in het algemeen, werd een theorie opgesteld die zegt dat pentobarbital zijn stralingsbeschermend effect uitoefent door tussenkomst in bepaalde celkinetische veranderingen, die optreden tijdens bestralingen van een normale rustende stamcel populatie. Omtrent het mechanisme van de beschermende invloed op de bloedvormende stamcel van anaesthetica en tranquillizers, zowel tijdens bestraling als behandeling met cytostatica, werd een gezamenlijke hypothese opgesteld. Deze hypothese zegt dat anaesthetica en tranquillizers een remmende werking uitoefenen op cellulaire controle systemen die een rol spelen bij de regulatie van belangrijke processen zoals celgroei en celdifferentiatie. De twee controle systemen die hierbij herkend worden zijn de membraan receptor-adenyl cyclase-cyclische necleotidenketen en het systeem van microtubuli en microfilamenten.

De nauwe verbondenheid tussen beide systemen is bekend. De beperkingen van de hypothese werden eveneens aangegeven. Samenvattend werd geconcludeerd dat verder onderzoek van het geziene be-

schermingseffect de moeite waard is. 1. voor eventuele klinische toepassing en 2. omdat de mogelijkheden tot het manipuleren van normale cellen in hun celcyclus meer inzicht kunnen opleveren in het reguleringsmechanisme van groei en differentiatie van cellen.

LIST OF ABBREVIATIONS

Ara-C	1-β-D-Arabinofuranosylcytosine
ATP	Adenosinetriphosphate
cAMP/Cyclic AMP	Cyclic Adenosinemonophosphate
cGMP/Cyclic GMP	Cyclic Guanosinemonophosphate
CFU	Colony Forming Unit = cell which gives rise to a visible spleen colony after injection into a lethally irradiated mouse
CNS	central nervous system, including the cerebrum, cerebellum and spinal cord
DNA	Deoxyribonucleic acid
D_0/D_{37}	Dosage of a cytotoxic agent which reduces survival to 37 (1/e) per cent at the exponential part of the dose-survival curve
G_0	Term used to describe a rest phase in the mitotic cycle of a cell from where it can be triggered into DNA synthesis
G_1	Phase in the mitotic cycle of a cell, immediately after cell division and before doubling of the genetic material (DNA synthesis)
G_2	Phase in the mitotic cycle of a cell, immediately after doubling of the genetic material and before cell division
^{3}HTdR	Tritium-labelled Thymidine
HU	Hydroxyurea
^{125}IUdR	Radioactive labelled Iododeoxyuridine
i.p.	intraperitoneally
LD_{50}	A dose which is lethal to 50 per cent of the treated specimen
M	Phase in the mitotic cycle of a cell during which a cell divides and produces two equivalent daughter cells
MF	Microfilament
MT	Microtubule
MTX	Methotrexate
OER	Oxygen enhancement ratio. Ratio of radiation dose needed to obtain a certain effect under hypoxic conditions to the radiation dose which

	gives this effect under well-oxygenated conditions
rad	Radiation absorbed dose
S	Phase in the mitotic cycle of a cell during which a cell doubles its genetic material (DNA synthesis)
s.c.	subcutaneously
Tc	Duration of one mitotic cycle
Ts	Duration of the phase of DNA synthesis
VLB	Vincaleucoblastine

ACKNOWLEDGEMENTS

I would like to express my gratitude to Prof. Dr. L. M. van Putten under whose direction the work described in this thesis was done and who introduced me to a number of fields of biomedical research. Thanks are due to Prof. Dr. D. W. van Bekkum for the use of all the excellent facilities of the Institute.

The dedicated and skilled assistance of Mrs. Metha M. Pijpers-de Bruin is greatly acknowledged.

The careful typing of the manuscript was done by Mrs. Mea van der Sman and Miss Hetty Jense. This is deeply appreciated.

The author is also grateful for the support of Peter Lelieveld, Joke de Ruiter, Truus Smink, Jannes Mulder, Cock van der Velde, Wil Akkerman and Steve Cramer.

Thanks are due to Mrs. Leonie van de Burg and Els N. Over for performing the irradiation.

The excellent drawings and photographs were prepared by Mr. Jan Ph. de Kler, Mr. Henk J. van Westbroek and Mr. Erik J. van der Reyden. Valuable help with collecting literature came from the librarian of the Institute, Mr. Paul G. M. van Rossum. Prof. Dr. E. L. Noach and Prof. Dr. P. Thomas and Dr. A. C. Ford read through the complete manuscript. Their valuable comments are greatly acknowledged.

Finally, I would like to express special gratitude for the efforts of Dr. Mark B. Edelstein, who read several times through the successive versions of the manuscript.

The work described in this thesis was done at the Radiobiological Institute TNO, in a joint appointment with the Interuniversity J. A. Cohen Institute for Radiopathology and Radiation Protection.

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