Effects of Ionizing Radiation

THE MEASURABLE CONSEQUENCES AT THE LEVEL OF THE CELL

The exposure of an organism to ionizing radiation has measurable consequences not only for DNA, but also for other cellular structures. Since the demonstration of the existence of received dose indicators based on the examination of affected chromosomes, newer techniques in molecular biology and their greater sensitivity have made it possible to develop biological indicators of cellular effects. Although these do not display the specificity of the chromosomal indicators they will help us gain a fuller understanding of radio-induced effects and afford criteria for the diagnosis and prognosis of radiation damage.

C. Cieutat/IPSN

High performance liquid phase chromatography (HPLC) apparatus. This analytical instrument is used to separate the complex organic macromolecules (lipids and proteins) that constitute, for example, cell membranes. HPLC can supply information that helps us understand the modifications of the physical properties of the cytoplasmic membrane due to ionizing radiation.

The need for biological indicators of cellular effects

The exposure of **cells** to **ionizing radiation** can have adverse consequences for **DNA** and other cell structures (box D, ***The cell, the essential link***). These consequences may affect not only the behavior of neighboring cells but the whole tissue affecting the functioning of the organism at some later stage. The probability of the damage and the rapidity of its **effect** depend on many factors, including the received **dose**, the type of radiation, the **dose rate** and the nature and volume of the tissues exposed.

It very soon became important to find biological parameters to evaluate or even better to quantify the damage done to different constituent parts of the organism. The usefulness of these parameters depends greatly on the need: risk of genetic damage due to natural radiation, estimation of received dose in the event of accidental overexposure, effects on healthy tissues and **tumors** during therapy, etc.

These indicators have to display certain intrinsic qualities: they must be **specific**, **easy** to identify and measure, **reproducible**, **sensitive**, **stable** and **repeatable**, so that the exposure can be reconstituted after the event. In addition, the demonstration of a dose-effect relation differentiates between biological *indicators* and *dosimeters*.

No current bio-indicator meets all these criteria. Those most fully developed are **cytogenetic** dose biomarkers. The effects of ionizing radiation on DNA and its impact on cell survival have long been known. The range of observed **chromosomal** damage has afforded a basis for devising various tests including the *a posteriori* reconstitution of the exposure and the estimation of the heterogeneity of the dose. Since the physical damage caused by the ionization should be distributed evenly throughout the cellular structures, the corresponding biological effects ought to be measurable elsewhere than in DNA. Recent scientific advances, especially at the molecular level, have enabled the identification of bio-markers of these effects in the **cytoplasmic membrane**, the

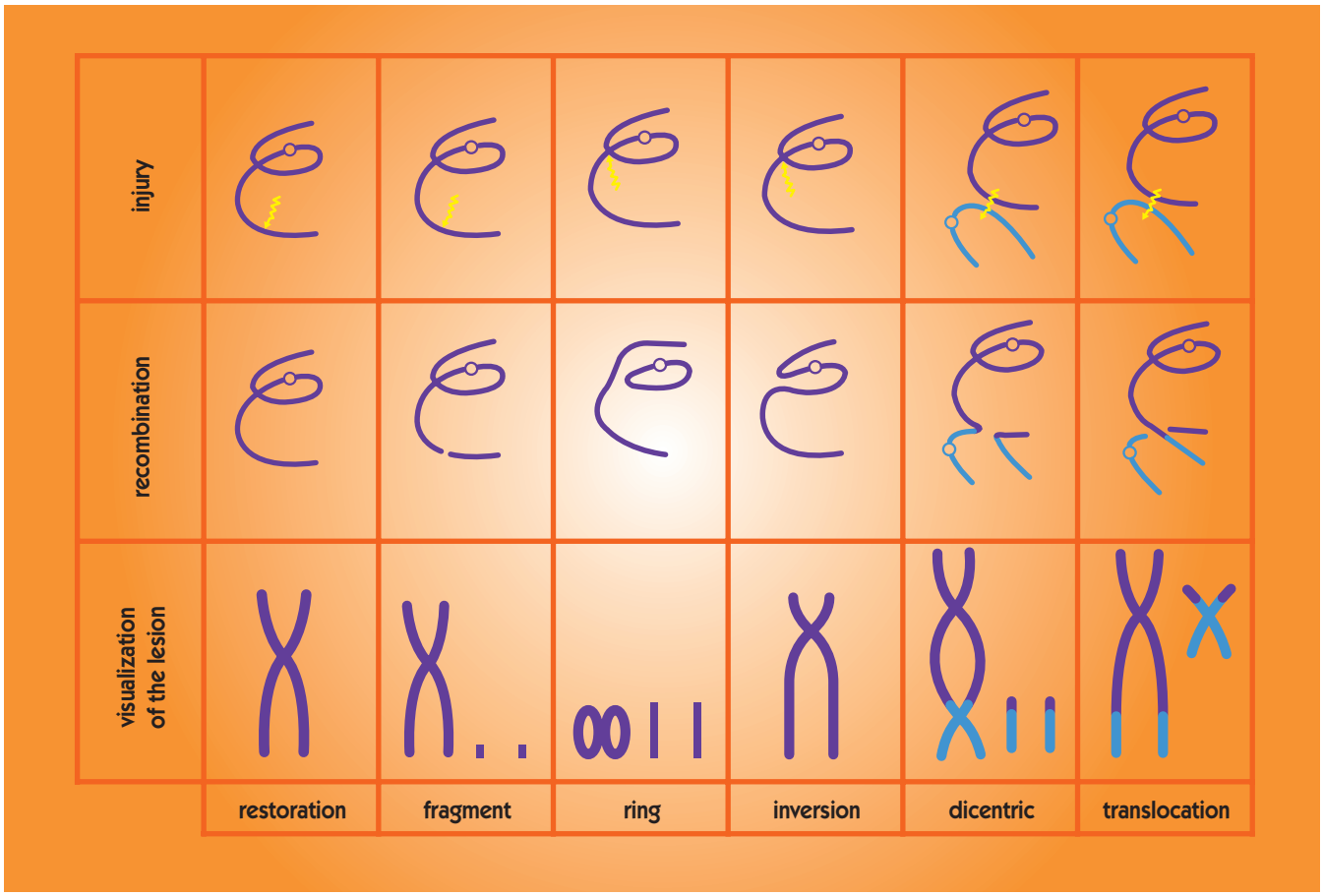


Figure 1. Nature and formation of radio-induced chromosomal aberrations. According to the position and number of DNA lesions, and according to the quality of the repair or the recombination of the chromosome with itself or with another one, either normal chromosomes or various types of aberration will be observed.

mitochondria and even in their influence on radio-induced cell death (**apoptosis**).

Consequences measurable in DNA

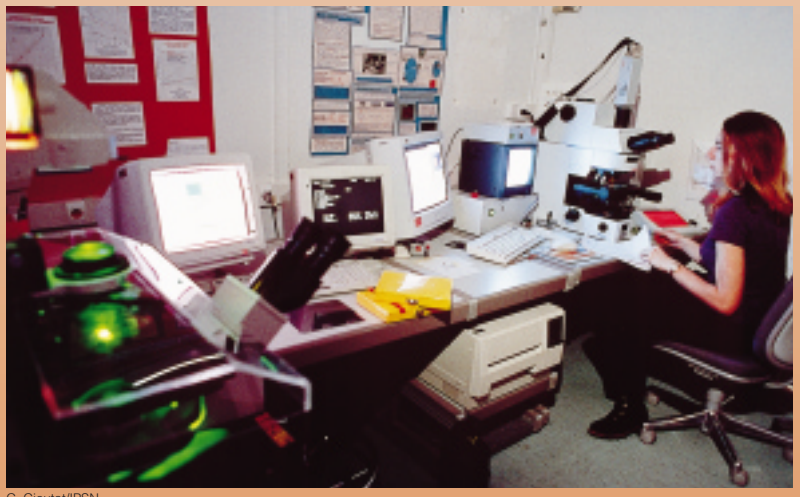
The wide spectrum of DNA damage due to ionizing radiation is the ultimate consequence of processes described above. Despite extremely efficient corrective action, a small number of errors can subsist. The most important of these are observed during the **metaphase** (box E, ***The cell division cycle: under control duplication***). These chromosomal aberrations (Figure 1) result from an unrepaired break (fragment, **deletion)**, misrepair of one chromosome **(inversion**, centric ring), or an exchange of material between at least two chromosomes (**translocation, dicentric chromosome**).

With their atypical shapes, the dicentric chromosomes and the centric rings are easily identified under the microscope after simple DNA staining. These chromosomal aberrations favor the elimination of the affected cell. They are termed *unstable*. In contrast, inversions and translocation, the shapes of which do not obviously differ from those of normal chromosomes, are able to «slip through» cell division and so are termed *stable*. Their visualization requires specific staining. Fragments, also resulting from chemical **mutagens**, are not characteristic of ionizing radiation. They can be eliminated directly in the form of intracytoplasmic micronuclei, the incidence of which is a valuable indicator of mutagenic effect. Finally, an experimental device makes it possible to observe the appearance of radio-induced chromosomal aberrations within hours or even days after irradiation, with no prior cell culture. More than just a way of measuring effects, the observation of these prematurely condensed chromosomes (PCC) constitutes a sophisticated method for understanding repair mechanisms.

Only some aspects of these techniques developed at the Multiparametric Biological Dosimetry Laboratory of the Nuclear Protection and Safety Institute (LDBM/IPSN) will be discussed here (box).

A high performance system for image analysis

Whether for plotting reference curves from *in vitro* experiments or for estimating doses in the event of accidental irradiation, or for research purposes, the detection of dicentric chromosomes and translocations requires carefully examining samples of hundreds of cells. To take some of the tedium out of this routine work the LDBM uses a system of image analysis adapted to biological dosimetry, jointly with the French company that developed it. At a suitable work station slides for inspection are placed on a motor-driven stage that helps locate (or relocate) lymphocytes in their metaphase state, by means of a camera equipped with automatic focusing. The positioning of the metaphase chromosomes is achieved using a controller connected to a computer. Validation tests have shown that the device successfully finds at least 80% of the metaphase stages utilizable for conventional or fluorescence cytogenetics. Appropriate software is used to help count chromosome aberrations semi-automatically. The system takes only a half to a quarter of the time needed for manual counting, allowing for the quality of the preparations and the radiation dose.

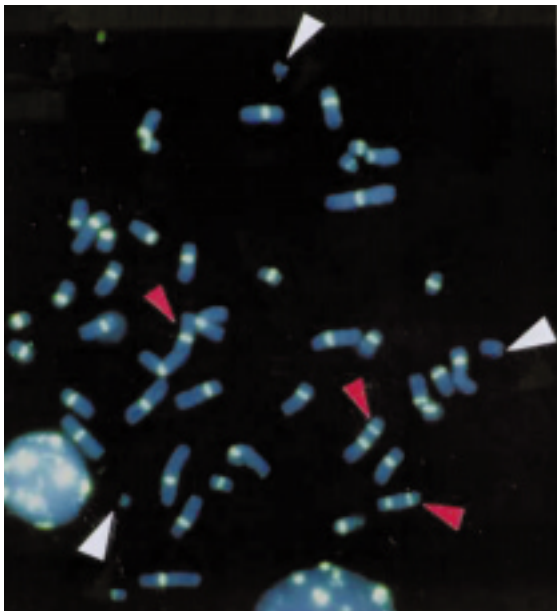


C. Cieutat/IPSN

The dicentric chromosome, specific indicator of cell irradiation

When accidental irradiation occurs the received dose is evaluated by counting the number of unstable chromosomal anomalies in the **lymphocytes**, from a simple blood sample.

A dose-effect curve (Figure 2) relates the recorded incidence of aberration to a dose of irradiation of the bone marrow. These curves are plotted by ***in vitro*** irradiation of blood samples and differ according to the type of radiation. The dose rate is also important, particularly for low energy radiation (gamma, X). The precision of the estimation depends on the number of metaphases observed.



IPSN

Microphotograph of the nucleus of a heavily irradiated lymphocyte grown in culture in a suitable medium and blocked at the metaphase stage (magnification x 1,000). Chromosomal aberrations, dicentric chromosomes (pink arrows) and fragments (white arrows) can be seen.

Thirty years of research have shown that apart from a few chemicals such as bleomycin (an antibiotic), only ionizing radiation will cause the appearance of dicentric chromosomes. Their rate of spontaneous formation in the French population is about 1 per 1,500 cells and does not vary significantly with age, sex or tobacco use.

After global *homogeneous* irradiation the incidence of chromosomal anomalies in the blood lymphocytes remains fairly stable for several months. Conversely, after *heterogeneous* irradiation, irradiated and non-irradiated lymphocytes mix rapidly, leading to an underestimation of local radio-induced damage due to the dilution effect. This can be partly corrected by mathematical models based on the kinetics of the disappearance of the aberrations over time.

Cases of suspected irradiation to which this method is applied are rare, both in France and elsewhere. Fewer than 200 cases have been analyzed by the IPSN in the last eight years, either among operatives directly or indirectly exposed in industry, research or health services, or among the general public. In most of these cases this procedure was able to show that the individuals had fortunately not been significantly irradiated.

Translocation, a specific indicator of old irradiation?

Accidental irradiation is sometimes evaluated years after the event, in particular in the case of the survivors of the Hiroshima and Nagasaki nuclear explosions (Japan, 1945), and the «liquidators» of the Chernobyl reactor meltdown (Ukraine, 1986). An analysis of stable chromosomal aberrations such as translocations was hitherto carried out using a difficult and cumbersome labeling technique. A recent method, derived from molecular biology and adapted to biological dosimetry, called fluorescent *in situ* hybridization (FISH), has greatly simplified their detection and is applied systematically to assess old irradiation damage. It reveals special zones on chromosomes, or allows them all to be selectively labeled (*painting*). FISH techniques are also used in clinical practice to detect hereditary or acquired anomalies. The aim is to form a molecular association (hybridization) between artificial **sequences** of single-**strand** DNA, specific to the chromosomal zones that the experimenter wishes to study, and metaphase chromosomes spread on a microscope slide. The fluorescent signal is visualized at the level of the hybridized zones. Given the available labels, only two or three of the 23 pairs of human chromosomes are generally labeled.

Analogous curves relating the incidence of translocation to the irradiation dose have also been plotted *in vitro*, like dicentric chromosomes. Overall, the results obtained according to the **radia-**

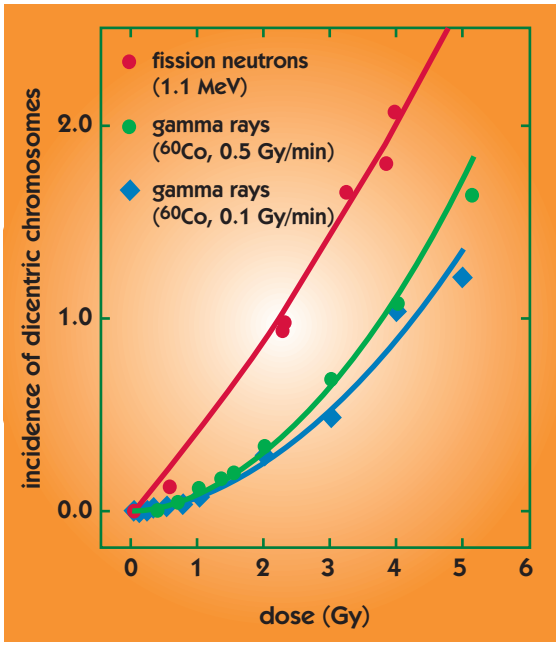
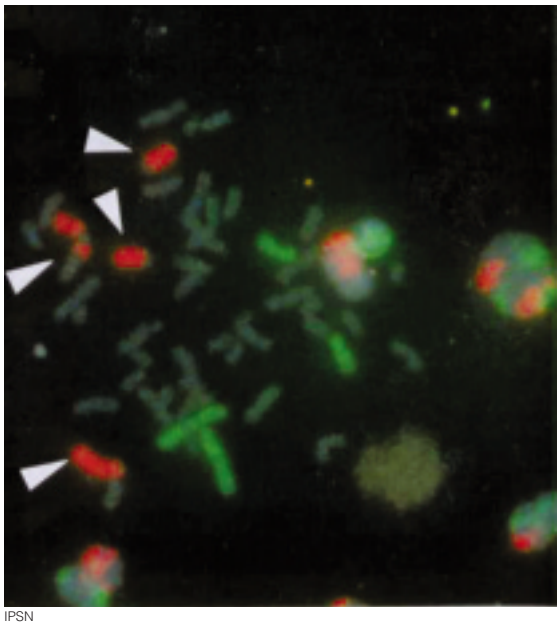


Figure 2. Dose-effect relation plotted at the LDBM by counting the dicentric chromosomes induced in peripheral blood lymphocytes after in vitro irradiation of normal blood with different types of ionizing radiation at different dose rates. For the same dose of radiation the number of aberrations produced by the high energy radiation (neutrons) is greater than that due to the low energy rays (gamma, X). In addition, the dose rate effect falls off markedly when the radiation is of high energy (neutrons, heavy ions).

tion quality and the dose rate are very similar. Although the principle of the evaluation of translocations is not recent, the ease with which they are detected by FISH has raised new questions. What is their specificity in relation to ionizing radiation? Does the basal rate of translocation increase with age and with factors such as alcohol abuse? And above all, what is the effective stability of translocations over time? The IPSN is conducting systematic studies, in particular of old accidents, to assess the true validity of this approach.

Prematurely condensed chromosomes

The PCC technique is based on the possibility of visualizing chromosomes without prior culture, by cell fusion. Lymphocytes from a blood sample are fused with dividing cells of an ovocyte **line** of a Chinese hamster. The substances they release induce the dissolution of the membrane of the **nucleus** and premature condensation of the lymphocytal chromosomes in the form of a single individual strand, before the **duplication** of the DNA can take place. The chromosomal aberrations take the form of extra fragments, so that the number of objects formed is greater than the 46 chromosomes of a normal human cell. A linear relation is established between this number and the received radiation dose. Unlike conventional cytogenetics, which allows the observation only of lymphocytes that have reached their metaphase, this technique applies to a random sample of cells. This method, which is faster than the counting of dicentric chromosomes, also makes it possible to study the kinetics and yield of DNA repair processes, by varying the time elapsed between irradiation and cell fusion. Dose-effect curves plotted for gamma radiation after different repair times show that for the same dose the number of radio-induced supernumerary fragments falls off very rapidly during the first 24 hours, and then more and more slowly during the following 48 hours. Other experiments have demonstrated that variations in the temperature of the blood between sampling and analysis do not influence the results. The PCC technique is applied to cases of accidental irradiation assessed by the IPSN, in parallel with conventional cytogenetics, in order to evaluate its scope and limits.

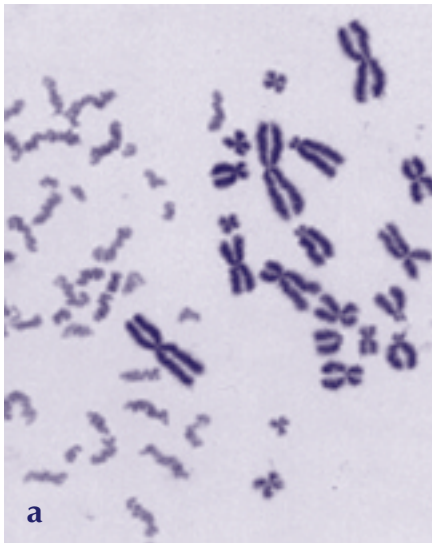


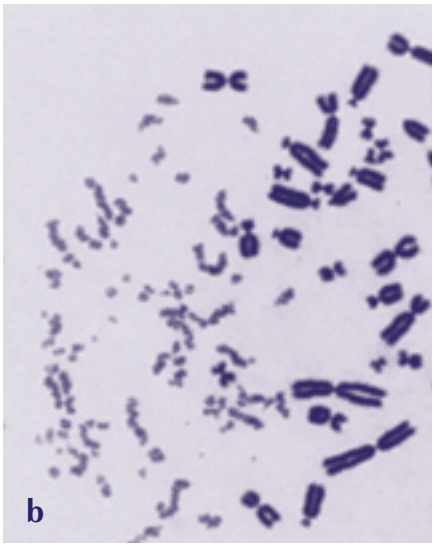
IPSN

Fluorescence microphotograph of an irradiated lymphocyte metaphase in which three pairs of chromosomes have been stained by specific DNA probes using the FISH triple painting technique. Chromosome pairs 2 and 12 are green and chromosome pair 4 is orange. The other chromosomes have been visualized by the binding of a non-specific fluorescent stain. The arrows indicate parts of chromosome 4 that have been cut on the painted parts and inserted in a non-painted chromosome (magnification x 1,500).

Impact on other constituents

Recent work has confirmed the essential role of the membrane and other cell constituents in radio-induced apoptosis. The chemical composition of the membrane makes it a prime target for free radicals produced by deposited energy, leading to early disturbances, of ranging duration, of its structure and functions. The membrane plays a role as an interface in the transfer of signals to the nucleus, and so influences cell functions. Radio-induced damage to it can have





IPSN

The fusion of a Chinese hamster ovocyte from a maintained line, blocked at the metaphase stage (on the right in each microphotograph), with a resting lymphocyte, non-irradiated (a), and irradiated (gamma rays ^{60}Co, 4 Gy, 0.5 Gy/min) (b), causes premature condensation of chromosomes in the form of single strands of DNA (on the left in each microphotograph). The irradiation leaves DNA breaks giving supernumerary fragments additional to the 46 DNA strands of a normal human cell.

consequences that may ultimately lead to apoptosis.

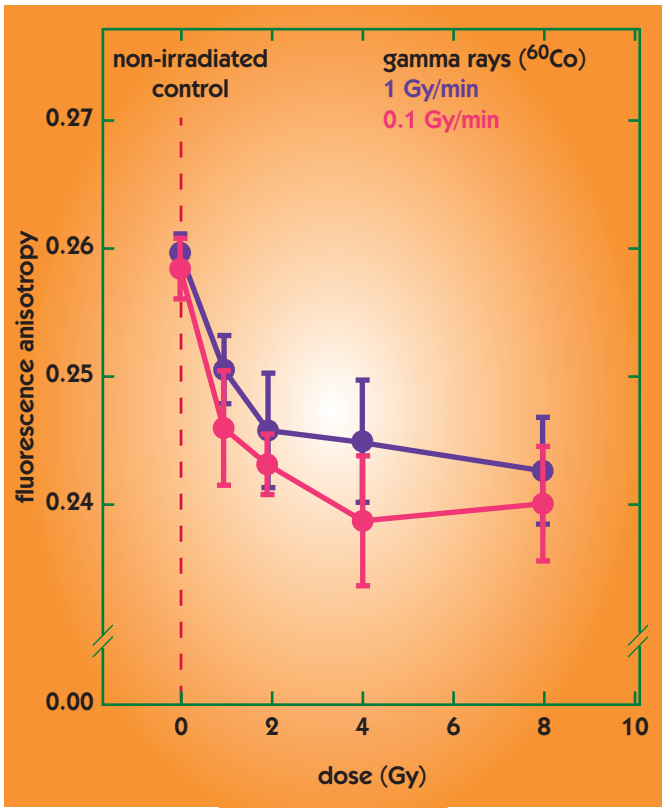
In this cascade of events three mechanisms seem to be candidates for the role of indicators of the cellular effects of ionizing radiation: (i) modifications of the physical properties of the membrane, (ii) the release, before apoptosis, of certain components into the extracellular medium as a result of breakdown of the membrane, and (iii) apoptosis itself.

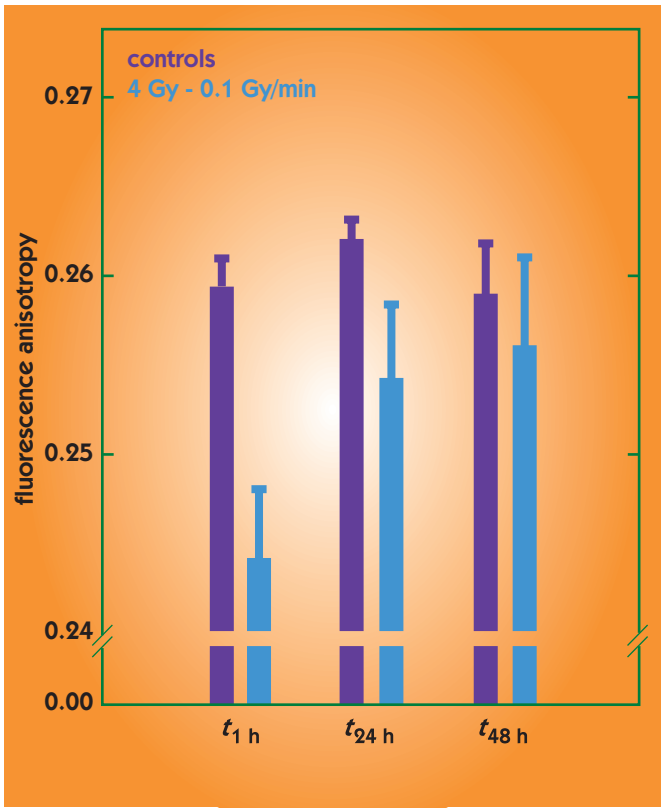
Membrane fluidity

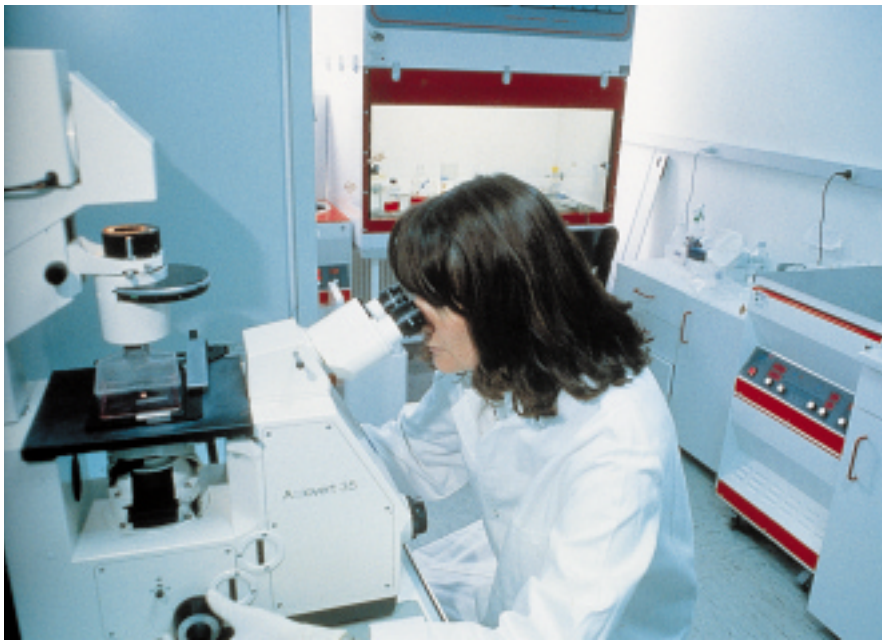
The molecules that compose the membrane move, separately or in groups, laterally, transversally or rotationally. This fluidity is in fact tightly controlled, the functional properties of the cell depending in part on the ability of the membrane to remain thermodynamically stable. To assess the biophysical modifications of the membrane the LDBM has chosen a technique based on the specific incorporation of fluorescent molecules, either in the lipid compartment, or at the lipid-**protein** interface. Illuminated with polarized light, these probes send back fluorescent light, according to their mobility, in a direction that will differ to a variable degree from that of the incident light. Comparison of the intensity of the fluorescence of the incident polarized light with that of the re-emitted depolarized light gives a measure of the movement of the probes and affords a parameter characteristic of the fluidity of the membrane to be evaluated: the fluorescence anisotropy.

Our research group has now demonstrated in living cells that it is possible to assess biophysical modifications of the membrane after irradiation at relatively low doses, in the range 0 to 8 Gy (Figure 3). The membrane fluidity varies in direct proportion to the received dose and in inverse proportion to the dose rate irrespective of the probe. The membrane is organized in micro-domains that react differently. The lipid compartment of the membrane seems to become more fluid, this fluidity varying according to the depth in the membrane, whereas the lipid-protein interface becomes stiffer. The effect can persist for up to 48 hours after the irradiation. This technique is applicable to several types of cell: lymphocytes, **red blood cells**, **fibroblasts**, and even intestinal cells. However, experiments have so far only focused on *in vitro* models from human blood or cultures of irradiated cells. These first findings await confirmation in various ***in vivo*** models.

Figure 3. Left: relation between the parameter characterizing the fluidity of the membrane and the dose received by blood lymphocytes for various dose rates, after incorporation of a fluorescent probe in the lipid part of the membrane. The dose-effect relations show that the membrane fluidity increases with the dose, the fluorescence anisotropy falling off, owing to the degradation of the lipid environment of the probe. The extent of this degradation increases as the dose decreases. Right: the membrane alterations persist for up to 48 hours after the irradiation.







C. Cieutat/IPSN

Inspecting a preparation using an inverted microscope. In this case the illumination comes from above the preparation, and the objectives are under the stage. This system is particularly well suited to examining dishes containing cell cultures.

Radio-induced apoptosis

Three techniques have been applied simultaneously to the same biological samples to estimate radio-induced apoptosis at different stages of the cell's progression toward its programmed death:

- **Fluorescent labeling with annexin V**, which affords an estimation of the transverse diffusion of phosphatidylserine, from the inner membrane surface where it is normally found, to the outer surface on apoptosis.
- **Labeling with $DiOC_6$**, a fluorescent molecule that penetrates specifically into mitochondria and whose intensity variation allows the determination of the drop in mitochondrial transmembrane potential occurring during apoptosis.
- **The FADU test (Fluorescence Analysis of DNA Unwinding)**, a spectrofluorimetric method that offers the possibility of evaluating the degradation of DNA properties directly linked to the number of radio-induced strand breaks.

In vitro studies on irradiated human blood grown in culture have shown, in the lymphocyte population, dose-effect relations that persist at least for seven days post-irradiation. The first two techniques provide reproducible and comparable results. A second *in vivo* study on rats from which blood samples were taken at different times after irradiation, has demonstrated that the detection of radio-induced apoptosis is possible up to 48 hours afterwards using mitochondrial labeling. The other two methods are applicable only in the hours following the irradiation.

The difference between the results obtained *in vitro* and *in vivo* can be explained by the cascade of events leading to apoptosis. Experiments show that exposure of phosphatidylserine outside the membrane, a signal for the elimination of the apoptotic cells by the **macrophages**, occurs *in vivo* within 48 hours of irradiation. In contrast, no elimination of apoptotic cells takes place *in vitro*, in the absence of macrophages in the cell suspension. Accordingly the same phenomenon is observed for much longer.

Eventually all these methods of investigation will give us a complete multiparametric picture of the effects of ionizing radiation on the cell and the organism. Adaptation of some of these techniques to cells other than blood cells is planned in order to be able to respond more specifically to the problem of localized irradiation.

Philippe Voisin
Department of Human Health Protection and Dosimetry
Nuclear Protection and Safety Institute
Fontenay-aux-Roses - France