Radiation and Bleomycin induced cell cycle delay, apoptosis and DNA damage in mammalian cells with respect to the endogenous glutathione status

By

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Submitted

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DECLARATION

I, Aparajita Dutta, hereby declare that the subject matter of this thesis is the record of work done by me, that the contents of this thesis did not form the basis of the award of any previous degree to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other university / institute.

This is being submitted to the North-Eastern Hill University for the degree of doctor of Philosophy in Zoology.

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Abbreviations used

Abt.M Aberrant Metaphases
AGT Average Generation Time
ALP Alkaline Phosphatase
ATP Adenosine triphosphate
BLM Bleomycin
BMC Bone marrow cells
BrdU 5'-bromodeoxyuridine
BSO D, L-Buthionine- (S, R)-sulfoximine
CAs Chromosomal aberrations
Chtd. Bk Chromatid break
Del Deletion
DL Dalton's *Lymphoma cells*
DNA Deoxyribonucleic acid.
DPX Distrin phthalate in xylene
Dsb double strand break
DTNB 5-5'-dithiobis 2'-nitrobenzoin acid
EDTA Ethylene diamine tetra actic acid.
Exch. Exchanges
FPG Fluorescence plus Giemsa staining
GGT γ-glutamyl transpeptidase
GR Glutathione reductase
GSH Reduced glutathione
GSSG Glutathione disulfide
GST Glutathione-S-transferase
Gy Gray
HDR High dose-rate
HPBLs Human Peripheral Blood Lymphocytes
i.p. Intraperitoneal
IAEA International Atomic Energy Association
IgG Immunoglobulin G
isochtd. Bk Isochromatid break
LDR Low dose-rate
LET Linear energy transfer
M1 First cycle metaphase
M2 Second cycle metaphase
M3 Third cycle metaphase
MI Mitotic Index
NADPH Nicotinamide adenine dinucleotide phosphate

NHEJ	Non-homologous end joining
NHR	Non-homologous recombination
NPSH	Non-protein thiols
PAGE	Polyacrylamide gel electrophoresis
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PHA	Phytohaemagglutinin
RI	Replicative Index
RIPA	Radio Immuno Precipitation Assay buffer
RPMI	Rosewell Park Memorial Institute
SCU	Sister chromatid union
SDS	Sodium dodecyl sulfate
SSA	5-sulfosalicylic acid
Ssb	single strand break
SSC	Saline sodium citrate
tGSH	Total GSH
TNB	5-thio-nitrobenzoic acid
UV	Ultra-violet
WBC	White blood corpuscles

Introduction

Radiation biology had its start in the year 1895 with the discovery of X-rays by Prof W.C. Roentgen. Almost simultaneously, within a period of about 4 months of their discovery, the ability of radiation to damage cells had been detected. In 1897, Dr Freund first used radiation for therapeutic purposes. In the recent context, radiobiology had become a very complex science because of its ambition to understand every step leading from the absorption of energy to death or final injury.

Ionizing radiation of every type is a form of energy. In order to act upon living or non-living systems, it has to be absorbed. Thus the way in which the various types of ionizing radiations are absorbed constitutes the first step in the process of interaction of radiation with matter.

An entire spectrum of electromagnetic radiation, which consists of oscillating electric and magnetic fields, is present in our environment in the form of radio waves, infrared visible, UV, X and gamma rays. The radiations of wavelengths in the visible regions are important to life. UV, too, is important for a few biological processes and produces its effects largely by the process of excitation. The electromagnetic radiations with shorter wavelengths produce both ionization and excitation in the media through which they travel. The biological effects of this radiation apparently result from the ionizations produced largely however. There are essentially three mechanisms by which energy can be transferred from these radiations to their surrounding media: the Photoelectric effect, the Compton effect and Pair production.

Ionizing radiation is detrimental to life. The energy absorbed from them induces changes at the molecular level. Most cell constituents including macromolecules (like DNA and enzymes) or even small molecules (like ATP or co-enzymes) are changed by radiation. The biological effects of radiation are the end products of a long series of phenomenon, which are set in motion by the passage of radiation through the cell. A time scale of the radiolytic events is outlined in table 1.

Radiation damage involves two kinds of mechanisms, which however cannot be separated in the living system. The two mechanisms by which radiation produces it's effects on the cell are a) the ***direct action*** where the molecular damage occurs by the

absorption of energy in the molecule itself directly without the involvement of any intervening medium and, b) ***indirect action*** where the energy is first absorbed by the surrounding water molecules leading to the generation of highly reactive free radical species. These free radicals then react with the tissue molecules. Absorption of radiation energy takes place within $10^{-18} – 10^{-16}$ seconds followed by ionization or excitation ($10^{-15} – 10^{-13}$ seconds) and free radical generation ($10^{-13} – 10^{-11}$ seconds). Since cells consist of about 85% water, to a great extent, the biological effects are mainly mediated through the action of radiation on water. The damage due to the indirect effect of radiation is supposed to be caused by the free radicals produced in the water sheath around the DNA molecule containing the bound water. The width of this water sheath is assumed to be several nm (Hutchinson, 1985; Ward, 1988). In cells, the DNA is partly protected from diffusible water radicals (Warters and Childers, 1982) and this protection generally diminishes the lifetime of the free radicals in a cellular environment.

Interaction	Time (sec)	Events and processes
Physical	10^{-18}-10^{-15}	Energy absorption, excitation and ionization.
Physico-chemical	10^{-15}-10^{-10}	Rearrangement of ionized or excited molecules, formation of diffusible radicals such as $H^{\cdot}$, $OH^{\cdot}$, $e^{-}_{aq.}$
Chemical	10^{-10}-10^{-3}	Free radical reactions molecular alterations, formation of bioradicals by indirect action, long lived lesions in macromolecules.
Biochemical	10^{-3}-10^{4}	Enzymatic reactions, recognition of lesions, repair, fixation of damage.
Cellular level	10^{4}-10^{7}	Cell death, cell loss, division kinetics, mutation.
Systemic	10^{8}-10^{10}	Hormonal effects, immune reactions, Vascular changes, functional impairment, adaptation, carcinogenesis, ageing and death.

Table 1. Time scale of radiolytic events in biological system

The yields of the free radicals depend on the density of ionization events and it can be expected to decrease with increasing ionization density as experiments (Appleby and Schwartz, 1969; Sauer *et al.*, 1977) and theoretical predictions show (Magee and Chatterjee, 1980). The radiolytically formed free radicals and molecular products from the reactions outlined above react with biomolecules and bring about the changes in their structure and function. Therefore, free radical formation processes are considered to play a very important role in radiation induced cellular lethality. The free radical species are very reactive and short lived. It is important that most of the free radical action in irradi- ated cells is fast and completed within a fraction of second leading to the fixation of damage which could be expressed immediately within hours, days or years depending on its mode and magnitude. Thus the free radical products formed initially, quickly decreases and an equilibrium concentration is set up which does not alter as the amount of radiation is increased. The magnitude of this equilibrium values however depends on the intensity (dose-rate) at which the radiation is delivered (Bacq and Alexander 1961). When considering the radical combination process, it is in general only necessary to deal with radicals formed within the same track since the distance between tracks is much greater than the distance between ionization (or excitation) within the track. For this reason, the dose-rate does not influence the number of primary chemical events. Consequently, only the radiochemical reactions in which the dose-rate effect can be determined are those in which a relatively long-lived intermediary is produced which is capable of reacting with other radicals. When dose-rate becomes extremely high, then the instantaneous concentration of radicals produced will go up since the different tracks overlap.

From the biological point of view, the effects produced by ionizing radiation depends on the energy absorbed by the system, and among other factors, largely, this depends on the rate at which this energy is deposited (Hall, 1991). This dose-rate effects covers significant areas in radiobiology applied to radiation therapy as well as radiation protection. Dose-rate effects have been widely studied and numerous examples quote the effect of variation of dose-rates of radiation on different endpoints within the biological system.

Deoxyribonucleic acid is a critical cellular target for the cytotoxic, mutagenic and carcinogenic effects of ionizing radiation (Alper, 1979, Grosch and

Hopwood 1979, Biaglow, 1981). Radiation induced chemical modifications of DNA involve the formation of ionic, radical and excited intermediates as a result of the deposition of energy within the biopolymers and indirect process involving water radiolysis species (Adams and Jameson, 1980). The radiation chemistry of DNA is extremely complex. One reason for this is the fact that ionizing radiation is not selectively absorbed by the molecule alone, but the solvent and other solutes, so that indirect reactions may interfere. In DNA, radiation induced alterations may be broadly classified as:

1. *Single strand breaks* – The scission in the sugar-phosphate backbone in one strand of DNA double helix causes single strand breaks.
2. *Double strand breaks* – Breaks in both the strands of DNA double helix, which may be caused either by single energy deposition event or by the interaction of two single strand breaks formed individually in close proximity.
3. *Base damage and base loss* – Alterations of the nitrogenous bases or loss of the bases by breakage of the glycosidic bonds upon irradiation. Some of these base damages even may result in the rupture of the sugar-phosphate backbone.
4. *Denatured zones* – This consists of breaking of the hydrogen bonds between the between the base pairs of the two strands of the double helix.
5. *Crosslinks* - Radiation induces the formation of various types of crosslinks. Intermolecular crosslinks between two molecules of DNA, or intramolecular crosslinks within the same molecule of DNA can be formed.

Despite the diversity of damages in DNA quoted by ionizing radiation as outlined above, there is evidence that the double strand breaks (dsb) is the lesion which if unrepaired or misrepaired is most likely to lead to cell death (Frankenberg *et al*, 1981, Iliakis, 1991). Many studies have pointed out a good correlation between dsb induction and radiosensitivity in human tumor cell lines (Ruiz de Almodovar 1994c, Whitaker *et al* 1995). There is evidence, however, that a correlation between remaining dsb and radiosensitivity may be found after exposure of human cells to radiation at 37^oC at different dose-rates in human cells (Blocher *et* al 1991, Wurm *et al* 1994). From several studies it seems that the dsbs remaining after exposure to radiation at 37^oC predict intrinsic cell radiosensitivity more accurately than measurements of dsb induction or

rejoining after an acute irradiation. Using alkaline unwinding technique, Dikomey (1988) had shown that DNA singe-strand breaks repair kinetics do not differ when cells were irradiated at 37°C or at 4°C. However, there are no similar studies on dsb, and results with all the strand breaks may not be directly extrapolated to dsb. The level of DNA dsbs immediately after exposure of a non-transformed human fibroblast cell line (HF-19) to γ-rays (0-40Gy) at four dose-rates (10, 1, 0.1 and 0.01 Gy min^{-1}) at 37°C was demonstrated using clamped homogeneous electric field gel electrophoresis (Forray *et al* 1996). This study showed that the rate of dsb rejoining changes continuously with repair time and that it is independent of dose and dose-rate in the range 10-40 Gy.

There is an interest in the development of assays that might predict response to radiotherapy is based upon observations that there is a range of radiation responses of cultured tumour and normal cells from different individuals. So far, there is no simple method has been devised that can rapidly and accurately predict patient sensitivity to ionizing radiation. Because measurements of rejoining of DNA strand breaks can be performed rapidly, they are appealing for use in a predictive assay. While a correlation between radiosensitivity and dsb repair capacity for tumour cells has been observed by some workers (Zaffaroni *et al* 1994, Schwartz *et al* 1996), others have failed to find one (Olive et al 1994, McKay and Kefford 1995). Moreover, the normal tissue response limits the total radiation dose that can be administered to a patient.

The ability of human peripheral lymphocytes to repair DNA damage by ionizing radiation has been shown to vary with the individual. This variability may depend on many factors including individual age (Licastro *et al* 1982, Mayer et al 1991, Fenech *et al* 1993) and DNA repair-status (McCurdy *et al* 1997). Rejoining of radiation induced ssb and dsb in HPBLs is slow relative to that observed for proliferating cells or stimulated lymphocytes (Tobi and Itzhaki 1993, Chukhlovin *et al* 1995). This observation can be explained at least in part by small pool sizes for deoxyribonucleotides (Green et al 1996) and low levels of at least some repair enzymes (Tashiro *et al* 1996). However, some rejoining always occurs, and perhaps the rate of rejoining in WBC may be indicative of repair capacity in other quiescent normal tissues that are radiation dose-limiting. In contrast, in proliferating cultured cell lines, kinetics of rejoining of ssb generally fail to account for differences in radiosensitvity (Szumiel 1981).

The idea of using PBL cells in the present study is largely because of the ready accessibility of these cells and well established and suitable for the assessment of cytogenetic effects (Adler 1984). Several laboratories have reported inter-individual differences in lymphocyte response to radiation damage when measured using clonogenicity (Geara *et al* 1992, Elyan *et al* 1993), or using the micronucleus assay (Ban *et al.,* 1993, Floyd and Cassoni 1994). However, whether there are substantial inter-individual differences in the ssb or dsb rejoining rates and whether observed differences are related to intrinsic radiosensitivity are still uncertain. At least a part of the difficulty in addressing these questions is that methods used to measure DNA repair may not be completely reliable since WBC are susceptible to rapid radiation-induced apoptosis and necrosis.

Apoptosis and necrosis are two distinct forms of cell death, which are triggered among other insults by ionizing radiation. These two forms of cell death have different morphological and molecular features and implications for the surrounding tissue. Apoptosis is an active process of cellular destruction characterized by cell shrinkage, chromatin aggregation with extensive genomic fragmentation and nuclear pyknosis (Kerr *et al.*, 1972, Wyllie *et al.,* 1980). In vivo, phagocytes normally sequester antigenically modified apoptotic cells, preventing inflammation and damage to the surrounding tissue (Savill *et al.,* 1995). Necrosis is instead characterized by passive cell swelling, intense mitochondrial damage with rapid energy loss and generalized disruption of internal homeostasis. This swiftly leads to membrane lysis and the release of intracellular constituents, which evoke an inflammatory reaction with local cellular infiltration, vascular damage, edema and injury to surrounding tissues.

As mentioned above, a number of factors may be mediating the differences in radiosensitivity found amongst both normal and tumour cell lines. The first one is the efficiency with which the external beam low-LET ionizing radiation induced DNA dsb, thought to be the critical lesion leading to cell death, may differ between cell types. The dose-response for DNA dsb could vary markedly between cell lines, mirroring differences in survival response, and suggesting a common relationship between the level of response in the non-denaturing filter elution assay and the level of cell killing for the cell lines examined (Radford 1986). Although there has been support for the conclusion that

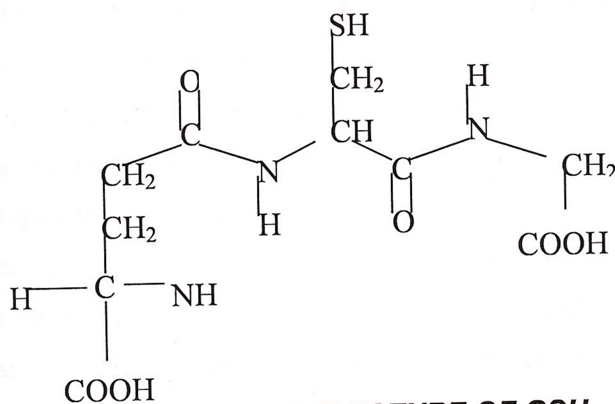
differences in the dose-response for induction of dsb can be important cause of differences in radiosensitivity (Peacock *et al.* 1989), there has been questioning of the validity of the non-denaturing filter elution assay as a measure of DNA dsb (Hutchinson, 1989). The second factor that may contribute to differences in radiosensitivity is mode of cell death after irradiation. It has long been known that some normal cell populations (eg. Lymphoid cells), which undergo apoptosis after irradiation, can show extreme radiosensitivity. It has been shown that non-lymphoid (predominantly fibroblast-like) cell lines underwent necrosis whereas lymphoid and myeloid lines tested showed evidence consistent with apoptotic death (Radford 1994). These differences in radiosensitivity appeared to correlate with the rapidity with which apoptosis was induced after lethal radiation insult, which in turn appeared to reflect differences in the pathways used to induce apoptosis in different cell lines (Radford and Murphy 1994).

Apoptosis is in fact required to destroy errant cells that pose a threat to the organism. It eliminates cells that have accrued genetic mutations and may thereby become cancerous. An important paradigm of the ontogeny of cancer came from the discovery of the roles of dormant proto-oncogenes that mutate into growth promoting oncogenes typified by such genes as *src, ras* and *raf*. However, on the other hand there is another class of genes known as tumor suppressors or anti oncogenes that suppress oncogenesis. For example, cell fusion experiments demonstrated that the malignant potential of cells could be abrogated by fusing them with normal cells. Some tumor suppressors are able to counteract the proliferative consequences through their ability to induce apoptosis. Our present understanding of cancer pivots on the understanding of these opposing influences and how this knowledge may be used for therapeutic intervention. Indeed induction of apoptosis is the mode of action of many anti tumor therapeutic agents that are currently in use.

Thiols dominated the field of radiation protection from the 19540s through to the 1980s. In recent years, studies on radioprotectors appeared to be a turning point: only a few new candidate drugs have been proposed and non-thiol protectors, including protease inhibitors, vitamins, metallo-elements, and calcium antagonists are actually playing a much larger role in radioprotection. In the 1990s, interest increased in endogenous

protective systems as opposed to chemical radioprotectors. Some of these biological agents act best when given prior to radiation; others can modulate radiation injury when given after irradiation. Aminothiols represent the most important group of radioprotective compounds. The endogenous aminothiol which has far-out the highest intracellular concentration is glutathione (GSH) (Vos,1992). GSH (L-γ-glutamyl-L-cysteainyl-glycine) is widely distributed in animal tissues and body fluids and is found also in many plants and microorganisms. Its concentration in most animal tissues is in the range of 0.5 – 10 mM (Meister, 1988) and is thus substantially higher than many other intracellular metabolites. GSH is characterized by its reactive thiol group –SH and its γ-glutamyl bond that makes it resistant to normal peptidase activity (Moldeus and Jiang 1987).

Features of GSH intimately associated with its diverse and important functions include: (1) protection of cells against oxidative damage, (2) existence as an important component of a system using pyridine nucleotides to provide a reducing atmosphere essential for the integrity of the cell membranes; (3) a key role in amino acid transport and multiple metabolic pathways such as synthesis of proteins, nucleic acids and leukiotrienes; (4) regulation of enzyme activation and the immune response and (5) acting as a reservoir of cysteine. GSH is also proposed to be involved in the homeostasis and detoxification of metal ions in biological systems (Freedman *et al* 1989, Gardener *et al* 1993, Kang *et al* 1988)



STRUCTURE OF GSH

Reaction of GSH with oxidants converts it to either glutathione disulfide (GSSG), an oxidized glutathione form or to a mixed disulfide (RSSG) (Moldeus and Jiang 1987). Thus three forms of GSH may exist in a cell. The reduced glutathione/oxidized glutathione (GSH/GSSG) complex is the major buffer in the cell (Meister 1988). Under normal physiological conditions, the intracellular environment is highly reducing due to a high GSH/GSSG ratio of 30:1 to 100:1 (Hwang *et al* 1992). Thus most of the cellular glutathione (90%) is present in its reduced form, while GSSG and RSSG constitute ~5% each. The bulk of intracellular glutathione is found in the cytosol but the existence of a minor mitochondrial pool has also been demonstrated (Moldeus and Jiang 1987). Tirmenstein and Reed (1988) found high concentrations of GSH in the nucleus, the values of the pool being similar to those of cytosolic pool. GSH is present in various body fluids including plasma, bile, glomerular filtrate, and in the lung bronchoalveolar lavage fluid (Moldeus and Jiang 1987). The distribution of GSH in different tissues is different. GSH is transported into the extracellular space and cleaved to its constitutent amino acids, which are subsequently utilized for the synthesis of GSH. This complete cycle is known as the γ- glutamyl cycle. Regulation of GSH biosynthesis may occur through feedback inhibition mechanism (Rahman *et al* 1999).

Information about the physiological functions of GSH is obtained by the observations on *in vivo* systems in which there is marked deficiency of glutathione. One of the many ways in which such expectation has been realized is by the use of selective inhibitors of glutathione synthesis. Depletion of glutathione in biological systems has been achieved by the use of oxidants such as hydroperoxides (Flohe *et al.* 1974, Sies and Wendel 1979) and diamides (Kosower and Kosower 1976). However, the effects of these compounds are generally short lived and are associated with markedly increased concentrations of glutathione disulfide. Since these reagents are non-specific, oxidation of other cellular components may occur, and also their effects may be of short duration because the rate of glutathione synthesis may increase sharply after the depletion of glutathione (Meister 1983). An alternative way intitially developed was by inhibition of glutamine synthetase by L-methionine-S-sulfoximine (Meister 1968, Meister 1974, Meister 1978), however, this was not found to be suitable since this compound was found to inhibit both glutamine synthetase as well as γ-glutamylcysteine synthetase, but

it's interaction with glutamine synthetase offers severe complications on studies in glutathione metabolism. Later on, prothionine sulfoximine and buthionone sulfoximine were found to selectively inhibit γ-glutamylcysteine synthetase, and it was notable that buthionine sulfoximine was much more effective than the other compounds in the depletion of glutathione levels (Meister and Anderson, 1983).

Since GSH constitutes approximately 90% of the non-protein thiols in the cell, considerable research has been directed to the understanding of its role in radiation response. It has already been proved that endogenous thiols of which GSH is the most prevalent one are important in cellular radiosensitivity (Edgren *et al.*, 1980, Clark *et al.*, 1984, Vos et al, 1986) and in general it was found that low intracellular GSH concentration cause sensitization especially under anoxic or hypoxic condition (Revesz, 1985). There is variation in the GSH content between different cell lines however, and this was reported by Mitchell *et al.* (1986) as well as Philips *et al.*(1986). The variation of GSH in different phases of the cell culture (Post *et al.*,1983) and in different culture conditions (Mitchell and Russo, 1983), have been reported.. GSH levels have also been reported to vary with different phases of the cell cycle (Ohara and Terasima, 1969) and that resistance to X-ray result synchronously with GSH content has been reported by Sinclair (1969). The role of glutathione in protecting the cells against radiation damage has been studied extensively by a number of workers. Most workers have used BSO for such studies. BSO inhibits GSH synthesis and very low levels of GSH can be obtained over a relatively short time periods without appreciable toxicity. When GSH levels are depleted very rapidly, cells are sensitized to radiation to a small extent (Mitchell *et al.*, 1983, Biaglow *et al.*, 1983). Prolonged exposure to BSO with subsequent chronic GSH depletion leads to greater radiosensitization (Biaglow *et al.*, 1986). However, it has been suggested that the compartmentalization of GSH within the cells take place and the GSH within the nucleus and in particular close to the DNA is important in determining cellular radiosensitivity (Edgren, 1987). GSH has also been hypothesized to act as a repair agent. Mitchell *et al.* have reported the role of GSH as a chemical repair agent in mammalian cells in 1986. In fact, a reduction in the chemical repair rate by about 2-3 fold has been determined on treatment with BSO. That GSH also plays a role as a cofactor in the enzymatic repair processes in the cell has also been outlined by Xue *et al.*, (1998).

The participation of GSH in the repair of single strand breaks in oxic conditions has been reported by Edgren *et al.* (1981) Thus on the basis of these background information, the **objectives** of the present investigation were:

- To evaluate the influence of different dose-rates of radiation on chromosome aberrations and cell proliferation in mammalian cells with respect to the endogenous GSH status.
- To study the influence of endogenous GSH on the interaction of the lesions produced by bleomycin and radiation at different dose-rates.
- To evaluate the apoptotic cell death by the combined treatment of bleomycin and radiation in mammalian cells.
- To examine the antitumour activity of bleomycin in combination with radiation at different dose-rates.

For the above-mentioned studies, the two model systems used were the mice bone marrow cells (BMCs) for the *in vivo* portion of the investigation and the human peripheral blood lymphocytes (HPBLs) for the *in vitro* portions. Both are well-established systems for the kind of work that was undertaken for the following investigations. The mice bone marrow cells are pleuripotent stem cells, which are in the continuous process of division. These cells are loosely attached to one another and therefore it is easy to prepare cell suspensions with them. However, the HPBLs are all quiescent cells i.e they are all in the G_0 phase. The HPBLs are a readily available source of cells (Adler, 1984) and they can be made to divide, by the addition of a mitogen, to obtain cells in large numbers and also the studies done on them could be easily correlated to humans *in vivo* (Evans and Buckton, 1982).