HPRT MUTANT FREQUENCIES FROM BRAZILIAN CHILDREN ACCIDENTALLY EXPOSED TO IONIZING RADIATION

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ABSTRACT

To examine the effects of ionizing radiation on somatic mutations *in vivo*, the *hprt* clonal assay was performed on children accidentally exposed during a radiological accident that happened in 1987, in Goiania, Brazil. The group of children exposed to ionizing radiation includes six males and four females ranging in age from 6 to 14 years. The radiation doses to which they were exposed ranged from 15 to 70 cGrays. A Brazilian control group, not exposed to ionizing radiation, was also analyzed under similar conditions for the purpose of comparison. The mean *hprt* mutant frequency for the exposed group was 4.6 times higher than the control group, although the cloning efficiency for the exposed group was significantly reduced. Linear regression analysis of the mutant frequency and ionizing radiation dose did not show a significant relationship between these two parameters. However, a reliable inverse relationship was demonstrated when the regression analysis was performed with cloning efficiency and ionizing radiation dose. It was demonstrated that cloning efficiency diminishes as ionizing radiation dose increases.

To verify the clonal relationship between the *hprt* mutant clones isolated, T-cell receptor analysis was performed with a number of mutant clones. It was verified that the majority of the mutants analyzed represented individual clones, thus validating the mutant frequencies obtained. The *hprt* clonal assay proved to be sufficiently sensitive

to detect somatic mutagenic effects caused by ionizing radiation in the exposed group of children. Nevertheless, a control group was required in order to compare the findings and to substantiate the observations.

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PREFACE

In Goiania, a mid-sized city in the central region of Brazil, a private radiotherapy clinic moved to new premises late in 1985. A caesium-137 teletherapy unit was discarded without the notification of the proper authorities, as required under the terms of the radiotherapy clinic's licence. Shortly thereafter, while the building was being demolished, the teletherapy unit became vulnerable to theft. Indeed, in September of 1987, two people attracted by the potential value of the scrap metal, but unaware of the risks, removed the lead source assembly from the radiation head of the unit. They then attempted to dismantle the source. This initiated a series of events that spawned one of the most serious radiological accidents yet recorded. During their attempts to dismantle the assembly, the source capsule was ruptured, and its integrity destroyed. The radioactive source was in the form of compressed caesium chloride salt pellets, not unlike grains of rice. Since caesium is highly soluble and readily dispersible, an extensive contamination of the local area occurred. This was accompanied by exposure to external irradiation and internal contamination of a large number of people.

The ruptured source assembly was subsequently sold as scrap to a local junkyard. The owner of the junkyard noticed that the material emitted a blue glow in the dark. This

phenomenon fascinated him, so he shared pieces of the source with his family and friends, so that over a period of several days some pieces of the source were distributed. About five days later, some of the people developed radiation-induced gastrointestinal symptoms. Initially, the symptoms were thought to be due to food poisoning, there being no obvious source of radioactive contamination in Goiania. Fortunately, one of the irradiated individuals connected the illness with the source capsule and took the remnant to the Department of Public Health in the city.

When the nature of the accident was finally recognized, a sequence of steps was initiated lead to the immediate establishment of an emergency response program by the local authorities. Physicists and physicians from the National Nuclear Energy Commission (CNEN), with the co-operation with the International Atomic Energy Agency (IAEA), carried out an intensive program comprising the assessment of individual radiation doses, the diagnosis, prognosis and treatment of the exposed persons, and the management of the severe environmental contamination.

From the oral descriptions provided by the victims of the accident, it was clear that complex exposures had occurred. Some had suffered continuous contact with the source for several days, while others, driven by their curiosity, even rubbed the material on their skin, and then ate with contaminated hands. Some individuals had been exposed to a mixture of external irradiation, skin contamination, and internal contamination. For these reasons, a variety of dosimetry techniques were used to obtain information on the

initial screening of potentially exposed persons. A whole-body counter was installed in the city and over 112.000 people were monitored. Of these, 249 were found to be contaminated, either internally, or externally, or both. More than 110 blood samples from individuals exposed in the accident were analyzed by cytogenetic methods. The level of chromosomal aberrations in cultured lymphocytes was determined, and an absorbed dose was estimated. Doses varied from 0 to 7 Grays (Gy). A statistical analysis (Poisson distribution) indicated that some individuals had suffered non-uniform exposures.

Individuals considered sufficiently exposed to require medical attention were transported to the Hospital Marcilio Dias in Rio de Janeiro. Urine samples from patients potentially suffering internal contamination were collected and analyzed. Internal contamination was identified in 46 patients. Isotope intakes and concomitant doses were estimated, using age specific mathematical models (The Radiological Accident in Goiania, IAEA, 1988). Patients with internal contamination were administered Prussian Blue (radiogardase) to promote the decorporation and excretion of caesium, with very successful results. Four patients with internal contamination died within four weeks of their admission to the hospital. The remaining patients recovered, but a group of about 120 persons who were exposed to ionizing radiation during the accident, are currently being monitored by the medical staff of the Fundação Leide das Neves Ferreira, a government institution created after the accident.

In 1989, a Canadian group, co-ordinated by Dr. Barry Glickman, visited Goiania with the primary objective of obtaining information about the radiological accident. By mutual agreement, the Canadian group, together with the Fundação Leide das Neves Ferreira and the Universidade Catolica de Goias, initiated a study of the genetic effects caused by ionizing radiation. The work has been in progress for the last four years, during which time four blood collections were undertaken. The blood samples were collected from people ranging in age from 0 to 66 years at the time of the accident. The group includes subjects exposed to a range of doses of ionizing radiation during the accident, as well as subjects presumably not exposed to radiation, i.e. the control group. Approximately 250 blood samples have been collected, half of which have already been processed in Dr. Glickman's laboratory in the Biology Department of the University of Victoria. The research involves the determination of mutant frequency and an evaluation of the spectra of mutation in the *hprt* gene of T-lymphocytes from blood samples collected from the Brazilian patients accidentally exposed to ionizing radiation. Blood samples were also collected from neighbours and relatives of the exposed patients, as well as from workers at the Fundação Leide das Neves Ferreira. These comprised the control group.

JUSTIFICATION AND STATEMENT OF GOALS

In March 1993, we began the analysis of T-lymphocytes collected in Brazil, from children accidentally exposed to ionizing radiation, and from a control group. The main objectives of this study are:

(1) To determine the *hprt* mutant frequency of T-lymphocytes in a group of Brazilian children accidentally exposed to low-dose ionizing radiation, and in an age matched control group.

(2) To investigate the effect of ionizing radiation on the mutant frequency of the group of children exposed to ionizing radiation.

(3) To investigate the clonal relationship between mutants isolated in both groups.

(4) To select and expand T-lymphocytes *hprt*-mutant-clones from both groups for further molecular analyses.

Samples from Brazilian children accidentally exposed to ionizing radiation were collected in 1990, two years after the accident. The exposed group includes eight children, six males and two females, ranging in age from 6 to 14 at the time of the blood collection. The doses detected for the group varied between 0.15 and 0.70 Grays. Samples from the control group were collected in 1991. The control group comprises eight children, four

females and four males, ranging in age from 4 to 16 at the time of blood collection. Children from the control group were relatives or neighbours of those in the exposed group, and according to the records of the Fundação Leide das Neves Ferreira, they did not receive any radiation during the accident. It was very appropriate to use this group as a control, since they had the same lifestyle and the same food habits as the exposed group at the time of the accident.

Ionizing radiation was the first known mutagen (Muller and Painter, 1929; Dobzhansky, 1929). However, many aspects of the molecular mechanisms that lead to ionizing radiation-induced mutagenesis remain unclear. The nature of ionizing radiation-induced mutations, as well as the relationship between dose and mutation induction *in vivo* are not established. The most prominent late effect of ionizing radiation exposure has been shown to be an increased incidence in tumorigenesis (Preston *et al.*, 1988). Although the relationship between mutagenesis and carcinogenesis is not completely explained, circumstantial evidence supports the idea that the two processes are related. Several molecular studies have been conducted on the analysis of mutations in humans exposed to ionizing radiation. However, because of the nature of the cases analyzed, those accidentally medically exposed to radiation tend to be adults, who already show increased levels of background (spontaneous) mutations as these accumulate with age (Trainor *et al.*, 1984; Branda *et al.*, 1993; Grist *et al.*, 1992). In addition, other factors including cigarette smoking, food habits, and lifestyle, can conceivably affect mutant frequencies.

Very little work has been done to determine the *hprt*-mutant frequency in T-lymphocytes obtained from children. From the few studies available, compared to adults, children have low mutant frequencies (McGuinnis *et al.*, 1990; Manchester *et al.*, 1992). The low mutation level in children provides an excellent advantage in this study, since lower background levels should facilitate the detection, and analysis, of minimal effects, even following low doses of ionizing radiation. In addition, it has been reported that lymphocyte proliferation decreases with age (Alder *et al.*,1982; Hallgren *et al.*, 1988; Lucivero *et al.*, 1988), making the children's potential high plating efficiencies an attractive benefit in the present study. As it is not yet possible to differentiate whether mutants are spontaneous in origin or not, a suitable control group was also analyzed for the purpose of comparison.

The Goiania accident provides an exceedingly rare opportunity for the analysis of the biological effects of radiation and to an improved understanding of the consequences of human exposure to ionizing radiation. We must emphasize that studies based upon accidents are bound to lack the refinement that could be achieved by experimental settings. Accidents also involve complex populations, with normal human heterogeneity, rather than pure bred laboratory animals. Some limitations concerning the small group of subjects available, the size and conditions of the samples obtained, and the low doses to which the subjects were exposed, are addressed in this work. It is considered that the present study, rather than presenting a single moment in time, can be extended into the future and will clarify some of the delayed effects of acute exposure to ionizing radiation.

CHAPTER I: INTRODUCTION

1.1 HISTORICAL BACKGROUND

The term "mutation" was introduced into the biological literature in 1901 by Hugo de Vries, a Dutch botanist, to account for sudden phenotypic changes that could be transmitted to successive generations. The theory of mutation, however, and its connection with the theory of the gene is due to Herman Joseph Muller. Muller's ideas were expressed in his papers of 1922 and 1923 where he addressed the nature of the gene and the concept of mutation as an alteration of the gene. The idea of the gene as the unit of mutation was later strongly supported by Muller's own observations (1927, 1928) that the rate of mutations in *Drosophila melanogaster* was markedly increased by X-rays. Muller's findings, suggesting a direct proportionality between X-ray dose and number of induced mutations, were later confirmed by a number of authors (Timofeef-Ressovsky, 1934; Timofeef-Ressovsky and Zimmer, 1947; Auerbach, 1976).

The effects of ionizing radiation on human health have been extensively investigated since the discovery of X-rays by Roentgen in 1895, and of radioactivity, immediately thereafter by Becquerel in 1896. Injuries such as eye irritation and skin burns following

radiation exposure were reported very early. However, a linear relationship between dose and induced mutation rate pointing towards a direct action of radiation quanta or released electrons upon the genetic material in the cell, only became feasible after Muller's discoveries. The concept of the gene as the unit of genetic material and, therefore, as the target of radiation, was reinforced as the radiation effects were quantitated. Mutagenic effects of X-rays provided a uniquely suitable tool for probing into the nature of mutations and thus into the concept of genes. Most of the findings of that time were difficult to understand since it was not known that genetic information was stored in the deoxyribonucleic acid (DNA).

The first indication for the connection between the chromosomes and a character difference showing Mendelian inheritance, came from work on sex determination in *Drosophila melanogaster* (Wilson, 1905). More evidence for the relevance of chromosome theory came from the work of Muller and Painter (1929), and from Dobzhansky (1929) involving structural changes in *Drosophila melanogaster* linkage groups, following X-irradiation. Conclusive demonstration that DNA was the carrier of hereditary characters was obtained by Avery, MacLeod and McCarty (1944) when cultures of *Diplococcus pneumoniae* bacterium were transformed with purified DNA. Convincing the entire scientific community, however, were the experiments of Hershey and Chase (1952) which showed that upon infection by bacteriophage T4, only the DNA entered the infected cells, while viral proteins remained outside. This refined but simple experiment demonstrated that DNA was responsible for bacteriophage multiplication, and

therefore, for the transmission of the genetic information.

Most of the findings made in the early period of mutation theory demanded a chemical model of the gene for their interpretation, and since no such model was available, the whole period was characterized by excitement in the experimental field coupled with frustration in the theoretical approaches.

In 1953, Watson and Crick combined the newly proven role of deoxyribonucleic acid (DNA) as the carrier of genetic information, Chargaff's chemical and physicochemical studies of DNA (1949), and the crystallographic studies of Rosalind Franklin and Wilkins (1953), and developed the double helix model for the structure of DNA. This model was immediately acknowledged with the expectation that mutations would be explainable in terms of nucleic acid chemistry, and was subsequently confirmed by both transmission and scanning tunnelling electron microscopical studies. The discovery inaugurated a new age of mutation research, one which was dominated by studies on the chemistry of DNA. The evidence that the nucleotide unit of the double-helix structure, as proposed by Watson and Crick, was indeed the target of mutations came from Benzer's work in 1955, with the fine-structure analysis of the bacteriophage T4 rII locus. This now classic work provided the missing link in the development of a molecular model for the study of mutations.

The study of molecular processes, such as DNA replication, translation and repair, have

helped to elucidate many of the several steps involved in the mutagenesis process. Molecular methods such as recombinant DNA techniques (Jackson and Berg, 1972), and DNA sequencing techniques (Maxam and Gilbert, 1977; Sanger *et al.*, 1977), can now be applied to mutagenesis studies, permitting the detailed analysis of changes in the structure of DNA. This new age of mutation research has been highly productive and detailed information on the action of several mutagenic agents on DNA has been elucidated. However, many basic questions about the cellular mechanism involved in the mutagenesis process remain to be answered.

1.2 THE NATURE OF IONIZING RADIATION

Radiation with sufficient energy to cause ionization of an atom or a molecule is called ionizing radiation. The interaction of ionizing radiation with the medium it traverses leads to the degradation and dissemination of radiation energy. The energy is absorbed by the medium, resulting in the expulsion of electrons from its atoms and molecules which became excited or ionized (Draganic *et al.*,1989).

Energy is clearly a very important component of ionizing radiation. It is critical for the determination of its properties. The unit used to describe radiation energy is the electron volt (eV) or multiples thereof (keV, MeV). For ionizing radiation to have significant biological effects, it must be above 124 eV (2.0×10^{-17}J) (Selman, 1974).

Ionizing radiation can exist as either particles or electromagnetic radiation. Particulate ionizing radiation includes electrons ($ß^-$), positrons ($ß^+$), alpha-particles, neutrons, and heavy ions. Electromagnetic ionizing radiation includes high energy photons such as X-rays and gamma rays. In general, alpha-particles have energies between 4 and 8 MeV. Beta-particles from natural radionuclides have energies ranging from about 0 to 3 MeV. The radiation energy spectrum covered by X-rays and gamma-rays is broad and overlapping. X-rays range in energy from 124 eV to approximately 500 KeV, while gamma-rays range from about 8.3 KeV to over 1 GeV. Except for man-made X-rays and accelerated ions, all these forms of radiation are products of nuclear decay, and their energy is typical of the radioisotope which emits them.

Excitation, as well as ionization, occurs in the electrons of the absorbing atoms when alpha- or beta-particles pass through, whereas photons lose energy almost exclusively by ionization (Upton *et al.*, 1986). The process by which radioactive particles (alpha- and beta-) react with matter (i.e. depositing energy) is basically through collisions (Coulomb interactions) with the absorbing atoms, but beta-particles also lose a small fraction of their energy through a radiative process known as bremsstrahlung (braking rays), whereby a low-energy photon is released when beta-particles are slowed down in matter. Photons lose energy through three mechanisms: photoelectric interaction, where all the energy is lost to a single electron in the absorbing atom; Compton interaction, where part of the energy is lost to a single electron of an absorbing atom, with the remainder being retained with the photon; and pair production, where energy is converted to the mass of

a negative and a positive electron pair. Ultimately, when subatomic particles pass through matter, their energy is transferred to electrons of the molecules with which they interact, thus creating excited molecules, ion radicals or free radicals.

1.3 IONIZING RADIATION AND DOSIMETRY

The biological effects of ionizing radiation are dependent on the absorbed dose delivered to the tissue, the dose rate, and the spacial distribution of energy (Svensson, 1988). Absorbed dose (D) is the mean energy (de) imparted by ionizing radiation per a unit mass of irradiated material at a point of interest, $D = de/dm$. The absorbed dose was traditionally expressed in rads (1 rad = 100 erg/g) (ICRU, 1980), but according to the International Standard (SI) system of units, the unit for the absorbed dose is currently the Gray (Gy). One Gray is equal to 1 joule (J)/Kg (1 Gy = 100 rads). The absorbed dose is generally calculated for the whole body, in the case of external gamma-radiation, and for individual organs, in the case of internal contamination. However, in radiation dosimetry the absorbed dose is of limited significance, since it does not reveal enough information about the way the energy is deposited.

Different types of radiation vary in their ionization density, or "Linear Energy Transfer" (LET). "Linear Energy Transfer" describes the relative amounts and distribution of ionization and excitation energy released along the track of a particle in a certain medium. For a given amount of energy absorbed, a radiation which creates the denser

distribution of ionizations will cause the greater extent of damage. LET is therefore defined as: LET = dE/dl, where dE is the energy lost through collisions by a charged particle in transversing a distance dl in the medium. The unit used to measure LET is KeV/μm. Photons (X-rays and gamma-rays) and electrons (beta-rays) of different energies are considered as low-LET radiation, whereas alpha-particles, n (neutrons), H^+, and heavy ions are high-LET radiation. Some examples for values of LET for different types of radiation are given in Table 1.

A quality factor (Q) is used to relate the biological effects of radiation with different values of LET. It gives an empirical scale of biological damage in relation to the local energy deposition, or the "Relative Biological Effectiveness" (RBE) of distinctive radiations. The quality factor has been established from estimates of the "Relative Biological Effect" of different types of radiation. RBE is a ration of a standard radiation dose (usually 250 KeV X-rays or gamma-rays) needed to produce a given magnitude of a certain biological effect compared to the dose of another radiation needed to produce the same magnitude. The RBE of various radiations depends on both the average rate of energy loss along the paths of individual ionizing particles or photons (LET), and the level of effect. In general, RBE values are observed to increase with increasing LET up to about 70-100 KeV/μm and then to decrease as LET becomes larger (i.e., as energy in excess of that required to induce aberrations is "wasted" after being deposited in critical targets by the high-LET tracks). As a practical value, all photons and electrons have a Q of 1, whereas, heavy particles have larger Q values (Table 1).

Table 1. Linear energy transfer (LET) and relative biological effectiveness (RBE) of various kinds of radiation (Dendy and Heaton, 1989).

RADIATION TYPE	LET (KeV/μm)	RBE
X-rays	3.5 or less	1
gamma-rays	3.5 or less	1
beta-rays	3.5 or less	1
neutrons	23 - 53	10
alpha-rays	175 and above	20

To account for the biological effects of different types of radiation, the dose-equivalent is provided. The dose-equivalent (H) is the product of absorbed dose (D) in rads, times the quality factor (Q), i.e. H = Q X D. In radiation protection, dose-equivalent was introduced in 1977 (ICRUM, 1986). The traditional unit of dose equivalent is the rem, however, in the SI system of units, the dose equivalent is expressed in Sievert (Sv) (1Sv = 100 rem), where 1 Sv = 1 J/Kg.

1.4 CAESIUM-137: PROPERTIES AND BASIC DATA

The teletherapy unit involved in the radiological accident in Goiania was a Caesium-137 source. It contained highly soluble Caesium Chloride salt, that had been compacted to form a cohesive mass which was doubly sealed within two stainless steel capsules. The Caesium-137 properties and basic data relevant for radiological protection purposes are given in Table 2.

Table 2. Caesium-137 properties and radiological protection data. (The Radiological Accident in Goiania, IAEA, 1988).

Caesium-137 Properties		
Gamma emissions		0.66 MeV (84%)
Beta emissions	Maximum energies	0.51 MeV (95%)
		1.17 MeV (5%)
	Mean energy	0.187 MeV
Half life	30 years	
Specific gamma ray constant	8.9 $mGy.h^{-1}$ at 1 m per GBq 0.33 $rad.h^{-1}$ at 1 m per Ci	
Data on the caesium source implicated in the Goiania accident (September 1987)		
Radioactivity	50.9 TBq (1375 Ci)	
Dose rate at 1 m	4.56 $Gy.h^{-1}$ (456 $rad.h^{-1}$)	
Radioactive material (source)		
Volume	3.1 X 10^{-5} m^3	
Mass	0.093 Kg	
Specific activity	0.55 $TBq.g^{-1}$ (15.1$Ci.g^{-1}$)	
Radiological protection data		
Dose rate at 1 m from uniform ground contamination		1.6 X $10^{-12}Sv.h^{-1}.(Bq.m^{-2})^{-1}$
Dose per unit intake (ingestion)		1.2 X 10^{-8} $Sv.Bq^{-1}$
Dose per unit intake (inhalation)		8.7 X 10^{-9} $Sv.Bq^{-1}$
Annual limit of intake (oral)		4.0 X 10^6 Bq
Annual limit of intake (inhalation)		6.0 X 10^6 Bq
Derived air concentration		2.0 X 10^3 $Bq.m^{-3}$

1.5 INTERACTIONS OF IONIZING RADIATION AND BIOLOGIC SYSTEMS

Energy and penetration are the principal properties of ionizing radiation. The energy of radiation is deposited at random in a volume of tissue and all tissues in the irradiated field are vulnerable. The initial event is the transfer of energy from the electromagnetic wave, or particle, to the tissue, resulting in the ejection of an orbital electron from molecules within the cells, leading to the ionization of these molecules. The ionization may directly affect and damage biologically important molecules in the cell, producing the direct effect of radiation (Ward, 1988). Alternatively, ionization can alter a water molecule in the cell leading to the generation of water free radicals (H● and OH●). As the predominant matter in the cells is water, most cellular damage results from reactions between the products of water radiolysis (aqueous free radicals) and other molecules within the cell, characterizing a mechanism termed as indirect effect of radiation.

Free radicals are atoms or molecules containing unpaired electrons, which are chemically highly reactive and unstable. They consist of hydrogen atoms (H●), hydrated electrons (e_{aq}^-), and hydroxyl radicals (OH●):

$$H_2O \xrightarrow{\text{ionizing radiation}} H\bullet,\ OH\bullet,\ H_2O^+,\ e_{aq}^- .$$

Radicals of biomolecules can be produced directly:

$$RH \xrightarrow{\text{ionizing radiation}} R\bullet + H\bullet,$$

or via the water radicals:

$$RH + OH\bullet \longrightarrow R\bullet + H_2O,$$

or: $R' + OH\bullet \longrightarrow R'OH\bullet$.

In the presence of oxygen, these radicals can be transformed into peroxiradicals:

$$R\bullet + O_2 \longrightarrow ROO\bullet.$$

Because the above reaction prevents recombination,

$$R\bullet + H\bullet \longrightarrow RH,$$

elevated oxygen levels increase the radical yield in the medium. Therefore, the biological effects of ionizing radiation strongly depend on the oxygen concentration (Koch, 1979) and can be extenuated by enzymes such as superoxide dismutase (SOD), catalase, and gluthatione (GSH) peroxidase as well as by dietary antioxidants such as ascorbate, α-tocopherol, ß-carotene, glutathione and ubiquitol (Frei and Ames, 1992).

1.6 THE TOXICITY OF IONIZING RADIATION

The toxicity of ionizing radiation on the human body is exerted through the chemical changes induced at the cellular level. These changes may affect individual cells in many different ways, causing cell death, the impairment or the delay of cell division, or permanent genetic modifications (i.e. mutations), which may be transmitted to daughter cells.

In humans, the harmful consequences of irradiation depend upon the dose, and become noticeable at different times after exposure. Early effects can take place in a matter of hours or days. These include symptoms such as bone marrow depletion, gastrointestinal

tract disturbance, and neuromuscular injuries. However, it may take years or decades for the late effects, such as leukaemia and other forms of cancer, to become noticeable. Hereditary effects involving chromosomal and genetic modifications may become apparent only in future generations (Schull *et al.*, 1981; Schull *et al.*, 1990; Yoshimoto *et al.*, 1990).

Factors which determine the type and extension of radiation toxicity, and radiation damage, depend on the absorbed dose, the dose rate, and LET as the main determinant of the damage potential of ionizing radiation (Bender, 1988). Other factors involve radiation conditions, and whether the entire body or part of the body is exposed.

Sensitivity of different mammalian cell lines to ionizing radiation does not generally vary by more than a factor of two, except for some DNA-repair deficient mutants that can be up to 10 times more sensitive than normal cells (Jeggo and Kemp, 1983; Thacker, 1989). However, when tissues are irradiated *in vivo*, a variation of radiosensitivity for each organ, and a variable interval before the effects are detected, can be observed. The cellular basis for tissue failure after ionizing radiation exposure is not completely established. There is a latency period varying from tissue to tissue that can be largely explained in terms of the cell kinetic parameters (Denekamp, 1989). Lesions can be rapidly detected in cells with short cell cycle times such as proliferative cells of the intestine, epithelial cells, and immune cells. However, lesions in lung, kidney, heart and other deep tissues which present very little natural turnover, can take months or years

to be observable (Denekamp, 1989).

In general, after low doses exposure, late tissue damage is seen more often than are acute reactions. Sometimes this reflects a true difference in radiosensitivity of cellular constituents. In other instances, however, this is simply due to the fact that only survivors of the early damage are available to express the late injury. Therefore, if death occurs early due to the sensitivity of a specific tissue, the animal cannot express late injury in a more radioresistant organ.

When the delayed effects of ionizing radiation exposure are taken into account, the development of neoplasia appears probable as the more harmful consequence. Various studies performed on atom bomb survivors (Beebe *et al.*, 1977; Schull *et al.*, 1981), radon exposed groups (Evans *et al.*, 1981; Samet, 1989; Samet *et al.*, 1989), and in uranium miners (Lubin *et al.*, 1990), demonstrate that populations exposed to high levels of ionizing radiation show increased incidence of neoplasia. The mechanism by which radiation causes cancer is not yet completely understood. Carcinogenesis is a multistep process, and each of these steps can be caused by a mutation (Nowell, 1976; Temin, 1988; Weinberg, 1989; Bishop, 1987; Volgstein and Kinzler, 1993; Little, 1993). A long and variable latency period may occur between exposure to radiation and the appearance of cancer. According to studies performed on victims of the atomic bomb in Japan, most of the leukaemia occurred 6 to 7 years after exposure, while the risk of all other forms of cancer becomes significant about 15 to 25 years after exposure (Kondo, 1993). While

somatic mutations are able to cause diseases like cancer, loss or modification of hereditary information in germ cells can possibly lead to genetic diseases in ongoing generations (Sankaranarayanan, 1991; 1994).

1.7 CYTOGENETIC EFFECTS OF IONIZING RADIATION

Ionizing radiation-induced chromosomal aberrations have been primarily studied in cultured human cells (Bender, 1957) and in human bone marrow cells *in vivo* (Tough *et al.*, 1960). However, the development of the short-term peripheral blood lymphocyte culture technique (Moorhead *et al.*,1960) permitted the rapid progress of human radiation cytogenetics and its application to biological radiation dosimetry (Clemenger and Scott, 1971; Lloyd *et al.*, 1986). In addition to ionizing radiation, many chemical and physical agents induce chromosomal aberration. It is important to recognize, as well, that aberrations can also arise spontaneously, without any known exposure to clastogenic agents (Awa and Neel, 1986). Indeed, clastogenic agents, including ionizing radiation, do not introduce any new or novel types of aberrations, but simply increase the frequency of those which occur at low frequency without any exposure (Brandom *et al.*, 1978a).

Chromosomal sensitivity may be understood according to the phase of the cell cycle in which the cell is irradiated. In the G1 phase, a chromosome contains a single DNA double helix running its length. The formation of aberrations involves direct or indirect breakage of the double helix and interactions between the broken ends. DNA double

helix chromosomes, containing deletions or other aberrations, are replicated as well, giving rise to the class known as "chromosome-type" aberrations, involving both chromatids identically. Once DNA has replicated (interphase), each double helix generally behaves as an independent target giving rise to aberrations called "chromatid-type" aberrations, involving only one or the other chromatid at any given location (Bender *et al.*,1988). In biological radiation dosimetry, human peripheral lymphocytes are the most used cells. Because the majority of these cells are in the blood in a nondividing pre-DNA-synthesis stage (G1 or G_o) of the cell cycle (Amneus and Erickson, 1986), radiation exposure induces exclusively chromosome-type aberrations.

Chromosomal aberrations are also classified according to the number of breaks involved and the subsequent interactions between broken ends (Bender *et al.*, 1988). Most of the lesions or breaks induced in interphase chromosomes are repaired and no aberration is seen at metaphase. If unrepaired (or misrepaired), single strand breaks lead to "deletions" whereas the deleted portion, no longer attached to the rest of the chromosome, is called "acentric fragment" (Bender *et al.*, 1988). Two or more unrepaired or misrepaired breaks can occur in the same cell, giving rise to multiple deletions. However, multiple breaks can also interact and rejoin, giving rise to chromosome forms called "rearrangements" or "exchanges" (Bender *et al.*, 1988).

Chromosomal aberrations may be modified or lost during cell divisions. Aberrations that are evident at the first metaphase following their induction may appear in different forms

in subsequent metaphase, or, they may definitely not be evident in subsequent metaphase, since the cell should not survive to enter another division. The destiny for chromosomal aberrations in the next cell generations depends mostly on the mechanical events related to cell division (Bender *et al.*, 1988). Acentric fragments may appear in the cytoplasm of daughter cells as micronuclei (Balasem, 1992; Cruz *et al.*, in press). When the two breaks involve a single chromosome, asymmetrical rearrangements result in ring chromosomes, while symmetrical rearrangements give rise to an inversion within a chromosome. If the two breaks are in different chromosomes, asymmetrical rejoining yields dicentric chromosomes, while symmetrical rejoining gives rise to translocation. Inversions and translocation can persist over many cell divisions and for this reason they are referred to as "stable " aberrations (Bender *et al.*, 1988).

The frequency of chromosomal aberrations in peripheral blood lymphocytes has been employed to estimate radiation dose in several cases of accidental (Kodama *et al.*, 1989; Lloyd *et al.*, 1986; Natajaran *et al.*, 1991; Ramalho *et al.*, 1990), occupational (Brandom *et al.*, 1978a, 1978b; Jha and Sharma, 1991; Sardas *et al.*, 1992), and medical exposures (Kelsey *et al.*, 1991c; Rigaud *et al.*, 1990). Ultimately, all types of ionizing radiation induce the same kinds of chromosomal aberrations in exposed cells. The number of aberrations induced, however, depends on the level of radiation exposure (dose) which is directly related to the amount of energy deposited by independent radiation tracks within the cells of the body (LET). This model assumes of course that the chromosomes are critical sites or targets within the nucleus. Cytogenetic dosimetry is straightforward

in the cases of external exposure to penetrating radiations such as gamma-rays or fast neutrons. It has been demonstrated that radiation exposure can be detected in populations when doses are low (Metalli, 1989) or, when doses are higher and the lymphocytes are sampled many years after exposure (Awa, 1983). However, for internal exposures, and especially for particulate radiation like alpha-particles, meaningful dosimetry is difficult, if not impossible. In the case of non-uniformly distributed internally deposited radionuclides, the radiation dose is not only non-uniform itself, but there is also a non-uniform distribution of lymphocytes in the body. Therefore, to estimate the radiation dose, both a relative distribution of the isotope, and the distribution of lymphocytes relative to the isotope, have to be considered.

1.8 IONIZING RADIATION AND DNA DAMAGE

DNA is supposed to be a very stable molecule, as it is committed to the maintenance and perpetuation of genetic information. However, DNA is subject to many structural changes. Changes in the chemistry, or specific sequence of particular nucleotide can arise as consequence of natural interactions, and natural transactions of DNA (such as those involved in replication, recombination and repair itself) (Friedberg, 1985). Changes can also result from the instability of chemical bonds present in the nucleotide or of inter-nucleotide bonds. Such bonds can be affected, for example, by the physiological conditions of temperature and pH. Furthermore, DNA is not an inert chemical in living cells. The DNA molecule is very reactive; it can react with a variety of chemical

compounds and physical agents that are present in any cellular environment. Changes resulting from the natural interactions or transactions of DNA are known as spontaneous damage, while those resulting from interactions between DNA and known chemical or physical agents are known as induced damage (Friedberg, 1985).

Unlike many DNA-damaging agents, ionizing radiation can be deleterious for all cellular components, and not only the DNA. However, most of the molecules located inside the cell have many replicate copies and their loss is not likely to be critical. The critical lesions caused by ionizing radiation are likely those in DNA, because of the singular nature of the information it carries. Ionizing radiation causes molecular disruption by exciting and ionizing molecules, displacing electrons, and hence breaking or weakening molecular bonds. The lesions can be caused directly, from primary ionizations in the DNA molecule, or indirectly by the attack of radicals generated by ionizations of other molecules. Most of the ionizing radiation energy is absorbed in the water compartment of the cells, since it represents about 70% of the cell weight (Alberts *et al.*, 1989). In many cases, the ultimate effects of radiation are essentially a consequence of reactions between species formed by the radiolysis of water (hydrogen peroxide, hydrogen atoms, hydrated electrons and hydroxyl radicals) with the chemical constituents of DNA. In the tissues, most of the ionized radiation-induced-damage involves OH-radicals. OH-radicals attack the DNA by abstracting covalently bound hydrogen atoms or by addition to double bonds in the DNA bases (Ahnstrom, 1988).

The effects of X-rays on DNA and its components have been investigated *in vitro* since the late 1940's (Scholes *et al.*, 1949). The role of oxygen on DNA degradation, the promotion of breaks in the ester bonds, sugar and bases rings, as well as the production of base derivatives were reported very early (Scholes *et al.*, 1949; Scholes *et al.*, 1960; Ward and Urist, 1967; Ward, 1975). This broad collection of DNA lesions has been investigated under many different conditions, and in different phases of the cell cycle (Friedberg, 1985; Friedberg, 1987; Ward, 1988), thus numerous details related to their generation, reactivity and consequences are nowadays understood.

REACTIONS WITH THE BASES MOIETIES

The predominant reaction with the bases is the addition of OH-radicals, e^{aq-} and H atoms to the double bonds. The double bonds are opened up and free radicals are formed on one of the carbon atoms. The C5-C6 double bond in pyrimidines is the favoured position for the attack of those radicals, while in purines the most vulnerable atoms are C4, C5 and C8 (Hutchinson, 1985; Dizdaroglu, 1993). OH-adduct radicals of purine and pyrimidine, as well as their derivatives, are well established in the presence and absence of oxygen (Hutchinson, 1985; Friedberg, 1985; Dizdaroglu, 1993). Some of these base products can also react with the sugar moiety in the DNA structure, giving rise to more complex derivatives. Figure 1 shows several base derivatives potentially resultant from the attack of oxygen-radicals to the base moieties in DNA structure.

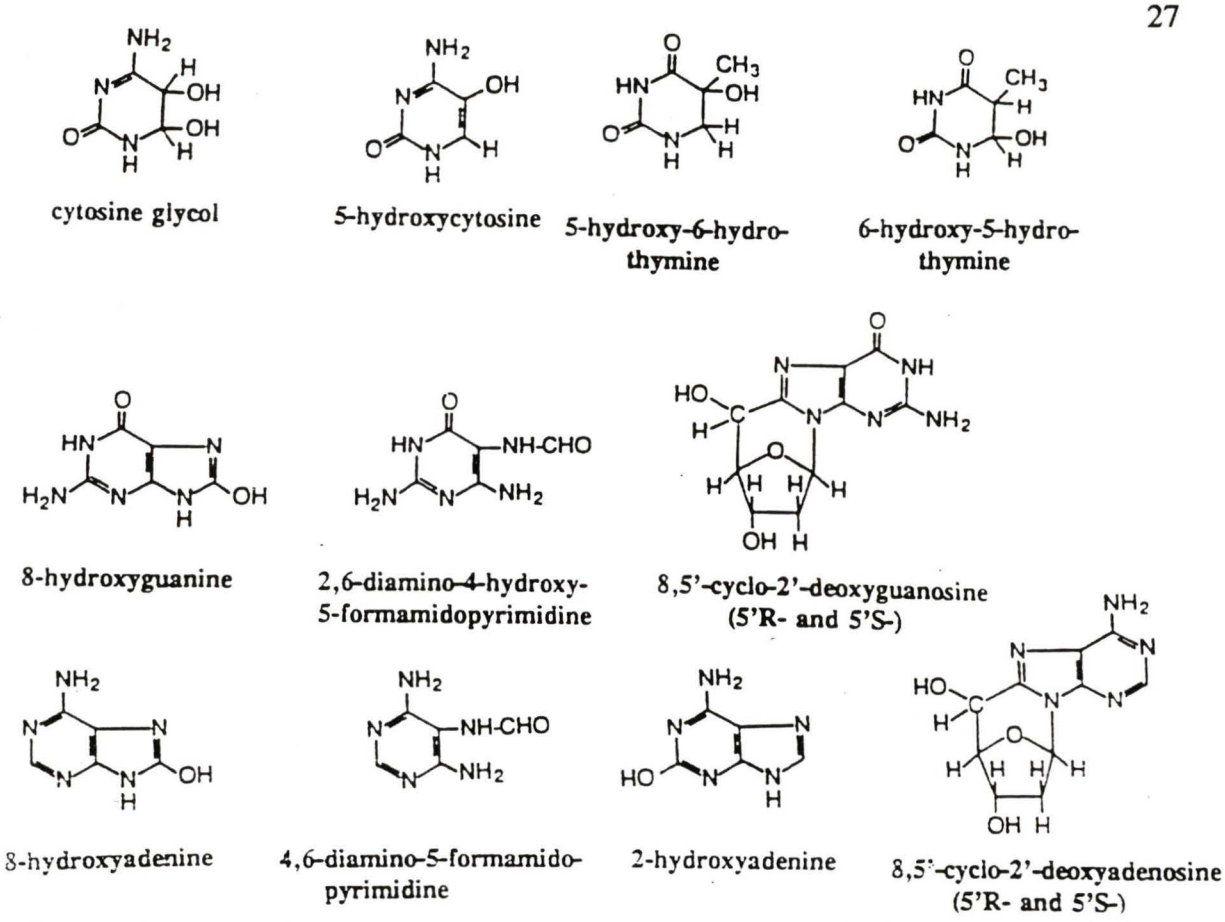

Figure 1. Base-derived products resultant from the attack of free oxygen-radicals to DNA. (Dizdarouglu, 1993)

REACTIONS WITH THE SUGAR MOIETIES

Abstraction of hydrogen from all five carbon atoms in the deoxyribose moiety is the primary radiation induced type of lesion in the sugar moiety of DNA (von Sonntag, 1987). Sugar radicals may undergo intra-molecular rearrangements such as beta-cleavage, giving rise to DNA strand breakage followed by release of an intact base and the formation of altered sugars (Dizdaroglu *et al.*, 1975; Beesk *et al.*, 1979). The oxidation of the C1-entered radical gives rise to a sugar lactone and to the release of an intact base (Dizdaroglu *et al.*, 1977). In the absence of oxygen, base radicals can also abstract H

atoms from a neighbouring sugar moiety leading to sugar radicals, and subsequent strand breakage (von Sonntag, 1987). In oxygenated systems, peroxyl radicals are formed by the addition of molecular oxygen to carbon-entered sugar radicals. Sugar peroxyl radicals are also converted into oxyl radicals, which undergo ß-cleavage giving rise to DNA-strand breakage and to the release of an altered sugar and an intact base (Dizdaroglu *et al.*, 1975), or to DNA strand breakage with the formation of an aldehyde group at the C5' end (Goldberg, 1987). It is important to note that the ultimate damage caused by ionizing radiation in the deoxyribose moiety is that one that leads to DNA strand breakage. Figure 2 shows some sugar products potentially obtained from the attack of oxygen-radicals to the sugar moieties in the DNA structure.

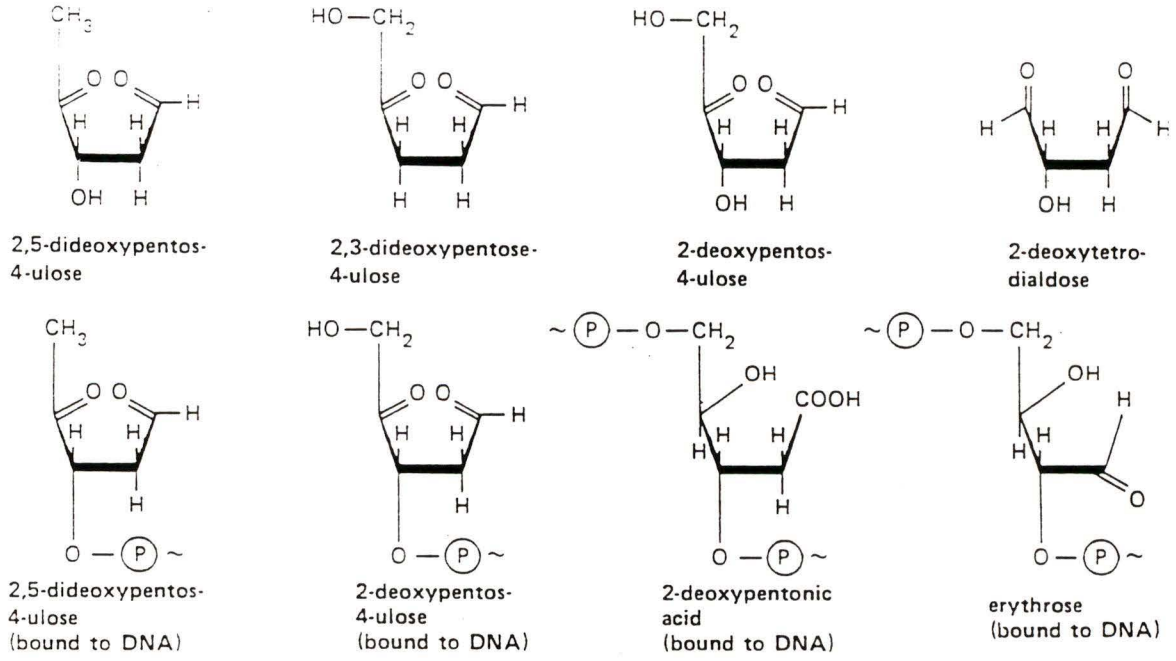

Figure 2. Sugar derived products resultant from the attack of free oxygen-radicals to the DNA molecule. (Dizdarouglu, 1993)

DNA-PROTEIN CROSSLINKS

In addition to DNA sugar and base products induced by free radicals after exposure to ionizing radiation, DNA-protein crosslinks in nucleoproteins are also detected (Oleinick *et al.*, 1987). Hydroxyl-radicals seem to be the major mediators of such DNA-protein cross-links that can be formed by combination of two radicals, one DNA radical and one protein radical. Radical addition reactions may also lead to DNA-protein cross-link by the addition of a DNA radical to an aromatic amino acid in the protein, followed by oxidation of the formed adduct radical. DNA-proteins crosslinks involving diverse amino acids have been identified in nucleoprotein exposed to ionizing radiation in the absence of oxygen. However, in the presence of oxygen, the formation of such DNA-protein crosslinks was inhibited except for those involving thymine and tyrosine (Nackerdien *et al.*, 1991). Decondensed protein near actively transcribed genes appears to be a better crosslink substrate than condensed protein (Chiu *et al.*, 1986).

STRAND BREAKS

Strand breaks can arise either by the direct or indirect effect of ionizing radiation (Friedberg, 1985). In *Escherichia coli* K-12, it was demonstrated that the deposition of approximately 9 eV of energy is sufficient to break the deoxyribose-phosphate backbone (Youngs *et al.*, 1976), while the indirect effect of ionizing radiation induced strand breaks seems to be mediated by hydroxyl-radicals (Bonura *et al.*, 1976). The breakage

of phosphodiester linkages in one of the polynucleotide chains, and the destruction of the deoxyribose rings are, the main cause of DNA strand breakages. A single strand break can result in a very localized denaturation in the neighbourhood of the break, which increases the probability for the attack of further free radicals in the same site. It has been suggested that the accumulation of strand breaks in a close vicinity is related to the formation of double strand breaks (Ward *et al.*, 1985). The mechanism by which DNA double strand breaks are generated is not completely explained. Because energy deposition events occur in clusters, more than two damage moieties can occur in close proximity in the DNA molecule leading to the generation of double strand breaks (Ward, 1988). For gamma-radiation, the distance between ionizations within a cluster has similar dimensions as the diameter of the DNA-helix. After gamma-ray exposure, a small percentage of the lesions appear as double strand breaks. The fraction of double strand breaks increases when a more densely ionizing type of radiation is used, such as alpha-rays, in which the distance between the formed clusters is so small, that it results in a continuous track of ionizations (Ahnstrom, 1988). The most destructive effect of ionizing radiation on biological systems is its ability to form DNA double strand breaks. It has been suggested that double strand breaks can also arise as repair intermediates when bases in opposite sites of the DNA complementary strands are damaged. If the modified bases are removed at the same time from the two strands, by the action of glycosylases, and their overlapping regions become gapped, double strand breaks can be therefore produced (Salganik and Dianov, 1992). Double strand breaks are typically caused by ionizing radiation and their formation correlates directly with chromosome aberrations

(Natarajan and Obe, 1978; Natarajan *at al.*, 1980) and cell lethality (Ritter and Cleaver, 1977) after exposure.

1.9 THE REPAIR OF IONIZING RADIATION DNA DAMAGE

Fortunately, ionizing radiation DNA-lesions are not necessarily permanent. For every 1 Gy of absorbed dose approximately 1000 single strand breaks, 40 double strand breaks and other assorted lesions are produced (Frankenber and Schwager, 1989; Ward, 1987). All cells seem eminently capable of repairing single strand breaks, using the opposite helical strand as a template. They are even capable of repairing most of the double-strand breaks, but a small fraction of these lesions are irreparable (perhaps 2-5%) (Denekamp, 1989). It is thought that this small percentage is responsible for cell death (Bonura *et al.*, 1975; Ritter and Cleaver, 1977; Frankenber and Schwager, 1989) and for the later effects of ionizing radiation.

The repair of DNA lesions in mammalian cells relies on two main pathways. Both of them require the preincision recognition of the lesion, incision of the damaged DNA strand at, or near the site of the defects, excision of the defective site and the local degradation of the affected strand, repair replication to replace the excised region with a corresponding stretch of normal nucleotide, and finally, ligation to join the repair patch at its 3' end to the contiguous parental DNA strand (Bohr *et al.*, 1987).

The first pathway utilizes a multiprotein complex (in *E. coli*, the UvrABC nuclease complex) with endonucleolitic activity for the recognition of the damage and incision of the strand probably on both sides of the lesion. The damaged nucleotide(s) and 12 to 45 additional nucleotide (Schrader, 1992) are removed and the gap is subsequently filled by DNA polymerase. The newly synthesized segment is joined to the old strand by ligase. This process is called nucleotide excision repair. The recognition signal for the endonuclease complex is probably a major helical distortion such as cross links or bulky adducts (Friedberg, 1991). Pyrimidine dimers induced by ultraviolet light and agents such as polyaromatic hydrocarbons are predominantly repaired by this pathway. There is evidence that this pathway also repairs some of the ionizing-radiation-induced lesions such as abasic sites, and thymine glycol, at least in *E. coli* (Czeczot, 1991). However, to what extent it repairs the same types of lesions in human cells is not known.

The second pathway involves a combination of glycosylases and apurinic/apirimidinic (AP)-endonuclease to achieve the first two steps in the repair, i.e. the recognition and incision steps (Bohr *et al.*, 1987). Glycosylases remove the damaged base and AP-endonuclease cleave the strand at the abasic site. Resynthesis and ligation completes the repair. Evidently the repair polymerase is different from the one used in the repair of bulky lesions. The recognition signal may be different for different glycosylases (Breimer and Lindahl, 1985). Some glycosylases recognize obviously specific chemical alteration in the base. In this case, the recognition signal may be more physico-chemical, such as change in the base stacking which induces minor helical distortions (Friedberg, 1985).

This pathway which starts by removing the damaged base is called base excision repair, and seems to take care of lesions induced by methylating agents which introduce rather small adducts into DNA. Most damage induced by ionizing radiation also seems to be processed via this pathway.

In eukaryotic cells a preferential distribution for DNA lesions and their repair has been described (Bohr *et al.*, 1987; Mullenders *et al.*, 1991; Carreau and Hunting, 1992; Wu and Lichten, 1994). Since the chromatin is a very compact structure with limited access to the DNA, it has been shown that repair of UV-induced pyrimidine dimers by the endonuclease complex is performed at a higher rate in regions with a more open chromatin configuration, such as in active genes (Bohr *et al.*, 1987). In contrast, a mutant in which the endo complex has been replaced by pyrimidine glycosylase, repairs dimers in all regions with the same rate (Bohr *et al.*, 1987). Its is likely that because of its size, the endonuclease cannot easily find lesions hidden in compact regions of the chromatin, while the glycosylases, rather small proteins, are able to penetrate the chromatin and start the repair process. However, in order for the ligase, a fairly large enzyme to complete the repair process, a local chromatin modification seems to be required. This modification may involve the participation of poly (ADP-ribose) synthetase. It has been suggested that this enzyme is stimulated by DNA strand breaks (Friedberg, 1985), and adds negatively charged groups to histone 1. When enough charge has accumulated, the histone are repelled from the DNA and the chromatin opens up (Ahnstrom *et al.*, 1988).

Single strand breaks that have intact 3'hydroxyl and 5'phosphate intact groups can be repaired by direct ligation (Friedberg, 1985). In mammalian cells, three different ligases have been identified (Tomkinson *et al.*, 1992). Single strand breaks with damaged ends cannot be directly repaired by ligase, since intact 3'hydoxyl and 5'phosphate groups are an essential requirement for this enzyme (Friedberg, 1985). Damaged ends require an extra step, probably catalized by an exonuclease (Salganik and Dianov, 1992), before ligation occurs. However such a mechanism remains to be proven in mammalian cells. One gene, bovine Apurinic 1 (BAP1), shows extensive homology with two bacterial genes (ExoA and ExoIII), which are involved in oxidative damage repair, makes a good candidate for the repair of damaged ends (McKay and Hanawalt, 1992).

Double-strand-breaks cleave the DNA molecule so that, at least in solution, the contact between the two fragments is lost. In the cell, the situation may be quite different, and dependent upon how the DNA is structurally organized. The DNA molecule is supported by various structures which might prevent the broken ends from diffusing away from each other. It has been demonstrated that homologous DNA-recombination seems to play an important role in the repair of double strand breaks in yeast and mammalian cells (Wu and Lichten, 1994; Valancius and Smithies, 1991). *E. coli* grown under conditions which promote the presence of several chromosomes in the cell is able to repair double strand breaks with high efficiency. In contrast, slow-grown *E. coli* cells with essentially one chromosome per cell are not able to repair DNA double-strand breaks (Hutchinson *et al.*, 1977). A parallel situation exists in yeast, where haploid cells are deficient in double

strand break repair while diploid cells have the capacity to repair such damage (Resnick *et al.*, 1978).

In mammalian cells, the role for p53 protein has also been described in the repair of single and double strand breaks. It was demonstrated that DNA strand breaks are sufficient, and probably necessary, to obtain p53 induction in cells exposed to DNA-damaging agents (Nelson and Kastan, 1994). It was also demonstrated that p53 may be directly involved in the alignment of complementary strands during the rejoining of single-strand DNA ends (Bakalkin *et al.*, 1994).

ADAPTIVE RESPONSE TO IONIZING RADIATION

A phenomenon related to ionizing radiation induced-repair, termed "adaptive" response has been reported in several studies (Olivieri *et al.*, 1984; Shadley *et al.*, 1987a, 1987b; Shadley and Wiencke, 1989). When mammalian cells cultured *in vitro* are pre-exposed to a low dose of ionizing radiation, they exhibit reduced biological effects following radiation. Reduced effects can include fewer chromosomal and chromatid aberrations, increased cell survivability, and a reduction in mutations (Kelsey *et al.*,1991a), following subsequent high dose exposure. The adaptation effect appears to depend on the dose, dose rate, and on the phase of cell cycle in which the exposures are performed (Shadley *et al.*, 1989). An optimal interval between the doses seems also to contribute to the efficiency of the response (Bai and Chen, 1993), which appears approximately 4 hours

after the first exposure and lasts for about 90 hours or three cell cycles (Shadley *et al.*, 1987b). The adaptive response can also be induced by clastogenic chemicals such as bleomycin and mitomycin C (Vijayalaxmi and Burkat, 1989, 1990), that mimic the effects of ionizing radiation, and also by low doses of hydrogen peroxide (Cortes *et al.*, 1990). The molecular mechanism involved in the adaptive response is not yet understood. It has been reported that the adaptive response is prevented when cells are cultured in the presence of cyclohexamide (an inhibitor of protein synthesis) (Youngblom *et al.*, 1989), 3-aminobenzamide (an inhibitor of poly-(ADP-ribose)polymerase) (Wiencke, 1986), or in a nicotinamide deficient medium (Wiencke, 1987). The existence of an ionizing radiation induced-adaptive response is still controversial, since the evidence for this response in humans is very sparse. Some authors were able to detect the response only when doses were delivered during specific phases of the cell cycle (Shadley *et al.*, 1987), while others were not able to detect it at all (Grosovski and Little, 1986; Hain *et al.*, 1992).

MAMMALIAN GENES ACTIVATED BY IONIZING RADIATION

During typical cell growth, four phases in the cell cycle can be distinguished: G_1, S (when DNA synthesis occurs), G_2 and M (when mitosis occurs). The G_0 phase comprises nongrowing cells that have left the cell cycle. A cell-cycle dependent variance in sensitivity to ionizing radiation is documented. Cells in M phase are the most sensitive to ionizing radiation, followed by cells in S phase. Cells in G_1 and G_2 are the most

resistant to radiation (Coggle, 1983). This sensitivity variance is assigned, at least in part, to DNA damage mechanisms that enhance protection, alter mutagenesis, alter cell growth, and ultimately enhance cell survival. In the case of cell growth, cell cycle delays are common responses to DNA damage, and are mediated by several genes, denoted *gadd* (growth arrest and DNA damage inducible) genes (Fornace *et al.*, 1989). A "G_2 checkpoint" induced by DNA damage, and responsible for the delay between the G_2 phase and mitosis, has been described in yeast (Weinert *et al.*, 1988; Rowley *et al.*, 1992) and in mammalian cells (Muschel *et al.*, 1991). This G_2 delay has a protective effect, presumably by allowing damage, such as radiation-induced double strand breaks, to be repaired prior to mitosis (Weinert *et al.*, 1988). Another checkpoint activated by DNA damage is in the G_1 phase of the cell cycle. This delay presumably protects by allowing time for repair of lesions that interfere with accurate replication. The tumour suppressor gene p53 is involved in this checkpoint (Diller *et al.*, 1990). In human cells, upon radiation-induced DNA damage, p53 protein levels increase, leading to activation of the G1 checkpoint. This permits the cell to repair its DNA, or if the damage is too elevated, to exit by apoptosis (Scott *et al.*, 1993; Lane, 1993). In addition to delaying the entry into S phase, DNA damage can also delay the progression through S phase, and part of this delay may be due to direct inhibition of DNA polymerase action by DNA damage, specially at high doses. In mammalian cells, activation of these check points by DNA damage is not due simply to the deleterious effects of the damage, but instead it represents active processes involving induction and regulation of different genes.

In mammalian cells, a very complex response is triggered by ionizing radiation, as well as by other genotoxic agents. It comprises the induction of several genes associated with many different cellular processes including signal transduction, intercellular signalling, growth control, responses to tissue injury, inflammation, DNA repair, responses to oxidative stress and other potentially protective responses (for a review see Fornace, 1992). The induction of such genes can be initiated by different types of DNA-damaging agents. Among them, oxidative stress appears as a major mediator. The study of DNA damage triggered by ionizing radiation has allowed the recognition of many processes involved in the cell response and their connection with the development of diseases like neoplasia, however, a remarkable number of questions still remains to be answered.

1.10 SPONTANEOUS MUTATION IN HUMAN POPULATION

There are several important reasons for the study of mutations occurring *in vivo* in human cells. According to numerous studies, it has been demonstrated that mutations in critical genes are related to the process of carcinogenesis (Vogelstein and Kinzler, 1993; Sommer, 1994). Furthermore, the study of gene mutations is presently used as a biomonitor of genotoxic exposure in human population, since the frequency and nature of mutations may reveal evidence into the origins of mutations (Sankaranarayana, 1993; Bridges, 1994; Ammenheuser *et al.*, 1993). Differences in the frequency and nature of mutations can also identify individuals who may present high risk of mutational damage from exposure, such as DNA-repair deficient individuals (Steingrimsdottir *et al.*, 1993;

Langlois *et al.*1989; Papadopoulo *et al.*, 1990). Therefore, *in vivo* mutational studies in the human population are important for both toxicological and biological purposes.

Since exposure to mutagenic agents has been correlated with the development of diseases (Fry, 1991; Little, 1993; Lubin *et al.*, 1990), attempts have been made to distinguish between spontaneous and induced mutations in humans. However, such discernment is almost impossible. Factors inherent in experimental limitations, intrinsic DNA instability, endogenous DNA damage products, and those related to the nature of the cells analyzed, might all interfere with the yield of mutations recovered without any known mutational treatment (Smith, 1992; Featherstone *et al.*, 1987; Kiefer, 1993). In addition, all types of mutations (base substitutions, frameshifts, insertions and deletions) seem to have the potential to be produced spontaneously. Therefore, the spontaneous mutation rate of an organism can be considered to be the result of all exogenous and endogenous factors that are either mutagenic or antimutagenic (Smith, 1992). Because of such discrepancies the term "spontaneous mutation" has been gradually replaced by "background mutation" particularly to account for data examined from human populations in the absence of specific treatment (Glickman *et al.*, 1994).

In humans, HeLa-cells, the lymphoblastoid cell line TK6, transformed fibroblast, and T-lymphocytes are the preferential cell models employed in the analysis of spontaneous mutagenesis. For studies *in vivo*, blood is the most frequently used tissue for monitoring DNA damage and mutations, since it can be obtained conveniently. Genetic endpoints

used to access mutant frequency in circulating blood cells include haemoglobin and glycophorin A variants in erythrocytes, and in lymphocytes, the frequency of mutations in the X-linked *hprt* gene, in the HLA locus in chromosome 14 and in the CD3/T-cell receptor complex.

A significant number of studies have been carried out to determine the mutant frequency in blood cells from people not exposed to any known mutagenic agent, and also from people known or believed to have been exposed to mutagenic or carcinogenic agents. Cancer patients, and patients having genetic disorders with deficient DNA repair or with increased sensitivity to mutagenic agents, have also been extensively analyzed. Cells analyzed in these studies consist mostly of T-lymphocytes and erythrocytes. In T-lymphocytes, three genetic endpoints have been studied, including the X-linked *hprt* gene, the alleles of the HLA A locus on the short arm of chromosome 6, and the CD3/T-cell receptor complex located on chromosome 14 (alpha) and 7 (beta). In erythrocytes, the frequency of haemoglobin and glycophorin A variants has also been evaluated. A recent review (Cole and Skopek, 1994) comparing results obtained from several different laboratories, demonstrates that a wide range of variance in mutant frequency exists for the different endpoints analyzed (Table 3). Factors affecting this wide range of variation are related to the size of the target gene analyzed, the chromosomal location of such a gene, the complex kinetics of proliferation and differentiation of the cells used in each study, and finally to experimental factors, since different methods have been used in those measurements.

Table 3. Average spontaneous mutant frequencies for different endpoints in human population (data from Cole and Skopek, 1994).

Cell Type	Genetic endpoint analyzed	Mean Mutant Frequency
T-lymphocytes	*hprt*	$> 5 \times 10^{-6}$
T-lymphocytes	HLA-A	$> 1 \times 10^{-5}$
T-lymphocytes	CD3/TCR complex	$> 1 \times 10^{-4}$
Erythrocytes	Haemoglobin	$\sim 5 \times 10^{-8}$
Erythrocytes	Glycophorin A	1×10^{-5}

The data available demonstrates that mutant frequency in human subjects is low at birth (McGinnis *et al.*, 1990; Manchester *et al.*, 1992). Mutant frequency increases with age, achieving values about tenfold higher in young adults (Trainor *et al.*,1984; Grist *et al.*, 1992, Branda *et al.*, 1993; Tates *et al.*, 1991). The increase in mutant frequency is not very well understood, but it has been attributed to different factors, such as an elevation in the spontaneous mutation rate, a decrease in the ability for DNA-repair, effects of endogenous mutagens produced in normal cellular processes, or an effect of prolonged exposures to low doses of environmental mutagens (Cariello and Skopek, 1993). Mutant frequency has been found to be elevated in smokers (Tates *et al.*, 1991; Cole *et al.*, 1989), although such differences have not always been significant (Branda *et al.*, 1993). Studies carried out on groups who have been exposed to known mutagens and carcinogens (Caggana *et al.*, 1992; Dempsey *et al.*, 1985; Hakoda *et al.*, 1988a, 1988b;

Nicklas *et al.*, 1990, 1991; Sala-Trepat *et al.*, 1990), as well as on patients with known or suspected defects in DNA repair (Tates *et al.*, 1989; Steingrimsdottir *et al.*, 1993; Papadopoulo *et al.*, 1990) demonstrate that higher mutant frequencies are detected in such groups. It is also known that for each mutational end-point the level of intra- and inter-individual variation within any donor category is clearly significant (Cole and Skopek, 1994). For this reason, in attempt to detect small increases in the *in vivo* mutation rates induced by low levels of "environmental" mutagen, stringent controls and the sources of variation within each system have to be well established.

1.11 IONIZING RADIATION AND MUTATIONS

Mutations are the consequence of rare failures in the mechanism used to replicate and maintain DNA and in those designed to repair DNA following a damage event (Glickman, 1985). Despite the fact that mutations provide the necessary basis for genetic and biological diversity in the evolutionary process (Arber, 1990, 1991; Drake, 1991), replication fidelity and repair efficiency must be taken into account, since mutations also play a major role in many genetic disorders (Cooper and Krawczak, 1990, 1991; Krawczak and Cooper, 1991), in the process of carcinogenesis (Bishop, 1987; Loeb, 1989; Ames and Gold, 1991; Vogelstein and Kinzler, 1993; Sommer, 1994) and in the aging process (Kirkwood, 1989; Mullart *et al.*, 1990; Ames and Gold, 1991).

When organisms are exposed to a mutagenic treatment such as ionizing radiation, the

resulting DNA damage (base damage, adduct formation, depurination, and strand breakage) is considered the initial step in the series of events that lead to the adverse effects of the exposure. If the cells survive, a subsequent step is the induction of mutations. In this case, mutations are introduced predominantly from the so called error-prone DNA repair, or misrepair. Once a mutation is established, i.e., once it has been fixed, which usually takes no more than one or few cell cycles, it cannot be reversed, except by the extremely rare event of a reversion (Auerbach, 1976). Based on this observation, many techniques have been developed making possible the determination of mutation rates and the molecular characterization of mutations arising spontaneously or under specific treatments. Such methods employ a variety of cell models such as bacteria, yeast, transformed and non-transformed cell lines, transgenic animals, and humans.

While ionizing radiation is a very strong cytotoxic agent, it is however considered to be a relatively weak mutagen. This is because a reasonable level of induced mutation occurs only at radiation doses that kill a significant fraction of the cells (Little, 1993). *In vitro* studies using mammalian cells, demonstrated that ionizing radiation-induced mutations increase as linear function of dose (Albertini and De Mars, 1973; Cox and Masson, 1976; Liber *et al.*, 1987), with total doses of acute irradiation up to approximately 4 to 6 Gy. Higher doses produce so much cell killing that it is difficult to quantify directly the frequency of mutants among the small fraction of surviving cells. Dose-rate effect for the induction of mutation in humans cells by fractionated exposure seems to be relatively small (Grosovski and Little, 1986; Tabocchini *et al.*, 1989; O'Neil *et al.*, 1990).

However, a significant fraction of mutants may accumulate in cell populations receiving fractionated radiation exposure, since cellular recovery processes greatly reduces the cytotoxic effects. As a consequence, protracted fractionated exposures may be relatively more mutagenic than acute exposures.

In humans, studies *in vivo* have been carried out on individuals accidentally, therapeutically, or occupationally exposed to ionizing radiation. A significant number of people with known exposure to ionizing radiation, such as atomic bomb survivors and cancer patients undertaking radiotherapy, have been analyzed for the glycophorin A locus in erythrocytes and for the *hprt* gene in T-lymphocytes. However, these studies present some limitations. Dose estimations for subjects exposed to ionizing radiation in the workplace or during accidents are always questionable and in these cases the validation of the study requires the establishment of suitable non-exposed control groups for comparative purposes. Moreover, because of the extensive individual variation (Cole and Skopek, 1994), the comparison of two different groups can be very complex. Cancer patients undertaking radiotherapy have been examined in several studies. Although many of these studies present clearly defined doses and offer the possibility of following the patient before and after treatment, limitations also arise here, since many of the patients have been exposed to several highly toxic treatments.

Survivors of the Hiroshima atomic bomb have been monitored for glycophorin A (GPA) mutations some 40 years after the initial exposure (Langlois *et al.*, 1987; Kyoizumi *et*

al., 1989). Despite inter-individual variations and experimental limitations, a significant linear relationship between variant frequency (VF), and radiation exposure, was demonstrated for three of the variant cell phenotypes (Nϕ, Mϕ and MM) of the GPA locus in the subjects analyzed. Further studies on the GPA variant frequencies were carried out on victims from two other radiological accidents, the Chernobyl nuclear power plant accident (Langlois *et al.*, 1990a; Jensen *et al.*,1990), and the Goiania, Brazil, ^{137}Cs radiological accident (Langlois *et al.*, 1990b; Straumer *et al.*, 1991). These last studies presented similar results to those obtained from the Hiroshima analysis, and with a few exceptions, they demonstrated an excellent correlation with the dicentric frequencies which were used for the dose estimations.

A large amount of data is available for the *hprt* locus in T-lymphocytes from people exposed to ionizing radiation. Because of the wide range of variation obtained with different methods, only those obtained with the so-called "*hprt* clonal assay" will be addressed here.

Cancer patients treated with chemotherapy and/or radiotherapy have shown remarkable increases in *hprt* mutant frequency, as demonstrated in Table 4. When the results are analyzed before, and immediately after treatment, mutant frequency was found to increase significantly (Messing and Bradley, 1985; Dempsey *et al.*, 1985; Sala-Trepat *et al.*, 1990). In patients where the effects of chemotherapy and radiotherapy, either alone or in combination, were analyzed, it has been suggested that the increase in mutant

frequency was almost wholly accounted for by the effect of radiotherapy (Sala-Trepat *et al.*, 1990). Previous studies employing non-clonal techniques for the *hprt* assay (Ammenheuser *et al.*, 1988; Ammenheuser *et al.*, 1991a, 1991b) had demonstrated that within 2 months following radiotherapy the high mutant frequency reported immediately following treatment returned to the pretreatment levels in most of the subjects analyzed. During a prospective study using the *hprt* clonal assay, where cancer patients were analyzed 3 to 48 months after treatment, persistent high mutant and mutation frequencies were demonstrated. None of the patients, however, were analyzed before the study (Caggana *et al.*, 1992). Although an average induction of mutant frequency of about 7 X 10^{-8} mutants/locus/cGy was suggested for breast cancer patients treated with gamma radiation (Messing and Bradley, 1985), no induction rates could be substantiated in later studies. Several studies comparing the *hprt* mutant frequency from control groups and cancer patients undergoing radiotherapy, have also been developed in different laboratories (Nicklas *et al.*, 1990, 1991; Kelsey *et al.*, 1991), showing that higher *hprt* mutant frequencies were always detected in radiotherapy patients.

Table 4. *hprt* mutant frequencies from cancer patients treated with chemotherapy and/or radiotherapy. Data obtained with the *hprt* clonal assay.

GROUP DESCRIPTION	AGE (years)			CLONING EFFICIENCY(%)			MUTANT FREQUENCY (X 10^{-6})			REFERENCES
	Mean	SD	range	Mean	SD	range	Mean	SD	range	
12 Breast cancer patients undergoing radiotherapy (Dose=50Gy, 2Gy/day during 5 weeks)	54.3	-	25 - 70							Messing and Bradley, 1985.
. Before treatment				6.5	-	1.6 - 14.7	8.6	-	0.1 - 54.7	
. After treatment (1 to 2 months)				2.5	-	0.9 - 7.9	36.0	-	4.9 - 160.8	
31 Breast cancer patients undergoing chemotherapy and radiotherapy	46.6	-	32 - 66							Sala -Trepat, *et al.*, 1990
. 28 cancer patients before treatment				24.3	-	4.2 - 49.8	20.6	-	6 - 73.0	
. 11 after radiotherapy only				22.0	-	5.2 - 44.8	36.4	-	18 - 80.0	
. 11 after chemotherapy only				33.7	-	4.6 - 45.5	26.2	-	11 - 38.0	
. 10 radiotherapy followed by chemotherapy				24.7	-	5.6 - 105	37.9	-	21 - 76.0	
. 17 chemotherapy followed by radiotherapy				21.6	-	8.6 - 47.6	38.7	-	7 - 142.0	
25 Cancer patients undergoing radioimmunoglobulin therapy (Dose=21Gy)										Nicklas *et al.*,1991
. 13 Patients before treatment	58	±11	40 - 76	23.0	±9	8.0 - 34	11.5	±5	0.9 - 20.3	
. 12 Patients after treatment	51	±20	17 - 77	14.0	±9	3.5 - 35	27.8	±16	7 - 70.8	
69 Cancer patients untreated or treated with chemotherapy and/or radiotherapy										Dempsey *et al.*, 1985
. 28 untreated patients with solid tumours	59	±3	-	20.9	-	-	6.3	-	-	
. 14 untreated patients with lymphomas	52	±4	-	14.6	-	-	7.8	-	-	
. 9 patients with solid tumours treated with chemotherapy and/or radiotherapy	45	±6	-	5.1	-	-	25.7	-	-	
. 18 patients with lymphoma treated with chemotherapy and/or radiotherapy	55	±3	-	6.5	-	-	24.3 -	-	-	
Obs. All the results are given as the geometrical mean										
6 Hodgkin's disease patients analyzed 3.4 to 20 years after x-ray therapy (Dose 30-40Gy)	8.6	-	25 - 57	19.4	±9	8.2 - 37.3	19.7	±11	3.7 - 35.0	Kelsey *et al.*, 1991

Table 04.(cont.)										
21 Hepatoma patients receiving ^{131}I radioimmunotherapy 2 to 7 prior to the analysis	49.2	-	20 - 83	8.2	-	1.0 - 43.0	90.2	±67	13.0 - 235.0	Nicklas *et al.*, 1990
11 Cancer patients treated with chemotherapy and/or radiotherapy 28 to 41 months prior to analysis (Dose 66-70Gy)										Caggana *et al.*, 1992
	34.3	-	24 - 48	16.3	-	9.3 - 32.1	14.3	-	6.0 - 27.2	
. 6 Hodgkin's disease patients	55	-	37 - 66	16.0	-	5.0 - 22.4	13.0	-	5.6 - 30.0	
. 5 squamous cell carcinoma patients										
Obs.The results presented here correspond to the average of the values obtained during a prospective study comprising various analysis of the patients over a 6-7-month period.										

Atomic bomb survivors have been studied for their *hprt* mutant frequencies in T-lymphocytes (Hakoda *et al.*, 1988 a, 1988b). When the results were compared with non-exposed controls, values significantly higher were detected for those exposed (Table 5). It was thus observed that *hprt* mutant frequencies persistently higher than controls can be detected even forty years after exposure. A relatively weak correlation between mutant frequency and estimated dose was described for the group. In contrast, a significant correlation was revealed when mutant frequencies were related with chromosomal aberrations (Hakoda, 1988b).

In order to investigate potential mutagenic effects of *in vivo* exposure to low levels of ionizing radiation during nuclear diagnostic tests using radionuclides (Thallium 201 and Technetium-99m), *hprt* mutant frequencies in T-lymphocytes have been determined by different groups (Table 6) (Seifert *et al.*, 1987; Kelsey *et al.*, 1991a, 1991b; Bachand *et al.* 1991; van Dam *et al.*, 1991). Despite the fact that the early studies (Seifer *et al.*, 1987) demonstrated a significant increase in *hprt* mutant frequency in lymphocytes from patients undergoing such treatments, no increase in mutant frequency was detected by other groups. It has since been suggested that a difference in the effective dose received by the patients lymphocytes accounted for the apparent contradiction with the earlier studies (Bachand *et al.*, 1991). Therefore, it is generally concluded that no detectable increase in *hprt* mutant frequency was detected for patients exposed to radionuclides used in nuclear medicine.

Table 5. *hprt* mutant frequencies from Atomic bomb survivors analyzed forty years after exposure. Data obtained with the *hprt* clonal assay.

GROUP DESCRIPTION	AGE (years)			CLONING EFFICIENCY(%)			MUTANT FREQUENCY ($X10^{-6}$)			REFERENCES
	Mean	SD	range	Mean	SD	range	Mean	SD	range	
37 subjects analyzed forty years after exposure . 30 subjects with doses: 1-300rads . 17 subjects with doses under 1 rad	58.5	-	-	44 43	- -	26-74 30-65	5.2 3.4	- -	0.8 - 14.4 1.3 - 9.3	Hakoda *et al.*, 1988.a
50 subjects analyzed forty years after exposure/Dose estimated according to the levels of chromosomal aberrations . 17 controls . 13 subjects from the high aberration group . 17 subjects from the low aberration group	58.5	-	-	43 43 49	- - -	- - -	3.4 6.7 3.7	- - -	- - -	Hakoda *et al.*, 1988.b

Table 6. *hprt* mutant frequencies from heart disease patients undergoing nuclear medicine diagnostic tests. Data obtained with the *hprt* clonal assay.

GROUP DESCRIPTION	AGE (years)			CLONING EFFICIENCY(%)			MUTANT FREQUENCY ($X 10^{-6}$)			REFERENCES
	Mean	SD	range	Mean	SD	range	Mean	SD	range	
10 Heart disease patients undergoing diagnostic test with one injection of Technetium 99-m (T1/2=6h) Dose:10-15mGy	-	-	25-65							Seifer *et al.*, 1987
. Before injection				35	±40	13 - 85	2.09	±3.2	0.32-10.84	
. 1 - 3 months after injection				16	±22	1 - 52	7.62	±21	0.83-68.83	
24 Heart disease patients undergoing diagnostic test with one injection of Thallium-291(T1/2=73h) Dose:2-30mCi	52	-	38-82							Kelsey *et al.*, 1991
. Before injection				15	-	1 - 67	5.2	±4.8	0.60-16.5	
. 24 hours after injection				21	-	1 - 64	5.7	±5.4	0.40-20.9	
. 1 month after injection				19	-	1 - 40	3.1	±1.8	0.30-56.50	
17 Heart disease patients undergoing diagnostic test with one injection of Technetium-99m	52	-	38-82							Kelsey *et al.*, 1991
. Before injection				16	-	2 - 47	4.1	±3.9	0.6-16.0	
. 24 hours after injection				14	-	2 - 36	3.5	±3.1	0.9-14.2	
. 1 month after injection				16	-	1 - 30	4.0	±6.4	0.2-18.0	
25 Heart disease patients undergoing diagnostic with one injection of Thallium-201	51.1	-	38-66							Bachand *et al.*, 1991
. Before injection				15	±16	1 - 76	32.9	±8.0	1.1-204.0	
. 1 - 3 months after injection				12	±9	2 - 33	34.3	±6.0	2.3-137.0	
13 Heart disease patients undergoing diagnostic test with one injection of Technetium-99m	51.5	-	33-67							van Dam *et al.*, 1991
. Before injection				16	±17	1 - 48	21.2	±20	5.7-71.5	
. 8 - 120 days after injection				33.4	±31	2 - 94	11.1	±7.2	1.7-22.4	

1.12 THE HPRT MODEL FOR THE STUDY OF MUTAGENESIS IN HUMAN CELLS

The measurement and molecular analysis of mutations arising *in vivo* in human populations has become possible during the last years (Albertini *et al.*, 1990; Cole and Skopek, 1994). However, genes that can be conveniently monitored *in vivo* are generally not those of particular biological interest. It is experimentally difficult to study, for example, genes related with the process of cancer in diverse tissues. Except for blood lymphocytes and transformed cells, it is impossible to carry out cultures of human cells obtained from normal tissues in an attempt to select those exceptionally rare ones bearing specific gene mutations. It is believed that mutations arising in blood cells in a certain indicator loci can reveal a pattern of mutation that may reflect mutagenic exposure. Since blood is an easily obtained tissue, many studies employing blood cells have been performed in the assessment of genotoxic exposure.

The most intensively investigated locus in human cells is the hypoxanthine-guanine phosphoribosyl transferase (*hprt*). The gene codes for the enzyme hypoxanthine phosphoribosyl transferase (HPRT: IMP:pyrophosphate phosphoribosyltransferase . 2.4.2.8) that catalyses one of the first steps in the salvage pathway for the purine bases hypoxanthine and guanine in mammalian cells. HPRT converts preformed purine bases, hypoxanthine and guanine, into their respective nucleotide (Figure 3), providing an alternate pathway to de novo synthesis of inosine-monophosphate (IMP) and guanosine-

monophosphate (GMP) (Stryer, 1988). The major advantage of HPRT in mutation studies is that it is a non essential enzyme, permitting the selection for and against gene expression in cultured animal cells. The existence of two pathways for the formation of purine nucleotide, grants mutant cells the ability to grow in medium containing purine derivatives, such as 6-thioguanine and 8-azaguanine, while wild type cells die. Resistance thus results from the inability of the mutant cells to synthesise toxic nucleotide from purine analogues, whose incorporation in the DNA impairs replication. On the other hand, cells lacking HPRT activity are unable to survive in HAT medium. HAT medium contains hypoxanthine (a purine source), aminopterin (an inhibitor of purine and thymidine synthesis) and thymidine (a pyrimidine source), consequently cells kept in this medium are rendered purine-deficient, and compelled to use the salvage pathway for purine acquisition.

In humans, HPRT deficiency is associated with two clinical disorders: a complete deficiency of HPRT results in the Lesch-Nyhan syndrome, characterized by developmental delay, spastic movements, postural tremors, mental retardation and behavioral abnormalities, while the partial deficiency is characterized by hyperuricemia, uric acid crystalluria and gouty arthritis (Stout and Caskey, 1988). HPRT has been detected in all somatic tissues, however, considerable differences in the distribution and activity of the enzyme have been demonstrated in different organs (Stout and Caskey, 1985).

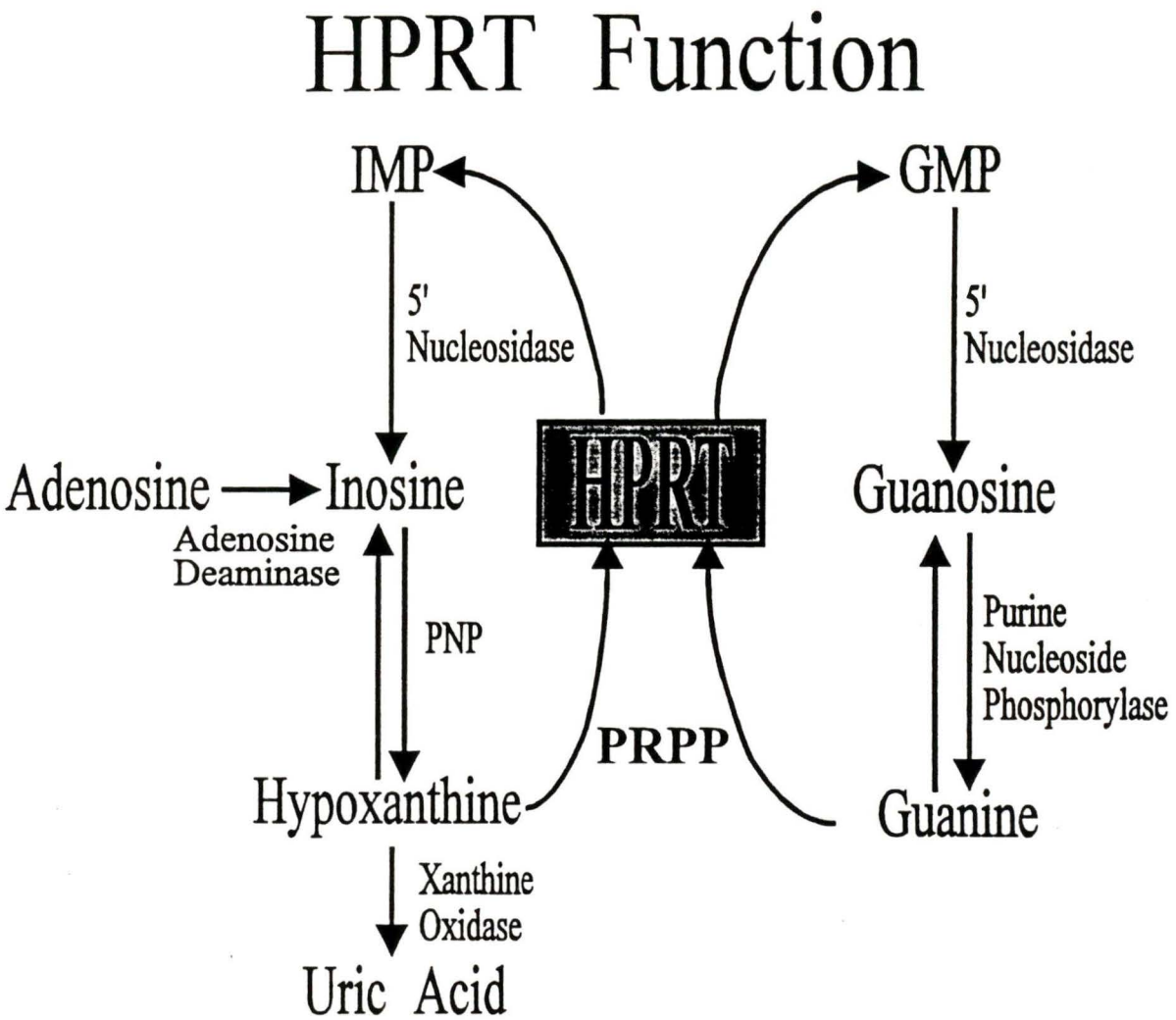

Figure 3. HPRT Function. HPRT is an enzyme from the salvage pathway of the purine metabolism. The salvage pathway recycles 90% of all free purine bases by nucleotide conversion (Stout and Caskey, 1985). HPRT catalyses the condensation of 5'-phosphoribosyl-1-pyrophosphate (PRPP) and the purine bases hypoxanthine and guanine to form 5'-inosine monophosphate (IMP) and 5'-guanosine monophosphate (GMP), respectively. The selection procedure used in the *hprt* clonal assay employs a base analogue, i.e. 6-thioguanine, that is converted into its respective analogue by the action of HPRT. The incorporation of such derivative nucleotides into DNA structure results in growth inhibition and death of wild type cells. *hprt* mutants are unable to catalyse such conversion and are therefore able to survive in the presence of 6-thioguanine.

The HPRT enzyme is not essential for cells growing in culture, which is one of the advantages of the system to select mutant cells (Caskey and Kruth, 1979). The levels of hprt-mRNA have been investigated in cultured human cells (Steen *et al.*, 1990; Steen *et al.*, 1992). It has been demonstrated that reduced, and occasionally null, *hprt*-mRNA levels are observed in the majority of the TG-resistant T cell clones, and also that many different types of *hprt* mutations can lead to this effect. HPRT m-RNA is expressed at very low levels in resting (Go) lymphocytes, but there is a relatively high, stable level of HPRT enzyme activity in such cells. In dividing T-lymphocytes, the levels of both m-RNA and enzyme increase considerably (Steen *et al.*, 1990, 1991).

In humans, HPRT is encoded by a single structural gene mapped to Xq26-27 (Figure 4). The gene comprises 42 Kb and is divided into nine exons (Kim *et al.*, 1986). Eight introns are removed during the processing of the primary transcript to yield an mRNA with approximately 1650 nucleotides which codes for a 218 amino acid monomer. Both cDNA (Jolly *et al.*, 1983) as well as the entire gene (Edwards *et al.*, 1990) have been sequenced.

The X-linked *hprt* gene has been widely used in mutation assays in animal and human cells for both *in vitro* and *in vivo* studies. As a result of the X-inactivation which occurs early in the development of females, the gene is functionally homozygous in both male and female cells. Since *hprt* is X-linked, only one functional copy is present in the cell and therefore a single mutational event can convert a given cell from wild type to a

mutant that can be detected in both genders. The *hprt* model is therefore different from autosomal genes where mutations in both alleles are necessary for the expression of the mutant phenotype. The *hprt* model allows the detection of a wide variety of mutations, including large and small deletions, frameshifts, single-base substitutions, as well as mutations that cause aberrant mRNA splicing. However, some caution is necessary when using an X-linked gene as a model for mutation studies. Since there is only a single copy of the *hprt* gene in male cells, mutational mechanisms that involve two alleles, such as gene conversion and recombination, cannot be studied in this type of system (Cariello and Skopek, 1993).

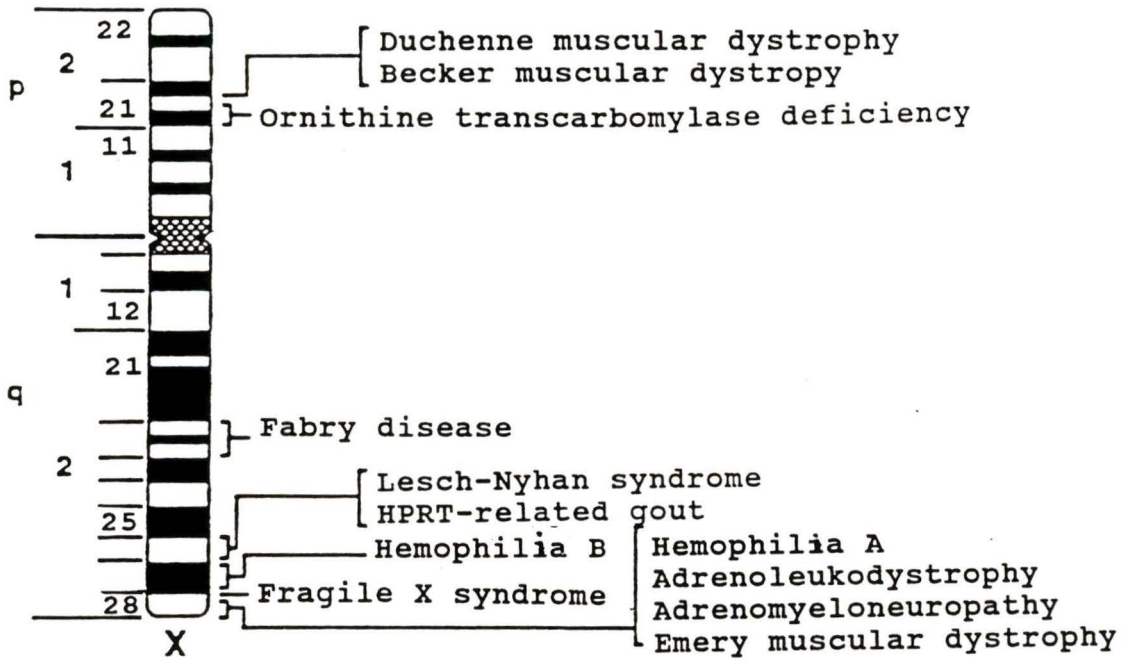

Figure 4. Chromosome location of the *hprt* gene.

The selection procedure for the *hprt* model is based on the fact that cells having HPRT activity can also convert purine analogues such as 8-azaguanine, used in many early experiments, or 6-thioguanine (6-TG), used in more recent years, to their respective nucleotide. Subsequent incorporation of such nucleotides in the DNA structure results in cell growth inhibition or cell death, since normal cells are sensitive to the derivative nucleotide. Cells with impaired or absent HPRT activity have impaired or absent ability to synthesize and incorporate such nucleotide analogues and are consequently resistant to them.

The original *hprt* assay for monitoring mutations in human populations is an autoradiographic assay (Strauss and Albertini, 1979). T-lymphocytes were stimulated to divide, using the mitogen phytohaemagglutinin (PHA), in a medium containing tritiated thymidine ^{3}H-TdR, in the presence and absence of 6-thioguanine (6-TG). Nuclei containing grains were then scored, assuming that only HPRT variants lacking enzyme activity could undergo the first mitogen-stimulated division in the presence of the selective agent. Very high "spontaneous" frequencies of variant cells, approximately 1 X 10^{-4} in normal donors, were reported in the initial studies (Strauss and Albertini, 1979). These very high estimates for *hprt* mutant frequencies were later explained by the fact that although the majority of circulating lymphocytes are in the Go phase of the cell cycle, some freshly isolated cells are already undergoing division, and these cells are able to incorporate the radioactive label in the presence of 6-thioguanione before they die. Later studies modifying the procedure of the assay (Morley *et al.*, 1983; Vijayalaxmi *et*

al., 1984; Everson *et al.*, 1985) and using cytometric cell sorting (Amneus and Erickson, 1986) reinforced this hypothesis. Freezing and thawing the cells before the test was performed rectified this artefact (Albertini, 1982), however the precise mechanism by which this modification operates remains to be explained. A different version of the *hprt* non-clonal assay, using 5-bromodeoxyuridine incorporation and Hoechst 33258 dye to identify the 6-thioguanine resistant cells by fluorescence microscopy is also available (Ostrosky-Wegman *et al.*, 1987). The non-clonal versions of the *hprt* assay have the advantage of being relatively simple, rapid and inexpensive. However, the disadvantage is that the mutant cells selected cannot be cloned and expanded for further molecular analysis of the nature of the mutational events.

The cloning version of the *hprt* assay which enables the selection and cloning of T-lymphocytes carrying *hprt* mutations directly from human peripheral blood was developed in the early 1980's (Albertini *et al.*, 1982, 1985; Morley *et al.*, 1983; Turner *et al.*,1985). Mononuclear cells obtained from blood samples are cultured in the presence of the mitogen, phytohemoaglutinin (PHA), which stimulates T-lymphocytes to divide. After about two days in culture, the cells are seeded in 96-well microtitre plates in the presence of irradiated feeder cells and T-cell growth factor interleukin-2 (IL-2). Under these conditions, T-cells divide to produce clones which can be scored after approximately 14 days incubation. When the selective agent 6-thioguanine is added to the medium, only cells mutant at the *hprt* gene can form colonies. Typically, 20-100 mutants are recovered from 10-20 ml of blood obtained from normal donors (Cariello and

Skopek, 1993). Cloning efficiencies in the presence and absence of 6-TG are calculated from the proportion of negative wells using the Poisson distribution, and the mutant frequency is given by the relationship between cloning efficiency in the presence of 6-thioguanine and cloning efficiency in the absence of 6-thioguanine (Morley *et al.*, 1985). Several laboratories have investigated, in detail, the culture conditions necessary to support good growth and cloning of T-cells (Morley *et al.*, 1985; Cole *et al.*, 1988a; Hakoda *et al.*, 1989; Beare *et al.*, 1993). The clonal version of the assay requires notably more resources, time and money than the non-clonal assay. However, it is the only available way to assess and analyze stable phenotypic alterations in human cells over several cell generations. It has the advantage that the mutant cells may be collected and propagated for confirmation of the mutant status and for analysis of the mutational spectrum. Furthermore, the *hprt* clonal assay also allows the molecular analysis of T-cell receptor (TCR) rearrangements (Nicklas *et al.*, 1987; Curry *et al.*1993). Since T-cells can undergo clonal expansion *in vivo* it is important to differentiate whether the several mutants selected in a given sample are related to the same clone, or if they really represent individual mutant events (Caggana *et al.*, 1992).

A large amount of sequence and frequency information exists for the *hprt* gene. In an attempt to centralize the data available, and facilitate the analysis and comparisons from different laboratories, a computerized *hprt* database with respective software became available in 1991 (Cariello and Skopek, 1992).

THE T-CELL RECEPTOR ASSAY

The human T-cell receptor (TCR) is a highly specific heterodimer protein expressed on the surface of T-lymphocytes. TCR recognizes foreign proteins and peptide presented by histocompatibility (MHC) proteins (Germain, 1994). The analysis of T-cell receptor (TCR) genes rearrangement in T-lymphocytes has been extremely useful in mutation studies employing the *hprt* clonal assay. High *hprt* mutant frequencies obtained with the clonal assay can be due to two different conditions. They can result from a large expansion of a small number of pre-existing *hprt* mutant cells, that might occur during the normal repopulation and antigenic stimulation of lymphocytes after recovery from cytotoxic exposure, or they can, in fact, result from a large number of independent mutations. The analysis of T-cell receptor gene rearrangement patterns can distinguish these two conditions, defining whether the individual really bears a high mutation frequency, or if he is only undergoing a clonal expansion of a T-lymphocyte precursor that sustained an *hprt* mutation.

T-cell-receptor rearrangement analysis of *hprt* mutant cells is also useful to determine the stage of cell differentiation in which the mutation occurred. TCR rearrangements occur early in the thymic ontogeny of T-lymphocytes during fetal development through early adolescence (Abbas 1991). During this stage, each T-cell rearranges its T-cell receptor and the resultant rearrangements are then fixed for the life of the cell, labelling a T-cell and its clonal siblings. T-cells show tremendous heterogeneity in the TCR and each T-

cell progenitor has the potential to be unique. Therefore, *hprt* mutant T-cells showing different TCR rearrangement patterns probably arose *in vivo* in different mature T-cells, representing independent mutational events. Despite the fact that *hprt* mutant T-cells with identical *hprt* mutations, but with different TCR rearrangement patterns, can represent past pre- or intra-thymic *hprt* mutation, this finding can also define a mutational hot spot within *hprt*. However, the term "hot spot" implies that the same mutation is often detected in different individuals.

The remarkable selectivity and diversity of T-cell response against foreign antigens is granted by the extraordinary diversity of amino acid sequence present in its T-cell receptor molecule (Ashwell and Klausner, 1990). Functional TCR genes are formed by somatic rearrangements of germline polymorphic gene segments, by a process similar to immunoglobulin gene rearrangements. The α and ß gene products combine to form the αß-heterodimer, while the products of gamma- and δ-genes combine to form the gamma-σ-heterodimer. TCR rearranged genes are formed from separate genes, the variable (V), joining (J), the constant (C), and in some cases the diversity (D) gene or random (N) nucleotide stretches. A remarkable polymorphism is observed for such genes that are able to generate numerous different combinations. Randomly added or deleted nucleotides are also detected in the N regions, making the number of possible rearrangements many times larger. The process of somatic rearrangement is a pre-requisite to TCR gene expression and is the basis for generation of TCR diversity (Abbas, 1991). TCR somatic rearrangements are mediated by V(D)J recombinase. This enzyme has endonuclease,

exonuclease and deoxynucleotidyl transferase activities (Gaus and Lieber, 1993). Recognition signals are conserved heptamer and nonamer sequences present in the genome adjacent to each rearranging segment (Gellert, 1992). The exonuclease and deoxynucleotidyl transferase activities of the enzyme are responsible for the random sequences at the junction between variable and joining segments (Lafaille *et al.*, 1989; Tonegawa *et al.*, 1983). At least three genes (ß-, gamma- and σ-genes) have been shown suitable for TCR analysis of T-lymphocytes clonal relationship (Kimura *et al.*, 1989; Nicklas *et al.*, 1986; de Boer *et al.*, 1992; Curry *et al.*, 1993). Since each gene rearranges independently, a distinct DNA restriction pattern, which persists for the life of the stem cell and its clonal descendants, is generated. This pattern thus operates as a fingerprint by which cells of common clonal origin can be identified. Ultimately, genomic DNA digests from the T-cell gamma-σ or α-ß heterodimers are extracted, and the products are analyzed by Southern blot (Nicklas *et al.*, 1987; 1989), single strain conformational polymorphism (SSCP) (Caggana *et al.*, 1992), or restriction fragment length polymorphism (RFLP) (Curry *et al.*, 1993; de Boer *et al.*, 1992).

CHAPTER II: METHODOLOGY

2.1 STUDY GROUPS

The study groups consisted of Brazilian children accidentally exposed to ionizing radiation and a control group presumably non-exposed. The control group includes eight children, four males and four females, ranging in age from 4 to 16 years. The control group lived in the neighbourhood where the radiological accident happened. Some are relatives of the exposed victims, and had similar lifestyles and food habits to those children accidentally exposed to ionizing radiation. According to the records of the Fundação Leide das Neves Ferreira, no radiation exposure was detected for children in the control group during the radiological accident. The exposed group comprises ten children, six males and four females. They range in age from 6 to 14 years, and according to the Fundação Leide das Neves Ferreira, the radiation doses, estimated by whole body-counting and cytogenetic assay, range from 15 to 70 cGy.

2.2 BLOOD COLLECTION AND CRYOPRESERVATION

In January, 1990, two years after the radiological accident, blood samples were obtained

from children accidentally exposed to ionizing radiation in Goiania, Brazil. Cells from the Brazilian control group were also collected in Goiania, in January, 1991, using the same procedure. Since most of the subjects were young children, the blood samples collected were often smaller than average, ranging from 10 to 20 ml.

Whole blood was collected by venipuncture using Leucoprep Vacuum Systems (Beckton-Dickinson). The systems contain sodium heparin and a Ficol/Hipaque column, i.e., a polyester gel plug under which lies a ficol gradient. Vacuntainer tubes containing whole blood were centrifuged 1800xg for 30 minutes, at room temperature. Centrifugation results in the migration of red blood cells and polymorphonuclear cells down through the plug, and in the formation of a dense band of mononuclear cells (MNC's) in the gradient. MNC's fractions consist of lymphocytes (T and B) and macrophages. MNC's were then removed and washed in blank RPMI 1640 (Professional Diagnostics). A second wash was performed in appropriate medium containing RPMI 1640, 10% bovine calf serum (CBS) (Professional Diagnostics) and antibiotics (100 U/ml penicillin and 100μg/ml streptomycin) (Sigma). The final washed pellets were resuspended in 2-5 ml of the same medium and counted for cell viability. Viable cells were then resuspended at densities ranging from 1 X 10^6 to 10 X 10^6 cells per ml in a freezing medium, containing RPMI 1640, 50% CBS, 10% dimethylsulfoxide (DMSO) (Sigma), and antibiotics. Cryopreservation was made in 1.8-ml plastic cryotubes. Controlled freezing by lowering the temperature approximately 1°C per minute was obtained by placing cryotubes in a freezing tray in the vapour phase of a liquid nitrogen tank. Cryotubes were

stored in liquid nitrogen and later transported to our laboratory in Canada. A considerable delay was incurred between the preparation of the cryotubes and the controlled freezing in the blood collection of the exposed children. Due to technical problems, cells remained at room temperature for about four hours, instead of being frozen immediately. We suspect that this delay reduced the survival of some cell samples during later experiments.

2.3 CLONAL ASSAY

The *hprt* clonal assay has been used by several groups to determine the mutant frequency in human populations, however, some variations are observed in the methods used in different laboratories. Since such variations can substantially affect the cloning efficiency of T-lymphocytes and therefore the mutant frequency for a given sample, we decided to evaluate some of the components present in the growth medium used in our procedure and to establish the optimal conditions for our experiments. Using appropriate concentrations for each component and the methodology previously used in our laboratory, one blood sample was split and plated under nine different medium conditions. Each fraction was plated in a distinct medium combination. After 15 days, the plates were scored for cloning efficiencies. The nine different medium combinations used in the experiment are described in Table 7. Combinations A, B, C and D were chosen to evaluate the effects of calf bovine serum (CBS), human serum (HS), fetal bovine serum (FBS), and the association of two of those supplements. Three different

sources of interleukin-2 (IL-2) were employed in the assay and the respective dilutions ere performed, giving rise to a final concentration of 5U/ml of growth medium. The effect of the three different interleukins can be evaluated by comparing combinations D, E and F. In the combination G, HL-1, a hybridoma medium specific for lymphocytes growth was omitted. Combination H can be compared with combination D, and was used to compare the effects of the antibiotics streptomycin and penicillin only, with the effects of "Four (+)", a mixture of the two antibiotics with glutamine and pyruvate. Combination I can be compared with combination D, and was performed to evaluate the effects of fungizone.

According to the results obtained for plating efficiency with the experiments above (Table 12), the medium composition was defined for our experiments. The procedure employed is based on the original method described by Albertini *et al.*, 1982. Some modifications were introduced in the original method, and the whole procedure is described here.

Table 7. Different medium compositions used to evaluate optimal T-lymphocytes cloning.

COMBINATION	MEDIUM COMPOSITION
A	RPMI, HL-1, IL-2(A), PHA, Fungizone, Four(+), CBS.
B	RPMI, HL-1, IL-2(A), PHA, Fungizone, Four(+), HS.
C	RPMI, HL-1, IL-2(A), PHA, Fungizone, Four(+), FBS, HS.
D	RPMI, HL-1, IL-2(A), PHA, Fungizone, Four(+), CBS, HS.
E	RPMI, HL-1, IL-2(B), PHA, Fungizone, Four(+), CBS, HS.
F	RPMI, HL-1, IL-2(C), PHA, Fungizone, Four(+), CBS, HS.
G	RPMI, IL-2(A), PHA, Fungizone, Four(+), CBS, HS.
H	RPMI, HL-1, IL-2(A), PHA, Fungizone, Penicilin/Streptomycin, CBS, HS.
I	RPMI, HL-1, IL-2(A), PHA, Four(+), CBS, HS.

Mononuclear cells from cryopreserved vials were thawed by placing them into a 37°C water bath and gently tapping them just before the last crystals melt. Cells were then washed in appropriate medium containing RPMI 1640 and 50% CBS. A second wash was

performed using a different medium containing RPMI 1640, 10% CBS and antibiotics (100U/ml penicillin and 100μg/ml streptomycin). After washing, cell pellets were counted for cell viability using trypan blue, resuspended to a concentration of 10^6 cells per ml and incubated for 36-40 hours at 37°C, 5% CO_2, in a pre-stimulating medium which contained RPMI 1640, 20% HL-1 (Professional Diagnostics), 5% CBS, 4% antibiotics (100 U/ml penicillin and 100μg/ml streptomycin), and 0.25 μg/ml phytohaemaglutinin (PHA) (Burroughs Wellcome Co.). This step permits the pre-stimulation of the T-lymphocytes with phytohemaglutinin, and also allows macrophages to attach to the walls of the flask, preventing those cells from being transferred to the next step.

After pre-stimulation, mononuclear cells were plated in selective and non-selective medium in microtitre plates (96 well, flat bottom) at a concentration of 10^4 and 3 cells per well, respectively. In addition, each well also received 10^4 lethally irradiated RJK 853 lymphoblastoid feeder cells. The RJK feeder cells which are required for the efficient growth of the primary T-lymphocytes, were initially obtained from a male Lesch-Nyhan patient carrying a complete deletion of the *hprt* gene (Stout and Caskey, 1985). The absence of the *hprt* gene in the feeder cells avoids any potential complications during PCR analysis of the *hprt* mutants isolated. Selective medium consisted of RPMI 1640 (Professional Diagnostic), 20% HL-1 (Professional Diagnostic), 5% human AB serum (HS) (Gibco), 5 U/ml interleukin-2 (IL-2 from Cetus Corporation), 0.25 μg/ml PHA (Burroughs Wellcome Co.), antibiotics (penicillin and streptomycin) (Sigma), and

2% Fungizone (Gibco). Selective plates also contained 10^{-5}M 6-thioguanine (Sigma). Plates were then placed on a sloped shelf (5°) in a 5% CO_2, 37°C incubator for a period of 14 to 18 days. Forty eight hours prior to scoring, plates were rotated 180° on the sloped shelf, and then scored at least two more times under an inverted phase contrast microscope. Cells from positive wells of the selective plates (when colonies had nearly filled the well) were transferred to 24-well plates and expanded in growth medium as far as possible. After a sufficient expansion, 6 X 10^4 cells were removed from each well, washed in PBS, counted, and diluted in cell pellets containing 2000 cells each for PCR preparations. The remaining cells were further expanded, and, when possible, cell pellets containing 2 x 10^6 cells were also prepared from the selected colonies for Southern blotting or Multiplex PCR preparations. Cell pellets obtained this way were stored at -20°C until required.

2.4 CLONING EFFICIENCY

The calculation of cloning efficiency under selective and non-selective conditions was based upon the Poisson distribution. The ratio of the number of wells where no T-cell colonies were detected to the total number of wells scored results in the Poisson ratio (Po).

Po = Negative Wells / Total Wells Scored

Cloning Efficiency (CE) was calculated by the negative natural log of the ratio (Po)

divided by the number of cells originally plated into each well. The cloning efficiency obtained from the non-selective plates, i.e. non-selective cloning efficiency (CE (-6TG)), generates the frequency of clonable T-cells present in the MNC population plated under non-selective conditions, i.e. in the absence of 6-thioguanine, while the cloning efficiency under selective conditions (CE (+6TG)), generates the frequency of *hprt* mutant cells present in the MNC population plated under selective conditions, in the presence of 6-thioguanine. In order to compare the results obtained for cloning efficiency in this study with those published by other laboratories all the values obtained for non-selective cloning efficiencies were multiplied by 10^2 and presented as percentage.

$$CE = - Ln (Po) / \text{Number of Cells per Well}$$

2.5 MUTANT FREQUENCY

The mutant frequency (MF) was determined by the ratio of the cloning efficiency obtained from the selective plates (CE+6TG) and the cloning efficiency obtained from the non-selective plates (CE-6TG). Mutant frequency yields the frequency of *hprt* mutant T-cells present in the clonable T-cell population.

$$MF = CE (+6TG) / CE (-6TG)$$

2.6 T-CELL RECEPTOR (TCR) ASSAY

The TCR assay was performed to verify the clonal relationship between some of the mutants isolated. The method used was previously developed in our laboratory (de Boer *et al.*, 1992; Curry *et al.*, 1993) but some modifications were necessary, due to the small number of cells available for the analysis. Cell pellets (about 2 X 10^4 cells in 2 μl of PBS) were removed from the freezer and placed on ice. 10 μl of nanopure water were added to each tube. Tubes were then vortexed, quickly spun down in a microfuge (about 2 seconds) and immediately placed back on ice until PCR mixture (Table 8) was prepared. 40 μl of the first PCR mixture and 10μl of the cell preparation were transferred to Microamp reaction tubes (Cetus) and thermocycled in a Perkin Elmer-Cetus 9600 thermocycler programmed according to Table 9. Primers V1 and J1 (described in Table 10) were used in the first reaction.

A second PCR, using 1 μl of the product obtained from first reaction as a template, plus 49μl of the second PCR mixture (Table 8), was carried out in a total volume of 50 μl. The second PCR was performed with nested primers V2 and J2 (described in Table 10), and the thermocycler program used as described in Table 9. After the second PCR round, 1 μl gel-loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water) was added to 5 μl of each reaction product and run on an agarose gel for verification (0.7-1.0% agarose (Dalton Chemicals); 1 μl 10mg/ml ethidium bromide for a 30 ml gel). A photograph of the gel was taken using a UV transilluminator. If the

PCR was positive, as discernible from presence of a band in the agarose gel, another PCR round was carried out in 50 μl using 1 μl from the first PCR product as template. The TCR/PCR product (100 μl) was precipitated by the addition of 20 μl of 10 M ammonium acetate and 2 volumes (240μl) of isopropanol at room temperature. The mixture was briefly agitated, left at room temperature for 15 minutes, and then spun for 30 minutes in a microfuge. The supernatant was discarded and the pellet washed with 1.0 ml 70% ethanol without mixing. After centrifugation for 15 minutes, the supernatant was discarded and the DNA pellet dried for 2 minutes in a speed vacuum drier (Savant). The DNA was subsequently resuspended in 40 μl of water for the restriction digestion step. When digestion was not performed immediately, the DNA was kept at -20°C. For restriction analysis, the purified DNA was split into two tubes to be digested with the restriction enzymes, BstN1 and RsaI (New England Biolabs, MA). Restriction digestion was performed overnight. One half of the DNA (20μl) was digested with 20 units of RsaI at 37°C, and the other half (20μl) with 20 units of BstN1 was digested at 60°C, using buffers provided by the manufacturer (New England Biolabs, MA). Restriction fragments from the two assays were examined on a 5% NuSieve (FMC Bioproducts) low melting point (LMP) agarose gel stained with ethidium bromide. The separation of the different restriction fragments was improved when the gel was run overnight with the power supply programmed to keep a constant 40 V. A photograph of the gel containing the restriction fragments was taken on a UV transilluminator and the clonal relation between the different mutants was analyzed according to their respective band patterns.

Table 8. Composition of PCR mixtures used in the TCR assay

First PCR Round	Stock Concentration	Volume (μl)
H_2O	-	28.0
10x PCR buffer	600 mM KCl 27.5 mM MgCl2 150 mM Tris-HCl(pH8.5)	5.0
Primer V1 (Dalton Chemicals)	0.5 OD_{260}	3.0
Primer J1 (Dalton Chemicals)	0.5 OD_{260}	3.0
dNTP (Pharmacia)	25mM each dNTP	0.5
Taq Polymerase (Cetus)	5 U/μl	0.5
		Total 40.0μl
Second PCR Round		
H_2O	-	37.0
10 x PCR buffer	600 mM KCl 27.5 mM MgCl2 150 mM Tris-HCl(pH 8.5)	5.0
Primer V2 (Dalton Chemicals)	0.5 OD_{260}	3.0
Primer J2 (Dalton Chemicals)	0.5 OD_{260}	3.0
dNTP (Pharmacia)	25 mM each dNTP	0.5
Taq Polymerase (Cetus)	5 U/μl	0.5
		Total:49μl

Table 9. PCR programs for first and second round of TCR DNA amplification.

FIRST PCR ROUND				
95°C - 5min	5 cycles 94°C - 2min 55°C - 2min 72°C - 5min	30 cycles 94°C - 20sec 55°C - 30sec 72°C - 1 min	72°C - 10min	4°C - ∞
SECOND PCR ROUND				
95°C - 2min	30 cycles 94°C - 5sec 55°C - 5sec 72°C -10sec			4°C - ∞

Table 10. Sequences of DNA primers used for PCR amplification of TCR DNA.

First PCR
Primer V1 : 5'-GAAGCTTCTAGCTTTCCTGTCTC-3'
Primer J1 : 5'-CGTCGACAACAAGTGTTGTTCCAC-3'
Second PCR
Primer V2 : 5'-CTCGAGTGCGCTGCCTACAGAGAGG-3'
Primer J2 : 5'-GGATCCACTGCCAAAGAGTTTCTT-3'

2.7 STATISTICAL ANALYSIS

The kwikstat statistical analysis program (Copyright 1991, Allan C. Elliot) was used for data management and statistical analysis. Descriptive statistics were performed for the different data obtained for each group. Since only two groups are analyzed in this study, t-test analysis of variance was performed to compare the two independent groups, using an alpha level of 0.05 (i.e. $P=0.05$). To analyze the potential correlation between two different variable obtained from the studied groups, linear regression analysis was performed. In general, linear regression analysis is used to describe the nature of the relationship between two variables, and to predict the value of one variable (y) according to the other (x). The idea of regression is closely associated with that of correlation. Correlation, like regression, considers the linear relation between two variables. It concerns the strength of the relationship between the values of two variables. The

correlation tells if and the degree changes in one variable are accompanied by changes in the other variable, and also if such changes are in the same or in different directions. A positive correlation means that larger values on one variable go with larger values on the other, whether a negative correlation means that larger values on one variable tend to go with smaller values on the other. Correlations vary in direction (+ and -) and in strength. The strength of a correlation is given by its correlation coefficient. The correlation coefficient measures the closeness with which the pairs of values fit a straight line. The correlation coefficient (r) ranges between +1 and -1. When r is close to zero, it means that there is no correlation at all. The closer the correlation coefficient gets to +1 or -1, the strongest the correlation; the closer it gets to zero, the weaker the correlation is (Rowtree, 1981). So, the correlation coefficient describes the strength and the direction of the relationship between pairs of values for two different variables. However, the reliability of a correlation doesn't depend only on the size of its coefficient. It depends also on the size of the sample. The more pairs of values are present in the same sample, the more likely it is to verify a similar correlation coefficient in other samples, and in the population as whole. Since the number of the samples in this study is very small and for different reasons very restricted, the correlations presented in the results section are those obtained for the group as a whole. However, as it is shown later, those correlations must be carefully analyzed in order to avoid generalization and possible misunderstandings.

CHAPTER III: RESULTS

In this study, ten children who had been accidentally exposed to ionizing radiation were analyzed for their *hprt* mutant frequencies in T-lymphocytes. A control group comprised of eight children was also studied for the purpose of comparison. Both groups were analyzed using the *hprt* clonal assay, and the same conditions for cell growth and mutant selection were provided. Age, sex, and dose corresponding to each subject are listed in Table 11.

3.1 MONONUCLEAR CELLS AVAILABLE FOR THE *HPRT* ASSAY:

Table 11 provides a description of the study groups as well as the number of mononuclear cells (MNC) available for each subject and used for the *hprt* clonal assay. The number of cells shown in Table 11 corresponds to the fraction recovered after thawing the samples and pre-incubating the cells with phytohaemoaglutinin (PHA) for a period of 24 to 48 hours. MNC from the exposed group were collected two years after the accident, and were kept frozen in liquid nitrogen for three years. MNC from the control group were collected three years after the accident, and were frozen in liquid nitrogen for two years.

Table 11. Description of the subjects analyzed for the *hprt* mutant frequency in T-lymphocytes, and number of mononuclear cells available for each subject.

CONTROL GROUP				
Subject	Age (years)	Sex	Dose (cGy)	MNC recovered after pre-incubation
B-176	04	M	-	6.12 X 10^6
B-33	05	F	-	6.40 X 10^6
B-177	09	F	-	4.56 X 10^6
B-111	11	M	-	3.84 X 10^6
B-156	13	M	-	3.29 X 10^6
B-172	14	F	-	4.60 X 10^6
B-123	14	M	-	4.76 X 10^6
B-121	16	F	-	15.28 X 10^6
Mean	10.8	-	-	6.10 X 10^6
EXPOSED GROUP				
Subject	Age (years)	Sex	Dose (cGy)	MNC recovered after pre-incubation
B-50	06	M	15	1.62 X 10^6
B-45	09	M	60	2.08 X 10^6
B-20	09	M	20	7.20 X 10^6
B-26	09	F	70	2.88 X 10^6
B-68	10	F	20	6.72 X 10^6
B-25	10	M	50	1.36 X 10^6
B-22	10	M	40	2.24 X 10^6
B-48	11	F	40	6.16 X 10^6
B-59	13	M	30	3.84 X 10^6
B-34	14	F	30	3.68 X 10^6
Mean	10.1	-	37.5	3.78 X 10^6

According to our records of the blood collections, similar amounts of blood were obtained from each of the two groups. Although the mean number of cells recovered for the exposed group after thawing and pre-incubation, i.e. 3.8 X 10^6 cells, was apparently lower than the mean number, i.e. 6.1 X 10^6 cells, recovered for the control group, the difference between the two means was not statistically significant ($p=0.0573$).

3.2 CLONING T-LYMPHOCYTES

The optimization of growth medium for cloning T-lymphocytes was investigated in an experiment in which different combinations of components were tested, using a single blood sample, under otherwise same conditions. The results obtained from such an experiment are shown below (see Table 12).

- Media A, B, C and D were used to analyze the effects of the serum supplement (CBS, FBS and HS). The highest plating efficiency was obtained with combination D where calf bovine serum (CBS) was used along with human serum (HS).
- Three different sources of interleukin-2 were tested in the combinations D, E and F. Although a higher cloning efficiency was found for the combination F, the difference between the three combinations was hardly significant.
- The effect of HL-1, a growth medium specific for lymphocyte cultures, was tested by using combinations D and G. A small difference was observed between the two media, with the highest plating efficiency being found with combination D.
- An increase in cloning efficiency was noted when the antibiotics penicillin and

streptomycin (combination H) were added, replacing the blend "Four(+)" (combination D). This media produced a higher plating efficiency (19.2%), compared to 12.5% as obtained with the combination D.

- The highest cloning efficiency was obtained with combination I, which has all the components of H, but from which the fungicide (fungizone) was omitted. However, this combination was not adopted, since the risk of fungal contamination was considered worth being avoided. Medium-condition H was therefore chosen as the standard medium for the clonal assay performed in this study.

Table 12. Cloning efficiencies obtained for a single blood sample using media of different composition.

COMPOSITION	TOTAL WELLS	POSITIVE WELLS	CELLS/ WELL	CLONING EFFICIENCY(%)
A	192	39	03	7.56
B	192	16	03	2.90
C	192	36	03	6.92
D	192	60	03	12.48
E	192	61	03	12.74
F	192	68	03	14.57
G	192	52	03	10.52
H	192	84	03	19.17
I	192	102	03	25.25

3.3 NON-SELECTIVE CLONING EFFICIENCY FOR THE STUDY GROUPS

The results obtained for cloning efficiency and mutant frequency from both groups are listed in Tables 13 and 14. Table 13 contains information regarding number of wells

plated in selective and non-selective conditions, number of cells per well, number of positive wells obtained in both conditions, and the calculated results for cloning efficiency and mutant frequency. Table 14 summarizes the data from both groups, including the means and standard deviations obtained for the respective results.

Table 13. Cloning Efficiency and Mutant Frequency from Brazilian children accidentally exposed to ionizing radiation and controls.

Subject	Cloning Efficiency (-6tg)			Cloning Efficiency (+6-tg)			Cloning Efficiency (-6tg)(%)	Cloning Efficiency (+6-tg) ($X10^{-6}$)	Mutant Frequency ($X10^{-6}$)
	Total wells	Positive wells	Cells/ well	Total wells	Positive wells	Cells/ well			
EXPOSED									
B-50	192	55	03	162	0	10^4	11.25	0	0
B-45	192	18	03	208	01	10^4	3.30	0.48	14.60
B-20	192	60	03	720	41	10^4	12.48	5.86	46.94
B-26	192	12	03	288	01	10^4	2.15	0.35	16.27
B-68	192	14	03	672	03	10^4	2.50	0.44	17.89
B-25	192	37	03	136	01	10^4	7.13	0.74	10.03
B-22	192	78	03	224	08	10^4	17.37	3.63	20.92
B-48	192	36	03	616	04	10^4	6.92	0.65	9.41
B-59	192	22	03	384	06	10^4	4.05	1.57	38.77
B-34	192	83	03	368	04	10^4	18.80	1.09	5.79
CONTROLS									
B-176	192	159	03	612	02	10^4	58.69	0.32	0.55
B-33	192	112	03	640	08	10^4	29.18	1.26	4.31
B-177	192	125	03	456	03	10^4	35.09	0.66	1.88
B-111	192	146	03	384	04	10^4	47.62	1.04	2.20
B-156	192	66	03	329	01	10^4	14.00	0.30	2.17
B-172	192	150	03	460	10	10^4	50.66	2.20	4.33
B-123	192	52	03	476	07	10^4	10.52	1.48	14.72
B-121	192	120	03	1528	04	10^4	32.69	0.26	0.80

Cloning efficiencies obtained for control and exposed groups are listed in Table 13. For the control group, non-selective cloning efficiencies ranged from 10.5% to 58.7%. The mean non-selective cloning efficiency for the control group was 34.8% (SD = $\pm$17.09). Such results are comparable to those obtained for adults in different laboratories (Branda *et al.*, 1993; Tates *et al.*,1991; Davies *et al.*,1992). Similar results for non-selective cloning efficiencies obtained from children and adults have also been reported in a study in which 49 healthy children were analyzed for the *hprt* clonal assay (Finette *et al.*,1993).

Comparatively low values for non-selective cloning efficiencies were found for the group of children accidentally exposed to ionizing radiation. Non-selective cloning efficiencies ranged from 2.15% to 18.8% with a mean value of 8.6% (SD = $\pm$6.10). These cloning efficiencies appear low. However, they cannot readily be compared with the results obtained in different laboratories since the conditions and the characteristics of the group being analyzed appears unique. Nevertheless, it is remains important to mention that low cloning efficiencies were also obtained in our laboratory for the group of adults similarly exposed to ionizing radiation during this accident (data not published).

When the means for non-selective cloning efficiency were compared between the two groups of children, a remarkable difference was demonstrated ($p=0.0032$). As shown in Figure 5, the mean non-selective cloning efficiency for the exposed group was about 25% of the mean obtained for the control group.

Table 14. Descriptive statistical data for cloning efficiency and mutant frequency obtained from Brazilian children accidentally exposed to ionizing radiation and controls

Study Group	Cloning Efficiency (-6-tg) (%)			Mutant Frequency ($x10^{-6}$)		
	Mean	SD	Range	Mean	SD	Range
Control	34.80	± 17.09	10.52 - 58.69	3.87	± 4.61	0.55 - 14.72
Exposed	8.59	± 6.10	2.15 - 18.80	18.06	± 14.54	0.00 - 46.94

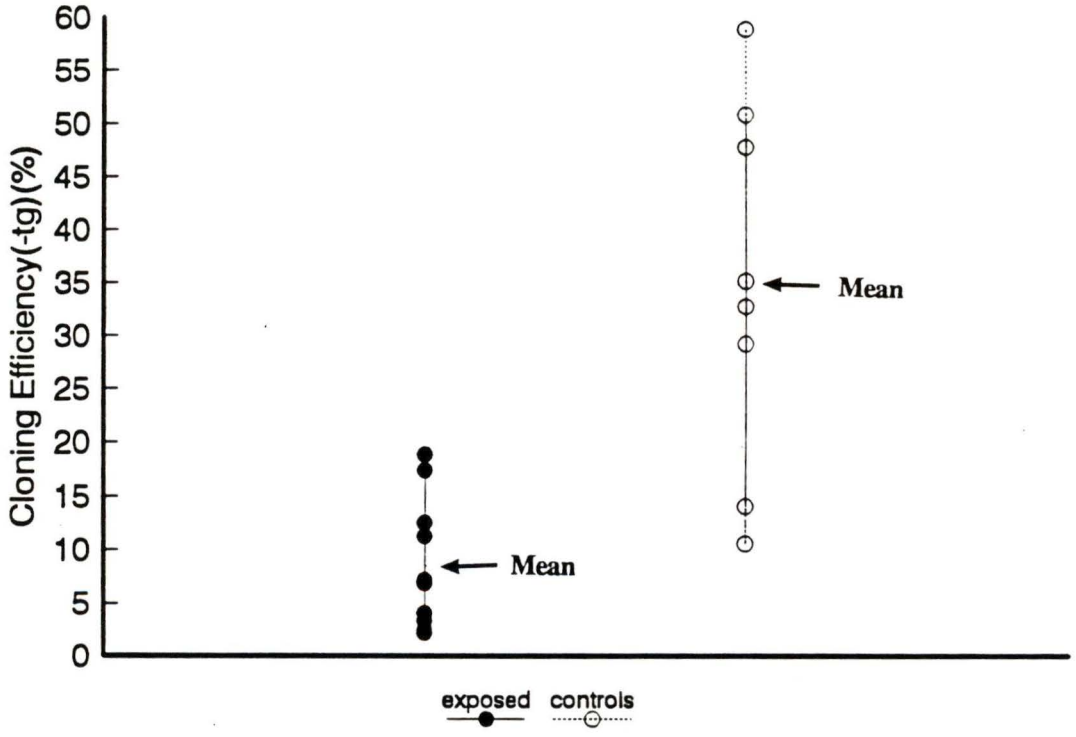

Figure 05. Cloning efficiencies obtained from the Brazilian children accidentally exposed to ionizing radiation and controls. The mean cloning efficiencies for the exposed and control group were 8.6% and 34.8%, respectively.

3.4 MUTANT FREQUENCIES

Hprt mutant frequencies were obtained for both groups of children. Mutant frequencies are calculated as the ratio between cloning efficiency in the presence of 6-thioguanine (selective) and cloning efficiency in the absence of 6-thioguanine (non-selective). For the control group, the mutant frequency ranged from 0.55 X 10^{-6} to 14.7 X 10^{-6}. The mean mutant frequency for this group was 3.87 X 10^{-6} (SD = $\pm$4.60). These results are very similar to the few data published by other laboratories for *hprt* mutant frequencies in children (Cole *et al.*, 1988; Cole *et al.*, 1991; Trainor *et al.*, 1984). It is, however, significantly lower than the mean mutant frequencies obtained from adults by different laboratories (Branda *et al.*, 1993; Tates *et al.*, 1991, Davies *et al.*, 1992). The mean mutant frequency obtained for the control group was higher than the mutant frequency observed in studies of newborns using cord blood cells (McGuinnes, 1990; Manchester *et al.*, 1992). The values obtained for the control group are therefore intermediate between those found for newborns and those found for adults.

For the exposed group, remarkably higher mutant frequencies were detected, as shown in Figure 6. In the exposed group, mutant frequencies ranged up to 47.0 X 10^{-6}. The mean mutant frequency detected for the exposed group was 18.1 X 10^{-6} (SD = $\pm$14.5). This value is significantly different from the one obtained for the control group ($p=0.0031$). It is approximately 4.6 times higher than the average mutant frequency obtained for the unexposed control group.

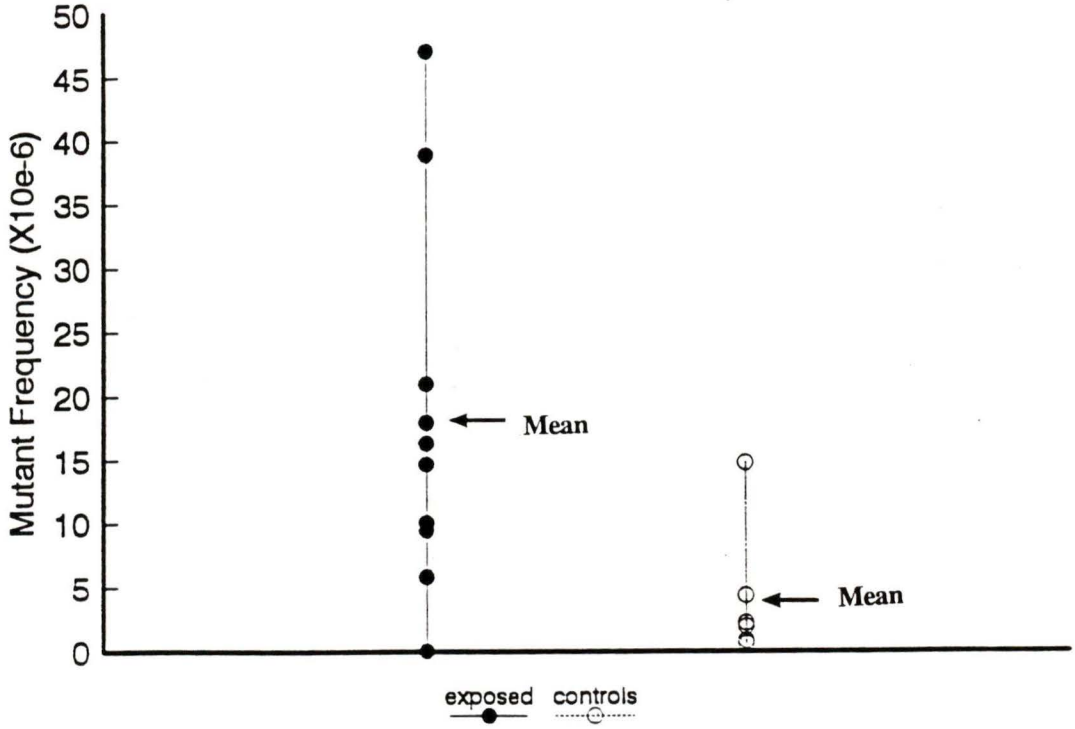

Figure 6. *hprt* mutant frequencies obtained from Brazilian children accidentally exposed to ionizing radiation and controls. The mean mutant frequencies for the exposed and control groups were 18.06 X 10^{-6} and 3.87 X 10^{-6}, respectively.

In both the exposed and control groups, exceptionally high mutant frequencies were found in some subjects. For example, in the control group, subject B-123 showed the highest mutant frequency of 14.7 X 10^{-6}, which is approximately 4 times higher than the average. However, it was noted that this subject also demonstrated the lowest value for cloning efficiency under non-selective conditions in the whole group. Such an inverse relationship between non-selective cloning efficiency and mutant frequency has been demonstrated by the majority of laboratories that employ the *hprt* clonal mutation assay.

In this study, the question of the relationship between cloning efficiency and mutant frequency will be addressed later. In the exposed group, remarkably high mutant frequencies were observed in subjects B-20, B-22 and B-59. In the case of subject B-59, a low non-selective cloning efficiency (4.05%) was observed. However, subjects B-20 and B-22 did not show such depressed values for non-selective cloning efficiency. The cloning efficiency in non-selective conditions for B-20 and B-22 was 12.48%, and 17.37%, respectively. In these cases, further experiments were carried out in an attempt to understand the origins of these high observed mutant frequencies.

3.5 FACTORS THAT POTENTIALLY INFLUENCE MUTANT FREQUENCY

In an attempt to analyze the factors that could potentially influence the mutant frequencies obtained in the groups studied, as well as those specified by different laboratories as correlated to mutant frequency, linear regression analysis was performed with each of the possible dependent variates. Since the number of subjects in each group is small and the groups are characterized by a very narrow age and dose interval, the correlation coefficients provided here are those obtained when the whole group was analyzed. Using a "stripping down" method (Gilbert, 1973) where the regression on all the variates is made together, and then step-by-step each variete is removed from the group, different possible regressions were obtained. The correlation coefficients for such regressions are presented in the appendices section. A large number of possible regressions can be considered, however, because it is already established that a broad inter-individual

variance exists for *hprt* mutant frequency, only the regressions obtained for the whole groups studied will be addressed in this section. It must be also emphasized that the linear regression was used in this study not to predict a certain variete according to another, but to verify the linear relation between two varietes.

AGE AND NON-SELECTIVE CLONING EFFICIENCY:

Reduced non-selective cloning efficiencies and a reduced response to PHA and feeder cell stimulation have been reported for T-lymphocytes from elderly individuals (Chrysostomou *et al.*, 1984; Hallgren *et al.*, 1988; Luciervo *et al.*, 1988). Since it has also been reported that non-selective cloning efficiency can inversely influence mutant frequency (Branda *et al.*, 1993; Tates *et al.*, 1991), the effect of age on non-selective cloning efficiency was investigated for both groups in this study. Although the subjects analyzed here were very young, and the age interval of the whole group is very narrow, a weak, but significant inverse correlation between age and non-selective cloning efficiency was observed for the control group (correlation coefficient = -0.40) with an estimated decrease in cloning efficiency of 1.56% per year. However, when subject B-176 that presented the highest cloning efficiency in the control group (58.69%) is excluded from the analysis, the correlation disappears, i.e. the correlation coefficient drops to -0.08). In contrast, no significant correlation was detected for the exposed group (correlation coefficient = 0.18), as shown in Figure 7.

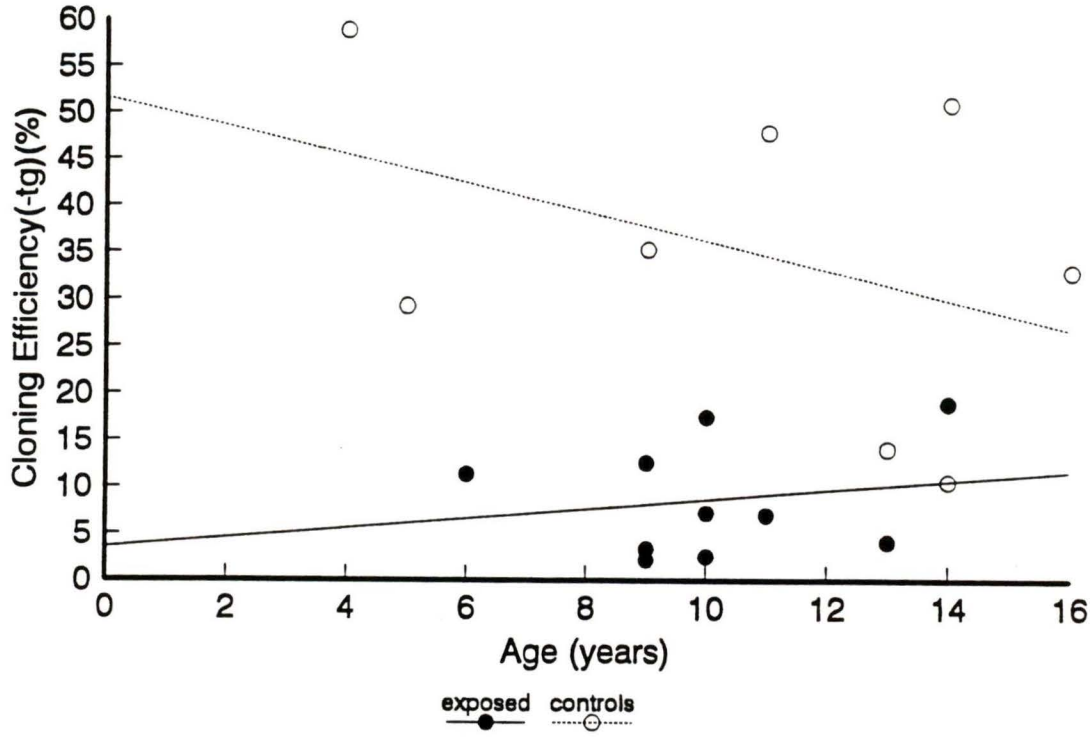

Figure 7. Linear regression analysis showing the correlation between non-selective cloning efficiency and age. A modest, but significant inverse relationship (correlation coefficient = -0.4018) was observed for the control group, while no significant relationship was observed for the exposed group (correlation coefficient = 0.1853).

AGE AND MUTANT FREQUENCY

A direct correlation between age and mutant frequency has been reported by different laboratories that use the *hprt* clonal assay for mutant frequency analysis (Tates *et al.*, 1991; Trainor *et al.*, 1984; Branda *et al.*, 1993). A weak correlation between age and mutant frequency was observed for the control group (correlation coefficient = 0.28). However, such correlation is given mostly by the values obtained for mutant frequency for the subjects B-176 (0.55×10^{-6}) and B-123 (14.72×10^{-6}). Such subjects provided,

respectively the lowest and highest values for mutant frequency and their correspondent ages were also in the lowest and highest intervals. By excluding one of those subjects, the correlation disapears. For the exposed group, the correlation was negligible (correlation coefficient = 0.18). The analysis of these variables is shown in Figure 8.

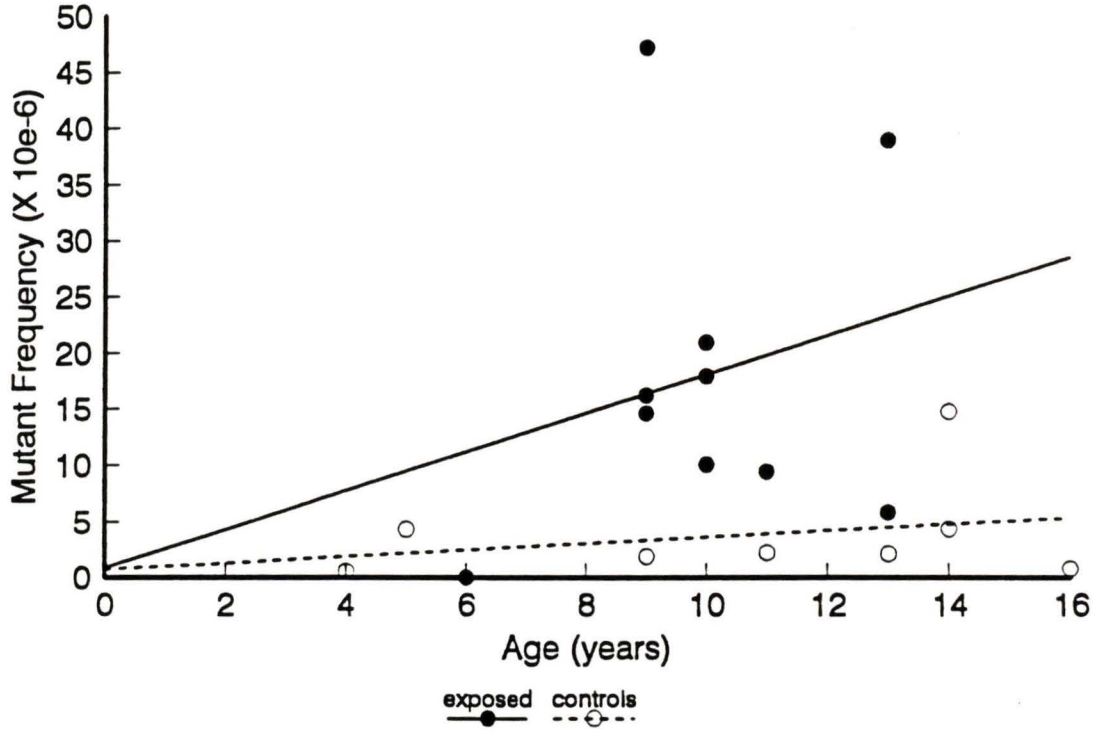

Figure 8. Linear regression analysis showing the correlation between mutant frequency and age. A weak, but significant correlation (correlation coefficient = 0.2806) was observed for the control group, while no significant relationship was observed for the exposed group (correlation coefficient = 0.1814).

NON-SELECTIVE CLONING EFFICIENCY AND MUTANT FREQUENCY

The correlation between non-selective cloning efficiency and mutant frequency for the two groups studied is shown in Figure 9. An inverse correlation between non-selective

cloning efficiency and mutant frequency was demonstrated for the control group (correlation coefficient = -0.58). Here, once more, the correlation is dependent on subject B-123. Subject B-123 showed the lowest cloning efficiency in the group (10.52%) and the highest mutant frequency (14.72 x 10^{-6}). If this subject is excluded from the analysis, the correlation disappears. The effect of cloning efficiency on mutant frequency was not observed for the exposed group (correlation coefficient = -0.09).

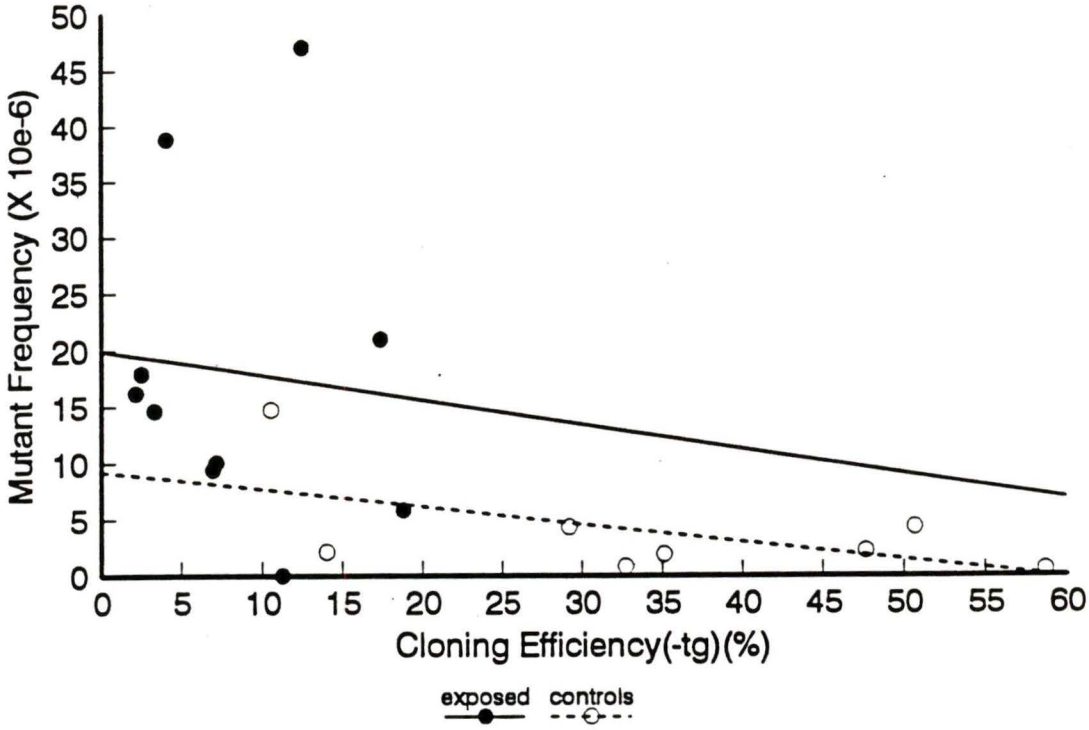

Figure 9. Linear regression analysis showing the correlation between non-selective cloning efficiency and mutant frequency. A significant inverse correlation (correlation coefficient = - 0.5778) was observed for the control group, while no significant correlation was observed for the exposed group (correlation coefficient = - 0.0912).

IONIZING RADIATION DOSE AND NON-SELECTIVE CLONING EFFICIENCY

Low, non-selective cloning efficiencies have the potential for producing artificially

elevated mutant frequencies (Branda *et al.*, 1993; Tates *et al.*, 1991). In contrast, low, non-selective cloning efficiencies can also be indicators of toxic exposure. It was, therefore, important to examine the effect of dose of ionizing radiation received by the exposed subjects. We thus examined the correlation between dose and non-selective cloning efficiency. Although the radiation doses reported are low, a slight, but significant inverse correlation between dose and non-selective cloning efficiency was detected for the exposed group (correlation coefficient = -0.40), as shown in Figure 10. This observation indicates that as the radiation dose increases, cloning efficiencies diminish.

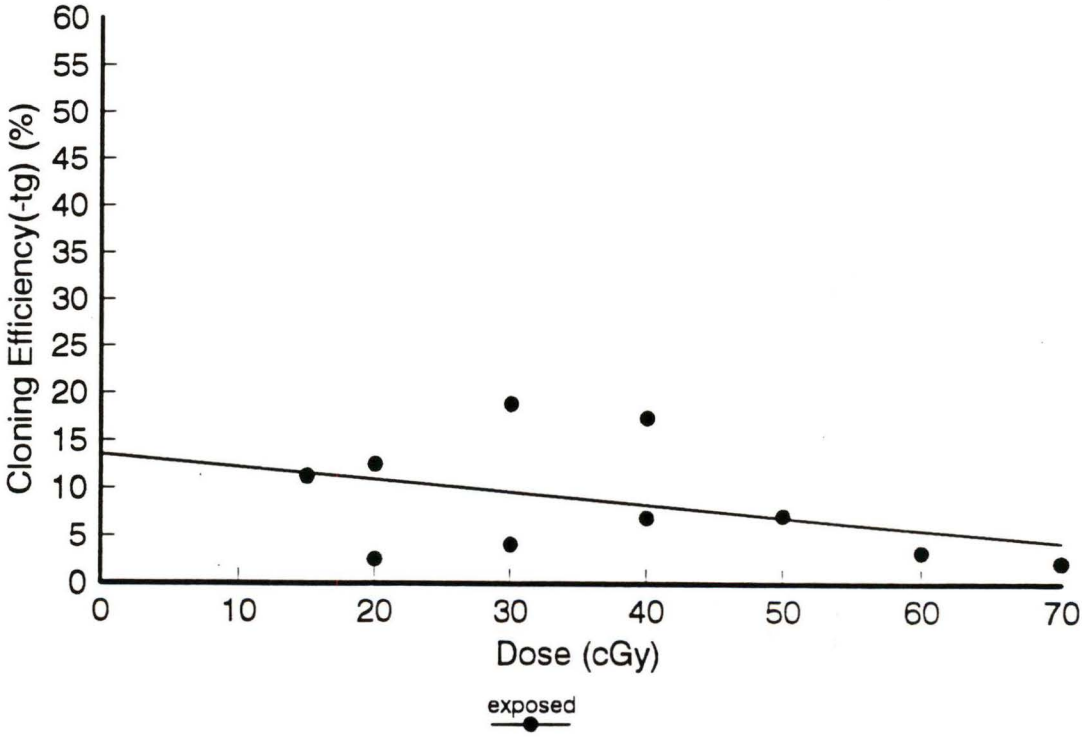

Figure 10. Linear regression analysis showing the correlation between non-selective cloning efficiency and ionizing radiation dose. A significant, but slight inverse correlation (correlation coefficient = - 0.3938) was observed for the exposed group.

Although the mean mutant frequency for the exposed group was significantly higher than the mean mutant frequency obtained for the control group, a dose-response relationship could not be demonstrated (Figure 11). Linear regression analysis showed no significant correlation between dose and mutant frequency (correlation coefficient = - 0.17).

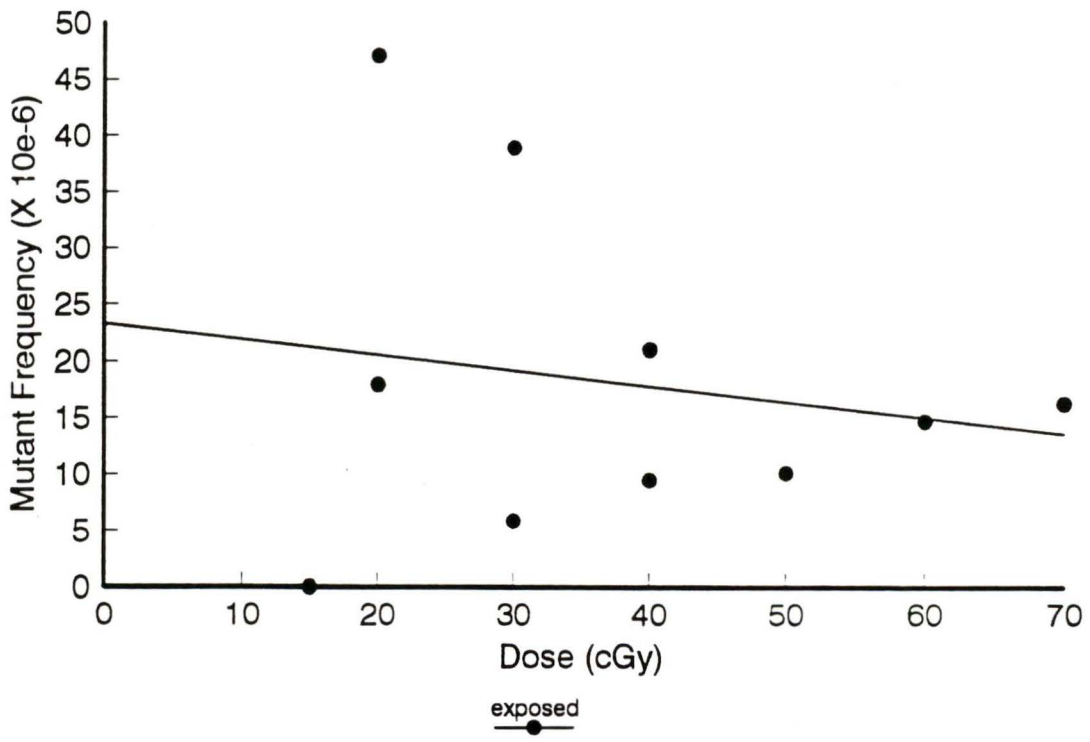

Figure 11. Linear regression analysis showing that no significant correlation between mutant frequency and ionizing radiation dose could be observed in this study.

3.6 MOLECULAR ANALYSIS OF THE T-CELL RECEPTOR

We had previously reported in this study that a high *hprt* mutant frequency was found in some of the subjects. To verify that such high mutant frequencies were truly elevated, i.e. whether they represented independent mutants, or whether they resulted from the *in vivo* expansion of individual mutant clones, T-cell receptor analysis was carried out in some of the 6-tg-resistant clones. Because T-cell clones present a variable growth rate under PHA stimulation, the number of cells scored after 15 days incubation also varies among different clones (Hakoda *et al.*, 1989; Caggana *et al.*, 1991; Morley *et al.*, 1985; Bradley *et al.*, 1987). Therefore, a long-term expansion (about 30 days) of 6-tg-resistant clones is necessary to obtain a sufficient number of cells required for the molecular analysis of the mutants isolated. Although the TCR method used here is economical with regards to the number of cells demanded by the assay, in some cases it was not possible to carry the on long-term culturing of the mutants selected, since some clones became unresponsive to PHA and feeder cell stimulation. Initially, 108 6-tg-resistant clones were selected from the 18 subjects analyzed in this study after 15 days od incubation. 69 clones were isolated from the exposed group, while 39 clones were isolated from the control group. Further clonal expansion was tried with all of the selected clones, however, only 59 clones were responsive to subsequent stimulation. TCR assay was performed up to three times with each of the 59 clones expanded. In the cases in which the TCR/PCR product was not obtained after the third repeat, the case was neglected. The results obtained from the TCR assays are shown in Table 15. It is important to

emphazise that in this study, the TCR assay was not used in an attempt to correct the mutant frequencies to their respective mutation frequencies. Since it was not possible to expand and analyze all the 6-tg-resistant clones isolated from the *hprt* clonal assay, nor to determine the sequences of the *hprt* mutants isolated, the TCR analysis was only useful to simply verify the clonal relationship among the mutants isolated. Furthermore, it was important to certify that in the majority of the cases, such as B-111, B-172, B-20, B-22, B-48, the mutant frequencies detected really reflected individual 6-tg-resistant clones.

Table 15. T-cell receptor analysis from 6-tg-resistant clones isolated from Brazilian children accidentally exposed to ionizing radiation and controls

Subject	**Number of Mutants Analyzed**	**Number of Individual Clones**
Controls		
B-111	02	02
B-172	08	05
Exposed		
B-20	14	14
B-22	03	02
B-48	04	04
B-34	03	01
Total	34	28

CHAPTER IV: DISCUSSION

The relationship between the incidence of cancer and known exposure to ionizing radiation has been well documented (Pierce and Vaeth, 1991; Schull *et al.*, 1981; Samet, 1989). With regard to public health, the detection and quantification of the risk arising from environmental and occupational exposure are of major importance. Genetic risk assessment of exposure to ionizing radiation implies a number of essential steps, ranging from the detection of cell cytotoxicity, chromosomal aberration, DNA damage and mutagenic effects in experimental animals and in humans, to the final extrapolation to epidemiological studies comprising the incidence of genetic diseases, the development of tumours and mortality. Qualitative and quantitative heterogeneity between species in response to genotoxic exposure, together with the natural differences between individual sensitivities, render this endeavor particularly difficult. Such difficulties lead to the demand of methods with increased sensitivity, able to detect the early effects of exposure, and to specify the causative factors and the quantitation of its risks.

In classical toxicology, the effects of a toxin generally do not appear until in excess of some critical threshold concentration (Ehrenberg, 1989). In contrast, in the cases where cellular genetic damage plays an essential role, such as the development of cancer, or in

the potential cases of induction of germline mutations (Sankaranarayanan, 1993), dose-response relationships are characterized by the absence of definable no-effect thresholds, in the sense that the genetic effects are stochastic and their frequencies increase with increasing dose. If no threshold exists, especially in the cases of mutations and cancer, complete protection is never achievable by keeping exposures below threshold limit values (TLVs), and even the very lowest doses will be associated with increased risk, in the sense of an increased probability of disease (Ehrenberg, 1989).

In the establishment of radiation safety policies (ICRP Publication 26, 1977), any activity leading to exposure has to be justified by the benefit of activity to human health or well being. Furthermore, a permissible exposure level must be established as a function of the risk considered to be acceptable when compared with its benefits. Additionally, the "ALARA principle", i.e. as low as reasonably achievable, has to be applied. Because of the absence of definable no-effect thresholds, the detection of genotoxic effects following ionizing radiation exposure are of major concern.

In order to determine the biological effects following exposure to ionizing radiation, great efforts have been made, both to define the genetic effects induced by exposure, as well as to understand the late consequences of such findings. To date, the assessment of genotoxic risks following ionizing radiation exposure has been based on data from epidemiological studies (Boice, 1988, 1986, 1990; Shimizu and Schull, 1990), and on numerous laboratory experiments. Relevant information has been generated from these

approaches, however, some drawbacks that hamper the effectiveness of risk assessment, especially for human exposure to low dose radiation, remain to be solved.

At the experimental level, much progress has been observed in the detection of genotoxic effects following ionizing radiation exposure, however, in some cases, the low sensitivity of the methods used may lead to a high probability of factors associated with non-acceptable risk remaining undetected. Since no threshold exists for ionizing radiation exposure, high sensitivity methods able to detect exposure from low to high doses, must be developed. In epidemiological studies, low sensitivity tests, characterized by low statistical power, are still aggravated by confounding factors that make the interpretation of the results even more difficult. Another complication that limits the assessment of ionizing radiation genotoxic risks is the late appearance (long latency time) of the effects. Development of cancer can vary from years to decades, while the establishment of somatic mutations may take one to several generations. The consequence of this latency period is translated into the additional difficulties in characterizing exposure situations in retrospective epidemiological studies. It can create considerable psychological and sociological harm before the detection of such consequences, and aggravates the whole situation. The lack of difference between the genotoxic effects generated after ionizing radiation exposure and those arising spontaneously is probably the most aggravating factor in the assessment of genotoxic risks. Exposure to ionizing radiation is expected to lead to an increase in the incidence of those genotoxic effects which already occur in populations, rendering the detection of a previous exposure extremely difficult.

It has been well established that ionizing radiation exposure leads to an increase in the individual frequency of somatic mutations (Hakoda *et al.*, 1988a,b; Caggana *et al.*, 1992; Nicklas *et al.*, 1990, 1991; Sala-Trepat *et al.*, 1990). An increased incidence of neoplasia has also been detected in populations exposed to ionizing radiation (Beebe *et al.*, 1977; Schull *et al.*, 1981; Evans *et al.*, 1981; Samet *et al.*, 1989). Despite the fact that carcinogenesis is considered a multistep process driven by somatic mutations (Vogelstein and Kinzler, 1993), the inter-relationship between somatic mutations detected in circulating blood cells and carcinogenesis remains unclear.

The use of circulating lymphocytes from human peripheral blood constitutes one of the few and certainly the most convenient approache in the assessment of spontaneous and induced somatic cell mutation frequencies. Circulating lymphocytes from human peripheral blood have long been used for chromosome studies. With the advance of techniques using crude T-cell growth factor, and more recently, interleukin-2, the cloning and propagation of human lymphocytes *in vitro* became possible, making feasible the determination of mutant frequencies arising spontaneously or induced by genotoxic agents. The *hprt* gene is the most investigated marker in mutation studies using human T-lymphocytes. After several improvements, the clonal version of the *hprt* assay has enabled a number of laboratories to investigate the mutant frequencies in circulating T-lymphocytes, and to recover the mutants selected for molecular characterization at the DNA level.

In a radiological accident that occurred in Goiania, Brazil, about five years ago, 249 people were exposed to ionizing radiation. The group was monitored for chromosomal aberration soon after the exposure and some medical assistance was provided to the victims. However, the trauma caused by the accident is still alive in the minds of the people, creating a constant concern about their future health conditions. In particular, anxiety is demonstrated by the parents of the children who were exposed to ionizing radiation. Since very few investigations have been conducted relating to mutant frequencies in children, we decided to study the small group of exposed children from Goiania. A non-exposed control group was also studied.

It is difficult to obtain large blood samples from children, especially from a group that has already been living under stress for a few years. The small size of the veins, in addition to the smaller volume of blood compared to adults and the stress generated by the venupuncture makes the whole undertaking very difficult. Therefore, the criteria used to select the group was very simple, and was based upon the consent of the children in donating the blood. Blood samples from the exposed group were frozen for three years, while blood samples from the control group, except for sample B-33, were frozen for two years. Using the *hprt* clonal assay, we analyzed the *hprt* mutant frequency in those blood samples.

As demonstrated in Table 16, very few studies using the *hprt* clonal assay have been carried out in children. The majority of such studies was performed in newborns using

cord blood cells. The general mean non-selective cloning efficiency obtained for newborns was 33.75% and the mean mutant frequency obtained from those studies was 0.88 X 10^{-6}. Eighteen children ranging in age from 1 to 17 years old were analyzed in three different studies, giving rise to a mean non-selective cloning efficiency of 39.3% and a mean mutant frequency of 3.10 X 10^{-6}. Although a reasonable number of newborns have been analyzed using the *hprt* clonal assay, the number of children with higher ages is small. In our study, the values obtained for the control group are similar to those shown in Table 16. In contrast, a remarkable difference is observed for the exposed group. As shown in the results section, the mean non-selective cloning efficiency for the exposed group was 8.6%. Since mutant frequency is calculated by the ratio between cloning efficiencies obtained under selective and non-selective conditions, it is important to obtain high consistent cloning efficiencies for all the subjects analyzed, in order to make accurate inferences about the final results. It is known that in the fraction of mononuclear cells separated from whole blood, only a proportion (70-75% in normal people) of the cells are T-lymphocytes. The remainder are B-lymphocytes (10-15%) and monocyte/macrophage cells (10-20%) (Abbas, 1991).

Most of the T-cells in circulating blood have been demonstrated to be potentially cloned under PHA and interleukin-2 stimulation (Moreta *et al.*, 1983). When low cloning efficiencies are obtained in a given study, the first concerns arise from the experimental conditions used in the analysis. In our study, one such difference was registered between the blood collection and separation of mononuclear cells from the two groups that were

performed on different dates. However, the blood sample from subject B-33 in the control group was obtained and processed on the same occasion as the blood collection of the exposed group. Nevertheless, a reasonably high non-selective cloning efficiency (29%) was obtained for this blood sample. This observation, in addition to the range observed for non-selective cloning efficiency in the exposed group, leads us to conclude that the differences in processing and freezing the cells did not really affect the results obtained.

Table 16. *hprt* mutant frequency in children. Data obtained with the *hprt* clonal assay. (From Cole and Skopek, 1994)

GROUP DESCRIPTION	AGE (years)		CLONING EFFICIENCY(-tg)(%)		MUTANT FREQUENCY($X10^{-6}$)		REFERENCE
	Mean	Range	Mean	Range	Mean	Range	
45 Newborns	0	-	42	9-100	0.64	0-160	McGinnis *et al.*, 1990
60 Newborns	0	-	-	-	1.65	0.3-14.7	Lippert *et al.*, 1990
10 Newborns	0	-	-	-	0.71	-	Manchester *et al.*, 1992
63 Newborns	0	-	23	2-98	1.43	0.2-14.7	Albertini *et al.*, 1991
9 Newborns	0	-	38	20-51	0.5	0.15-0.52	Cole *et al.*, 1994
10 Newborns	0	-	32	18-51	0.4	0.18-1.87	Cole *et al.*, 1991
Mean			33.8		0.88		
6 children	-	1-17	56	17-152	-	0.8-5.0	Cole *et al.*, 1991
7 children	5	1-10	47	11-92	2.24	1-39.5	Cole *et al.*, unpubl.
5 children	-	8-17	15	-	3.97	3.4-4.4	Vijayalaxmi *et al.*, 1985
Mean			39.3		3.10		

Since the experimental differences observed in the study were not sufficient to explain the variation obtained for the groups analyzed, we have to consider factors that could explain our results. Several physiological factors can influence the inter-individual variances observed for cloning efficiency. In normal individuals, a considerable intra- and inter-individual variance in the total number of T-cells and T-cell subsets has been reported by different laboratories (Levi *et al.*, 1988; Kornefeld *et al.*, 1982; Smart *et al.*, 1986). Some attempts have been made to distinguish the T-cell subsets that are more promptly clonable under selective and non-selective conditions (Albertini *et al.*, 1985; Hakoda *et al.*, 1988a, 1988b; McGinnis *et al.*, 1990). According to those studies, most of the mutant and wild-type cells cloned by the assay are CD4-positive/CD-8-negative cells. It has also been reported that CD8-positive clones are usually smaller and more difficult to expand (Baron *et al.*, *in press*). The intra- and inter-variability of T-cell subsets could entirely explain the inter-individual variance observed for non-selective cloning efficiencies obtained in this kind of study, however, it is not sufficient to explain the difference in cloning efficiency obtained from the two groups analyzed here. Although the number of cases analyzed was small, as well as the doses to which the subjects were exposed, a significant (cor. coef. =-0.39), but slight inverse relationship between dose and non-selective cloning efficiency was observed using linear regression analysis. Some factors can be considered in order to justify such results. A substantial decrease in the number of white blood cells (leucopenia) was demonstrated for the victims of the Goiania accident soon after the accident (Brandao, 1993). It has also been demonstrated that changes in immunological status of the donor can result in reduced T-

lymphocytes number in circulating blood, and also in a poor response to PHA and interleukin-2 stimulation *in vitro*. However, the extent to which such changes persisted in the body of the victims after exposure is not very clear. Another interesting observation which can be taken into account relates to the effects of stress on the immune system (Khansari *et al.*, 1990). It had been shown that while stress results in no changes in absolute T- and B- cell numbers in circulating blood, it does result in a significant suppression of the ability of T-lymphocytes to respond to mitogen stimulation *in vitro* (Khansari *et al.*, 1990). We conclude that the lower average obtained for non-selective cloning efficiency in the exposed group probably resulted, not only from the exposure to ionizing radiation, but also from the considerable stress to which those children have been long submitted.

In the exposed group, the mean mutant frequency was 4.6 times higher as in the control group. Although an inverse relationship between cloning efficiency and mutant frequency has been reported by many different groups (Cole *et al.* 1989; Albertini *et al.*, 1985), in our study, such a relationship was detected only for the control group (cor. coef. = -0.58), but not for the exposed group (cor. coef. = 0.09). For technical reasons such a correlation can be considered as an artefact. Since mutant frequency is calculated by the ratio between cloning efficiency under selective and non-selective conditions, low non-selective cloning efficiencies can raise mutant frequency, projecting extremely high results for mutant frequency. If that was the case for the high mutant frequency obtained for the exposed group, such an inverse correlation should be substantiated using linear

regression analysis. However, this was not the case. Another possible explanation of the high mutant frequency demonstrated for the group could be that individual mutant clones were undergoing clonal expansion *in vivo*. Unfortunately, it was not possible to analyze all the mutant clones selected in the assays. However, in the exposed group, from 24 mutant clones analyzed for the T-cell clonal assay, 21 were shown to represent individual clones, assuring therefore the high number of individual mutant clones obtained as well as the high mutant frequencies. Persistent high mutant frequencies have been reported by several groups studying individuals exposed to ionizing radiation (Hakoda *et al.*, 1988a, 1988b; Sala-Trepat *et al.*, 1992; Caggana *et al.*, 1992). In such studies, high *hprt* mutant frequencies have been demonstrated in all the exposed groups compared to normal non-exposed controls, however no clear relationship between dose and mutant frequency could be verified. Similarly, in our study, no correlation between dose and mutant frequency was observed.

We conclude therefore that the *hprt* clonal assay used in this study has been demonstrated to be sufficiently sensitive to distinguish and to differentiate the two groups of children analyzed, the exposed and the control group. The low background mutant frequency observed for the control group certainly facilitates the analysis, since even with the small doses to which the individuals were exposed, a reasonable difference between the groups was confirmed. Nevertheless, such differentiation was only confirmed by comparisons with an age matched control group. Despite the fact that the assay has been shown to be sensitive to radiation exposure, even to small doses, such an assertion was only possible

by comparing the two groups. The assay is therefore not able to detect these levels od radiation exposure without a reliable control group. Another concern with the assay is the lack of a dose-response relationship. The lack of a dose-response greatly diminishes the test sensitivity, an observation which has been also confirmed by other groups (Hakoda *et al.*, 1988a; Caggana *et al.*, 1992). When the specificity of the method is taken into account, problems can also be highlighted. It is impossible to differentiate in the study groups, wheter the mutations found really arose from ionizing radiation exposure, or if they were induced by other factors. Many attempts have been made to sequence and analyze the mutants found following different exposures and to classify those mutants establishing specific signatures for different genotoxic agents, however, for studies *in vivo* such a goal has not yet been achieved. Another drawback with the method is the relationship between the consequences of its findings, i.e. the consequences between high *hprt* mutant frequencies in circulating lymphocytes and the later development of diseases, such as neoplasia. It is possible that in the future such problems can be solved and that the assay can be better understood and therefore more useful.

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APPENDIX

PEARSON'S CORRELATION COEFFICIENTS
AGE X CLONING EFFICIENCY

SUBJECT EXCLUDED	CORRELATION COEFFICIENT
CONTROLS	
B-176	- 0.0799093
B-33	- 0.5608117
B-177	- 0.4059795
B-111	- 0.4289903
B-156	- 0.3523186
B-172	- 0.5804814
B-123	- 0.2948465
B-121	- 0.4316042
EXPOSED	
B-50	0.3757927
B-45	0.1413138
B-20	0.2332995
B-26	0.1324114
B-68	0.1919833
B-25	0.1868555
B-22	0.2238832
B-48	0.2018937
B-59	0.3546186
B-34	0.2736974

PEARSON'S CORRELATION COEFFICIENTS
AGE X MUTANT FREQUENCY

SUBJECT EXCLUDED	CORRELATION COEFFICIENT
CONTROLS	
B-176	0.131837
B-33	0.353729
B-177	0.258518
B-111	0.285690
B-156	0.321884
B-172	0.281474
B-123	- 0.013003
B-121	0.486626
EXPOSED	
B-50	- 0.084167
B-45	0.233030
B-20	0.514272
B-26	0.057311
B-68	0.243059
B-25	0.247772
B-22	0.243631
B-48	- 0.019798
B-59	- 0.060922
B-34	0.481766

PEARSON'S CORRELATION COEFFICIENTS
CLONING EFFICIENCY X MUTANT FREQUENCY

SUBJECT EXCLUDED	CORRELATION COEFFICIENT
CONTROLS	
B-176	- 0.519776
B-33	- 0.5749114
B-177	- 0.5823458
B-111	- 0.5626347
B-156	- 0.7563874
B-172	- 0.6401392
B-123	- 0.1237061
B-121	- 0.6147564
EXPOSED	
B-50	- 0.026466
B-45	- 0.385170
B-20	- 0.353226
B-26	- 0.115550
B-68	- 0.098889
B-25	- 0.109992
B-22	- 0.146067
B-48	- 0.114130
B-59	0.072015
B-34	0.197659

PEARSON'S CORRELATION COEFFICIENTS
DOSE X CLONING EFFICIENCY

SUBJECT EXCLUDED	CORRELATION COEFFICIENT
EXPOSED	
B-50	- 0.367888
B-45	- 0.2990735
B-20	0.2936711
B-26	- 0.2050929
B-68	- 0.5888189
B-25	- 0.3903370
B-22	- 0.5793452
B-48	- 0.3844520
B-59	- 0.4445538
B-34	- 0.3852274

PEARSON'S CORRELATION COEFFICIENTS
DOSE X MUTANT FREQUENCY

SUBJECT EXCLUDED	CORRELATION COEFFICIENT
EXPOSED	
B-50	- 0.447200
B-45	- 0.153790
B-20	0.092760
B-26	- 0.187620
B-68	- 0.185300
B-25	- 0.132302
B-22	- 0.176868
B-48	- 0.166640
B-59	- 0.116980
B-34	- 0.228480

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PUBLICATIONS

Glickman, B.W., V. Saddi and J. Curry (1994). Spontaneous mutations in mammalian cells. Mutation Research, 304:19.

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PRESENTATIONS

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August 09, 1994