To the Graduate Council:

I am submitting herewith a dissertation written by Marilyn Elizabeth Johnson Aardema entitled "A Study of the Mechanisms Involved in the Increased Sensitivity of Mouse Myeloid Leukemic Cells to the Induction of Chromosome Aberrations." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

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A Study of the Mechanisms Involved in the Increased Sensitivity of Mouse Myeloid Leukemic Cells to the Induction of Chromosome Aberrations

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Marilyn Elizabeth Johnson Aardema

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DEDICATION

TO MY PARENTS HERBERT AND NITA

TO MY HUSBAND CHUCK

TO MY DAUGHTER LAUREN

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ABSTRACT

X ray-induced chromosome aberrations were analyzed in *in vitro* normal mouse RFM bone marrow cells and in an RFM mouse myeloid leukemia cell line, ML-1. Chromosome aberrations were analyzed in cells that were irradiated in G_1 of the cell cycle. Dicentrics and terminal deletions were induced in greater frequencies in ML-1 cells than in normal cells, and chromatid-type aberrations were induced only in ML-1 cells.

The induction of chromatid-type aberrations in ML-1 and not in normal cells suggested: 1) a difference between the normal and ML-1 cells in cell cycle progression after irradiation and/or 2) a difference between the cells in the repair of X ray-induced DNA damage. Flow cytometric analyses showed that cells of both cell types in G_2 at the time of irradiation were similarly delayed in progression into mitosis. However, normal cells in G_1 were delayed in progression into and through the S phase while the ML-1 cells progressed without delay into the S phase. The X ray-induced delay in G_1 normal cells may serve as a protective mechanism allowing extra time for DNA repair before DNA replication in the S phase. It was concluded that the lack of a cell cycle delay after irradiation in G_1 ML-1 cells allowed cells to progress into the S phase with unrepaired DNA damage where a misrepair event results in the formation of chromatid-type aberrations.

These results indicated that the ML-1 cells had possible repair deficiencies resulting in X ray-induced DNA damage which persisted until the cells entered the S phase. Experiments utilizing the excision repair inhibitor cytosine arabi-

noside (ara C) showed that the ML-1 cells had a slower rate of excision repair resynthesis than the normal cells.

In order to determine if the differential sensitivity between ML-1 and normal cells was present for cells in other cell cycle phases, aberrations were analyzed in cells that were treated in G_2 with X rays and ara C. ML-1 cells had a higher frequency of aberrations induced by X rays, and ara C, and an increased sensitivity to a combined ara C and X ray treatment compared to normal cells. This suggests that the DNA in ML-1 cells has fragile sites. These results provide an explanation of the mechanisms leading to the increased sensitivity to chromosome aberration formation in the ML-1 cells.

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CHAPTER 1

INTRODUCTION

Exposure to various chemicals and radiations can induce genetic damage. Alteration of the genetic material, DNA, can potentially have adverse genetic effects including mutations, chromosome aberrations, toxicity or cellular transformation. There is an increasing amount of evidence to indicate that these effects lead to other genetic hazards of great biological significance such as birth defects, genetic diseases, and cancer. Because DNA damage is so deleterious to the cell, many cellular mechanisms have evolved to deal with it, the most notable being the many DNA repair processes. These protective mechanisms greatly influence the consequences of DNA damage and variations in the capacity to perform these mechanisms can influence susceptibility to genetic damage by a particular agent.

It is apparent that there are individuals who have different sensitivities to DNA damage induced by the same agent. Cells from ataxia telangiectasia (AT), Bloom syndrome (BS), and Fanconi anemia (FA) patients, compared to normal individuals, have a higher rate of spontaneous as well as induced chromosome aberrations. Cells from xeroderma pigmentosum (XP), some forms of retinoblastoma (RB) and Down syndrome patients have a higher rate of induced chromosome aberrations (for a review, see German, 1972). In addition to having chromosome instability, these patients have a predisposition to develop various neoplasms (for a review, see German 1983). Thus, one of the factors influencing the differential sensitivity of individuals to the induction of

cancer may be genetic instability. While these syndromes are probably examples of extremes in differential sensitivity, they spark an interest in the relationship between the increased sensitivity to chromosome aberration formation and the induction of cancer as well as other biological endpoints.

Identification of other populations of cells which have differences in sensitivity to an agent allows for the further investigation into mechanisms leading to differential sensitivity. Chapter 2 of this thesis describes the experiments to determine the differential sensitivity of a mouse myeloid leukemia cell line (ML-1) and normal mouse bone marrow cells to X ray-induced chromosome aberrations. Chapter 3 describes experiments on some mechanisms involved in the differential sensitivity of cells to form chromosome aberrations. Chapter 4 describes the experiments to determine if the differential sensitivity observed between G_1 normal and ML-1 cells is found in G_2 cells and to determine the effect of ara C on X ray-induced aberrations in both cell types. The following portion of Chapter 1 presents information used in designing the experiments and interpretations that can be made from them.

Mouse Myeloid Leukemia ML-1 Cell Line

Cell lines established from various species are used extensively in scientific investigations and specific cell lines have been shown to be effective in answering many scientific inquiries. Classically, cells which differ in their ability to perform cellular functions, such as mutants or cell types with specific deficiencies, often provide the most useful information. The studies presented here on factors that influence sensitivity to chromosome aberration induction utilized a

mouse myeloid leukemia cell line designated ML-1. The ML-1 cell line was established from an X ray-induced leukemia in an RFM mouse (Upton et al., 1979) and has the characteristics described below.

The ML-1 cells have a stable diploid chromosome number of 40. Thirty-nine of the chromosomes are acrocentric and one chromosome is a metacentric (Figure 1.1). ML-1 cells, as analyzed by the trypsin-Giemsa banding (G-banding) technique were found to have the following consistent chromosomal alterations: deletion of chromosome 2 bands C-1 and F1-3, trisomy 19, no Y chromosome, and the metacentric chromosome is an isochromosome 8 which is probably the product of a Robertsonian translocation between the two homologs of chromosome 8 (Figure 1.2) (Au et al., 1983). The metacentric chromosome is recognized easily in cytological preparations and allows for the unequivocable identification of the cell as being an ML-1 cell. The ability to distinguish between normal and leukemic cells is especially useful for in vivo studies. Work by Au and Goldenthal (1985) has shown that an accurate analysis of SCE frequencies in coexisting normal bone marrow and leukemic cells can be made, and they found that the spontaneous SCE frequency of ML-1 cells in vivo did not differ from the spontaneous SCE frequency of coexisting normal bone marrow cells.

The deletion in chromosome 2, band C, found in the ML-1 cell line is similar to deletions of chromosome 2 observed by Azumi and Sachs (1977) in other mouse myeloid leukemia cells. Hayata et al.(1983) observed a deletion in chromosome 2 bands C and D in 49/52 cases of mouse myeloid leukemia. This is especially interesting since there is increasing evidence which indicates that certain cancers are associated with specific chromosomal alterations (for a

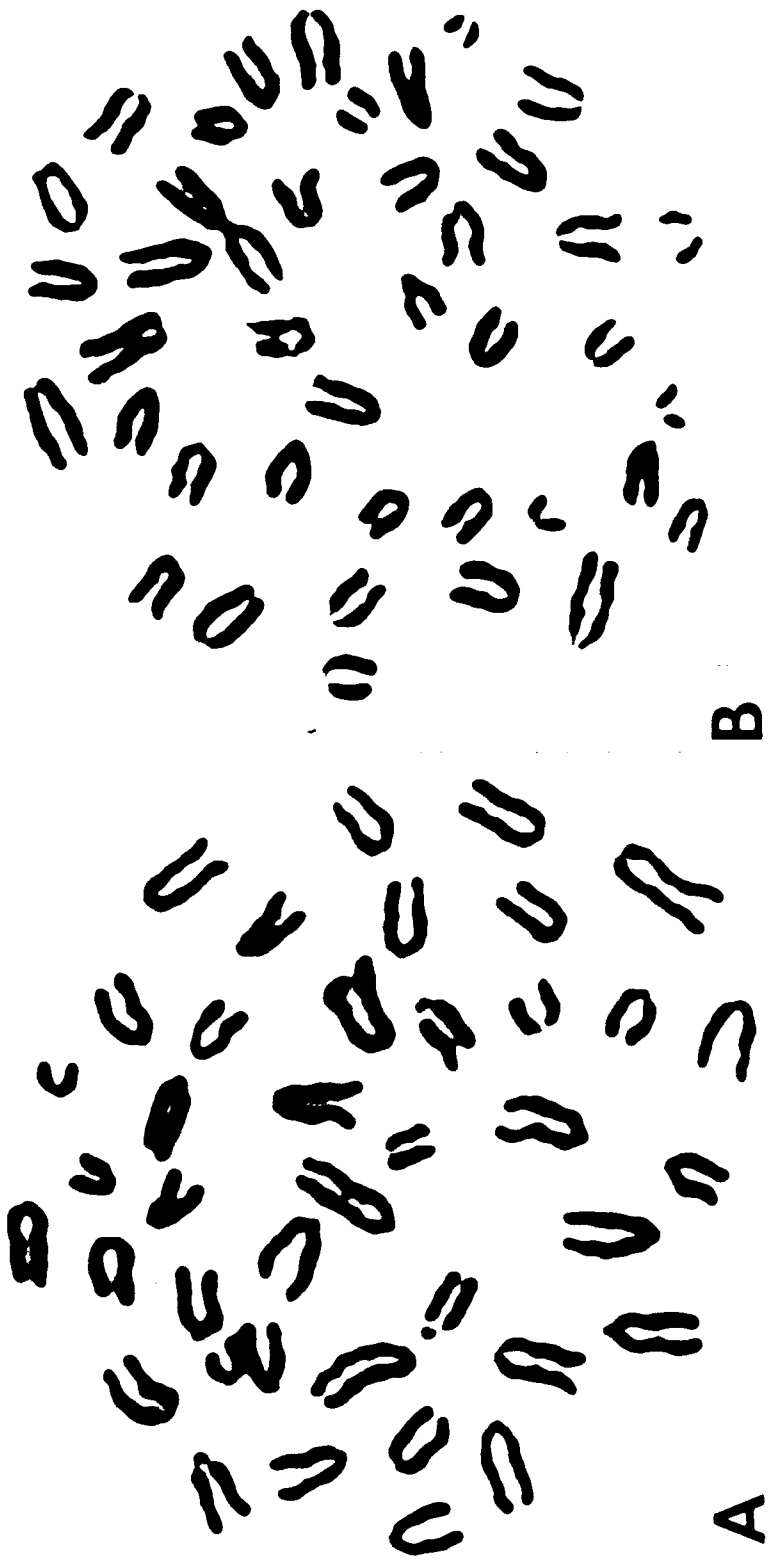

Figure 1.1. Normal Mouse Bone Marrow (A) and ML-1 (B) Metaphases.

Figure 1.2. G-banded karyotype of the ML-1 cell line. G-banded karyotype of the Ml-1 cell line showing the following abnormalities: deleted chromosome 2 bands C-1 and F1-3, trisomy 19, no Y chromosome, isochromosome 8.

review, see Klein, 1981). For example, mouse plasmacytoma has a specific 15;12 translocation which involves the *myc* oncogene (Taub et al., 1982) and all human Burkitt lymphomas studied to date have either an 8;14, 8;2, or 8;22 translocation also involving the myc gene (Dalla-Favera et al., 1982; Taub et al., 1982). The consistent association between deletions in chromosome 2 and mouse myeloid leukemia as described above suggests that this is another example of a cancer which involves a specific chromosomal alteration and that these deletions may be involved or are important in the development of mouse myeloid leukemia.

Cell Cycle

Chromosome aberrations are studied in cell populations that are cycling and the cell cycle stage at the time of treatment directly influences both the type and frequency of the production of chromosome aberrations. Howard and Pelc (1952) first described the cell cycle as consisting of four successive stages, G_1, S, G_2, and D. This division was based upon two major processes, DNA replication which defines the S phase and mitotic division which defines the D phase-now known as the M phase. The G_1 and G_2 periods are defined as the gap periods before and after DNA synthesis, respectively (Figure 1.3). Aberrations can be observed, using the light microscope, in metaphase cells where the chromosome is greatly condensed. Metaphase cells are accumulated in a population by the addition of the spindle poison colchicine or its synthetic derivative, colcemid.

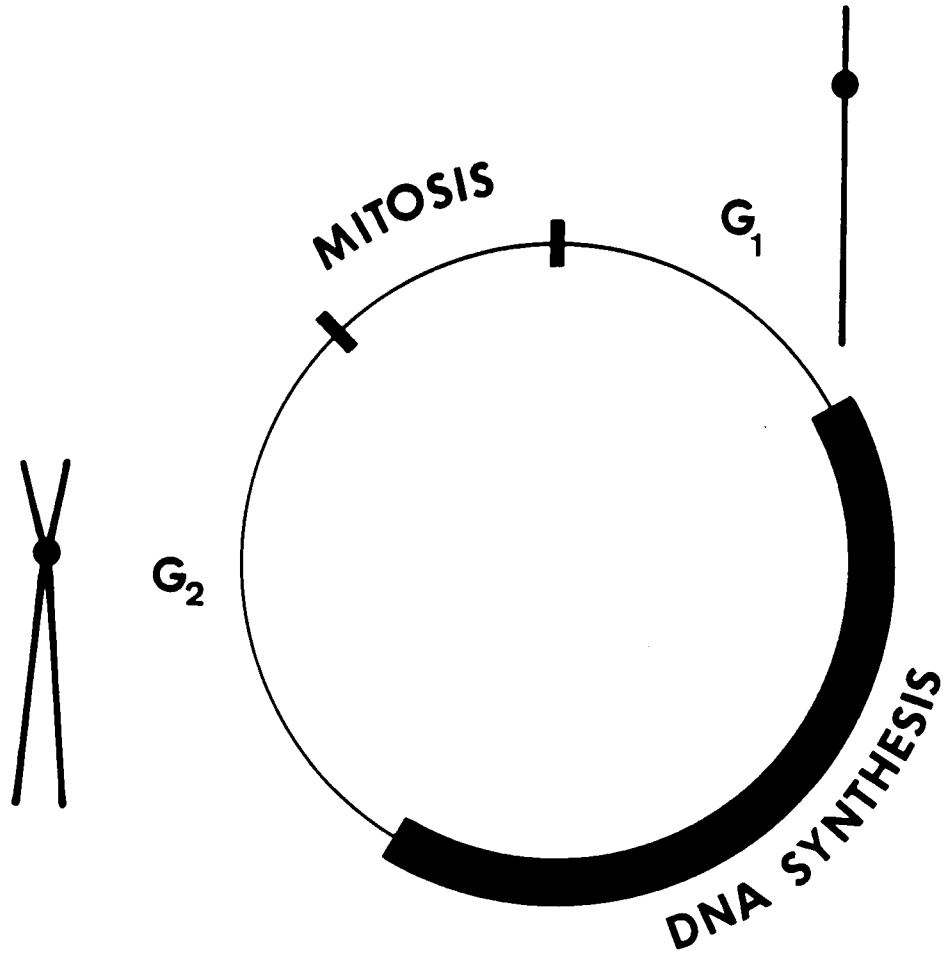

Figure 1.3. The Cell Cycle.

As mentioned above, one of the factors influencing the types of chromosome aberrations that are produced by an agent is the stage of the cell cycle that the cell is in at the time of exposure. In G_1, the chromosome consists of a single DNA double helix and cells treated in G_1 primarily produce chromosome-type aberrations; that is, both chromatids are involved in the aberration (Figures 1.4 and 1.5). After DNA replication, the chromosome consists of two sister chromatids each containing a single DNA double helix. Aberrations in cells treated in S or G_2 are primarily chromatid-type; that is, one chromatid of the chromosome is involved in the aberration (for a review, see Savage, 1975) (Figures 1.4 and 1.5).

The types of chromosome aberrations produced by an agent are also influenced by repair of the DNA damage. For instance, a DNA lesion induced in a G_1 chromosome which persists until DNA replication can result in a chromatid-type aberration. Thus, the interval of time in which DNA repair occurs between treatment and DNA synthesis can affect the types and frequencies of aberrations observed.

In order that an accurate estimate of the induced frequency of chromosome aberrations can be made, cells in their first mitosis after treatment must be analyzed. A determination of the cell cycle kinetics of a population of cells allows for the development of an experimental protocol to insure that first division metaphases are analyzed. This is accomplished by growing cells in the presence of the nucleoside analog 5-bromodeoxyuridine (BrdU) which is incorporated into replicating DNA in place of thymidine. Treatment of cells that have incorporated BrdU with the dye Hoechst 33258 black light, heat and

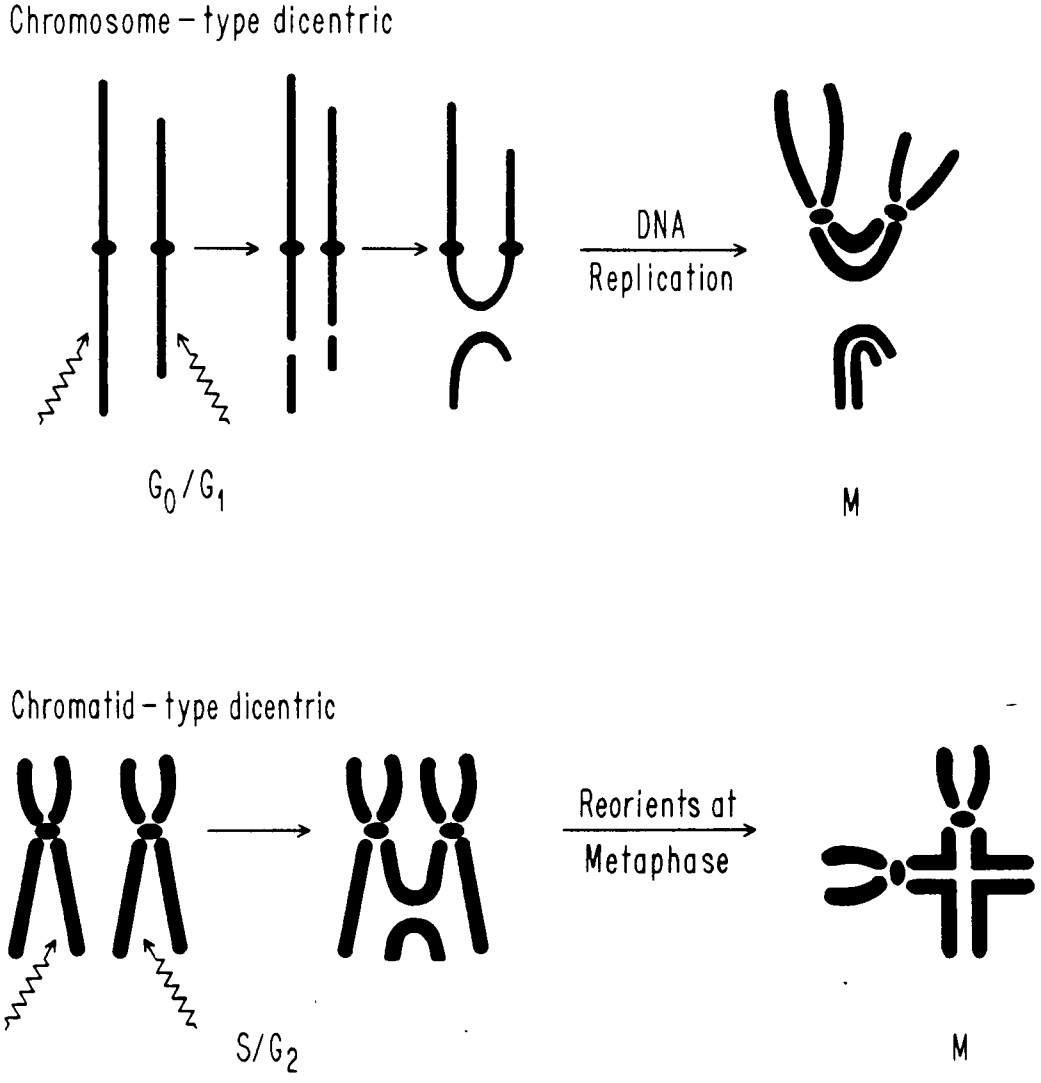

Figure 1.4. Schematic of Chromosome- and Chromosome-type Aberrations.

Figure 1.5. Examples of chromosome- and chromatid-type aberrations in ML-1 cells. C-banded metaphase ML-1 cells with A) no aberrations, B) chromatid-type exchange (arrow) and deletions (arrow head), C) chromosome-type dicentrics (arrow) and deletions (arrow head).

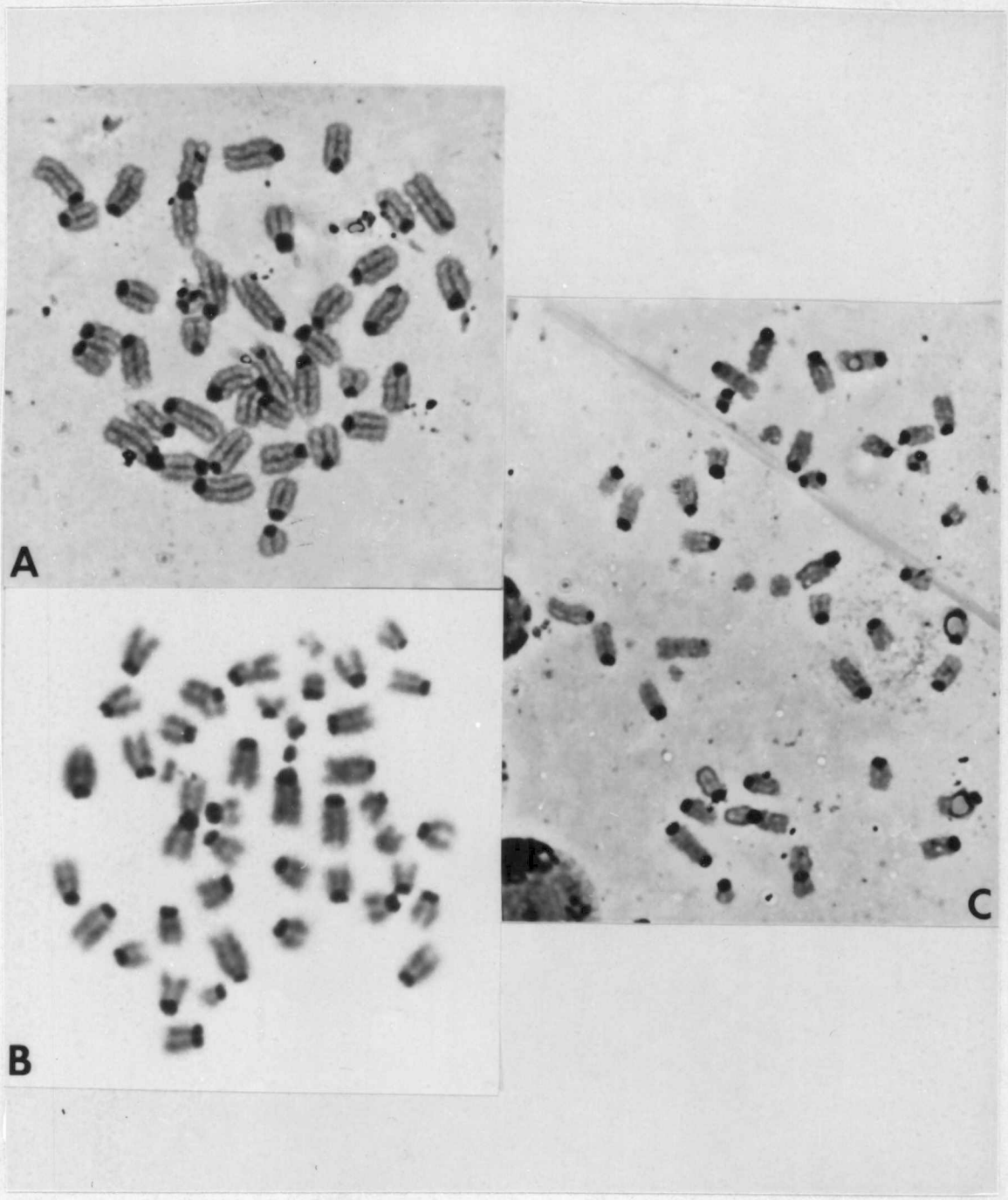

Figure 1.5

Giemsa stain [fluoresence-plus-Geimsa (FPG) technique, Goto et al., 1978] results in a differential staining of the chromatids. As shown in Figure 1.6, replication through one complete cycle in the presence of BrdU results in metaphase chromosomes with both sister chromatids having unifilarly substituted DNA. These chromosomes stain uniformally lighter than those which contain no BrdU. Cells which have chromosomes with this staining pattern are designated M1 signifying that the cells have replicated once in the presence of BrdU. Cells which have not replicated in the presence of BrdU are designated MO. A second round of replication in the presence of BrdU results in one sister chromatid with unifilarly substituted DNA and one chromatid with bifilarly substituted DNA. These chromosomes have a characteristic harlequin staining pattern of one light and one dark chromatid. Cells which have chromosomes with this pattern are designated M2 indicating that the cell has passed through two complete rounds of replication during the BrdU treatment. A third round of replication in the presence of BrdU results in one half of the chromosomes in a cell staining totally light and the other half with the harlequin pattern. This type of cell is given the designation M3.

There are certain features of the mouse chromosome which allow for a more detailed anaylsis of the cell cycle. The centromere of mouse chromosomes is adenine and thymine rich and these bases are asymmetrically distributed in the centromeric DNA (Figure 1.7). One complete round of replication in the presence of BrdU results in half of the centromere staining darkly and the other half, which has incorporated considerably more BrdU, staining lightly (Figure 1.7, number 1). This helps distinguish M1 cells from MO cells. If BrdU incorporation begins while a cell is already in the DNA synthesis phase,

Figure 1.6. Schematic of the incorporation of BrdU into replicating DNA. Each pair of parallel lines represents a DNA double helix, the set of four lines represents replicated DNA. Solid lines: DNA containing thymidine. Dotted lines: DNA containing BrdU. Chromosome figures represent various FPG staining patterns. Dark chromatids: dark staining. Hatched chromatids: intermediate staining. White chromatids: light staining. MO: cell which has not incorporated BrdU. M1: cell which has gone through one complete round of replication in the presence of BrdU. M2: cell which has gone through two complete rounds of replication in the presence of BrdU. M3: cell which has gone through three complete rounds of replication in the presence of BrdU. A sister chromatid exchange (SCE) is depicted in the lower portion of the figure.

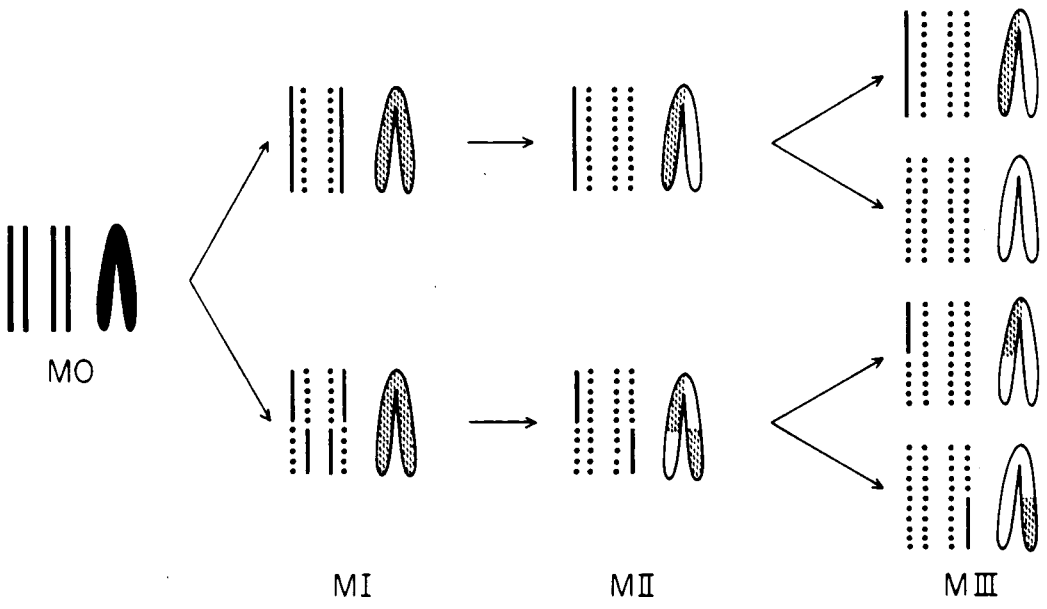

Figure 1.6

Figure 1.7. Schematic of the mouse chromosome incorporating BrdU. Schematic of the incorporation of BrdU into the DNA of the mouse chromosome when BrdU is present for: (1) the entire S phase, (2) part of the S phase, (3) only during the replication of the centromeres and the X and Y chromosomes, (4)only during the replication of the Y chromosome. The chromosome figures represent the FPG staining pattern observed in metaphase cells that have incorporated BrdU as shown in 1-4. The A-T base pairs shown in 1 indicate that the centromeric DNA in the mouse chromosome has an asymmetrical distribution of thymidine.

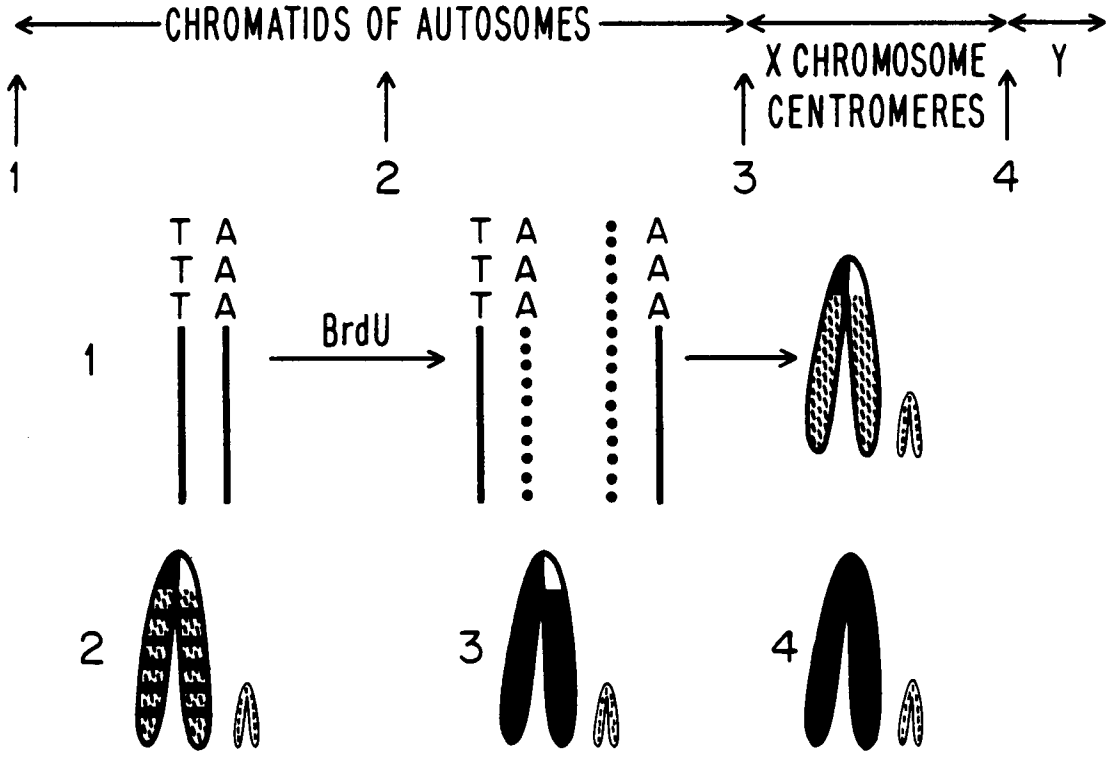
CHROMATIDS OF AUTOSOMES
X CHROMOSOME CENTROMERES
Y
1
2
3
4
T A
T A
T A
BrdU
1
2
3
4

Figure 1.7

there will be DNA replicons which have completed replication in thymidine along with replicons which have incoporated BrdU. This results in a chromosome with a banded staining pattern and allows the identification of those cells that were in the S phase at the time BrdU was added (Figure 1.7, number 2). These cells are designated M1S. The Y chromosome is the last chromosome of the mouse genome to relicate and if BrdU is present only at the end of the S phase, the Y chromosome will incorporate the analog and stain lightly as shown in Figure 1.7, number 4. These cells are designated MOy. Cell cycle kinetics are measured by analyzing the percentages of each of the cell types, MO, M1S, M1, M2, or M3 after varying BrdU treatment times (Figure 1.8).

The Mechanism of Chromosome Aberration Formation

Understanding the mechanisms by which chromosome aberrations are formed provides a basis for the interpretation of many cytogenetic findings. There are two major opposing theories concerning the mechanism of chromosome aberration formation, particularly by ionizing radiation (for a review, see Evans, 1962; Revell, 1974). The first theory, or classical theory, is called breakage-and-reunion and was proposed by Sax in 1938. According to this theory, the ionizations produced by X rays at the instant of irradiation, result in primary breaks in the DNA. These DNA breaks have one of three outcomes: restitution of the DNA to its original form where no aberration is produced; rejoining of the DNA pieces which are close spatially and temporally to produce an exchange aberration; failure to rejoin in any form resulting in a break.

Figure 1.8. Metaphase cells after different BrdU incorporation times. (A) cell which did not incorporate BrdU (MO). (B) cell which incorporated BrdU during part of S phase (M1S). (C) cell which incorporated BrdU for one complete round of replication (M1). (D) cell which incorporated BrdU for two complete rounds of replication (M2).

The important point of this theory is that the DNA break is primary and all aberrations are derived from the initial break.

The alternate theory is Revell's exchange hypothesis (Revell, 1955; 1959). According to this hypothesis, the initial radiation-induced damage is an unstable lesion. This lesion is said to either "decay" to a stable, undetectable state or interact with another lesion close by to form an exchange aberration. During the exchange process, there is a chance that the rejoining process will be incomplete. This results in a chromosome break. The major interpretive difference between Revell's theory and the breakage-first theory is the nature of the primary lesion involved in the formation of chromosome aberrations, particularly breaks. Revell proposed that a DNA lesion is the primary event of damage and breaks are secondary, while the breakage-first theory contends that the break is the initial event.

Both theories have been challenged by many different investigators and it is generally agreed that chromosome breaks arise both from incomplete exchanges as well as from directly induced DNA breaks (Duncan and Evans, 1983). The exchange hypothesis proposes that DNA lesions "decay" which suggests that DNA repair processes, other than simple ligation, are involved in aberration formation. Therefore, the exchange hypothesis serves as a good basis for the interpretation of data concerning the formation of chromosome aberrations which involve repair processes.

Biological Consequences of Chromosome Aberrations

The biological consequences of most chromomsome aberrations are well known (Joshi et al., 1982) Aberrations which result in the formation of an acentric fragment are generally cell lethal since the chromosome segment lacking a centromere usually does not segregate properly at the time of cell division and is lost. Cell lethal aberrations in which an acentric fragment is formed include: chromosome-type deletions, dicentrics, rings; chromatid-type deletions, asymmetrical exchanges (for a review, see Savage, 1975).

The types of chromosome aberrations that cause adverse health effects are those that are transmissable. Aberrations which cause a genetic risk are those induced in germ cells and are of the type that either do not result in the loss of genetic material at cell division (example: chromosome-type and chromatid-type reciprocal translocations and inversions) or result in small, submicroscopic deletions that are transmissable and can give rise to specific locus mutations. The relationship between induced chromosome aberrations and deleterious health effects, in particular cancer, is the subject of much current research. The aberrations types known to be related to specific tumors are reciprocal translocations, inversions and deletions (catalogued by Mitelmen, 1983).

X Ray-Induced DNA Damage and Repair In The Formation Of Chromosome Aberrations

Ionizing radiation is an environmental hazard which induces damage in DNA leading to cell death and other types of cellular injury. X rays are an

example of ionizing radiation with a low linear energy transfer (LET) for which there is a large body of background information. X rays have served as an experimental tool in many studies involving DNA damage and repair, and is the agent used in the studies presented in the following sections of this thesis. The damage induced by ionizing radiation can be divided into three classes: single strand breaks, double strand breaks, and base damage (for a review, see Ward, 1975). Ionizing radiation induces these forms of damage either directly by the deposition of radiation energy on the DNA double helix or indirectly from the radicals produced by water radiolysis such as $OH\cdot$, $O\cdot_2^-$, and $H\cdot$.

The first class of radiation-induced damage, single strand breaks, results from damage to the sugar-phosphate backbone of the DNA. Single strand breaks are repaired rapidly and 50% are repaired within 2 min (Van der Schans et al., 1983). It is assumed from this extremely fast rate of repair that single strand breaks are not involved in the formation of chromosome aberrations (Preston, 1983; Bender, 1980). Directly induced double strand breaks result from two single strand breaks less than five bases apart in the DNA helix. One or two ionization tracks can induce a double strand break but it is believed that a single track is usually involved. DNA double strand breaks can also be induced indirectly by the action of a single strand endonuclease in the DNA across from a single strand gap (Natarajan and Obe, 1978). Fifty percent of double strand breaks are repaired within 10 min (Van der Schans et al., 1983). There is a certain amount of controversy concerning the involvement of double strand breaks in the formation of chromosome aberrations. While it is accepted that chromosome aberrations ultimately result from double strand

damages, the controversy lies in whether these are directly induced breaks (Natarajan et al., 1982) or the result of repair processes (Preston, 1983).

DNA base damages constitute the majority of lesions induced by ionizing radiation (for a review, see Cerutti, 1976; Wallace, 1983). Some of the types of base damages induced by ionizing radiation are apurinic sites, apyrimidinic sites, urea fragments and 5,6-dihydroxy-5,6-dihydrothymine (thymine glycol) (Figure 1.9). There have been many studies on radiation damage to thymine with the most attention being given to thymine glycol (Hariharan and Cerutti, 1972). The other DNA bases are expected to be modified by radiation, yet information on these is scarce. Cytosine glycol and the products of the break-down of the glycols are examples of radiation damage to cytosine (for a review, see Ward, 1972).

One mechanism for the repair of base damage involves an excision via nuclease digestion of the damaged area followed by polymerization and ligation (for a review, see Wallace, 1983). Studies using a monoclonal antibody to thymine glycol showed that the majority of radiation-induced glycols are removed in about two hours (Leadon and Hanawalt, 1983). Thus, the repair of base damages is a progressive process which takes longer than the repair of radiation-induced strand breaks.

One of the experimental approaches used to investigate the contribution of the repair of base damages to the production of chromosome aberrations involves the use of the DNA replication/repair inhibitor, cytosine arabinoside (ara C). When ara C is converted into ara CTP, it becomes a competitive

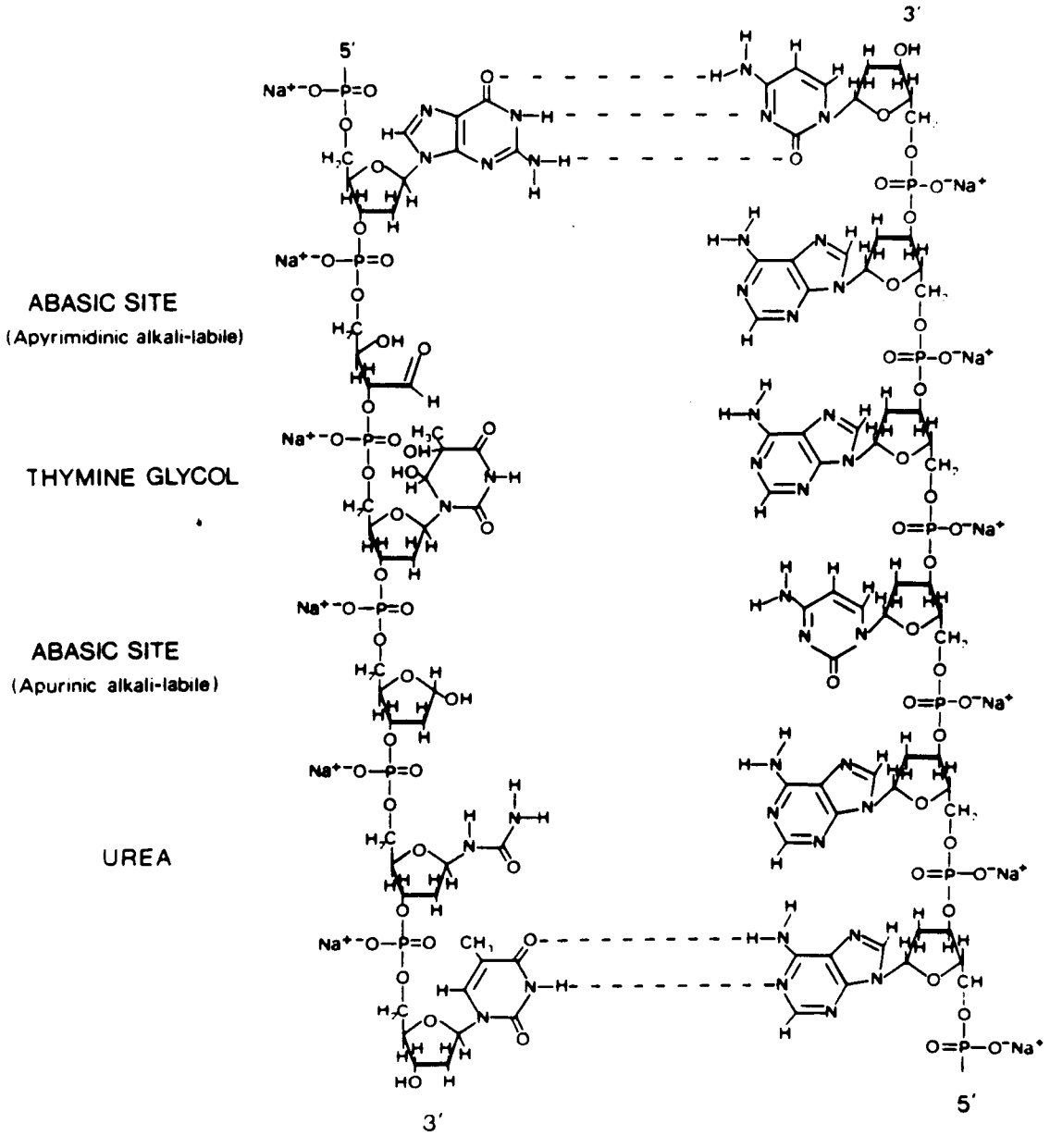

Figure 1.9. Examples of X Ray-Induced Base Damage.

inhibitor of DNA polymerase α. Ara C also becomes incorporated into repairing regions where it serves as a poor primer terminus for further DNA synthesis. One or both of these mechanisms lead to the inhibition of excision repair resynthesis. Incubation of cells undergoing DNA excision or excision-like repair with ara C, causes the accumulation of DNA gaps at repairing regions. The inhibition of ara C can be reversed by the addition of deoxycytidine (dC). When X irradiated cells are incubated with ara C for varying lengths of time followed by the addition of dC, the frequency of chromosome aberrations greatly increases. From results such as this, it is proposed that interactions between coincidently repairing base damages lead to the formation of aberrations (for a review, see Preston, 1983). Some of the experimental results presented in the following chapters will be interpreted in light of Preston's findings.

CHAPTER 2

DIFFERENTIAL SENSITIVITY OF A MOUSE MYELOID LEUKEMIA CELL LINE AND NORMAL MOUSE BONE MARROW CELLS TO X RAY-INDUCED CHROMOSOME ABERRATIONS

Summary

ML-1 cells and *in vitro* normal mouse bone marrow cells were analyzed to determine if there was a differential sensitivity to X ray-induced chromosome aberrations in G_1 cells and/or differences in post-irradiation cell cycle progression. Cells in G_1 at the time of irradiation, identified by their staining pattern after replication in BrdU, were analyzed for all types of chromosome aberrations following X ray doses of 0.5, 1.0, 1.5 and 2.0 Gray (Gy). ML-1 cells showed a greater sensitivity to the induction of both chromosome-type aberrations (dicentrics and terminal deletions) and chromatid-type aberrations (exchanges and deletions) compared to normal mouse bone marrow cells, which only contained chromosome-type aberrations. The presence of chromatid-type aberrations in the ML-1 cells but not normal bone marrow cells suggested a differential progression through the cell cycle for the two cell types after irradiation. Mitotic index and flow cytometric analyses were performed and showed that both cell types have a delay in progression from G_2 into mitosis but only the normal mouse bone marrow cells have a delay in progression from G_1 into S, as well as delayed progression through the S phase following X irradiation. These results indicate that the ML-1 leukemia cells

have an increased radiosensitivity. This may be due to a defect in their ability to respond to DNA damage as evidenced by their lack of a G_1 and S phase delay which allows normal cells an increased time to repair DNA damage before replication. These same characteristics have been observed in ataxia telangiectasia (AT) cells and may well represent a general feature of cells with increased radiosensitivity.

Introduction

The relationship between cellular sensitivity to cytotoxic agents, cell cycle delay, and DNA repair has been investigated using mammalian cell lines which show a differential sensitivity to the induction of chromosome damage, cell killing, and/or mutation compared to normal cells. Cells derived from inherited human disorders which show an increased sensitivity to various agents have been examined for possible repair and/or replication defects. These include AT cells which are sensitive to X rays (Higurashi and Conen, 1973; Taylor et al., 1975; 1976; Taylor, 1978), bleomycin (Taylor et al., 1979; Zampetti-Bosseler and Scott, 1985), 4-nitroquinoline-1-oxide (Smith and Paterson, 1980) and neocarzinostatin (Shiloh et al., 1982); Fanconi anemia (FA) cells which are sensitive to cross-linking agents (Sasaki and Tonomura, 1973; Cohen et al., 1982), bleomycin (Cohen et al., 1982) and X rays (Higurashi and Conen, 1973); Bloom syndrome (BS) cells which are sensitive to X rays (Higurashi and Conen, 1973); xeroderma pigmentosum (XP) cells which are sensitive to ultraviolet light (UV) (Cleaver, 1968; Parrington et al., 1971). There are conflicting reports concerning repair or replication defects in these syndromes but

no specific defect which could completely account for the differential sensitivity has been found except for XP cells which clearly show a deficient repair of UV-induced pyrimidine dimers (Cleaver, 1968; 1969).

Rodent cell lines have also been used to study mechanisms of differential sensitivity. The V79/79 Chinese hamster cell line has been shown to be more sensitive than the parental V79 line to X rays (Sinclair et al., 1964), UV, methyl nitrosourea, ethyl methanesulphonate (Fox et al., 1982), methyl methanesulphonate and nitrogen mustard (HN2) (Graham and Fox, 1983). Mouse lymphoma L5178Y sublines show an increased sensitivity to X rays compared to the parent L5178Y line (Scott et al., 1974; Ehmann et al., 1974; Rosenberg et al., 1976; Scott and Zampetti-Bosseler, 1980; Ockey et al., 1983). The rat Yoshida lymphosarcoma cell line has an increased sensitivity to methylene dimethane sulphonate , UV (Fox and Fox, 1971/1972,) X rays, HN2, and sulfur mustard (Scott et al., 1974). As with the human cell lines, no specific defect has been found in these rodent cell lines which can account for the increased cross-sensitivity to these agents.

The increased radiosensitivity of AT cells compared to normal cells has recently been associated with differences in cell cycle progression for both cell types following X irradiation (Painter et al., 1980). AT cells are reported to have less radiation- or bleomycin-induced delay in the initiation of DNA synthesis compared to normal cells, (Painter et al., 1980, Cramer and Painter, 1981; Jaspers et al., 1982). Therefore, AT cells do not show the normal radiation-induced delay in progression from G_1 to S or S to G_2 (Nagasawa and Little, 1983; 1985; Imray and Kidson, 1983). FA fibroblasts, following treatment with HN2, showed no delay in S phase progression (Dean and Fox,

1983) and HN2-treated V79/79 cells showed reduced S phase delay (Graham and Fox, 1983). The above reports support the idea of an association between increased cellular sensitivity and reduced G_1 and/or S phase delay. However, results with the human lymphoblastoma cell line TK6, which is sensitive to HN2, showed more S phase delay than the less sensitive Raji cell line (Dean and Fox, 1984) and mouse lymphoma L5178Y cells showed an X ray-induced delay in DNA synthesis similar to that for resistant cells (Ockey, 1983).

The present study was carried out to compare the sensitivities of a mouse myeloid leukemia cell line (ML-1) and normal mouse bone marrow cells to X ray-induced chromosome aberrations, and to examine the effects of X rays on the cell cycle progression of both cell types. Flow cytometric and mitotic index analyses were used to study the effects of radiation on the cell cycle.

Materials and Methods

Cell Culture

These studies employed the ML-1 cell line derived from X ray-induced RFM mouse myeloid leukemia (Upton et al.,1964) and normal mouse bone marrow cells grown *in vitro*. ML-1 cultures were maintained in RPMI 1640 (Gibco) containing 10% heat-inactivated (56°, 30 min.) fetal bovine serum (Flow Laboratories) at 37° in an incubator humidified 95-100% in an atmosphere of 5% $C0_2$ in air. Cultures were initiated at 4×10^5 cells per ml for each experiment.

Normal mouse bone marrow cell cultures were established prior to each experiment using a modified version of the technique described by Nakamura (1972). Male RFM mice 9-11 weeks old were sacrificed by cervical dislocation and the femurs were removed. Bone marrow cells were flushed out of the femurs with a 22g needle using Hanks' balanced salt solution adjusted to pH 7.0 with CO_2 at 37°C containing 5 units per ml of sodium heparin (Lilly). Pooled bone marrow cells were centrifuged at 350 $\times$ g for 4 minutes and resuspended in 37° RPMI 1640 plus 25% heat-inactivated fetal bovine serum. An aliquot of cells was treated with Zapoglobin IIR (Coulter Diagnostics) to lyse the red blood cells, and white blood cell counts were determined using a Coulter Counter (Coulter Electronics, model ZM). The cell concentration was adjusted to approximately 3.5 $\times$ 10^6 nucleated cells per ml. Cell cultures were established with 7 $\times$ 10^6 nucleated cells per 2ml in 35 mm plastic culture dishes and incubated as described for the ML-1 cells. The bone marrow cells established *in vitro* are a heterogeneous population and the metaphases analyzed at the selected fixation times, as described below, represent only a small proportion of the total heterogeneous population. However, as a cell population for comparison with the ML-1 leukemia cell line, this would seem to be a reasonable choice due to the presumed derivation of the leukemia from a bone marrow progenitor cell. In addition, the cells analyzed at metaphase are more homogeneous than the total bone marrow population because they are cycling cells with a cell cycle duration of about 20 h (Figure 2.1). Although the use of these particular cell systems complicate the interpretation of specific differences in radiosensitivity of the ML-1 cells and the cell type from which they

Figure 2.1. Percentages of M1 and M2 division metaphases in normal and ML-1 cells. Percentages of first (M1) (●), and second (M2) (○) division metaphase cells from: A, invitro normal mouse bone marrow cells; B, mouse myeloid leukemia (ML-1) cells. T_c is the average cell cycle time and is calculated, as shown, from the point where 50% of the cells are in the ascending portion of the M1 curve to the point where 50% of the cells are in the ascending portion of the M2 curve. T_c for the normal cells is 20 h, and T_c for the ML-1 cells is 15 h.

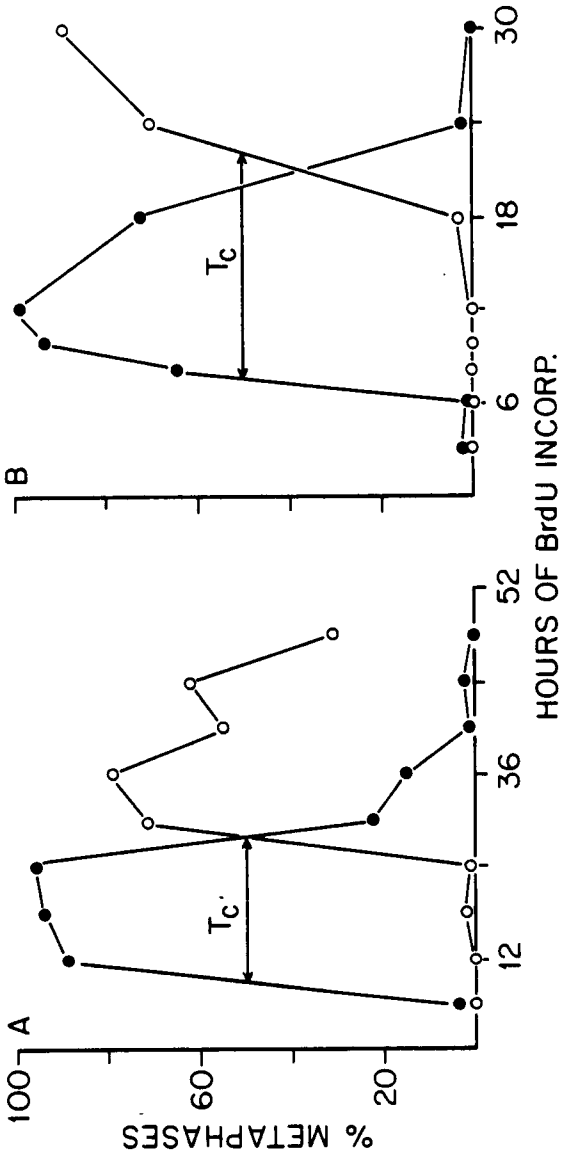
% METAPHASES
100
60
20
A
B
T_c'
T_c
12
36
52
6
18
30
HOURS OF BrdU INCORP.

Figure 2.1

were derived, it is very useful to answer questions of general differences in radiosensitivity in leukemic and normal cycling cells.

Slide Preparation

For the cell cycle kinetics and chromosome aberration experiments, colcemid at a final concentration of 1×10^{-7} M was added 1 h prior to fixation. For the cell cycle kinetics, chromosome aberration and MI experiments, cells were centrifuged, resuspended in 10 ml of 0.075 M KCl and incubated at 37° for 20 min. Cells were centrifuged and approximately 1 ml of fixative (3 parts methanol:1 part glacial acetic acid) was slowly added to the pellet. Cells were washed a total of three times with fixative and single drops of cell suspension were air-dried onto wet microscope slides. The slides were stained by the technique of Goto et al. (1978) was performed to achieve differential staining of the chromatids.

Determination of Cell Cycle Kinetics

An analysis of the cell cycle kinetics for each cell type was made to determine the time at which each culture should be fixed in order to analyze metaphases that were in G_1 at the time of irradiation. To obtain the cell cycle kinetic data shown in Figure 2.1, 10 μM BrdU was added at the time the cultures were established and the ML-1 cells were fixed 4, 6, 8, 10, 12, 18, 24 or 30 h later. The normal bone marrow cells were fixed 6, 12, 18, 24, 30, 36, 42 or 54 h later. The cell cycle kinetic data indicated that at the following fixation times, 90% or more of the metaphases were in G_1 when the BrdU was

added:10-14 h after initiation for the ML-1 cells and 12-25 h after initiation for the bone marrow cells.

To determine the time of irradiation which would allow both cell types an equivalent length of time for repair of DNA damage before entering the S phase, the position of the G_1/S border in the cell cycle was determined as shown in Figure 2.2. The ML-1 cells are a continuous culture and the data from the experiment described above are replotted in Figure 2.2 to show the percentages of M1 and M1S cells. Because the normal bone marrow cells are not a continuous culture, the following protocol was used to determine the time after irradiation that cells reach the G_1/S border, for those G_1 cells which reach mitosis at 24 h. Normal bone marrow cells were fixed 24 h after being established in culture and BrdU was added 4, 6, 8, 10, 12, 18 or 24 h prior to fixation. That is, cells were in culture 20, 18, 16, 14, 12, 6 or 0 h before BrdU was added.

Slides were prepared and differentially stained as previously described. At each time point a minimum of 100 metaphases were analyzed to determine their cell cycle stage at the time of BrdU addition. Cells were classified in a manner similar as described by Tice et al.(1976) into MO, M1S, M1, M2, M3, or greater, to indicate whether they were in G_2, S, or G_1, respectively, at the time BrdU was added.

X Irradiation

For the chromosome aberration, mitotic index (MI) and flow cytometry studies, cell cultures were incubated 12 h before being irradiated with 0.50,

Figure 2.2. Percentages of metaphases from G_1 at the time of BrdU addition in normal and ML-1 cells. Percentage of metaphases from G_1 at the time BrdU was added (M1) (●), and from S at the time BrdU was added (M1S) (○) for A, normal cells and B, ML-1 cells. The arrows indicate the X ray treatment times at 12 h and 14 h before fixation.

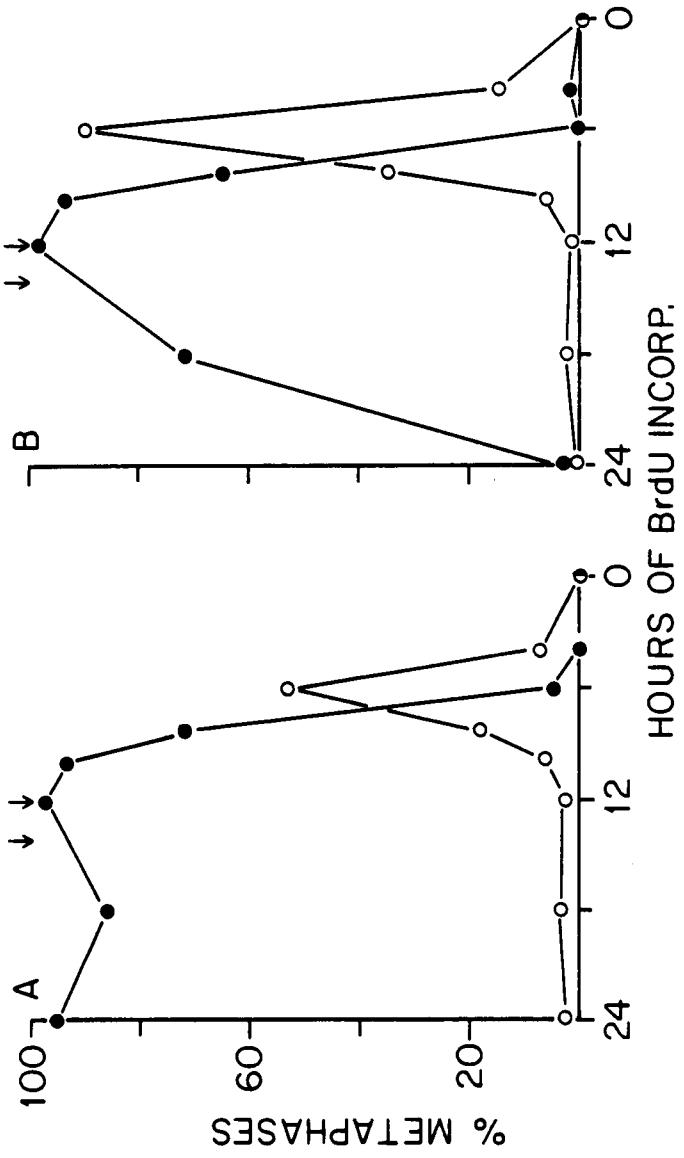

Figure 2.2

1.0, 1.5, or 2.0 Gray (Gy) of X rays at 1 Gy per min. X irradiations were performed with a 320 kVp Gemini Industrial X ray Unit (TFI Corp. New Haven, CT) operated at 250 kV and 12 mA with 2.3 mm of aluminum filtration.

Chromosome Aberration Study

Cultures were fixed 12 and 14 h after X irradiation for the analysis of chromosome aberrations. Slides were prepared and differentially stained as described previously. 100 metaphases with the G_1 staining pattern were analyzed for each X ray dose, and all classes of aberrations were recorded. The classes of aberrations were reported as: chromosome-type aberrations which includes dicentrics, rings, translocations and deletions (both terminal and interstitial deletions). Chromatid-type aberrations include all types of deletions and exchanges. Acentric fragments associated with nonunion proximal and distal (NUPD) isochromatid deletions were included in the terminal deletion category.

Mitotic Index (MI) Determination

Giemsa stained slides were prepared for analysis of the MI every 2 h from 0-14 h following irradiation with 0.5, 1.0, 1.5, or 2.0 Gy. For each sample 2000 cells were counted and the MI expressed as a percentage of the control.

Flow Cytometry Study

A minimum of 1 $\times$ 10^6 cells were fixed every 2 h from 0-14 h post-irradiation with 0.5, 1.0, 1.5, or 2.0 Gy for the ML-1 cells and 2.0 Gy for the normal cells, for analysis by flow cytometry. The samples were prepared for analysis by the technique described by Crissman et al., 1982). Basically, each sample was resuspended in 2 ml of room temperature saline/EDTA and this solution was then added to 5 ml of 95% ethyl alcohol (4°C) and refrigerated at 4°C until analysis. Cells were centrifuged for 10 min at 350 g and then resuspended in saline EDTA at 4°. Pancreatic RNAase 100ug per ml was added to each cell sample and solutions were left at room temperature for 30 min. Cells were stained with 50μg per ml of propidium iodide for 10 min. Samples were passed through a 20 μ filter, then stored on ice until they were analyzed. At least 50,000 cells per sample were analyzed for DNA content using an Ortho 50/H cytoflurograph (Ortho Instruments Westwood, MA). For the normal bone marrow, cycling cells were selected for analysis using a combined scatter versus fluorescence plot as described by Mann et al. (1984). Cell cycle phase distributions were computed from the DNA histograms on a Digital Equipment PDP 11/70 computer (Digital Equipment Corp. Maynard, MA) using the method described by Fried (1976).

Results

Chromosome Aberrations

It is possible to obtain apparent differences in sensitivity to the induction of chromosome aberrations for different cell types if the experimental protocol

does not insure that for both cell types: 1) first division metaphases are scored and 2) the time for repair of DNA damage between treatment and replication is equivalent (Preston, 1982b). To this end, an analysis of the cell cycle kinetics for both cell types was made. Figure 2.1 shows that the population of dividing normal bone marrow cells analyzed in these experiments has a cell cycle time (T_c) of 20 h and the ML-1 cells have a shorter cell cycle time of 15 h. In order to irradiate both cell types in G_1 and allow each the same length of time for repair of damage before entering the S phase, a determination was made of the average time that it took cells to pass from the G_1/S border to metaphase as described in Materials and Methods. Figure 2.2 shows that the difference in the cell cycle times can be attributed primarily to the longer G_1 of the normal bone marrow cells. This finding is in agreement with other reports of cells with different cell cycle times (Prescott,1976). From Figure 2.2 it can be seen that treatment of both cell types 12 or 14 h before fixation would yield cells in mitosis that were from the same region in G_1, approximately 6 or 8 h, respectively, before the midpoint of S.

The frequencies of aberrations are given in Tables 2.1 and 2.2. For purposes of comparison, chromosome-type and chromatid-type aberrations are graphed in Figure 2.3. Both cell types showed a dose dependent increase in chromosome-type aberrations, with the ML-1 cells showing a significantly greater sensitivity to the induction of chromosome-type aberrations at all X ray doses and at both 12 and 14 h time points (Figure 2.3). Table 2.1 shows that there was a two-fold or greater increase in terminal deletions in the ML-1 cells

Table 2.1. X ray-induced chromosome aberrations in normal mouse bone marrow cells in vitro

Treatment	Number of cells analyzed	Chromosome-type aberrations per cell ± s.e.		Chromatid-type aberrations per cell ± s.e.
		Dicentrics	Terminal deletions	
Control	100	0.0	0.01 ± 0.01	0
		12 h harvest		
0.50 Gy	100	0.04 ± 0.02	0.11 ± 0.03	0.02 ± 0.01
1.00 Gy	100	0.12 ± 0.03	0.19 ± 0.04	0.0
1.50 Gy	100	0.07 ± 0.03	0.33 ± 0.06	0.01 ± 0.01
2.00 Gy	100	0.20 ± 0.04	0.29 ± 0.05	0.02 ± 0.01
		14 h harvest		
0.50 Gy	100	0.03 ± 0.02	0.06 ± 0.02	0.01 ± 0.01
1.00 Gy	100	0.06 ± 0.02	0.11 ± 0.03	0.02 ± 0.01
1.50 Gy	100	0.13 ± 0.04	0.23 ± 0.05	0.01 ± 0.01
2.00 Gy	100	0.23 ± 0.05	0.37 ± 0.06	0.02 ± 0.01

at all doses and time points compared to the normal cells. Many of these terminal deletions could be isochromatid NUPD deletions which are indistinguishable from chromosome-type terminal deletions. The terminal deletions contribute the most to the observed increase in the chromosome-type aberrations for the ML-1 cells, but there was a significant increase in the frequency of dicentrics at the 14 h fixation following 2.0 Gy of irradiation. The ML-1 cells in contrast to the normal cells also had a significant number of chromatid-type aberrations (deletions and exchanges). The true frequency of chromatid-type aberrations is probably higher because isochromatid NUPD deletions were classified as chromosome-type terminal deletions as described in Materials and

Table 2.2. X ray-induced chromosome aberrations in ML-1 cells

Treatment	Number of cells analyzed	Chromosome-type aberrations per cell ± s.e.		Chromatid-type aberrations per cell ± s.e.
		Dicentrics	Terminal deletions	
Control	100	0.0	0.01 ± 0.01	0.0
		12 h harvest		
0.50 Gy	100	0.03 ± 0.02	0.26 ± 0.05	0.11 ± 0.03
1.00 Gy	100	0.06 ± 0.02	0.59 ± 0.08	0.09 ± 0.03
1.50 Gy	100	0.10 ± 0.03	0.92 ± 0.10	0.12 ± 0.03
2.00 Gy	100	0.19 ± 0.04	0.93 ± 0.10	0.13 ± 0.04
		13 h harvest		
0.50 Gy	100	0.03 ± 0.02	0.22 ± 0.05	0.05 ± 0.02
1.00 Gy	100	0.09 ± 0.03	0.62 ± 0.08	0.12 ± 0.08
1.50 Gy	100	0.20 ± 0.04	0.64 ± 0.08	0.11 ± 0.03
2.00 Gy	100	0.44 ± 0.07	0.80 ± 0.09	0.09 ± 0.03

Methods. The presence of many chromatid-type aberrations in ML-1 cells irradiated in G_1 indicates that the cells are progressing into the S phase with unrepaired DNA damage that is then being converted into chromatid-type aberrations. Since both cell types were treated at the same time prior to entering S, the presence of only chromosome-type aberrations in the normal cells indicates: 1) that they are delayed in G_1 where mis-repair of the X ray-induced damage produces chromosome-type aberrations and/or, 2) that the normal cells have different DNA repair kinetics compared to the ML-1 cells. To investigate the first possibility, an analysis was made of the X ray-induced perturbations of the cell cycle. Studies on the repair kinetics of the ML-1 and normal cells are discussed in Chapter 3.

Figure 2.3. Aberration frequencies in normal and ML-1 cells following X irradiation. Aberration frequencies per cell following 0.5, 1.0, 1.5, or 2.0 Gy of X ray. A and B present the results from the 12 h fixation time , C and D present the results from the 14 h fixation time. In A and C, the chromosome-type aberrations are shown; in B and D, the chromatid-type aberrations are shown for the normal cells (●) and the ML-1 cells (○).

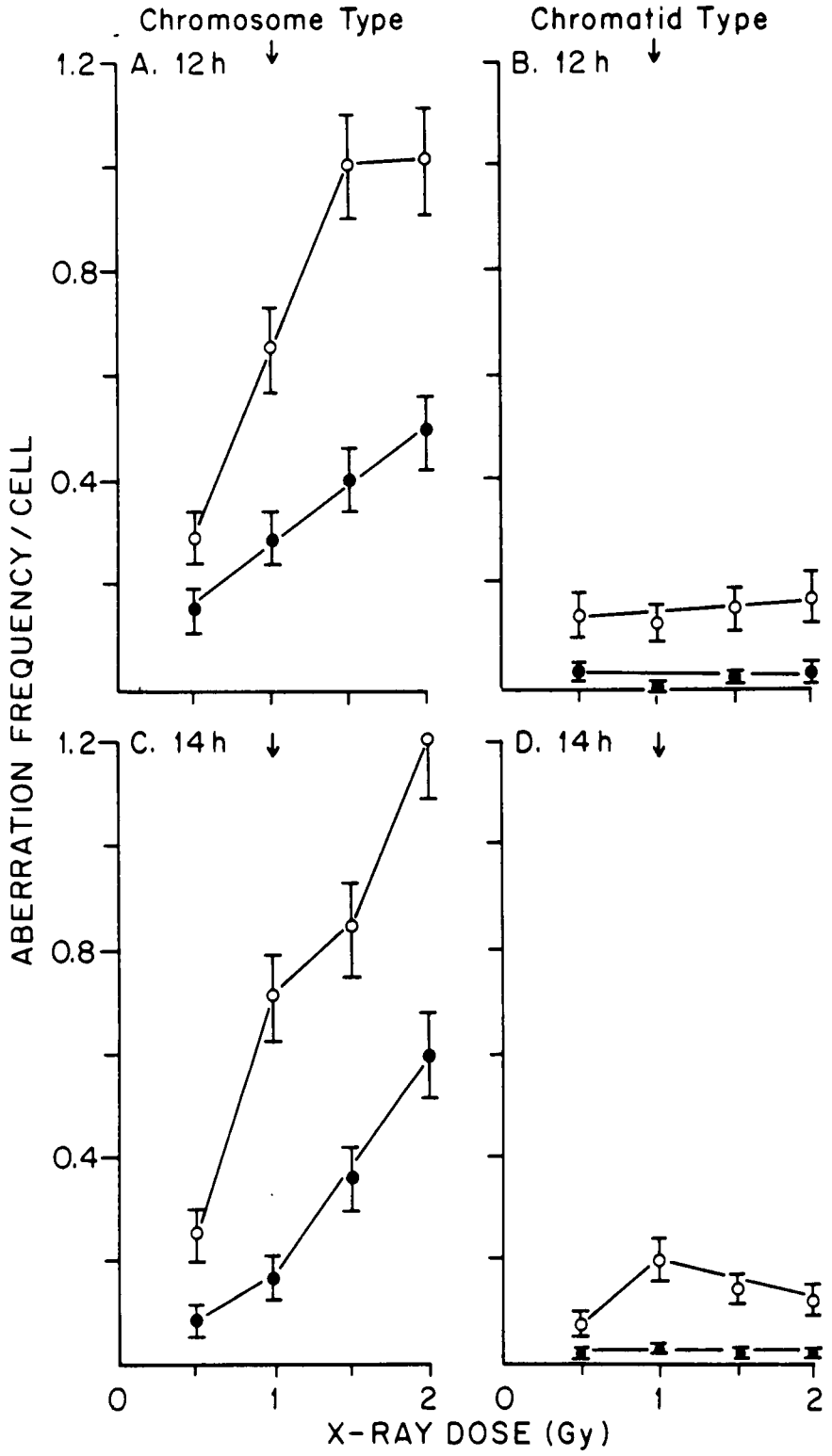
Chromosome Type
Chromatid Type
A. 12 h
B. 12 h
C. 14 h
D. 14 h
1.2
0.8
0.4
0
1
2
ABERRATION FREQUENCY / CELL
X-RAY DOSE (Gy)

Figure 2.3

Mitotic Delay

Figure 2.4 shows the results of the MI analysis. Both cell types showed a similar delay in the progression of cells into mitosis following irradiation and this delay was more pronounced with increasing X ray dose. For example, 2 h after irradiation of the ML-1 cells, the MI decreased from 54% of the control at 0.50 Gy to 25% at 1.0 Gy, 15% at 1.5 Gy and finally 0% at 2.0 Gy. With time, the MI of both cell types recovered to control levels, and except for the normal cells at 1.50 Gy, the MI exceeded the 100% level due to the temporarily delayed cells progressing through mitosis in a synchronous wave.

Flow Cytometry

Flow cytometry was used to further investigate the X ray-induced perturbations of the cell cycle. Figure 2.5 shows the DNA histograms for the ML-1 cells following X irradiation. The cell cycle phase distributions from these histograms were computer analyzed and for comparative purposes, the control, 0.5, and 2.0 Gy data are graphed in Figure 2.6. The first time point shown in Figure 2.5 is 2 h after irradiation and the DNA histogram has a normal distribution which can be used as a comparison to later time points. It can be seen in Figure 2.5 that very little perturbation of the cell cycle occurred at the lowest dose of 0.5 Gy, but at increasingly higher doses there was a rise in the G_2/M peak which returned to normal at later times (data not shown). The MI at these time points was very low (Figure 2.4), therefore the increase in the

Figure 2.4. The mitotic index in normal and ML-1 cells following X irradiation. Mitotic index (MI) as % of control. MI in A is after 0.5 Gy, B is after 1.0 Gy, C is after 1.5 Gy and D is after 2.0 Gy of X ray for normal cells (●) and ML-1 cells (○).

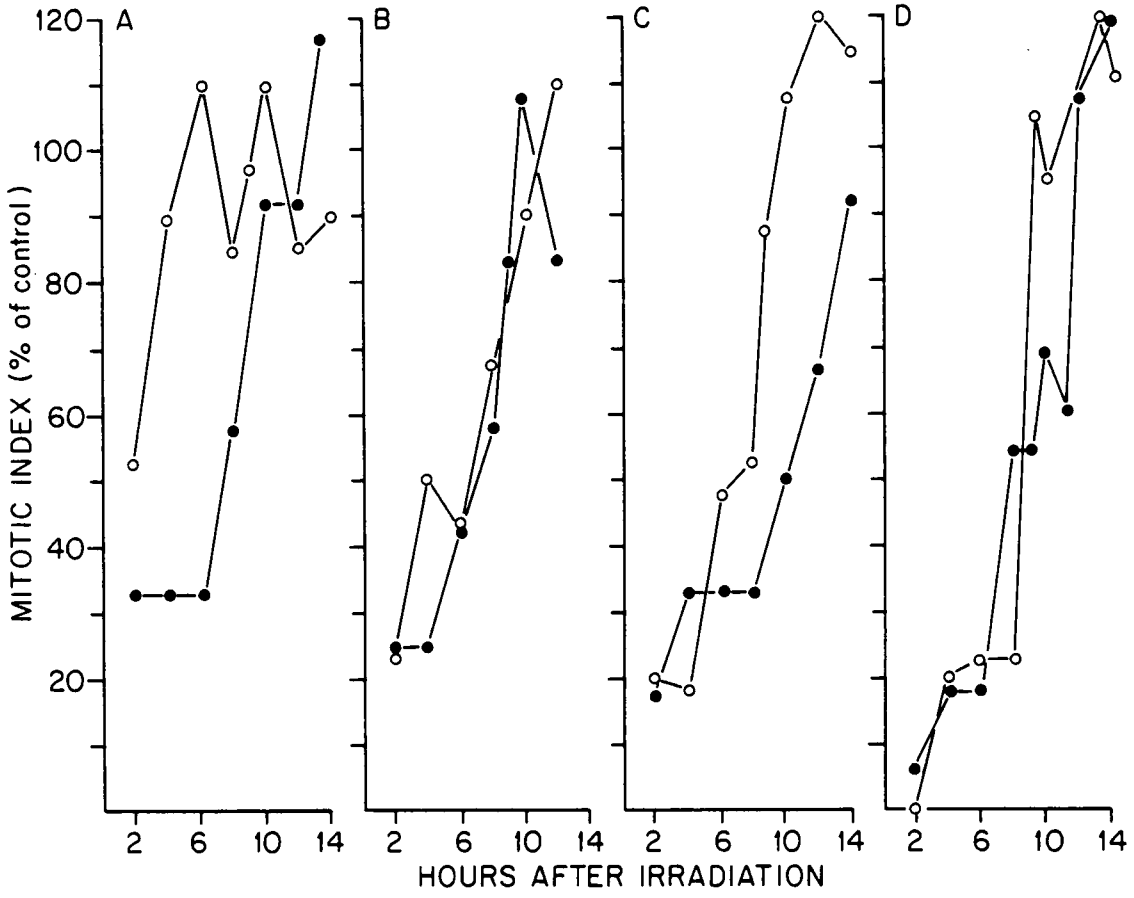
A
B
C
D
120
100
80
60
40
20
MITOTIC INDEX (% of control)
2
6
10
14
HOURS AFTER IRRADIATION

Figure 2.4

Figure 2.5. DNA histograms of ML-1 cells following X irradiation. Representative DNA histograms showing cell cycle distributions of ML-1 cells 2, 4, 6, 9, and 12 h after exposure to 0.5, 1.0, 1.5, or 2.0 Gy of X ray. The left-hand peak contains G_1 cells, the right-hand peak contains G_2/M cells and the shaded region contains the S phase cells. For each individual histogram, the ordinate is the frequency of cells and the abscissa is the fluorescence.

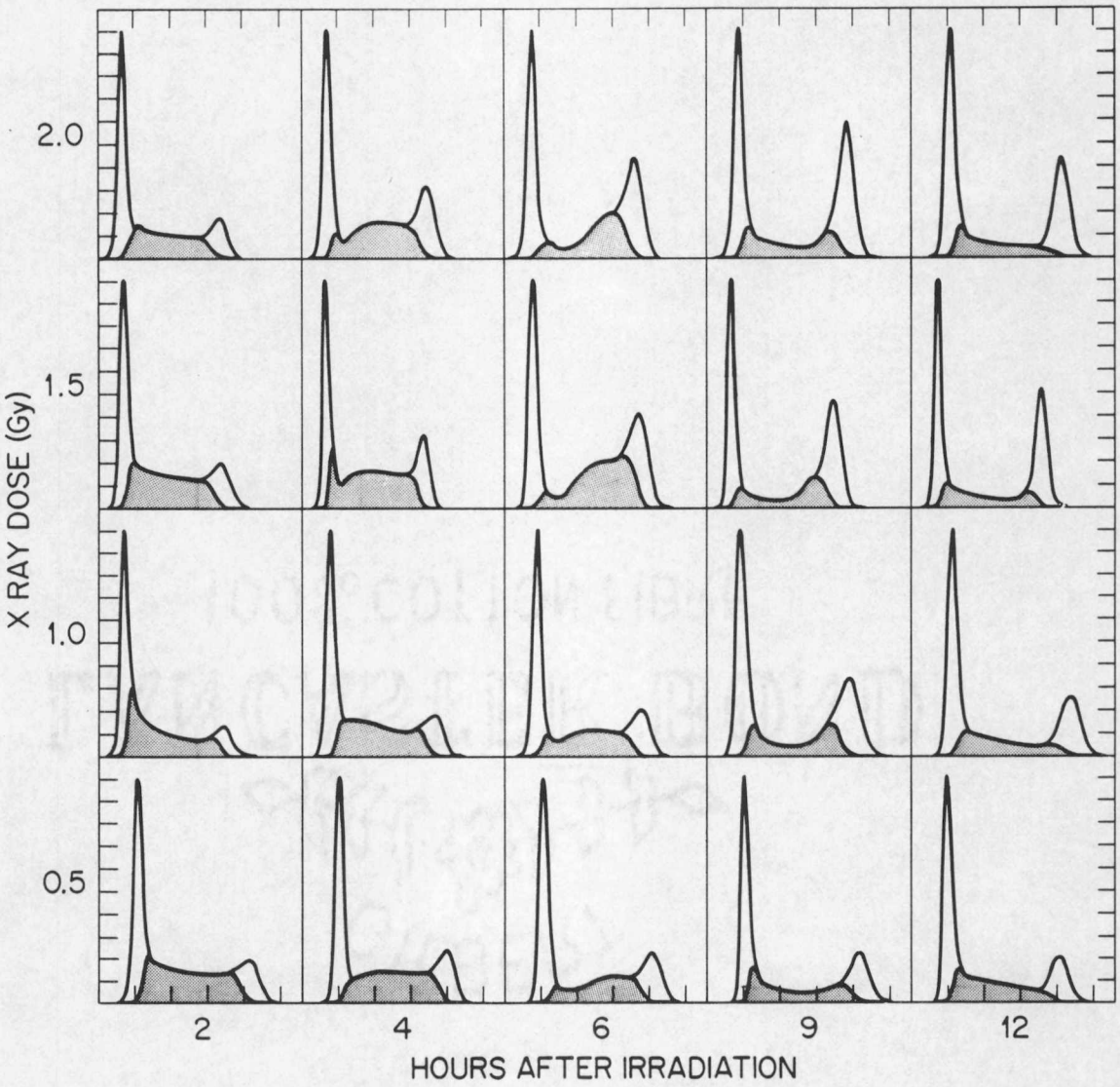

Figure 2.5

Figure 2.6. Cell cycle phase distributions of ML-1 cells following X irradiation. The proportion of ML-1 cells in G_1, S, G_2/M after 0 Gy(●), 0.5 Gy(○),or 2.0 Gy(Δ) of X ray. Data plotted are the average of three experiments ± standard error (s.e.)

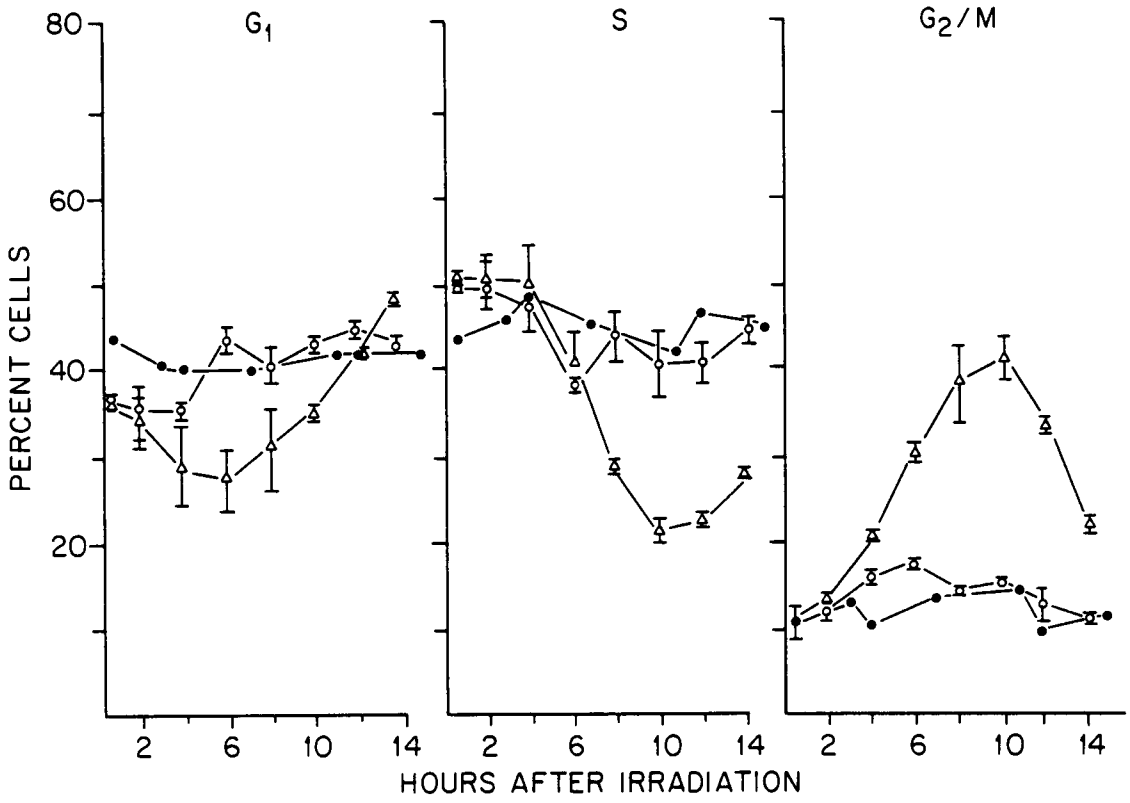
80
60
40
20
PERCENT CELLS
G1
S
G2/M
2
6
10
14
HOURS AFTER IRRADIATION

Figure 2.6

G_2/M peak was caused by G_2 cells which were being delayed in progressing into mitosis. Thus the flow cytometry data confirm the MI data, showing a G_2 delay in the ML-1 cells following irradiation. The continued progression of G_1 cells into S, together with the inhibition of the progression of G_2/M cells through mitosis and back into G_1, resulted in a decrease in the proportion of cells in G_1. For example, 6 h after irradiation with 2.0 Gy, the percentage of cells in G_1 was lowered to 28% of the control population (Figure 2.6). As cells continue through the cell cycle, there are fewer G_1 cells moving into the S phase, resulting in a decrease in the proportion of S phase cells with time (Figure 2.6). Figure 2.5 shows that, as expected, the decrease in the proportion of S phase cells begins with the late G_1/early S phase cells. This is represented in the histogram by the "dip" in the population of cells in the left-hand region of the shaded S phase area. As this population of cells passes through the S phase, there is a subsequent decrease in the proportion of cells in late S phase of the cell cycle. These results along with the aberration data, indicate that the ML-1 cells are progressing into the S phase with unrepaired DNA damage where mis-repair of this damage can lead to chromatid-type aberrations.

Figures 2.7 and 2.8 show the DNA histograms and cell cycle phase distributions, respectively, for the normal bone marrow cells following irradiation with 2.0 Gy. The X ray-induced perturbation of the cell cycle is not as dramatic in the normal bone marrow cells as it was in the ML-1 cells. The G_2 delay indicated by the MI data is not evident in these DNA histograms (Figure 2.7). With the normal bone marrow cell cycle time being 20 h, only 2-3%

Figure 2.7. DNA histograms of normal cells following X irradiation. Representative DNA histograms showing cell cycle distributions of normal cells 0, 2, 4, 6, 8, 12, or 14 h after irradiation with 2.0 Gy of X ray. For each individual histogram, the ordinate is the frequency of cells and the abscissa is the fluoresence.

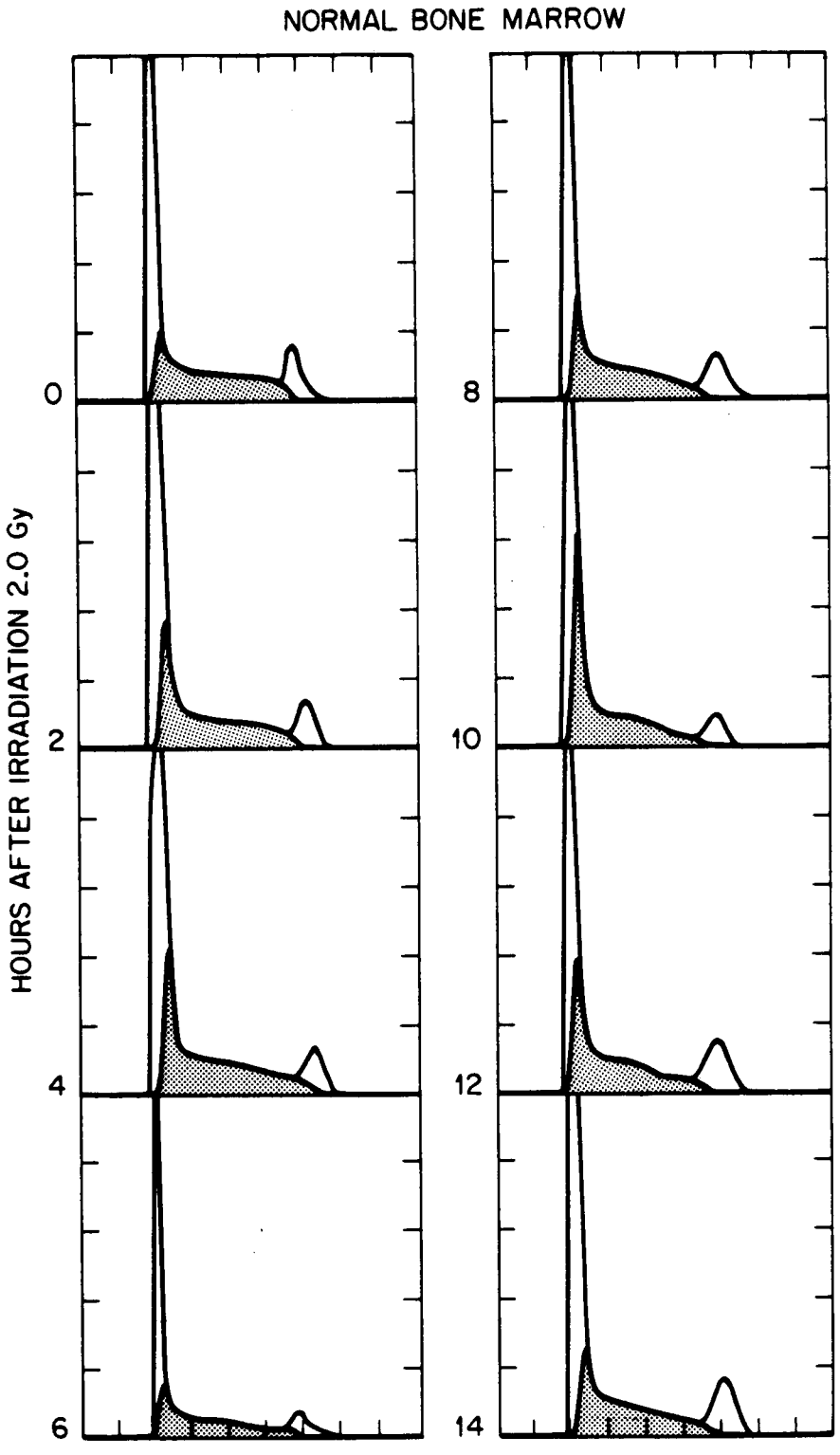
NORMAL BONE MARROW
HOURS AFTER IRRADIATION 2.0 Gy
0
2
4
6
8
10
12
14

Figure 2.7

Figure 2.8. Cell cycle phase distributions of normal cells following X irradiation. The proportion of normal cells in G_1, S, G_2/M after 0 Gy (●) and 2.00 Gy (Δ) of X ray. Data plotted are the average of three experiments ± s.e.

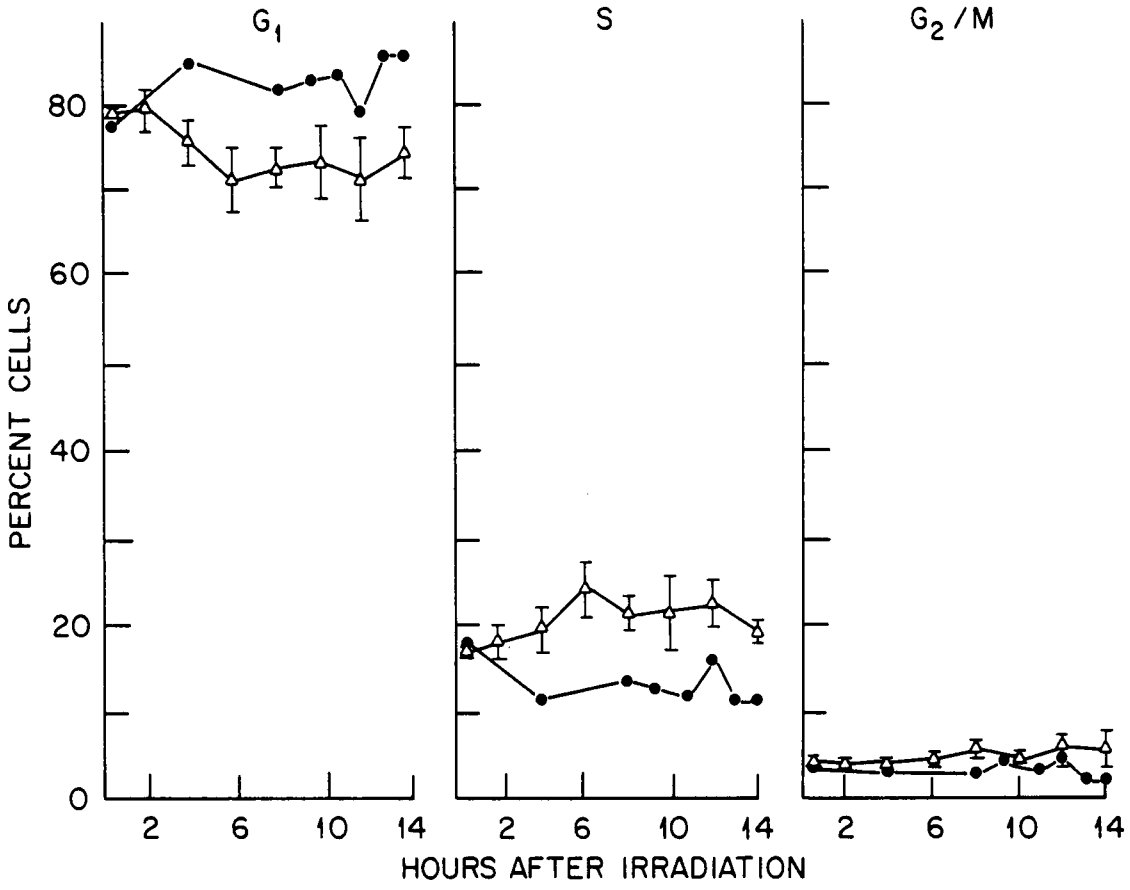
G1
S
G2 / M
80
60
40
20
0
PERCENT CELLS
2
6
10
14
HOURS AFTER IRRADIATION

Figure 2.8

of the cells occupy the G_2/M region and the X ray-induced G_1 and S phase delays, discussed later, caused fewer cells to progress into the G_2/M phase, therefore any X ray-induced G_2 delay would cause only a very small change in the G_2/M peak and this will not be observed in the DNA histograms. In contrast to the ML-1 cells, which show a decrease in the proportion of S phase cells after irradiation, the proportion of normal cells in the S phase increased with time after irradiation indicating that the cells are delayed in their progression through this phase. From Figure 2.7, it appears that the accumulation of late G_1/early S cells is contributing the most to the overall increase in the proportion of S phase cells seen in Figure 2.8. These results along with the aberration data indicate that the normal bone marrow cells are delayed in G_1 and early S, where mis-repair of the X ray-induced damage can lead to chromosome-type aberrations.

Discussion

ML-1 cells, established from an X ray-induced myeloid leukemia in an RFM mouse, showed an increased sensitivity to X ray-induced chromosome aberrations compared to *in vitro* normal mouse bone marrow cells. Specifically, G_1 irradiated ML-1 cells had twice as many chromosome-type aberrations than normal cells many of which were in the terminal deletions category. ML-1 cells also had a significant number of chromatid-type aberrations and the true frequency is probably higher since isochromatid NUPD deletions are included in the terminal deletion category. This is analogous to X-irradiated G_1 AT cells which are reported to have chromatid-type aberrations and a high frequency of

fragments reported as terminal deletions (Taylor et al.,1976; Taylor, 1978; Natarajan and Meyers, 1979; Bender, 1980; Nagasawa and Little, 1983).

Normal cells after treatment with a DNA damaging agent, have been shown to undergo an inhibition of DNA synthesis, (Wantanabe, 1973; Painter and Young, 1980; Cramer and Painter,1981; Jaspers et al., 1982) and G_1 and S phase delays (Leeper et al., 1972; Gerner et al., 1977; Imray and Kidson, 1983; Nagasawa and Little, 1983; Nagasawa et al.,1985). Retinoblastoma (RB) cells, which do not have a greater sensitivity to X ray-induced chromosome aberrations and may or may not be sensitive to killing by X rays (Zampetti-Bosseler and Scott, 1981; Nagasawa and Little, 1983), also show a radiation-induced delay in the initiation of DNA synthesis and a G_1 block (Nagasawa and Little, 1983). In contrast, AT cells do not show the same radiation-induced inhibition of DNA synthesis or G_1 and S phase delays (Painter and Young, 1980; Cramer and Painter,1981; Jaspers et al., 1982; Imray and Kidson, 1983; Nagasawa and Little, 1983; Nagasawa et al., 1985). Painter and Young (1980), proposed that cell cycle delays may serve as a protective mechanism which allows normal cells, or cells with normal radiosensitivity like RB cells, additional time to repair DNA damage and that AT cells have increased radiosensitivity because they fail to undergo normal X ray-induced cell cycle delays. However, this is not consistent with the increased S phase delay in the HN2 sensitive TK6 cell line (Dean and Fox, 1984) or the similar X ray-induced delay in DNA synthesis in the mouse L5178Y cells (Ockey, 1983), suggesting that there may be different mechanisms for the increased cellular sensitivity of these cells.

The results obtained by flow cytometry with the ML-1 and normal bone marrow cells following irradiation, indicate that the normal cells are delayed in G_1 as well as S while the ML-1 cells do not have the same G_1 and S delays. These results explain the presence of chromatid-type aberrations in the ML-1 cells and not the normal cells by showing that the normal cells undergo a G_1 and S phase delay following irradiation which allows them extra time in G_1 where mis-repair of the X ray-induced damage leads to chromosome-type aberrations. The ML-1 cells, like AT cells, progress into the S phase with unrepaired DNA where mis-repair of that DNA damage leads to chromatid-type aberrations. Experiments on the repair kinetics of the ML-1 and normal cells discussed in Chapter 3 show that a repair defect exists in the ML-1 cells which results in more DNA damage remaining when the cell enters the S phase, thus contributing to the formation of chromatid-type aberrations and the overall increased sensitivity observed for these cells.

There are conflicting reports concerning the relationship between a G_2 delay and cellular sensitivity. X ray sensitive L5178Y sublines have a greater X ray-induced G_2 delay than the parent line (Rosenberg et al., 1976; Scott and Zampetti-Bosseler, 1980). FA cells after mitomycin C treatment have a greater G_2 delay than normal cells (Kaiser et al., 1982) but HN2 treated FA cells have less G_2 delay (Dean and Fox, 1983). AT fibroblasts have been shown to have less X ray-induced mitotic delay (Zampetti-Bosseler and Scott, 1981; Scott and Zampetti-Bosseler, 1982) while AT lymphoblastoid cells do not show this decreased G_2 delay (Imray and Kidson, 1983; Ford et al., 1984). The MI and flow cytometric analyses in this report, showed that the ML-1 and normal cells have a similar dose-dependent delay in the progression of cells

from G_2 into mitosis and the MI recovered in about the same time after irradiation for both cell types.

We describe here a mouse myeloid leukemia cell line, ML-1, which has an increased sensitivity to X ray-induced chromosome- and chromatid-type aberrations and decreased X ray-induced cell cycle delays compared to normal mouse bone marrow cells. ML-1 cells, in response to radiation, have characteristics similar to irradiated AT cells suggesting that these cells may have similar defects contributing to their increased radiosensitivities. In addition, it would be expected that these differences in aberration induction between the ML-1 and normal cells would be paralleled by differences in cell killing, as for AT cells versus normal human fibroblasts, since the majority of aberrations observed are cell lethal. It is also expected that there would be differences in sensitivity betweeen the two cell types to the induction and transmission of non-cell-lethal aberrations. This could be analyzed by examining banded chromosome preparations from irradiated cells.

CHAPTER 3

DNA REPAIR KINETICS AND THE SENSITIVITY OF CELLS TO X RAY-INDUCED CHROMOSOME ABERRATIONS: A MOUSE MYELOID LEUKEMIA CELL LINE AND NORMAL MOUSE BONE MARROW CELLS

Summary

The frequency of X ray-induced chromosome aberrations in G_1 ML-1 mouse myeloid leukemia cells and normal mouse bone marrow cells increased with post-irradiation incubation with the DNA repair resynthesis inhibitor, 1-B-D-arabinofuranosylcytosine (ara C), but the frequency of aberrations in the leukemic cells increased with quite a different time response compared to the normal cells. Irradiated normal mouse bone marrow cells had a rapid increase in the frequency of chromosome exchanges and deletions with increasing ara C incubation time, for example, an increase was observed with 0.5 h ara C incubation. In contrast, the ML-1 cells did not have a significant increase in aberrations until 1 to 2 h post-irradiation incubation with ara C. These results suggest that the ML-1 cells, per unit time, initially undergo less repair of the X ray-induced DNA damage that can be converted into chromosome aberrations. We previously showed that the ML-1 cells have a higher frequency of X ray-induced chromosome aberrations compared to normal cells and the results presented here indicate that a slower rate of repair resynthesis is contributing to the increased sensitivity of the ML-1 cells.

Introduction

We previously described a mouse myeloid leukemia cell line, ML-1, which showed an increased sensitivity to X ray-induced chromosome aberrations compared to normal mouse bone marrow cells grown *in vitro* (Aardema et al., 1985). G_1 irradiated ML-1 cells were found to have a significantly higher frequency of chromatid-type aberrations than normal cells. This was explained, in part, by cell cycle analyses that showed that the ML-1 cells did not undergo the X ray-induced delay in progression from G_1 into and through the S phase exhibited by the normal cells (Aardema et al., 1985). This X ray-induced cell cycle delay allowed the normal cells extra time to repair or misrepair the X ray-induced damage thereby leading to the formation of only chromosome-type aberrations in the normal cells. In contrast, the ML-1 cells progressed into the S phase with unrepaired or incompletely repaired DNA damage. This damage could then be converted into chromatid-type aberrations either by a misrepair event following replication, or by interactions between coincidently replicating regions which contain incompletely repaired damage (Evans, 1977). These results also indicated that there was some X ray-induced DNA lesion in the ML-1 cells which persisted until the cells enter the S phase. This suggested the possiblity of repair differences between the ML-1 cells and the normal bone marrow cells.

Preston (1980b, 1981) showed that the increased sensitivity of G_0 Down syndrome lymphocytes to X ray-induced chromosome aberrations could be explained by a more rapid rate of initiation of excision repair in Down cells than in normal cells. To investigate whether differences in DNA repair

between the ML-1 cells and normal mouse bone marrow cells are contributing to the differential X ray sensitivity of the two cell types, the frequency of X ray-induced chromosome aberrations with increasing post-irradiation incubation in the presence of ara C was examined using an experimental protocol similar to that used with the Down syndrome cells. The results indicate that a slower rate of DNA excision repair, possibily at the resynthesis step, is contributing to the increased sensitivity of the ML-1 cells to X ray-induced aberrations.

Materials and Methods

Cell Culture

Normal mouse bone marrow cultures were established prior to each experiment using a modified version of the technique described by Nakamura et al. (1972) as previously described (Aardema et al., 1985). Briefly, male RFM mice 9-11 weeks old were sacrificed by cervical dislocation and the femurs were removed. Bone marrow cells were flushed out of the femurs with 37° RPMI 1640 (Gibco) containing 25% heat inactivated fetal bovine serum (Flow Laboratories) using a 22g needle. Pooled bone marrow cells were centrifuged and an aliquot of cells was treated with Zapoglobin IIR (Coulter Diagnostics) to lyse the red blood cells. White blood cell counts were determined using a Coulter Counter (Coulter Electronics, Model ZM). The cell concentration was adjusted to approximately 3.5×10^6 nucleated cells per ml. Cell cultures were established with 7×10^6 nucleated cells per 2 ml of media in 35 mm plastic

culture dishes and maintained in an incubator humidified 95-100% in an atmosphere of 5% CO_2 in air.

The ML-1 cell line used in these experiments was established from an X ray-induced myeloid leukemia of an RFM mouse as previously described (Au et al., 1983). ML-1 cells were subcultured prior to each experiment, at 4×10^5 cells per ml of RPMI 1640 containing 10% heat inactivated fetal bovine serum. Cultures were incubated as described for the normal cells.

X Ray Experiments

X irradiations were performed with a 320 KVp Gemini Industrial X Ray Unit (TFI Corp. New Haven, CT) operated at 250 kV and 12 mA with 2.3mm of aluminum filtration. All cell cultures were exposed to 2.0 Gy at a dose rate of 1.0 Gy per minute.

Cell cultures were irradiated 11 h after initiation and harvested 15 h after irradiation. One hour prior to fixation, colcemid (1×10^{-7} M, Sigma) was added to each culture. As previously described (Aardema et al., 1985), this protocol permits the analysis of cells that were in G_1 at the time of irradiation. Fifteen minutes prior to irradiation, ara C (5×10^{-5} M, Sigma) was added to each culture. Post-irradiation incubation in the presence of ara C was for 0.5, 1.0, 2.0, or 3.0 h at 37°. After incubation with ara C, cultures were washed twice with RPMI 1640 plus fetal bovine serum and returned to fresh medium. Deoxycytidine (dC) (1×10^{-4} M, Sigma) was added to each culture after it

was returned to fresh medium in order to reverse the inhibitory effects of ara C on DNA repair. The control cultures were: one untreated; one incubated with ara C for 3 h; one irradiated with 2.0 Gy but not treated with ara C.

Analysis of Cells

Fixation and slide preparation were performed according to standard methods (Brewen and Gengozian, 1971). Slides were centromere-stained (C-banded) using the method described by Salamanca and Armendares (1974). A minimum of 100 metaphases were analyzed for each treatment, and all classes of aberrations were recorded. The classes of aberrations were reported as: chromosome-type exchanges (dicentrics, rings, and translocations); chromosome-type deletions (terminal, interstitial, and acentric fragments associated with nonunion proximal and distal (NUPD) isochromatid deletions); chromatid-type aberrations (deletions and all types of exchanges).

Results

The data for the effect of ara C on the frequencies of X ray-induced chromosome aberrations for the normal mouse bone marrow cells are shown in Table 3.1 and the data for the ML-1 cells are shown in Table 3.2. The frequencies of chromosome-type deletions and exchanges are depicted graphically in Figure 3.1.

The frequencies of chromosome aberrations induced by 2.0 Gy X rays alone were greater in the ML-1 cells than in the normal cells. For example, after 2.0 Gy of X rays, the frequencies of chromosome-type deletions and

Table 3.1. The frequencies of chromosome aberrations in normal bone marrow cells following X irradiation in G_1 with or without a post-treatment with Ara C

Treatment	Number of cells	Chromosome-type aberration frequencies (± s.e.)		Chromatid-type aberration frequency (± s.e.)
		Deletions	Exchanges	
Control	100	0.02 ± 0.01	0.0	0.0
ara C, 3 h	100	0.01 ± 0.01	0.0	0.0
X ray 2.0 Gy	300	0.38 ± 0.04	0.22 ± 0.02	0.11 ± 0.02
X ray 2.0 Gy + ara C, 0.5 h	200	0.55 ± 0.05	0.365 ± 0.04	0.07 ± 0.02
X ray 2.0 Gy + ara C, 1 h	200	0.85 ± 0.06	0.430 ± 0.05	0.06 ± 0.02
X ray 2.0 Gy + ara C, 2 h	200	0.89 ± 0.07	0.430 ± 0.05	0.11 ± 0.02
X ray 2.0 Gy + ara C, 3 h	200	0.92 ± 0.07	0.375 ± 0.04	0.09 ± 0.02

Table 3.2. The frequencies of chromosome aberrations in ML-1 cells following X irradiation in G_1 with or without a post-treatment with Ara C

Treatment	Number of cells	Chromosome-type aberration frequencies (± s.e.)		Chromatid-type aberration frequency (± s.e.)
		Deletions	Exchanges	
Control	100	0.02 ± 0.01	0.0	0.01 ± 0.01
ara C, 3 h	100	0.03 ± 0.02	0.0	0.02 ± 0.01
X ray 2.0 Gy	200	0.49 ± 0.05	0.29 ± 0.04	0.115 ± 0.02
X ray 2.0 Gy + ara C, 0.5 h	200	0.50 ± 0.05	0.335 ± 0.04	0.135 ± 0.03
X ray 2.0 Gy + ara C, 1 h	200	0.63 ± 0.06	0.275 ± 0.05	0.215 ± 0.23
X ray 2.0 Gy + ara C, 2 h	200	0.70 ± 0.06	0.390 ± 0.04	0.105 ± 0.02
X ray 2.0 Gy + ara C, 3 h	200	0.74 ± 0.06	0.485 ± 0.05	0.145 ± 0.03

Figure 3.1. Aberration frequencies in normal and ML-1 cells following X irradiation in G_1 with or without a post-treatment with ara C. The effect of postirradiation incubation with 50μm ara C for 0, 0.5, 1, 2, or 3 h on the frequency of X ray-induced chromosome aberrations in normal mouse bone marrow cells (●) and ML-1 cells (○). Panel A shows chromosome-type deletions and Panel B shows chromosome-type exchanges. The data points represent the chromosome aberration frequency ± standard error.

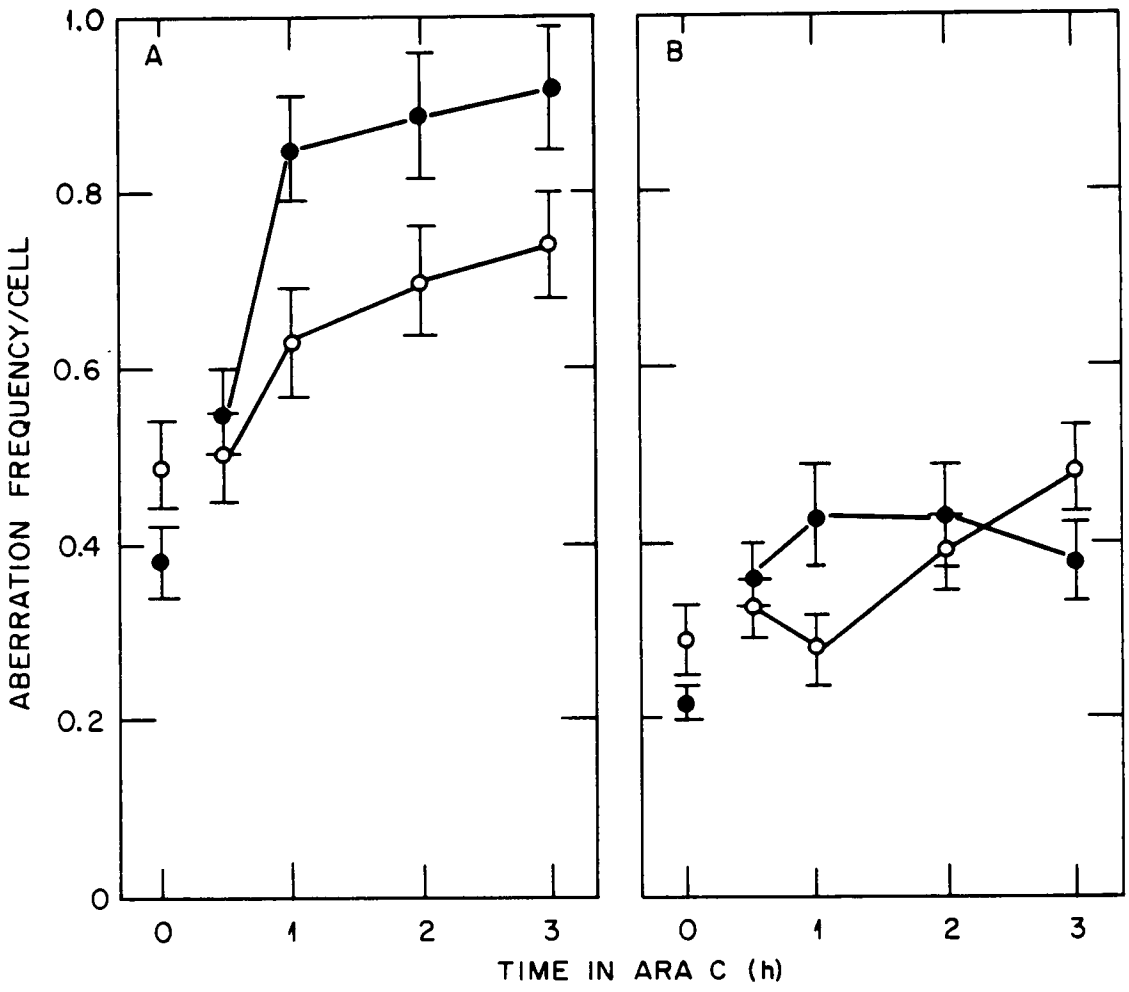
1.0
0.8
0.6
0.4
0.2
0
A
B
ABERRATION FREQUENCY/CELL
0
1
2
3
0
1
2
3
TIME IN ARA C (h)

Figure 3.1

exchanges in the ML-1 cells were 0.49 and 0.29, respectively, and in the normal cells the frequencies were 0.38 and 0.22, respectively. It can be seen that the frequencies of chromosome-type aberrations increased in both cell types with postirradiation incubation with ara C. However, the time response of the increase in aberrations in the two cell types was quite different. The increase in the frequencies of aberrations with increasing postirradiation incubation time in ara C appears to be more rapid in the normal cells compared to the ML-1 cells (Figure 3.1). The frequencies of chromosome-type deletions and exchanges in the normal bone marrow cells showed a rapid increase with 0.5 and 1 h post-irradiation incubations in ara C, with little further increase for 2 and 3 h ara C incubations. In contrast, the frequency of deletions in the ML-1 cells did not increase until 1 h ara C incubation, and there was a slight increase at longer ara C incubation times. The frequency of exchanges in the ML-1 cells did not show an increase until 2 h ara C incubation and this frequency increased with 3 h ara C incubation. For the 3 h incubation in ara C, the frequencies of exchanges were similar in the ML-1 and normal bone marrow cells, whereas there is still a significant difference for deletions. We cannot yet offer an explanation for this latter observation.

There was little effect of post-irradiation incubation with ara C on the frequency of chromatid-type aberrations in either cell type (Tables 3.1 and 3.2).

Discussion

It has been shown that ara C inhibits the repair of DNA damage induced by chemicals and radiation (Karran, 1973; Hiss and Preston, 1977; Collins et

al., 1977; Dunn and Regan, 1979). This inhibition can be reversed by the addition of deoxycytidine (Karran, 1973; Hiss and Preston, 1977). One proposed mechanism for the inhibitory action of ara C involves the competitive binding of the ara C metabolite, ara CTP, at the d CTP binding site on the DNA polymerase molecule thus inhibiting repair resynthesis (Graham and Whitmore, 1970). Other proposed mechanisms involve the incorporation of ara CMP into a repairing region (Momparler, 1969; Graham and Whitmore, 1970). This is thought to either distort the DNA helix in such a way as to prevent further resynthesis (Graham and Whitmore, 1970), or resynthesis is halted because a terminal ara CMP residue is a poor primer for further polymerase action (Major et al., 1982).

Any of these mechanims would predict the accumulation of DNA single strand gaps at repairing regions with increasing time in ara C, and that the greater the number of regions that initiate repair per unit time, the greater the number of gaps that will be accumulated. Thus, post-irradiation incubation with ara C enhances the frequency of DNA gaps which are a normal intermediate during DNA excision repair. For X ray-induced DNA damage, single strand gaps accumulate in the presence of ara C primarily as a result of the inhibition of repair of base damage which, unlike directly-induced strand breaks, does require the excision and subsequent resynthesis of a number of bases. Such excision repair is inhibited by ara C (Hiss and Preston, 1977; Preston, 1980a; 1980b). A variety of investigators have used ara C to accumulate DNA gaps as a measurement of the amount of DNA repair occuring over a period of time (Hiss and Preston, 1977; Collins, 1977; Erixon and Ahnstrom, 1979; Dunn and Regan, 1979).

When ara C inhibition is reversed by the addition of dC, the greater the number of accumulated gaps, the greater the probability of a misrepair event during the completion of repair. This increases the probability for the formation of chromosome aberrations (Preston, 1980a; 1980b; 1981; 1982a). Similarly, in the absence of ara C, the more excision repair events that are initiated per unit time, the greater the probability of there being an interaction between DNA strands containing coincidently repairing regions leading to the formation of chromosome aberrations. Preston (1980b; 1981) has shown with Down syndrome lymphocytes, that an estimate of differences in repair kinetics betweeen cell types can be made from differences in the rate of increase of aberration frequency with ara C incubation after X irradiation.

To understand how differences in repair kinetics could lead to differences in sensitivity to X ray-induced chromosome aberrations, it is necessary to consider how variations in the kinetics of different steps of excision repair could affect the probability of the formation of a chromosome aberration. This is shown diagrammatically in Figure 3.2. This model assumes that cells with a similar amount of DNA will have a similar amount of X ray-induced DNA damage. The closed circles in Figure 3.2 represent base damage which, as discussed before, is subject to excision repair. For ease of presentation, the amount of repair is considered to be that which occurs in a unit of time from $t=0$ to $t=1$. Although total repair of all the induced damage is a sequential process, this model concentrates on those repairing regions which were initiated at $t=0$. Figure 3.2, step 1, shows the effect of differences in the rate of incision betweeen two cell types. In the example, twice the number of incisions per

Figure 3.2. Schematic of excision repair of X ray-induced base damage. Schematic of differences in excision repair of X ray-induced base damage in three hypothetical cell types: A, B, C, from time (t)=0 to time (t)=1. The figure shown at t=0 represents a double strand of DNA containing X ray-induced base damage (●). (A) Cell type with a rate of incision=4 incisions per unit time. (B) Cell type with a rate of incision=2 incisions per unit time. (C) Cell type with a resynthesis rate of 1 repairing region per unit time. (See text for further description).

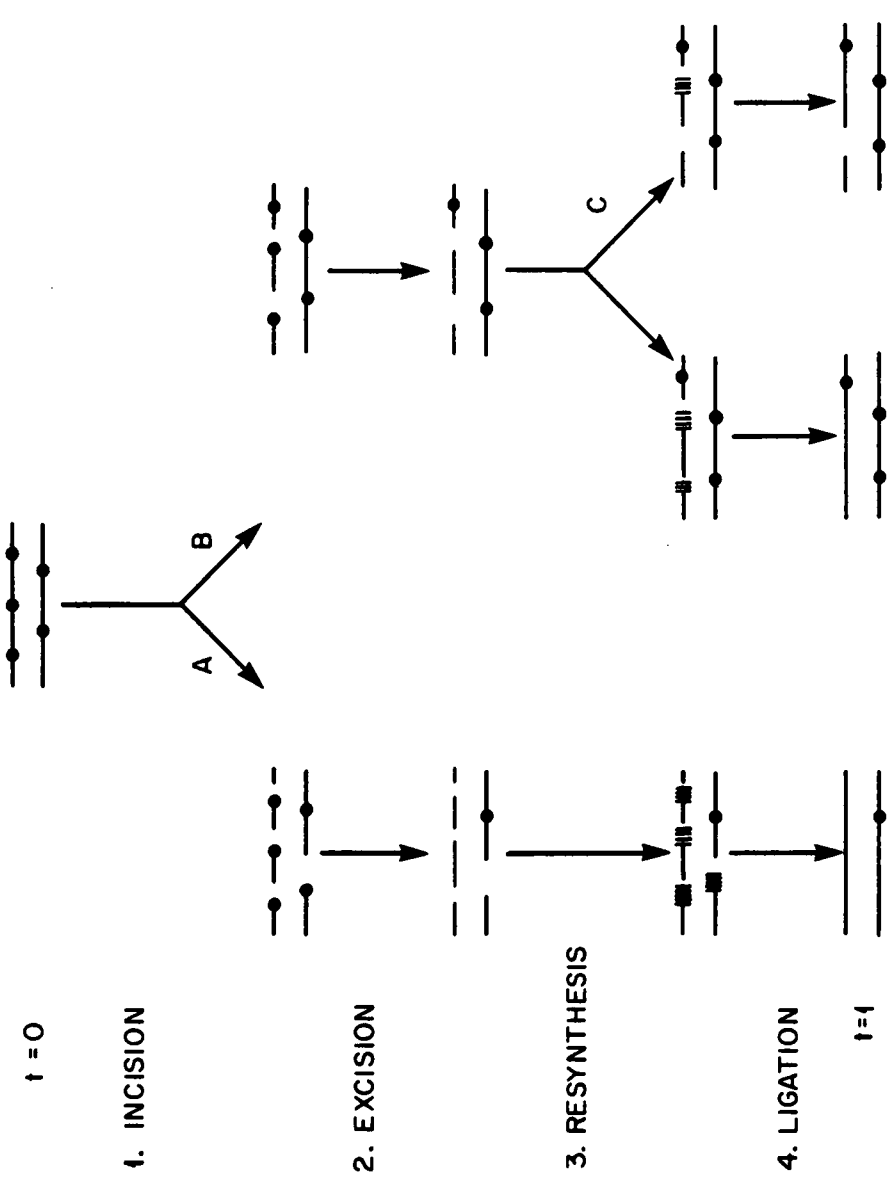

Figure 3.2

unit time results in the initiation of 4 repairing regions in A, versus only 2 in B. The DNA damage in the 4 repairing regions in A and the 2 in B next undergo excision, step 2. Cells conceivably can differ in the rate of excision, where a slower rate of excision would decrease the number of regions continuing through the repair process per unit time, but this model considers the number of repairing regions undergoing excision to be equivalent to the number of initiations. After excision, the resynthesis step 3 fills in the gaps in the DNA after which ligation, step 4, completes repair. If cells differ in their ability to resynthesize, as shown in C, it can be seen that repairing regions will be left open for a longer period of time in the cell where resynthesis occurs at a slower rate.

During the repair of DNA damage, a cell can either repair the DNA correctly such that no chromosome aberrations are formed, or misrepair the damage which involves an interaction between repairing regions. When misrepair occurs chromosome aberrations are formed. The probability of a misrepair event increases with the number of coincidently repairing regions (Preston, 1981). From Figure 3.2, cell type A versus B, it can be anticipated that cells of type A will have a greater number of coincidently repairing regions than cell B, due to the greater number of incisions initiating the process of excision repair. This increases the probability of an interaction occurring between repairing regions during the resynthesis step, leading to the formation of more aberrations in A than B with X rays alone. This can be tested utilizing ara C to inhibit resynthesis. The inhibition of resynthesis can be considered to be equivalent in both cell types provided both have the same ara C uptake and phosphorylating abilities along with similar competing levels of deoxynucleoside

pools, factors shown by Snyder et al.(1984) to be important in ara C inhibition. Because cell A has more repairing regions able to be inhibited by ara C per unit time compared to B, more gaps are accumulated in A than in B per unit time in ara C. When dC is added, the inhibition is reversed and because cell A has more accumulated gaps and these are now able to interact to form an aberration, A has a much greater increase in the frequency of aberrations with increasing time in ara C compared to B.

An example of type A cells is Down syndrome lymphocytes when compared to normal human lymphocytes (Preston, 1981). Down syndrome G_0 lymphocytes are more sensitive to X ray-induced aberrations than normal cells (Sasaki and Tonomura, 1969; Evans and Adams, 1970). Preston (1980b; 1981) found that Down cells show a more rapid increase in the frequency of chromosome aberrations with increasing ara C incubation time compared to normal cells. It was concluded that Down cells have a more rapid rate of incision of that X ray-induced DNA damage that can be converted into chromosome aberrations. Due to this faster rate of incision, there are more repairing regions per unit time in Down cells than in normal cells. This leads to the increased frequency of chromosome aberrations in Down cells compared to normal cells with X ray alone, as well as to the more rapid increase in the frequency of aberrations with increasing ara C incubation time.

We previously reported on the increased sensitivity to X ray-induced chromosome aberrations of ML-1 cells compared to normal mouse bone marrow cells (Aardema et al., 1985). In order to determine if repair differences were contributing to the observed differential X ray sensitivity between the two cell types, the repair kinetics were analysed using a similar experimental approach

to that with the Down versus normal lymphocytes. After 2.0 Gy of X rays, it was found that the frequencies of aberrations increased in both cell types when incubated with ara C, but the response with time was markedly different. For example, the normal cells had a rapid increase in chromosome aberrations with 0.5 h post-irradiation incubation in ara C while the ML-1 cells showed a slower increase, where no increase in aberrations was observed until 1 or 2 h ara C incubation. These results are different from those observed for the Down cells where X ray sensitive Down cells showed a more rapid increase in the frequency of chromosome aberrations with post-irradiation incubation in the presence of ara C compared to the normal cells. Thus, the increased sensitivity of ML-1 cells seems to be the result of a different mechanism from that in Down cells where a more rapid rate of incision could account for enhanced frequencies of X ray-induced aberrations.

The results with the ML-1 cells seem to be consistent with the idea that ML-1 cells function as a type C cell (Figure 3.2). A decreased rate of resynthesis or initiation of resynthesis as shown in step 3, type C, would leave repairing regions open longer which increases the probability of coincidently repairing regions being available to misrepair and form aberrations. Type C cells should be more sensitive to the induction of chromosome aberrations by X rays than type B. As previously reported (Aardema et al., 1985), ML-1 cells X irradiated in G_1, have an increased frequency of aberrations compared to normal bone marrow cells. In addition, many of these aberrations are of the chromatid-type. This is expected if the resynthesis step is sufficently delayed or slower in the ML-1 cells such that there is a greater chance that some of the X ray-induced damage will remain unrepaired when the cell enters the S phase.

Misrepair or misreplication of DNA damage in the S phase leads to the formation of chromatid-type aberrations. It has been previously shown (Aardema et al., 1985) that the ML-1 cells do not undergo an X ray-induced cell cycle delay in progression from G_1 into and through S. This would further increase the chances of forming a chromatid-type aberration by allowing less time for repair before replication occurs.

It was suggested above that ara C inhibition of excision repair requires, in part, incorporation of ara CTP during resynthesis. If resynthesis is slower, less ara C inhibition can occur; the repairing region in the absence of resynthesis will be similar to a gap resulting from ara C inhibition. There will be no effect on X ray-induced aberrations until sufficent resynthesis is underway to allow ara C to produce its inhibitory effect. The aberration frequency will then increase with increasing post-irradiation incubation with ara C. Our results presented here are consistent with this idea and show that the ML-1 cells have a slower increase in the frequency of aberrations with time in ara C compared to normal mouse bone marrow cells.

In conclusion, these results suggest that the ML-1 cells have a slower rate of excision repair resynthesis of the X ray-induced damage that can be converted into chromosome aberrations. This slower rate of resynthesis might account, in part, for the previously reported X ray sensitivity of the ML-1 cells compared to normal bone marrow cells.

CHAPTER 4

THE INDUCTION OF CHROMOSOME ABERRATIONS IN G_2 CELLS OF A MOUSE MYELOID LEUKEMIA CELL LINE AND NORMAL MOUSE BONE MARROW CELLS BY X RAYS AND CYTOSINE ARABINOSIDE

Summary

The frequency of X ray-induced chromosome aberrations was higher in G_2 ML-1 mouse myeloid leukemia cells compared to G_2 normal mouse bone marrow cells. Incubation of both cell types with ara C alone for 3 h until fixation resulted in an increase over untreated control levels of the frequency of chromatid-type deletions but not interchanges. The ML-1 cells were considerably more sensitive to ara C-induced aberrations compared to the normal cells. The ML-1 cells had an increase in deletions from 0.08 deletions per cell in untreated cells to 4.14 deletions per cell in ara C treated cells. This is compared to the normal cells which had 0.05 deletions per cell in untreated cells and 0.21 deletions per cell in ara C treated cells.

When G_2 cells were X irradiated and then incubated in the presence of ara C for 3 h until fixation, the deletion frequencies in both cell types were increased with a greater increase for ML-1 cells. However, no interchanges were observed in either cell type following the combined ara C and X ray experiment. These results indicate that G_2 ML-1 cells are more sensitive to

aberrations induced by X rays, ara C or the combined X ray and ara C treatment compared to normal cells. These results also show that ara C alone induces chromosome breaks either directly or indirectly. The deletions observed in metaphase cells after a combined ara C and X ray treatment could be produced either directly by ara C or by the inhibition of repair of X ray-induced damage by ara C.

Introduction

We previously reported in Chapter 2 that ML-1 cells irradiated in G_1 of the cell cycle have more chromosome aberrations than G_1 irradiated normal mouse bone marrow cells grown *in vitro*. In order to determine if this difference in sensitivity was present for cells irradiated in other phases of the cell cycle, the frequency of aberrations induced by X rays in G_2 ML-1 and normal cells was investigated. It was found that G_2 ML-1 cells had a higher frequency of X ray-induced aberrations compared to G_2 normal cells. A slower rate of DNA excision repair resynthesis was found in G_1 ML-1 cells as described in Chapter 3 and it is assumed that G_2 ML-1 cells also exhibit this repair deficiency. This slower rate of resynthesis could account, in part, for the higher frequency of X ray-induced aberrations in G_1 ML-1 cells and could also be contributing to the higher frequency of X ray-induced aberrations observed in ML-1 cells from G_2 of the cell cycle.

The increased radiosensitivity of G_1 and G_2 ML-1 cells is similar to that observed in fibroblasts from patients with ataxia telangiectasia (AT) and Fanconi anemia (FA) fibroblasts (Higurashi et al., 1973; Bigelow et al., 1979; Rary et al., 1974; Bender et al., 1985). AT and FA patients, along with other

chromosome breakage syndrome patients, show a predisposition to develop various types of neoplasms (for a review see German 1983). This points to some relationship between an increased sensitivity to chromosome aberration formation and the development of cancer. Investigating the sensitivity of the ML-1 cells to X rays and ara C may provide a means for further understanding some of the mechanisms leading to the increased sensitivity to the formation of chromosome aberrations.

Cytosine arabinoside (ara C) is an inhibitor of DNA synthesis and DNA excision repair resynthesis (Karran, 1973;Hiss and Preston, 1977;Collins et al., 1977; Dunn and Regan, 1979). Ara C also induces chromosome aberrations, either directly or indirectly, in G_O, G_1, S, or G_2 of the cell cycle (Kihlman et al., 1963; Brewen, 1965; Brewen and Christie, 1967; Preston, 1980a). We studied the effect of ara C and a combined ara C and X ray treatment on the frequency of chromosome aberrations in both ML-1 and normal mouse bone marrow cells. The ML-1 cells were found to have a higher frequency of ara C-induced aberrations compared to the normal cells. The combined X ray and ara C treatment resulted in an increase in aberration frequencies in both cell types but the ML-1 cells had the greater increase compared to the normal cells.

Materials and Methods

Cell Cultures

Normal mouse bone marrow cultures were established prior to each experiment using a modified version of the technique of Nakamura et al. (1972) as

previously described (Aardema et al., 1985). Briefly, male RFM mice 9-11 weeks old were sacrificed by cervical dislocation and the femurs were removed. Bone marrow cells were flushed out of the femurs with 37° RPMI 1640 (Gibco) containing 25% heat inactivated fetal bovine serum (Flow Laboratories) using a 22g needle. Pooled bone marrow cells were centrifuged and an aliquot of cells was treated with Zapoglobin IIR (Coulter Diagnostics) to lyse the red blood cells. White blood cell counts were determined using a Coulter Counter (Coulter Electronics, Model ZM). The cell concentration was adjusted to approximately 3.5×10^6 nucleated cells per ml. Cell cultures were established with 7×10^6 nucleated cells per 2 ml of media in 35 mm plastic culture dishes and maintained in an incubator humidified 95-100% in an atmosphere of 5% CO_2 in air.

The ML-1 cell line used in these experiments was established from an X ray-induced myeloid leukemia of an RFM mouse as previously described (Au et al, 1983). ML-1 cells were subcultured prior to each experiment, at 4×10^5 cells per ml of RPMI 1640 containing 10% heat inactivated fetal bovine serum. Cultures were incubated as described for the normal cells.

X Ray Experiments

X irradiations were performed with a G.E. Maxitron 300 kVp X ray Unit (General Electric, Milwaukee, WI) operated at 250 kVp and 15 mA with 3 mm of external aluminum filtration (h.v.l. 0.46 mm Cu). Cell cultures were exposed to 1.0 Gy at a dose rate of 1.0 Gy per minute. Normal bone marrow

cultures were irradiated 20 h after being established. The ML-1 cells were irradiated approximately 25 h after being subcultured.

In Series #1 experiments, ara C (5×10^{-5} M, Sigma) was added to each culture fifteen minutes prior to irradiation. The ara C remained in the cultures until fixation. The control cultures were: one untreated; one incubated with ara C for 3 h; one irradiated with 1.0 Gy but not treated with ara C. Tritiated thymidine ([3H]-dT) (specific activity 2.0 Ci/mmol, New England Nuclear) was added to each culture immediately after irradiation at a final concentration of 1 μCi/ml of culture media. The [3H]-dT remained in the cultures until fixation. 30 min after irradiation, colcemid (1×10^{-7} M, Sigma) was added to each culture. Cell cultures were harvested 2.5 h later, i.e.3 h after irradiation.

In Series #2 experiments, [3H]-dT Colcemid was added immediately after the addition of ara C and cultures were harvested 3 h later. Fixation and slide preparation were performed according to standard methods (Brewen and Gengozian, 1971)

Autoradiography

Slides were prepared for autoradiography using a technique similar to that described by Gude (1968). Briefly, slides were dipped in NTB2 liquid nuclear track emulsion (Kodak). The slides were air dried 1 h then stored in plastic slide boxes containing a desiccant. The boxes were covered and sealed with black tape and stored in the refrigerator at 4°C. Slides from Series #1 were exposed for 2 days and slides from Series #2 were exposed for 5 days. The slides were developed in Kodak D19 developer for 3 minutes, washed, then

fixed in Kodak Acid Fixer for 3 minutes. At least 100 metaphases or interphases were analyzed for the presence or absence of label.

Analysis Of Cells

Slides were centromere-stained (C-banded) using the method described by Salamanca and Armendares (1974). This technique does not significantly remove autoradiographic grains from the cells and allows for the analysis of aberrations in unlabeled metaphases. One hundred metaphases were analyzed from each treatment, and all classes of aberrations were recorded. The classes of aberrations were reported as: chromatid deletions, isochromatid deletions, chromatid intra- and inter-changes.

Results

Autoradiography

The frequencies of labeled and unlabeled metaphases for various treatments for the normal cell are given in Table 4.1 and for the ML-1 cells in Table 4.2. An analysis was made of the percentages of labeled and unlabeled metaphases in control and X irradiated normal and ML-1 cells 3 h and 4 h after 1.0 Gy of X rays. The results indicate that a 3h harvest yielded approximately the same ratio of labeled to unlabeled metaphases in both cell types and a larger percentage of labeled cells were observed in both cell types at the 4 h harvest (Tables 4.1 and 4.2). After irradiation of both cell types with 1.0 Gy and harvesting 3 h later, 100% of the normal cells and 91% of the ML-1

Table 4.1. The frequency of labeled and unlabeled metaphases and interphase nuclei in normal mouse bone marrow cells following various treatments

Treatment	Time	Percentage metaphases[a]		Number of interphases scored	Percentage interphases	
		L	U		L	U
Control	3 h	20	80	148	7	93
Control	4 h	25	75	*b*	*b*	*b*
1.0 Gy	3 h	0	100	141	6	94
1.0 Gy	4 h	4	96	*b*	*b*	*b*
ara C[c]	3 h	0	100	134	0.7	99.3
ara C + 1.0 Gy[c]	3 h	0	100	138	0	100
ara C [d]	3 h	0	100	150	6	94

[a]100 metaphases were analyzed for each treatment.

[b]Not determined.

[c]ara C was added 15 min. prior to tritiated thymidine, Series #1 experiments, Materials and Methods.

[d]ara C was added 30 min. after tritiated thymidine, Series #2 experiments, Materials and Methods.

cells were unlabeled (Table 4.1 and 4.2). The 4 h harvest after irradiation yielded 96% and 71% unlabeled cells in the normal and ML-1 cells, respectively, indicating that as expected, more S phase cells were progressing to mitosis at the 4 h harvest compared to the 3 h harvest. A 3 h harvest was chosen for the remainder of the experiments to insure that in both cell types greater than 90 % of the cells in mitosis were from G_2 and that the length of time for DNA repair to take place before mitosis is equivalent.

Table 4.2. The frequency of labeled and unlabeled metaphases and interphase nuclei in ML-1 cells following various treatments

Treatment	Time	Percentage metaphases[a]		Number of interphases scored	Percentage interphases	
		L	U		L	U
Control	3 h	51	49	142	65	35
Control	4 h	75	25	*c*	*c*	*c*
1.0 Gy	3 h	9	91	162	62	38
1.0 Gy	4 h	29	71	*c*	*c*	*c*
ara C[c]	3 h	0	100	170	0	100
ara C + 1.0 Gy[c]	3 h	0	100	162	0	100
ara C [d]	3 h	2	98	350	60	40

[a]100 metaphases were analyzed for each treatment.

[b]Not determined.

[c]ara C was added 15 min. prior to tritiated thymidine, Series #1 experiments, Materials and Methods.

[d]ara C was added 30 min. after tritiated thymidine, Series #2 experiments, Materials and Methods.

X Ray-Induced Aberrations

The frequencies of aberrations in unlabeled metaphases are given in Table 4.3. It can be seen that G_2 ML-1 cells have a higher frequency of chromosome aberrations induced by X rays than normal cells. X irradiated ML-1 cells had more deletions and twice as many interchanges as the normal cells; 0.10 interchanges per cell were observed in the normal cells after 1.0 Gy of X rays compared to 0.23 interchanges per cell in the ML-1 cells. We have previously shown that G_1 ML-1 cells are more sensitive to X ray-induced chromosome aberrations compared to G_1 normal cells (Chapter 2, Aardema et al., 1985)

Table 4.3. Effect of 1.0 Gy irradiation, ara C, and X irradiation plus ara C on the frequency of chromatid aberrations in normal mouse bone marrow and ML-1 cells in G_2

Cell type	Treatment	Abnormal metaphases[a] (%)	Chromatid aberrations per cell ± s.e.				
			Chromatid	Isochromatid	Combined deletion types	Interchanges	Gaps
NORMAL	Control	5	0.05 ± 0.02	0	0.05 ± 0.02	0	0
	1.0 Gy	45	0.62 ± 0.08	0.17 ± 0.04	0.79 ± 0.09	0.10 ± 0.03	0
	ara C	18	0.19 ± 0.04	0.02 ± 0.01	0.21 ± 0.05	0	0
	1.0 Gy + ara C	40	1.50 ± 0.12	0.38 ± 0.06	1.88 ± 0.14	0	0.14 ± 0.04
ML-1	Control	7	0.06 ± 0.02	0.02 ± 0.01	0.08 ± 0.03	0	0.01 ± 0.01
	1.0 Gy	67	0.85 ± 0.09	0.20 ± 0.04	1.05 ± 0.10	0.23 ± 0.03	0.06 ± 0.02
	ara C	95	3.10 ± 0.18	1.31 ± 0.11	4.41 ± 0.21	0	1.56 ± 0.12
	1.0 Gy + ara C	100	9.28 ± 0.30	3.09 ± 0.17	12.37 ± 0.35	0	1.94 ± 0.14

[a]100 metaphases were analyzed for each treatment.

and the above results indicate that G_2 ML-1 cells show the same increased radiosensitivity.

Ara C-Induced Aberrations

Treatment of both ML-1 and normal cells with ara C for 3 h until fixation resulted in an increase in the frequencies of chromatid deletions and isochromatid deletions compared to control levels (Table 4.3). No interchanges were observed in either cell type after ara C treatment (Table 4.3). The ML-1 cells showed a dramatic 55 fold increase in the frequency of deletions from 0.08 deletions per cell in untreated cells to 4.41 deletions per cell in ara C treated cells compared to the normal cells which had a four fold increase in the frequency of deletions from 0.05 deletions per cell in untreated cells and 0.21 deletions per cell in ara C treated cells (Table 4.3). Ara C clearly induced a low frequency of aberrations in the normal cells and 18% of the cells had aberrations. In contrast, the ML-1 cells had a high frequency of aberrations after 3 h of ara C and 95% of the cells had aberrations. In both cell types those deletions classified as isochromatid were apparently non-union proximal and distal (NUPD) where there was no rejoining of the broken portions of the chromosomes as has been described before (Preston, 1980a).

The results presented in Tables 4.1 and 4.2 show that the addition of ara C 15 minutes prior to the addition of [3H]-dT, as discussed in Series #1 in Materials and Methods, results in 0% labeled metaphases or interphases for either cell type. Ara C is an inhibitor of DNA synthesis, and therefore, it inhibits the incorporation of [3H]-dT into the replicating DNA of S phase cells.

This explains the absence of labeled interphases and metaphases in the above experiments. To confirm that only G_2 phase cells were being analyzed after ara C treatment in Series #1, [3H]-dT was added 30 min prior to the addition of ara C as described in Series #2 of Materials and Methods, and the cultures were harvested 3 h later. The results in Tables 4.1 and 4.2 show that 98% of the ML-1 metaphases and 100% of the normal metaphases were unlabeled after ara C treatment, indicating that all the analyzed metaphases were from G_2 treated cells.

One observation during the scoring of aberrations was that many of the deletions in the ML-1 cells induced by ara C seemed to be at specific chromosomal locations, as was observed by Kihlman et al. (1963). The ML-1 cells have one metacentric chromosome in the karyotype which is easily and unequivocably identifiable and the frequency of aberrations involving this chromosome were recorded. This analysis gives only a rough estimation of the specificity of chromosome breaks and the analysis of G-banded karyotypes is required for more detailed information. The deletions seemed to fall into four categories; small terminal deletions were given the designation type 1, a small terminal deletion on one arm combined with a larger deletion closer to the centromere on another arm was classified as type 2, the larger deletion alone was classified as type 3, an isochromatid NUPD was classified as type 4 (Table 4.4). The results in Table 4.4 show that treating ML-1 cells with ara C increases the percentage of aberrations involving the metacentric chromosome from 2 % with X rays alone to 7.7% with ara C and 5.9% with X rays plus ara C. Forty-four percent of the aberrations involving this chromosome were small

Table 4.4. Involvement of the Metacentric chromosome in ML-1 cells in aberrations induced by various treatments

Treatment	Number of metaphases analyzed	Percentage of total aberrations involving the metacentric	Percentage of different aberration categories[a]			
			1	2	3	4
Control	100	0	0	0	0	0
1.0 Gy	100	2.0	50	0	50	0
ara C	100	7.7	44	6	38	12
1.0 Gy + ara C	100	5.9	34	15	41	10

[a]Aberration categories: 1—small terminal deletion; 2—small and large terminal deletions; 3—large terminal deletion; and 4—isochromatid deletion. (See text for further description.)

terminal deletions of type 1; 38% were large terminal deletions of type 3. These results suggest that ara C may be affecting specific DNA regions leading to specific aberrations. This will be elaborated upon in the Discussion.

X Ray Plus Ara C-Induced Aberrations

When G_2 cells were X irradiated and incubated with ara C for 3 h until fixation, the frequencies of deletions were increased in both cell types (Table 4.3). In ML-1 cells, ara C alone induced 4.41 deletions per cell and X rays alone induced 1.05 deletions per cell (additivity=5.46) while 12.37 deletions per cell were induced by the combined X ray and ara C treatment. This was a greater increase than observed in the normal cells which had 0.21 deletions per cell with ara C, 0.79 deletions per cell with X rays (additivity=1.00) but 1.88 deletions per cell with X rays combined with ara C. Again, those deletions

scored as isochromatid were NUPD. With X rays alone, there were 0.10 and 0.23 interchanges per cell in the normal and ML-1 cells respectively, but no interchanges were observed in cells after irradiation combined with ara C. These results are in agreement with previous work which showed that X irradiation of G_2 cells followed by ara C incubation until fixation inhibits the production of interchanges (Preston, 1980a;Bender and Preston, 1982)

Table 4.4 shows the involvement of the ML-1 metacentric chromosome in aberrations induced by the combined X ray and ara C treatment. The metacentric chromosome was involved in 5.9% of the total aberrations induced and 34% of the aberrations involving this chromosome were type 1 small terminal deletions; 41% were type 3 larger terminal deletions. As with the ara C treatment, type 1 and type 3 aberrations were the most common aberration types in which the metacentric chromosome was involved.

Discussion

X Ray-Induced Aberrations

We previously reported that G_1 cells from the ML-1 cell line were more sensitive to the induction of chromosome aberrations by X rays compared to normal mouse bone marrow cells grown *in vitro,* (Chapter 2, Aardema et al., 1985). The results presented here are an extension of that work and show that ML-1 cells X irradiated in G_2 of the cell cycle have a higher frequency of chromatid aberrations compared to G_2 normal mouse bone marrow cells. Specifically, G_2 irradiated ML-1 cells had more chromatid-type deletions and

twice as many interchanges compared to the normal cells. Previous work (Aardema et al., 1985) described in Chapter 2 showed that both ML-1 and normal cells had a similar cell cycle delay in progressing from G_2 into mitosis after irradiation. Therefore, the higher frequency of aberrations in G_2 ML-1 cells after irradiation does not seem to be due to the ML-1 cells progressing into mitosis faster than the normal cells with unrepaired DNA damage forming deletions as the chromosome condense.

ML-1 cells in G_1 have been shown to have a slower rate of excision repair resynthesis of X ray-induced base damage as discussed in Chapter 3. It is assumed that G_2 ML-1 cells also undergo excision repair resynthesis more slowly than the normal cells. As discussed in Chapter 3 a slower rate of repair resynthesis in the ML-1 cells compared to normal cells, would leave DNA repairing regions open for a longer period of time. This would increase the probability of an interaction or misrepair event occurring between coincidently repairing regions leading to the increased probability that an aberration will be formed in the ML-1 cells compared to the normal cells. This explains, in part, the higher frequency of aberrations formed in G_1 ML-1 cells after irradiation than in normal cells and can also explain the increased frequency of aberrations observed in X irradiated G_2 ML-1 cells. In particular, a slower rate of repair resynthesis in the ML-1 cells would increase the chances of a cell entering mitosis with unrepaired or incompletely repaired DNA damage thus contributing to the high frequency of deletions and incomplete exchanges observed in the ML-1 cells.

Results reported for various L5178Y sublines which show sensitivities to different agents as discussed below, and the ML-1 cells reported here, support

the idea that there may be some general features found in cancer cells leading to an increased sensitivity to various agents as measured by the induction of chromosome aberrations as well as other endpoints such as mutation and cell killing. One subline of the mouse lymphoma L5178Y cells that was sensitive to X ray-induced killing, was shown to have a higher frequency of X ray-induced aberrations compared to an L5178Y X ray resistant subline (Scott et al., 1974; Scott and Zampetti-Bossesler, 1980). Another L5178Y subline, M10, which is sensitive to killing by ionizing radiation and methyl methanesulphonate (MMS), had a higher frequency of gamma ray- and 4-nitroquinoline oxide (4NQO) induced aberrations compared to parental cells (Takahashi et al., 1982; 1983) L5178Y subline M13 is sensitive to UV and 4NQO killing and had a higher frequency of UV- and 4NQO-induced chromosome aberrations compared to parent cells (Takahashi et al., 1982; 1983).

Evidence for a relationship between increased sensitivity to chromosome aberration formation and the induction of cancer comes from studies of human syndromes described as "chromosomal breakage syndromes" (German, 1969). Higurashi et al. (1973), showed that G_0 cells from ataxia telangiectasia (AT), Bloom syndrome (BS) and Fanconi anemia (FA) patients had a higher frequency of radiation-induced aberrations compared to control cells and it is known that these patients have a predisposition to develop various neoplasms (for a recent review, see German, 1983). It has also been shown that G_2 cells from FA (Bigelow et al., 1979) and AT cells (Rary et al., 1974; Bender et al., 1985) have high yields of chromatid aberrations after irradiation. Xeroderma pigmentosum (XP) patients are sensitive to the induction of skin cancer by

sunlight and fibroblasts from these patients have been shown to be more sensitive to the induction of chromosome aberrations by UV than normal fibroblasts (for a review, see Cleaver and Bootsma, 1975). Comparative studies between cancer cells and cells from patients which have a predisposition to develop cancer offer an opportunity to elucidate some of the mechanisms involved in cells becoming neoplastic. Since both cell types seem to have an increased chromosomal instability, studies of the factors contributing to the increased aberration formation in these cells may help define some common defects. The results described in Chapters 2, 3 and 4 of this thesis suggest that the lack of cell cycle delays and differences in DNA repair kinetics may be characteristics in common in cells with increased sensitivity to chromosome aberration formation.

Ara C-Induced Aberrations

The ML-1 cells treated with ara C for 3 h until fixation showed a much higher frequency of deletions compared to the normal cells. The sensitivity of the ML-1 cells to ara C could be due to a variety of reasons as discussed below and further experiments are required to provide definitive answers. It is possible that the Ml-1 cells have more replication errors which could be due to a less efficient 3′-5′ proofreading exonuclease. Errors in DNA replication may persist until the cell enters G_2 at which time they may undergo repair that can be inhibited by ara C. This could lead to the chromosome deletions observed at metaphase. It is possible that the addition of ara C disturbs the nucleotide

pools to a greater extent in the ML-1 cells than in the normal cells and interrupts or inhibits some maintainence repair that normally corrects spontaneously arising DNA lesions or those DNA errors left from replication.

Another possibility is that the ML-1 cells have fragile DNA; perhaps they have specific fragile sites as suggested by the results in Table 4.4 which show that aberrations in the ML-1 metacentric chromosome occur in localized regions. It has been suggested by Pantelias and Wolff (1985) that ara C directly induced breaks. Fragile sites may be more susceptible to this ara C-induced breakage. Another possibility is that the fragile DNA may break naturally and the presence of ara C until fixation prevents the fragile sites from rejoining or condensing properly leading to chromosome breaks at those sites.

It has been shown by Pantelias and Wolff (1985) that treatment of G_0 human lymphocytes with ara C results in a high frequency of chromosome fragments when the chromosomes are examined by premature chromosome condensation (PCC). It has been shown, however, that G_0 lymphocytes treated with ara C and analyzed at metaphase have very low yields of aberrations (Brewen and Christie, 1967; Preston, 1980a; Bender and Preston, 1982). Pantelias and Wolff explained this discrepancy as being the result of selection against damaged cells progressing from G_0 into mitosis. Another explanation is that ara C-induced breaks are normally rejoined rather efficiently such that only a few misrepair or non-repair events can take place, leading to the low frequencies of aberrations observed at metaphase. The chromosome breaks observed in the PCC technique after treatment of G_0 cells with ara C may be breaks which normally are not involved in aberration formation. If ara C

induces single strand lesions or breaks as suggested by Pantelias and Wolff, then the PCC technique itself may be inducing "artificial" deletions at these sites of ara C-induced breakage. The unrejoined lesions could be pulled apart by the chromosome condensation such that they will be unable to rejoin, leading to an elevated frequency of chromosome deletions. In a similar manner, ara C damage induced in G_2 in ML-1 and normal cells can be resolved into chromosome deletions the chromosomes condense in mitosis. If ML-1 cells have more ara C-induced damage because of fragile regions or repair deficiencies, than more chromosome deletions will be produced. This is what was observed, although the specific mechansim has not been identified.

X Ray Plus Ara C-Induced Aberrations

The combined treatment of G_2 cells with X rays and ara C gave a greater yield of chromosome deletions than the additive effects of 1.0 Gy and ara C given separately. This is in agreement with other studies where X rays and ara C treatments were combined (Preston, 1980a; Bender and Preston, 1982). The ML-1 cells had a higher frequency of aberrations induced by the combined treatment than the normal cells.

With 1.0 Gy of X rays alone, 0.10 and 0.23 interchanges per cell were observed in the normal and ML-1 cells respectively. When ara C was present until fixation, no interchanges were produced. This indicates that ara C inhibits the repair of all the X ray-induced damage which results in interchanges (Preston, 1980a; Bender and Preston, 1982). It has been proposed that ara C inhibits the repair of X ray-induced base damage (Preston, 1980a) and the

inhibition of repair in G_2 by ara C, directly or indirectly results in double strand breaks that appear as chromosome deletions at metaphase. Thus, only deletions and not interchanges will be observed.

It seems that the deletions observed at metaphase, particularly in ML-1 cells that were irradiated in G_2 and treated with ara C until fixation, result from at least two mechanisms based on the clastogenicity of and repair inhibition by ara C. The deletions can either result directly or indirectly from ara C or from the inhibition by ara C of the repair of X ray-induced damage.

The ML-1 cells were shown previously to be sensitive to X ray-induced aberrations in G_1 and are also shown here to be more sensitive than normal cells to aberrations induced by X rays in G_2. ML-1 cells also show an unusually high frequency of ara C-induced aberrations as well as an increased sensitivity to a combined ara C and X ray treatment compared to the normal cells. These results further define the increased sensitivity of the ML-1 cells described in Chapters 2 and 3 of this thesis and suggest the possibility that the DNA in these cells has an inherent fragility.

CHAPTER 5

EPILOGUE

There were two major objectives of the experiments described in Chapter 2. The first objective was to determine if the mouse myeloid leukemia cell line, ML-1, was differentially sensitive to X ray-induced chromosome aberrations compared to normal mouse bone marrow cells grown *in vitro*. In carrying out the first objective, a second objective was established and that was to determine the effect of X irradiation on the cell cycle progression of both cell types.

The experiments designed to fulfill the first objective led to the identification of the ML-1 cells as an X ray sensitive cell line where a higher frequency of chromosome aberrations were induced by X rays in the ML-1 cells than in the normal cells. Several important observations were made from these results. The cells analyzed in the study in Chapter 2 were in G_1 of the cell cycle at the time of irradiation and, therefore, only chromosome-type aberrations were expected. The ML-1 cells had a higher frequency of chromosome-type aberrations than the normal cells but chromatid-type aberrations were also observed in the ML-1 cells. This was an interesting finding since it has been reported that X irradiated G_1 AT cells also have chromatid-type aberrations. It is possible that ML-1 and AT cells have characteristics in common that contribute to their increased radiosensitivity.

As discussed in Chapter 1, the interval of time in which DNA repair occurs between treatment and DNA synthesis affects both the types and frequencies of aberrations observed. With this in mind, the presence of

chromatid-type aberrations in the ML-1 cells and not in the normal cells suggested that the ML-1 cells were progressing more rapidly into the S phase following irradiation than the normal cells and/or that there was a repair defect in the ML-1 cells which left unrepaired DNA damage in the ML-1 cells at the time of replication. Experiments were designed to satisfy the second objective of Chapter 2, to study the X ray-induced cell cycle delays in both cell types. Two experimental techniques were used in the analysis of the effect of X rays on cell cycle progression, flow cytometry and mitotic index analyses. These experiments demonstrated that X irradiated normal cells were delayed in progression into and through the S phase. This type of delay has been observed in other normal cell types by various investigators and it has been proposed that cell cycle delays serve as a protective mechanism which allows normal cells extra time to repair DNA damage before DNA replication takes place. In contrast, the ML-1 cells did not have the same delay of progression into the S phase after irradiation. It was concluded that the lack of a G_1 cell cycle delay allowed the ML-1 cells to progress into the S phase where unrepaired DNA damage can be converted into chromatid-type aberrations, thus contributing to the increased X ray sensitivity of the cells.

These results suggest the following areas for further research. Since the ML-1 cells progress into the S phase with unrepaired DNA damage, it would be interesting to analyze the SCE in these cells. It would be expected that the replication of damaged DNA in the ML-1 cells would increase replication errors leading to the induction of SCE. It would also be interesting to determine if the ML-1 cells *in vivo* have the same increased radiosensitivity and cell cycle progression after irradiation. An *in vivo* study would allow for a direct

comparison between ML-1 and coexisting normal bone marrow cells and the presence of a marker chromosome in the ML-1 cells makes this sort of experiment feasible.

Having demonstrated that the normal cells undergo a cell cycle delay after irradiation, it would be important to know what serves as the "signal" for cells to slow their progression through the cell cycle. Perhaps of even greater importance would be to determine what causes cancer cells and other abnormal cells not to be delayed. Is the "signal" missing? Are the cells unable to respond to the "signal"? In speculating about the possible answers to these questions, it is tempting to think in terms of the inducible SOS system in bacteria which is called into action by DNA damage or anything that interferes with DNA replication. The inducing signal in the SOS system is believed to be DNA damage in the form of single-stranded regions. Some system perhaps even more elaborate than the SOS cascade, could function in mammalian cells. A logical inducing signal is DNA damage.

A mammalian system with some features in common with the SOS system involves poly(ADP-ribose). It is known that DNA or damaged DNA is involved in the synthesis of the polymer, and the polymer has been implicated in many cellular functions including DNA repair. Normal cells have an increased level of poly(ADP-ribose) after irradiation and some AT cells lines have been shown not to have an increased level of the polymer after irradiation (Edwards and Taylor, 1980; Zwelling et al., 1983). It is possible that, like AT cells, ML-1 cells do not have an increased level of poly(ADP-ribose) after irradiation which may relate to a DNA repair defect in AT as well as ML-1 cells.

One study would be to measure poly(ADP-ribose)polymerase activities in the ML-1 cells and normal cells. Another study would be to analyze the effect of 3-aminobenzamide, an inhibitor of poly(ADP-ribose)polymerase, on the induction of chromosome aberrations after various treatments in the two cell types.

Taking a slightly different approach, it would be interesting to determine how damaged DNA affects the S phase of the cell cycle by analyzing the rates of DNA synthesis in both cell types after irradiation. This could be accomplished using synchronized cells prelabeled with [14C]-thymidine, treating them with X rays, then growing the cells in media containing [3H]-thymidine. A measurement could then be made of the tricholoroacetic-acid-precipitable radioactivity and the ratio of 3H dpm to 14C dpm would give a comparison of the rates of DNA synthesis in the two cell types. It would be interesting to know if irradiation affects replicon initiation and/or chain elongation in the ML-1 cells as it must in the normal cells.

The presence of chromatid-type aberrations in G_1 ML-1 cells is similar to what has been reported for AT cells as described in Chapter 2. It has also been reported that some AT cells have altered repair kinetics for X ray-induced base damage, therefore, it seemed possible that there are possible repair differences between the ML-1 and normal cell used in these studies. The results suggested that there was X ray-induced DNA damage persisting in the ML-1 cells as they entered the S phase. The objective of the experiments described in Chapter 3 was to define the repair differences between the two cell types. Experiments utilizing the excision repair inhibitor, ara C, were conducted and it was determined that the ML-1 cells had a slower rate of excision repair resynthesis than the normal cells. This slower rate of resynthesis would leave

repairing regions open for a longer period of time in the ML-1 cells than in the normal cells which increases the probability of an interaction (misrepair) occurring between coincidently repairing regions leading to the formation of chromosome aberrations. This repair deficency may explain, in part, the increased frequency of aberrations in the ML-1 cells after irradiation compared to the normal cells.

To further define the repair capacities of the normal and ML-1 cells, it would be informative to examine the kinetics of DNA repair of X ray damage more directly in both cell types by using the alkaline elution technique of Kohn et al., (1976) to measure the accumulation of breaks from cells irradiated and with or without ara C incubation. One assumption made in the interpretation of the results in Chapter 3 was that the inhibition of DNA repair by ara C was equivalent in both cell types. To test this, an analysis could be made in both cell types of 1) differences in inherent sensitivity of the DNA polymerase to inhibition 2) differences in uptake and incorporation of ara C into DNA 3) differences in levels of competing deoxynucleoside pools.

The experiments described in Chapter 4 had two main objectives: 1) to determine if the differences in sensitivity between ML-1 and normal cells observed for G_1 treated cells was present in other stages of the cell cycle 2) to determine the effect of ara C on X ray-induced aberrations. Chromosome aberrations induced in G_2 cells following treatment with X rays, ara C and the combination of ara C and X rays were analyzed. The following conclusions can be made from the results. G_2 ML-1 cells had a higher frequency of aberrations induced by X rays than normal cells. This indicated that G_2 ML-1 cells had the same increased radiosensitivity relative to normal cells as was observed in

G_1 cells. Ara C and ara C plus X rays induced an unusually high frequency of aberrations in G_2 ML-1 cells than in normal cells. One area for furture study would be to look at the effect of ara C treatment in S phase cells and measure another endpoint, SCE frequencies. It is known that inhibitors of DNA replication like ara C, increase the frequency of SCE. Since the ML-1 cells seem to be more sensitive to chromosome breakage by ara C, it can be hypothesized that S phase cells would show an increased frequency of SCE after ara C treatment.

One of the main conclusions that was made from these results was that the ML-1 cells may have DNA which is inherently fragile. Fragile DNA has been described previously in the X chromosome of patients with a particular mental retardation syndrome now known as the fragile-X mental retardation syndrome (for a review, see Tariverdian and Weck, 1982). Fragile sites are detected as gaps or breaks in the chromosome when cells are grown in media free of folic acid orthymidine. One logical approach to further investigate if the ML-1 cells have fragile DNA would be to analyze the karyotype of cells grown in media free of folic acid and thymidine. Another approach would be to study G-banded karyotypes from cells that were irradiated and incubated with ara C to determine if there are specific chromosome locations involved in the aberrations as was indicated by the results presented in Chapter 4.

We conclude from the results presented in Chapters 2-4 that the ML-1 cells lack a cell cycle delay after irradiation, have an excision repair deficiency, and may have fragile DNA. It is known that AT patients have a recessive mutation which results in deficient DNA repair and a predisposition to develop cancer. The similarities between ML-1 and AT cells, as described in this thesis

(Table 5.1), suggest that ML-1 cells also have a genetic defect and this defect may have been present in a progenitor cell prior to leukemogenesis contributing to the induction or developement of the myeloid leukemia. It is hoped that the analysis and identification of some specific differences between the ML-1 and normal cells presented here will contribute to the further understanding of the complexities of cellular transformation and cancer.

Table 5.1. Comparison of different characteristics observed on ML-1 and ataxia telangiectasia cells

Characteristic (compared to respective normal type)	Cell type	
	ML-1	AT
Increased aberration formation after X irradiation—G_1 cells	+	+
Chromatid-type aberrations observed in cells irradiated in G_1	+	+
Increased aberration formation after X irradiation—G_2 cells	+	+
Less cell cycle delay in progression from G_1 into S	+	+
Less cell cycle delay in progression from G_2 into M	−	+/−
Less inhibition of DNA replication after irradiation	n.d.	+
Higher spontaneous SCE frequency	−	−
Sensitivity to other agents—bleomycin, 4NQO neocarzinostatin	n.d.	+

n.d. = not determined

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