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Mechanisms for Induction of Mutations and Chromosome Alterations

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Genotoxic agents induce chromosomal alterations, such as aberrations, micronuclei, and sister chromatid exchanges as well as mutations both *in vivo* and *in vitro*. Ionizing radiation and typical radiomimmetic agents such as bleomycin are very efficient inducers of chromosomal aberrations. The type of aberrations induced by these agents are cell-cycle dependent, i.e., chromosome type in pre-replication stages and chromatid type in post-replication stages of the cell cycle. Under optimal DNA repair conditions, DNA double-strand breaks (DSBs) appear to be the most important lesion responsible for the production of aberrations. In human lymphocytes, fast-repairing DSBs lead to exchange-type aberrations. The fact that the dose–response curves for induction of exchange aberrations induced by ionizing radiation are similar *in vitro* and *in vivo* allows one to use the yield of induced aberrations to estimate absorbed radiation dose in the case of accidents. In this respect, frequencies of translocations detected by the chromosome painting technique appear to be more sensitive. Mutations do not express immediately after exposure and require an expression time before they can be detected. In humans, it is estimated that for the mutations induced in bone marrow, it takes about 2 months for them to express and to be detected in peripheral blood lymphocytes. Hence, frequency of mutations is of limited value for estimating radiation doses immediately after an accident. This holds true for chemical exposure as well. Most of the lesions induced by chemical mutagens (such as alkylating agents) are converted into aberrations only during the S phase of the cell cycle, and therefore an intervening DNA synthesis following exposure is necessary for the visualization of aberrations. These agents induce mainly the chromatid-type aberrations and are also very efficient in inducing sister chromatid exchanges. Chemical mutagens are more efficient in inducing gene mutations than ionizing radiations. In monitoring human populations, mutations in the *hprt* locus in peripheral lymphocytes and hemoglobin mutations as well as glycophorin mutations in erythrocytes can be studied. The *hprt* mutations and glycophorin mutations can arise from single base-pair changes to large deletions, whereas hemoglobin mutations arise from changes in a single codon.

Introduction

Exposure of the human population to genotoxic agents can be monitored by measuring DNA and protein adducts as well as by assessing biological effects such as chromosomal alterations and gene mutations. All genotoxic agents induce these events *in vitro* and *in vivo*. Because both chromosomal alterations and gene mutations are known to be involved in inherited disorders as well as in etiology of human neoplasms, it is important to develop methods to assess these events after human exposure to known genotoxic agents. In this paper the basic mechanisms of induction of these events and methods to detect them in human cells are reviewed.

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Chromosomal Alterations

Chromosomal alterations can be assessed by the frequencies of chromosomal aberrations (CA), micronuclei (MN), and sister chromatid exchanges (SCEs). Ionizing radiation and typical radiomimetic agents such as bleomycin are efficient inducers of chromosomal aberrations. The type of aberrations induced by these agents depends on the stage of cell cycle at the time of treatment. They induce chromosome-type aberrations in prereplicative stages and chromatid-type aberrations in S-phase and post-replicative stages. Among the different types of DNA lesions induced by ionizing radiation [namely, single-strand breaks (SSBs), double-strand breaks (DSBs), base damages, and DNA–protein cross links], DSB appears to be the critical lesion leading to chromosomal aberrations. Biochemical and cytological evidences leading to this conclusion are *a*) high LET radiation induces more DSBs than low LET radiation and for a given dose induces more chromosomal aberrations; *b*) when X-irradiated Chinese hamster fibroblasts are post-treated with Neurospora endonuclease, a treatment which converts SSBs into

DSBs, the frequencies of DSBs and chromosome aberrations increase to a similar extent (*1,2*); and *c*) restriction endonucleases, which induce exclusively DSBs efficiently induce chromosomal aberrations (*3,4*), and the cell-dependent pattern of the types of aberrations induced by REs is similar to ionizing radiation (*4*).

Human peripheral lymphocytes, because of their availability, are usually employed to monitor exposure to genotoxic agents. The lymphocytes are in a dormant G_0 stage and divide *in vitro* on stimulation. Two major classes of aberrations are recognized after irradiation of human lymphocytes, namely, exchanges (dicentrics, translocations, rings) and fragments. The kinetics of repair of DNA DSBs induced by ionizing radiation in human cells is biphasic, with a fast component in which about 80% of breaks are repaired within 10 min and a slow component that lasts for some hours (*5*). X-ray-induced dicentrics appear to be formed by misrepair of fast-repairing DSBs. Evidence for this comes from three types of experiments. First, when irradiated lymphocytes are post-treated with cytosine arabinoside (araC), the frequencies of dicentrics increase by a factor of 2, and this increase is confined to the first 15–30 min after irradiation (*6,7*). Second, there is no influence of araC on the repair of DNA strand breaks when challenged 1 hr after irradiation (*7*). Finally, the frequencies of dicentrics induced are similar in lymphocytes that were fused with mitotic CHO cells to induce premature chromosome condensation (PCC) immediately after irradiation to those that were stimulated and grown for 48 hr and scored in mitotic metaphases (*8*). When the fusion was carried out after different repair periods, the frequencies of dicentrics remained the same, whereas the frequencies of acentric fragments declined with time, indicating that most of the dicentrics were formed immediately after irradiation [(*8*), Fig. 1].

Similar frequencies of dicentric chromosomes are induced in human lymphocytes by ionizing radiation for a given dose under *in vitro* and *in vivo* conditions. Dose–response curves for induced frequencies of dicentric chromosomes in lymphocytes generated after *in vitro* irradiation (of different qualities) have been successfully used to estimate absorbed radiation dose of individuals in the case of radiation accidents, based on the frequencies of dicentrics observed in lymphocytes (*9*). The distribution of dicentrics among the lymphocytes can be used to discern partial body exposure, high or low LET exposure, acute or chronic exposure, external or internal exposure, etc. (*10*). This technique was effectively used in a recent radiation accident in Goiania, Brazil (*11*).

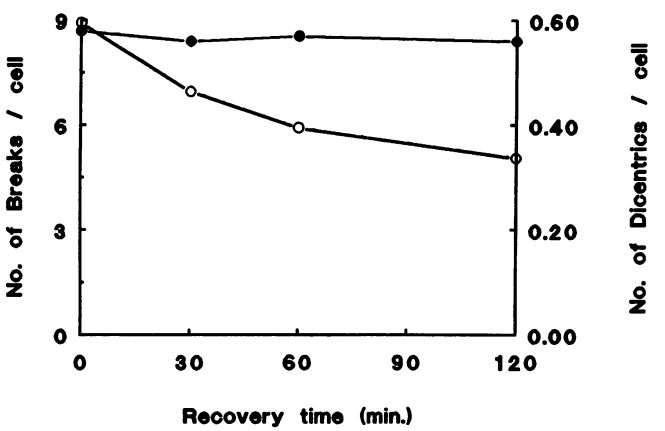

FIGURE 1. Frequencies of chromosome breaks and dicentrics detected in premature chromosome condensation after irradiation of lymphocytes with 3 Gy of X-rays at different recovery times. Note that frequency of dicentrics remains constant while the frequency of breaks declines with time. Data from Vyas et al. (*8*).

In case of external exposure, the frequencies of dicentrics decrease with time, whereas in the case of internal exposure the frequencies increase with time up to a certain period (*11,12*). Dicentrics are unstable aberrations and will be eliminated during cell divisions. Though from the radiation dosimetric point of view, dicentrics are very important, from the genetic risk point of view they are not important. On the other hand, stable aberrations like balanced chromosome translocations are very important from the risk point of view, as they are not eliminated during cell divisions and thereby perpetuate.

Unlike dicentrics, translocations are difficult to detect unless one uses special staining techniques such as G or Q banding to identify individual chromosomes in every cell. This procedure is very slow and laborious. Recently, human chromosome-specific DNA libraries have become available. This has made it possible by *in situ* hybridization with biotinylated chromosome-specific probes and immunochemical staining ("chromosome painting") to specifically stain individual chromosomes (*12,13*). This technique offers the possibility to detect chromosomal translocations very efficiently. In earlier studies using chromosome banding techniques, it has been reported that the frequencies of radiation-induced dicentrics and reciprocal translocations are formed with equal frequencies (*14*). With the chromosome painting technique, it is possible to discern translocations involving very small segments. In addition, types other than reciprocal translocations, such as interstial and terminal translocations, can be identified (*15*).

In human lymphocytes irradiated *in vitro*, using specific probes for six different chromosomes, we have estimated the frequencies of translocations and found that they are about two times more than the dicentrics depending on the dose used (*15*). The dose–response curve for X-ray-induced dicentrics and translocations is presented in Figure 2. These increased frequencies open up the possibility of using translocation frequency as a more sensitive method for biological dosimetry compared to the use of dicentric frequency (*15*).

In a follow-up study of the victims of the radiation accident in Goiania, we have detected balanced translocations involving specific chromosomes in their lymphocytes. Some individuals had several cells (up to 6%) with the same translocation involving chromosome no. 2, indicating the existence of clones containing that specific aberration (*16*). Taking into consideration translocations detected by the chromosome painting technique, estimates of doses of

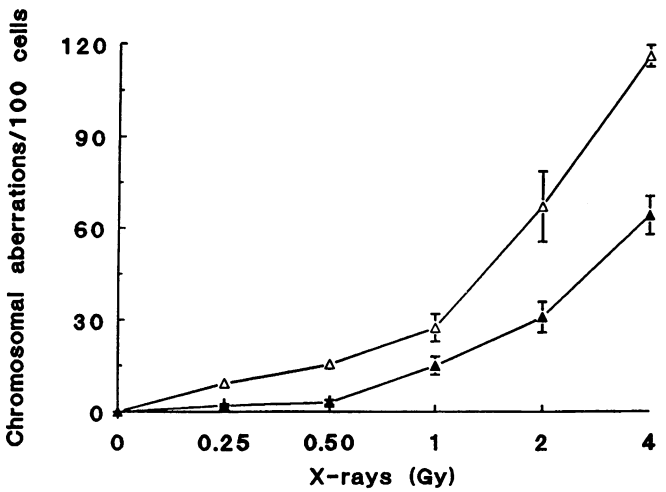

FIGURE 2. Frequencies of dicentrics (lower curve) and translocations (upper curve, as detected by *in situ* hybridization) induced by different doses of X-rays. Data from Natarajan et al. (*15*).

past radiation exposures in the victims of Hiroshima and Nagasaki have been reported (*17*). But the presence of clones containing the same translocation in some individuals may make dose estimation of past exposures difficult.

Many of the chromosome fragments that lag during anaphase movement will form micronuclei, which can be detected in the interphase of the daughter cells. However, for quantitative evaluation of the frequencies of micronuclei, it is essential to estimate the number of cells that have undergone one cell division. This has been made possible by the use of cytochalasin B, which inhibits cytokinesis, giving rise to binucleated cells after division (*18*). If one restricts scoring to micronuclei in binucleated cells, it is possible to accurately estimate the frequencies. The dose–response curve for induction of micronuclei in human lymphocytes induced by X-rays is curvilinear, indicating that part of the micronuclei arise from the acentrics associated with two-hit events, i.e., dicentrics (*19*). A parallel analysis of chromosome aberrations and micronuclei in irradiated lymphocytes indicated that about 70–90% of fragments form micronuclei at low radiation doses (up to 2 Gy), whereas at higher doses about 40% of fragments formed micronuclei (*19*). This low efficiency of the micronucleus technique at higher radiation doses appears to be due to the facts that *a*) some of the fragments are included into the main nuclei and *b*) cells carrying no or a lower number of fragments divide earlier than highly affected cells. The frequencies of spontaneously occurring micronuclei vary between individuals, and this makes it difficult to use frequencies of micronuclei as a biological dosimeter to estimate absorbed radiation dose, especially at low exposure levels.

Micronuclei can be formed by lagging whole chromosomes or acentric fragments. These two types of micronuclei can be distinguished by immunological staining of centromeres by CREST antibodies or by *in situ* hybridization with centromere-specific probes. It can be shown that most of the micronuclei induced by ionizing radiation originate from acentric fragments, whereas those induced by agents that interfere with chromosome separation (such colchicine, vincristine, etc.) originate from lagging chromosomes.

Chemically Induced Chromosomal Alterations

Genotoxic chemicals induce a wide variety of DNA lesions in different proportions. Unlike ionizing radiation-induced chromosome aberrations, which are formed immediately after exposure irrespective of the treated cell-cycle stage, most of the chemically induced aberrations are formed only during the DNA synthesis phase, probably due to misreplication. *In vivo* human exposure to chemical mutagens induce lesions in the DNA of lymphocytes, most of which are repaired by cellular repair enzymes. Unrepaired lesions give rise to chromatid-type aberrations during the S phase, when the lymphocytes are stimulated *in vitro*. However, sometimes chromosome-type aberrations can also be found, which are probably formed before stimulation in the G_0 stage by a different mechanism, (e.g., apurinic or apyrimidinic sites converted into strand breaks and misrepaired).

A sister chromatid exchange (SCE) is a cytological manifestation of DNA breakage and rejoining at apparently homologous sites on the two chromatids of a single chromosome. SCEs are efficiently induced by those agents that form covalent adducts to DNA or otherwise interfere with DNA metabolism or repair. The baseline frequency of SCEs varies between individuals, and smokers have increased baseline frequencies in their lymphocytes. Elevated frequencies of SCEs have been found in humans exposed to known genotoxins. A subpopulation of lymphocytes with high frequencies of SCEs (high-frequency cells) has been found in many human population studies (*20,21*). These cells may represent persistent DNA lesions in long-lived lymphocytes or a sensitive subpopulation. In biomonitoring human populations, consideration of only high-frequency cells appears to be a more sensitive index than consideration of overall SCE values, as demonstrated in a recent study on ethylene oxide-exposed workers (*21*).

Gene Mutations

Mutations can arise due to single base-pair changes (transition, transversion, frame shift) and small or large rearrangements (deletions, translocations, etc.). In humans, mutations can be studied in lymphocytes and erythrocytes. In erythrocytes, hemoglobin mutations and glycophorin mutations can be detected. Hemoglobin mutations arise as single base-pair change in one of the codons (transition, transversion or deletion) in the α or β chain. Many hemoglobin mutations such as sickle cell anemia and others occur in nature. Monospecific polyclonal antibodies have been raised against several of these mutations, which can be used to detect such mutations in a population of erythrocytes (*22*). Because the frequencies of such muta-

tions are very low, millions of cells have to be screened to detect these rare events, and the automated image analysis system has been developed for this purpose (*23*). A large increase in the frequency of hemoglobin mutants has been detected in radiation accident victims from Goiania (*11*).

Erythrocytes can also be screened for mutations in glycophorin A locus. This cell-surface glycoprotein occurs in two allelic forms (M and N) and is codominantly expressed. Mutation in this locus can be studied only in heterozygous (M/N) individuals. Monoclonal antibodies for individual allelic forms are conjugated with a different fluorescent dye and used to label fixed erythrocytes. Flow cytometry and sorting are used to estimate the frequency of cells that lack the expression of one of the GPA alleles. Because this system is based on loss of gene expression, single base-pair or gross changes can lead to this mutations. Increases in the frequency of mutants have been found in atom bomb survivors and chemotherapy patients (*24*).

There are three systems available to screen for mutants in human lymphocytes, namely, HPRT, HLA, TCR-CD3 assays. Of these, HPRT mutations, which are selected as resistance to 6-thioguanine, have been used in many laboratories (*25–27*). Using this method, increases in mutations have been found in the lymphocytes of cancer patients treated with cyclophosphamide, adriamycin, isophosphamide, etc. It is feasible to clone T-lymphocytes *in vitro* by adding growth factors such as interleukin 2 to the growth medium. HPRT mutants isolated by the clonal method can be used to characterize the exact molecular nature of these mutants (*28*). Spontaneously occurring mutations have been found at all regions of the gene and comprise base-pair changes (transitions, transversions), frame shifts, small deletions, and large deletions involving one or more exons (splice mutations). Though it has been found that smokers have an increased frequency of mutants compared to nonsmokers, the mutation spectrum appears to be similar in both (*29*).

In a recent biomonitoring study of workers exposed to ethylene oxide, a significant increase in the frequencies of HPRT mutants was found (*21*). Though there was no difference in the frequencies of different classes of mutants induced, a hot spot for mutation at position 617 of the HPRT coding region (amino acid residue 206), which was not present in the control spectrum, was found. The fact that exclusively GC to AT transitions were observed at this position makes it possible that this hot spot is caused by ethylene oxide adducts, since alkylation of guanine at the O^6 position of ethylating agents predominantly gives rise to this type of base substitution (*30*).

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