Assay of new systems on *in vivo* mutagenesis for determining the effects of low doses of ionizing radiation

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ABSTRACT

Ionizing radiation reacts directly and indirectly with the genetic material in living cells and produces DNA damage. Processing of this damage by correcting enzymes may result in appearing of mutations which, in turn, may lead to carcinogenesis.

We have focused on the determination of *in vivo* mutagenesis induced after exposure to X-rays, aiming at establishing methods to evaluate the effect of low doses of radiation. *In vivo* mutagenesis has been addressed in the Muta Mouse model that carries a lacZ marker gene and provides a relatively simple assay of appearance of mutations.

Mutation frequencies were determined in the lacZ gene copies recovered from mice irradiated with 1 Gy or 4Gy of Xrays, acute or fractionated. Liver, spleen and bone marrow DNA samples were isolated at different times after irradiation, ranging from 1 day to 2 months, and evolution of mutations was studied.

Results showed different responses depending on the organ and especially on the time of analysis, suggesting that the mutagenic proccess *in vivo* is much more complex than previously deduced from *in vitro* experiments. Therefore, determination of the relationship between dose and mutagenic effect *in vivo* will require additional studies.

INTRODUCTION

Ionizing radiation is capable to interact with the living matter producing changes in intracellular molecules, both in the cytoplasm and the nucleus. Chemically reactive molecules produced by ionizing radiation in the cytoplasm may, in turn, react with the nuclear material. As a result, changes in the DNA molecular structure may arise directly or indirectly after irradiation.

Although changes can also occur in other biomolecules, it is generally believed that the biological effects observed after irradiation are mainly caused by changes in DNA structure. This is particularly true for the effects observed at long times after low-dose irradiation, especially for cancer.

In order to maintain cellular continuity, the genetic information contained in the DNA molecules must be passed on to daughters cell after cellular division. It is essential that the DNA structure and sequence are strictly preserved during the cell cycle, especially at DNA replication. In addition, since cellular functions depend upon adequate DNA

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transcription and translation into proteins, changes in DNA sequence may also disturb cell growth and survival.

Cells are provided with a series of enzymatic mechanisms devoted to correction of possible mistakes occurring during replication and/or damages induced after external injury by radiation or chemicals. Mostly, enzymatic repair of damaged DNA occurs correctly, in the sense that both DNA structure and coding sequence are restored. However, removal of certain types of damage or at certain points during cell cycle may be particularly difficult and the repair proceeds with error. In this case, the original sequence of DNA is changed and it is said to have mutations.

Mutations may be classified as *point mutations* (affecting to single base pairs in the DNA sequence), *insertions and deletions* (affecting to one or more consecutive bases which are added or lost, respectively, with respect to the original sequence). The type of mutation is highly dependent on the type of damage induced by a particular agent. In the case of ionizing radiation all types of mutations have been found to occur, although large deletions appear to be the most important.

The biological effects derived from changes in the DNA sequence depend on the relevance of the protein affected by mutations and even on the relative importance of the mutated region with regard to protein functionality. In other words, similar mutations may range from unimportant to crucial, depending on the region of the gene(s) affected. Normally, large deletions will compromise the function of one or several proteins and will appear more deleterious than point mutations, but certain point mutations are known to be sufficient to produce a strong effect.

Radiation-induced cancer is supposed to be due to mutations occurring on critical genes coding for proteins involved in the control of cellular growth and differentiation. The higher the dose, the higher the probability of those critical genes being affected.
Most of the knowledge on the mechanisms for induction of mutations after irradiation comes from experiments in which cells, cultured *in vitro*, had been irradiated at high doses of X or γ rays. However, isolated cells do not behave as cells within a tissue and it is possible that the results obtained do not reflect the actual mutagenic changes that would lead to a carcinogenic process in a living organism. More recently, mutations at particular genes have been studied after *in vivo* irradiation. These experiments usually involved *in vitro* culture of cells during several cell generations, in the presence of a selecting medium. Apart from the limitations due to cell type and possible artefacts due to selection, these methods provide valuable results. There is the problem, however, that results appear highly variable depending on the gene studied and on the experimental conditions used, and there are not yet clear conclusions regarding the mutation frequency induced by a given dose of radiation.

In the last years, transgenic animals have played an important role on the elucidation of the molecular mechanisms involved in carcinogenesis and other pathological processes.
In this regard, animal models for the evaluation of *in vivo* mutagenesis have been designed and are being used to study the mutagenic properties of many chemicals. These models appear to be useful also to determine the mutation frequency induced by ionizing radiation under different conditions of *in vivo* exposure, thus giving a better approach to the knowledge of the mutagenic mechanisms operating *in vivo* and allowing for

determination of the number and type of mutations remaining after various times after irradiation.

OBJECTIVES AND EXPERIMENTAL APPROACH.

Within a collaborative project CIEMAT-CSN, we focused on the study of the mutagenic process induced *in vivo* by ionizing radiation with particular interest on determining the effect of low doses of X rays.

Aiming at this, we decided to use the transgenic model "Muta Mouse" consisting of an otherwise normal mouse, carrying the bacterial gene LacZ integrated in both copies of chromosome number 3. This gene serves as a molecular marker in which it is relatively simple to determine the frequency of mutations appearing after irradiation of the whole animal; therefore, this mouse model gives the potential to determine induced mutation frequencies in any tissue, under any irradiation conditions.

Female Muta Mice (10-week old) were subjected to irradiation with X rays from a Phillips equipment (MG324). Mice were introduced into a metacrylate chamber designed to this purpose and situated onto a rotating platform to ensure uniform irradiation. Within this system, we were able to vary either dose (varying time of irradiation) or dose rate (varying distance).

In order to first establish conditions for mutation analysis, two moderate doses of X rays (1Gy and 4 Gy) were assayed in these experiments. Future experiments will address the effect of lower doses. Doses were given either acute or fractionated in five consecutive days. Mice were sacrificed at different times after irradiation, ranging from 1 day to two months. Relevant organs were extracted, deep frozen and stored at -70°C until analysis.

Mutation frequencies in the lacZ genes isolated from tissues of irradiated mice were determined as follows :

Genomic DNA was isolated from the tissue of interest by incubation at 50°C in lysis buffer (containing SDS, proteinase K and RNAse), phenol extraction and alcohol precipitation. About 6 to 10 µgs of genomic DNA were incubated with Muta Plax extracts (Epicentre) at 32 °C to package fragments containing the lacZ marker gene into infective lambda phages. Packaged mixtures were used to infect an engineered strain of E. coli (lacz$^-$galE$^-$) unable to grow in the presence of galactose. Plating was performed in the presence of P-gal, a product which is metabolized to toxic galactose when a functional copy of the lacZ gene is present in E. coli. In this assay, phages carrying intact lacZ genes are unable to form plaques, whereas phages carrying mutant copies of the lacZ gene do not transform the P-gal and, therefore, are able to survive and form plaques. Thus, only mutant copies of the marker gene are detected in this way. Plating was also performed, in parallel, in the absence of P-gal to allow for total packaged phages to be scored. Mutation frequency in a given sample was determined as the relation between mutant and total phages scored for that sample.

RESULTS AND DISCUSSION.

Mutation frequencies were determined in the lacZ gene of DNA samples isolated from liver, spleen and bone marrow of mice irradiated with either one single dose of 1 Gy or 4 Gy, or with 5 doses (consecutive days) of 0.2 Gy or 0.8 Gy. Thus, comparisons can be made between organs from the same animals and between doses, both acute and fractionated. Control animals (non irradiated) were also analyzed in order to determine the background mutation frequency in the lac Z gene of the three selected tissues.

Available results showed that the mutation frequency increases up to about 5-fold in liver and spleen after acute irradiation of 4 Gy ; values not significantly higher than the background mutation frequency were found after 1 Gy of X-rays. On the contrary, preliminary results obtained in bone marrow showed an increase in the mutation frequency at 1 Gy, indicating the possibility of strong differences in "sensitivity" to mutagenic effects between organs. Most probably, this accounts for differences in growth rates of different cell populations *in vivo*, rather than for different sensitivities to damage by radiation. Since it is the total mutagenic effect what may be relevant for carcinogenesis, confirmation of these results would be in good agreement with the observed high frequency of hemopoietic cancers induced by radiation.

Differences were also observed between organs with regard to the kinetics of the mutation frequency. Liver and spleen showed a detectable increase of mutations after a few days, which lasted until about 20 days after irradiation; after that time, the mutation frequencies returned to background values. In the bone marrow, the mutation frequencies appeared to increase at later times but remained higher for longer times. The reason of the decay of the mutation frequencies might be related to elimination of cells with high number of mutations, perhaps through an apoptotic phenomenon, but confirmation of this hypothesis would require complementary experiments.

It is interesting to remark that the results shown here, although not yet conclusive, indicate that mutagenesis occurring in an organ *in vivo* is a complex process that varies very much with time after irradiation and may display a different pattern within different organs. Variation with time should be taken into account when comparing the effect due to different irradiation conditions such as dose rate or fractionated doses, since maximal mutation rates may appear at different times. Similarly, comparisons between organs should consider differences in their kinetics for the accumulation of mutations; otherwise, it would be possible that organs with a delayed increase in the mutation frequency were regarded as less sensitive for this effect.

Finally, we would like to stress that the mutagenic process *in vivo* appears to be much more complex than what is deduced from older *in vitro* experiments and it does not seem likely that a simple relationship between dose and mutation frequency will apply to all cases. Therefore, much work has to be done to gain further knowledge on this process, and especially to relate it to tumor appearance, in order to be able to use mutation frequency as an early indicator of carcinogenic risk after radiation injury.