8 Monitoring Cytogenetic Damage *In Vivo*

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8.1 INTRODUCTION

Chromosomal alterations have been used as an important biological endpoint to study the mutagenic effects of ionizing radiation and chemicals. Some basic principles in radiobiology were based on chromosome aberration data obtained in plant cells and *Drosophila* (Lea, 1946).

Structural and numerical chromosomal aberrations are encountered often in human newborns (0.6%) and in spontaneous abortions (30 to 40%). In addition, many tumors are associated with chromosomal aberrations that may be involved in the initiation or promotion stages of carcinogenesis. These facts emphasize the importance of studying chromosome aberrations *per se* as a relevant biological endpoint to assess the risks involved in exposure to mutagenic carcinogens. The origin and significance of chromosomal alterations have been discussed earlier (Natarajan, 1984). The techniques to detect chromosomal changes have been improved constantly to increase the ease and accuracy of recognition of aberrations, and in some cases automation has become available. In this review, the authors summarize some of the recent significant advances in this field.

Biological endpoints to be discussed include chromosomal aberrations, micronuclei, sister chromatid exchanges, and aneuploidy. In *in vivo* studies, only

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some tissues are amenable to chromosomal analysis, the prerequisite being that the cells are capable of proliferation. This requirement limits the number and type of cells that can be studied, and is thus usually confined to bone marrow cells, blood lymphocytes, regenerating liver cells, or proliferating male germ cells. Techniques to study non-dividing cells by premature chromosome condensation have also become possible (Hittelman, 1990).

8.2 CHROMOSOMAL ABERRATIONS

The types and frequencies of induced chromosomal aberrations depend on the mutagen used and the stage of the cell cycle treated. Ionizing radiation and chemicals which directly produce DNA strand breaks induce chromatid aberrations in the G_2 stage and chromosome aberrations in G_1 stage. Short-wave UV, alkylating agents, and most of the other chemical mutagens induce chromatid-type aberrations in G_1 (detected in the following mitosis) and G_2 (detected in the second division following treatment), as the chemically induced lesions require a DNA-synthesis dependent repair process for the formation of aberrations. Experimental protocols should be designed to take into account these points.

The classical method to assess *in vivo* exposure to mutagens in laboratory animals is to study the frequency of chromosomal aberrations in bone marrow cells. Usually several fixation times are used to discern effects on the stages of the cell cycle as well as to compensate for the expected mitotic delay due to treatment with a mutagen. The scoring of aberrations is very time consuming, and requires expertise. This method can easily be replaced by the use of inducer micronuclei in polychromatic erythrocytes. The doubling dose (DD) is calculated from the linear equation

$$y = a + bD \tag{8.1}$$

as the ratio of the spontaneous rate (a) and the linear regression coefficient

$$DD = \frac{b}{a} \tag{8.2}$$

Adler (1990) measured the induction of chromosomal aberrations and micronuclei following treatment with six known clastogens, and found the doubling doses to be very similar.

Chromosomal aberrations in peripheral blood lymphocytes have been used to monitor human exposure to genotoxic agents as well as from patients treated with cytostatic agents. Blood samples are cultured *in vitro* in the presence of a mitogen, such as phytoheamagglutinin, and dividing lymphocytes are evaluated for the presence of chromosomal alterations. In cases of radiation accidents, where no physical dosimetry is feasible, chromosome aberrations (especially the frequencies

of dicentrics and rings) have been used as a biological dosimeter. This technique can evaluate cases such as partial body irradiation and exposure to a mixture of high and low LET radiations (International Atomic Energy Agency, 1986). This technique was found to be very useful in a recent accident involving a ^{137}Cs gamma source resulting in a heavy exposure to several individuals in Goiania, Brazil, in 1987 (Ramalho *et al.*, 1988a).

In cases where chromosomal aberrations have to be determined immediately after exposure using non-proliferating cells, the use of premature chromosome condensation technique (PCC) is feasible. This technique has been exploited to study the induction of breaks and their repair in cells grown *in vitro* (Hittelman, 1990). The technique involves fusing non-dividing treated target cells with mitotic cells of Chinese hamster ovary cells (CHO) or HeLa cells in the presence of inactivated Sendai virus or polyethylene glycol. In the hybrids formed, the mitotic factors present in the donor cell condense the nucleus of the target cell. G_1 chromosomes appear as single chromatin threads; G_2 chromosomes appear as two chromatid threads. Dose–response curves for induction of fragments in lymphocytes of rats irradiated *in vitro* and lymphocytes derived from rats irradiated *in vivo* (assessed by the PCC technique) were very similar, indicating that the response of lymphocytes *in vivo* and *in vitro* is very similar. In acute radiation accidents involving large doses, the PCC technique could be useful to determine quickly whether the exposure is partial or whole body, as such information is necessary when making decisions concerning a possible bone-marrow transplantation to the victims.

Improved staining methods have become available recently, which can increase the resolution power of conventional chromosomal aberration evaluation. Chromosome specific libraries have been generated that can be used to stain specific individual chromosomes by *in situ* hybridization. The technique involves: (a) amplification of chromosome specific probes; (b) nick translating using DNAase, DNA polymerase, and four deoxynucleoside triphosphates of which one is biotinylated; (c) denaturation of the mitotic preparations and renaturing with the labelled probes; and (d) detection of the sites of hybridization with avidin and FITC. When one chromosome is exclusively stained, translocations involving this chromosome can be detected easily and accurately. Using this "chromosome painting" technique, translocations involving chromosome 2 in the lymphocytes of individuals involved in the radiation accident in Goiania, Brazil, have been detected accurately (Natarajan *et al.*, 1991). Another approach using the same technique employs telomeric and centromeric specific probes of a chromosome, such as chromosome 1 region 1q12 and 1p36. Detection of dicentrics and translocations involving this segment of chromosome 1 can be achieved with greater accuracy and ease than by conventional methods of C or G banding to detect dicentrics and translocations, respectively (Lucas *et al.*, 1989).

Automated scoring of chromosomal aberrations has been attempted. The synthesis of automated computerized devices that scan cytological preparations and find metaphases that are well spread was the first step toward this objective. The

frequency of dicentrics scored from selected metaphases does not differ from the frequency obtained by conventional manual selection techniques. In principle, many image analysis systems available for complete karyotyping of metaphases can be used to score chromosomal aberrations. However, this method is not used because of the equipment cost and the long time for an analysis. Rather, systems are being developed to rapidly analyze chromosome spreads to detect dicentrics (Piper *et al.*, 1988; Lorch, *et al.*, 1989).

8.3 SISTER CHROMATID EXCHANGES

Sister chromatid exchange (SCE) is a cytological manifestation of DNA double-strand breaking and rejoining at apparently homologous sites between two chromatids of the same chromosome. The occurrence of SCEs was first detected from the transformation of small ring chromosomes to large ring chromosomes in maize following cell divisions (McClintock, 1938). Using tritiated thymidine as a marker and micro-autoradiography for detection, Taylor *et al.* (1958) demonstrated the presence of SCEs from the silver grain patterns. This method was replaced eventually by cytochemical methods, using 5-bromodeoxyuridine (BrdU) and fluorochrome or fluorochrome plus Giemsa staining (Latt, 1973; Perry and Wolff,1974).

SCEs are induced efficiently by numerous mutagenic or carcinogenic agents, especially those which form covalent adducts to the DNA or interfere directly or indirectly with DNA replication. The induction of SCEs has been correlated with the induction of point mutations (Carrano *et al.*, 1978) and cytotoxicity (Natarajan *et al.*, 1984). While most of the mutagens induce SCEs very efficiently, not all agents which induce SCEs also induce point mutations. Nonmutagens such as inhibitors of DNA synthesis, (e.g., cytosine arabinoside and hydroxyurea) as well as inhibitors of poly (ADP-ribose) synthetase, (3-aminobenzamide) also induce SCEs efficiently (Natarajan *et al.*, 1981).

The frequency of SCEs has been correlated with the extent of BrdU incorporated in DNA, and most spontaneous SCEs occur when the BrdU-containing DNA is used as a template for replication. Attempts have been made to minimize the incorporation of BrdU and to detect the SCEs by reacting with antibodies against BrdU (Pinkel *et al.*, 1985; Natarajan *et al.*, 1986; Tucker *et al.*, 1986). Using this method, the baseline frequency of SCEs averages about two per cell cycle, a value similar to that obtained in systems using ring chromosomes without the incorporation of BrdU (Natarajan *et al.*, 1986).

In animals, the frequencies of SCEs *in vivo* can be measured by injecting or infusing solutions of BrdU or by implanting tablets containing BrdU, and examining dividing bone marrow or spermatogonial cells. The frequency of spontaneous SCEs obtained *in vivo* is usually very low compared with that obtained *in vitro*.

The frequency of SCEs following *in vivo* exposure to genotoxic agents can also be determined by culturing peripheral blood lymphocytes obtained from exposed

animals or humans for two cell cycles in a medium containing BrdU. Numerous studies exist in which human populations exposed to known mutagens or patients undergoing chemotherapy have been studied for the frequencies of SCEs. Several confounding factors may influence the frequency of SCEs in humans, and these have been discussed by Carrano and Natarajan (1988).

8.4 MICRONUCLEI

Micronuclei are formed in anaphase by chromosomal fragments or lagging chromosomes not included in the nucleus of the daughter cells. The presence of micronuclei in eukaryotic cells has been known for over six decades (Wilson, 1925). In the ensuing years, micronuclei have been observed in plants (Evans *et al.*, 1959) and in numerous tissues of many animal species (Heddle *et al.*, 1983). Presently, micronuclei serve as an important endpoint to detect the genetic damage by chemicals or radiation in cultured cells and intact organisms. Compared to more traditional approaches involving the analysis of metaphase chromosomes, micronucleus methods are rapid and easy to learn, and have comparable sensitivity. For these reasons, micronucleus assays are being used with increasing regularity. In this section, various forms of this assay are presented, their uses discussed, and possible future developments raised.

8.4.1 ERYTHROCYTES

8.4.1.1 Bone marrow

The most common form of micronucleus assay uses rodent bone marrow for several reasons:

1. Rodents are frequently used as models for human biological responses. Their small size and ease of handling makes them a natural subject of *in vivo* testing. Mice in particular are favored, primarily because murine bone marrow lacks the leukocytic granules, which can be confused easily with micronuclei found in rat preparations.
2. Bone marrow cells can be removed with little effort, smeared on to slides and then fixed and stained. No tissue culture is necessary, and the slides can be viewed within minutes of obtaining the tissue. The presence of numerous erythrocytes in microlitre volumes of bone marrow and the enucleate nature of these cells facilitate observation of micronuclei, and enhance scoring speed and accuracy.
3. Erythrocyte precursors in bone marrow are constantly undergoing cell division, making them sensitive to the effects of agents that interfere with DNA replication or cell division.

4. As with most procedures examining genetic damage through the use of proper staining techniques, only those cells that were actively growing during the exposure period are to be scored. Hayashi *et al.* (1983) demonstrated that the fluorescent dye acridine orange can distinguish mature, RNA negative "normochromatic erythrocytes" (NCEs) from immature, RNA positive "polychromatic erythrocytes" (PCEs). While earlier Giemsa-based techniques (Schmid, 1975) also make this distinction, acridine orange offers substantially superior contrast. With the proper selection of sampling time (approximately 24 to 48 hours after exposure depending on the agent and dose), the RNA positive erythrocytes are derived from cells undergoing their final division (and enucleation) during the exposure period.

The use of bone marrow erythrocytes as an endpoint for genetic toxicity testing has been well validated. Guidelines for the bone marrow micronucleus test have been published (MacGregor *et al.*, 1987). Heddle (1973) showed that micronuclei could be induced in the mouse bone marrow by X-rays. Hayashi *et al.* (1983) demonstrated that bone marrow micronuclei reflect chromosomal breakage phenomena. The USEPA Gene Tox Program has conducted an extensive review of the literature, and showed this system to be sensitive to chemical clastogens and to spindle disrupting agents (Heddle *et al.*, 1983). A series of publications has shown that micronuclei can be induced efficiently by different exposure routes including oral gavage and intraperitoneal injection (Hayashi *et al.*, 1989); others have shown induction following inhalation (Erexson *et al.*, 1986; Odagiri *et al.*, 1986; Jauhar *et al.*, 1988; Tice *et al.*, 1988) and exposure to 50-Hz electric fields (El Nahas and Oraby, 1989).

Thus, the preponderance of evidence is in agreement that the bone marrow erythrocyte micronucleus assay is a valid and sensitive means to detect genetic damage resulting from many routes of exposure to a wide variety of chemical and physical agents.

8.4.1.2 Peripheral blood

With increasing frequency, peripheral blood is being used to assess frequencies of micronuclei. In rodent studies, the most obvious advantage is that the test animal does not have to be euthanized to obtain blood samples. A simple tail vein nick or puncture of the periorbital sinus is sufficient to obtain the few microlitres of blood needed for a smear. This permits the investigator to obtain multiple sequential samples from the same animal, and enables more accurate determination of the effects of various agents upon the kinetics of erythrocyte and micronucleus development.

In human studies, obtaining bone marrow samples to measure the effects of exposure is generally not practical or ethical, although such sampling has been reported (Hogstedt *et al.*, 1983). Peripheral blood is much easier to obtain. The

disadvantage of using peripheral erythrocytes in humans is that micronuclei are observed only in people who have no functional spleen (Schlegel *et al.*, 1986), making the method generally not useful to monitor exposed populations. In large urban areas, substantial numbers of splenectomized people are present. Everson *et al.* (1988) studied 20 individuals 6 months after splenectomy, and described the effects of folate intervention on a person who had a high spontaneous frequency of micronuclei. Some regimens of cancer therapy, including Hodgkin's disease, involve routine splenectomy prior to radiotherapy. In such populations, monitoring the effects of therapy using peripheral blood micronuclei, and correlating the micronucleus response with other markers of exposure is considered relatively easy to accomplish.

8.4.1.3 Automation

The popularity of the erythrocyte micronucleus assay in bone marrow and peripheral blood has led to efforts to automate the scoring of these preparations using image analysis (Oleson, 1989; Romagna and Staniforth, 1989; Hayashi *et al.*, 1990) and flow cytometry (Hutter and Stohr, 1982; Ishidate *et al.*, 1987; Hayashi *et al.*, 1990; Tometsko and Leary, 1990). Both image analysis and flow cytometric approaches have been successful (Tucker *et al.*, 1989). Several image analysis systems are on the market (Romagna and Staniforth, 1989). Although manual scoring of micronuclei is relatively rapid compared with the analysis of metaphase chromosomes, the advantages of automation are significant, including increased speed of producing results and increased numbers of cells scored per sample enabling detection of lower doses and reliance on smaller cohorts.

One primary difficulty encountered in the early phases of automation was distinguishing erythrocytes from the large number of leucocytes. To solve this problem, Romagna and Staniforth (1989) used a cellulose column to purify erythrocytes, which has the advantage of removing leukocytic granules that are problematic in rat bone marrow, thereby making the rat amenable to erythrocyte micronucleus assays. A limitation of existing image analysis systems is that it is based on Giemsa-stained preparations. Although at least one commercial system is able to distinguish between RNA-positive and RNA-negative cells, the use of acridine orange would almost certainly improve erythrocyte characterization. In flow systems as in manual scoring, the use of acridine orange is relatively easy. In the near future, both flow cytometry and image analysis are expected to be used regularly to assess micronucleus frequencies in human and rodent erythrocytes.

8.4.2 NUCLEATED CELLS

Micronuclei in nucleated cells have been used to measure exposure since Obe and Beek (1975) treated human lymphocytes with radiation and observed a significant

increase. This approach was employed for the next 10 years to assess the effects of various agents, in spite of a fundamental problem: No adequate means existed to determine which cells had undergone division since exposure. Because not all lymphocytes respond to a mitogen, distinguishing between proliferating and non-proliferating cells was impossible. Nevertheless, the procedure was used with considerable success *in vitro* (Countryman and Heddle, 1976; Heddle and Carrano, 1977), and *in vivo* with lymphocytes (Aghamohammadi *et al.*, 1984; Maki-Paakkanen, 1987), buccal mucosa cells (Stich and Rosin, 1983; Sarto *et al.*, 1987), fibroblasts (Rudd *et al.*, 1988), and exfoliated cells (Rosin and German, 1985; Reali *et al.*, 1987; Stich, 1987).

The micronucleus assay, however, did not work with combination protocols (e.g., X-rays plus caffeine; Natarajan *et al.*, 1982). Several years ago, however, two procedures were introduced that offered potential solutions. Pincu *et al.* (1984) used BrdU followed by fluorescence and Giemsa staining to distinguish between proliferating and non-proliferating cells. The second solution, proposed by Fenech and Morley (1985a), used cytochalasin B which arrests cytokinesis yet does not interfere with karyokinesis. The resulting multinucleated cells may be centrifuged directly on to microscope slides and stained with May–Grunwald–Giemsa. With this procedure, only cells with intact membranes are scored. The primary advantage is that each binucleated cell represents the products of a single mitosis. In a direct comparison between the BrdU and cytokinesis block (CB) procedures, Ramalho *et al.* (1988a) concluded that the CB method detected chromosome fragments more efficiently than the BrdU procedure. For these reasons, the use of cytochalasin B is now the preferred method of measuring micronuclei in nucleated cells.

8.4.2.1 Application of the CB procedure *in vitro*

Early work with the CB procedure on human peripheral lymphocytes showed significant yields following radiation exposure (Fenech and Morley, 1985a, 1986). The micronucleus frequencies appeared to be consistent with the production of acentric fragments (Littlefield *et al.*, 1989), although agreement on this issue is incomplete (Prosser *et al.*, 1988). Others have shown dose–response to chemicals (Eastmond and Tucker, 1989a). The CB method has also been applied successfully to established cell lines. Wakata and Sasaki (1987), Eastmond and Tucker (1989b), and Krishna *et al.* (1989) demonstrated the utility of cytochalasin B on Chinese hamster cells for *in vitro* genotoxicity testing. Significant increases in micronuclei have been observed following treatment with clastogenic and spindle disrupting agents. Application of the CB procedure to *in vitro* testing gives every indication of being a rapid and sensitive means of measuring exposure.

8.4.2.2 Application of the CB procedure *in vivo*

The CB procedure has also been used following exposure *in vivo*. Experiments involving mice treated with radiation (Erexson *et al.*, 1989) and chemicals (Erexson *et al.*, 1987) have indicated that this procedure works very well even in small animals where limited amounts of peripheral blood are available for sampling.

In humans, the simplicity and utility of this procedure makes it applicable to monitor exposed populations. Sorsa *et al.* (1988) and Yager *et al.* (1988) examined people exposed to alkylating agents, and found a positive trend between exposure and micronucleus frequencies. Fenech and Morley (1985b, 1986, 1987) demonstrated that the frequency of micronuclei increases significantly with age, being about four times higher in people aged 80 than in newborns. Other confounding variables such as smoking and sex may exist, but have not yet been demonstrated.

8.4.2.3 Automation

Several investigators have attempted to automate the scoring of micronuclei in cytokinesis-blocked cells. The requirements for automating this method are much more complex than for erythrocytes. Such a system should:

1. determine the number of micronuclei in each binucleated cell;
2. distinguish between binucleated cells with an intact cytoplasmic membranes and other artifact cellular and non-cellular objects;
3. distinguish micronuclei from nuclei and nuclear blebs;
4. have the option of determining the average number of nuclei per cell as a means of assessing toxicity; and
5. operate quickly and reliably with a minimum of user interaction.

The need for multiple morphological assessments of each cell indicates that image analysis would be more suitable than flow cytometry, despite of the results of Nusse and Kramer (1984). In preliminary studies using image analysis, Fenech *et al.* (1988) demonstrated the utility of automating the BrdU incorporation method (Pincu *et al.*, 1984). Fenech's procedures to automate the (CB) method have been unsuccessful.

Studies directed towards automated scoring of micronuclei in binucleated lymphocytes using the CB method and the LeyTAS-MIAC system (Leyden Texture Analysis System using the Leitz Modular Image Analysis Computer and a multichannel Leitz Autoplan microscope) have been reported (Tates *et al.*, 1990). These investigators indicated that the requirements for automated detection of micronucleated binucleated cells can be fulfilled, provided more time was invested in fully developing this method.

Microcomputer-based image analysis system with microscope control for slide scanning has been developed as a less costly alternative. Using this system, an image analysis procedure was developed successfully (Tates *et al.*, 1990). This

procedure involves sequential analysis of micronuclei and nuclei (stained with Gallocyanin) and cytoplasm (stained with Naphtol Yellow S). Initial results of validation tests indicated that about 60% of the micronuclei and about 65% of binucleated cells can be detected automatically. The artifact rejection procedure is capable of eliminating most kinds of non-relevant cellular and non-cellular objects present on slides. Nuclear blebs, when present, do not result in false positive micronuclei. The present system still needs further refinement.

8.4.2.4 Aneuploidy

Aneuploidy is a frequent cause of fetal wastage, birth defects, and mental retardation, and also appears to play an important role in the pathogenesis of malignancy; yet, its molecular mechanisms are poorly understood. Until recently, acceptable procedures to measure aneuploidy in mammalian cells were limited to counting metaphase chromosomes (Galloway and Ivett, 1986; Dulout and Natarajan, 1987). This procedure is not only tedious and prone to artifact chromosome loss, but is limited to measuring hyperploidy and not hypoploidy. However, the procedure developed by Dulout and Natarajan (1987), using embryonic diploid fibroblasts of Chinese hamster grown on cover glasses and fixed *in situ*, overcomes these difficulties. Recently, several other excellent reviews of aneuploidy have been published (Cimino *et al.*, 1986; Dellarco *et al.*, 1986; Parry and Parry, 1987; Vig and Sandberg, 1987). Some of these have stressed the need new cytogenetic approaches to measure aneuploidy in mammalian cells. In this section, recent developments are described, and their uses and limitations discussed.

Kinetochore staining in micronuclei

Micronuclei can result from spindle disruption or clastogenic processes. Until recently, the ability to discriminate between these processes was limited to comparing the size of micronuclei induced by various agents (Hogstedt and Karlsson, 1985). This approach works reasonably well when large numbers of micronuclei are examined; yet, it is unable to determine the mechanism of origin for individual micronuclei. Recently several laboratories have begun to use antikinetochore antibodies obtained from the serum of people with the crest form of scleroderma. These antibodies are highly specific for the kinetochore region, and appear to react equally well with human, mouse, and Chinese hamster cells. The use of kinetochore staining in micronucleated cells is based on the assumption that kinetochore-positive and kinetochore-negative micronuclei contain whole chromosome(s) and chromosome fragment(s), respectively. This method has now been applied by several laboratories to measure the effects of exposure *in vitro*. Both human lymphocytes (Thomson and Perry, 1988) and fibroblasts (Hennig *et al.*, 1988) have been used, as well as cultured hamster cells (Degrassi and Tanzarella,

1988). These studies have demonstrated that this procedure discriminates efficiently between aneuploidogenic and clastogenic responses in individual cells. Other studies have combined the use of antikinetochore antibodies with the CB method of obtaining micronuclei (Eastmond and Tucker, 1989a, b; Fenech and Morley, 1989). This combination of techniques is especially powerful because it enables analysis of the products of a single mitosis and avoids the problem of artifact loss of chromosomes. The procedure is only slightly more difficult than the analysis of micronuclei alone, yet it permits the characterization of both aneuploidogenic and clastogenic processes.

The future of kinetochore labelling as an assay for aneuploidy appears promising, especially if coupled with the CB procedure. This method should be of interest to those performing standardized toxicity tests and to regulators in need of a simple and reliable procedure to measure the induction of aneuploidy. The method is also capable of screening populations of exposed individuals.

DNA probes

With *in situ* hybridization using chromosome specific probes, aneuploidy is determined by counting the number of hybridization signals present in each cell. Scoring is rapid, minimal training is needed, and the cells of interest need not be cultured. This latter characteristic has significant implications for solid-tumor cytogeneticists. Cells from solid tumors are difficult to grow; metaphase preparations are difficult to obtain, and are often of poor quality. *In situ* hybridization avoids these limitations; consequently, significant advances are being made in this area (Cremer *et al.*, 1988; Devilee *et al.*, 1988; Hopman *et al.*, 1988).

In spite of such success, several disadvantages remain for this procedure. The number of different fluorophores available for simultaneous use, and investigators can measure aneuploidy for only a few chromosome domains per slide. In addition, hybridization signals in interphase nuclei have a significant chance of overlapping, partly because the three dimensional structure of the nucleus is viewed in only two dimensions, and partly because two separate chromosome domains may truly be adjacent. Consequently, hypoploidy is difficult to detect, and investigations must be limited to examining the effects of hyperploidy.

In the future, fluorescent *in situ* hybridization may be used to answer specific and fundamental questions concerning aneuploidy in both normal and malignant tissues. Monitoring human exposure using sperm to investigate potential reproductive effects and cytokinesis-blocked cells to address the specificity of chromosome loss may also be possible. Most available probes are human in origin; but as material from other species becomes available, animal models to mimic humans may be developed.

Transgenic animals

Transgenic mice carrying large sequences of foreign DNA in one or more chromosomes have been used to study aneuploidy *in vivo* (Natarajan, 1989; Natarajan *et al.*, 1990). In these mice, either multiple copies of lambda sequences and/or c-*myc* sequences have been introduced. The chromosomes carrying these sequences can be detected by *in situ* hybridization in both metaphase and interphase nuclei. Aneuploid events can easily be detected in different organs; therefore, this system offers the advantage of studying organ-specific changes.

Automation

Presently, no automated systems are capable of detecting aneuploidy in mammalian cells. The antikinetochore antibody procedure as applied to binucleated cells could be automated by modifying the micronucleus system to include the ability to detect the presence of kinetochore label. The DNA probe method could be automated by developing an instrument to quantify the number and intensity of chromosome-specific labels in interphase cells. Each approach is probably amenable to image analysis, while a flow system scheme would appear less feasible.

Table 8.1. Spontaneous frequencies of chromosome aberrations in human spermatozoa

Authors	Subjects (*n*)	Sperm (*n*)	Chromosome abnormalities aneuploid	structural	total
Rudak *et al.* (1978)	1	60	3	1	4
Martin *et al.*(1983)	33	1000	52	40	92
Brandriff *et al.*(1985)	11	2468	41	190	231
Martin *et al.*(1982)	18	240	18	7	25
Kamiguchi and Mikamo (1986)	4	1091	10	142	152
Mikamo *et al.* (1989)	26	9280	125	1357	1482
Total	93	14139	249 (2%)	1737 (12%)	1986 (14%)

8.5 SPERM CYTOGENETICS

The ultimate objective of genetic risk assessments is the quantitative estimation of genetic damage transmitted to subsequent generations. Recently, the direct study of chromosomes in human spermatozoa has been made possible by using interspecific *in vitro* fertilization with zona-free golden hamster oocytes (Table 8.1; Rudak *et al.*, 1978; Martin *et al.*, 1983; Brandriff *et al.*, 1984, 1985). With this

technique, the hamster egg unfolds the human sperm chromatin, and processes it into microscopically analyzable mitotic chromosomes amenable to microscopic analysis. Presently, this method is the only one for direct assessment of chromosomal effects of mutagens in human germ-line cells.

Spontaneous frequencies of chromosome aberrations (Table 8.2), particularly structural aberrations, in human spermatozoa are much higher than those in mouse and Chinese hamster. The basis of the high frequency of chromosome aberrations in human sperm is unclear. The possible effect of interspecies cross fertilization should be examined. However, considerable variation among donors suggests involvement of environmental factors such as cigarette smoking. An increase in chromosome aberrations in spermatozoa has been found in cancer patients receiving radiotherapy and chemotherapy (Martin *et al.*, 1986, 1989; Brandriff *et al.*, 1987; Jenderny and Rohrborn, 1987; Genescà *et al.*, 1990).

Table 8.2. Species difference in spontaneous chromosome aberrations in spermatozoa

	% Chromosome abnormalities		
Species	Aneupoloid	Structural	Total
Humans (Table 8.1)	1.8	12.3	14.1
Mouse[1]	0.6	0.9	1.5
Chinese hamster[2]	0.7	1.4	2.1

[1]Fraser and Maudlin (1979)
[2]Mikamo and Kamiguchi (1983)

The frequency of chromosome aberrations in human spermatozoa by *in vitro* exposure to ionizing radiation have been studied by Kamiguchi *et al.* (1987, 1989) and Brandriff *et al.* (1988). The aberrations were mostly chromosome breaks and fragments, and their frequency increased linearly with radiation dose. The interspecies comparison of chromosomal radiosensitivity indicated that the human spermatozoa were highly vulnerable and about three times more radiosensitive than those of mouse (Table 8.3).

Chromosome analysis in spermatozoa can also be used to determine the segregation of translocations in meiotic processes (Martin, 1989; Pellestor *et al.*, 1989). Segregation properties vary among different types of translocations, and thus the analysis of sperm chromosomes provides a useful means to assess risks arising from the induction of translocations in spermatogonial stem cells as well as the heritable translocations.

8.6 INTERSPECIES COMPARISONS

Interspecies comparisons of cytogenetic response to chemical mutagens have been carried out to a limited extent, although data exist for radiation responses. Rodent:human comparisons are difficult for the following reasons:

1. most human data come from studies on resting (G_0) peripheral lymphocytes, whereas rodent data are obtained from proliferating bone marrow cells;
2. karyotypes of rodents and humans are different, and karyotype configurations are known to influence the yield and/or detection of aberrations;
3. the extent and distribution of heterochromatin, which also influences the yield and distribution of aberrations, is different between rodents and humans; and
4. metabolic activation and repair capacities differ between human and rodents.

Table 8.3. Chromosome radiosensitivity of spermatozoa of different species

Species	Relative radiosensitivity
Guinea pig	0.5 (DL)
Rabbit	0.5 (DL)
Golden hamster	2 (DL)
Mouse	1 (SCA)
Chinese hamster	1 (SCA)
Human	3 (SCA)

DL = dominant lethal
SCA = sperm chromosome abnormality

Some interspecies comparative studies have been reported. Following treatment with benzo[*a*]pyrene, for instance, more SCEs were induced in rat compared with mouse for equivalent amounts of adducts (Kligerman *et al.*, 1989). Dichloromethane-induced chromosomal aberrations and SCEs in mice (Allen *et al.*, 1990), but had no effect in rats and Chinese hamsters (Burek *et al.* 1984). Diaziquone induced more SCEs in humans than in mice (Kligerman *et al.*, 1988). More comparative studies of this nature are needed.

8.7 CONCLUSIONS

Chromosomal alterations can be analyzed directly in humans using proliferating bone marrow cells and stimulated peripheral blood lymphocytes. The significance of chromosomal aberrations is twofold: chromosomal rearrangements (a) are associated with cancer and hereditary diseases and (b) may indicate exposure to carcinogenic or mutagenic agents.

Combining molecular biological techniques with cytogenetic methods has led to

easy and rapid detection of chromosomal rearrangements. Classical cytogenetic techniques can be used easily to detect chromosome or chromatid aberrations (unstable aberrations), the frequency of which is a quantitative index of exposure to ionizing radiation and a qualitative index of exposure to chemical mutagens. To detect stable chromosomal rearrangements, however, chromosome painting (*in situ* hybridization with chromosome specific probes) can be used with ease and accuracy. In addition, the PCC technique (hybridization of interphase cells to mitotic cells) allows one to study chromosomal aberrations particularly in non-proliferating cells. Similarly, cross-fertilization by interspecies hybridization *in vitro* (e.g., human sperm in golden hamster egg) enables one to study sperm chromosomes directly. Using this technique, human spermatozoa have been found to have many more background aberrations than those of rodents.

To assess rapidly the frequency of chromosomal fragments, micronucleus assays have been developed. Of special significance is the cytocholasin-B block method of generating binucleated cells, which possesses increased sensitivity and ease of quantification of micronuclei frequency in proliferating cells.

Numerical aberrations (aneuploidy) lead to important genetic consequences of exposure to environmental agents. Direct detection of aneuploid cells in different organs (including germ cells) has become feasible by use of transgenic mice carrying large insertions of foreign DNA. The chromosome carrying such insertions can be detected by *in situ* hybridization with the appropriate probes. In addition to aneuploidy, the recombination events can be detected in transgenic mice. Kinetochore-specific antibodies can be used to determine whether micronuclei contain a centromere (an indicator of a whole chromosome loss), and can be used as a measure of aneuploidy.

Many of the techniques discussed in this chapter can be, or are being, automated. When automation is achieved, large numbers of cells can be scored quickly, enabling detection of effects produced at relatively low doses.

Though a large amount of data are available for interspecies comparison of cytogenetic response to ionizing radiation, the database for chemicals is comparatively small. Perhaps this situation is due to inherent difficulties in comparing experimental animals to humans following chemical exposures. Certainly more effort is needed in this area.

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