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Biological Monitors for Low Levels of Ionising Radiation

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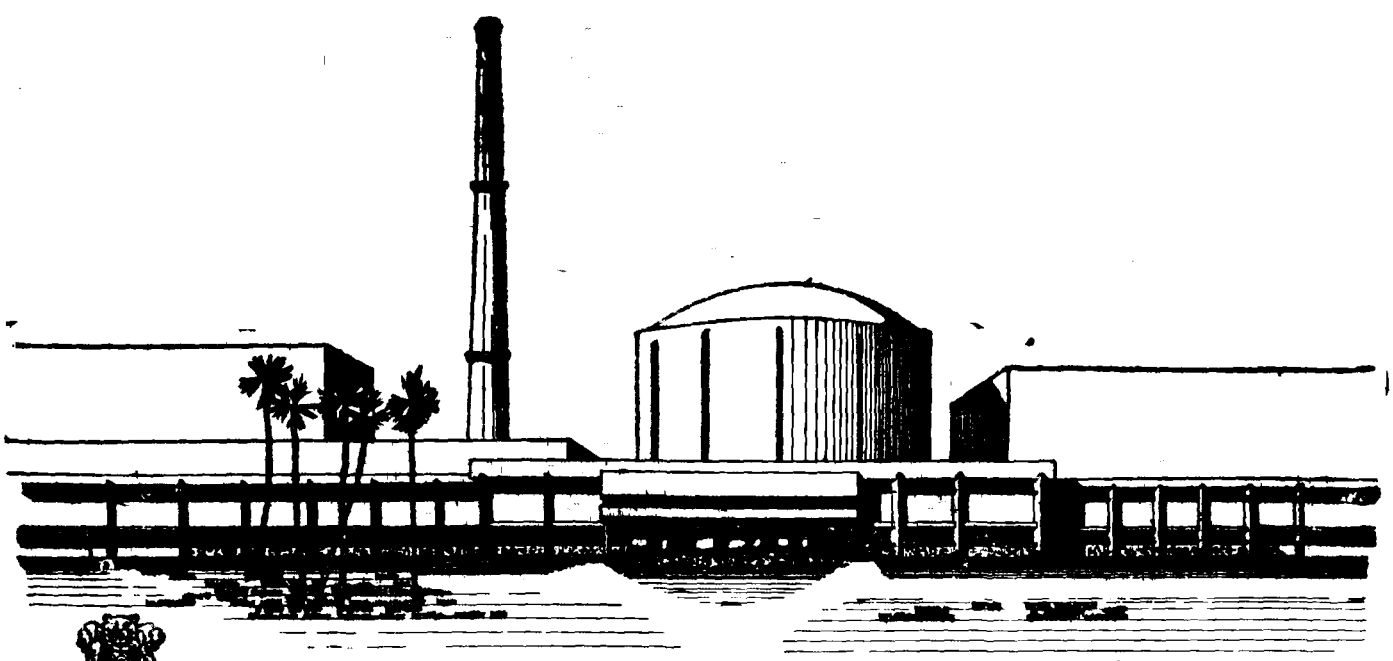

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BIOLOGICAL MONITORS FOR LOW-LEVELS OF IONISING RADIATION*

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ABSTRACT

The biological effects of high doses of ionising radiation are well understood and the methods of measurement of these doses well established. However the effects due to extremely low doses remain by and large uncertain. This is because of the fact that at such low doses no gross symptoms are seen. In fact, at these levels the occurrence of double strand breaks leading to the formation of chromosomal aberrations like dicentrics is rare and chances of mutation due to base damage are negligible. Hence neither chromosomal aberration studies nor mutational assays are useful for detecting doses of the order of a few milligray. Results of exhaustive work done by various laboratories indicate that below 20 mGy the chromosomal aberration technique based on scoring of dicentrics cannot distinguish between a linear or a threshold model. However indirect methods like unscheduled DNA synthesis (UDS) and sister chromatid exchanges (SCEs) appear to be promising for the detection of radiation exposures due to low levels of radiation. This report reviews the available literature on the biological effects of low levels of ionising radiation and highlights the merits and demerits of the various methods employed in the measurement of UDS and SCE. The phenomenon of radio-adaptive response(RAR) and its relation to DNA repair is also discussed.

Key Words: Low level radiation, Unscheduled DNA synthesis (UDS), Sister chromatid exchanges (SCE), Radio-adaptive response (RAR).

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* This report forms a part of the doctoral work of Smt.Mary N.Mohankumar to be submitted to the University of Madras, under the supervision of Dr.R.K.Jeevanram.

CONTENTS

1. INTRODUCTION

The health effects of low level ionising radiation have been viewed with much concern and are receiving considerable attention in both the public and the academic spheres. This is mainly because of the fact that current research has not yet succeeded in providing definitive indications as to whether 'low dose' radiation is detrimental to health or not, and if so to what degree. With a global increase in the use of nuclear energy for power generation and radiation sources in medicine as well as in industry, the environmental levels of radioactivity are likely to increase measurably. Hence there is need to obtain a better understanding on the biological effects of low level radiation exposures.

In this context it should be noted that the major share of human radiation exposure would still come from natural sources like cosmic rays, radon isotopes, which are the daughter products of uranium and thorium present in rocks and soils and potassium-40 found in body cells. These sources contribute on an average a dose of about 2.4 mSv per individual per year (Mukherjee and Mircheva 1991). This indicates that all living organisms have always been subjected to inevitable small doses of radiation during periods of normal growth and reproduction. The fear about radiation, however, arises because harmful effects have been observed at high doses of the order of Sieverts. The consequences of exposures of the order of a few mSv are bound to be ambiguous since

1. changes at the cellular level at these exposures are not directly observable and
2. other factors like diet, tobacco, viral infection and sexuality (Fig 1) are far more likely to lead to the same consequences

Recent developments suggest the possibility of indirect methods for observing the effects of even very low levels of ionising radiation. Low levels of radiation have been found to cause a quickening of the DNA repair process in human lymphocytes when they are subjected to damage from a clastogen like UV radiation. Similarly, there appears to be a decrease in the sister chromatid exchange frequency caused by chemicals like Mitomycin C in cells that have received prior low level exposures. Quantification of these indirect effects seems to offer a promising method to detect low doses in biological systems.

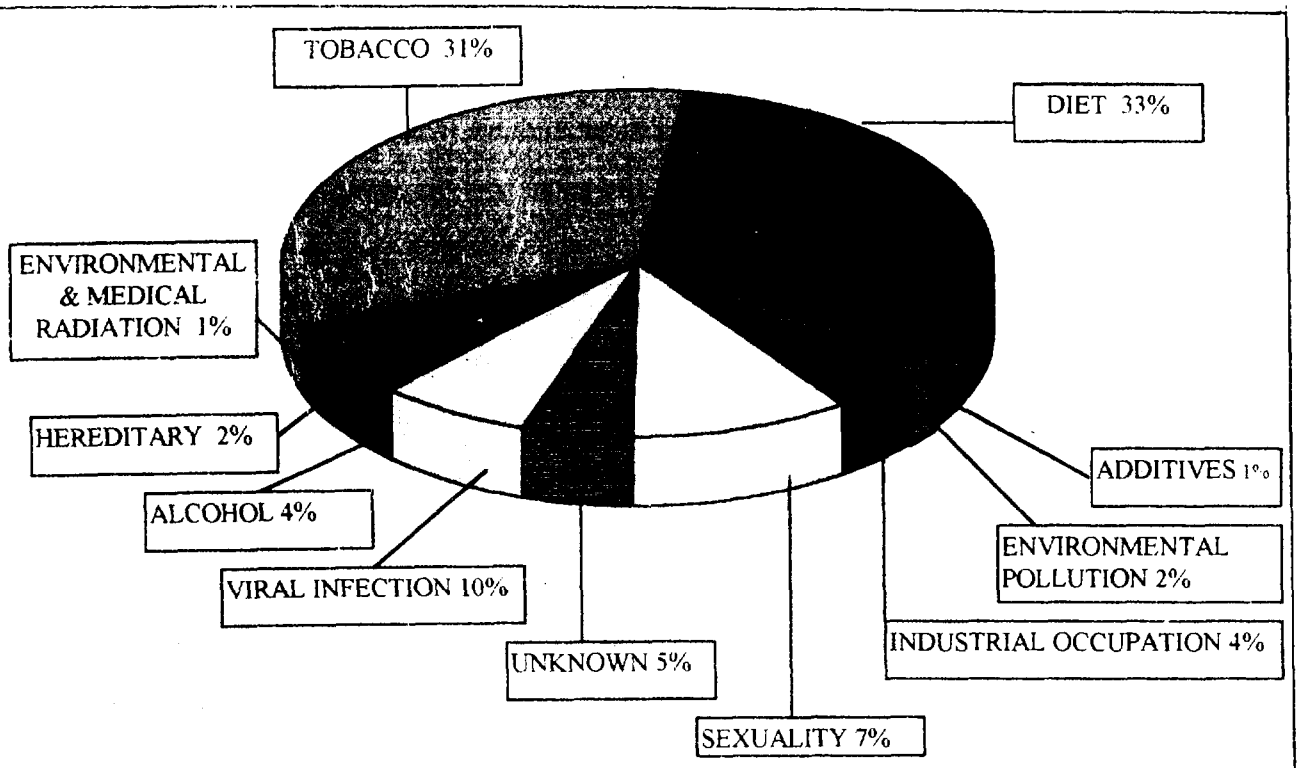

FIG. 1 CANCER RISK FACTORS IN DAILY LIFE
(E.H.Rosenbaum: Can You Prevent Cancer)

2. LOW DOSE RADIATION

The term low dose is used relatively in different situations. In radiation biology a dose range from a few hundreds of mGy to several Gy is regarded as being low since it constitutes the lowest range where the investigated effect is still measurable. However, in radiation protection this could mean environmental levels. Microdosimetric considerations, based on energy absorption events and target volumes of cells, have been applied by some workers (Booz et al 1988, Bond et al 1988) who define 0.3 mGy as a low dose and doses above 30 mGy as high. In this report a low dose refers to a dose which ranges between the annual natural background radiation levels and a dose below which cytogenetic effects (like chromosomal aberrations) are no longer detectable. This low dose range in quantitative terms lies between 2 and 20 mGy. This incidentally, is also the typical annual range of occupational exposures in the nuclear industry.

3.RADIATION EFFECTS ON BIOLOGICAL SYSTEMS

The interaction of radiation with the biomolecules can be considered to occur in four stages. The first one is the physical stage lasting about 10^{-15} to 10^{-10} sec when molecules are excited and ionised. This is followed by the chemical stage occurring in time scales of 10^{-7} sec when free radicals are produced. Next comes the biological stage, in which damage occurs to molecules like proteins affecting cell functions and to nucleic acids giving rise to chromosomal aberrations. This takes place in a period which may last from a few seconds to a few hours, depending on the dose. The final stage observable over several years is the cumulative expression of the effects as mutation or cancer.

Among the four stages, the last two are extremely complex in that, for a given type of radiation and for a given type of lesion the effect may depend on several factors such as, the nature of the molecule involved, the location of the lesion in the molecule, the tissue and cellular kinetics, the developmental stage of the species, inter and intra cellular environmental condition. Hence it has not yet been possible to give a precise quantitative description of the biological effects and to give a one-to-one correspondence between the initial energy absorption and the final effect.

The biological effects of radiation can be broadly classified as somatic and genetic effects.

3.1. Somatic Effects: These effects arise due to damage caused by ionising radiation to cells in the body other than the reproductive cells. Somatic effects may be immediate or late. Immediate effects caused only by very high acute doses include skin erythema, depletion in blood counts, nausea, vomiting, diarrhoea and sterility. Depending on the degree of exposure prognosis for recovery may be good or mortality may ensue. Persons who survive the immediate effects may also exhibit delayed effects like cancer or cataract formation.

Somatic effects may be further classified as deterministic effects and stochastic effects

3.1.1. Deterministic Effects: In some cases the biological response to radiation increases in severity with increase in dose, exhibiting a threshold dose below which the effect is not manifested (Fig 2). Typical examples include skin erythema which has a threshold dose of 2 Gy. Most deterministic effects are immediate with the exception of cataract formation which is normally expressed only after a latent period of a few years.

3.1.2. Stochastic Effects: When the probability of induction of a biological effect is dose dependent without a threshold (Fig 3), it is termed a stochastic effect. Observed stochastic effects

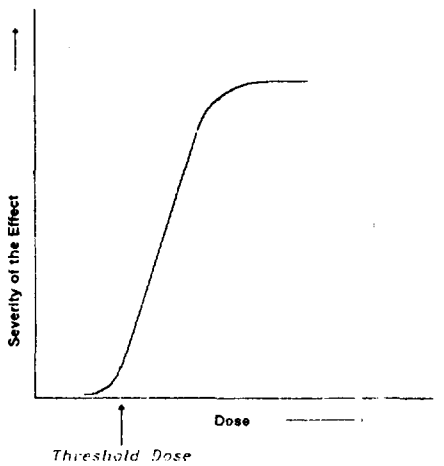

Fig 2. Deterministic Effects of Ionising Radiation

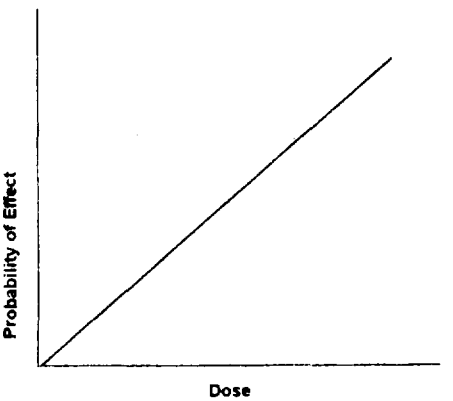

Fig 3 Stochastic Effects of Ionising Radiation

are mutation and cancer. The induction of radiogenic cancer, however, is a dualistic phenomenon consisting of a primary effect on the target cell followed by secondary reactions caused by several environmental factors which ultimately result in the expression of the disease. Induction of cancer by radiation, is therefore, a probabilistic event which has an increased frequency with increasing dose.

For doses above 1 Gy well documented analyses are available through reports published by Radiation Effects Research Foundation(RERF 1992a, RERF 1992b), UNSCEAR (UNSCEAR 1988) and BEIR,(BEIR III, BEIR V) on the probabilities of occurrences of different types of malignancies, expressed in terms of the doses and time after exposure to radiation. Data obtained on observations on atomic bomb survivors indicate that the risk of cancer is in linear proportion to dose between 0.1 and 4.0 Gy (Doll 1991). However, for doses below 0.2 Gy most epidemiological survey data do not always document cancer risks. (Mukerjee and Mircheva 1991).

Nevertheless, the standards laid for radiation protection are all based on direct linearly intrapolated results of the effects at high doses. Hence it has been concluded that however low the dose may be, there is always some risk associated with radiation exposure. This is also the basis for the concept of ALARA in radiation protection which aims at controlling the exposure to levels as low as reasonably achievable.

3.2. Genetic Effects: If radiation causes mutation in the germ cells of an individual, then there is a probability that the damaged cells may participate in zygote formation which will ultimately be expressed as a genetic defect in the progeny. Such effects are termed as genetic effects. There is no proven incidence of such radiation induced genetic effects on humans, although statistical correlations have been reported as for instance by Gardner (Gardner et al 1987). Experiments performed on insects and mice however, have indicated that such a probability exists for humans as well.

4. EPIDEMIOLOGICAL STUDIES

Epidemiological studies still remain the only source of information to assess the biological effects of low-dose radiation on human beings. Study groups can be classified as follows.

(a) Populations living in areas with high natural background radiation.

(b) Populations exposed to man made sources of nuclear radiation such as fallout from weapons tests or those living in the vicinity of nuclear reactors and

(c) Patients exposed to radiation for diagnostic purposes.

The data obtained from such epidemiological studies can relate to effects at cellular levels or to gross consequences such as oncogenic effects.

4.1. EFFECTS AT CELLULAR LEVELS

The most widely studied cellular effect is that of chromosomal aberration. Nevertheless, data obtained from chromosomal aberration studies conducted on human lymphocytes are equivocal (Table 1). To cite a few examples, a 10 year study of peripheral blood lymphocytes of 200 UK naval dockyard workers with chronic gamma ray dose of less than 50 mSv per annum, showed a linear no-threshold increase in gross chromosome aberration, suggesting significant

cytogenetic damage at doses well below permissible occupational limits compared to non-radiation workers (Evans et al 1979).

Chromosome aberration studies investigated in peripheral blood lymphocytes of people living in areas with high natural background radiation (HNBR) and radiation workers, show significant increase in aberration frequencies for exposures ranging between 2 and 10 mGy per year. However for exposures ranging between 10 and 30 mGy per year aberration yields possibly decrease and remain constant (Pohl Ruling 1992). Chromosomal aberration studies conducted on lymphocytes irradiated in-vitro have also yielded mixed results. While some authors reported a threshold effect some others failed to observe any (Table 1).

Table 1 CHROMOSOME ABERRATION DATA FOR RADIATION EXPOSURES

S. No.	Population	Radiation Dose	Type of Exposure	Dose Response	Reference
1	UK Naval Dockyard Workers	< 50 mGy/Yr	Chronic γ dose	Linear	Evans et al., 1979
2	High Natural Background Radiation, Austria	Upto 300 mGy	Chronic α & γ dose	Linear increase, plateau between 100-300 mGy	Pohl Ruling et al 1978
3	In-vitro Exposure	0-500 mGy	Acute X-irradiation	Threshold at 150 mGy	Kucerova, 1972
4	In-vitro Exposure	50-400 mGy	Acute γ-irradiation	Plateau between 100-300 mGy, 50 mGy threshold	Luchnik and Sevankaev, 1976
5	In-vitro Exposure	50-250 mGy	Acute X-irradiation	Linear	Lloyd et al., 1986
6	In-vitro Exposure	4-300 mGy	Acute X-irradiation	Linear	Edwards et al. 1989
7	In-vitro Exposure	0-300 mGy	Acute X-irradiation	> 20 mGy Linear response . < 20 mGy Dicentric Yield below background	Lloyd et al., 1992

Attempts to estimate dicentric yields for low doses with X-rays were undertaken by scoring a large number of cells by a joint effort of various laboratories. It was reported that below 20

mGy, the observed dicentric yields were generally lower than background, though not significantly (Edwards et al 1989). Although the authors interpreted this anomaly for a probable role of stimulation of repair mechanisms, Lloyd et al (1992) conclude that for doses below 20 mGy, due to statistical uncertainties, the chromosomal aberration technique is unable to distinguish between a linear or a threshold model.

Several problems do exist in correlating invivo dose effects with results of experiments performed in vitro. In the first place the contribution due to alpha dose (mainly due to radon and thoron in the environment) is difficult to estimate. Secondly, invivo aberration effects monitored in persons living in areas with high natural background radiation, are all due to cumulative doses delivered over a period of many years and hence net effects may not necessarily relate to true doses if dicentrics are scored as end points. This difficulty can perhaps be overcome by scoring stable aberrations like translocations, which in turn is a strenuous procedure using currently available banding techniques. The emerging fluorescence in-situ hybridisation (FISH) technique is likely to overcome this difficulty in the near future (Popp and Cremer 1992).

4.2. ONCOGENIC EFFECTS

On the basis of currently accepted estimates, cancer risks due to low-level radiation exposure are very much lower compared to various other environmental factors (Fig1). Exceedingly large populations are therefore needed to demonstrate true effects at low doses. Such studies are hindered by factors like

(1) Lack of proper controls,

(2) Impossibility of discriminating a true radiation effect from the effects due to other established carcinogens in the same subjects and of distinguishing between the role of radiation and other agents,

(3) Socio-geographical factors like lifestyle, hereditary, type of dwelling, diet etc.

Interestingly, surveys on oncogenic mortality incidents in HNBR areas have more frequently indicated decreased mortality rates (Frigerio et al 1976, Liu 1989, Wei et al 1990, Nambi et al 1987) (Table 2b), although there are some publications which relate rise in cancer mortality to increased background radiation (Baum 1973). Hence most radiation biologists believe that

potential health risks of low level exposures are relatively small in line with the linear model. However, some researchers argue that there exists a threshold below which the risk is effectively zero, others contend that the risks are disproportionately lower or higher than those predicted by the linear model.(Fig 4, Mukherjee and Mircheva, 1991).

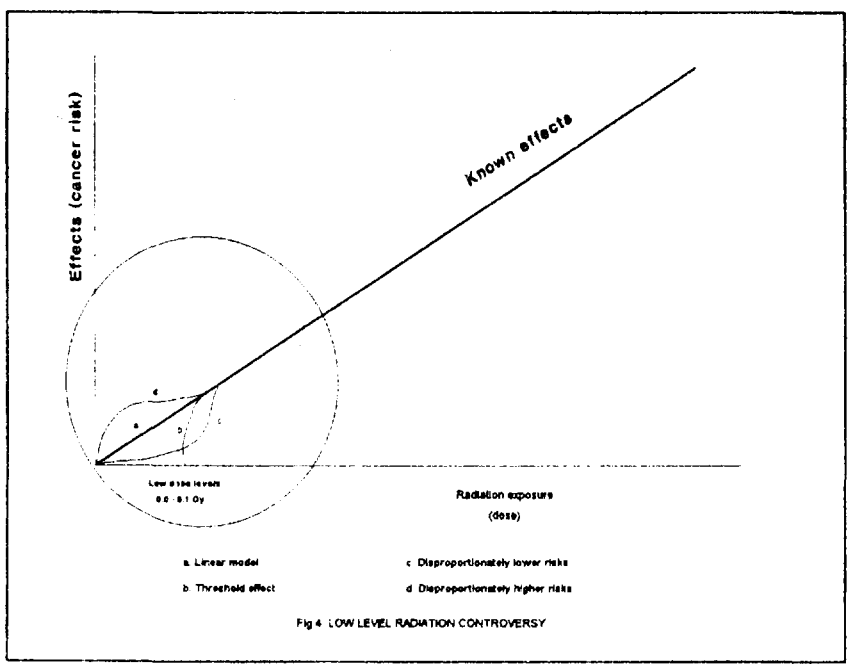

Fig 4 LOW LEVEL RADIATION CONTROVERSY

Thus, with controversial predictions of the biological effects of radiation exposures below 50 mGy, in depth study at molecular and cellular levels is necessary to establish with certainty the true nature of the effect of low level ionising radiation and ascertain if it is detrimental or otherwise.

5. RADIATION HORMESIS

No discussion on the biological effects of low levels of radiation will be complete without mentioning the phenomenon of radiation hormesis (Sagan 1987), which has of late attracted the attention of radiation biologists and authorities concerned with radiation protection. Hormesis is the stimulation of body cells by low doses of any potentially harmful agent. Claims for radiation hormesis have been based on experiments performed with organisms ranging from unicellular ones to plants, insects, fish, vertebrates and primates (Luckey 1980, Luckey 1982, Parsons 1990). Reported hormetic effects include lengthening of lifespan, improved resistance to infection, enhanced healing of wounds, general growth and decrease in cancer mortality (Tables 2a & 2b). Observations of hormesis are in apparent contradiction to the stochastic theory of carcinogenesis. The hormetic effect is however, best explained by the linear-quadratic model of Hickey (Hickey et al 1983).

Table 2a RADIATION HORMESIS ON VARIOUS ORGANISMS				
S.No.	Species	Dose Range	Effect	Reference
1.	Plants	0.01 - 2 Gy (X-Rays)	Increased germination of wheat, barley and corn	Suess 1965
2.	Plants	0.2 Gy/day (X-rays) for 3 weeks	Increased plant height	Evans 1961
3.	Birds	6.4 Gy (Rays, embryo)	Chicks grew faster than controls	Shebaita 1975
4.	Mice	< 0.01 Gy/day X-Rays life time dose)	Greater mean life span than controls	Lorenz et al 1955
5.	Monkeys	0.6 - 1.6 Gy (gamma & neutron dose)	Showed better concentration and learnt better discrimination than controls	Mc Dowell 1958

Table 2b RADIATION HORMESIS ON HUMAN POPULATIONS				
S.No	Population	Dose Range	Effect	Reference
1.	Atomic Bomb Survivors	0.01 - 0.09 Gy	Lower cancer frequency than unexposed controls	Hickey 1983
2.	HNBR area, China	3.3 mGy/yr	Lower cancer mortality compared to controls	He et al 1985
3.	HNBR area, S.E.Finland	296 Bq/m^3 radon burden*	Decreased incidence of female lung cancer	Castren et al 1984
4.	Cumberland county USA	340 Bq/m^3 radon burden	Lower incidence of lung cancer	Cohen 1987
5.	HNBR area Kerala, India	10 - 30 mGy/yr radon, thoron burden	Decrease in cancer mortality by a factor of 0.03 μSv	Nambi 1987
6.	HNBR areas USA	1- 25 mGy/yr	Decrease in cancer mortality with increase in dose	Daglish 1983

* Average indoor radon burden (unventilated area) - 40 Bq/m^3

6. EFFECTS AT MOLECULAR LEVELS

Among all the constituents of a living cell, the DNA has been established to be the target molecule of greatest interest. That is to say that any detriment to the functioning of the cell essentially results from the damage to the DNA. The DNA molecules of all living cells are constantly attacked and damaged by both exogenous environmental and endogenous cellular factors. The exogenous factors include chemicals, viruses and radiation while endogenous events are the depurination and deamination of bases as well as replication errors caused by malfunctioning of DNA polymerases and their associated proteins.

It has been estimated that every hour, human and other mammalian cells undergo at least 50-100 times as much spontaneous or natural DNA damage as would result from exposure to 10 mGy of ionising radiation (Billen 1990).

The fact that normal human beings who are invariably exposed to such spontaneous DNA damage do not suffer from its effect, suggests that all organisms are equipped with a wonderful network of DNA repair mechanisms which faithfully and promptly repair and restore the damaged DNA to its original form. Hence it may be reasonable to assume that cells are constantly at work trying to maintain the integrity of the genetic code. DNA repair thus represents an important factor in the prevention of undesirable mutations that can cause cancer. This is well established while studying repair deficient diseases like xeroderma pigmentosum, ataxia telangiectasia and retinoblastoma. Patients suffering from such diseases are easily susceptible to cancer and it has been irrefutably proved that these patients show enhanced sensitivity to the action of environmental mutagens. (Cleaver 1977).

The two main DNA repair processes occurring in human cells are (1) excision repair and (2) post replication repair. In addition to the above, two other repair mechanisms namely photoreactivation and inducible SOS repair, which are known to exist in prokaryotic cells are thought to occur in eukaryotic cells as well. However the existence of these repair mechanisms in human cells is still not confirmed.

Whatever may be the repair process, it should be noted that it is the error-free repair mechanism that is required to protect all organisms from the effects of DNA damaging agents.

For example, the well studied SOS repair mechanism, induced by large doses of radiation is known to occur via an error-prone mechanism, whereas an error-free repair pathway may be induced when the damage to DNA is small (Samson & Cairns 1977).

One of the major objectives of cancer research is to determine the molecular mechanisms underlying the initiation and propagation of chemically induced cancer. For example, activation of the ras proto-oncogene resulting from mutations in the ras- coding sequence is thought to play an important role in initiating cancer. It is suggested that in such cases an error-prone repair might initiate cancer while an error-free repair might play a direct role in its prevention (Walker 1985)

7.0 BIOLOGICAL END-POINTS FOR DETERMINING LOW LEVELS OF IONISING RADIATION

While some data do exist on the induction of chromosomal aberration for doses as low as 20 mGy, there appears to be no definite end points to serve as indices for biological effects in the dose range of 1 mGy (general average background radiation level) and 20 mGy.

Cells that have been previously exposed to low doses of ionising radiation are reported to exhibit changes in their DNA repair capacity when they are subjected to proven mutagens. Studies on such indirect effects could throw some insight on the effect of ionising radiation at this low dose range.

The indicative end points for the above type of repair are

(a) enhancement in the rate of UV radiation induced unscheduled DNA synthesis (UDS) and

(b) decrease in Mitomycin C induced sister chromatid exchange (SCE) frequency.

8.0 UNSCHEDULED DNA SYNTHESIS

Among various DNA repair measurements, unscheduled DNA synthesis (UDS) has been widely used to quantify excision repair and cell recovery following DNA damage. UDS is generally known to represent a stage of the DNA excision repair process during which on a single strand fragment of the DNA molecule, the missing part of the strand is synthesised complementally by denovo synthesis (Karpfel 1983, Fig 5). UDS was first discovered by Rasmussen and Painter (1964), however it was Djordjevic and Tolmach (1967) who gave the phenomenon its name, as they found that unlike semiconservative DNA synthesis which occurs

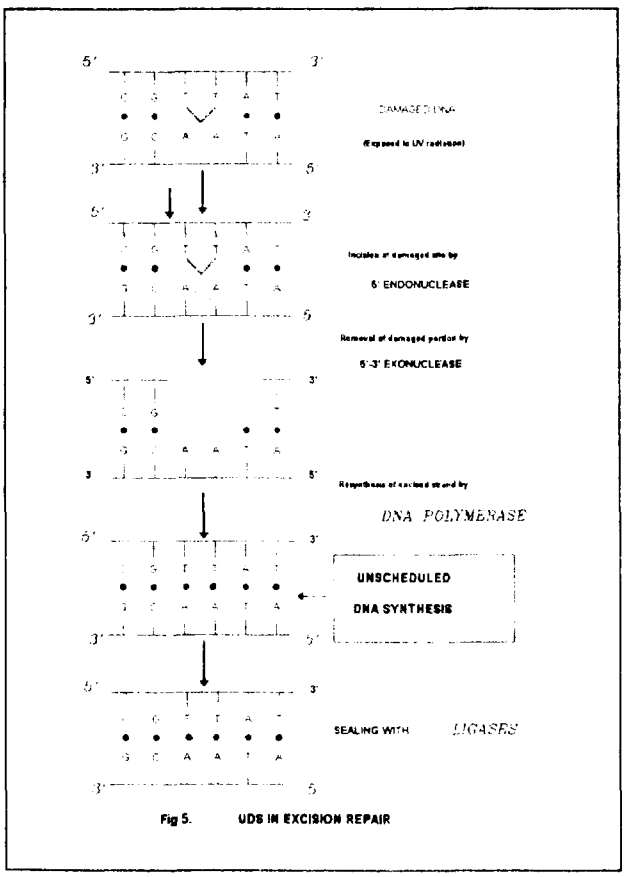

Fig 5. UDS IN EXCISION REPAIR

only in the S phase of the cell cycle, UDS occurred in all phases (i.e, G_1, G_2 and M phases as well).

Subsequently many others recorded their observations of UDS in different cell types such as liver (Galingani et al 1983) lung (Rasmussen et al 1981), gonads(Sega et al 1990, Mirsalis 1989), brain (Hollatz and Temple 1978) and fibroblast (Ichihashi and Ramsay 1976) of several organisms including rodents and humans. Insects are also known to exhibit UDS (Koval 1976). UDS is induced by UV radiation, high doses of gamma radiation (Cleaver and Thomas 1981) and chemical mutagens. The study of UDS has found application in understanding DNA excision repair mechanism and its kinetics (Spiegler and Norman 1969).

Nevertheless, the field in which UDS finds the most interesting application is in monitoring potential carcinogens and mutagens, since there appears to be a good correlation between the carcinogenicity of a chemical compound and its ability to trigger UDS in cultured mammalian cells(San & Stich 1982). Monitoring UDS, therefore provides an effective and easy way of identifying chemical carcinogens like pesticides and other chemicals (Mirsalis 1989) including cigarette smoke (Rasmussen et al 1981). The other important area in which UDS has brought insight and understanding is in the study of repair deficient diseases like Xeroderma Pigmentosum (XP), Ataxia Telangiectasia, Retinoblastoma (Cleaver 1977) and inflammatory rheumatic diseases. XP is the best studied disease among the above and is a striking illustration of the mutational theory of malignancy. It is caused by defective repair of UV induced damage in skin cells. (Cleaver 1977, Walker et al 1985). UDS experiments conducted on cells derived from XP patients have helped to determine the decisive step in the repair mechanism which is found

lacking in cells of persons affected by these diseases. UDS is also used in screening potential radiosensitising compounds (Voiculetz et al 1975)

8.1. UDS AND LOW-LEVEL RADIATION

The rate of UV radiation induced UDS in lymphocytes of persons living in an area with a high natural background radiation was found to be higher compared to that of persons living in normal levels of background radiation (Tuschl et al 1980). This enhanced repair capacity has been attributed to the prior very low exposure of higher levels of natural radiation. Similar finding has been observed by these authors in radiation workers as well (Tuschl et al 1983). It has also been shown that there exists a significant correlation between the relative amount of UV radiation induced UDS and the quantity of gamma radiation received by a radiation worker over a period of 1 to 3 months. Doses exhibiting this UDS enhancement, ranged between 0.14 and 0.98 mGy per month. (Tuschl et al 1983). The observations made by Tuschl et al have been confirmed by Liu et al who also observed similar enhancement in UDS levels in lymphocytes of persons living in an area with high natural background radiation levels in China (Liu et al 1985). Enhancement in the rate of UDS of the above type was observed by us in radiation workers one month after receiving gamma ray exposures in the range of 0.35 and 7 mGy (Mohankumar and Jeevanram 1990).

Liu et al (1987) observed that when mouse spleenocytes were irradiated with small doses (25 to 250 mGy) of X or gamma rays there occurred an increase in DNA repair capacity following UV irradiation. However, for doses above 500 mGy there was a dose dependent decrease in UDS levels. These authors attribute the enhanced repair capacity to radiation hormesis. Wojcik and Tuschl(1990) observed similar enhanced UDS effects in spleen lymphocytes of mice.

Several enzymes are thought to play a role in the induction of this phenomenon. Notable among them is the chromatin bound enzyme poly ADP-ribose polymerase (PARP) which is known to be produced in response to DNA strand breaks. The exact mechanism by which PARP facilitates DNA repair has not been clearly understood. However, it is postulated that the polymer plays a structural and/ or functional role in DNA repair process. With regard to the probable structural role, the enzyme can be rapidly poly ADP-ribosylated with as many as 15

branched polymer chains per protein molecule. Rapid synthesis of such a large polyanion provides a mechanism to loosen the tightly packed chromatin structure so as to facilitate access of repair enzymes to complete repair. Once the repair is complete the structure is restored (Fig 6). The importance of a chromatin modification is well established in relation to repair processes in XP patients who normally lack the capacity to repair DNA containing pyrimidine dimers. It has been found that cell extracts of some complementation groups of xeroderma pigmentosum can excise pyrimidine dimers from naked DNA but not from chromatin (Cleaver 1977). The probable role of PARP in UDS enhancement is indicated by an experiment in which significant increase in PAR levels was observed in lymphocytes of occupational workers (Klein et al 1985). Similar observations were made in lymphocytes of persons exposed to ^{222}Rn (Altmann 1982). These studies indicate that measurement of UDS in human lymphocytes can be utilised to monitor low level radiation exposures.

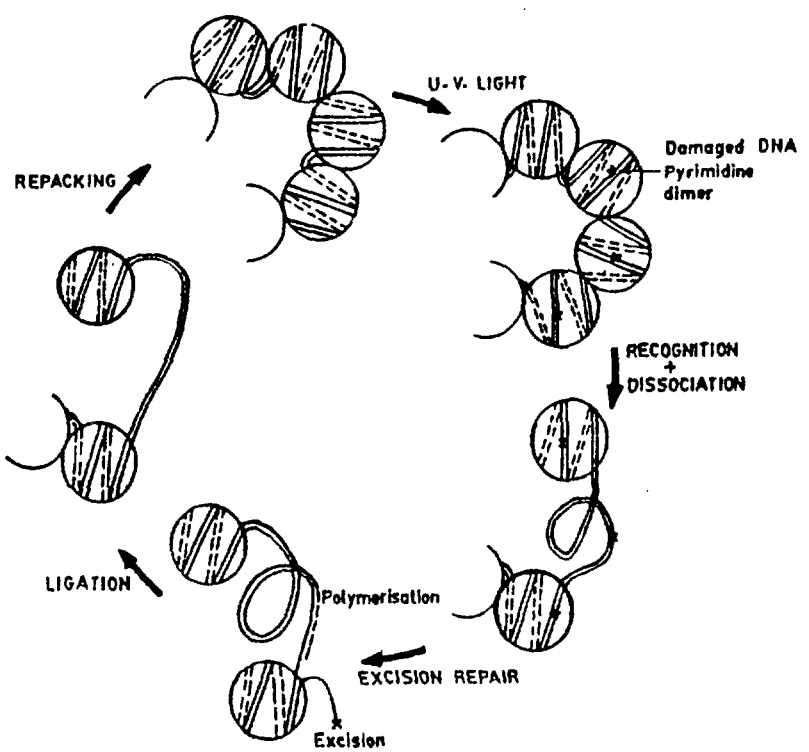

Fig.6. Chromatin modifications in excision repair
From J.E. Cleaver (1977)

8.2. MEASUREMENT OF UNSCHEDULED DNA SYNTHESIS

8.2.1. AUTORADIOGRAPHY

This is one of the original methods by which UDS was detected (Rasmussen and Painter 1964). In this method, cells are exposed to the UDS inducing agent (chemicals, UV or high levels of gamma radiation) and immediately labelled with high specific activity tritiated thymidine (^{3}HTdR) in a suitable medium and incubated at 37°C. Repair begins immediately after DNA damage by UV radiation and the labelled DNA precursor gets incorporated during this period. After a stipulated time (90-120 mins) the cells are fixed in methanol- acetic acid, rinsed in 70% ethanol and are air-dried. Slides prepared thus are coated with a photographic emulsion and are kept in dark at 4°C for about two weeks. Slides are then carefully developed with appropriate developing medium, stained with Giemsa and scanned under a light microscope. Grains indicative of the incorporated 3-HTdR, are counted on interphase cells. UDS is quantified by measuring excess grains in comparison with control.

8.2.2. LIQUID SCINTILLATION COUNTING

Since autoradiography is a laborious and time consuming procedure, liquid scintillation counting of incorporated tritium is preferred for screening large number of samples.

In this method, a known number of cells after tritium incorporation are directly transferred into liquid scintillation vials containing a suitable cocktail and then counted. However, unlike the autoradiographic method, cells undergoing UDS cannot be visually identified and hence artefacts like counts due to incorporation of tritium in cytoplasmic components cannot be discriminated from true incorporation in nuclear material (Maurer 1981). This problem can be overcome by hydrolysing the cells, isolating the DNA and estimating the DNA content. Results can then be expressed in counts / μg of DNA (Mirsalis et al 1989).

8.2.3. BND(BENZOYLATED NAPHTHOYLATED DEAE) CELLULOSE METHOD

Yet another method to measure repair replication is by BND cellulose method (Scudiero et al 1975). In this method repair synthesis in cells can be separated from semiconservative DNA synthesis using benzoylated naphthoylated DEAE cellulose (BND- cellulose) column (Fig 7).

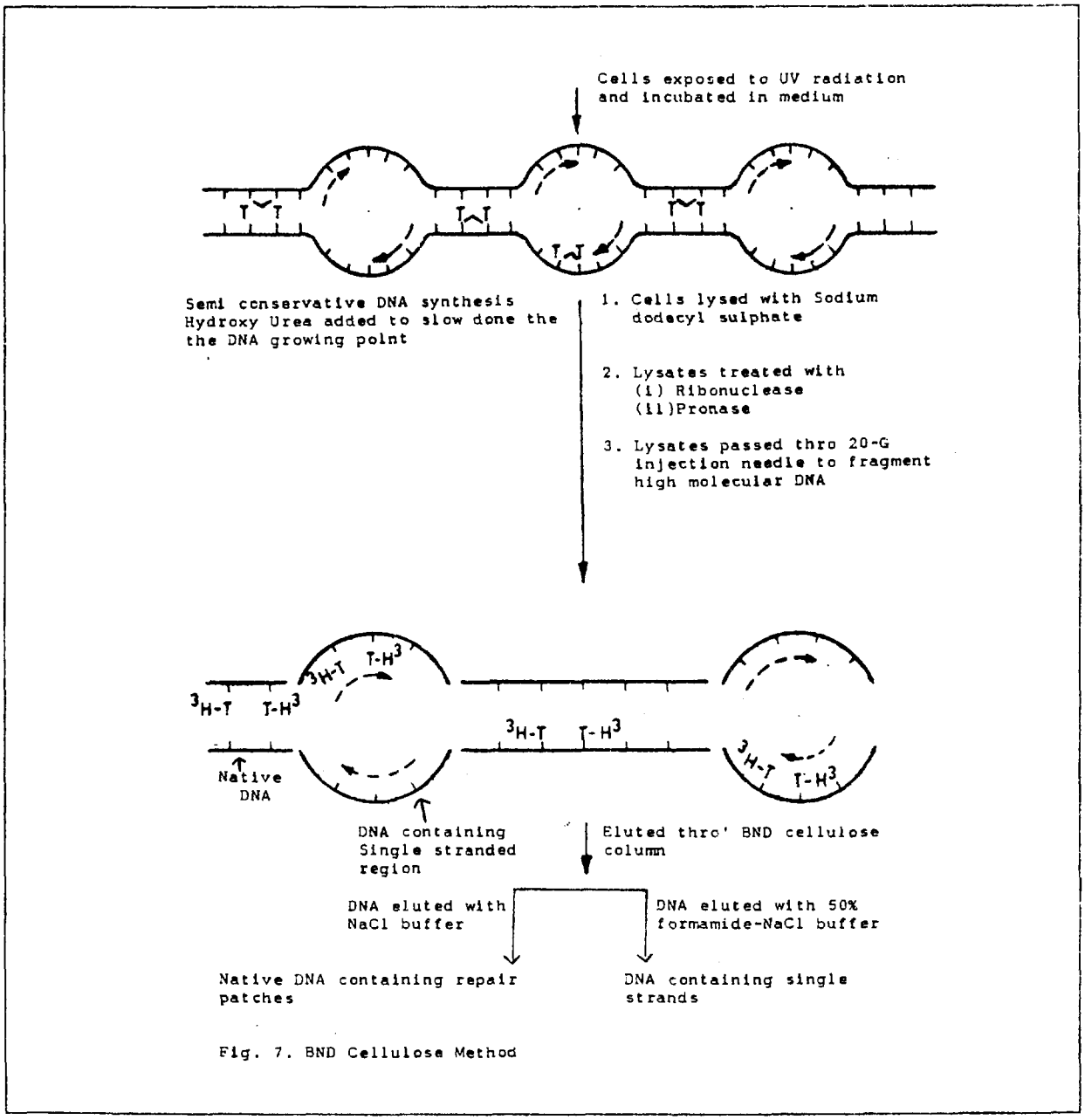

Fig. 7. BND Cellulose Method

Cells exposed to a clastogen like UV radiation are incubated with ^{3}H-TdR in the presence of hydroxyurea which helps to inhibit semiconservative DNA synthesis. The cells are washed and re-suspended in standard saline-citrate (SSC) and lysed with sodium dodecyl sulphate (SDS). Lysates are treated with RNase for 1h and pronase for 2 h at 37°C in order to remove RNA and proteins. After fragmentation of high molecular DNA by pressing the DNA solution through a 20 G-injection needle, lysates are layered on BND cellulose columns.

Native DNA is eluted with NaCl buffer and DNA containing single-stranded regions eluted with 50% formamide-NaCl buffer.

Any increase in radioactivity in the native DNA is due to repair synthesis. The specific repair activity (nucleotides inserted per μg of DNA) can be determined from radioactivity and absorbency measurements.

8.2.4. EQUILIBRIUM SEDIMENTATION TECHNIQUE

This technique (Smith et al 1981) has certain advantages over the earlier methods in that, UDS can be measured in replicating cells as well. This is due to the fact that in this method the DNA synthesis due to repair replication can be resolved from that due to semiconservative DNA synthesis. Repair can hence be measured without the addition of agents like hydroxyurea needed to suppress semiconservative DNA synthesis which are also known to interfere with repair synthesis. Moreover using this method one can also measure the size of the repair patches. The method in brief is as follows.

Mammalian cells are grown in medium containing ^{32}P orthophosphate or ^{14}C-thymidine for 2 or 3 generations. Cells grown thus are then depleted of the medium and washed in phosphate buffered saline at 37°C. Cells are next treated with the test agent like UV radiation or reactive chemicals, followed by incubation in medium containing ^{3}H 5-BrdU. After a desired incubation time, cells are harvested, lysed with sodium dodecyl sulphate(SDS) in presence of proteinase K to digest proteins. The molecular weight of the double stranded DNA in the digests is reduced to about 10^7 by two passages through a 25 gauge needle. The digest is then made to a density of 1.72 g/ml with CsCl, centrifuged to equilibrium. The heavier DNA fragments (hybrid density) due to the presence of large amounts of BrdU incorporated during semiconservative DNA synthesis settle at the bottom of the density gradient tube while the normal density fraction remains at the top. A minute hole is made at the bottom of the tube and various fractions are collected and are assayed for both ^{3}H and ^{32}P or ^{14}C radioactivity (Fig 8).

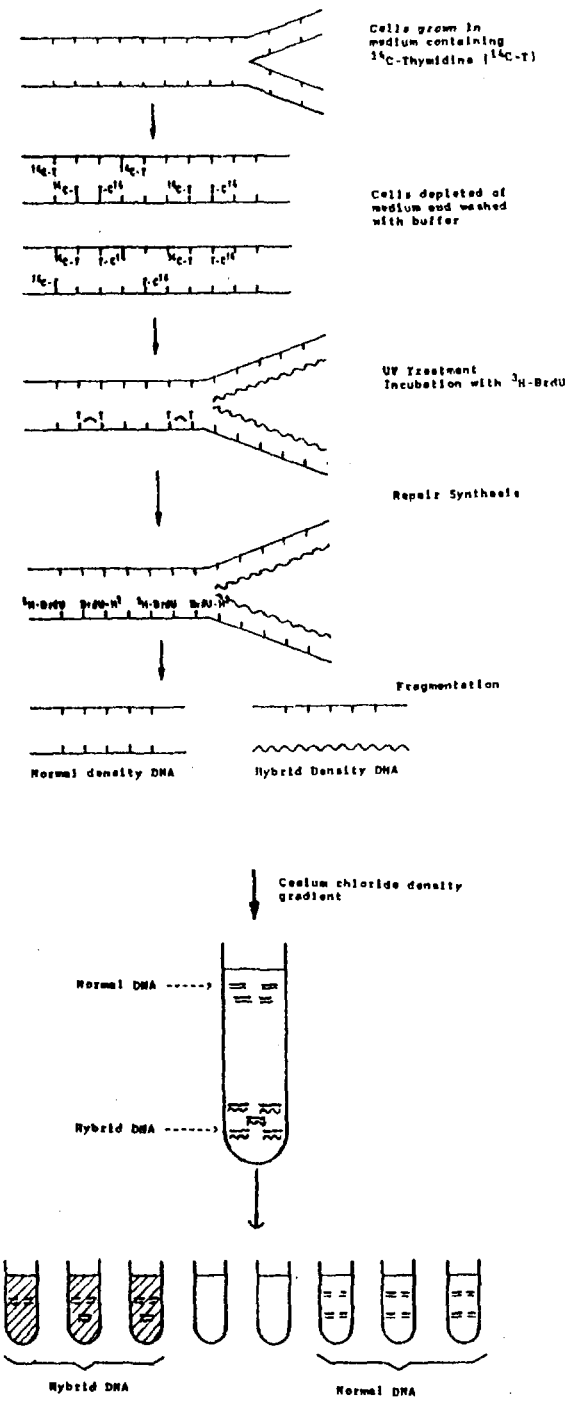

FIG.8. EQUILIBRIUM SEDIMENTATION TECHNIQUE

In most experiments with mammalian cells, good separation of normal density DNA and hybrid density DNA is achieved. The fractions containing unreplicated parental density DNA (normal density), are rebanded in a second CsCl equilibrium density gradient to resolve repair synthesised DNA from semiconservatively replicated DNA. Fractions are collected and assayed as before.

The ratio of ^{3}H to ^{32}P or ^{14}C in the DNA of parental density in the second gradient provides a measure of repair. If the specific activity of the prelabelled DNA is determined, the amount of label incorporated due to repair, per unit DNA can be calculated.

8.2.5. FLUORESCENCE TECHNIQUE

Recent studies (Beisker et al 1988) have shown that UDS can be easily quantified by flow cytometry after immunochemical staining of incorporated bromo-deoxyuridine (BrdU).

In this method, quiescent cells are treated with a specified amount of 5-BrdU for a brief period in order to cause equilibration of BrdU with intracellular nucleotide pools. The cells are rinsed in PBS and are UV irradiated followed by suspension in medium containing BrdU and incubation at 37°C for stipulated periods. Cells are then harvested, fixed in ethanol, treated with RNase to remove cellular RNA, and with 0.1M HCl and 0.5% Triton to remove histones. Since the available antibodies against BrdU are only able to detect BrdU in single stranded DNA, the cellular DNA is partially denatured prior to antibody incubation. This is accomplished by heating the cell suspension in distilled water for 10 mins at 95°C. This provides a careful balance to cause enough DNA to become single stranded for immunological reactivity and yet allow sufficient DNA for efficient staining of total DNA.

Anti-BrdU antibody is added to the dehistonised and denatured cells, followed by washings in PBT (PBS+0.5% Tween 20) and staining with FITC conjugated goat anti-mouse IgG. Cells are then washed and counter stained with 10 μg/ml propidium iodide for staining total DNA (Fig 9). Cells that have incorporated BrdU show green fluorescence while the rest appear red. The mean green fluorescence of G_o/G_1 cells due to incorporated BrdU is a measure of UDS which can be quantified by flowcytometry.

It has been shown that significant indication of BrdU uptake during UDS could be obtained after doses of UV light as low as 0.1 to 0.2 J/m^2.

9.0. SISTER CHROMATID EXCHANGE

When cells are irradiated with UV radiation or treated with chemical mutagens, damages are produced in the DNA; if these damages are not repaired before the cell progresses into the S phase, then sister chromatid exchanges (SCEs) appear at metaphase. SCEs are formed due to symmetrical interchange at one locus between sister chromatids on a chromosome and thus do not result in any alteration in chromosome morphology.

Using the technique of autoradiography Taylor et al (1957) first demonstrated the occurrence of SCE in plant somatic cell chromosomes observed at the M2 mitosis following a

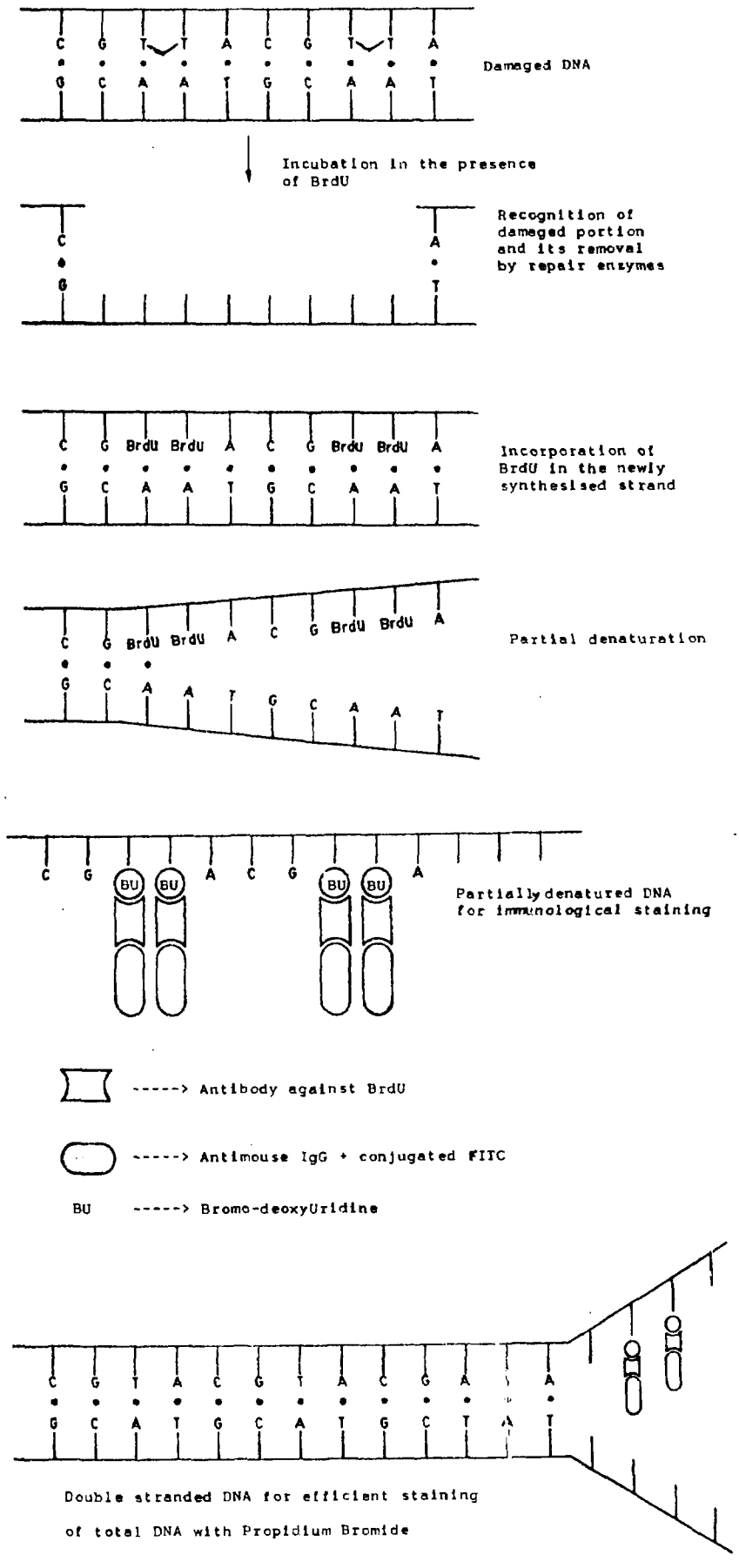

Fig.9. Unscheduled DNA Synthesis using 5-Bromo-2'-deoxyuridine (BrdU) and flow cytometry.

pulse exposure to ^{3}H Thymidine during the S phase of the M1 cycle. In recent years the study of SCE has become an extremely useful method for measuring the effects of DNA damaging agents (NTIS 1978 -1989). This is mainly due the fact that it has now become possible to differentially stain the sister chromatids by simpler non-isotopic labelling techniques. The fluorescence plus Giemsa technique (Perry and Wolff 1974) utilises the fact that chemicals like 5-bromodeoxyuridine which is a chemical analogue of thymidine, when made available in the culture medium are incorporated by the dividing cells to produce chromosomes containing chromatids that can be stained differently from each other (Fig 10). This differential staining can be accomplished if the preparations are stained with fluorescent dyes like Hoechst 33258 followed by exposure to Giemsa.

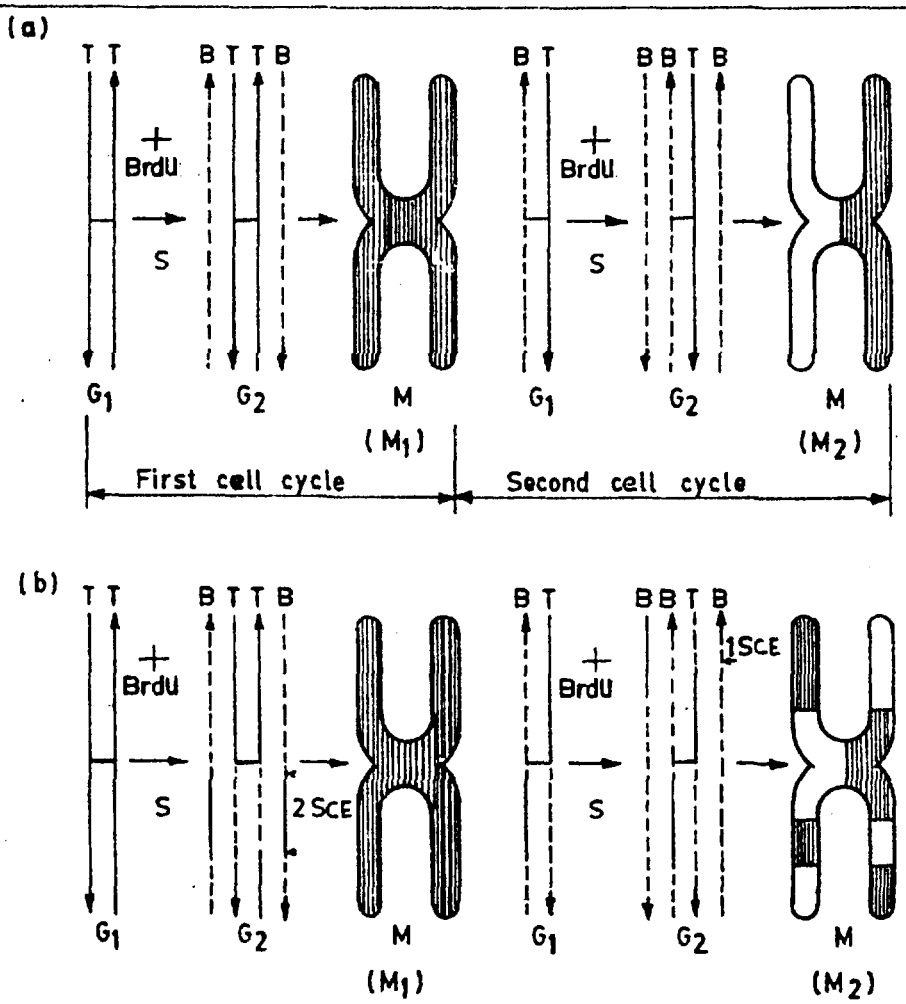

Fig.10. Metaphase Chromosomes from cells cultured in the presence of BrdU for two cell cycles. Dashed lines represent BrdU incorporated DNA Strand showing the staining patterns in which BrdU reduces the intensity.

a) Normal Chromosome without SCE

b) Chromosome with SCE

From T. Ikushima (1989 b)

The ease of preparation and scoring, coupled with the fact that for many DNA damaging agents, SCEs are produced by concentrations of chemicals far lower than those necessary to bring about chromosome aberrations, has made this technique widely favoured for determining cytogenetic effects of DNA damaging agents and environmental mutagens. Comparison of SCE results with UDS data generally helps to confirm the mutagenic and carcinogenic property of the test compound.

The molecular basis of SCE formation as well as the biological significance of the exchanges are not completely understood. SCEs occur during the process of semiconservative DNA synthesis, perhaps at the growing point of the replication fork (Fig 11)

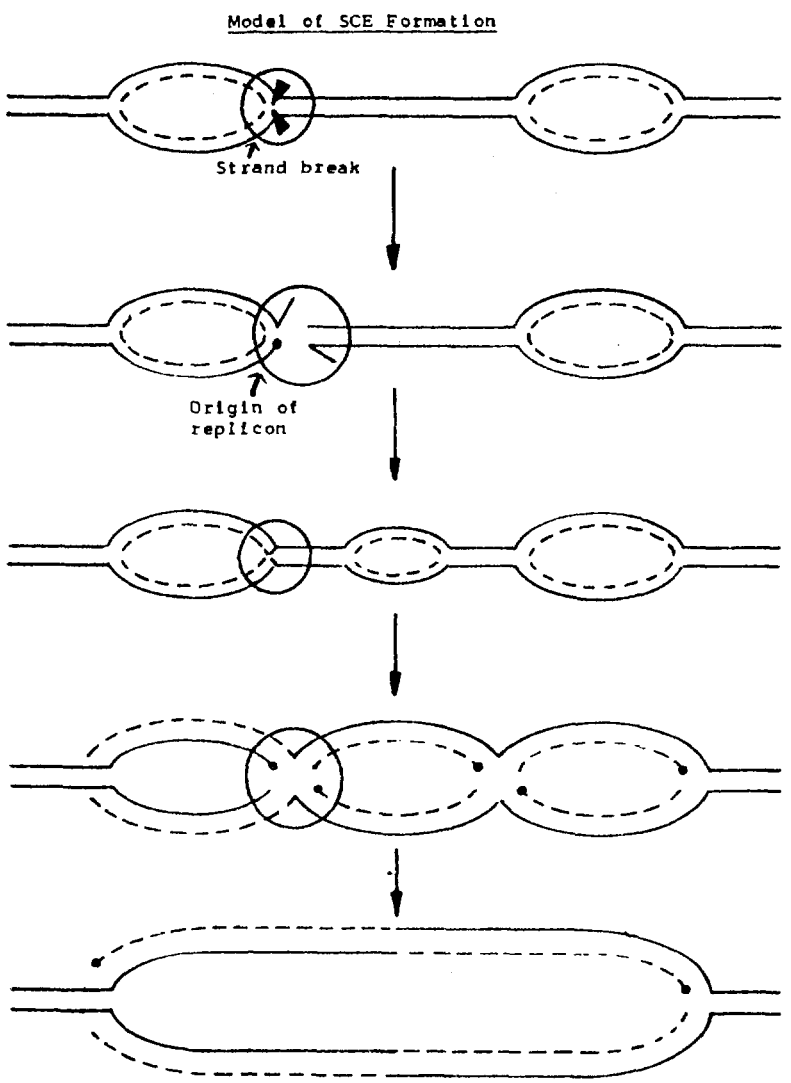

Fig.11. SCE formation at junction between contiguous replicons with different temporal order of DNA replication. Broken lines indicate newly synthesised strands and solid lines parental strands of DNA

From T. Ikushima (1989 b)

9.1 SCE AND LOW-LEVELS OF IONISING RADIATION

While the induction of SCEs by very low concentrations of mutagenic chemicals is well established, the role of ionising radiation in producing a similar effect remains ambiguous and unresolved. Direct measurement of SCE frequency caused by X or gamma radiation has yielded contradictory observations, although alpha particles and neutrons have been proved to be positive inducers of SCEs (Table 3).

The finding that prior exposure to low levels of ionising radiation results in a decrease in mitomycin C (MMC) induced SCE frequency, has been reported by many authors. This phenomenon is a typical example of radiation induced cytogenetic adaptive response (section 10). Tuschl et al (1983) have reported significant decrease in MMC induced SCE frequency among occupationally exposed personnel and in persons exposed to about 2 mSv during the Chernobyl accident (Tuschl and Kovac 1990). Experiments performed with mice exposed to whole body exposures at the rate of 0.05 Gy/ day also reveal similar reduction in SCEs (Wojcik & Tuschl 1990). However such an effect has not been observed in lymphocytes of the atomic bomb survivors. SCE studies carried out on the atomic bomb survivors decades after the initial exposure is unlikely to evoke the response due to a fading effect. Such a fading effect has been reported by Tuschl et al while studying enhanced UDS effects on occupational workers (Tuschl et al 1983).

Irrespective of the molecular mechanism underlying the above observations, the estimation of MMC induced SCEs appears to be a sensitive test to demonstrate population exposures to low doses of ionising radiation.

9.2. MEASUREMENT OF SISTER CHROMATID EXCHANGE

9.2.1. AUTORADIOGRAPHY

In this method cells are mitogenically stimulated to divide in culture medium in the presence of tritiated thymidine for one cell cycle. The second cell cycle is cultured in the absence of the radioactive probe. Due to the semiconservative replication of the DNA double helix and the semiconservative distribution of the label into the daughter chromatids, this treatment results in the formation of chromosomes containing one chromatid having the radioactive label and a

Table 3. SISTER CHROMATID EXCHANGES DUE TO IONISING RADIATION					
S.No.	Subject	Radiation Dose	Occurrence of SCE	Other details	Reference
1	Lymphocytes of Atomic Bomb Survivors	Neutron and gamma	No increase in SCE frequency		Pant et al
2	Lymphocytes of Atomic Bomb Survivors	Neutron and gamma	No increase in MMC induced SCE frequency		Nakano & Awa 1980
3	Rat bone marrow cells	0.44 Gy gamma rays	Significant increase in SCE frequency	100% increase than control	Rodriguez 1981
4	Human Lymphocytes	0.14 - 0.98 mGy/month gamma dose	Significant decrease in MMC induced SCE frequency	Occupational workers	Tuschl et al 1983
5a	Human Lymphocytes	0.4 mGy/ month	Significant increase	Occupational workers	Gundy et al 1984
5b	Human Lymphocytes	0 - 5 Gy gamma rays	Significant increase	Invivo exposure. Increase upto 0.25 Gy	Gundy et al 1984
6	Lymphocytes of high background group	3.3 mGy/yr	Significant increase		Li Jinsheng 1985
7a	Human Lymphocytes	Alpha rays (0.3 - 0.7 Gy)	Significant increase	Pu-238, invitro exposure	Aghamohammadi et al 1988
7b	Human Lymphocytes	X-rays 3.12 Gy	No significant increase	Invitro exposure	Aghamohammadi et al 1988
8	Human Lymphocytes	42 Mev neutrons	Significant increase	Dose dependent	Savage et al 1988
9	Human Lymphocytes	0 - 2 Gy alpha rays	Significant increase	Response only in G_1 state	Moquet et al 1989
10	Human Lymphocytes	X rays (0.08 - 4 2 mSv)	Increase in SCE frequency	Occupational workers	Tsoneva et al 1989
11	C57BL mice spleenocytes	0.05 Gy/day for 4 days, gamma rays	Significant decrease in MMC induced SCE frequency	Invivo exposure	Wojcik & Tuschl 1990
12	Human Lymphocytes	2 mSv	Significant decrease in MMC induced SCE frequency	Exposure 150 Km from Chernobyl	Tuschl & Kovac 1990

sister chromatid with no labelled DNA. Thus the two sister chromatids are physically different. Cells thus treated and processed by autoradiography are visualised by the presence of silver grains above the radioactive chromatid. Exchanges that have taken place are apparent as switches of label from one chromatid to the other.

Autoradiography however has proved to be a laborious and time consuming process and the advent of chemical analogues of thymidine coupled with fluorescent dyes have made the above method obsolete.

9.2.2 FLUORESCENCE PLUS GIEMSA TECHNIQUE

In this method about 0.5 ml whole blood is cultured in a suitable medium in the presence of PHA and 10μM BrdU. The culture vials are incubated for 70 hours after which 0.1 μg colcemid is added and incubation continued for two more hours. Good yields of second division metaphases are generally obtained after about 72 hours of total culture time. Agents to be tested for potential SCE induction are either added at the start of the culture or 24 hours before harvest. Care is taken to observe that all manipulations of cells after addition of BrdU to the cultures are done in subdued light, since light causes disintegration of BrdU.

At the end of 72 hours leukocytes are harvested and subjected to hypotonic treatment. RBCs are then lysed by washings in 3:1 methanol acetic acid mixture Cells are resuspended in fresh fixative and are cast on clean dry slides and air dried.

Cast slides are then dipped successively in PBS, 0.5 μg/ml Hoechst 33258 in PBS and water (2-3 washings). After this step the differentially stained chromatid can be observed for its fluorescence under a fluorescence microscope. However this fluorescence fades rapidly and hence it is further stained with Giemsa for permanent records. For this purpose, the Hoechst stained and washed slides are dipped in McIlvaines buffer and mounted with a cover slip and exposed to sunlight for 4 to 5 hours or to a 20 watt cool white bulb for 6 to 12 hours. This treatment causes the excitation of Hoechst fluorescence and promotes selective destruction of the chromatid which has been extensively substituted with BrdU. This is followed by incubating the slide in 2X SSC at 65°C for 15 to 30 minutes followed by staining with 4% Giemsa in Sorensons buffer. Slides are washed, dried and mounted with DPX. Slides prepared thus can be viewed by light microscopy and chromatids are visible as pale and darkly stained. Any exchanges that have taken place due to the effect of the clastogen can then be easily detected and scored.

10. ADAPTIVE RESPONSE TO IONISING RADIATION

It was observed that pre-exposures of human lymphocytes to low levels of ionising radiation (5 - 20 mGy) either from incorporated radioisotopes like tritium (Olivieri et al 1984) or from X-rays (Shadley et al 1987) could decrease the chromosome damage caused by a subsequent larger challenge dose of radiation. This phenomenon is considered as an adaptive response of human lymphocytes to low-dose radiation also known as Radio-Adaptive Response (RAR) (Szumiel 1990 and Wojcik & Streffer 1994).

Adaptive response to ionising radiation has been observed in cells of various species. Prospective end points observed were, decrease in chromatid aberrations, SCEs and mutation frequency (Table 4).

Table 4. ADAPTIVE RESPONSE

S.No	Species	Adapting dose	Challenge dose	Effect	Reference
1	Human Lymphocytes	>0.14 - 0.98 mGy/month (invivo exposure)	Mitomycin C	Reduction in SCE frequency	Tuschl et al 1983
2	Human Lymphocytes	Low dose of tritiated thymidine	1.5 Gy X-rays	Reduction in chromatid breaks	Olivieri et al 1984
3	Human Lymphocytes	0.01 - 0 05 Gy X-rays	1.5 Gy X -rays	25 - 50 % reduction in chromatid breaks	Shadley et al 1987
4	Chinese hamster cells	10 - 50 mGy gamma rays	1 Gy gamma rays	25% reduction in micronuclei frequency	Ikushima 1989
5	C57BL/6 mice bone marrow cells	20 mGy gamma rays	750 mGy gamma rays	Reduction in number of chromatid breaks	Cai & Liu 1990
6	Human Lymphocytes	30 mGy X-rays	3 Gy gamma rays	50 % reduction in chromatid breaks	Khandogina et al 1991
7	Human Lymphocytes	10 mGy x-rays	3 Gy x-rays	reduction in HPRT mutations	Kelsey et al 1991
8	Fish cell lines	19 -190 mGy X-rays	1.9 Gy X-rays	12 -45% reduction in chromatid breaks	Kurihara et al 1992

This adaptive response to ionising radiation which can occur after exposures that are so low that they do not themselves cause any aberrations, has been attributed to the induction of a

repair mechanism that causes the restitution of chromosome breaks caused by the challenge dose (Wolff et al 1988).

Five main parameters are known to play a decisive role in RAR, they are,

1. the initial or priming dose
2. the interval between the initial and the challenging doses
3. the challenging dose.
4. the phase of the cell cycle and
5. the variability of the human population.

It appears that the adaptive response is dependent on the dose and the dose rate of the initial exposure. If doses are administered acutely, the effect could not be induced by doses less than 5 mGy or greater than 200 mGy (Wolff 1992). Also doses delivered at the rate of 50 - 200 mGy/min could induce RAR, while slower dose rates did not. For mammalian cells irradiated invitro RAR has been found to be induced by 10 - 100 mGy of ionising radiation. However, bone marrow cells of mice given whole body irradiation with doses ranging between 2 and 50 mGy followed by a challenge dose of 650 mGy showed a significant decrease in chromosomal aberration (Liu et al 1990) suggesting, that for invivo exposures AR is expressed for much lower priming doses. On the whole it appears that there exists a window of doses for the phenomenon to occur.

It has been experimentally verified that a time gap of about 4 to 6 hours between the initial and challenge dose is required for the full expression of RAR (Shadley et al 1987) and this expression has been found to decay with the progression of the cell cycle exhibiting the effect for about three cycles after which the effect ceases. However, the effect can be reintroduced by re-administering the priming dose.

As for the challenging dose RAR is best seen for X and gamma doses of the order of 1 Gy. Chemicals such as mitomycin C are also known to cause RAR.

Most researchers have observed RAR to occur only in cycling cells in their G_1, S and G_2 stages (Shadley et al 1987, Moquet 1989). However few of them have reported RAR in cells exposed in their resting phase (G_o stage) as well (Liu 1989, Khandogina et al 1991).

The variability of the human population in the expression of adaptive response is another important factor to be considered. Bosi and Olivieri(1899) found that 4 out of 18 donors failed

to exhibit any RAR and in some cases the adapting and the challenging doses interacted synergistically. In another experiment these authors observed that lymphocytes of some individuals previously not exhibiting RAR, showed the response at a later time. Similarly some strains of mice failed to exhibit the response (Wojcik et al 1992). Further, growth factors and even the pH of the culture medium could affect RAR (Bosi et al, 1991).

RAR seems to be different from that due to alkylating agents (chemical adaptive response) and is thought to occur via an unknown inducible DNA repair mechanism (Ikushima 1989). While AR due to chemicals is evoked by the induction of DNA glycosylases and alkyl transferases, the activation of genes coding for some enzymes like ligase, DNA glycosylase, topoisomerases and poly(ADP-ribose) polymerase are thought to be responsible for RAR. However the phenomenon at present, is explained by the hypothesis that the priming dose induces certain enzymes which are responsible in efficiently repairing the large number of DNA breaks caused by subsequent high doses thus resulting in less total damage being left in the cell. This hypothesis is supported by the observation that RAR could be inhibited by 3-aminobenzamide, an inhibitor of poly (ADP-ribose) polymerase and also by cycloheximide, a protein synthesis inhibitor. Poly (ADP- ribose) polymerase is known to be produced in response to the production of strand breaks and is thought to be involved in the repair of chromosomal breaks. The involvement of PARP in both RAR and UDS effects indicates that the enhanced UDS may be due to adaptive response initiated by low levels of radiation. However the identification of inducible proteins and the isolation of the genes coding for the proteins are necessary for the full understanding of the process of adaptation to radiation damage as one of the protective systems for a variety of environmental stress. Such studies may provide basic information for the elucidation of the mechanism of radiation hormesis.

11. CONCLUSION

The various methods and the mechanisms involved in the measurement of end-points useful in monitoring low-levels of radiation in biological samples have been discussed in this report. The quantum of work carried out in this field, to-date, is substantial. Yet further developments in the above techniques will help us to understand better the biological effects of low level ionising

radiation. Quantification of these effects for dosimetry purposes will also be a challenging venture.

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