

**THE ANALYSIS OF RADIATION-INDUCED MICRONUCLEI IN
PERIPHERAL BLOOD LYMPHOCYTES FOR PURPOSE OF
BIOLOGICAL DOSIMETRY**

JACQUES LE ROUX

Thesis presented for the Degree of
Master of Science
Faculty of Medicine
University of Cape Town

December 1994

The University of Cape Town has been given
the right to reproduce this thesis in whole
or in part. Copyright reserved for the author.

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

ACKNOWLEDGEMENTS

I sincerely wish to express my thanks and appreciation to the following persons, who have contributed in many different ways to the realization of this dissertation.

Prof G H Blekkenhorst, (Promoter) for his interest, valuable suggestions and for many stimulating discussions in the course of this study.

Prof B J Smit, the staff and donor patients at the Department of Radiotherapy, Tygerberg Hospital. Without their co-operation, this study could not have been carried out.

Dr D Reitmann for making available the radiation- and laboratory facilities at the National Accelerator Centre, and staff members Dr J P Slabbert and Ms H L Jones for their competent technical assistance.

Drs A A Edwards and D Murray who devised the method of deriving the localized dose and irradiated fraction.

Ms B Beck for her everlasting patience in typing and retyping the manuscript.

My parents, for their support and inspiration over the years.

My wife, Karen, for her encouragement and moral support during the difficult and frustrating periods.

The Department of Health is gratefully acknowledged for the time granted and financial support for this project.

Cape Town
South Africa

J le Roux
December 1994

ABSTRACT

In the investigation of radiation accidents it is of great importance to estimate the dose absorbed by exposed persons in order to plan their therapy. Although occasionally in these situations physical dose measurements are possible, most often biological methods are required for dose estimation.

The aim of this investigation was to assess the suitability of the cytokinesis blocked (CB) micronucleus assay as a biodosimetric method using lymphocytes irradiated *in vivo*. The approach adopted to achieve this was to estimate whole body doses by relating micronuclei yields in patients undergoing radiotherapy treatment with an *in vitro* radiation dose-response curve. These biologically derived estimates were then compared with the corresponding doses obtained by physical measurement and calculation.

As a first approach a study was performed of the *in vitro* dose-response of gamma-ray induced micronuclei following cytokinesis-block in the lymphocytes of peripheral blood samples obtained from 4 healthy donors. The results indicated that the distribution of the induced micronuclei were overdispersed. Furthermore a linear dose-response relationship was established when a curve was fitted to the data by an iteratively reweighted least squares method. By means of an analysis of covariance it was demonstrated that this result is in agreement with the dose-response relationships found by various other workers (Fenech *et al.*, 1985; Fenech *et al.*, 1986; Fenech *et al.*, 1989; Balasem *et al.*, 1992, and Slabbert, 1993).

To assess the suitability and accuracy of dose assessment using the CB micronucleus assay for *in vivo* exposure of lymphocytes, blood samples obtained from 8 patients undergoing radiotherapy before, during and after treatment were examined. The physical doses of these patients were determined according to conventional radiation treatment plans and cumulative dose-volume histograms. The dose-volume histograms permitted calculation of integral doses and

subsequently the estimate of equivalent whole-body doses. The results of the CB micronucleus assay applied to peripheral blood lymphocytes of 6 patients undergoing fractionated partial-body irradiation showed a dose-related increase in micronucleus frequency in each of the patients studied. This demonstrated that micronuclei analysis may serve as a quantitative biological measure of such exposures. The pooled data of these patients compared to the pooled data of the healthy donors show that there was no statistically significant difference between *in vitro* and *in vivo* results, however a slightly lower induced micronuclei frequency was observed after *in vivo* exposure.

When the biological dose estimates for equivalent whole body doses obtained from the *in vitro* dose response curve were compared with calculated physical doses, it was found that : biologically estimated dose = 0.936 physical dose. However there was inadequate statistical evidence to discard the hypothesis that the gradient of the equation was equal to one. Therefore the analysis of micronuclei induced in lymphocytes *in vivo* yields highly quantitative information on the equivalent whole-body dose.

The negative binomial method was used for analysing the micronucleus data from two patients who received single, relatively larger tumour doses of 10 Gy each, with the objective to obtain estimates of the exposed body fraction and the dose to this fraction. The dose estimates to the irradiated volume were found to be within 30% of the physical tumour dose. The irradiated volume estimates seemed to be higher than the physically calculated volumes but by discarding the correction for the loss of cells due to interphase death the agreement was good between the physically and biologically determined integral doses.

This study has revealed that the CB micronucleus assay appears to offer a reliable, consistent and relatively rapid biological method of whole body dose estimation. It is recognised that further corroborative work using the techniques described in this thesis is required for estimating localized exposure.

CONTENTS

Acknowledgements	i
Abstract	ii
Contents	iv

CHAPTER I

A PERSPECTIVE ON THE BASIC PHYSICS AND BIOLOGICAL CONSEQUENCES OF RADIATION ABSORPTION

1.	Introduction	1
2.	Radiation and matter	1
2.1	Radiation theory - the paradox	1
2.2	The electromagnetic spectrum	2
2.3	Ionising radiation	2
2.4	Types of radiation and radiation sources	2
2.5	Natural radiation sources	3
2.6	Radiation dosimetry	4
2.7	Interaction between electromagnetic radiation and biological matter	6
3.	Basic concepts regarding the biological effects of radiation	6
3.1	Molecular effects of radiation	6
3.2	Cellular effects of radiation	7
3.3	Factors modifying effects of radiation on cells	8
3.4	Quantifying biological damage	9
3.5	Deterministic and stochastic effects of radiation	10
3.6.	Conceptual framework	11
3.7	Radiation risk	11

CHAPTER II

THE PHYSIOLOGY AND RADIOPATHOLOGY OF THE HAEMOPOIETIC SYSTEM

1.	The physiology of the haemopoietic system	13
1.1	The bone marrow as haemopoietic organ	13
1.2	Haemopoiesis	14
1.2.1	Erythropoiesis	15
1.2.2	Leucopoiesis	16
1.2.3	Thrombopoiesis	17
2.	Radiopathology of the haemopoietic system	18
2.1	The radiosensitivity of the haemopoietic system	18
2.1.1	Single high dose whole body exposure	19
2.1.2	Chronic or repeated low dose whole body exposure	22

2.2	Difference in radiosensitivity of the various blood cell lines	24
2.3	Problems regarding partial-body exposure	27

CHAPTER III

BIOLOGICAL INDICATORS TO DETECT AND QUANTIFY RADIATION EXPOSURE

1.	Introduction	29
2.	Biophysical indicators	30
2.1	Electron spin resonance (ESR)	30
2.2	Neutron activation analysis	31
3.	Biochemical indicators	31
3.1	Enzymes	32
3.2	Amino acids and other metabolites or catabolites	32
3.3	Changes in cellular metabolism and membranes	33
3.4	Reduction in the diameter of human hairs	34
4.	Immunologic indicators	34
4.1	Absolute and relative numbers of lymphocyte subpopulations	35
4.2	Production of specific antibodies in vitro	35
4.3	Lymphocyte stimulation test (LST)	35
4.4	Somatic mutation assays	36
5.	Cytological indicators	37
5.1	Spermatogenic indicators	37
5.2	Haematological indicators	37
5.2.1	Erythema	38
5.2.2	Telethermography	39
5.2.3	Vascular scintigraphy	39
5.2.4	Peripheral blood elements	39
5.2.5	Stem cell and progenitor cell compartments	40
5.2.6	Bone marrow cellularity	41
5.2.7	Bone marrow mitotic index	41
6.	Cytogenic indicators	42
6.1	Chromosome aberration analysis	42
6.2	The nucleoid sedimentation technique	43
6.3	Enumeration of micronucleated erythrocytes in peripheral blood	44
6.4	Assessment of micronuclei	44

CHAPTER IV

RADIATION INDUCED MICRONUCLEI IN BLOOD LYMPHOCYTES : *IN VITRO* TECHNIQUE

1.	Introduction	46
2.	Objective	46
3.	Experimental protocol	47
3.1	Donors of lymphocytes	47

3.2	Radiation source	47
3.3	Materials and methods	48
3.3.1	Lymphocyte separation	48
3.3.2	Cell culture	49
3.3.3	Cell harvest	49
3.3.4	Nuclear staining	50
3.3.5	Scoring of micronuclei	50
4.	Identification of micronuclei in human lymphocytes	50
4.1	Criteria for the identification of micronuclei	50
4.2	Observation of micronuclei	51
5.	Results	52
6.	Discussion	57
7.	Comparison of dose-response relationships	59

CHAPTER V

PHYSICAL ESTIMATE OF THE EQUIVALENT WHOLE-BODY DOSE OF DONOR PATIENTS FOLLOWING FRACTIONATED PARTIAL-BODY RADIOTHERAPY

1.	Introduction	63
2.	Objective	64
3.	Selection of patients	64
4.	Details regarding individual patients	66
4.1	Patient : a	66
4.2	Patient : b	66
4.3	Patient : c	66
4.4	Patient : d	66
4.5	Patient : e and f	67
5.	Dose-volume histograms (DVH)	67
5.1	Method for generating and plotting DVHs	71
5.2	Three-dimensional representation of the patient	71
5.3	Computational accuracy	71
5.4	Application of DVHs	72
6.	Physical dosimetry results	72
7.	Validity of the calculated equivalent whole-body doses	75

CHAPTER VI

BIOLOGICAL DOSIMETRY : USING THE CYTOKINESIS BLOCKED MICRONUCLEUS ASSAY FOR QUANTIFYING THE DOSE TO RADIOTHERAPY PATIENTS WHO RECEIVED FRACTIONATED PARTIAL-BODY IRRADIATION

1.	Introduction	78
2.	Objectives	79

3.	Experimental protocol	80
3.1	Laboratory procedure	80
3.2	Methods of calculation of the dose to the irradiated body fraction and the volume of this fraction	81
4.	Results	84
4.1	Half-body irradiations	84
4.2	Fractionated partial-body exposure	84
4.2.1	Dose-response relationship of micronuclei in CB lymphocytes from patients receiving fractionated radiotherapy	86
4.2.2	Biological dose estimates	87
4.2.3	Calculations of localized dose and irradiated body fraction	88
5.	Discussion	90
5.1	Factors responsible for modification of the experimental protocol	90
5.2	MN in unexposed human lymphocytes	90
5.3	MN yield as qualitative biomarker of radiation exposure	91
5.4	MN in human lymphocytes irradiated <i>in vitro</i> and <i>in vivo</i>	92
5.5	Application of the <i>in vitro</i> results for <i>in vivo</i> dosimetry	95
5.6	Estimates reflecting non-uniformity of exposure	98
5.7	Applying the negative binomial method to MN data obtained from fractionated partial-body exposure	101
6.	Conclusions with regard to future studies	102
6.1	Overall reliability of the equivalent whole-body dose concept	102
6.2	Extending localized dose estimates to fractionated partial-body exposure	103
6.3	Future prospects of the MN assay	104
	References	105

CHAPTER I

A PERSPECTIVE ON THE BASIC PHYSICS AND BIOLOGICAL CONSEQUENCES OF RADIATION ABSORPTION

1. Introduction

In the event of radiation accidents involving large population groups there are normally no physical procedures for personal dose measurement, thus other procedures to assess body doses are required. During the past two decades it has become increasingly possible to correlate biological effects quantitatively to the absorbed dose. Various types of biological indicator systems, of which the micronucleus assay is one, can be used in an attempt to estimate absorbed doses to irradiated persons (Biological Indicators Working Group, 1991).

The aim of this study is to evaluate the use of micronuclei in the peripheral blood lymphocytes of exposed persons as a tool for biological dosimetry. For such an evaluation it is necessary to examine the basic principles regarding radiation, radiation dosimetry and radiation biology as well as the physiology and radiopathology of the haemopoietic system.

2. Radiation and matter

2.1 Radiation theory - the duality

Radiation is a form of energy in motion, which has a duality in its basic physical properties. It has both quantum and wave properties. It can be thought of as discrete packets of waves called quanta or photons. Individually the quanta act like particles ; in large numbers they act like waves. When radiation travels from one place to another without losing energy the wave aspect (e.g. reflection, interference and diffraction) appears. But when radiation interacts with matter and gives up energy, then the particle aspects are of major importance.

However, x-rays and γ -rays do differ in their origin, in that x-rays are produced extranuclearly and γ -rays intranuclearly. These radiations are emitted spontaneously by radioactive nuclides, and can also be produced by radiation equipment like x-ray units.

Particulate radiation includes protons, electrons, neutrons, alpha particles, heavy charged ions and π -mesons. Particulate radiation such as beta particles (electrons), alpha particles and neutrons may be spontaneously emitted by radionuclides, or produced in devices such as linear accelerators or cyclotrons.

2.5 Natural radiation sources

The sun emits the entire spectrum of radiation from radio waves to cosmic rays, the latter consisting of both subatomic particles and electromagnetic radiation. Other major contributors to background radiation are the nuclides uranium-235 and uranium-238, thorium-232 and potassium-40, and their associated decay products, present in the earth's crust.

Radiation from these natural sources contributes about 87% of the average individual's total exposure (Fig. 1.1). About 50% is due to the inhalation of radon daughter products. Radon is a natural radioactive gas which emanates from any material containing uranium, thorium and their radium decay products. The next most important source of radiation to the average individual (about 12%) derives from radiography for medical diagnosis (x-rays). Other man-made sources of radiation such as nuclear tests and nuclear power, contribute only about 1% of the average individual's total radiation exposure. (Fig. 1.1).

2.2 The electromagnetic spectrum

Radio waves, radiant heat, light waves, ultraviolet rays, x-rays and gamma rays are all examples of electromagnetic radiation. The electromagnetic spectrum includes all this radiation from the very long radio waves with energies in the order of 10^{-11} eV to the exceedingly short, penetrating gamma rays with energies of the order of 10^{10} eV at the other end of the spectrum. It should be emphasized that the regions of the spectrum overlap, so there is no sudden change in properties as we move from the region of radio waves into the infrared region or from the ultraviolet into the x-ray region. (le Roux *et al.*, 1992).

2.3 Ionising radiation

When radiation interacts with matter, including living tissue, its energy is absorbed by and dissipated in the atoms and molecules. Radiation in the high-frequency range (10^{15} Hz) with energy in excess of 10 eV transfers sufficient energy to eject an electron from the outer shells of atoms with which it interacts, resulting in a positively charged ion and a negatively charged electron. This process, known as ionisation, can ultimately result in biological effects and can best be quantified by radiation measuring devices.

Radiation with quantum energy less than 10eV, such as ultraviolet-, visible-, and infra-red, light waves, microwaves and radio waves do not dissipate enough energy to cause ionisation.

Although the term *radiation* is in fact very broad, it is most often (and particularly in this text) used to mean *ionising radiation*.

2.4 Types of radiation and radiation sources

Ionising radiation can be classified as electromagnetic or particulate radiation. Electromagnetic radiations, as exemplified by x- and γ -rays are identical in nature and properties.

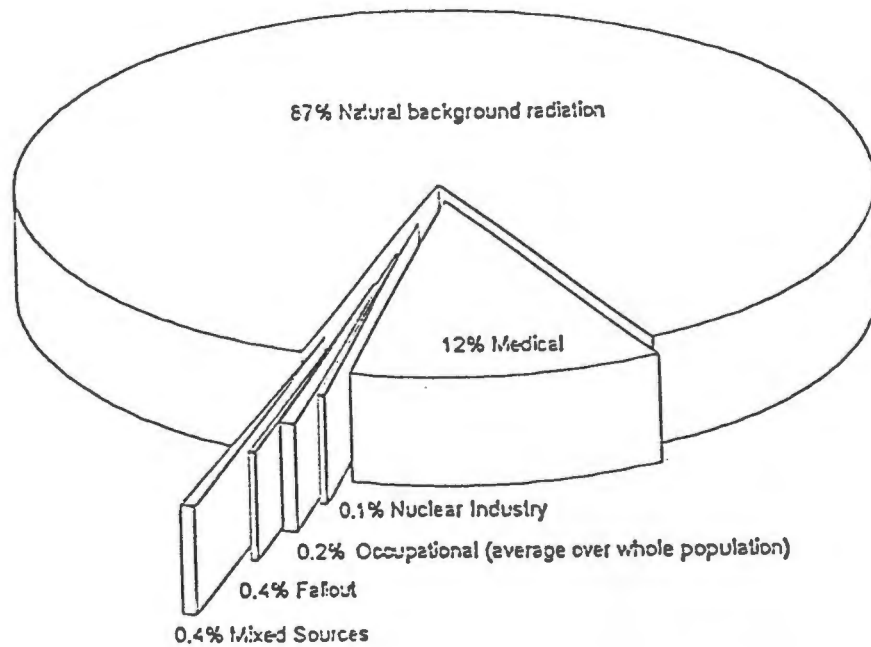


Fig 1.1 Contribution of various sources to the average individuals total exposure. (NRPB, 1989)

2.6 Radiation dosimetry

Radiation dosimetry is concerned with the determination of the energy absorbed in a medium exposed to radiation. From 1895 to 1937 a wide variety of radiation units were proposed and almost every known physical action of x-rays and the radiations from radium were made the basis for a suggested unit.

The units were often associated with the names of their proposers. The names of Curie and Röntgen were given to units during and after this period, but with the adoption of Systeme International (NBS, 1977) these famous names has been abandoned. However SI does resurrect the names of Becquerel, Gray and Sievert to be used in association with units.

Other units long redundant were the *uranie* (1905), the *milligram-hour* (1921), the *millicurie destroyed* (1914) and *skin erythema dose* (Mould, 1980). The latter were used independently for both x-rays and radium. Other biological units such as the *epilation dose* (Hampson, 1911) and *rad unit*, (Russ, 1918) based on material such as rabbit muscle, (Cutler, 1926) ascari eggs, *drosophila* eggs and tadpoles were used for radiation experiments. However, biological-based dosimeters and units were considered impractical since they required too many tests for each measurement.

The *x-unit*, (Belot, 1906) based on the ionisation effect, was used for measurements of relative intensities soon after Röntgen's discovery in 1895, and Villard (1908) is generally credited as the first to quantify this unit. Several units e.g. the *e-unit* and the *R-unit* (Solomon, 1925) were based on this effect before the *röntgen* was finally adopted in 1928, as unit of *exposure*. During the years ionisation chambers of different types shapes and sizes have been designed to measure exposure originally in röntgens and more recently in Coulombs per kilogram.

Several of the physical properties of radiation which have been used for dose measurement almost 100 years ago are applied in modern dosimetry, e.g. scintillation detectors, photographic film, chemical and calorimetric indicators, and calorimetric methods (Knoll, 1979).

It is interesting to note that the idea of a quantity to specify the amount of energy deposited by the radiation per unit of mass (or of volume) was proposed by Christen in 1912, but that it was not until 1953 that the quantity absorbed dose, and its relevant unit the rad [which in SI units became the gray (Gy)] was adopted. The absorbed dose can be derived from a measurement of exposure in air, however, more recently ionisation chambers are calibrated directly in gray (Gy).

2.7 Interaction between electromagnetic radiation and biological matter

When a beam of photons passes into a biological medium the energy of the beam is finally converted into biological damage and heat. The sequence of events has been reviewed by a number of authors (Johns and Cunningham, 1977 and ICRP, 1990). In brief, the initial step in the process involves the collision between a photon and an orbital electron, resulting in the scattering of some radiation and the setting in motion of a high speed electron. The high speed electron produces a track along which ionisations occur, excitation of atoms takes place, and molecular bonds are broken. The amount of energy which is transferred in such a way is relatively small, about 100 eV per interaction for x-rays.

Most of the electron energy, however, is converted into heat, producing no biological effect. Some of the high speed electrons may suffer a radiation collision and produce bremsstrahlung. This bremsstrahlung, as well as the scattered radiation, can then undergo an interaction in the same way as the original photon. Usually, some 30 interactions are required before all the energy of the photon is converted into electronic motion. (ICRP, 1990).

3. Basic concepts regarding the biological effects of radiation

3.1 Molecular effects of radiation

Energy deposited in biological matter by radiation will primarily be the cause of damage to important cell structures. This can happen by direct ionisation of the macromolecules and subsequent formation of free radicals. Since tissue cells are however more than 70% water most of the energy is absorbed by water. The net result of this is the production of diffusible radical products of the radiolysis of water molecules. These radicals are chemically very active. They can move rapidly in the medium from the site of the original event and indirectly cause further chemical changes in the molecules of the

medium before they are inactivated (in times of the order of 10^{-6} sec. or less). (ICRP, 1990).

Molecular changes reflecting breakage of chemical bonds can manifest themselves over various periods of time and in a variety of ways depending on the nature of the medium.

3.2 Cellular effects of radiation

The eventual morphologic changes induced within cells by irradiation will depend on the cell type and radiation dose and may include cellular swelling, cytoplasmic vacuolization, mitochondrial enlargement, and distortion, disruption, swelling, and fragmentation of the endoplasmic reticulum. (Robbins and Kumar, 1987).

Cell damage from radiation results primarily from the direct- and indirect molecular effects which may cause single-strand and double-strand breaks in the DNA structure. Such induced damage may be removed and the original DNA structure is restored. Alternatively repair processes may yield errors such that base sequence changes (point mutations) or more gross changes such as gene deletions or rearrangements (chromosome aberrations) occur (ICRP, 1990). These misrepair events may have long term consequences for the cell and can result in cell reproductive death or stable genetic changes in surviving cells.

If enough cells are killed in an organ or tissue the function of the organ or tissue may become impaired. In extreme cases the organism itself may die. These effects constitute what are defined as *deterministic effects*, and with whole-body exposure are classically divided into three general patterns : (1) hematopoietic, (2) gastrointestinal, and (3) cerebral.

Stable changes in the genome of a surviving irradiated cell can have one of two effects. If the changes take place in the germinal cells of the reproductive tissues, they may result in transmissible lesions,

which are passed on to and may be manifest as hereditary disorders in succeeding generations.

A second effect is the modification of a normal cell by neoplastic transformation. Cancer, which is currently accepted as the most dangerous result of exposure to relative low doses of ionising radiation, may result. These effects of radiation (where the cell structures are changed, but the cells survive) are known as *stochastic effects*.

3.3 Factors modifying effects of radiation on cells

Any type of cell in the body can be damaged or inactivated by radiation, depending on the following factors :

Radiation quantity (Dose) : Damage increases with an increase in the radiation dose. The dose-response relationship is, however different for *deterministic* and *stochastic effects* respectively (see par. 2.5)

Radiation energy : Although the effects of all types of radiation are qualitatively the same, they differ in their penetrability. For example, the penetrability of electromagnetic radiation is far greater than that of particulate radiation and directly proportional to the photon energy.

Dose rate : The rate of delivery of radiant energy significantly modifies its effect, especially when it is delivered in divided doses or fractions, as is the practice in radiotherapy. With low dose rates and with dose fractionation, cells are able to repair more of the sustained damage, than at high dose rates.

Linear energy transfer (LET) : For equal radiation dosage, radiation delivering more energy per unit path length (high LET radiation) is more likely to kill cells than low LET radiation, dissipating less energy per unit path length.

Oxygen effect : Another variable that modifies the effect of radiation is the oxygenation of cells and tissues, Molecular oxygen has two

unpaired electrons which may interact with radiation-induced free radicals to amplify their damaging effect.

3.4 Quantifying biological damage

The average energy transfer per unit mass of the irradiated medium is known as the average absorbed dose (D_T) in the medium T. This dose, measured in Gray, is a measure of the biological damage caused by a specific exposure. However, the biological damage also depends on the linear energy transfer of the specific type of radiation.

Consequently, biological damage may be more accurately quantified by the so-called equivalent dose, H_T where

$$H_T = w_R D_T \quad (\text{Equ 1.1})$$

The weighting factor for this purpose is now called the radiation weighting factor, w_R , and is selected for the type and energy of the radiation incident on the body. The equivalent dose is measured in sievert (Sv) and is therefore the absorbed dose averaged over a tissue or organ and weighted for the radiation quality that is of interest.

The total detrimental effect due to exposure of an individual is the sum of the effects attributable to the exposure of the individual tissues. The radiosensitivity of different tissues differs. To allow for this the ICRP has defined a further quantity by which the equivalent dose in tissue or organ T is weighted, namely the tissue weighting factor w_T (ICRP, 1990). The tissue weighting factor is then used to calculate the effective dose E. The effective dose is the sum of the doubly weighted absorbed dose in all the tissues and organs of the body and is given by the expression :

$$E = \sum_T w_T H_T \quad (\text{Equ 1.2})$$

It is apparent from this that radiation weighting factors are tissue independent and tissue weighting factors are radiation independent.

The effective dose is also given in Sv. The 1990 ICRP recommendations propose the use of the Effective Dose as an adequate surrogate for radiation detriment to an individual and Collective Effective Dose for a population.

3.5 Deterministic and stochastic effects of radiation

Deterministic effects in humans can result from whole body or localised irradiation causing an amount of cell killing that cannot be compensated for by proliferation of viable cells. However, as most organs and tissues are unaffected by the loss of even a substantial number of cells, this mechanism will cause observable (deterministic) effects only when such organs are irradiated above a specific threshold dosage. Above the threshold the severity of the effect increases with dosage, as illustrated in Fig. 1.2.

Killing is not the only process involved in deterministic effects. Functional disorders can also result from direct alteration of other cellular processes such as membrane permeability. Despite the fact that in slowly dividing cell populations (e.g. nerve cells) death may not occur for months or years, deterministic effects are mainly short term effects which become manifest in rapidly dividing cell populations (e.g. stem cells) a few hours or days after exposure. (Scherer, Streffer and Trott, 1991).

The second process, taking place in much longer overall times, is the modification of a normal cell, presumably the result of molecular DNA changes by neoplastic transformation. One characteristic result of this change is the potential capability of the transformed cells for unlimited cellular proliferation eventually giving rise to an occult tumour or hereditary disorders in descendants. These stochastic (statistical) effects may be induced even at low irradiation with a proportionality equal to the dose. Although no stochastic effects have been noted at very low doses, a linear relationship between dose and effect, as indicated in Fig. 1.2, is assumed (ICRP, 1990). The interval

between exposure and the detection of a radiation induced cancer, are usually many years, and is referred to as the latency period. This period varies with the type of cancer and the age at exposure. e.g. 4 years or less for leukaemia and up to 20 years or longer for solid tumours. (Scherer, Streffer and Trott, 1991).

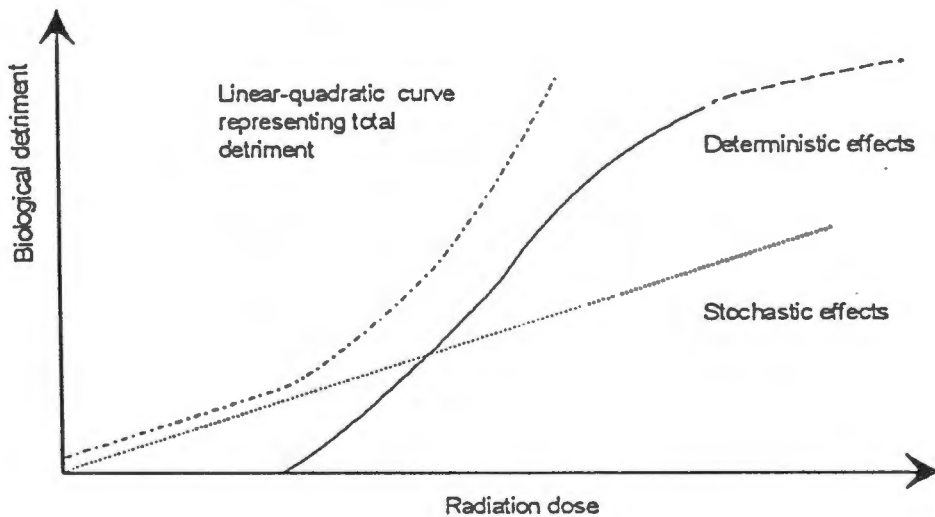


Fig 1.2 Dose response relationship

3.6 Conceptual framework

The basic physical-, chemical- and biological effects of radiation are not exact phenomena, and clinical effects are predicted with some degree of error. Because of inter-individual variation in radiation response, it is only possible to talk about a *mean effect* of a given dose of radiation to which a specific tissue/organ has been exposed (UNSCEAR, 1988).

3.7 Radiation risk

Radiation effects are quantified in terms of a risk.

Probabilistic risk analyses are undertaken to estimate the probabilities of e.g. death, defined as the product of the probability of

receiving a dose as a result of a precisely defined sequence and the conditional probability of death directly as a result of that dose, were it to be received.

It is necessary to project the estimate of probability of e.g. cancer induction for the period of observation to the lifetime of the exposed population, in order to obtain the full lifetime risk. Among many possible choices two principal models have been used for that purpose, one the absolute (risk) or additive projection (ICRP, 1977) and the other the relative (risk) or multiplicative projection model (ICRP,1990). The former predicts, in its simplest form, a constant excess of induced cancer throughout life unrelated to the spontaneous rate of cancer while the latter predicts that the excess of induced cancers will increase with time as a constant multiple of the spontaneous or natural rate of cancer and consequently will increase with age in that population.

The main reason why the new risk estimates are higher than those from 1977 is the use of the multiplicative rather than the additive projection model. For example the 1977 estimate for fatal cancer induction due to whole body irradiation was $1.25\%Sv^{-1}$ for a population including all age groups, as against $5\%Sv^{-1}$ estimated in 1990.

CHAPTER II

THE PHYSIOLOGY AND RADIOPATHOLOGY OF THE HAEMOPOIETIC SYSTEM

1. The physiology of the haemopoietic system

1.1 The bone marrow as haemopoietic organ.

For an understanding of the haemopoietic system and in particular the radiopathology of the system it is necessary to consider briefly the anatomical structure and functional organization of the bone marrow.

The production of the different types of blood cells including erythrocytes, leucocytes and thrombocytes (platelets) is called haemopoiesis and is normally confined, during extra-uterine life, to the bone marrow.

Bones are composed of cortex and medulla. The bone marrow is part of the medulla which is a honeycomb of cancellous bone. As the child ages haemopoietic marrow contracts centripetally, to be replaced by fatty marrow. By early adult life haemopoietic marrow is largely confined to the skull, vertebrae, ribs, clavicles, sternum, pelvis and the proximal half of the humeri and femora.

Bone-marrow can be divided into a vascular and haemopoietic compartment.

The vascular compartment consist of the central nutrient artery which enters long bones at mid shaft and bifurcates to two longitudinal central arteries. The branches of the central artery give rise to arterioles and capillaries which eventually re-enter the marrow and open into a network of thin-walled sinusoids. The sinusoids drain into the central venous sinusoid which accompanies the nutrient artery. The sinusoids are large thin-walled vessels. The walls of sinusoids consist of endothelial cells, an incomplete basement membrane and

an adventitia. Mature haemopoietic cells enter the circulation by passing transcellularly, through sinusoidal endothelial cells.

The haemopoietic compartment of bone-marrow include the haemopoietic cells which lie in cords or wedges between the sinusoids. The cells are embedded in a connective tissue stroma which occupies the intertrabecular spaces of the medulla. Stroma cells include cells which have been designated reticulum or reticular cells and have a support function. The phagocytic ability of these cells is very limited.

1.2 Haemopoiesis

There are different views regarding the development of blood cells from the so-called stem cells in the bone-marrow. According to the polyphyletic theory there is a separate stem cell for each type of circulated blood cell. According to the monophyletic theory, however, there is a common stem cell for all the different types of circulated blood cells - the presently accepted theory (Meyer, 1983).

Functionally, two main stages (phases) can be identified during the development of all blood cell types, namely an undifferentiated and a differentiated stage. Undifferentiated cells or stem cells can proliferate and deliver a large amount of descendants. Differentiated cells on the contrary are specialised to perform specific functions, for example, to transport oxygen (red cells), to phagocytose organic material (neutrophils and monocytes) or to produce anti-bodies (lymphocytes).

By means of modern techniques it has been demonstrated that the so-called three-phase model for haemopoiesis and stem cell differentiation (Fig. 2.1) is realistic (Meyer, 1983).

The upper section of the diagram represents the pluripotent stem cells or resting (S) cells. The middle section represents the precursor cells. The lower section represents the maturing cells which will move into the blood outside the haemopoietic organ.

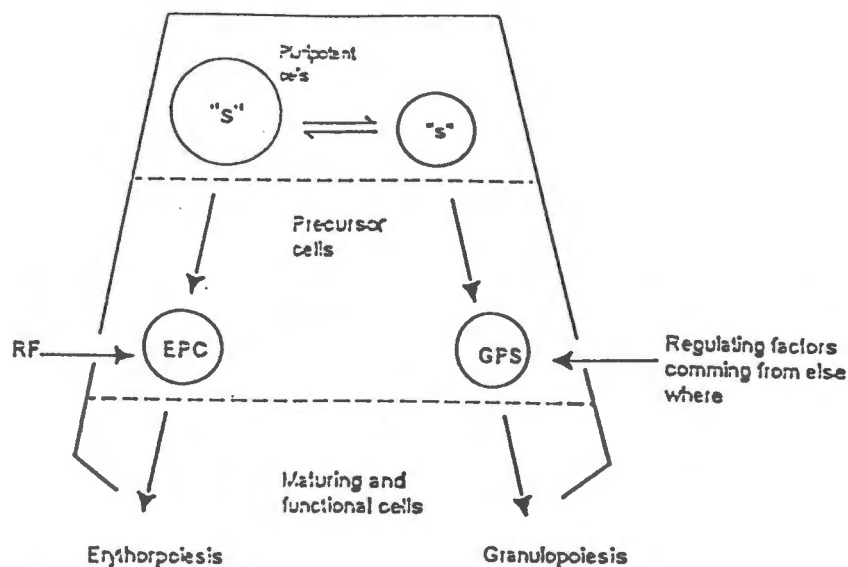


Fig. 2.1 The three-phase model of haemopoiesis

1.2.1 Erythropoiesis

The earliest red cell precursor in the bone marrow is the *proerythroblast*, a large cell with a large nucleus and a thin rim of cytoplasm. As the cell divides haemoglobin synthesis starts in the cytoplasm, and with successive cell divisions the amount of haemoglobin increases.

After successive cell divisions cells are called normoblasts and are classified as early, intermediate, or late, according to (1) the amount of haemoglobin present, and (2) the degree of contraction of the originally large nucleus. When the cell contains its full complement of haemoglobin the small dense nucleus remaining is extruded, leaving a *reticulocyte*. After 24-48 hours in the peripheral blood the reticulocyte becomes an erythrocyte.

The diameter of the mature erythrocyte is between 6,7 and 7,7 μm . The number of erythrocytes depends on age and sex, and in humans there are 4,5 to 6,5 million per mm^3 in men, and from 3,9 to 5,6 million per mm^3 in women.

The red cell normally survives in the blood stream for about 110 days after which time it is removed by the phagocytic cells of the reticulo-endothelial system, broken down and some of its constituents reutilized for the formation of new cells.

1.2.2 Leucopoiesis

Leucocytes can be divided in three main types, the polymorphonuclear cells (granulocytes), monocytes and lymphocytes. The development of each type differs from each other.

Granulopoiesis : The first recognizable granulopoietic cell is the myeloblast. This is a large cell with a large nucleus and little cytoplasm. As development takes place through successive cell divisions the nucleus becomes smaller and loses its nucleoli, at the same time the characteristic granules begin to appear in the cytoplasm and the cell is then called a myelocyte. As the single nucleus becomes bean shaped, and then horse-shoe shaped, the cell is called metamyelocyte. When this horse-shoe nucleus forms separate lobes, the cell is a true polymorph.

There are three types of polymorphonuclear cells namely, neutrophils, eosinophils and basophils. It is possible to differentiate between these cells according to the staining reactions of the granules in their cytoplasm.

Monocytopoiesis : Monocytes are derived from a morphologically unrecognizable common granulocytic-monocytic precursor. The earliest morphologically recognizable precursor is a monoblast, which is larger than the myeloblast with a large nucleus and abundant cytoplasm. Monoblasts mature into promonocytes which in turn mature into monocytes, which migrate rapidly into the peripheral blood.

Lymphopoiesis : Mature lymphocytes are derived from lymphoblasts which are found both in the bone marrow and lymphoid tissues

(Gamong, 1993). Lymphocytes appear to concentrate about arterial vessels near the centre of the haemopoietic cords. Mainly two types of lymphocytes are distinguishable on the ground of their maturation processes. Firstly B-lymphocytes, which are thymus-independent and secondly T-lymphocytes which are thymus-dependent (Meyer, 1983). B-lymphocytes migrate to the tissues without passing through or being influenced by the thymus. These lymphocytes play a major role in humoral immunity, and on the stimulation of antigens on the cell surfaces, they mature into plasma cells that synthesize humoral antibodies. T-lymphocytes either pass through the thymus or are influenced by it on their way to the tissues. T-lymphocytes can suppress or assist the stimulation of antibody production in B-lymphocytes in the presence of antigen and can kill such cells as tumor and transplant tissue cells. They are largely responsible for cell-mediated immunity and have the property of anamnestic response. Several subsets of T-lymphocytes are recognized on the basis of surface markers and function, e.g. memory T-cells, helper T-cells and suppresser T-cells. Other types of lymphocytes that may be present in minimal quantities are killer lymphocytes, null lymphocytes, transformed lymphocytes and Rieder's lymphocytes. Approximately 75% of blood lymphocytes are T-lymphocytes and 25% B-lymphocytes (Heyns, 1981).

Leucocytes are, in contrast with erythrocytes, nucleated cells. They are present in normal blood in smaller numbers than red cells, the normal adult range being between 4000 and 11 000 per mm³ of blood. Their main function is to act as one of the body's defences and some are also thought to be connected with antibody formation.

1.2.3 Thrombopoiesis

The earliest morphologically recognizable precursor in the bone marrow is the megakaryocyte. The megakaryocytes are classified on the basis of their nuclear and cytoplasmic characteristics into three stages of maturation. Group III megakaryocytes are mature cells, and platelets (thrombocytes) are formed by the process of "budding" or "pinching off" of the cytoplasm of this giant multi-nucleated

megakaryocyte. Megakaryocytes lie directly outside the sinusoid. They discharge platelets by protruding cytoplasmic processes through the endothelial cells. Platelets are small non-nucleated oval or round cells, 2-3 μ in diameter.

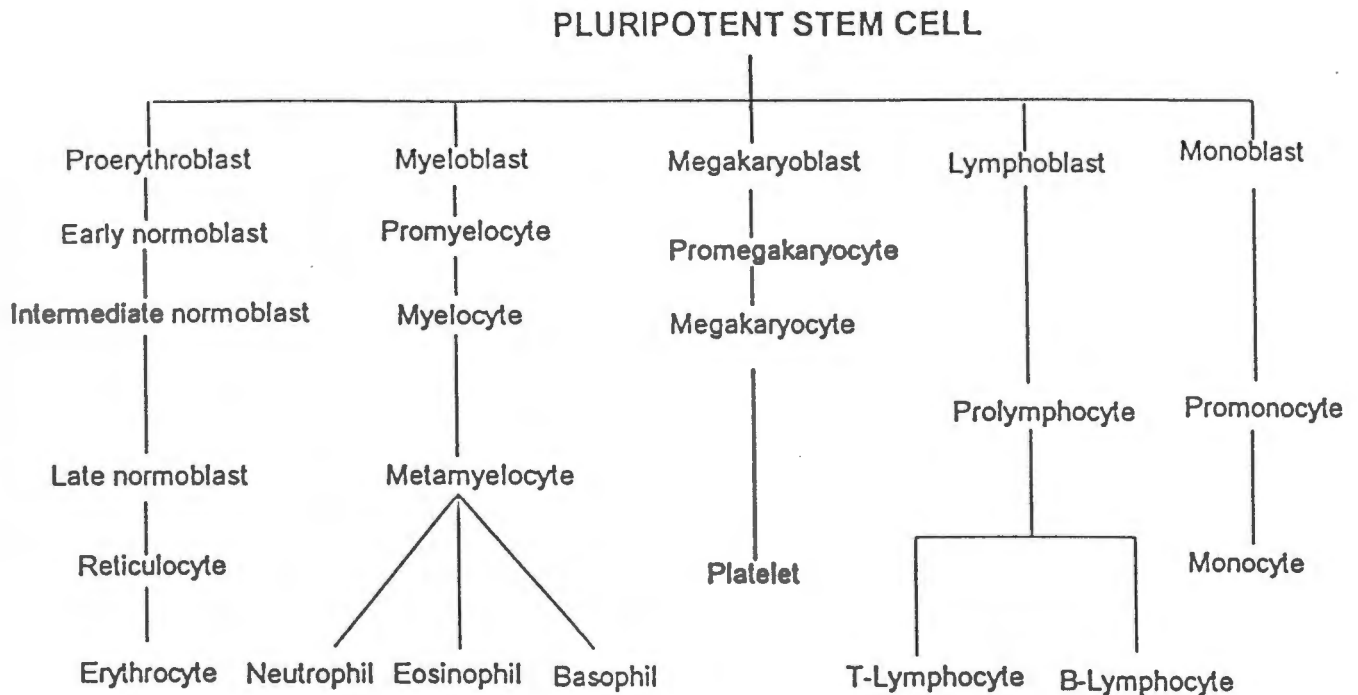


Fig. 2.2 Diagram illustrating haemopoiesis

2. Radiopathology of the haemopoietic system

2.1 The radiosensitivity of the haemopoietic system

Ranked according to radiosensitivity, haemopoietic cells in circulation are second only to the germinal cells of the testes (Jandl, 1987). Many of the early radiation effects observed in organs and systems are mediated by radiation injury of the vascular system. During the immediate post-irradiation period, blood vessels may show only dilatation, accounting for the erythema of the skin. Later (or with higher levels of exposure) endothelial cells undergo swelling, vacuolation and even destruction. Heavily damaged vessels may rupture, or undergo progressive fibrosis and narrowing of their lumen. In this way the dependent parenchymal cells are deprived of their

nutrition and thus undergo atrophy and die, much like what is encountered in aging.

Each site of the haemopoietic tissue is in a certain sense primarily an autonomous subunit of the whole organ. But in addition, all parts of the haemopoietic tissue are interconnected with each other via the bloodstream. Consequently, due to the persistent interchange of signals and the cellular traffic between all the bone marrow sites, the haemopoietic tissue in the whole skeleton is able to respond as a single entity to any damage, whether local or involving the whole body.

Perhaps the most important radiobiological principle involved in the understanding of radiation damage to the haemotopoietic system involves a 'law' postulated as early as 1906 by Bergonie and Tribondeau which states: *cells that normally often divide are radiosensitive; cells that do not divide normally are radioresistant.* This law holds remarkably well among cell systems, although, as with essentially all laws, exceptions do exist, e.g. all lymphocytes appear to be extremely sensitive to radiation (see par. 2.2).

With regard to the radiopathology of the haemopoietic system it is further necessary to distinguish between single high dose whole body exposure and prolonged chronic or repeated low dose whole body exposure.

2.1.1 Single high dose whole body exposure.

This type of exposure is identified by the so-called *haemopoietic* or *bone marrow syndrome*. This syndrome is clinically characterised with mild gastrointestinal symptoms related to injury to the gut, followed by changes in the peripheral blood and bone marrow.

Since the pioneer work of Heineke (1903) many workers have studied the effect of radiation on haemopoiesis. The subject continues to stimulate much interest but is now more concerned with the effects of radiation on the pluripotent stem cell pool than on radiation damage

to the haemopoietic cells themselves. It is suggested (Bond 1971) that the renewal system should be seen as two separate compartments made up of dividing and non-dividing cells. The immature dividing cells can be regarded as radiosensitive and the more mature non-dividing cells can be regarded as more radioresistant. That this is in fact true is clear from the survival curves of stem cells as obtained with numerous experiments (McCulloch and Till, 1962; Sinclair, 1968). All these curves indicate that a relatively small dose of approximately 1Gy is required to reduce the number of surviving stem cells to 50% of the normal and at LD₅₀ values only of the order of 2 to 3 stem cells per thousand retain the potential for proliferation. Dividing cells are rapidly reduced, due to either direct- or mitotic cell death, and by maturation of the remainder. The chain of events in this case is illustrated in Fig. 2.3. In this illustration it is assumed that the non-dividing maturing cells continue to mature. The feed into the peripheral blood will therefore be constant for a time period approximately equal to the time taken for the cells of the last cell divisions to reach the peripheral blood.

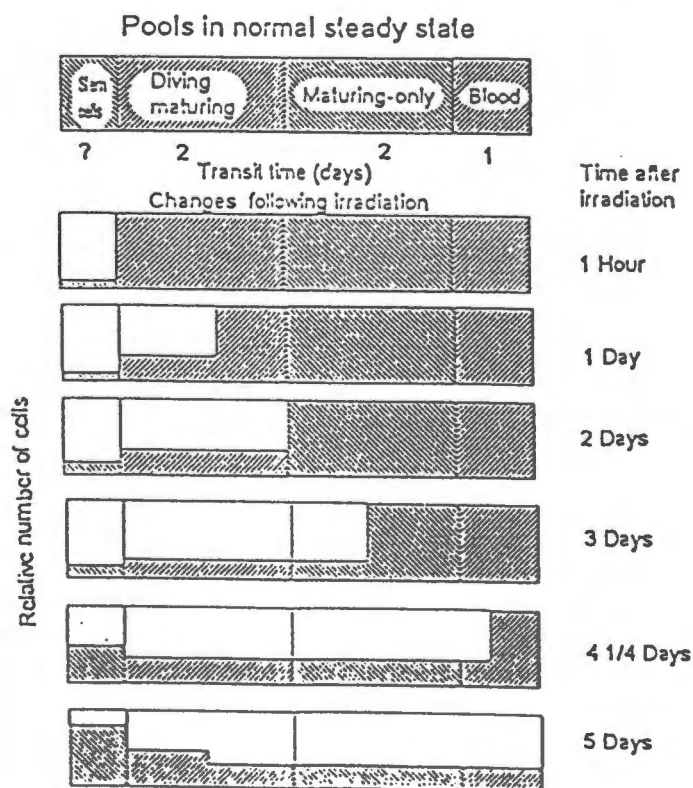


Fig 2.3 Schematic representation of the events if an earlier "factory" cell compartment is selectively depleted, while the more mature "supply" cells remain intact. (Adapted from Bond 1971)

Effects of radiation on the peripheral blood : The situation described above is illustrated diagrammatically in Fig. 2.4 showing the percentage of circulating cells plotted against time following whole-body exposure. (Real values will depend on the specific type of cell dose rate and total dose). According to Bond (1971) the curve can be divided into 3 sections.

The degenerative section consisting of firstly the initial shoulder of which the width represents the time span from when mitosis ends in the dividing maturing bone marrow compartment until the exit of mature elements from the bone marrow into the peripheral blood. Secondly the initial rapid drop representing the typical exponential disappearance time of normal cells from the peripheral blood (the half-time depending on the kind of cell). This correlates with the sudden and almost total interruption of the supply of normal cells from the bone marrow.

The abortive rise section of the curve, the mechanism of which is not clear. The most acceptable explanation is based on stem cells damage to the extent that they do not die, but have a limited proliferative potential. These cells, having lost their normal proliferative capacity, initially proliferate and would result in the abortive regeneration. After the limited potential for proliferation is exhausted, they and their daughter cells disappear and the supply of mature cells is again reduced.

The recovery section of the curve is related to the relatively small number of cells in which proliferative capacity is essentially unaffected. These cells will repopulate the stem cell pool and in time will produce cells that can mature and feed into the peripheral blood. Thus the initial rate of rise of cells in the blood is very slow, but increases gradually as the stem cell compartment is repopulated.

The primary changes will determine the fate of the individual following acute exposure. The resultant neutropenia and thrombopenia, with subsequent haemorrhage and infection lead to the haemopoietic or bone marrow syndrome.

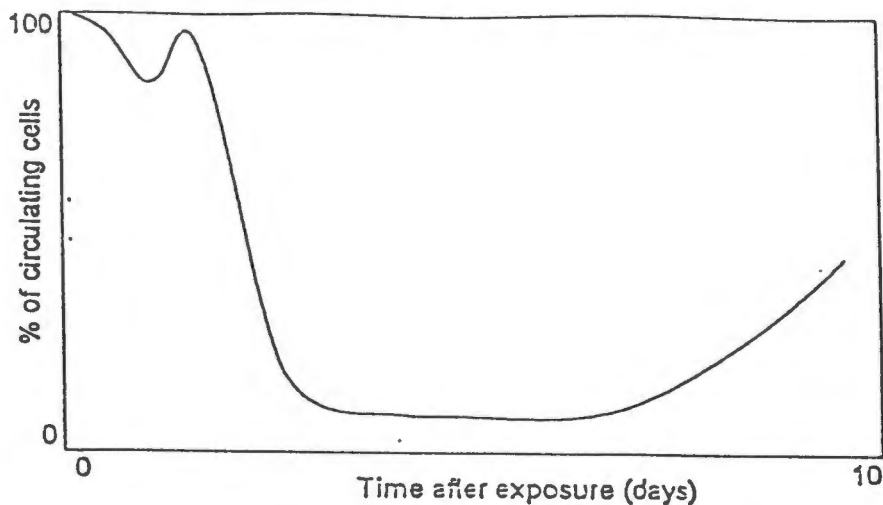


Fig 2.4 Percentage of circulating cells as a function of time after exposure

2.1.2 Chronic or repeated low dose whole body exposure

The effect of continuous exposure is the same as that following on single lethal exposures. With decreasing dose rate there is a progressive reduction in the haematological injury until the only significant change is a small reduction in the number of lymphocytes in the circulation. The dose rates considered in this section are, however, of such a low level that the bone marrow syndrome will not be observed.

Many elaborate methods have been employed to detect minimal haematological changes in workers chronically exposed to radiation and different clinical parameters have been used for this purpose.

Peripheral blood counts : The relation between haematological changes and dose is difficult to ascertain. Sievert (1947) found changes in persons exposed to x-rays at levels estimated to be as low as 0.02 to 0.05 cGy per day. Mayneord (1951) reported a leukopenia with lymphocytosis in patients exposed to 0.125 cGy per day.

Such results seem to indicate a high sensitivity to radiation. However, one must be very careful in ascribing these modifications in the blood to radiation alone. More recent experimental results (Bain *et al.*, 1992) indicate that in general haemopoietic tissue is not particularly sensitive to low-level long-term irradiation and that peripheral blood counts may not provide a reliable estimate of the amount of radiation received

Chromosome aberrations : Chromosome aberrations have been studied in radiation workers exposed for many years to x- and gamma-rays. In leukocyte cultures for these workers the frequency of aberrations is significantly higher than that for the population at large (Sasaki *et al.*, 1963 and Norman *et al.*, 1964). It appears difficult to relate the dose to the frequency of the aberrations. Chromosome studies reveal radiation damage, while other criteria are still within normal limits.

Aplastic anemia : With prolonged exposure to moderate doses of radiation, the differentiating marrow cells begin to show dysplastic nuclear changes that signify ineffective haematopoiesis (Wilson *et al.*, 1991). As exposure continues, the rate of stem cell death increases until aplastic anemia is observed. Long-term exposure to small amounts of radiation led to an increased incidence of aplastic anemia, as was found in radiologists and other occupationally exposed persons (Wilson *et al.*, 1991).

Radiation-induced leukemia : The leukemogenic action of radiation first suggested in 1911 by von Jagiè, Schwarz and von Siebenrock, have since been confirmed by many authors (Betz *et al.*, 1971).

Numerous observations indicate a close relationship between radiation exposure and the occurrence of neoplasms, amongst which leukemia (either acute or chronic granulocytic leukemia) has attracted the greatest attention. The raised incidence of leukemia observed among radiologists in the US is but one example (Warren and Lombard, 1966).

All the above effects, induced by chronic exposure, are only observed a long time (years) after exposure started and are therefore termed the late effects of irradiation. Identical late effects are also induced by single high dose exposures, as observed amongst others in the atomic bomb survivors, patients treated with x-rays for ankylosing spondylitis and children irradiated for thymic enlargement.

2.2 Difference in radiosensitivity of the various blood cell lines

Lymphocyte count : The white cells and particularly the circulating lymphocytes is one of the most sensitive cell types in the body. Within minutes to hours after significant radiation exposure the lymphocyte count drops. A drop has been demonstrated with whole body exposures as low as 0.5Gy, (Gerstner, 1958) and a mild decrease was found with doses less than 1Gy (Cyonkite *et al.*, 1964). The most recent survival curves, obtained from *in vitro* exposures indicates a D_0 value between 0.25 and 0.35Gy (Scherer *et al.*, 1991).

Moreover, while most cells do not die until they attempt to divide, lymphocytes are killed without entering the mitotic cycle i.e. their death is non-mitotic.

Other white cell types : The change in the counts of the other types of white cells differ from that of lymphocytes. Whilst mature lymphocytes are killed by radiation, this is much less likely to happen to granulocytes, resultantly no drop and even an abortive rise in count may occur before a fall begins. Their numbers drop only when the disruption of mitotic activity in the bone marrow is expressed in the circulating cells. Usually the low point of the granulocyte count in humans is about the 30th day after irradiation with a dose of approximately 2Gy. A severe depression can be induced in this period with exposures between 3 to 5Gy (Saenger, 1981).

Platelets : A moderate decrease in the platelet count during the 3rd and 4th week has been correlated with an exposure of less than 2Gy reaching its lowest value about 28 days after exposure and approaching the normal value in approximately 40 days. A severe

depression is induced within 3 to 5 weeks after whole body doses between 2 and 5Gy. In this instance the count drops to about 10% of the normal value and recovery starts between the 30th and 35th days. The recovery rate is somewhat variable, reaching normal values 6 to 8 weeks after exposure. After sublethal levels of whole body exposure (from 4 to 9Gy) the platelet count begins to drop much earlier and may approach zero by the end of the first week (Wald, 1971).

An effort has been made to compare the radiosensitivity of the various peripheral blood elements with the dose of radiation to the bone marrow (Scherer *et al.*, 1991). From the data obtained from humans accidentally exposed to radiation, graphs illustrated in Figs. 2.5, 2.6 and 2.7 have been constructed to show the average time-response of lymphocytes, neutrophils and platelets respectively with four different dose levels. Whole blood counts and particularly lymphocyte counts, are the most sensitive parameter to detect radiation exposure.

Red cells : Mature erythroid or myeloid cells are several orders of magnitude less radiosensitive than that of actively dividing cells. The low point in their count is reached between 40 and 60 days after exposure and results exclusively from bone marrow effects, which inhibits their production. However due to the fall in platelets, there may be bleeding, resulting in a loss of red cells from circulation. The drop in red cells may therefore be more rapid than that caused by mitotic delay alone. A fall in the reticulocyte count five days after irradiation has been found with doses of approximately 3Gy. The immediate effects of sublethal doses of radiation on erythropoiesis is considerable, as revealed in the depression of the reticulocyte counts (Saenger, 1981).

Exposure of erythrocytes *in vitro* to very high doses of irradiation does produce observable effects such as haemolysis, loss of intracellular potassium, and increased osmotic fragility. However, even a dose of 20Gy caused no change in their survival, which again demonstrates that changes observed in the red cell count level must therefore be secondary to events occurring in the bone marrow (Harris, 1971).

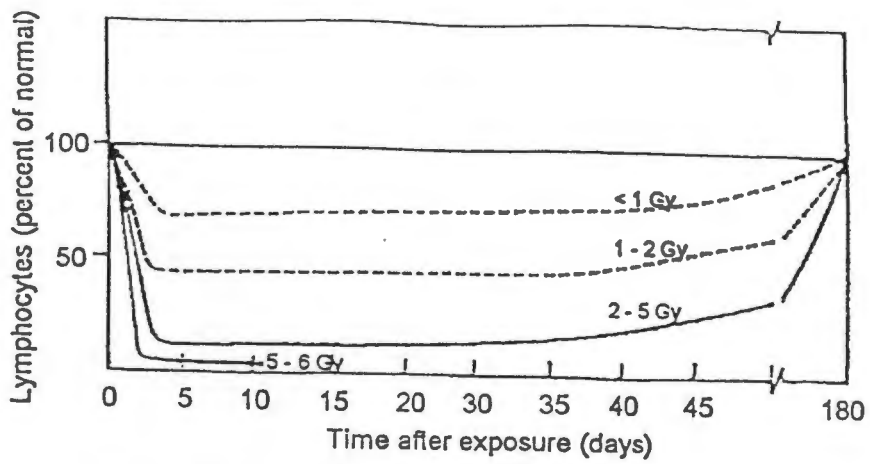


Fig 2.5 Average time-course of lymphocyte changes
(adapted from Saenger 1981)

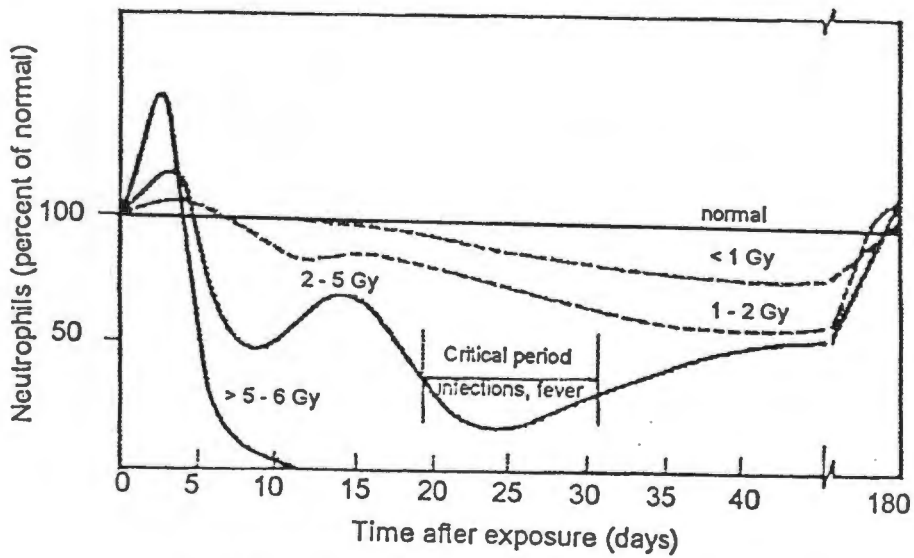


Fig 2.6 Average time-course of neutrophil changes
(adapted from Saenger 1981)

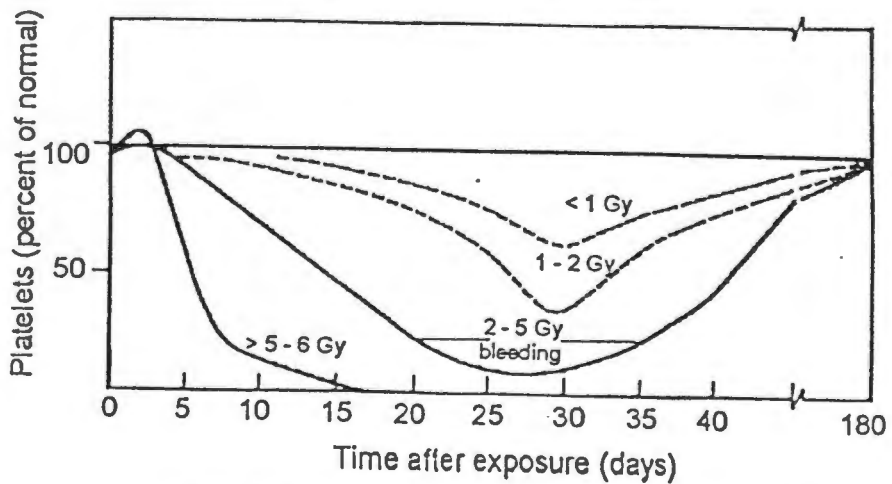


Fig 2.7 Average time-course of platelet changes
(adapted from Saenger 1981)

2.3 Problems regarding partial-body exposure

Exposure of human beings almost always results in an unevenly distributed absorbed dose. Knowledge in this respect is mainly based on cases of incidental overexposure. With heterogeneous exposures it is important to distinguish between biological parameters indicating total dose, regardless of the proportion or anatomical region of the body being exposed, and those which are only a measure of the clinical outlook in cases of suspected radiation injury.

The stem cells : If the decisive factor regarding the haemopoietic syndrome is the degree of radiation damage induced in the stem cell population, the prognosis of the exposed individual will be the same for the same fraction of surviving cells, irrespective of partial or whole body exposure. Theoretically the fraction of surviving stem cells could be calculated if the following are known (Cronkite, 1971) :

- (a) the absorbed dose within the active marrow in the various parts of the body.
- (b) the survival curve for stem cells of the marrow.

If this approach is correct, the fraction of surviving stem cells at a lethal dose point (say LD₅₀) should be the same whether the exposure was uniform or not. Experimental results indicate that the approach seems to be valid and may provide a technique for handling the difficult problem of non-uniform exposure (Cronkite, 1971). These results also lend weight to the theory (see 2.1) that the haemopoietic system, consisting of independent compartments, acts as a single integrated system in as far as supplying mature blood cells to the common blood pool.

It has been suggested that in cases of acute exposure the lymphocyte can serve as an integrating dosimeter providing an indication of total dose regardless of the part of the body exposed (Whitfield *et al.*, 1965 and Fenech *et al.*, 1990). Whether radiation effects on lymphocytes is helpful or potentially misleading, in evaluating specific cases of radiation exposures, will depend ultimately on the information

regarding the absorbed dose-response relationship for non-uniform exposures.

However, experimental results indicate that the consequences of marked inhomogeneities in the absorbed dose are variable, making it difficult to estimate the exposure dose (Lloyd *et al.*, 1991). It seems to be essential that the biological limitations of such *integrating biological dosimeters* be recognized when one is attempting to quantify the radiation exposure.

00

CHAPTER III

BIOLOGICAL INDICATORS TO DETECT AND QUANTIFY RADIATION EXPOSURE

1. Introduction

In addition to physical methods of dose assessment, appropriate biological indicators have become available to detect and quantify the extent of radiation exposure. Minimum requirements for a useful biological dosimeter are the following:

- (a) Easy collection of test sample
- (b) Reproducibility
- (c) Dose effect relationship
- (d) Persistence of effect
- (e) Sensitivity
- (f) Specificity
- (g) Early availability of results

The various types of biological indicator systems that have been used can be divided in four main groups namely, biophysical indicators, biochemical indicators, immunological indicators and cytological indicators.

In this Chapter it is envisaged to deal with the changes in these parameters in a quantitative manner, whereby a dose-response can be characterized with sufficient accuracy to allow a valid assessment of the physical dose.

The difficulty in interpreting biological data obtained from non-uniform exposure or after multiple exposures makes the limitations of this approach even worse.

2. Biophysical indicators

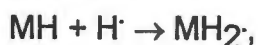
2.1 Electron spin resonance (ESR)

Ionizing radiation produces free radicals in almost any material. By analyzing these radicals by means of ESR a reconstruction of the accident can be made which will help to obtain post exposure dose assessments (Swartz *et al.*, 1972).

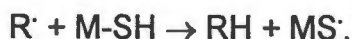
Radiation induced radicals can only be identified by ESR spectroscopy and used for dosimetry if they are stable for some hours or days. Consequently only radiation effects in solid state substances are of interest for dosimetry, because the majority of reactions of radiation induced radicals in water-containing samples generally occur within a time of less than 10^{-2} sec (Brady *et al.*, 1968).

Typical radiation induced radicals produced in solid substances are :

- (a) radicals resulting from abstraction or addition of hydrogen radicals :



- (b) sulphur radicals insofar as SH-groups are present in the substances :



- (c) electrons or holes, trapped in the lattice of crystals.

In man, these radicals may occur after irradiation of the bone and teeth, which both contain hydroxyapatite and other inorganic components (Desrosiers, 1991).

For both bone and teeth a linear correlation was found between the radical concentration and the radiation dose applied over a wide range using electron spin resonance. The lowest detectable doses are approximately 0.5 Gy for teeth and 1 Gy for bone with a linear dose effect relationship up to 1.5×10^4 Gy (Dziedzic-Goclawska and Ostrowski, 1974).

2.2 Neutron activation analysis

For neutron activation, the subject or sample needs to be exposed to either a neutron beam or a mixed beam, of which the neutron-gamma ratio is known. Nuclear reactions take place, producing several radioactive products, which decay with half-lives ranging from a few seconds to several days. The decay is usually associated with gamma radiation. By measuring the gamma ray energies and intensities specific elements can be identified and the induced activity thereof be determined (Sims, 1989; Straume *et al.*, 1994; Straume *et al.*, 1992). The induced activity is directly proportional to the neutron flux to which the sample material was exposed.

For dosimetry purposes the concentration of sodium-24 in the body is usually determined, either by whole body counting, or in various body fluids. Other radionuclides activated and conceivably available for analysis would be ^{32}P , or for instance ^{198}Au in tooth fillings or in jewelry (rings, etc.) worn by the accident victim. The neutron activation analysis technique has been successfully employed in the dosimetry of the Oak Ridge criticality accidents (Auxier, 1961) and more recently in the dosimetric analysis of a serious reactor accident in Argentina (Hübner *et al.*, 1986).

3. Biochemical indicators

Biochemical changes are known to occur in the body following radiation exposure, e.g. changes in enzyme activities, in the metabolism of nucleic acid, as well as changes in cell membranes and of the concentration of different metabolites in blood and urine. Some of the problems with this type of biological indicators are its time-dependency and limited duration after exposure, as well as the lack of a consistent dose response relationship for humans (Biological Indicators Working Group, 1991). In addition there are considerable metabolic differences between animals and man, so that animal experiments may therefore only be extrapolated to man to a limited extent (Gerber 1986). However, a brief description is given of some of the potential biochemical indicators.

3.1 Enzymes

Radiation-induced chemical changes are reflected by measurable changes of certain enzyme levels in the blood serum and urine as a function of dose and time after exposure (Biological Indicators Working Group, 1991). These changes are the result of the radiation-induced release of enzymes from the cell nucleus, the mitochondria and cell membranes because of sufficient damage to the cells or even cell death. Enzymes may be released and others may not be synthesized following irradiation. Consequently there may be an increase in the level of some enzymes and a decrease in the level of others.

Increases of enzyme levels after irradiation have been reported for esterases, alkaline phosphatase, β -glucuronidase and creatine kinase (Stamm *et al.*, 1981).

One of the more promising potential enzyme indicators is amylase, which is released into the blood after the parotid gland has been irradiated (Stamm and Bögl 1982). A relationship between irradiation of the parotid gland with doses of 2 to 20 Gy and the increase of amylase in the blood serum and, concomittantly, in the urine was demonstrated. The amylase levels in blood and urine peak about 30 h after exposure and returns to normal after about 72 h. This suggests that serum amylase may be a biological indicator as long as the irradiated tissue volume includes the parotid gland.

3.2 Amino acids and other metabolites or catabolites

Amino acids and other smaller compounds which are either metabolised or catabolised have been examined as potential candidates for developing biochemical dosimetry. Taurine, for example is an amino acid which appears in the blood serum and in the urine as a function of time and dose following irradiation. For taurine a peak concentration was observed 24 h after absorption of doses up to 8 Gy (Feinendegen, 1986). More recent data appear to confirm a linear dose effect relationship for taurine (Biological Indicators Working Group, 1991). This may, however, not be generalized or extrapolated to other amino acids.

Another, and possibly more promising indicator in the serum is the thymidine level. It was established (Stamm *et al.*, 1983) that total body irradiation causes an increase of the serum thymidine level in a dose-dependent fashion at low doses. In mice the assay is sensitive to between 0.005 to 1 Gy. If standardized, this assay certainly holds promise to be a sensitive and useful biological indicator.

Other amino acids which are transiently present in the serum after irradiation, such as cysteine, valine and leucine as well as aminoisobutyric acid a metabolite of thymidine, were found to be elevated in radiation accident patients (Catravas 1982). A dose dependency could however, not clearly be shown, especially for doses of less than 1 Gy.

3.3 Changes in cellular metabolism and membranes

Altered cellular metabolism has been studied as another possible biological indicator. This method is based on the rate of incorporation of labeled DNA precursors, such as ^3H or ^{14}C labeled thymidine or ^{125}I labeled iododeoxyuridine, into DNA of cultured cells. A depression of the rate of incorporation of these precursors was observed as a function of absorbed dose. The lower region of dose, where significant effects could still be observed was close to 0.5 Gy (Hüber *et al.*, 1986). It was predicted that with improvement of experimental procedures this lower limit may eventually be less than 0.01 Gy.

A reduction in the electrophoretic mobility was observed in the cell membranes of several different types of cells after radiation exposure. This effect shows a linear dose dependence from 0.25 Gy up to about 2 Gy and depends on the ionic strength of the medium. The mechanism of this effect is based on the displacement of acid sugars on the membrane. The reduction in the electrophoretic mobility reaches a minimum value approximately 4 hours after exposure. Cells can repair and normal mobility restored in intact cells within 24 h (Koteles, 1982).

The lectin-binding technique which reveals early dose- and time-dependent alterations was suggested as another potential procedure for dose determination (Kubasova *et al.*, 1981). Different types of cells,

irradiated either in vitro or in vivo, were used, and functional alterations of lectin-binding sites on cell surfaces investigated. The lectin ^3H -Concanavalin A (^3H -Con A) was used. The amount of bound radioactivity was measured by liquid scintillation spectrometry, and it was proved that radiation causes a rather early and dose-dependent increase in the binding of radioactive lectin.

3.4 Reduction in the diameter of human hairs

The measurement of damage to growing human hairs exhibits potential for use in biological dosimetry, especially in cases of non-uniform overexposure. Hairs have been proposed as a potential dosimeter because they are located over much of the body surface, they are rapidly proliferating and damage will be expressed in a short time-scale (Wells and Charles, 1982).

Geng and Potten (1990) found that reduction in hair diameter to be dose dependent over the range from 3 to 14 Gy. When the diameter was reduced by $\approx 50\%$ the hairs broke and were lost from the study, thus marking the upper limit of sensitivity of the test. According to the available data points, the percentage reduction in diameter for human hair is of the order of 2,4% per Gy (Sieber *et al.*, 1992). At present it is not possible to say whether measurement of the reduction in hair diameter will have a role in biological dosimetry, however, for localized exposure in the range from 3 to 14 Gy it might be useful in an accident situation.

4. Immunologic indicators

A number of immunological tests have been evaluated as potential biological indicators of radiation dose. The value of these tests depends in part on the duration of the effect, a demonstrable dose response, individual variability and other confounding factors (Doria *et al.*, 1982). Some of the more important immunologic indicators are detailed in the following sections.

4.1 Absolute and relative numbers of lymphocyte subpopulations

Lymphocytes consist of two major groups, namely B lymphocytes and T lymphocytes. T lymphocytes can be divided into several subpopulations which differ with regard to their radiosensitivity. Consequently changes in the ratio of B and T lymphocytes as well as changes in the ratios of the various subsets of T cells have been identified after irradiation (Wasserman *et al.*, 1982). B cells appear to be particularly sensitive although repopulation occurs rapidly (days to weeks) if haematopoiesis has not been irreversibly damaged by the radiation. It is therefore not possible to use the subpopulations of lymphocytes as an indicator of radiation dose long after exposure.

4.2 Production of specific antibodies in vitro

The pokeweed mitogen (PWM) induced immunoglobulin (Ig) production test involves the isolation of lymphocytes from peripheral blood and their incubation in vitro in the presence of PWM. After seven days the immunoglobulin production is measured. This test is difficult to standardize and has not yet proved itself as useful for dosimetry purposes. The suppressive production effect of the radiation is likely to depend on the dose. In radiotherapy patients it was found that the decreased production of immunoglobulin classes IgM, IgG, IgA recover slowly and the phenomenon was still present after 12 to 18 months (Wasserman, 1986).

4.3 Lymphocyte stimulation test (LST)

This test (previously mentioned in par. 3.3) is based on the measurement of thymidine incorporation into DNA after stimulation of the lymphocytes with either Concanavalin-A or by nonproliferated lymphocytes from another individual (Petrini *et al.*, 1982). A dose-effect relationship has been shown for human lymphocytes *in vitro* after high doses of radiation (1 Gy to 6 Gy). These tests are however only of value soon after radiation exposure, and dose estimation of exposures that occurred years earlier is not possible.

4.4 Somatic mutation assays

Somatic mutations may be induced by ionising radiation. Methods for the quantitative detection of somatic mutations in man are limited, although scientists in California adopted a novel approach to this problem (Conner, 1988).

The technique exploits the fact that human erythrocytes have two forms of glycoprotein A located in the membrane of the cells. These two forms called GPA(M) and GPA(N) differ slightly in their structures, with different genes coding for the two proteins.

Radiation damages the gene responsible for GPA(M), so stem cells that receive a dose of radiation will divide to produce daughter cells that lack the GPA(M) protein in their cell membranes, and only have GPA(N). The researchers from Lawrence Livermore have devised a technique for labelling and counting cells that possess GPA(M) and GPA(N) protein. Monoclonal antibodies are used, which recognise and bind to specific proteins. The antibodies binding to GPA(M) are also bound to a red fluorescent dye, while those bound to GPA(N) are labelled green. Those cells damaged by radiation, and which therefore do not have GPA(M), are only green. Each type of labelled cell is counted by means of a fluorescence activated cell sorter.

Recently, other methods for the detection of somatic mutations at specific loci have been established (Akiyama *et al.*, 1992). These somatic mutation assays will have possible applications for use as biological dosimeters. However the restricted availability of the specific monoclonal antibodies, and the need for well-trained personnel to perform the analyses are major disadvantages for the use of these assays in a large-scale survey.

5. Cytological indicators

5.1 Spermatogenic indicators

The high degree of germinal epithelium sensitivity to radiation has been substantiated by methodical investigations after acute (Oakberg and Heller, 1966) and chronic (Wendt, 1971) exposure to radiation. The spermogram is a sensitive cellular assay for radiation exposure, and changes in sperm count, sperm motility, and sperm morphology following exposure to ionising radiation have been described (Oakberg and Heller, 1966; Suzuki and Withers, 1978).

A decrease in sperm count may not be observed until approximately 45 days after exposure. This delay provides for baseline data to be obtained before the radiation effect occurs, however, at the same time it is the basic reason why a sperm count is of no practical value for early dosimetry or diagnosis of radiation injury. Methods based on change in spermatogenesis are useful in estimating the dose following exposures as low as 0,15 Gy, which still cause a severe decrease in the sperm count within seven weeks (Oakes and Lushbaugh, 1952; Hasterlik and Marinelli, 1956; Heller, 1967).

Hacker *et al.* (1982) found that with regard to spermatogenesis the DNA synthesizing cells are extremely radiation sensitive and are best suited as indicator for radiation exposure. Results from a quantitative relationship between radiation exposure and a reduction in the number of DNA synthesizing cells are so far only available from mice experiments. The authors, however, suggest that the dose effect curve obtained by them is adaptable to man.

Recovery of sperm numbers and fertility would make this biological parameter unsuitable for the estimation of dose years after exposure.

5.2 Haematological indicators

Haematological parameters are unequivocally the most popular biological indicators to evaluate the severity of injury resulting from radiation

exposure in man. Measured haematological changes as a direct indicator of the severity of radiation injury (i.e. the response-injury relation) provides useful information to the clinician for diagnosis and prognostic evaluation. Dose-response relationships based on different blood parameters have been accumulated from various sources during the past decades. A brief summary of these is given below.

5.2.1

Erythema : As mentioned before erythema is a dose-related phenomenon due to the dilatation of blood vessels. Skin erythema doses have been discussed by Failla *et al.* (1922) for radium, and by Schall (1932) for x-rays. This effect corresponds to a minimal absorbed dose of 3 to 8 Gy to the skin. The energy of the radiation will determine the exact threshold dose required to produce this effect. Less energetic radiation is more effective in this regard. With low energy radiation (140 kV), the dose decreases with depth from the surface reaching about 80% at 6 mm. More energetic radiation (200 kV) shows a region of almost constant dose for the first 2 to 4 mm followed by an exponential decrease. Ionising radiation of energy of about 1 MeV exhibits a rise in dose for the first 2 mm to reach a broad maximum extending over several mm. As the energy is still further increased, the surface dose becomes smaller and the maximum occurs at greater and greater depths, with a resultant skin sparing effect. The phenomenon is explained in terms of electronic "buildup". With high energy radiation the electrons set in motion by the radiation are projected primarily in the forward direction. Hence, the number of these electron tracks will increase with depth until a depth equal to the range of these electrons is reached. From this point on, the dose decreases with depth, due to the attenuation of the radiation. Hence, the dose rises from a small value on the surface to a maximum and then decreases. With low energy radiation, the range of the electrons is so small that this effect is not observed and the dose falls continually with increase in depth.

In addition to the radiation energy dependence skin erythema is inconsistent. It may appear after approximately 72 h, vanish and

reappear. Normally, the earlier the appearance of erythema the higher the dose.

5.2.2 Telethermography : Two methods, i.e. thermal and radioisotopic methods, provide valuable information on the response of skin blood flow to irradiation (Flury-Hérard, 1986; Lushbaugh *et al.*, 1986; Jammet and Gongora, 1981). Dilatation of blood vessels as well as radiation injury of tissue is indirectly responsible for an increased blood flow in exposed areas of the body. This causes, from exposure onwards and before the appearance of any clinical symptoms, a hyperthermia (2°C or more) of the irradiated areas. The thermal methods can be very simple, using liquid crystal thermography (Jammet *et al.*, 1980). Telethermography is more accurate, and may exhibit differences of temperature up to 8°C between two symmetric areas, exposed and non-exposed (Flury-Hérard, 1986). This method enables isothermic curves to be drawn up which can be compared with isodose curves of physical dosimetry, but clearly only provides for a relative dose evaluation.

5.2.3 Vascular scintigraphy : With the use of radioactive nuclides the recording of the vascular circulation in irradiated regions and the establishment of vascular circulation curves is possible. Changes in the rate of flow precede any clinical evidence of exposure. The radioisotopic method consists in recording the vascular circulation in the irradiated regions by means of a scintillation camera after intravenous injection of ^{99m}Tc (300-800 MBq) (Flury-Hérard, 1986).

The severity of vascular changes depends on the radiation dose. A severity (isoactivity) gradient can be drawn up but it is not possible to establish absolute dosimetry unless the physical dosimetry is known. This method also enables long-term surveillance of radiation lesions.

5.2.4 Peripheral blood elements : Because the blood-forming organs are extremely radiosensitive changes of cell concentrations in

blood and bone marrow might be considered obvious and most useful biological indicators for radiation exposure. As indicated in Chapter 2 the haematologic response to doses of ionising radiation has been extensively described (World Health Organization 1961; IAEA 1967, 1969; Bond *et al.*, 1965).

The system is approximately quantitative and dose related above 0.5 to 1 Gy whole body dose. An estimate of dose can be obtained by using the typical granulocyte, platelet or lymphocyte response after total body irradiation (Thoma and Wald 1959; Andrews 1967; National Academy of Sciences, 1967). Due to recovery from radiation exposure (following repopulation of the bone marrow) the reduction in numbers of blood cells is transient. Therefore, the utility of this biological indicator is limited to days or a few weeks after exposure, depending on dose and recovery of the bone marrow.

5.2.5

Stem cell and progenitor cell compartments : Another dose-response approach has been to observe changes in the pluripotent stem cells and the progenitor cells of the different cell lineages from where blood cell production originates. Due to their proliferative state these cells are the most sensitive to ionising radiation. Therefore, one may assume that the analysis of stem- and progenitor cell alterations in principle will be the appropriate approach to an assessment of radiation damage to the bone marrow. This concept is based on the fact that haemopoietic stem cells and progenitor cells are located not only in the bone marrow itself, but are also present in the circulating blood. Their concentration is in the order of 100 per ml blood. These haemopoietic blood cell populations are in a dynamic equilibrium with the corresponding compartments located in the different bone marrow sites that are distributed over the whole skeleton.

Nonuniformity of exposure is biologically significant if one considers that the bone marrow is spread over the entire body. Clearly 1 Gy to 10% of the body will cause less damage to the total bone marrow mass than 0,1 Gy over the whole body. Sequential

studies on the changes of the stem cells in the blood after whole body irradiation are quite rare, due to the technical problems associated with the assay in this special case (Barnes and Loutit, 1967). The results obtained show for most stem cells a simple exponential dose response in the dose range from 0 to 3 Gy (Grilli *et al.*, 1982). In the case of partial body irradiation the decrease is proportional to the irradiated bone marrow volume. Regeneration of the stem cell and progenitor cell populations in the blood occur more slowly than in the bone marrow (Nothdurft *et al.*, 1983).

5.2.6 Bone marrow cellularity : The assessment of bone marrow cellularity can be performed only under conditions where bone marrow samples of a defined volume can be obtained by biopsy or resection of pieces of bones. The determination of the bone marrow cellularity can be absolute, i.e. total cell number contained in a certain volume of bone marrow, or by the assessment of cell density, i.e. counting the cells per mm² of bone marrow (Stein 1972).

In dogs the early changes in bone marrow cellularity after whole body irradiation were determined in several samples of bones representative for different regions of the skeleton (Wilson *et al.*, 1978). The depression in cell number 24 h after irradiation showed an approximately exponential dose response.

5.2.7 Bone marrow mitotic index : The mitotic index (MI) in normal bone marrow, although subject to a relatively wide variation, has a value of the order of 0.8% for all haemopoietic cells together (Fliedner *et al.*, 1959).

Studies of bone marrow preparations from the patients in the Oak Ridge accident carried out by Fliedner *et al.*, (1960) showed significant deviations from the normal mitotic rate at four days after exposure. An initial depression and a temporary normalisation of the MI has been observed at about the 8th day, after which a renewed reduction took place, followed by a progressive increase.

There was an apparent dose dependency in the depression. On this basis, it has been proposed that bone marrow examinations performed promptly after an assumed exposure and at daily intervals for a period of one week would be most useful in detecting exposure to radiation in the 0.5 - 3 Gy range (Cronkite, 1966).

6. Cytogenic indicators

6.1 Chromosome aberration analysis

A more specific approach for estimating dose to humans was developed in the 1960s and involves culturing lymphocytes (Moorhead *et al.*, 1960) and subsequently determining changes present in their chromosomes (Bender and Gooch, 1962; Bender 1964). Since then, many studies in animals and man have been performed, whereby this technique has been validated as a method for dose estimation in cases of whole body exposure (Biological Indicators Working Group Report, 1991). Chromosome changes appear dose-dependent from doses of 0.15 Gy for gamma-rays (0.05 Gy for neutrons) to lethal dose values.

Various researchers employ slightly different preparative techniques. Lymphocytes, are separated from whole blood by centrifugation, stimulated by the drug, phytohaemagglutinin, to become actively dividing cells, and are arrested during their first division by another drug, colcemid. This procedure takes approximately 2 days during which period the cells are maintained at 37°C in a mixture of culture medium and serum. After 48 h the cells are fixed, dispersed onto slides, stained and examined through a conventional light microscope for chromosome damage. Usually, for statistical reasons, several hundred cells need to be scored.

Chromosome analysis after radiation exposure produces highly reproducible results which are very closely related to the dose. The technique has a number of disadvantages, as detailed below :

- (a) Large inter-laboratory variations in the relationship between radiation dose and number of chromosome aberrations, (Lloyd *et al.*, 1987).
- (b) Chromosome analysis is a labour intensive technique and not readily applicable when it comes to evaluating a large number of victims.
- (c) Nonuniform exposure presents somewhat of a challenge in terms of resolving the question of how to improve the cytogenetic dosimetry in this situation.

However, recent developments such as the introduction of different staining techniques, research into automated analysis and flow cytometry and a better understanding of the kinetics of human lymphocytes suggests that chromosome analysis for radiation dosimetry will be further refined.

6.2. The nucleoid sedimentation technique

This technique is also based on induced chromosome damage in peripheral lymphocytes. The induced damage is evaluated by nucleoid sedimentation.

This method is based on the gentle lysis of cells in the presence of non-ionic detergents and high salt concentrations resulting in the release of protein (histone) depleted nuclei. The extraction of histones from the nuclei causes the DNA and nuclear matrix to collapse into compact structures called nucleoids. Nucleoids are therefore DNA superstructures, containing all the nuclear RNA and DNA, but no histones. The degree of chromosome change is reflected by alterations in the sedimentation co-efficient of the nucleoids.

It was established that the nucleoid density as well as the corresponding sedimentation rate gradually decreases as radiation induced strand breaks increases (Cook and Brazell 1976, Fillippovich *et al.*, 1982, van Rensburg 1987).

Doses as low as 0.05 Gy have been estimated, which entails approximately one single-strand break per chromosome (Rydberg 1984). It is the most sensitive of commonly used methods for quantitating DNA strand breakage, although it has been suggested to be too complicated for practical use (Cook and Brazell 1976 and Pitout *et al.*, 1982).

6.3 Enumeration of micronucleated erythrocytes in peripheral blood

The occurrence of nucleated red cells in the peripheral blood following irradiation does not have a ready explanation (Ingram, 1960; Ingram and Preston, 1964). Dose-response experiments show a significant increase in the micronucleated erythrocytes in bone marrow and peripheral blood of mice with exposures as low as 0.01 Gy. It was found that in the low-dose region (0.02 Gy) the relevant dose-response is approximately linear. (Grawe *et al.*, 1992).

With the advent of automated techniques for quantitative cytometric measurements, based on flow cytometry, methods for automated enumeration of micronucleated erythrocytes have been described, (Hutter and Stöhr 1982). By applying these methods it may be feasible to use the observed increase in nucleated erythrocytes in peripheral blood as a parameter for estimating radiation dose.

6.4 Assessment of micronuclei

In addition to chromosome aberrations, radiation causes deletions which are responsible for acentric chromosomal fragments generally called micronuclei. They are observed in the cytoplasm of interphase cells which have passed through at least one cycle of mitosis after irradiation.

Quantitative relationships between radiation doses and micronuclei yields were reported for the first time by Countryman and Heddle (1976). Since then a number of researchers (reviewed by Almassay *et al.*, 1987) have suggested counting micronuclei in blood lymphocytes as an alternative to counting chromosome aberrations.

The reliability of conventional micronucleus assays is diminished owing to the inclusion of non-dividing cells in the estimate. This problem has, however, been overcome by the development of the cytokinesis-block method (Fenech and Morley 1985, 1986). These authors incubated proliferation-stimulated lymphocytes with cytochalasin B in such a concentration that karyokinesis can take place but cytokinesis is inhibited. By scoring binucleate cells only, analysis is restricted to those cells that have undergone only one mitotic division of the cell nucleus.

The micronucleus assay circumvents some of the problems of chromosome dosimetry. Microscopic examination is simpler, quicker and therefore more cost-effective, and also may be easier to automate. This technique and the associated statistical analysis will be described in greater detail in the ensuing chapters.

CHAPTER IV

RADIATION INDUCED MICRONUCLEI IN BLOOD LYMPHOCYTES: *IN VITRO* TECHNIQUE

1. Introduction

The scientific and medical response to accidental overexposure to ionising radiation should include an attempt to estimate absorbed doses to irradiated persons. This information may be gained from physical and biological methods, although as stated before, in many situations information from physical techniques may be scant. At Chernobyl, for example, the initial sorting of casualties was done by observations of the speed of onset and severity of the prodromal response. More precise biological dosimetry was then performed using the well established technique of analysing peripheral blood lymphocytes for dicentric chromosomal aberrations. In the event of another Chernobyl-like accident, chromosomal studies would probably again be the method of choice for biological dosimetry.

However, as chromosomal analysis is particularly labour intensive, time consuming and needs experienced manpower there may be considerable logistical problems in dealing with large numbers of patients. The newer micronucleus assay that utilises cytochalasin B to block cytokinesis offers an alternative to scoring for dicentrics. Because the images are far simpler, the analysis time could be completed significantly faster and by less skilled technicians.

2. Objective

The initial experiments were conducted to ensure that the micronuclei frequencies observed for blood samples irradiated *in vitro* were compatible with those reported by other investigators and particularly those previously obtained at the National Accelerator Centre (NAC), situated at Faure, South Africa (Slabbert, 1993). This was considered necessary to confirm that the experimental protocol and assessment criteria for micronuclei *in vivo* (which will be dealt with in Chapter VI) would concur in all respects with that used in the *in vitro* technique.

Only consistency of the assay would provide sufficient accuracy to allow a valid intercomparison between the *in vitro* and *in vivo* results.

3. Experimental protocol

3.1 Donors of lymphocytes

To evaluate the accuracy of dose assessment by means of the cytokinesis blocking (CB) micronucleus assay, a systematic study was performed of the *in vitro* dose response of gamma-ray induced micronuclei for a selected group of healthy donors. This group consisted of 4 persons ages 28, 30, 37 and 57 years randomly selected from a heterogeneous working population. The donors were all non-smoking, non-drinking male volunteers. Blood was obtained only once from each donor.

3.2 Radiation source

For exposure of the blood samples obtained from the donors to gamma radiation a modified Siemens ^{60}Co radiotherapy unit was used. This unit, shown in Fig 4.1, contains a cobalt source which has decayed to an activity of 70.3 TBq. The beam was directed vertically upwards. The dose rate was measured with a Baldwin Farmer dosimeter at a source to object distance of 32.4 cm, and was found to be, 88.63 cGy min⁻¹. The measurement was done with a 6 mm thick perspex plate in the beam. The blood samples were exposed in thin wall glass tubes with an external diameter of 9 mm placed in a circular radiation field of 15 cm diameter on the perspex plate, so as to ensure electron equilibrium within the samples. The dose response relationship was determined in the dose range 1 to 4 Gy.

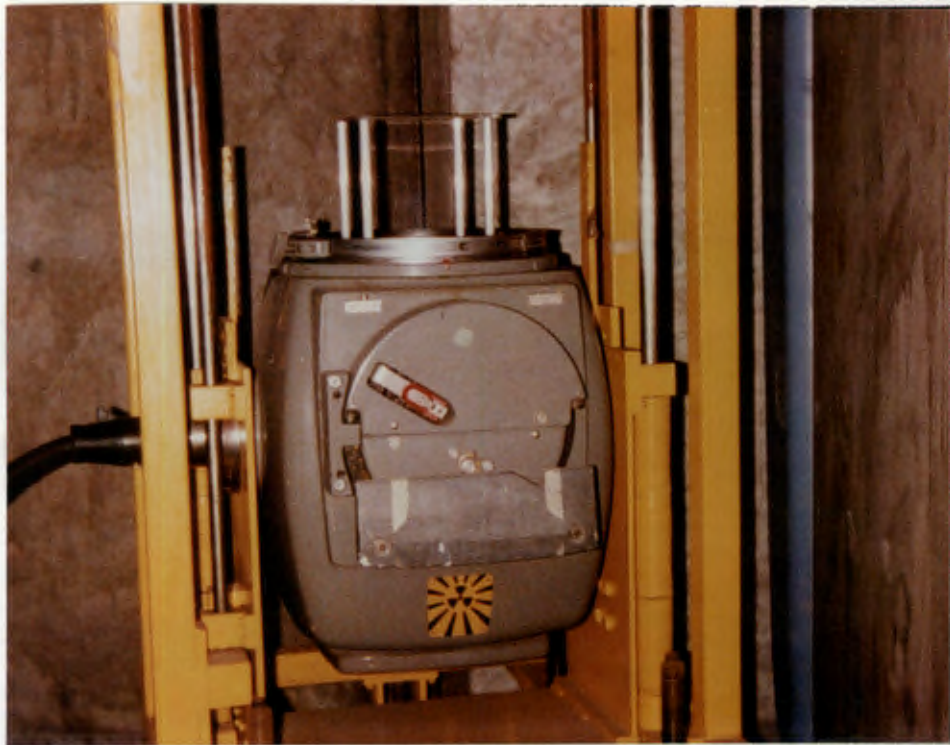


Fig 4.1. ⁶⁰Co Irradiator

3.3 Materials and methods

Five heparinized whole blood samples of 10 ml each, were drawn from the donors. One sample of each donor served as a control for determining the spontaneous micronucleus frequency. The other samples were exposed to the ⁶⁰Co gamma-ray source. Doses of 1,2,3 and 4 Gy were delivered at a constant dose rate to the four samples respectively. After exposure, the temperature of the blood was maintained for a further 1 h at 37°C and then allowed to fall to room temperature.

3.3.1 Lymphocyte separation

Following methods by Fenech and Morley (1985), samples were prepared for culture by first separating the lymphocyte population from the other blood elements. After the blood samples were centrifuged for 10 minutes at 190g the serum was partially removed so that only 1 cm of serum remained above the cell components.

The remaining cells and serum were diluted (1:1) with phosphate buffered saline (PBS) and layered on 3 ml Histopaque (Sigma). The layers were then centrifuged for 30 minutes at 190g. Following centrifugation, the lymphocyte layer was clearly visible, and the supernatant was removed so that about 1 cm remained above the cloud of lymphocytes. Using a Pasteur pipette, the lymphocytes were selectively transferred to a clean centrifuge tube, diluted with an equal volume of PBS and centrifuged for 10 minutes at 190g. The supernatant was discarded and the button of cells, resuspended in 10 ml RPMI-1640 growth medium supplemented with penicillin (0.1 mg/ml) streptomycin (0.1 mg/ml) and 15% heat inactivated foetal calf serum.

3.3.2 Cell Culture

The isolated lymphocytes were stimulated to divide by adding 100 µl of a 1.3 mg/ml solution of phytohaemagglutinin (PHA) to the growth medium.

The cells were incubated in an upright 25 cm² Falcon tissue culture flask at 37°C. After 44 hours of incubation, cytochalasin B at a concentration of 3 µg/ml was added to the growth medium to block cytokinesis. The cultures were then incubated for an additional 28 hours.

3.3.3 Cell Harvest

After 72 h the cell suspensions were removed from the incubator and centrifuged at 93.2g for 5 minutes. All but 0.5 ml of the supernatant was removed and the lymphocytes resuspended in the remaining medium using a vortex mixer. About 10 drops of the cell concentrate were placed on a microscope slide which was then centrifuged for 7 minutes at 93.2g. Because a cytospin was not available, the slides were placed on filter paper at the bottom of the centrifuge bucket (Heraeus Christ, Digifuge model). The slides were left to dry on the laboratory bench after which the lymphocytes were fixed by flooding the slides with methanol:acetic acid (3:1).

3.3.4 Nuclear staining

The lymphocytes were stained with acridine orange for fluorescence microscopy. The staining solution consisted of 0.5 ml of a 0.1g/100 ml solution of acridine orange diluted in 50 ml phosphate buffer (pH 6.8). Slide preparations were left in this solution for 3 minutes, washed in distilled water and then destained for 2 minutes in the phosphate buffer (pH 6.8). A coverslip was mounted on a thin layer of phosphate buffer and the sides of the coverslip were sealed using Entellan (Merck).

3.3.5 Scoring of micronuclei

Fluorescence microscopy was performed using a Zeiss microscope, model IM 35. The microscope was fitted with a blue epi-fluorescence exciter filter (450–490 nm) and a 520 nm barrier filter.

Micronuclei were scored in binucleated cells only, using a 400 x magnification. Some observations were also made using a 100 x objective (oil) giving a final magnification of 1000 x in cases of uncertainty. Binucleated cells in cell groups required particularly careful visual examination of the individual cell boundaries of cytoplasm.

An average of 500 binucleate cells for any data point were scored, with a minimum of 449 and a maximum of 1236 cells, depending on the frequency of binucleate cells.

4. Identification of micronuclei in human lymphocytes

4.1 Criteria for the identification of micronuclei

The criteria for scoring micronuclei in human lymphocytes were largely based on the rules prescribed by Countryman and Heddle (1976), Krepinsky and Heddle (1983), Lasne *et al.*, (1984) and Almassy *et al.*, (1987). Using fluorescence microscopy, the following standards were adhered to, namely :

- (a) Micronuclei were scored only in cells containing two main nuclei with a well preserved cytoplasm.

- (b) A micronucleus must have the same granular structure as the main nucleus, i.e. the staining intensity should be equal to that of a main nucleus.
- (c) A micronucleus must be smaller than the main nucleus and must not exceed about one third of the size of the main nucleus.
- (d) A micronucleus must be a round or nearly round body visibly separated from the main nucleus.
- (e) Cells with more than two main nuclei were excluded from the count. These cells have been reported to constitute less than 5% of the population (Gantenberg *et al.*, 1991), although they frequently contain a micronucleus.
- (f) A micronucleus must show a DNA-specific reaction, similar to that of the main nuclei, i.e. the green-yellow fluorescence with the acridine orange fluorochrome used for these observations (Hayashi *et al.*, 1983) should be the same.
- (g) Micronuclei touching, but not overlapping each other or a main nucleus were counted (Huber *et al.*, 1992).

4.2 Observation of micronuclei

In the cytokinesis-block (CB) method, enumeration of micronuclei is restricted to cells that are blocked from undergoing cytokinesis and which are consequently easily recognisable as large binucleate cells. CB cells were found to be at an ideal stage for scoring micronuclei because they are certain to have divided and micronuclei can still be easily recognised. The levels of CB cells accumulated depend on the proportion of cells in culture that respond to mitogen but it is generally possible to accumulate several hundred CB cells from a 1 ml culture.

Huber *et al.*, (1983) claimed that the conventional micronucleus assay as applied to lymphocyte cultures was too imprecise to detect exposures

to x-ray doses less than 30 cGy. By contrast, the results obtained by Fenech and Morley (1986) indicate that exposures to 5 cGy of x-rays are readily detected by the CB method and that such detection can be done rapidly because hundreds of CB cells can be visually scored within a few hours.

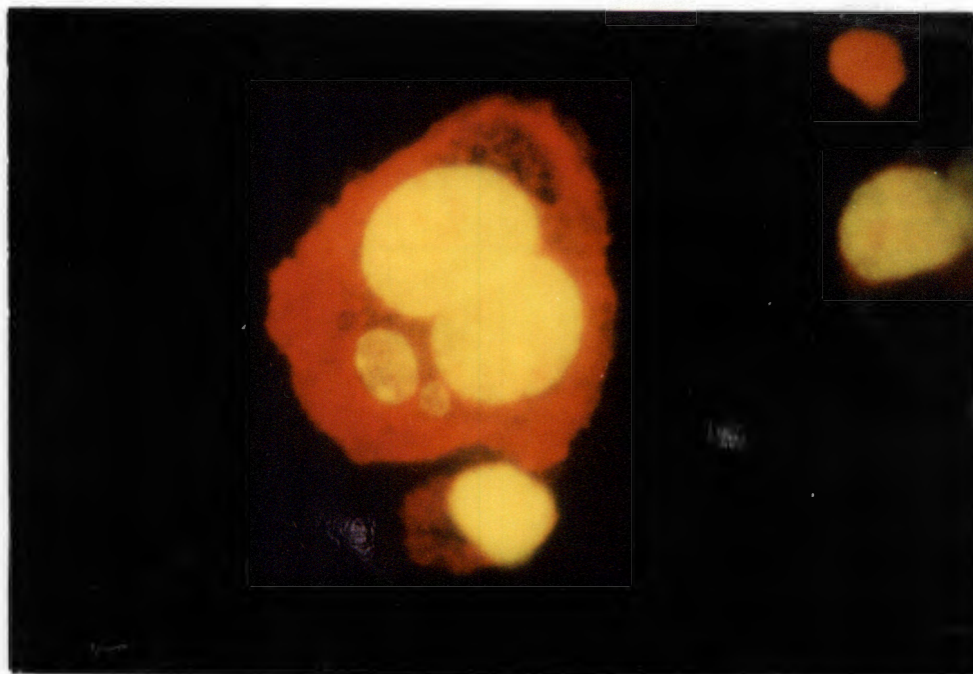


Fig 4.2 Photomicrograph of a binucleate lymphocyte containing micronuclei

5. Results

The dose response data obtained by scoring micronuclei in CB cells of 4 donors are summarized in Table 4.1. The Poisson standard errors are given in brackets. Included in the table is the percentage of binucleated cells (BNC) observed in each culture.

The consistency of yield between donors at each dose was checked. In contrast to the extensive interindividual variability noted by Gantenberg *et al.*, 1991 and others, the present data was found to be in very good agreement. It was therefore considered reasonable to pool the data. By averaging the mean yields of each donor the data in Table 4.2 was obtained.

Table 4.1: Mean micronuclei (MN) frequencies observed in cytokinesis blocked (CB) lymphocytes following exposure to Co-60 gamma-rays, with standard errors given in parenthesis

Donor	Dose (Gy)	No. CB cells observed	No. MN	MN per 500BNC	BNC (%)
A	0	1126	13	5.8 (±1.6)	54
	1	470	36	38.3 (±4.5)	49
	2	1011	199	98.4 (±7.0)	53
	3	1087	279	128.3 (±7.7)	46
	4	1236	448	181.2 (±8.6)	48
B	0	988	10	5.1 (±1.6)	51
	1	644	67	50.5 (±6.2)	55
	2	484	86	88.8 (±6.8)	54
	3	690	205	148.6 (10.4)	47
	4	1006	347	172.5 (±9.3)	45
C	0	449	6	6.7 (±7.7)	52
	1	538	41	38.1 (±6.0)	50
	2	871	192	110.2 (±8.0)	43
	3	689	188	136.4 (±9.8)	51
	4	704	269	191.5 (11.6)	49
D	0	696	11	7.9 (±2.4)	48
	1	1232	109	44.2 (±4.2)	52
	2	971	155	79.8 (±6.4)	47
	3	655	185	141.2 (10.4)	53
	4	574	234	203.8 (13.3)	46

Table 4.2: Mean values of pooled data given in Table 4.1, with standard errors given in parenthesis

Dose (Gy)	0	1	2	3	4
Mean MN per BNC	1.24 E-2 (± 1.8 E-3)	8.56 E-2 (± 9.8 E-3)	1.88 E-1 (± 2.3 E-2)	2.75 E-1 (± 1.6 E-2)	3.69 E-1 (± 2.2 E-2)

A linear relationship appears evident between dose and mean micronuclei frequency. Consequently, as a first approach, and in accordance with previous work (Slabbert, 1993) linear-regression fitting was done. The analytical expression of the linear-regression fit to these data, represented in Fig 4.3 by the solid line, is given by :

$$y = 9.022E-2 (\pm 2.326E-3)D + 5.482E-3 (\pm 5.698E-3) \quad (\text{Equ 4.1})$$

with $r^2 = 0.998$

Whilst there is no difficulty in deriving a dose from a measured yield of micronuclei, there is no generally accepted way of deriving its uncertainty. The uncertainty is usually expressed as a 95% confidence interval, which defines an interval such that the limits will encompass the true dose on 95% of the occasions.

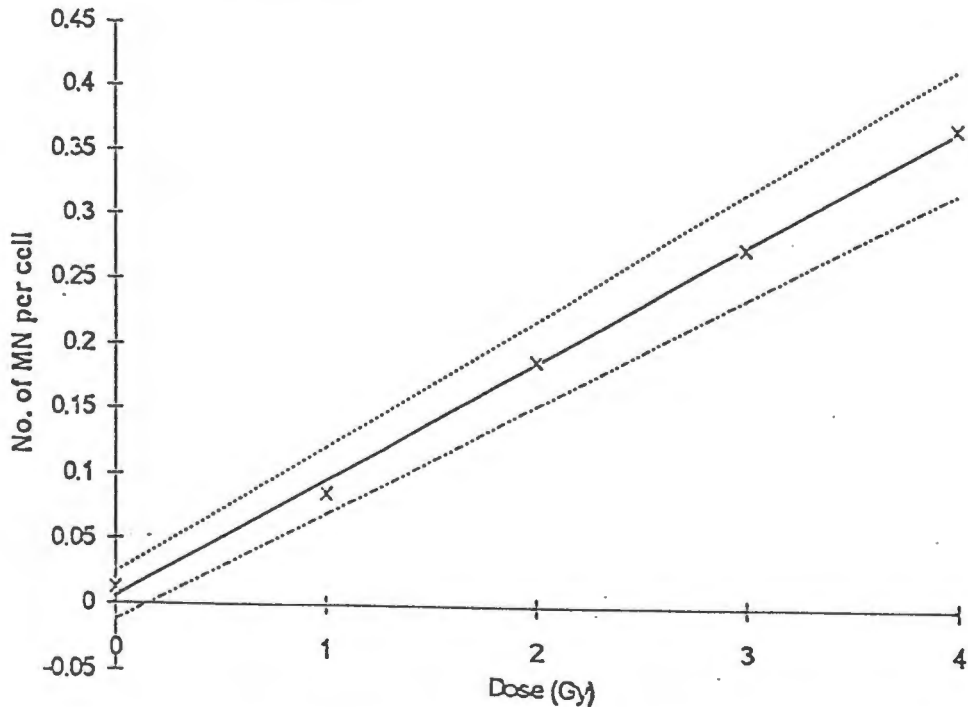


Fig 4.3: Dose response curve fitted by linear regression. (95% confidence intervals are given by the broken lines)

In the regression fitting equal weights were given to each point. At the lower doses i.e. at 0 and 1 Gy, there appears to be more marked differences between the observed and calculated values of the number of micronuclei per binucleate cell than at the high doses. It might be suggested that low dose points should be weighted more than high dose points.

The data were then fitted by an iteratively reweighted least squares method using a 486 personal computer and a statistical software package, SAS/STAT. The program gives the maximum likelihood estimates of the parameters exactly. This method, described by

Papworth (1975) has been used extensively by others (Lloyd *et al.*, 1987; Balasem *et al.*, 1993 etc.) in this regard.

The average yields of MN and their distribution in BNC from irradiated blood samples of the 4 donors are shown in Table 4.3. To test whether the distribution was Poissonian, Papworth's U test was used (Papworth, 1970).

The variance is given as :

$$\sigma^2 = \frac{\sum_{l=1}^{N_i} (k_l - \bar{y})^2}{(N_i - 1)} \quad (\text{Equ 4.2})$$

where k_l is the number of micronuclei in the l^{th} of N cells (Huber *et al.*, 1992). The ratio is σ^2/\bar{y} known as the dispersion index, where \bar{y} is the mean micronuclei frequency. Over dispersion is indicated when σ^2/\bar{y} is greater than unity.

To determine if the mean and the variance of the observed distributions were significantly different, the standard unit normal deviate of (σ^2/\bar{y}) (μ -parameter in Table 4.3) was calculated according to Savage (1970).

$$\mu = d - (N - 1) / \sqrt{2(N - 1)(1 - 1/N\bar{y})} \quad (\text{Equ 4.3})$$

where $d = (N - 1) \sigma^2/\bar{y}$ (coefficient of dispersion)

Over dispersion is indicated by a positive value of μ , and under dispersion by a negative value. If μ exceeds 1.96 the over dispersion is significant at the 95% confidence level, which means that with a Poisson distribution there is only a one in 20 chance that the magnitude of μ will exceed 1.96.

The observed distributions of micronuclei deviated from a Poissonian distribution; they were significantly over dispersed for all the donors except at the zero dose values (Table 4.3).

Radiation induced micronuclei in general tend to exhibit an over dispersed distribution even for an homogeneous exposure, whereas dicentric chromosome aberrations tend to show a Poissonian distribution (Prosser *et al.*, 1989).

Table 4.3: Intracellular distribution of MN in CB cells (Micronuclei frequencies determined in blood samples obtained from 4 donors are pooled)

Dose (Gy)	No. of CB cells observed	MN distribution in CB cells							σ^2/\bar{y}	μ
		0	1	2	3	4	5	6		
0	3259	3219	40	-	-	-	-	-	0.987	-0.489
1	2904	2678	200	25	1	-	-	-	1.13	5.14
2	3337	2867	348	87	30	5	-	-	1.46	19.05
3	3121	2435	552	104	23	7	-	-	1.23	8.99
4	3520	2527	756	191	30	10	6	-	1.25	10.47

The way in which this over dispersion was taken into account when the data were fitted to a curve by iteratively reweighted least squares is as follows. There seems to be no systematic change of variance with dose although the zero point could be regarded as showing a Poisson distribution. It can be assumed that the variance/mean ratio is independent of dose and averages 1.2. The Poisson standard errors are then increased by $\sqrt{1.2}$ for all points and this is included when weighting factors are calculated. The weighting factors used were $1/\sigma^2$.

As expected this analysis indicates a polynomial of the first degree of which the analytical expression, represented by the solid line in Fig 4.4(a), is given by the equation:

$$y = 8.73E-2(\pm 2.68E-3)D + 1.14E-2 (2.6E-3) \quad (\text{Equ 4.4})$$

with a probability, $p = 0.1231$

Alternatively one could say that the Poisson distribution applies at zero dose and the variance to mean micronuclei frequency ratio is 1.25 at all other doses. This leads to the dose-response relationship represented by the solid line in Fig 4.4 (b) for which the following equation applies:

$$y = 8.72E-2(\pm 2.66E-3) D + 1.16E-2(2.36E-3) \quad (\text{Equ 4.5})$$

with $p = 0.1337$

In both figures 4.4(a) and (b) the 95% confidence limits are indicated by the broken lines.

Both methods result in very similar fits to the data. The fit to a straight line is very good, particularly in view of the large number of micronuclei scored at high doses.

The method of calculation of standard errors for the curves comes directly from the reweighted least squares fitting routine (SAS/STAT software package). A routine which fits data using orthogonal polynomials was used. The standard errors on the coefficients of the orthogonal polynomials were supplied by the program and these were combined assuming independence, to obtain the standard errors on the coefficients and on the curves. The standard errors are based on a variance which is calculated using the deviations of the points from the fitted curve.

6. Discussion

There is very little difference between the curves illustrated in Figs 4.3 and 4.4. However, in theory the reweighted least squares analysis should provide a more accurate fit to the observed data than the linear - regression procedure.

Despite the fact that there is also little to choose between the dose-response curves obtained by the above analysis it was decided to utilise the dose-response curve given in Fig 4.4(b) for later calculations of estimated dose. The reasons for this choice are the following:

- (a) The assumption that the Poisson distribution applies at zero dose was verified for all the individual donors and was also found consistently in the results of other researchers. (Lloyd *et al.*, 1991).
- (b) The predicted zero dose value obtained by the second analysis (Fig. 4.4 (b)), $1.16\text{E-}2 (\pm 2.36\text{E-}3)$, appears to be a better estimate of the observed value of $1.24\text{E-}2 (\pm 1.8\text{E-}3)$.

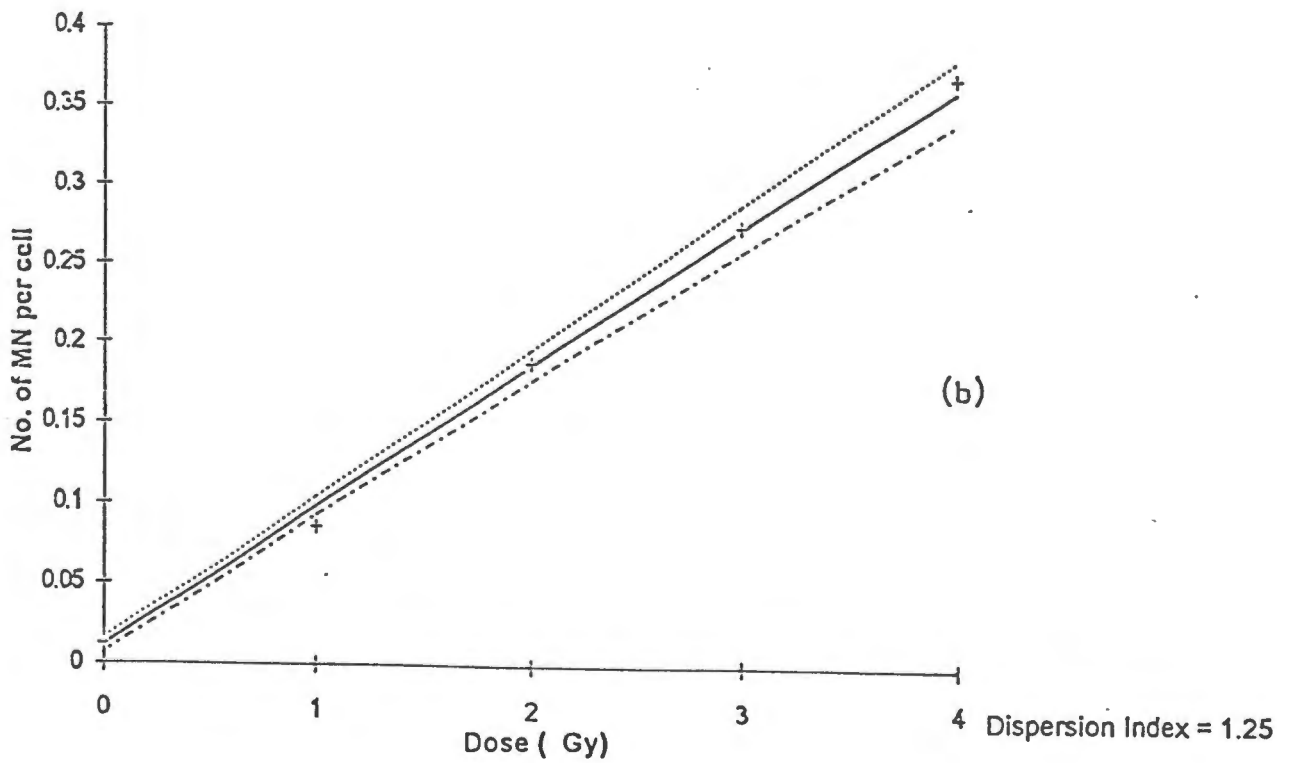
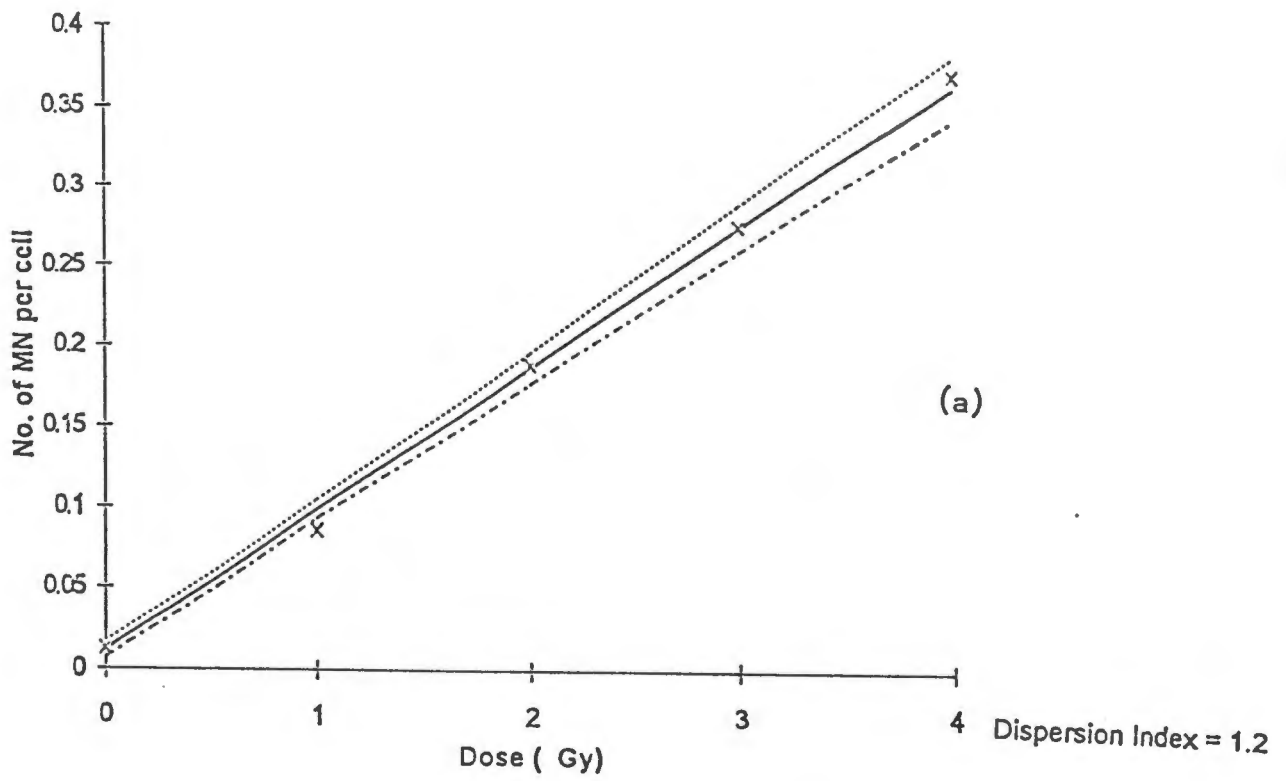


Fig 4.4 (a) & (b): Dose response curve fitted by maximum likelihood estimates (Broken lines indicate 95% confidence intervals)

7. Comparison of dose-response relationships

There are numerous descriptions of dose-response relationships with micronuclei as end-point described in the literature. Since the studies of Evans *et al.* (1959) many authors have shown that radiation induced micronuclei in stimulated peripheral blood lymphocytes is a sensitive biological indicator of chromosomal damage (Countryman and Heddle, 1976; Fenech and Morley, 1985; Huber *et al.*, 1983; Pincu *et al.*, 1984; Ramalho *et al.*, 1988). However, comparing the type of dose-effect relationships reported in the literature extensive interlaboratory differences appear.

The relationships have been described as linear (Huber *et al.*, 1983; Bauchinger, 1984; Pincu *et al.*, 1984; Fenech *et al.*, 1985; Fenech *et al.*, 1986; Fenech *et al.*, 1989; Fenech *et al.*, 1990; Balasem *et al.*, 1992 and Slabbert 1993.), linear-quadratic (Bettega *et al.*, 1980; Huber *et al.*, 1983; Krepinisky and Heddle, 1983; Almassy *et al.*, 1986 a,b; Almassy *et al.*, 1987; Kormos and Koteles, 1988; Prosser *et al.*, 1989; Littlefield *et al.*, 1989 and Gantenberg *et al.*, 1991), or conforming to a power-law expression (Countryman and Heddle, 1976).

Prosser *et al.* (1988) have demonstrated, what may be seen as a compromise, i.e. that up to 1 Gy of exposure the frequency of induction of micronuclei is linear-quadratic and beyond 1 Gy it remains virtually linear.

The results of the various workers can be compared to assess the suitability and relative accuracy of dose assessment using this technique. When these different relationships are translated mathematically to the absolute number of micronuclei induced per unit dose, differences up to a factor of two or more appear. Moreover, the response seemed to vary with the type of radiation and the time of culturing (Huber *et al.*, 1983).

A disturbing feature was the difference in micronuclei yields reported by two laboratories that shared aliquots of the same samples of blood (Lloyd *et al.*, 1991). Interlaboratory disagreements apparently resulted from differences in technical procedures and different criteria for the identification of micronuclei.

Several research groups report that an increase of donor age resulted in an increase of the spontaneous incidence of micronuclei (Fenech and Morley, 1986; Huber *et al.*, 1989), which could also be partly responsible for the observed difference in micronuclei yield. Thierens *et al.* (1991) stated unequivocally that the low accuracy of the dose assessment in the dose range up to 1 Gy and the detection limit of 30 cGy, in this range could be attributed to the poor statistics for the number of micronuclei and to the variability of the spontaneous incidence of micronuclei within a heterogeneous population. This phenomenon would only make a difference in the very low levels of the yield of micronuclei induced by relatively low doses of radiation, but will hardly be of any importance with doses expected in screening programs following a radiation incidence exceeding 1 Gy.

The above authors came to the conclusion that a higher degree of international co-operation and standardisation of the CB micronucleus assay was required to eliminate the reported differences. Attempts in this regard had been made earlier, for example, by substituting microscopic observation with flow cytometric analysis (Nüsse and Kramer, 1984) and computerised image analysis (Callisen *et al.*, 1984). It was expected that automation would reduce statistical uncertainty, and also improve the sensitivity of the micronucleus test, simply because of the possibility of screening many thousands of cells in a very short time. By scoring for instance 4000 - 5000 cells, Fenech and Morley (1986) showed that doses as low as 5 cGy could be detected with this technique.

Despite the fact that efforts to standardise the technique were started more than a decade ago, the differences in the results of more recent studies still point to the need that interpretation of micronuclei yields in terms of dose should be done using *in vitro* calibration curves constructed in the same laboratory where the dose determination is to be done.

In more recent studies (similar to the present one) the linear dose-effect relationship was confirmed, which is in contrast to the linear-quadratic relationship usually observed for chromosome-type aberrations (IAEA, 1986). Consequently it was decided to compare some of the available linear dose-response data obtained with similar experimental protocols,

to that of the present one, with the results obtained in this study. For this purpose data given by the following authors were used, namely Fenech *et al.* (1985); Fenech *et al.* (1986); Fenech (1989); Balasem *et al.* (1992); and Slabbert *et al.* (1993). The data was first mathematically translated to induced micronuclei per 500 CB cells for different exposure values in cGy.

Linear-regression analysis of these data indicated that the relationships could be described by the following equations:

$y=0.50 (\pm 0.20)D - 0.28 (\pm 3.27)$	with $r^2 = 0.97$	Fenech <i>et al.</i> (1985)
$y=0.40 (\pm 0.03) D + 4.85 (\pm 0.58)$	with $r^2 = 0.88$	Fenech <i>et al.</i> (1986)
$y=0.38 (\pm 0.05) D + 24.84 (\pm 10.43)$	with $r^2 = 0.61$	Fenech <i>et al.</i> (1989)
$y=1.10 (\pm 0.02) D + 6.39 (\pm 0.32)$	with $r^2 = 0.99$	Balasem <i>et al.</i> (1992)
$y= 0.52 (\pm 0.02) D+ 4.26 (\pm 4.14)$	with $r^2 = 0.99$	Slabbert (1993)
$y= 0.45 (\pm 0.01) D + 2.74 (\pm 2.85)$	with $r^2 = 0.99$	This study.

By means of an analysis of covariance it was tested whether the six regression equations, based on the six different sets of data significantly differ from each other. In the first stage of the analysis a test was performed to determine if the regression coefficients differ significantly, and it was found that the null-hypothesis of equal regression coefficients could not be discarded at a 5% significance level.

In the second part of the analysis a common gradient was assumed and a test was performed to determine if the y- intercepts differ significantly. Once again the null-hypothesis could not be discarded ($p < 0.05$).

Because of the generally poor statistics at low doses the results of curve fitting sometimes lead to negative estimates of the background value which obviously have no biological basis. Opinions vary as how to treat the background level of micronuclei in fitting dose response data. Some investigators resolve this problem by ignoring zero-dose data points and constraining the curve to pass through the origin. There are, however, sufficient data published from surveys of subjects exposed only to background radiation to show that there is a marked positive background level of micronuclei. Ideally a laboratory should generate its own background data.

In conclusion, however, it may be considered that all six regression lines are in fact representative of the same universal set. In view of the diversity in the dose-response relationship (measured in terms of micronuclei induced in CB cells) described in the literature, it was reassuring to find agreement between six different sets of data. The induced micronuclei frequency determined in this study could therefore be substituted with confidence in the equations for calculating the radiation dose equivalent due to partial body irradiation, which will be described in the following chapters.

-----00-----

CHAPTER V

PHYSICAL ESTIMATE OF THE EQUIVALENT WHOLE-BODY DOSE OF DONOR PATIENTS FOLLOWING FRACTIONATED PARTIAL-BODY RADIOTHERAPY

1. Introduction

Experience over more than 25 years has shown that most suspected overexposure incidents have involved just one, or occasionally a few subjects, usually inhomogeneously exposed. Such incidents involve either chronic or fractionated exposure. When considering the use of MN evaluations as a tool for biological dosimetry this as well as the question whether there are any differences between the dose-response relationships after *in vitro* and *in vivo* exposure of lymphocytes, should be considered.

Since there is no significant difference between the dose-response curve for chromosome aberrations in lymphocytes obtained following *in vivo* whole-body irradiation and that obtained following *in vitro* irradiation of lymphocytes (Buckton *et al.*, 1971; Clemenger and Scott, 1971 and Fabry and Lemaire, 1986) it is reasonable to expect the same for micronuclei which are derived from chromosomal aberrations. Gantenberg *et al.* (1991) found that there was indeed no statistically significant difference between *in vitro* and *in vivo* results obtained with MN. However, the results observed for whole-body irradiation do not necessarily apply to fractionated partial-body exposures. In the latter case there is usually sufficient time between radiation fractions for the lymphocytes to redistribute themselves either within or outside the radiation field, which could influence the distribution of damage within the lymphocyte pool. This and other considerations such as interphase cell death, have to be taken into account in applying the cytokinesis-blocked (CB) micronucleus assay for dosimetric purposes.

Although several laboratories have reported data on *in vitro* MN dose response, there have been few reports of MN evaluations in persons who have received radiation exposure (Fenech *et al.*, 1990). In these *in vitro* experiments, for each dose, volumes of irradiated and unirradiated blood from the same donor were mixed in differing proportions in order

to simulate the mixing of blood that would occur after acute partial-body irradiation (Lloyd *et al.*, 1987; Lloyd *et al.*, 1991 and Vral *et al.*, 1992). It is therefore a more simplified situation than that which applies *in vivo* where the distribution of doses over the body would form a continuum rather than two homogeneous fractions. The simplification also has taken no account of the distribution and circulation characteristics of lymphocytes or any other physiological processes that may operate on lymphocyte populations *in vivo*. Nevertheless, these experiments demonstrated that it should be possible to use biological dosimetry to give a more refined indication of a high, but non-uniform irradiation, over and above quoting an estimate of averaged equivalent whole-body dose. Lloyd *et al.* (1991) stated that *in vivo* studies of partially or inhomogeneously irradiated persons are necessary as a follow up to the *in vitro* work on which his group reported.

2. Objective

The primary objective of the present work was to follow up the above-mentioned recommendation of Lloyd and his co-workers (1991). For this accurate physical dosimetry of the irradiated persons is necessary, to calculate an equivalent whole-body dose which could serve as reference to determine the validity of the biological measurements. Consequently the first objective was to determine integral doses to irradiated sites from computer-derived dose-volume histograms, and to use these doses to calculate the appropriate equivalent whole-body doses.

3. Selection of Patients

To evaluate the use of the CB micronucleus method for radiation dosimetry following *in vivo* exposure of lymphocytes, a similar study was designed to that described by Buckton (1983) for irradiated ankylosing spondylitis patients. The main difference between the present study and that of Buckton (1983) was that the patients had a variety of tumour sites and integral doses.

The project was approved by the Ethics Committee of Tygerberg Hospital and the informed consent of each individual patient selected was obtained.

Eight patients with different types of cancer were studied prospectively. As a first approach it was decided to perform the MN assay on 2 patients who received a single-dose of half-body irradiation. This would exclude

complications for which dose fractionation could possibly be responsible and should provide an equivalent whole-body dose of approximately 3 to 4 Gy.

Two patients treated for multiple metastases of prostate cancer were selected. It was not possible to find a sufficient number of binucleated cells for assay in lymphocyte cultures of blood samples obtained from these patients after exposure. This was probably due to prior treatment and medication that accompanied half-body irradiation of the patients (see Chapter VI).

Six patients with cancer undergoing fractionated partial-body irradiation were therefore selected on the following basis :

- (a) that they had not undergone any previous radio- or chemotherapy;
- (b) to represent relatively large irradiated volumes and consequently a wide range of integral doses;
- (c) that they would survive treatment; and
- (d) that the treatment plan consists of single planar fields.

In four patients conventional radiotherapy was carried out according to a standard protocol of fractionated daily irradiation over a period of 5 to 6 weeks. It was planned to collect blood samples just before radiation treatment, 24 h after completion of the first treatment and thereafter once weekly for 4 consecutive weeks during treatment. The experimental protocol was limited, to the first 4 weeks of treatment, because beyond 4 weeks of treatment, patients may receive a blood transfusion and/or, the treatment plans are modified, due to tumour shrinkage.

Two patients were planned to receive palliative treatment, in 3 fractions over a period of 3 weeks. It was not possible to uphold the treatment protocol for these patients because both of them died within a few days after receiving the first dose fraction. However, the single blood samples obtained from both of these, 24 h after the first treatment, is nevertheless of importance for the previously mentioned refined calculations regarding partial-body exposure (see Chapter VI section 3.2).

4. Details regarding individual patients

In the following sections the relevant clinical history, treatment plans and other details regarding the radiation treatment of the individual donor patients are given.

4.1 Patient : a

This patient was treated for cancer of the prostate with a 16 MV linear accelerator. A total tumour dose of 64 Gy was given in 32 fractions at a dose rate of 2.5 Gy min⁻¹. The treatment plan included 4 radiation fields as indicated in Fig 5.1.

4.2 Patient : b

This patient had stage 3B cancer of the cervix and was given a total tumour dose of 50 Gy with a 8 MV linear accelerator. The dose was administered in 25 fractions at a dose rate of 2.2 Gy min⁻¹. The treatment plan was a combination of 4 radiation fields as indicated in Fig 5.2.

4.3 Patient : c

This patient received treatment for a carcinoma of the bladder. A total dose of 59.4 Gy was given in 31 fractions at a dose rate of 2.2 Gy min⁻¹. The treatment is the product of four 8 MV photon beams as illustrated in Fig 5.3. This patient received a blood transfusion and the treatment plan was also modified at the end of the fourth week of treatment, however the last blood sample was collected before the blood transfusion.

4.4 Patient : d

This patient was treated for a stage 3B cancer of the cervix. A total tumour dose of 50 Gy was administered in 25 fractions at a dose rate of 2.5 Gy min⁻¹. A combination of four 16 MV photon beams were used, as indicated in Fig 5.4.

4.5 Patients : e and f

Both these patients were treated for cancer of the cervix, stages 3B and 4A respectively. They were treated with a Co-60 unit delivering a dose rate of $0.921 \text{ Gy min}^{-1}$. In both cases two direct opposing fields were used [(13 x 13) cm with a separation of 19 cm, and (14 x 13) cm with a separation of 20 cm respectively] as illustrated in Fig 5.5. The total tumour dose of 30 Gy was to be given in three equal weekly fractions of 10 Gy each.

5. Dose-volume histograms (DVH)

Significant development has occurred in the technology of treatment planning in recent years. From central axis isodose curve distributions providing single planar treatment plans in the past, the spectrum of available dosimetry modalities has grown to include a number of techniques which directly produce three-dimensional (3D) dosimetric maps tailored specifically to the research task at hand.

One such an alternative method to isodose distributions of displaying the results of dose calculations, is by the use of dose-volume histograms. Treatment plans represent dose calculations in a single plane whereas dose-volume histograms require dose calculations in three dimensions. Although the latter do not replace isodose distributions, they are very useful when used in conjunction with them, particularly when comparing different treatment plans for the same patient. A drawback is that they are very time consuming to calculate.

A histogram may be plotted according to the usual mathematical definition, as the accumulated volume of those elements receiving dose in a specified dose interval against a set of equispaced dose intervals. This is referred to as a differential dose-volume histogram (DVH). Such a DVH graphically summarizes the simulated radiation distribution within a volume of interest, or within the total irradiated volume of a patient, which would result from a proposed radiation treatment plan. In most instances, the volume is specified as the percentage of total volume receiving dose within each percentage dose interval; however, it may be more appropriate to specify absolute volume and dose in some cases.

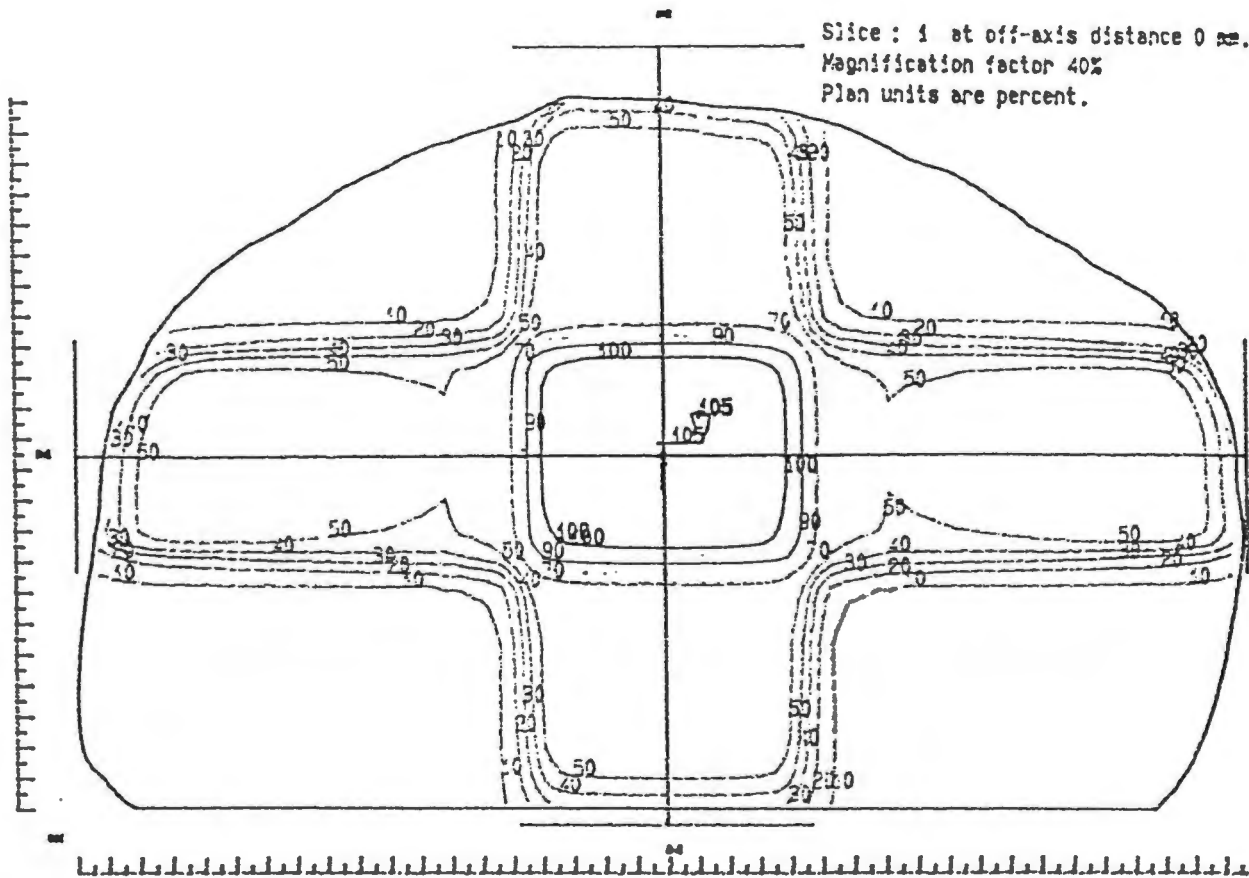


Fig 5.1 Treatment plan : a

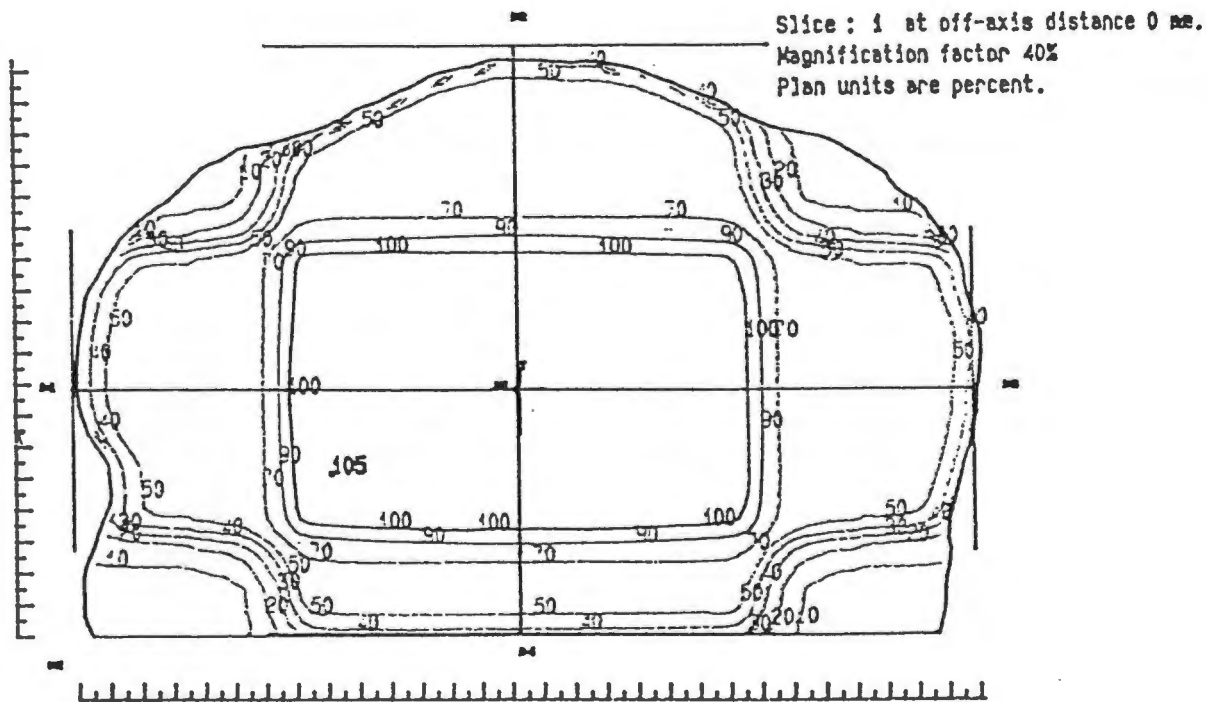


Fig 5.2 Treatment plan : b

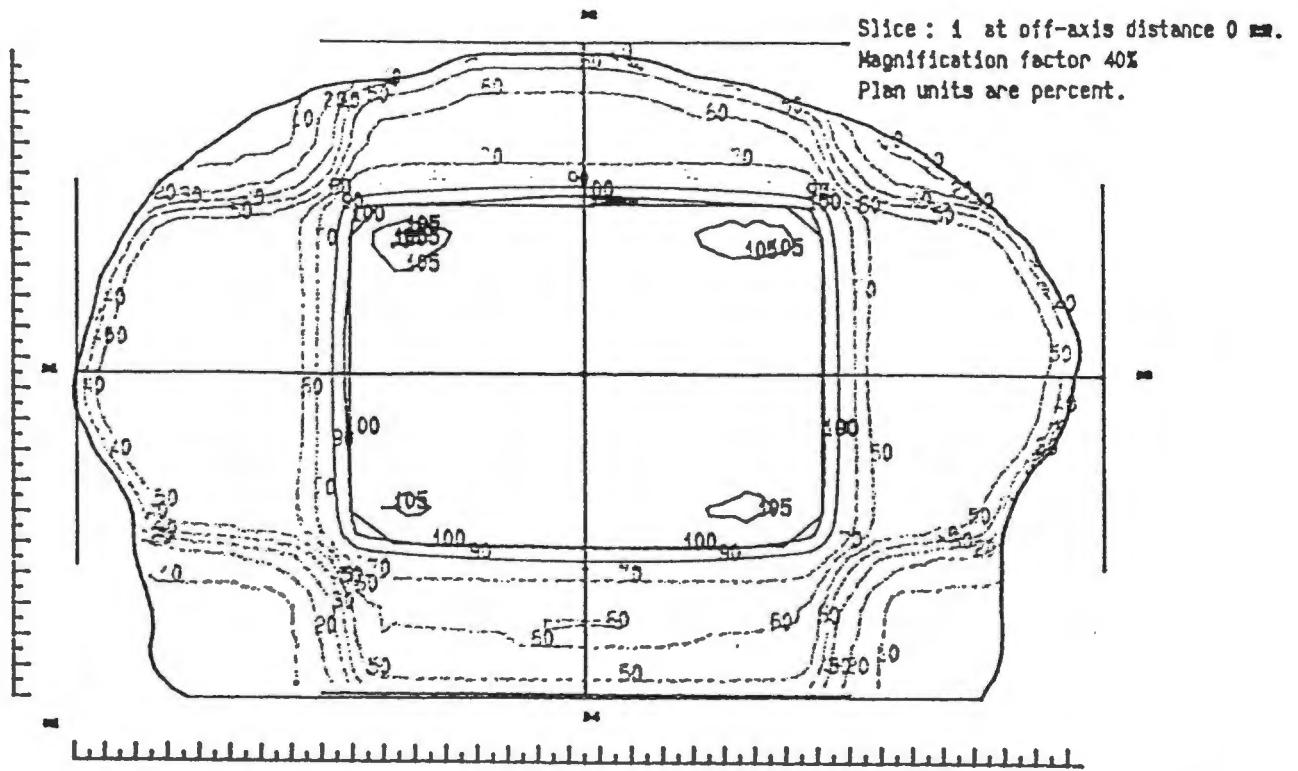


Fig 5.3 Treatment plan : c

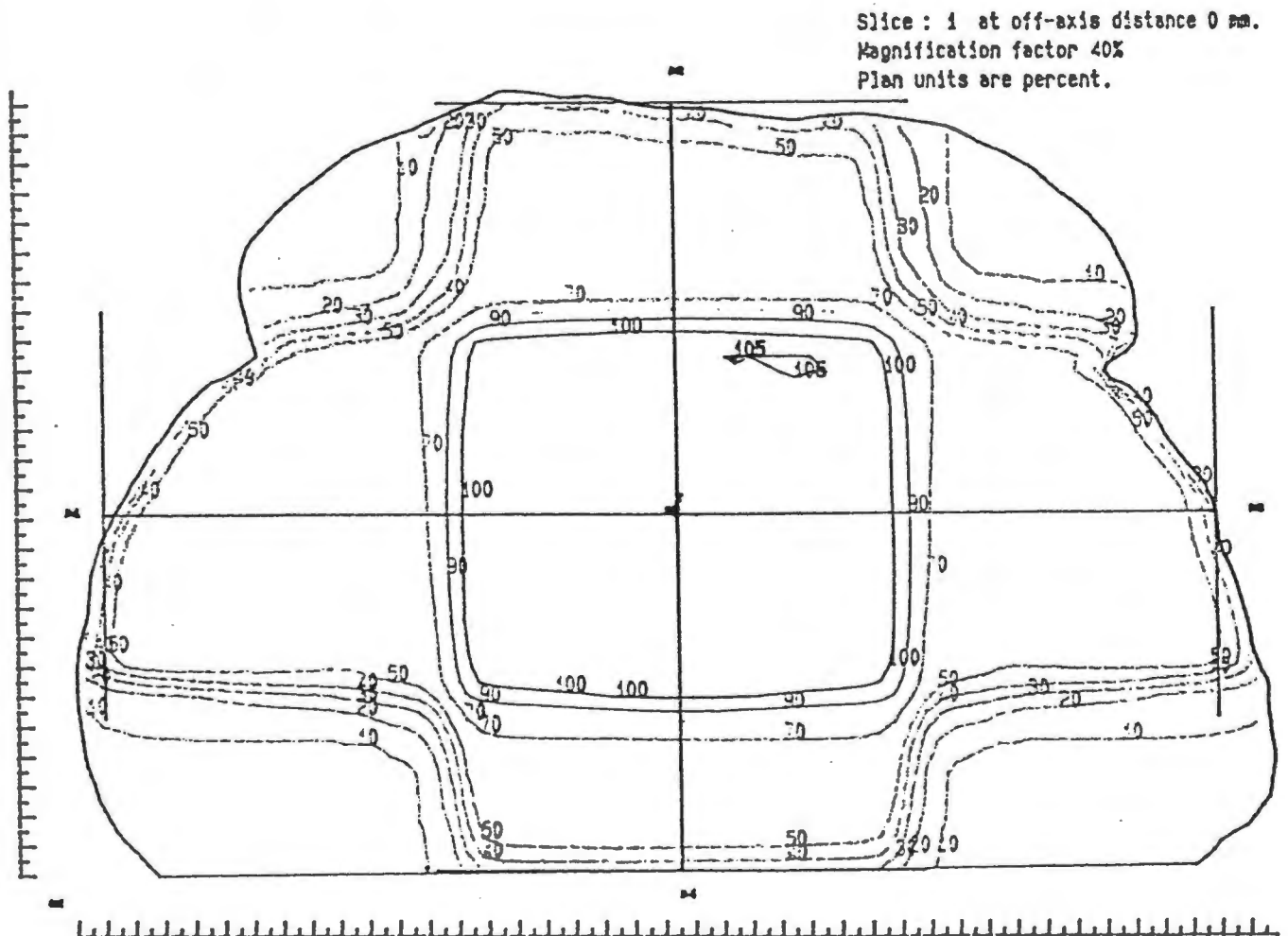
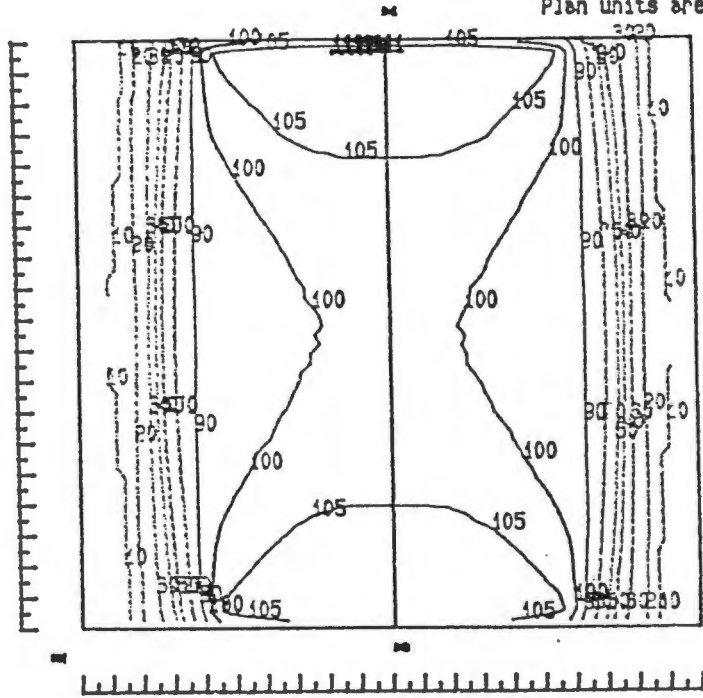


Fig 5.4 Treatment Plan : d

Slice : 1 at off-axis distance 0 mm.
 Magnification factor 40%
 Plan units are percent.



Slice : 1 at off-axis distance 0 mm.
 Magnification factor 40%
 Plan units are percent.

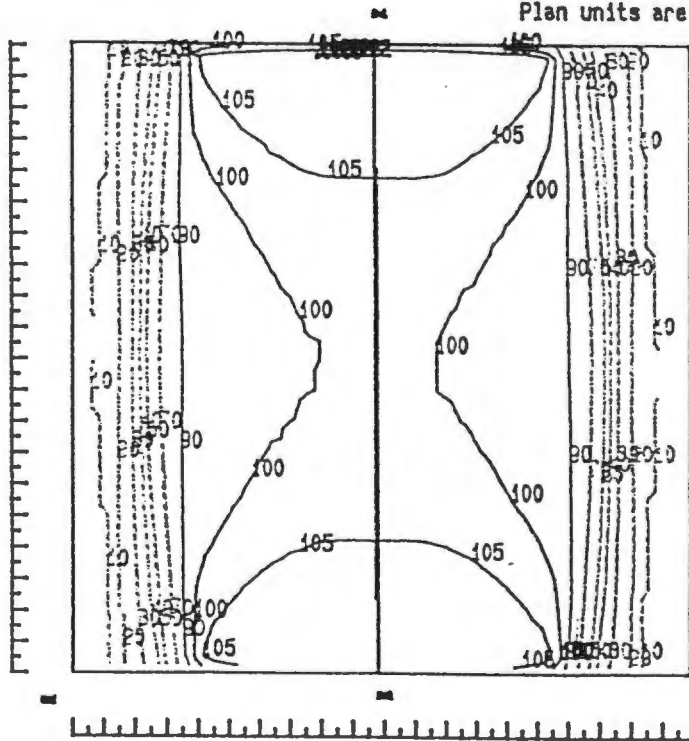


Fig 5.5 Treatment Plans : e and f

The use of DVHs as well as details of methods for their calculation has been described in the literature (Chen *et al.*, 1984; Drzymala *et al.*, 1987; Lyman, 1985; Mohan *et al.*, 1987; Kooy *et al.*, 1993; Webb, 1993.)

5.1 Method for generating and plotting DVHs

Initially, the boundaries of the relevant anatomical structure of the patient must be defined and the dose must be computed for the volume of interest (usually the tumour). The relevant anatomy is then subdivided into a volume grid of appropriate resolution. To arrive at the total volume of this anatomy or the volume of a particular structure within this anatomy, the relevant volume elements (voxels) are summed. Concurrently with voxel-summing the dose for each voxel can be determined so that, ultimately all contributing voxels are accumulated within the appropriate dose bin of the histogram. The bin values are then plotted.

5.2 Three-dimensional representation of the patient

The basic anatomical representation of the patient is provided by a contiguous sequence of computed tomographic (CT) scans. This volume may be visualized as being subdivided into a three-dimensional grid of cuboid volume elements which is equispaced in the X and Y directions but may or may not be equispaced in the Z direction. (In the present study, each CT slice lies at a constant Z). The volume of a voxel, therefore, is the product of the X and Y grid spacings and the slab thickness.

5.3 Computational accuracy

Computing a DVH involves three-dimensional volume and radiation dose matrices, which need not coincide. Dose calculations tend to be slow, consequently a relatively coarse matrix is usually employed, but the matrix required for accurate volume estimation may be of much finer resolution. The finer the grid, the greater the accuracy of dose

calculation, with the disadvantage, however of increased computation times and the creation of large disk files. The conflicting requirement of computing dose in a large total treatment volume yet with sufficient resolution to capture dose gradients that change within millimetres sometimes excludes computing dose values on a regular spaced grid. Such a grid would require on the order of 10^5 computations (10 cm^3 at better than 0.25 cm resolution). Goitein and Miller (1983) demonstrated, however that 2% dose accuracy could be achieved with a grid spacing of 5 mm.

5.4 Application of DVHs

Dose-volume histograms can be used for comparing alternative treatment plans for a specific patient or as input data to estimate tumour control probability and normal tissue complication probability. A plot of a cumulative dose-volume frequency (or DVH) could, however, also be computed which graphically represents the total radiation distribution within a patient's body. Such a plot can therefore be used to calculate the integral dose received by the patient.

The total energy absorbed from the beam by the patient is called the integral dose and was defined originally by Mayneord (1942). The integral dose to a mass of tissue is the product of the mass of tissue and the dose which it receives. Thus the unit of integral dose is the kg.Gy (i.e. $1 \text{ kg Gy} = 1 \text{ Joule}$).

The area under the DVH therefore represents the relative integral dose received by the patient.

6. Physical dosimetry results

A cumulative DVH has been computed by means of the "Target" (General Electric) non-coplanar radiotherapy treatment planning program for the total irradiated volume resulting from the treatment plan of each participating patient. Figures 5.6 through 5.11 are the graphic presentations of relative dose against volume for the various patients.

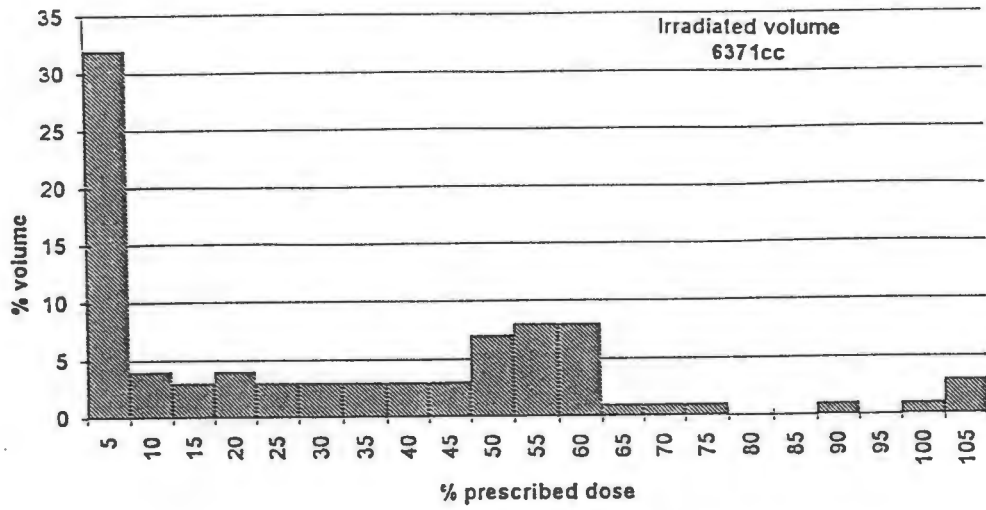


Fig 5.6 Histogram : a

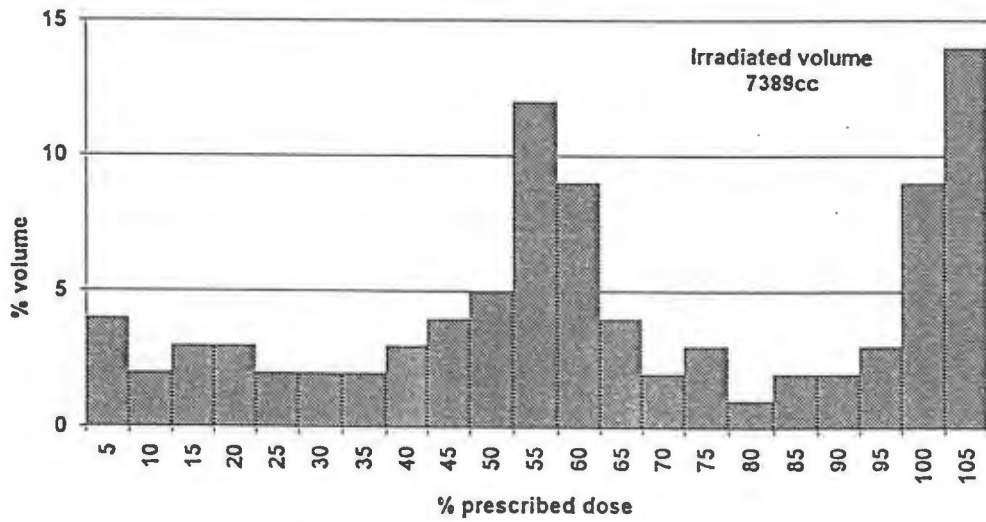


Fig 5.7 Histogram : b

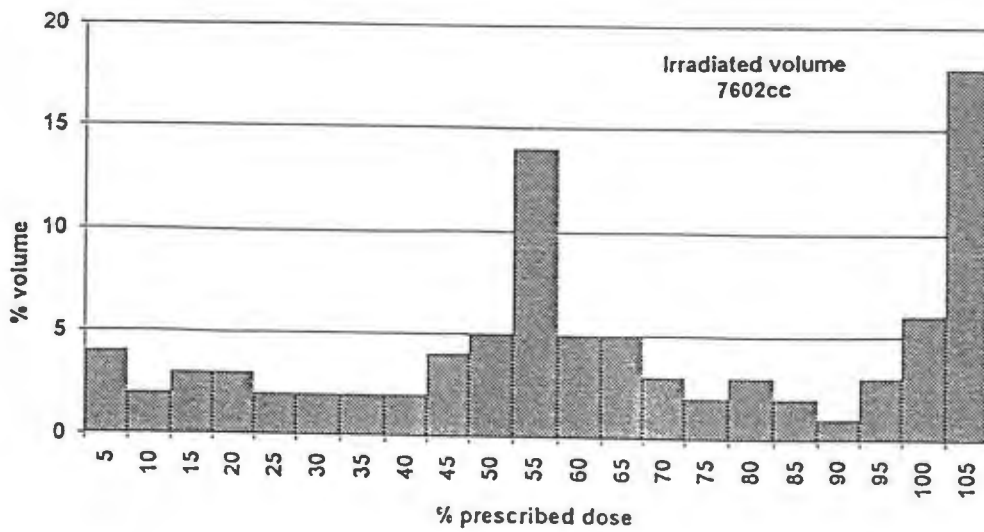


Fig 5.8 Histogram : c

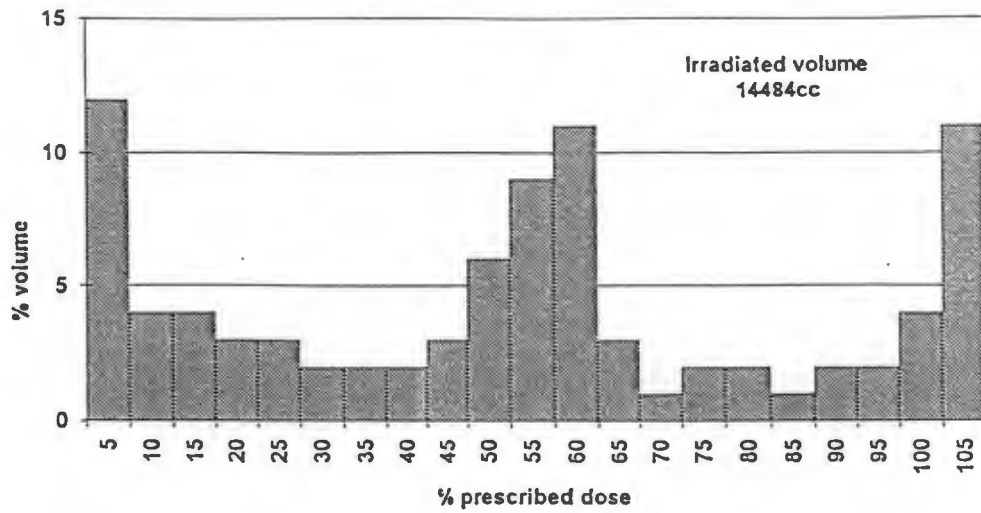


Fig 5.9 Histogram : d

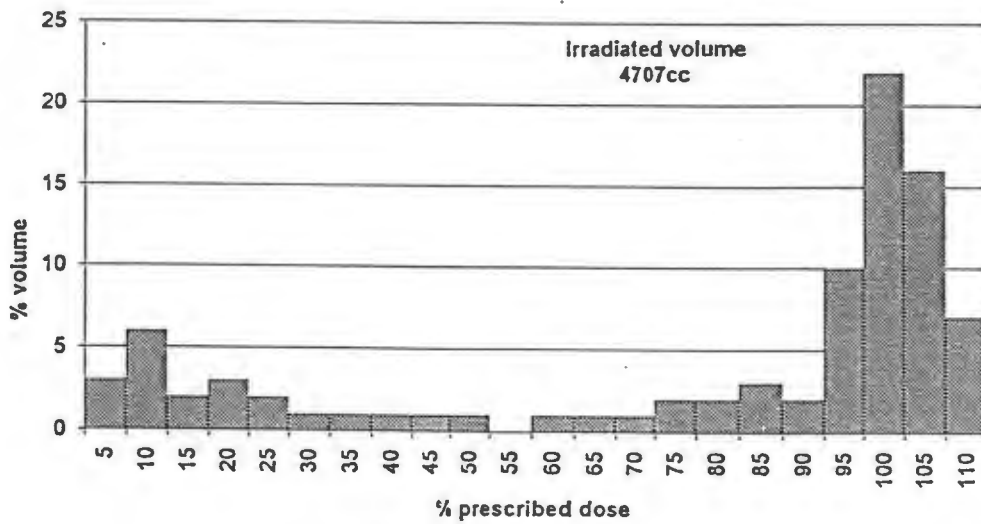


Fig 5.10 Histogram : e

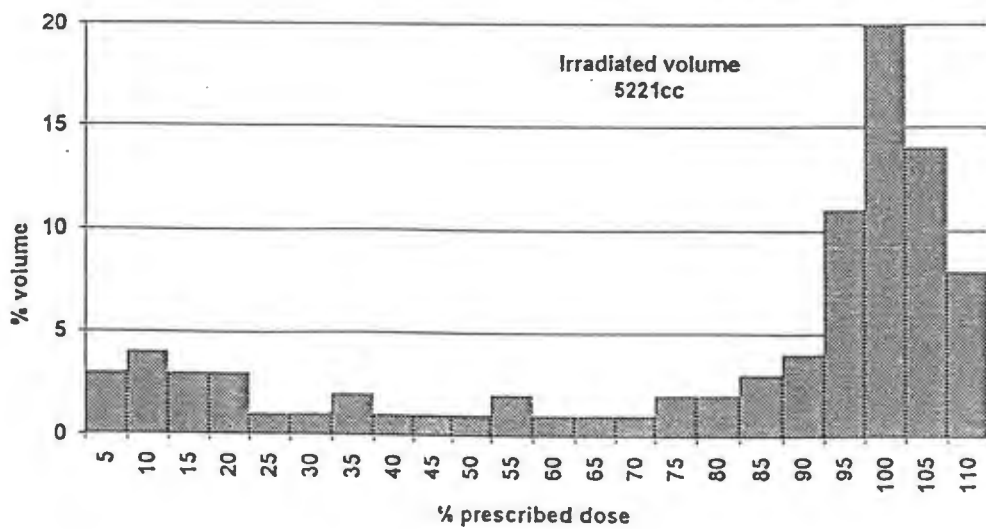


Fig 5.11 Histogram : f

It is convenient to use normalized relative values in these plots. For these computations X,Y and Z grid spacings of 5 mm, and dose intervals of 5% were used. Consequently for each histogram the dose was calculated at approximately 20 000 points providing an estimated dose accuracy of approximately 2%.

The relative integral dose is the sum of the products of the percentages of the volume irradiated and the corresponding dose frequencies (i.e. the different percentages with the relevant interval midpoints 2.5, 7.5 ---- - 97.5 etc.). The absolute integral doses were obtained by multiplying these figures with total irradiated volume and the applicable tumour doses given in Table 5.1

To derive an equivalent whole-body dose which could be related to dose estimates determined by the cytokinesis-blocked micronucleus assay for lymphocytes irradiated *in vivo*, use was made of the findings of Matsubara *et al.* (1974). In their extensive study of partial-body irradiation with varying single doses of gamma rays, they reported that the fraction of irradiated lymphocytes could be approximated by the volume of the irradiated site relative to body weight. This approximation, has to take into account some important variables (see 7 below).

In the present investigation, this approximation was applied, and an equivalent whole-body dose estimated by dividing the integral dose by the body weight of the patient. Full details of the tumour doses, integral doses and equivalent whole-body doses are listed in Table 5.1

7. Validity of the calculated equivalent whole-body doses

A comparison of whole-body dose estimates based on the MN frequency ratio in lymphocytes following fractionated partial-body radiation therapy with calculated whole-body doses from physical dose measurements will be valid with regard to the latter, if it is assumed that :

- (a) fractionation of radiotherapy results in a significant dispersal of the total dose over the lymphocyte pool;

Table 5.1 Physical dose calculations for donor patients

Patient related information	Tumour Dose (Gy)	Absolute Integral Dose (kgGy)	Equivalent Whole Body Dose (cGy)
Patient : a	2	3.33	4
Volume irradiated (cc) : 6371	10	16.64	18
Body weight (kg) : 90	20	33.28	37
Relative integral dose : 2612.5	30	49.92	55
	40	66.56	74
Patient : b	2	8.21	18
Volume irradiated (cc) : 7389	10	41.06	89
Body weight (kg) : 46.0	20	82.12	179
Relative integral dose : 5557.5	30	123.18	268
	40	164.24	358
Patient : c	2	8.57	15
Volume irradiated (cc) : 7602	12	51.43	93
Body weight (kg) : 55.4	19.8	84.86	153
Relative integral dose : 5637.5	28.8	123.44	223
	39.6	169.73	306
Patient : d	2	12.91	16
Volume irradiated (cc) : 14484	12	77.47	97
Body weight (kg) : 79.5	22	142.03	179
Relative integral dose : 4457.5	32	206.59	260
	42	271.15	341
Patient : e			
Volume irradiated (cc) : 4707	10	31.70	41
Body weight (kg) : 76.4			
Relative Integral dose : 6735			
Patient : f			
Volume irradiated (cc) : 5221			
Body weight (kg) : 81.8			
Relative integral dose : 6822.5	10	35.62	44

- (b) the variation in the concentration of lymphocytes within the different irradiated sites of patients is not significant; and
- (c) the densities of different tissues throughout the body are the same.

It must further be considered that there is also a variability in the proportion of lymphoid tissue and bone marrow such that the consequences of partial-body irradiation may be very site specific (Liniecki *et al.*, 1983; Savage and Breckon, 1985).

Antoine *et al.* (1981) and Deiner *et al.* (1988) suggested that exposure of a relatively small part of the body to a single fraction out of a series of fractions that make up the total fractionated treatment is unlikely to result in a uniform exposure of all lymphocytes. However, an entire course of fractions would result in a relatively uniform exposure over the lymphocyte pool.

With regard to the present study a further assumption is that the frequencies of the induced MN are independent of the photon energy and dose rate of the radiation inducing the MN. Although this is contrary to the results of some authors (Almassy *et al.*, 1987) it seems reasonable to accept these assumptions because :

- (a) there is no difference between the radiation weighting factors of the relevant radiations used (ICRP, 1990) and
- (b) the effect of various dose rates on the frequencies of MN was demonstrated only with low doses administered at extremely low dose rates (see Chapter VI).

CHAPTER VI

BIOLOGICAL DOSIMETRY : USING THE CYTOKINESIS BLOCKED MICRONUCLEUS ASSAY FOR QUANTIFYING THE DOSE TO RADIOTHERAPY PATIENTS WHO RECEIVED FRACTIONATED PARTIAL-BODY IRRADIATION

1. Introduction

Measurement of cytogenetic damage by simple techniques would be of great value in evaluating the radiation risk following fractionated partial-body, occupational or accidental exposure in circumstances when physical dosimetry was not available. The reliability of the conventional CB micronucleus assay for this purpose has never before been proved unequivocally. The findings of various authors (Littlefield *et al.*, 1991; Lloyd *et al.*, 1991 and Prosser *et al.*, 1989) suggest that in general the micronucleus assay does not give reliable estimates of equivalent whole-body dose as does chromosomal aberration analysis.

Accidental irradiation is usually due to a single inhomogeneous exposure. For highly irradiated patients the clinician requires information on the inhomogeneity of the exposure as management of casualties may require an early decision on whether treatment should include transplantation of allogenic bone marrow or treatment with growth factors. If there is a likelihood that some of the patient's own marrow will have survived because of inhomogeneous exposure, then transplantation should be avoided. Graft rejection was observed in several of the Chernobyl patients (Lloyd *et al.*, 1991) and in some cases probably contributed to their deaths.

Two methods have been proposed for analysing chromosomal aberration data from inhomogeneously exposed persons with the objective of improving the dose estimates to reflect non-uniformity, rather than quoting an averaged whole-body dose estimate that is obtained simply from relating a dicentric yield to an *in vitro* dose response curve.

The first method, known as the contaminated Poisson method was proposed by Dolphin (1969) and the second approach, termed the Qdr method, by Sasaki and Miyata (1968). It might be inferred that both these methods are applicable to MN data.

However, the Qdr method is based on the ratio of unstable chromosomal aberrations (dicentric and rings) consequently it is not applicable to MN data. The contaminated Poisson method is based on the overdispersed distribution of aberrations among the scored cells. This overdispersed distribution is considered to be the sum of a Poisson distribution which represents the irradiated fraction of the body and the remaining unexposed fraction. Micronuclei however have a natural underlying tendency for overdispersed distributions even with homogeneous irradiation. Consequently the assumption that the Poisson distribution applies to MN in cells which have been irradiated breaks down. Therefore the above methods do not unconditionally enable similar calculations for micronuclei as endpoints, but can possibly be modified to take the overdispersion into account.

With fractionated partial-body exposure a further problem arises which theoretically renders the dose estimate from MN data unsuitable for estimating the localized dose. Between dose fractions the lymphocytes mix thoroughly, so that after several fractions some cells have been irradiated once, some twice, some three times, etc. The population of lymphocytes is not a simple uniformly irradiated fraction, which is the basic assumption made when applying the contaminated Poisson method (Dolphin, 1969) and the Qdr method (Sasaki and Miyata, 1968).

2. Objectives

The objectives of the present study were the following :

- (a) To show that MN evaluations in patients who receive fractionated partial-body exposures can serve as a qualitative biological marker of their radiation exposures.

- (b) To determine whether MN analysis will yield statistically significant quantitative information on the radiation doses of these patients, with the objective of quoting averaged whole-body dose estimates obtained from relating MN yields to an *in vitro* dose response curve.
- (c) To attempt to confirm the findings of Gantenberg *et al.* (1991) that there is no significant difference between the induced MN frequencies obtained with *in vitro* and *in vivo* whole-body exposure respectively.
- (d) To derive a mathematical method for analysing MN data from persons having received a single inhomogeneous exposure, with the object of improving the dose estimates to take the fraction of the body irradiated and localized dose into account.

3. Experimental protocol

The experimental protocol was aimed to meet the objectives in 2 above following similar laboratory techniques as was used for the scoring of MN induced *in vitro*, as discussed in Chapter IV.

3.1 Laboratory procedure

Blood samples (10ml) were drawn from patients into heparinized tubes. In two patients receiving a single dose (6 Gy) half body exposure blood samples were collected before and 24 h after completion of radiation.

Blood samples were collected from the other six patients who were undergoing fractionated radiotherapy before and 24 h after their first exposure, and thereafter at weekly intervals for 4 weeks during the course

of treatment. Peripheral blood lymphocytes were isolated from the whole-blood sample and cultured using the technique described in Chapter IV section 3.3.2.

From each culture a minimum of four microscope slides were prepared and stained as described in sections 3.3.3 and 3.3.4 of Chapter IV. Following the set criteria for MN identification the MN frequency for any data point was determined by attempting to score at least 500 CB cells. For most of the data points, particularly at higher exposure values, it was necessary to scan all four slides to accumulate a sufficient number of CB cells.

3.2 Methods of calculation of the dose to the irradiated body fraction and the volume of this fraction

The two methods by which one may arrive at an estimate of the localized dose and irradiated body fraction have been reviewed in IAEA Report 260 (1986).

Qdr method. The Qdr method was proposed by Sasaki and Miyata (1968). This method assumes that all cells with unstable chromosome-type aberrations (dicentrics, rings and excess acentrics) were irradiated and it considers the ratio of dicentrics plus rings to the total number of damaged cells. This ratio, Q_{dr} can be related to dose by the following equation :

$$Q_{dr} = y_1 / 1 - \exp(-y_2) \quad (\text{Equ. 6.1})$$

where y_1 is the dose-response curve for dicentrics plus rings and y_2 the curve for total aberrations. In view of the dependance of the Qdr method on specific and total aberrations it is not suitable for application to MN data.

The contaminated Poisson method. The contaminated Poisson method described by Dolphin (1969) considers how the observed distribution of dicentrics deviates from the Poisson distribution that would be expected from a homogeneous exposure. It assumes that a fraction (f) of the scored cells have received a uniform dose and thus the distribution of aberrations among them is Poissonian. These are mixed with a fraction ($1 - f$) of

unexposed, and therefore aberration free, cells. Maximum likelihood estimates of f and the mean aberration yield (y) in this fraction are calculated by using equations 6.2 and 6.3 below. The mean dose to the fraction f may be obtained from y by reference to the dicentric dose-response curve.

$$y / 1 - e^{-y} = X/N - n_0 \quad (\text{Equ. 6.2})$$

$$yf = X/N \quad (\text{Equ. 6.3})$$

where N is the number of cells scored, X the number of dicentrics scored, and n_0 the number of cells free of dicentrics.

By applying a correction to fraction f to compensate for the selective loss of irradiated cells due to interphase death and mitotic delay, the originally exposed fraction of cells (F) can be calculated using equation 6.4, and this original fraction can be taken to indicate the proportion of the body exposed in an *in vivo* irradiation (IAEA 1986).

$$F = \frac{f/p}{1 - f + f/p} \quad (\text{Equ 6.4})$$

The surviving fraction (p) for the selective loss of cells is assumed to follow the equation :

$$p = \exp(-D/D_0) \quad (\text{Equ 6.5})$$

where D is the dose to the irradiated scored fraction (f) and D_0 the dose required to reduce the number of irradiated cells by a factor e due to interphase death. There is experimental evidence that the value of D_0 lies between 3 and 8 Gy as determined for *in vitro* irradiated lymphocytes (Lloyd *et al.*, 1991). It was decided to fix the value of D_0 at 8 Gy for the purpose of the present analysis because it was suggested that *in vivo* the lymphocytes may suffer less radiation induced interphase death than in *in vitro* (Lloyd *et al.*, 1991).

This technique applies very well to distributions of dicentric (Lloyd *et al.*, 1991). However if the technique is applied to a distribution of MN two problems arise. Firstly the unirradiated fraction has a level of MN which typically may be 1 or 2 in 100. These may not be negligible whereas for dicentric a typical background value is 1 in 1000 which can be ignored and a yield of zero assumed for the unirradiated fraction. Secondly because it is known that MN are overdispersed for whole-body exposure, as confirmed by the distributions at all the data points (except the zero dose value) shown in Table 4.3, (which may be considered equivalent to whole-body exposure) the assumption that the Poisson distribution applies to MN in cells which have been irradiated breaks down.

The negative binomial method. According to Lloyd *et al.* (1991) the deficiencies of the contaminated Poisson method may be overcome by assuming that the MN in the irradiated fraction follow a negative binomial distribution. To describe the overdispersion by the negative binomial two parameters are required, namely S and θ . θ is a dispersion parameter. The parameter $1/\theta$ is the ratio of variance to mean, so that θ must lie between 0 and 1 and will typically lie between 0.7 and 0.9 for MN (Lloyd *et al.*, 1991). The parameter S ought to be an integer but could be treated as a variable if the factorials are replaced by the appropriate Γ function. Lloyd and his co-workers (1991) derived the following maximum likelihood equations whereby the parameters f , θ and S can be solved :

$$\frac{\partial \text{Log } \mathcal{L}}{\partial f} = 0 \quad (\text{Equ 6.6})$$

$$\frac{\partial \text{Log } \mathcal{L}}{\partial \theta} = 0 \quad (\text{Equ 6.7})$$

$$\frac{\partial \text{Log } \mathcal{L}}{\partial S} = 0 \quad (\text{Equ 6.8})$$

(Where \mathcal{L} is the likelihood of the observations n_0, n_1, n_2 etc).

These equations were solved numerically by minimising routines developed by Edwards and Murray (personal communication) whereby $\text{Log } \mathcal{L}$ is minimised. The minimising routines, NAG routine EO4JBF and the

Levenberg-Marquardt were used for this purpose (Press W., 1986; Murray *et al.*, 1981). In an attempt to simplify the computation it was assumed that for the basic negative binomial the variance to mean ratio (1.25 in the present study) is fixed. When analysing the data, allowing all the parameters f , θ and S to float, it was not always possible to obtain a sensible solution. It was decided to fix the parameter θ at 0.8 for the purpose of the present analysis, the results of which are shown in Table 6.4. Having obtained the values of the parameters f , θ and S the mean yield of MN per cell could be calculated using equation 6.9 below (Lloyd *et al.*, 1991).

$$y = S (1 - \theta) / \theta \quad (\text{Equ 6.9})$$

By substituting y in the original, *in vitro* determined, dose-response curve, the dose D to the irradiated fraction, and the irradiated fraction F , was deduced.

4. RESULTS

4.1 Half-body irradiations

The lymphocyte cultures prepared from all four blood samples obtained from the two donor patients who received half-body irradiation failed. It was not possible to score 100 BNC on any four slides originating from either pre- or post-exposed blood samples.

4.2 Fractionated partial-body exposure

The average yields of MN and their intracellular distribution in BNC from blood samples obtained from six donor patients before treatment, and after administering different tumour dose fractions at regular time intervals, are

Table 6.1 Intracellular distribution of MN in CB lymphocytes of patients before and during radiotherapy. The dispersion index σ^2/\bar{y} and standard unit normal deviate (μ) are also listed

Equivalent whole-body dose (cGy) <i>(From table 5.1)</i>	Blood sampling intervals	MN distribution in BNC						Number of BNC observed	MN per 500 BNC (\pm 1SE)	σ^2/\bar{y}	μ
		0	1	2	3	4	5				
Patient : a											
0	Pre-irr	576	6					582	5.2 (\pm 2.1)	0.99	-0.16
4	24h	500	12	1				513	13.6 (\pm 3.6)	1.12	1.96
18	1w	574	18	2				594	18.5 (\pm 3.9)	1.15	2.58
37	2w	537	18	3	2			560	26.8 (\pm 4.9)	1.55	9.34
55	3w	521	20	6	3			550	37.3 (\pm 5.8)	1.66	11.07
74	4w	503	24	5	3	1		536	43.8 (\pm 6.4)	1.77	12.68
Patient : b											
0	Pre-irr	511	13					524	12.4 (\pm 3.4)	0.98	-0.38
18	24h	484	18	3				505	23.8 (\pm 4.9)	1.20	3.32
89	1w	506	32	6	2			546	45.8 (\pm 6.5)	1.39	6.52
179	2w	472	45	7	3	1		528	68.2 (\pm 8.0)	1.48	7.80
268	3w	391	44	9	5	2		451	94.2 (\pm 10.2)	1.66	9.99
358	4w	484	101	39	5	3	1	633	166.7 (\pm 11.5)	1.44	7.95
Patient : c											
0	Pre-irr	554	14					568	12.3 (\pm 3.3)	0.98	-0.40
16	24h	505	15	3				523	20.1 (\pm 4.4)	1.25	4.10
93	1w	444	31	6	1			482	47.7 (\pm 7.0)	1.30	4.68
153	2w	463	53	10	1	1		528	75.8 (\pm 8.5)	1.32	5.32
223	3w	476	59	16	2	1		554	91.2 (\pm 9.1)	1.37	6.26
306	4w	492	93	30	4	2	1	622	143 (\pm 10.7)	1.43	7.69
Patient : d											
0	Pre-irr	470	9					479	9.4 (\pm 3.1)	0.98	-0.27
16	24h	487	17	2				506	20.8 (\pm 4.5)	1.15	2.46
97	1wq	638	42	6	2			688	43.6 (\pm 5.6)	1.31	5.88
179	2w	535	50	10	3			598	66.1 (\pm 7.4)	1.35	6.11
260	3w	547	64	19	3			633	87.7 (\pm 8.3)	1.33	5.91
341	4w	529	79	33	11	3		655	145.0 (\pm 10.5)	1.59	10.81
Patient : e											
0	Pre-irr	489	9					498	9.0 (\pm 3.0)	0.98	-0.27
41	24h	652	12	5	2			671	20.9 (\pm 3.9)	1.75	13.92
Patient : f											
0	Pre-irr	424	8					432	9.3 (\pm 3.3)	0.98	-0.26
44	24h	401	13	3	1			418	26.3 (\pm 5.6)	1.49	7.34

given in Table 6.1. In this table tumour doses are substituted by the derived equivalent whole-body doses given in Table 5.1. To compare the distribution of MN among BNC to a Poisson distribution, [using Papworth's U test (Papworth, 1970)], the dispersion index (σ^2/\bar{y}) and the standard unit normal deviate of this ratio, i.e. the μ -parameter in Table 6.1, were calculated as shown in Chapter IV, section 5. As shown by others (Prosser *et al.*, 1988; Balasem *et al.*, 1993) and the *in vitro* results of this study (Table 4.3) MN were distributed in a non-Poisson manner. They were significantly overdispersed in all the data points with the exception of the zero-dose values. There was some indication of a dose dependence of overdispersion, however, this was not a consistent phenomenon throughout all the data, and appeared patient dependent.

4.2.1 Dose-response relationship of micronuclei in CB lymphocytes from patients receiving fractionated radiotherapy

To investigate whether the dose-response data of individual patients, given in Table 6.1 could be pooled, a linear regression model was fitted on the induced MN yield of patients a,b,c and d. Subsequently, by means of an analysis of covariance, it was tested whether the four regression coefficients and y- intercepts, (given in Table 6.2) significantly differ from each other.

Table 6.2 Regression coefficients and y- intercepts for induced MN yields in lymphocytes of patients receiving fractionated radiotherapy

Patient	Regression coefficient	y-intercepts
a	0,1058 (\pm 0,0126)	0,0148 (\pm 0,0031)
b	0,0743 (\pm 0,0071)	0,0261 (\pm 0,0073)
c	0,0800 (\pm 0,0037)	0,0250 (\pm 0,0033)
d	0,0690 (\pm 0,0057)	0,0214 (\pm 0,0060)

Standard errors are given in brackets

The null-hypothesis of equal regression coefficients and equal y- intercepts could not be discarded ($p > 0.05$). Consequently it was decided to pool the individual data sets in Table 6.1.

The overdispersion of the MN frequency distributions was taken account of as described in Chapter IV when the data were curve fitted by iteratively reweighted least squares (Papworth, 1975). A fit of the dose-response data to the equation $y = \alpha D + c$ obtained by reweighted analysis (using the SAS/STAT computer software package) yielded the following dose-response function :

$$y = 7.575 \text{ E-2 } (\pm 2.963 \text{ E-3}) D + 2.126 \text{ E-2 } (\pm 2.085 \text{ E-3}) \quad (\text{Equ 6.10})$$

(Standard errors are given in parenthesis)

The chi-squared value of 19.165, is approximately the same as the number of degrees of freedom 18, which points to an excellent fit.

4.2.2 Biological dose estimates

The relationship between measured doses of external radiation and the frequency of *in vivo* induced MN has been investigated (Fenech *et al.*, 1990; Gantenberg *et al.*, 1991). However, no study has been reported to date in which dose estimates were obtained by relating *in vivo* induced MN frequency to a previously *in vitro* determined calibration curve, with the objective of comparing the physically calculated, equivalent whole-body doses with corresponding biological estimates.

In the present study the biological estimates were calculated by substituting the *in vivo* data (MN frequencies) of Table 6.1 in the *in vitro* determined dose-response equation of Chapter IV, i.e.

$$y = 0.0872 (\pm 0.0027) D + 0.0116 (\pm 0.0024) \quad (\text{Equ 4.5})$$

The results are detailed in Table 6.3.

Table 6.3 Comparison of the physically determined equivalent whole-body doses with the biological dose estimates in patients undergoing radiotherapy

MN/BNC (E-2)	Physical dose (cGy)	Biological dose estimates (cGy)	MN/BNC (E-2)	Physical dose (cGy)	Biological dose estimates (cGy)
Patient : a			Patient : c		
1.04	0		2.46	0	15 (\pm 3)
2.72	4	18 (\pm 3)	4.02	16	32 (\pm 3)
3.70	18	29 (\pm 3)	9.54	93	96 (\pm 5)
5.36	37	48 (\pm 4)	15.16	153	160 (\pm 7)
7.46	55	72 (\pm 5)	18.24	223	196 (\pm 9)
8.76	74	87 (\pm 5)	28.62	306	315 (\pm 12)
Patient : b			Patient : d		
2.48	0	15 (+ 3)	1.88	0	8 (\pm 3)
4.76	18	41 (\pm 4)	4.16	16	34 (\pm 3)
9.16	89	92 (\pm 6)	8.72	97	87 (\pm 6)
13.64	179	143 (\pm 7)	13.22	179	138 (\pm 7)
18.84	268	203 (\pm 9)	17.54	260	188 (\pm 8)
33.34	358	369 (\pm 14)	29.00	341	319 (\pm 12)
Patient : e			Patient : f		
1.80	0	7 (\pm 3)	1.86	0	8 (\pm 3)
4.18	41	35 (\pm 4)	5.26	44	47 (\pm 4)

(Standard errors of biological estimates are given in brackets; physical doses are from Table 5.1)

4.2.3 Calculations of localized dose and irradiated body fraction

Contaminated Poisson method : Despite the discrepancies regarding the use of the contaminated Poisson method for calculating the irradiated body fraction, F and the localized dose, D to this fraction from MN data (see 3.2) as an exercise this method has been applied to the single exposure data points of patients e and f, as given in Table 6.4.

Negative binomial method : Applying the negative binomial method to the MN data obtained 24 h after the first exposures of patients a,b,c and d no solutions of the relevant equations were possible. Patients e and f received much larger single exposures and estimates of the localized dose, D and the irradiated body fraction, F were obtained (given in Table 6.4). This method was also applied to two sets of data points obtained after 4 weeks of fractionated exposure of patients b and c respectively.

Table 6.4 Estimates of localized dose D, irradiated fraction F, and integral dose ID, in patients having received radiotherapy

Physical parameters					
Patient	Weight (kg)	Vol. Irr (cc)	F	D (Gy)	ID(kgGy)
e	76.4	4707	0.06	10	31.70
f	81.8	5221	0.06	10	35.62
b	46.0	7389	0.16	40	164.24
c	55.4	7602	0.14	39.6	169.73
Biological estimates : Contaminated Poisson method					
Patient	D (Gy) ($\pm 1SE$)	F ($\pm 1SE$)	*f($\pm 1SE$)	ID (kgGy)	
e	9.31 (2.95)	0.15 (0.05)	0.05 (0.02)	35.57	
f	6.01 (2.62)	0.26 (0.08)	0.10 (0.03)	47.70	
Biological estimates : Negative binomial method					
Patient	D (Gy) ($\pm 1SE$)	F ($\pm 1SE$)	*f($\pm 1SE$)	ID (kgGy)	*s
e	12.10 (5.41)	0.13 (0.06)	0.03 (0.02)	28.66	4.25
f	5.91 (4.31)	0.16 (0.09)	0.08 (0.06)	39.64	2.10
b	17.49	0.88	0.46	370.30	6.15
c	15.92	0.85	0.44	387.50	5.60

* f = fraction of scored cells exposed

* s = binomial parameter

5. Discussion

5.1 Factors responsible for modification of the experimental protocol

Originally it was intended to perform the MN assay on approximately 15 patients who received single doses of half-body irradiation. However, after performing the MN assay in 2 patients who had received half-body radiation, it was noted that the number of micronuclei was too small to yield reliable results (Section 4.1). This may be caused by previous chemotherapy and post irradiation antiemetic therapy of the patients. This therapy included the administration of high doses of dexamethasone, a corticoid with a well-known anti-proliferative action.

As a second approach it was decided to select approximately 10 patients representing a range of relatively large irradiated volumes distributed throughout the body. Amongst these were 4 patients treated for advanced carcinoma of the breast. These were subjected to multi-planar treatment plans for which the required dose-volume histograms could not be calculated with the existing computational facilities. Consequently the breast patients had to be rejected.

Finally only the data obtained from 6 patients, selected according to the criteria given in Chapter V, Section 3, were used for MN analysis. Obtaining the data was a time consuming process which extended over more than two years and consequently precluded the inclusion of more patients in the study.

5.2 MN in unexposed human lymphocytes

The MN frequency in lymphocytes of patients, not exposed to irradiation given in Table 6.1, falls within the normal range described in previous studies (Fenech and Morley, 1986). However, the pretreatment baseline frequencies of the patients are not only higher than that of the healthy donors but there is also a much greater interindividual variability. When the average age of the patients tested in this study (70 years, ranging from 54 to 86 years) is compared with the average age of healthy donors (38 years ranging from 28 to 57 years) the difference in baseline MN frequency can most likely be attributed to age dependence (Norman *et al.*, 1985; Fenech and Morley, 1986 and Thierens *et al.*, 1991).

Other factors may also play a role in MN frequency. The results of Xue *et al.* (1992) show that smoking significantly increases MN frequency in lymphocytes as compared with healthy non-smokers. Furthermore alcohol significantly increases and tea decreases the MN frequency induced by smoking. It was also suggested (Gantenberg *et al.*, 1991) that the cancer itself could be responsible for a higher MN baseline frequency. It is important to note that Fenech *et al.* (1985) could find no evidence that the Cyt- B method in itself caused any increase in micronuclei.

It was shown by Thierens *et al.* (1991) that for *in vitro* irradiation of whole blood, doses lower than 0.3 Gy could not be unequivocally detected with the MN assay due to the variability of the baseline MN frequency within the donor population. The latter as well as the interindividual variability reduces the low-dose sensitivity of the test when, as usually will be the case, a population control value rather than an individual pre-irradiation frequency must be used. However with regard to accidental exposure where there is a large number of potentially exposed people, the initial requirement is to identify those who have received a large, possibly life threatening dose, so that treatment can be concentrated where it is most needed. In this case these inaccuracies of the MN test for detecting low doses are not important and are outweighed by the potential for rapid analysis afforded by the assay. Indeed, Thierens *et al.* (1991) have shown that the uncertainty of the dose is not seriously reduced at higher dose levels. On the other hand if the individual pre-irradiation baseline value is known, the absorbed dose could be deduced from the number of radiation induced MN instead of the total number of MN, thereby eliminating the variation in the baseline frequency within a normal population.

5.3 MN yield as qualitative biomarker of radiation exposure

The observed MN yield per 500 binucleated cells for measurements performed at different tumour doses are illustrated in Fig 6.1. For each of the four patients a pronounced dose-dependent increase in MN yield was observed. Consequently these findings indicate that MN evaluations in persons who receive fractionated high-dose, localized exposures can serve as a qualitative biomarker of radiation exposures.

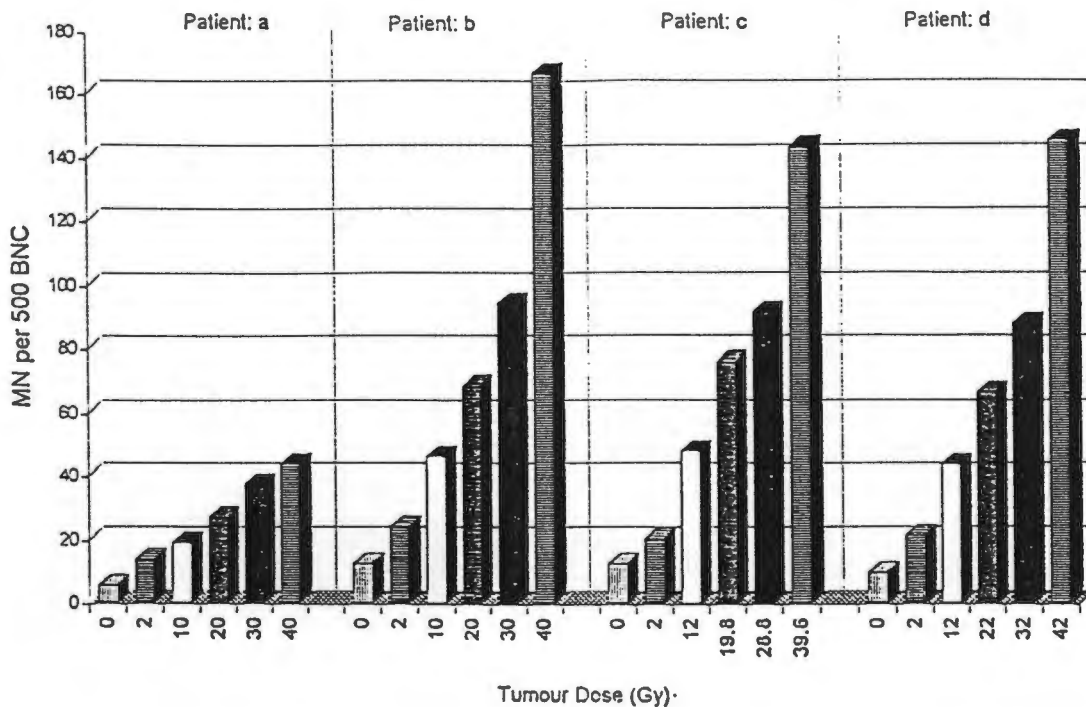


Fig 6.1 Dose-response relationship of MN in binucleated lymphocytes (BNC) exposed *in vivo* in patients receiving fractionated radiotherapy.

In principle the extent of MN induction should be similar in patients irradiated at the same site with similar integral doses. This was found to be the case with patients c and d of whom the integral doses were approximately the same.

5.4 MN in human lymphocytes irradiated *in vitro* and *in vivo*

Another requirement which the MN assay has to meet to be useful as biological dosimeter lies in the answer to the question as to whether there are differences in the dose-response relationship following *in vitro* and *in vivo* exposures.

Since there is no significant difference between the *in vitro* and *in vivo* radiosensitivity of human lymphocytes when chromosomal aberrations are

used as a measure of sensitivity (Buckton *et al.*, 1971; Clemenger and Scott, 1971) it is reasonable to expect the same for micronuclei, which are derived from chromosomal aberrations. Gantenberg *et al.* (1991) found that comparison of *in vitro* and *in vivo* results points to a slightly smaller increase in micronuclei after *in vivo* exposure, however there was no statistically significant difference.

A dose-response relationship was estimated from the pooled data of all the patients given in Table 6.1. It was assumed that :

- (a) fractionation of radiotherapy resulted in a significant dispersal of the total dose over the lymphocyte pool and,
- (b) the variation in concentration of lymphocytes within the different irradiated sites of the patients was not significant.

The data were fitted by the iteratively reweighted least squares method to derive maximum likelihood estimates of the parameters α and c in the equation $y = \alpha D + c$. These parameters as well as those previously obtained from *in vitro* exposure of donor blood samples are given in Table 6.5.

Table 6.5 Dose coefficients and y- intercepts obtained with in vitro and in vivo exposure, with standard errors in brackets.

Exposure	Coefficient (α)	y-intercept (c)
In vitro	0.0872 (\pm 0.0027)	0.0116 (\pm 0.0024)
In vivo	0.0757 (\pm 0.0030)	0.0213 (\pm 0.0021)

It was found that there was no statistical reason to discard the null-hypothesis of equal regression coefficients. However the hypothesis that the y- intercepts are the same should be discarded at the 5% confidence level. This difference might be expected, as the pre-treatment baseline micronucleus frequencies of the patients were higher than that of the healthy donors.

As reported by Gantenberg *et al.* (1991), the dose coefficient obtained with *in vivo* exposure was slightly lower than that from *in vitro* exposure, but the difference was not statistically significant. Gantenberg and his co-workers present as explanation for this difference the disparity in the dose rates of their *in vitro* (1Gy min^{-1}) and *in vivo* (0.033 Gy min^{-1}) exposures. The dose rates used in the present study were low for *in vitro* exposures (0.88 Gy min^{-1}) and high for *in vivo* exposures (2.5 Gy min^{-1}). It is suggested that two other explanations exist for the gradient of the dose-response curve obtained from *in vivo* exposures being lower than that obtained from *in vitro* exposures.

Firstly, the *in vitro* exposures were administered in a single exposure, compared to the conventional radiotherapy protocol of fractionated exposure. Radiation induced MN are mainly acentric chromosomal fragments from the cell nucleus, which are observed in the cytoplasm (Streffer, 1993). In several studies (ICRP, 1990) it was shown that the probability for chromosomal repair, particularly at higher doses when a damaged cell may be expected to contain two or more acentric fragments, was much higher with dose fractionation than with single exposures. This will have the effect of depressing the observable MN yield, resulting in a lower gradient of the dose-response curve with *in vivo* exposures.

Secondly, in the case of *in vivo* exposure, evaluation was difficult, because fewer binucleated cells were obtained, and the quality of the cells was inferior to that of cells from the *in vitro* exposures. This was probably due to medication that accompanied the radiation treatment of patients and possibly the disease itself. This phenomenon was also perceived by Gantenburg *et al.* (1991). Consequently the dose-response relationship obtain from the patient data, compared to that obtained from healthy donor data, may then be considered less reliable for dosimetry purposes.

5.5 Application of the *in vitro* results for *in vivo* dosimetry

The major objective of this study was to evaluate the usefulness of the MN assay as an *in vivo* dosimetry system for fractionated partial-body exposure. The equivalent whole-body dose estimates and the corresponding doses obtain from physical measurement and calculation are given in Table 6.3. To compare the two sets of data, whole-body dose estimates below approximately 40 cGy were discarded because :

- (a) the relatively high pre-treatment baseline, and interindividual variability of the MN frequency, renders the low dose estimates unreliable and,
- (b) at the 95% confidence level, doses lower than 30 cGy can not be unequivocally detected with the CB micronucleus assay due to the poor statistical accuracy when scoring approximately 500 cells (Thierens *et al.*, 1991).

In order to compare the two sets of doses (biological [BD] and physical [PD]) the conventional linear model was fitted to the data of individual patients. This was followed by a covariance analysis to determine if data of individual patients could reasonably be pooled to provide a single model from physical measurements to biological measurements.

The y- intercepts (β), and gradients (α), obtained from the linear regression model, $BD = \beta + \alpha (PD)$ are given in Table 6.6.

Table 6.6 y-intercepts and regression coefficients, with standard errors, for individual patients

Patient	Coefficient (α)	y-intercept (β)
a	1.06 (\pm 0.07)	10.1 (\pm 3.5)
b	0.99 (\pm 0.22)	-20.6 (\pm 52.9)
c	1.03 (\pm 0.15)	-5.3 (\pm 31.2)
d	0.92 (\pm 0.17)	-18.1 (\pm 40.9)

Data given in Table 6.6 indicated that the null-hypothesis of equal y-intercepts and equal regression coefficients could not be discarded.

A linear regression analysis of the pooled data of all the patients (excluding the very low dose data points) indicated that the relationship between the two sets of doses could be described by

$$BD = 0.896 PD + 6.258 \quad (\text{Equ 6.11})$$

The linear regression fit representing this expression is given by the solid line in Fig. 6.2. None of the points deviate appreciably from the regression line. Only one point (the second last point of patient d) deviated with approximately two standard deviations from the line. This was insufficient to discard this point.

Subsequently it was investigated if the intercept could be zero. It was found that such a hypothesis could not be discarded. When it was assumed that the line must go through the origin, the following expression was obtained :

$$BD = 0.936 PD \quad (\text{Equ 6.12})$$

The main reason for this comparison was to determine if the biological estimates fell within the statistical variation of the physical measurements. The results of the study suggested that the biological estimates were slightly low. There is, however, inadequate statistical evidence to discard the null-hypothesis that the gradient of Equ. 6.12 is equal to one. (The Bland and Altman statistical procedure yielded $p=0.1177$, and the 95% confidence limits for the mean difference between the two measurements ranged from -24.20 to 2.97. This interval includes the 0 value and this confirms the hypothesis).

There are no precise criteria for deciding on the accuracy that a biological estimate of dose needs to achieve. Generally, biological data carry more uncertainty than physical measurements because of factors such as individual variability and sampling statistics. When considering the needs of radiological protection, particularly at high doses, precision is not too important, as medical intervention tends to follow the appearance of

deterministic symptoms rather than anticipating them due to forewarnings from dosimetry.

Lloyd *et al.* (1987) suggested that a dose estimate within 30% of the estimated physical dose ("true" dose) is a good accuracy to attain. The limits of the interval presenting 30% of the "true" dose, are indicated by the broken lines in Fig. 6.2. Taking a generalised criterion that a dose estimate within 30% of the "true" dose is sufficiently accurate for radiological protection purposes then all the equivalent whole-body dose estimates obtained with fractionated partial-body exposure are acceptably close to the "true" dose values.

After a major radiation incident segregation of casualties into the three categories, namely below 1.5 Gy, between 1.5 and 5 Gy and above 5 Gy is a reasonable initial objective (Prosser *et al.*, 1989). From the results presented in this thesis it can be stated that MN data can provide dose estimates accurate enough to confirm the triage category into which the patients should be placed, based on symptoms.

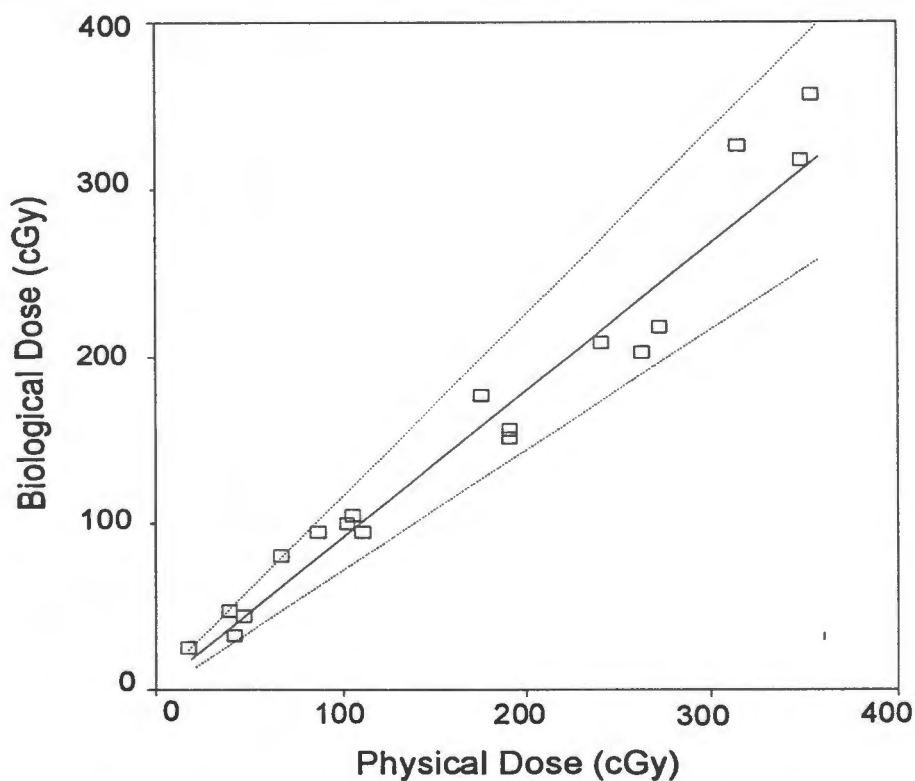


Fig 6.2 Correlation between estimated physical dose and biological dose in patients undergoing fractionated radiotherapy, with 30% tolerance lines.

5.6 Estimates reflecting non-uniformity of exposure

In Table 6.4 the dose to the irradiated body fraction and the size of this fraction, estimated according to the contaminated Poisson and negative binomial methods respectively, are compared with the corresponding doses obtained from physical measurement and calculation. In applying either of these methods there is the basic underlying assumption that there are only two types of cells i.e. a simple uniformly irradiated fraction and an unirradiated fraction.

During a localized single treatment only a fraction of the lymphocytes will lie within the radiation field. Lymphocytes are distributed throughout the body, principally in the lymph nodes, spleen, bone marrow, thymus, and the lymphoid tissue of the gut. Trepel (1974) has estimated the distribution of lymphocytes within these compartments and other tissue in the normal adult human. Changes in the physiological state of an individual can alter this distribution, but at present these alterations are known only qualitatively.

Aside from those that are circulating in the blood, the lymphocytes essentially remain fixed during a single radiation treatment and therefore, if the distribution of dose throughout the treatment volume is uniform, all the lymphocytes which are exposed will receive the same dose. Lymphocytes within the blood do not receive a uniform dose during a treatment since they move in and out of the field. Few lymphocytes are in the blood at any one time (2.2% of the total number, [Ekstrand and Dixon, 1982]) so that, because these cells receive only a small portion of the dose, these cells can be considered to be outside the radiation field.

The contaminated Poisson and negative binomial methods were applied to the data of patients e and f who each received a single localized exposure of 10Gy. Theoretically, the contaminated Poisson method is not applicable to this data, because the assumption that the Poisson distribution applies to MN in cells which have been irradiated, is not valid. It is known that MN are overdispersed compared with a Poisson distribution (Prosser *et al.*, 1988; Lloyd *et al.*, 1991 and the distributions given in Tables 4.1 and 6.1 respectively).

Notwithstanding this argument there is very little difference between the corresponding estimates of local dose and irradiated body fraction (Table 6.4) from the two methods. The reason for the unexpected suitability of the contaminated Poisson method for this analysis probably lies in the overdispersion-dose relationship. A quantitative estimate of how overdispersion varies with dose is not available. Some information on this is given by Prosser *et al.* (1988) and Littlefield *et al.* (1989) but the results were variable. Clearly further work is required to clarify this point.

Micronuclei data obtained from lymphocytes exposed *in vivo* to ionising radiation has to date not been analysed using the negative binomial method. In the analysis presented here it was necessary to find parameters of the contaminated negative binomial distribution and to compare these with the corresponding values obtain from *in vitro* results. The background yield of micronuclei was assumed to be 0.0116, which was the fitted control level for the *in vitro* observations. For θ a value of 0.8 was assumed, because $1/\theta$ (the ratio of variance to mean) according to the *in vitro* data points was 1.25. These values are similar to the corresponding background yield (0.012) and θ value (0.8) used by Lloyd and his co-workers (1991) in the analysis of their *in vitro* MN data.

Bearing in mind all the uncertainties, the doses to the irradiated volume are reasonable, taking a generalised criterion that a dose estimate within 30% of the "true" localized dose is sufficiently accurate for radiological protection purposes. It should be noted that even in the patients receiving a single exposure of 10 Gy there were between 97 and 98% of cells seen that did not contain any aberrations. This information in itself is of immediate qualitative value as an indication that the greater proportion of the cells has been spared. The implication from this is that there is likely to be natural recovery of bone marrow. In a highly irradiated subject whose lymphocytes are analysed by the micronucleus assay, this would be apparent after relatively few cells had been scored. The clinician could then be informed early on with the expectation that when a larger sample has been analysed a more quantitative estimate of inhomogeneous irradiation would be available using the methods described in this Chapter.

Data in Table 6.4 suggests a discrepancy with regard to the irradiated volume. For the latter the biological estimates seems to be consistently

higher than the physically estimated volumes. However using the negative binomial method the deviation from the physically estimated volumes is only approximately one standard error and with the contaminated Poisson method two standard errors.

There are three possible explanations for this discrepancy. The first is that the fraction of irradiated lymphocytes is greater than that estimated using Matsubara and co-workers' (1974) report as a basis. These workers suggested that the fraction of irradiated lymphocytes could be approximated by the volume of the irradiated site relative to body weight, i.e. an even distribution of lymphocytes throughout the body. From the data of Trepel (1974) on the distribution of lymphocytes this approximation does not apply in the strict sense to single partial-body exposure. In fact it may well be that the amount of lymphocyte-bearing tissues within the irradiated fraction is significantly greater than the approximated 6% (see Table 6.4) of the total. Furthermore the nature of the disease is such that the treated area may be infiltrated with lymphocytes. Thus the fraction of lymphocytes which were exposed in the treatments is possibly greater than that applicable to normal individuals.

The second explanation is that the value of D_0 may be incorrect. The survival curve for lymphocytes may be represented by a simple exponentially decreasing function (Equ 6.5) as described by Lloyd *et al.* (1973). In this equation D_0 is the dose for which the surviving fraction p , is $1/e$. Estimated values for D_0 are determined for *in vitro* irradiation of lymphocytes and vary considerably. The maximum of these values, i.e. 8 Gy, was applied in calculating the irradiated fraction, F . Nevertheless, considering these fractions given in Table 6.4, there is a tendency for the biological estimates to be somewhat high. This leads to the possibility that the assumed value for D_0 is too low. It is possible that in *in vivo* the lymphocytes may suffer less radiation induced interphase death than in *in vitro*. Sasaki (1978) has indicated that chromosome repair does not take place except in the case of activated lymphocytes or lymphocytes exposed in an artificial medium. Other researchers have disputed this (Virsik and Harder, 1980). They found that repair does occur and it is virtually complete within two hours of the irradiation. It was suggested by Lloyd *et al.* (1992) that *in vivo* some stem cells could multiply and in this way cause an apparent radioresistance of lymphocytes *in vivo*. Consequently a good

case can be made for more research to extend the experimental basis for D_0 values.

A third explanation for the discrepancy regarding the irradiated fraction (F) may lie in the basic assumption that when these methods for calculating F are applied to observations in patients the dose should be uniform throughout the irradiated volume. In general the dose-volume histograms (Chapter V) indicate that the treatment plans of the patients indicate reasonable dose uniformity. However, only about 20% of the irradiated volume received 100% of the tumour dose, i.e. 10 Gy. Consequently the average dose over the total irradiated area will be less than 10 Gy, but the product of the average dose and the irradiated volume (mass) i.e. the integral dose as calculated using the contaminated Poisson and negative binomial methods must still correspond with the physically determined integral dose.

In view of the uncertainty in quantifying D_0 for lymphocytes irradiated *in vivo*, it was decided to ignore the correction for the selective loss of cells due to interphase death and mitotic delay in calculating the integral dose. Consequently the fraction of cells irradiated and seen (f) was taken to be the originally exposed fraction of cells (F). This fraction (f) is therefore assumed to represent the proportion of the body exposed during *in vivo* irradiation.

There is a good agreement between the physically determined integral doses and the integral doses derived from the dose values obtained by means of the negative binomial method (Table 6.4). It is however, not recommended that the assumption ($F = f$) should be accepted without reservation for *in vivo* exposure of lymphocytes, because interphase death, although probably less than for *in vitro* exposure, must also occur with *in vivo* exposure of lymphocytes.

5.7 Applying the negative binomial method to MN data obtained from fractionated partial-body exposure

A basic requirement in the negative binomial method is that the irradiated volume (and therefore the fraction of lymphocytes within the irradiated field) is constant during treatment. During any single treatment, which usually is completed within about 3 - 5 minutes, the fraction of lymphocytes within the

irradiated volume will comply with this requirement. Typically the time between treatment fractions is at least 24 hours. This is sufficient for the lymphocytes to redistribute themselves, either within or outside the radiation field. Consequently estimates reflecting the non-uniformity of exposure, i.e. the localized dose and irradiated fraction, by means of the negative binomial method was not possible from data obtained with fractionated partial body exposure. To demonstrate this the MN data points of patients a and b obtained after 20 daily fractions of exposure were selected for analysis. As indicated in Table 6.4 this analysis provided estimates of localized dose and irradiated fraction which do not agree with the corresponding physically measured values.

6. Conclusions with regard to future studies

Although the procedures used in the present study have been available for several years, it is applied here for the first time to MN data obtained from lymphocytes exposed *in vivo*. This study has revealed two related problem areas for both of which a good case can be made for more research to extend the applicability of the relevant experimental methods and analysis used in this study, as discussed below.

6.1 Overall reliability of the equivalent whole-body dose concept

The fundamental objective of this study was to show that an equivalent whole-body dose estimate, obtained from relating an *in vivo* induced MN yield to an *in vitro* dose-response calibration curve, is sufficiently accurate for clinical management of accidental partial-body irradiation.

In estimating the equivalent whole-body dose values given in Fig 6.2 the fraction of irradiated lymphocytes is approximated by the volume of the irradiated site relative to the body weight. This approximation, however, has to take into account some important variables. For example, lung tissue is of a lower density than other tissues, so that the irradiated volume represents a relatively lower tissue mass. Another important factor is the

variability in the proportion of lymphoid tissue and bone marrow in the exposed site, so that the consequences of partial-body irradiation may be very dependent on the irradiation site (Liniecki *et al.*, 1983; Savage and Breckon, 1985).

The patients used in this study did have a variety of tumours (as regards pathology and volume) and integral doses, but they were all located in the pelvic area. Consequently it is necessary to confirm the repeatability of the graph shown in Fig. 6.2 for other tumour sites, before it can be accepted with confidence. Research in this regard may involve three-dimensional dose calculations from more complicated multi-planar treatment plans.

Applying the same techniques to obtain estimates of the equivalent whole-body dose from the MN frequency ratio following fractionated radiation therapy of body sections other than the pelvic area, may show the assumption of lymphocytes being evenly distributed throughout the body to be incorrect. An alternative approach may then be to develop a model whereby the amount of lymphocyte-bearing tissue within the irradiated volume can be estimated. The data of Trepel, (1974) on the distribution of lymphocytes within each lymphoid compartment could be used as a starting point to calculate the fraction of lymphocytes in the irradiated volume.

6.2 Extending localized dose estimates to fractionated partial-body exposure

Further research in this field should be directed at development of methods for analysis of MN data from persons having received fractionated inhomogenous exposure, with the aim of estimating the localized dose reflecting the non-uniformity. A possible means to describe data obtained after several fractions of exposure is to model the MN distribution after each fraction separately, assuming thorough mixing of lymphocytes.

This and other considerations, such as interphase cell death, have been taken into account by Ekstrand and Dixon (1982) when developing a mathematical equation describing the dose-response relationship for lymphocyte chromosome aberration yields occurring following fractionated

partial-body irradiation. The same technique could possibly be used to make an estimate of the localized dose from the MN frequency ratio, following fractionated partial-body radiation therapy. Results in this regard may prove useful in the interpretation of MN yields as a sophisticated form of *in vivo* dosimetry.

6.3 Future prospects of the MN assay

A number of biochemical and cytological assays have been proposed as biological indicators of exposure (see Chapter III). They suffer from drawbacks concerning factors such as poor sensitivity, particularly to doses below 1 Gy, and fluctuating inter- or intra-donor variability in response. In some instances the response to irradiation is too transient for practical application. Cytogenic dosimetry however stands out as being the most sensitive and widely used of the possible biological dosimeters.

However in this regard much work remains to be done on the yield of MN as a function of the distribution of dose in space and time and of the quality of radiation. Nevertheless, the comparison of biologically and physically recorded whole-body doses illustrates that useful estimates can already be made of the dose absorbed by the circulating lymphocytes in people accidentally exposed to acute doses of radiation. The relationship of MN frequency and medical risk for leukemia and other late effects of radiation remains a study for the future. A major drawback on progress in this area will be removed when the scoring of MN is fully automated.

REFERENCES

- Akiyama M., Kusunoki Y., Shigeko U., Yoko H., Nakamura N. and Kyoizumi S. (1992). Evaluation of four somatic mutation assays as biological dosimeter in humans. *Radiation research : A twentieth-century perspective*. San Diego, Academic Press, Inc. p 177 - 182.
- Almássy Z., Krepinsky A.B., Bianco A. and Köteles G.J. (1987). The present state and perspectives of micronucleus assay in radiation protection. A review. *Int. J. Appl. Radiat. Isot.* 38, 241 - 249.
- Almássy Z., Kanyár B. and Köteles G.J. (1986a). Frequency of micronuclei in x-irradiated human lymphocytes. *Int. J. Radiat. Biol.* 49, 719.
- Almássy Z., Kanyar B. and Köteles G.J. (1986b). Dose assessment through micronuclei in irradiated human lymphocytes. In : *Proceedings of the European Congress of IRPA, IVth, Salzburg*. International Radiation Protection Association, Eindhoven.
- Andrews G.A. (1967). Radiation accidents and their management. *Radiat. Res.* 7 (suppl), 390 - 397.
- Antoine J.L., Gerber G.B., Leonard A., Richard F. and Wambersie A. (1981). Chromosome aberrations induced in patients treated with telecobalt therapy for mammary carcinoma. *Radiat. Res.* 86, 171 - 177.
- Auxier J.A. (1961). Dosimetric considerations in criticality exposures. *Diagnosis and Treatment of Acute Radiation Injury*. WHO, International Documents Service, New York.
- Bain B.J., Clark D.M. and Lampert J.A. (1992). *Bone Marrow Pathology*. Blackwell Scientific. p 1 - 38.
- Baker F.J., Silverton R.E. and Luckcooc E.D. (1966). *Introduction to medical laboratory technology*, 4th edition. Butterworths, London. p 491 - 498.
- Balasem A.N., Ali A.S.K. and Abdul-Khalig J.J. (1993). The yield of micronuclei in human blood lymphocytes treated with radioactive caesium. *Radiat. Prot. Dosim.* 46, 295 - 297.
- Barnes D.W.H. and Loutit J.F. (1967). Effects of irradiation and antigenic stimulation on circulating hemopoietic stem cells of the mouse. *Nature*, 213, 1142 - 1143.
- Bauchinger M. (1984). Cytogenetic effects in human lymphocytes as a dosimetry system. In : *Biological Dosimetry*. Eds. W.G. Eisert and M.L. Mendelsohn. Springer Verlag, Berlin. p 15 - 23.

Belot J. (1906). The principal factors in radiotherapy and radiumtherapy. Archives of the Roentgen Ray, 11,36.

Bender M.A. (1964). Chromosome aberrations in irradiated human subjects. Ann. N.Y. Acad. Sci. 114, 249 - 251.

Bender M.A. and Gooch P.C. (1962). Types and rates of x-ray induced chromosome aberrations in human blood irradiated *in vitro*. Proc. Nat. Acad. Sci. Wash. 48, 522 - 532.

Bergonie J. and Tribondeau L. (1906). Interprétation de quelques résultats de la radiothérapie et essai de fixation d'une technique rationnelle, C.r. hebd. Séanc. Acad. Sci., Paris. 143, 983 - 985.

Bettega D., Bombana N., Pelucchi T., Poli A., Tallone L.A., Lombardi A. and Fuhrman Conti A.M. (1980). Multinucleate cells and micronucleus formation in cultured human cells exposed to 12 Mev protons and gamma-rays. Int. J. Radiat. Biol. 37, 1 - 9.

Betz E.H., Cottier H., Nakao K. and Odartchenko N. (1971). Clinical aspects of chronic radiation. In : Manual on Radiation Haematology, Technical Report Series No 123, International Atomic Energy Agency, Vienna. p 175 - 179.

Biological Indicators Working Group (1991). Current status of biological indicators to detect and quantify previous exposures to radiation. Health Phys. 60, 103 - 109.

Bond V.P. (1971). Radiobiological bases for the understanding of the haematological consequences of radiation exposure. In : Manual on Radiation Haematology, Technical Reports Series No. 123, International Atomic Energy Agency, Vienna. p 71 - 77.

Bond V.P., Fliedner T.M. and Archambeau J.O. (1965). Mammalian Radiation Lethality : A disturbance in Cellular Kinetics. Academic Press, New York.

Brady J.M., Aarestead N.O. and Swartz H.M. (1986). *In vivo* dosimetry by electron spin resonance spectroscopy. Health Phys. 15, 43 - 47.

Buckton K.E. (1983). Chromosome aberrations in patients treated with x-irradiation for ankylosing spondylitis. In : Radiation-induced Chromosome Damage in Man. Eds. T. Ishihara and M.S. Sasaki. R. Liss, New York. p 491 - 511.

Buckton K.E., Langlands A.O., Smith P.G., Woodstock G.E., Looby P.C. and Leiland J. (1971). Further studies on chromosome aberration production after whole body irradiation in man. Int. J. Radiat. Biol. 19, 369 - 374.

Callisen H., Norman A. and Pincu M. (1984). Computer scoring of micronuclei in human lymphocytes. In : Biological Dosimetry. Eds. W.G. Eisert and M.L. Mendelsohn. Springer Verlag, New York. p 171 - 179.

Catravas G.H. (1982). Biological indicators of radiation injury. In : Proceedings of NATO Working Group Meeting on the Assessment of Injury from Ionising Radiation in Warfare. Armed Forces Radiobiology Research Institute, Bethesda, MD. p 435 - 446.

Chen G.T.Y., Austin-Seymour M., Castro J.R., Collier J.M., Lyman J.T., Pitluck S., Saunders W.M. and Zink S.R. (1984). Dose volume histograms in treatment planning evaluation of carcinoma of the pancreas. In : Proceedings, Eight International Conference on Uses of Computers in Radiation Therapy, IEEE, New York. p 264 - 268.

Christen T. (1912). Absolute measure for quality of röntgen rays and its use in röntgen therapy. Verhandlungen der Deutschen Röntgengesellschaft, 13, 119.

Clemenger J.F. and Scott D. (1971). In vitro and in vivo sensitivity of cultured blood lymphocytes to radiation induction of chromosome aberrations. Nature (New Biol.) 234, 154.

Conner S. (1988). Protein reveals damage from radiation. New Scientist, 1, 41.

Cook P.R. and Brazell I. (1976). Conformational constraints in nuclear DNA. J. C. Sci. 22, 287 - 302.

Countryman P.I. and Heddle J.A. (1976). The production of micronuclei from chromosome aberrations in irradiated cultures of human lymphocytes. Mutat. Res. 41, 321 - 332.

Cronkite E.P. (1966). Radiation injury in man. In : The Biological Basis of Radiation Therapy. Ed. F.E. Schwartz. J.B. Lippincott, Philadelphia. p 163.

Cronkite E.P. (1971). Special problems of partial body exposure. In : Manual on Radiation Haematology, Technical Report Series No. 123, International Atomic Energy Agency, Vienna. p 191 - 192.

Cronkite E.P., Bond V.P. and Conard R.A. (1964). The haematology of ionizing radiation. Atomic Medicine. Ed. C.F. Behrens. Williams and Wilkens, Baltimore. p 192.

Cutler M. (1926). Comparison of the effects of filtered and unfiltered tubes barried in rabbit muscle. American Journal of Roentgenology and Radium Therapy, 16, 535.

Deiner A., Stephan G., Vogl T. and Lissmer J. (1988). The induction of chromosome aberrations during the course of radiation therapy for Morbus Hodgkin. *Radiat. Res.* 114, 528 - 536.

Del Regato A.J., Spjut H.J. and Cox J.D. (1985). *Cancer : diagnosis, treatment and prognosis*, 16th edition. St. Louis, Mosby.

Desrosiers M.F. (1991). *In vivo* assessment of radiation exposure. *Health Phys.* 61, 859 - 861.

Dolphin G.W. (1969). Biological dosimetry with particular reference to chromosome aberration analysis : A review of methods. *Handling of Radiation Accidents. Proc. Int. Symp. International Atomic Energy Agency, Vienna.* p 215 - 224.

Doria G., Agarossi G. and Adorini L. (1982). Selective effects of ionising radiation immunoregulatory cells. *Immunol. Rev.* 65, 23 - 54.

Drzymala R.E., Harms W.B. and Purdy J.A. (1987). Dose-volume histograms for 3-D radiation treatment plans. *Med. Phys.* 14, 460 - 464.

Dziedzie-Goclawska A. and Ostrowski K. (1974). Dosimetry of the absorbed dose of ionising radiation based on the electron spin resonance spectroscopy of stable paramagnetic centers induced in skeletal tissues. *Radiat. Res.* 59, 240 - 248.

Ekstrand K.E. and Dixon R.A. (1982). Lymphocyte chromosome aberrations in partial-body fractionated radiation therapy. *Phys. Med. Biol.* 27, 407 - 411.

Evans H.J., Neary G.J. and Williamson F.S. (1959). The relative biological efficiency of single doses of fast neutrons and gamma-rays on *Vicia faba* roots and the effect of oxygen. Part II. Chromosome damage: the production of micronuclei. *Int. J. Radiat. Biol.* 3, 216 - 229.

Fabry L. and Lemaire M. (1986). Dose response relationships for radiation induced chromosome aberrations in human lymphocytes *in vivo* and *in vitro*. *Strahlentherapie*, 162, 63 - 67.

Failla G., Quinley E.H. and Dean A. (1922). Some problems in radiation therapy. *American Journal of Roentgenology and Radium Therapy*, 9, 479.

Feinendegen L.E. (1986). Biochemical Indicators. In : *Biological Indicators for Radiation Dose Assessment*. Eds. A. Kaul, A. Dehos, W. Böhl, G. Hinz, F. Kossel, E. Schwarz, A. Stamm and G. Stepan. MMV Medizin Verlag, München. p 70 - 81.

Fenech M., Denham J., Francis W. and Morley A. (1990). Micronuclei in cytokinesis-blocked lymphocytes of cancer patients following fractionated partial body radiotherapy. *Int. J. Radiat. Biol.* 57, 373 - 383.

Fenech M. and Morley A.A. (1985). Measurements of micronuclei in lymphocytes. *Mutat. Res.* 147, 29 - 36.

Fenech M. and Morley A.A. (1986). Cytokinesis-block micronucleus method in human lymphocytes : effect of *in vivo* ageing and low dose x-irradiation. *Mutat. Res.* 161, 193 - 198.

Fenech M. and Morley A.A. (1989). Kinetochores detection in micronuclei : A alternative method for measuring chromosome loss. *Mutagenesis* 4, 98 - 104.

Filippovich I.V., Sorokima N.I., Soldatenkov V.A. and Romantzev E.F. (1982). Supercoiled DNA repair in thymocyte fractions differing in radiosensitivity. *Int. J. Radiat. Biol.* 42, 31 - 44.

Fliedner M.T., Cronkite E.P., Bond V.P. and Andrews G.A. (1960). Mitotic activity and cytology of human bone marrow after accidental exposure to ionizing radiation. In : *Proceedings of the Seventh Congress, European Society of Haematology, London, 1959, Vol. II, Pt. 1.* Karger, Basel. p 458 - 467.

Fliedner T.M., Cronkite E.P., Bond V.P., Rubini J.R. and Andrews G.A., (1959). The mitotic index of human bone marrow in healthy individuals and irradiated human beings. *Acta Haematol.* 22, 65 - 78.

Flury-Hérard A. (1986). Intérêt de la téléthermographie et de la scintigraphie vasculaire pour le diagnostic et le pronostic des surexpositions aiguës des mains (CEA-IPSN, Fontenayaux-Roses, France), *Rapport DPS 86/01 SEAPS. op. cit.* Nénot J.C. (1990). Medical and surgical management for localized radiation injuries. *Int. J. Radiat. Biol.* 57, 783 - 795.

Gamong W.F. (1993). *Review of Medical Physiology*, 16th edition. Appleton and Lange.

Gantenberg H.W., Wuttke K., Streffer C. and Müller W.U. (1991). Micronuclei in human lymphocytes irradiated *in vitro* or *in vivo*. *Radiat. Res.* 128, 276 - 281.

Geng L. and Potten C.S. (1990). Changes after irradiation in the number of mitotic cells and apoptotic fragments in growing mouse hair follicles and the width of their hairs. *Radiat. Res.* 123, 75 - 81.

Gerber G.B.(1986). Biochemical indicators of radiation injury. The current state of the knowledge and possible expectations for the future. In : *Biological Indicators for Radiation Dose Assessment.* Eds. A. Kaul, A. Dehos, W. Böhl, G. Hinz, F. Kossel, E. Schwarz, A. Stamm and G. Stepan. MMV Medizin Verlag, München. p 237 - 239.

Gerstner H.B. (1958). Acute clinical effects of penetrating nuclear radiation. *J. Am. Med. Assoc.* 168, 381 - 385.

- Goitein M. and Miller T. (1983). Planning proton therapy of the eye. *Med. Phys.* 10, 275 - 283.
- Grawe J., Zetterberg G. and Annéus H. (1992). Flow-cytometric enumeration of micronucleated polichromatic erythrocytes in mouse peripheral blood. *Cytometry*, 13, 750 - 758.
- Grilli G., Nothduret W. and Fliedner T.M. (1982). Radiation sensitivity of human erythropoietic and granulopoietic progenitor cells in the blood and bone marrow. *Int. J. Radiat. Biol.* 41, 685 - 687.
- Hacker U., Schumann J. and Göhde W. (1982). Mammalian spermatogenesis as a new system for biological dosimetry of ionising irradiation. *Acta Radiol. Oncol.* 21, 349 - 351.
- Hampson W. (1911). The epilation dose. *Archives of the Roentgen Ray*, 16, 85 - 89.
- Harris E.B. (1971). Effects of radiation on erythropoiesis. In : *Manual on Radiation Haematology, Technical Report Series No. 123, International Atomic Energy Agency, Vienna.* p 99 - 106.
- Hasterlik R.J. and Marinelli L.D. (1956). Physical dosimetry and clinical observations on four human beings involved in an accidental critical assembly excursion. *Proceedings of the International Conference on Peaceful Uses of Atomic Energy. United Nations, Geneva.* 11, 25- 34.
- Hayashi M., Sofuni T. and Ishidate M. (1983). An application of Acridine Orange fluorescent staining to the micronucleus test. *Mutat. Res.* 120, 241 - 247.
- Heineke H. (1903). Über die Einwirkung der Röntgenstrahlen auf Tiere, *Münch. Med. Wschr.* 50, 2090. *op. cit.* Betz E.H., Cottier H., Nakao K. and Odartchenko N. (1971). Late effects on Haemopoiesis and life-shortening. In : *Manual on Radiation Haematology, Technical Report Series No 123, International Atomic Energy Agency, Vienna.* p 175 - 179.
- Heller C.G. (1967). Effects on the germinal epithelium. In : *Radiobiological Factors in Manned Space Flight, NRC Publ. 1487, Washington, D.C.* p 124 - 133.
- Heyns A. du P. en Badenhorst P.N. (1981). *Bloedsiektes, 1ste uitgawe. Academica (Edms) Bpk., Pretoria.*
- Huber R., Braselmann H. and Bauchinger M. (1992). Intra- and inter- individual variation of background and radiation-induced micronucleus frequencies in human lymphocytes. *Int. J. Radiat. Biol.* 61, 655 - 661.

Huber R., Braselmann H. and Bauchinger M. (1989). Screening for interindividual differences in radiosensitivity by means of the micronucleus assay in human lymphocytes. *Radiat. Environ. Biophys.* 28, 113 - 120.

Huber R., Streng S. and Bauchinger S. (1983). The suitability of the human lymphocyte micronucleus assay system for biological dosimetry. *Mutat. Res.* 111, 185 - 193.

Hübner K.F., Littlefield L.G. and Du Grain R.J. (1986). Experience in the practical application of biological indicators. In : *Biological Indicators for Radiation Dose Assessment*. Eds. A. Kaul, A. Dehos, W. Böhl, G. Hinz, F. Kossel, E. Schwarz, A. Stamm and G. Stepan. MMV Medizin Verlag, München. p 17 - 34.

Hutter K.J. and Stöhr M. (1982). Rapid detection of mutagen induced micronucleated erythrocytes by flow cytometry. *Histochemistry*, 75, 353 - 362.

IAEA (1967). Effects of ionising radiations on the haematopoietic tissue. In : *Proceedings of a panel on the effects of various types of ionising radiations from different sources on haematopoietic tissue*. International Atomic Energy Agency, Vienna. p 17 - 20.

IAEA (1969). Symposium on the handling of radiation accidents. International Atomic Energy Agency, Vienna.

IAEA (1986). Report 260. *Biological Dosimetry: Chromosomal Aberrations Analysis for Dose Assessment*. International Atomic Energy Agency, Vienna.

ICRP (1977). Recommendations of the International Commission on Radiological Protection. ICRP Publication 26. *Annals of the ICRP*, Pergamon Press, Oxford.

ICRP (1990). Recommendations of the International Commission on Radiological Protection. ICRP Publication 60. *Annals of the ICRP*, Pergamon Press, Oxford.

Ingram M. (1960). The occurrence and significance of binucleate lymphocytes in peripheral blood after small radiation exposures. *Int. J. Radiat. Biol.* 1 Spec. Suppl.

Ingram M. and Preston K. (1964). The importance of automatic pattern recognition techniques in the early detection of altered blood production. *Ann. N.Y. Acad. Sci.* 113, 1066 - 1072.

Jammet H., Gongora R., Jockey P. and Zucker J.M. (1980). The 1978 Algerian Accident : Acute local exposure of two children. In : *The Medical Basis for Radiation Accident Preparedness*. Eds. K.F. Hübner and S.A. Fry. Elsevier Inc. Amsterdam. p 229 - 245.

Jammet H. and Gongora R. (1981). Radiolésions aiguës localisées. Intérêt de la dosimétrie physique et biologique; étude clinique. Il Sistema Tegumentario e le Radiozioni Ionizzanti: radiation and skin. Ed. E. Strambi. Associazione Italiana Radioprotezione Medica, 3e Congresso Nazionale, Saint Vincent, Italy. p 9 - 40. *op. cit.* Nénot J.C. (1990). Medical and surgical management for localized radiation injuries. *Int. J. Radiat. Biol.* 57, 783 - 795.

Jandi J.H. (1987). Blood (Textbook of Haematology). Little, Brown and Company, Boston.

Johns H.E. and Cunningham J.R. (1977). The Physics of Radiology. Charles C. Thomas, Illinois, USA.

Knoll G.F. (1979). Radiation Detection and Measurement. John Wiley and Sons, New York.

Kooy H.M., Lucien A.N., Alexander III E. and Loeffler J.S. (1993). Dose-volume histogram computations for small intracranial volumes. *Med. Phys.* 20, 755 - 760.

Kormos C. and Koteles G.J. (1988). Micronuclei in x-irradiated human lymphocytes. *Mutat. Res.* 199, 31 - 35.

Koteles G.J. (1982). Radiation effects on cell membranes. *Radiat. Environ. Biophys.* 21, 1 - 18.

Krepinsky A.B. and Heddle J.A. (1983). Micronuclei as a rapid and inexpensive measure of radiation-induced chromosomal aberrations. In : Radiation Induced Chromosome Damage in Man. Eds. T. Ishihara and M.S. Sasaki. R. Liss, New York. p 93 - 98.

Kubasova T., Varga L.P. and Koteles G.J. (1981). Surface alterations of mammalian cells upon ionising radiation as detected by a lectin-binding technique. I. binding of Concanavalin A by blood cells of x-irradiated mice. *Int. J. Radiat. Biol.* 40, 175 - 186.

Lasne C., Gu Z.W., Venegas W. and Chouroulinkov I. (1984). The *in vitro* micronucleus assay for detection of cytogenetic effects induced by mutagen-carcinogens : comparison with the *in vitro* sister-chromatid exchange assay. *Mutat. Res.* 130, 273 - 282.

Le Roux P.R., van As D., Burns Y., de Beer G. and van der Merwe M.G. (1992). Radiation. In : Radiation Environmental Management in South Africa. Eds. R.F. Fuggle and M.A. Rabie. Juta and Co Ltd Cape Town. p 544 - 568.

Liniecki J., Bajerska A. and Wysznska K. (1983). Dose-response relationships for chromosome aberrations in peripheral blood lymphocytes after whole - and partial body irradiation. Effects immediately after irradiation. *Mutat. Res.* 110, 83 - 101.

Littlefield L.G., Sayer A.M. and Frome E.L. (1989). Comparisons of dose-response parameters for radiation induced acentric fragments and micronuclei observed in cytokinesis arrested lymphocytes. *Mutagenesis*, 4, 265 - 270.

Littlefield L.G., Sayer A.M., Sallam F., Frome E.L. and Kleinerman R.A. (1991). Experience with the "cytochalasin-B method" for quantifying radiation induced micronuclei in human lymphocytes. *Mutagenesis*, 4, 183 - 188.

Lloyd D.C. and Edwards A.A. (1987). Doses in radiation accidents investigated by chromosome analysis - XVII. A Review of Cases Investigated in 1986. National Radiological Protection Board (NRPB) R207.

Lloyd D.C., Edwards A.A. and Leonard A. (1992). Chromosomal aberrations in human lymphocytes induced *in vitro* by very low doses of x-rays. *Int. J. Radiat. Biol.* 61, 335 - 343.

Lloyd D.C., Edwards A.A., Natarjan A.T. and Darroudi F. (1991). Biological dosimetry applied to *in vitro* simulated partial body irradiation. In : Treatment and Biological Dosimetry of Exposed Persons. Post-Chernobyl action. Eds. K. Chadwick and G. Gerber. Report No. EUR 12558. CEC : Luxembourg. p 51 - 80.

Lloyd D.C., Edwards A.A., Prosser J.S., Barjaktarovic N., Brown J.K., Harvan D., Ismail S.R., Koteles C.J., Almassy Z., Krepinsky A., Kucerova M., Littlefield L.G., Mukkerjee U., Natarjan A.T. and Sasaki M.S. (1987). A collaborative exercise on cytogenetic dosimetry for simulated whole and partial body accidental irradiation. *Mutat. Res.* 179, 197 - 208.

Lloyd D.C., Purrot R.J. and Dolphon G.W. (1973). Chromosome aberration dosimetry using human lymphocytes in simulated partial body irradiation. *Phys. Med. Biol.* 18, 421 - 431.

Lushbaugh C.C., Fry S.A., Ricks R.C., Hübner K.F. and Burr W.W. (1986). Historical update of past and recent skin damage radiation accidents. *Br. J. Radiol. Suppl.* 19, 7 - 22.

Lyman J.T. (1985). Complication probability as assessed from dose-volume histograms. *Radiat. Res.* 104, 13 - 19.

Matsubara S., Sasaki M.S. and Adachi T. (1974). Dose-response relationship of lymphocyte chromosome aberrations in locally irradiated persons. *J. Radiat. Res.* 15, 189 - 196.

Mayneord W.V. (1942). The measurement of radiation for medical purposes. *Proc. Phys. Soc.* 54, 405.

Mayneord W.V. (1951). Some problems of radiation protection. *Br. J. Radiol.* 24, 525 - 537.

- McCulloch E.A. and Till J.E. (1962). The sensitivity of cells from normal mouse bone-marrow to gamma radiation *in vitro* and *in vivo*. *Radiat. Res.* 16, 822 - 832.
- Meyer B.J. (1983). *Die Fisiologiese Basis van Geneeskunde*, 3de uitgawe. HAUM, Pretoria.
- Mohan R., Brewster L.J. and Barest G.D. (1987). A technique for computing dose volume histograms for structure combinations. *Med. Phys.* 14, 1048 - 1052.
- Moorhead P.S., Norwell P.C., Mellman W.J., Baptists D.M. and Hungerford D.A. (1960). Chromosome preparations of leukocytes cultured from human peripheral blood. *Exp. Cell. Res.* 20, 613 - 616.
- Mould R.E. (1980). *A History of x-rays and Radium*. IPC Building & Contract Journals Ltd., Surrey House, Sutton SM14QQ.
- Murray W., Gill P. and Wright B. (1981). *Practical Optimization*. Academic Press, London.
- National Academy of Sciences (1967). Report of the space radiation study panel of the life sciences committee. In : *Radiobiological Factors in Manned Space Flight*. Ed. W.H. Langham. NRC, Washington, D.C. p 1487.
- NBS. (1977). The metric system of measurement (SI). Federal register notice. National Bureau of Standards, Washington D.C. 20234.
- Norman A., Bass D. and Roe D. (1985). Screening human populations for chromosome aberrations. *Mutat. Res.* 143, 155 - 160.
- Norman A., Sasaki M., Ottoman R.E. and Veomett R.C. (1964). Chromosome aberrations in radiation workers. *Radiat. Res.* 23, 282 - 289.
- Nothdurft W., Steinback K.H. and Fliedner T.M. (1983). *In vitro* studies on the sensitivity of canine granulopoietic progenitor cells (GM-CFC) to, ionising radiation. *Int. J. Radiat. Biol.* 43, 38 - 52.
- NRPB. (1989). *Radiation Doses, Maps and Magnitudes*. National Radiological Protection Board, Chilton, Didcot, Oxon OX110RQ.
- Nüsse M. and Kramer J. (1984). Flow cytometric analysis of micronuclei found in cells after irradiation. *Cytometry*, 5, 20 - 25.
- Oakberg E.F. and Heller C.G. (1966). Radiation response of spermatogonia in man. In : *Proceedings of the Third International Congress of Radiation Research, Rome*. *op. cit.* Biological Indicators Working Group (1991). Current status of biological indicators to detect and quantify previous exposures to radiation. *Health Phys.* 60, 103 - 109.

Oakes W.R. and Lushbaugh C.C. (1952). Course of testicular injury following accidental exposure to nuclear radiations. *Radiology*, 59, 737 - 742.

Papworth D.G. (1975). Curve fitting by maximum likelihood. In appendix to paper by J.R.K. Savage, Radiation-induced chromosomal aberrations in the plant *Tradexantia* : dose response curves. *Radiat. Bot.* 15, 87 - 140.

Papworth D.S. (1970). In appendix to paper by J.R.K. Savage. Sites of radiation-induced chromosome exchanges. *Curr. Top. Radiat. Res.* 6, 129 - 194

Petrini B., Wasserman J., Blomgren H., Glas U., Baral E. and Strender L.E. (1982). The effect of irradiation on concanavalin A-induced suppression of mitogen responses in human lymphocyte cultures. *Int. Arch. Allergy Immunol.* 67, 57 - 60.

Pincu M., Bass D. and Norman A. (1984). An improved micronucleus assay in lymphocytes. *Mutat. Res.* 139, 61 - 65.

Pitout M.J., Louw W.K.A., Izatt H., van Vuuren C.J.J., Hugo N. and van der Walt J.J. (1982). A modified neutral sucrose centrifugation method for rapid detection of DNA damaged by gamma radiation and repair in human lymphocytes. *Int. J. Biochem.* 14, 899 - 904.

Press W. (1986). *Numerical Recipes : The art of scientific computing.* Cambridge Univ. Press.

Prosser J.S., Lloyd D.C. and Edwards A.A. (1989). A comparison of chromosomal and micronuclear methods for radiation accident dosimetry. In : *Proceedings of the 4th International Symposium, Malvern.* NRPB, Chilton, Didcot Oxon, OX11 ORQ, UK. p 133 - 136.

Prosser J.S., Moquet J.E., Lloyd D.C. and Edwards A.A. (1988). Radiation induction of micronuclei in human lymphocytes. *Mutat. Res.* 199, 37 - 44.

Ramalho A., Sunjevaric I. and Natarajan A.T. (1988). Use of the frequencies of micronuclei as quantitative indicators of x-ray induced chromosomal aberrations in human lymphocytes : Comparison of two methods. *Mutat. Res.* 207, 141 - 146.

Robbins S.L. and Kumar V. (1987). *Basic Pathology.* W.B. Saunders Company, London. p 351 - 404.

Russ S. (1918). A suggestion for a new x-ray unit in radiotherapy. *Archives of Radiology and Electrotherapy*, 23, 226 - 229.

Rydberg B. (1984). Detection of DNA strand breaks in single cells using flow cytometry. *Int. J. Radiat. Biol.* 46, 521 - 527.

- Saenger E.L. (1981). Radiation Accident Preparedness. Edison Electric Institute. Distributed by Science - Thru - Media, Inc., New York.
- Sasaki M.S. and Miyata H. (1968). Biological dosimetry in atomic bomb survivors. *Nature* 220, 1189 - 1193.
- Sasaki M.S., Ottoman R.E. and Norman A. (1963). Radiation induced chromosome aberrations in man. *Radiology*. 81, 652 - 656.
- Sasaki M.S. (1978). Radiation damage and its repair in the formation of chromosome aberrations in human lymphocytes. In: *Mutagen-Induced Chromosome Damage in Man*. Eds. J.H. Evans and D.C. Lloyd. Edinburgh Univ. Press. p 62 - 76.
- Savage J.R.K. (1970). Sites of radiation induced chromosome exchanges. *Cur. Top. Radiat. Res.* 6, 129 - 194.
- Savage J.R.K. and Breckon G.(1985). Chromosome aberration frequencies after parital-body irradiation of Syrian hamsters. *Br. J. Radiol.* 58, 1105 - 1110.
- Schall W.E. (1932). *X-rays : their origin, dosage and practical application*. John Wright and Sons, Ltd., Bristol.
- Scherer E., Streffer C. and Trott K. (1991). *Radiopathology of Organs and Tissues*. Springer-Verlag, Berlin.
- Sieber K., Sugden E.M., Alcock C.J. and Belten R.R. (1992). Reduction in the diameter of human hairs following irradiation. *Br .J. Radiol.* 65, 148 - 151.
- Sievert R.M. (1947). The tolerance dose and the prevention of injuries caused by ionising radiation. *Br. J. Radiol.* 20, 506 - 318.
- Sims C.S. (1989). Nuclear accident dosimetry intercomparison studies. *Health Phys.* 57, 439 - 448.
- Sinclair W. (1968). Cyclic-x-ray responses in mammalian cells *in vitro*. *Radiat. Res.* 33, 620 - 643.
- Slabbert J.P.(1993). Inactivation synergy between high energy neutrons and ⁶⁰Co gamma rays. Ph.D. Thesis, Faculty of Medicine, University of Cape Town.
- Solomon I. (1925). Über die Wahl einer Quantimertrischen Einheit. *Strahlentherapie*, 20, 642 - 648.

Stamm A. and Bögl W. (1982). Biochemische Indikatoren für Strahlenexpositionen. Teil II. Eine Literaturstudie über biochemische : Veränderungen an biologischem Material nach Exposition mit Röntgen- und Gammastrahlen. Report of the Institute for Radiation Hygiene of the Federal Health Office ISH 13.

Stamm A., Weitz B. and Bögl W. (1981). Biochemische Indikatoren für Strahlenexpositionen, Teil I. Eine Literaturstudie über Blutuntersuchungen nach Exposition mit Röntgen- und Gammastrahlen. Reports of the Institute for Radiation Hygiene of The Federal Health Office, ISH 4.

Stamm A., Bögl W., Willich N. and Lissner J. (1983). Biochemical indicators of radiation exposure. Assessment of serum thymidine concentration, serum amylase activity and isoenzyme activities of acid serum phosphatase. Proceedings of the Seventh International Congress of Radiation Research. Eds. J.J. Broerse, G.W. Barendsen, H.B. Kal and H.J. van der Kogel. Martinus Nijhoff Publishers, Amsterdam. E3 - 14.

Stein G. (1972). Zytologische Frühveränderungen der Knochenmarkszellsysteme bei Ratten nach einmaliger Röntgen-Ganzkörperbestrahlung in Abhängigkeit von Zeit und Dosis. Zugleich ein experimenteller Beitrag zur hämatologischen Diagnostik und Prognosestellung bei akutem Strahlenunfall. Inaugural - Dissertation, Ulm. *op. cit.* Fliedner T.M. and Nothdurft W. (1986). Cytological indicators : haematopoietic effects. In : Biological Indicators for Radiation Dose Assessment. Eds. A. Kaul, A. Dehos, W. Böhl, G. Hinz, F. Kossel, E. Schwarz, A. Stamm and G. Stepan. MMV Medizin Verlag, München. p. 123 - 152.

Straume T., Egbert S.D., Woonson W.A., Finkel R.C., Kubik P.W., Gove H.E., Sharma P. and Hoshi M. (1992). Neutron discrepancies in the DS 86 Hiroshima dosimetry system. Health Phys. 63, 421 - 426.

Straume T., Harris L.J., Marchetti A.A. and Egbert S.D. (1994). Neutrons confirmed in Nagasaki and at the Army Pulsed Radiation Facility : implications for Hiroshima. Radiat. Res. 138, 193 - 200.

Streffer C. (1993). Is the micronucleus assay predictive for cellular radiosensitivity. Br. J. Radiol. Suppl. 24, 70 - 73.

Suzuki N. and Withers H.R. (1978). Exponential decrease during aging and random lifetime of mouse spermatogonial stem cells. Science, 202, 1214 - 1215.

Swartz H.M., Bolton J.R. and Borg D.C. (1972). Biological Applications of Electron Spin Resonance. Wiley-Interscience, New York.

Thierens H., Vral A. and de Ridder L. (1991). Biological dosimetry using the micronucleus assay for lymphocytes: Interindividual differences in dose response. Health Phys. 61, 623 - 630.

Thoma G.E. and Wald N. (1959). The diagnosis and management of accidental radiation injury. *J. Occup. Med.* 1, 421 - 447.

Trepel F. (1974). Number and distribution of lymphocytes in man. A critical analysis. *Klin. Wochenschr.* 52, 511 - 515.

UNSCEAR (1988). Sources and Risks of Ionising Radiation. Annex G. Early Effects in Man of High Doses of Radiation. United Nations Scientific Committee on the Effects of Atomic Radiation. United Nations, New York. p 545 - 612.

van Rensburg E.J. (1987). Gamma-radiation induced DNA damage and repair in human peripheral lymphocyte subpopulations as studied by nucleoid sedimentation. PhD Dissertation, University of Stellenbosch.

Villard P. (1908). The radiosclerometer. *Archives d'electricité médicale, Bordeaux*, 14, 692.

Virsik R.P. and Harder D. (1980). Recovery kinetics of radiation-induced chromosome aberrations in human G₀-lymphocytes. *Radiat. Environ. Biophys.* 18, 221 - 238.

Vral A., Thierens H. and De Ridder L. (1992). Study of dose-rate and split-dose effects on the *in vitro* micronucleus yield in human lymphocytes exposed to x-rays. *Int. J. Radiat. Biol.* 61, 777 - 784.

Wald N. (1971). Haematological parameters after acute radiation injury. In : *Manual on Radiation Haematology, Technical Report Series No. 123, International Atomic Energy Agency, Vienna.* p 253 - 256.

Warren S. and Lombard O.M. (1966). New data on the effects of ionising radiation on radiologists. *Arch. Environ. Health*, 13, 415 - 421.

Wasserman J. (1986). Immunological indicators. In : *Biological Indicators for Radiation Dose Assessment.* Eds. A. Kaul, A. Dehos, W. Böhl, G. Hinz, F. Kossel, E. Schwarz, A. Stamm and G. Stepan. MMV Medizin Verlag, München. p 85 - 103.

Wasserman J., Petrini B. and Blomgren H. (1982). Radiosensitivity of T-lymphocyte subpopulations. *J. Clin. Lab. Immunol.* 7, 139 - 140.

Webb S. (1993). *The Physics of Three-Dimensional Radiation Therapy : Conformal Radiotherapy, Radiosurgery and Treatment Planning.* Inst. of Physics Publishing Ltd., Ledger Bldg., Independence Square, Philadelphia. p 18.

Wells J. and Charles M.W. (1982). Biological dosimetry of non-uniform radiation exposure. In : Proceedings of the Third International Symposium on Radiation Protection. Advances in Theory and Practice. Inverness. p 352 - 358.

Wendt E. (1971). Differential diagnosis of radiation injury. In : Manual on Radiation Haematology, Technical Report Series 123, International Atomic Energy Agency, Vienna. p 297 - 298.

Whitfield J.F., Kellerer S., Brohee H. and Youdale T. (1965). The Feasibility of a New Dosimeter for Biological Dosimetry. Rep. Eur. 2505-e. *op. cit.* Wald N. (1971). Haematological parameters after acute radiation injury. In : Manual on Radiation Haematology, Technical Report Series No. 123, International Atomic Energy Agency, Vienna. p 253 - 256.

Wilson F.D., Stitzel K.A., Klein A.K., Shifrine M., Grahm R., Jones M., Bradley E. and Rosenblatt L.S. (1978). Quantitative response of bone marrow colony-forming units (CFU-C and PFU-C) in weanling beagles exposed to acute whole-body irradiation. *Radiat. Res.* 74, 289 - 297.

Wilson J.D., Martin J.B., Isselbacher K.J., Braunwald E., Adams R.D. and Petersdorf R.G. (1991). *Harrison's Principles of Internal Medicine*, 12th edition. McGraw-Hill International.

World Health Organization (1961). Diagnosis and treatment of acute radiation injury. Proceedings of a scientific meeting, WHO, Geneva. International Documents Service, New York.

Xue K., Wang S., Ma G., Zhou P., Wu P., Zhang R., Xu Z., Chen W. and Wang Y. (1992). Micronucleus formation in peripheral-blood lymphocytes from smokers and the influence of alcohol- and tea-drinking habits. *Int. J. Cancer*, 50, 702 - 705.