IL NUOVO CIMENTO Vol. 31 C, N. 1 Gennaio-Febbraio 2008
DOI 10.1393/ncc/i2008-10279-3

PULEX: Influence of environment radiation background on biochemistry and biology of cultured cells and on their response to genotoxic agents

F. Antonelli([1]), M. Belli([1])(*), M. Pinto([1]), O. Sapora([1]), E. Sorrentino([1]), G. Simone([1]), M. A. Tabocchini([1]), F. Amicarelli([2]), L. Conti De Virgiliis([2]), M. C. Carbone([2]), M. Balata([3]), L. Ioannuci([3]), S. Nisi([3]) and L. Satta([4])

([1]) *Dipartimento di Tecnologie e Salute, Istituto Superiore di Sanità and INFN Sezione di Roma I Gr. coll. Sanità - 00161 Rome, Italy*
([2]) *Dipartimento di Biologia di Base ed Applicata, Università de L'Aquila Coppito (AQ), Italy*
([3]) *INFN, Laboratori Nazionali del Gran Sasso - L'Aquila, Italy*
([4]) *INFN, Laboratori Nazionali di Frascati - Rome, Italy*

(ricevuto l' 11 Giugno 2008; pubblicato online il 16 Settembre 2008)

Summary. — Some years ago we performed two experiments aimed at studying the influence of the background radiation on living matter by exploiting the low radiation background environment in the underground Gran Sasso Laboratory of the INFN. Their results were consistent with the hypothesis that the "normal" background radiation determines an adaptive response, although they cannot be considered conclusive. PULEX-3 (the third experiment of the series) is aimed at comparing the effects of different background radiation environments on metabolism of cultured mammalian cells, with substantial improvements with respect to the preceding ones. The experiment was designed to minimize variabilities, by maintaining two cultures of Chinese hamster V79 cells in exponential growth for up to ten months in the underground Gran Sasso Laboratory (LNGS), while two other cultures were maintained in parallel in a biological laboratory installed at the LNGS outside the tunnel. Exposure due to γ-rays was reduced by a factor of about 10 in the underground laboratory while the Rn concentration was small in both cases. After ten months the cells grown in the underground laboratory, compared to those grown in the external one, exhibited: i) a significantly lower capacity to scavenge reactive oxygen species (ROS), and ii) an increased sensitivity to the mutagenic effect of rays. Since the probability that this finding is due to casual induction of radiosensitive mutants is extremely low, it corroborates the hypothesis that cells grown in a "normal" background radiation environment exhibit an adaptive response when challenged with genotoxic agents, which is lost after many generations in a low background radiation environment.

PACS 87.53.-j – Effects of ionizing radiation on biological systems.
PACS 87.53.Ay – Biophysical mechanisms of interaction.

(*) E-mail: mauro.belli@iss.it

1. – Introduction

In spite of the many studies, there is still a large uncertainty on the health effects of exposure to low doses and low dose-rates of ionising radiation, as those usually encountered in the environment, in working activities, and in medical diagnostic applications. What is usually done is to extrapolate to low levels the risks known from epidemiological studies concerning acute exposures at high and moderate doses, notably the so-called "Life Span Study" of the Japanese survivors to the Hiroshima and Nagasaki atomic bombs. As from 1959 the International Commission for Radiological Protection (ICRP) has accepted a linear, no threshold, (LNT) relationship between dose and health effects to assess the risks of low levels exposures. However, to perform a scientifically sound extrapolation, a detailed understanding of the mechanisms by which radiation induces cancer and genetic disorders is required. Since this is not yet the case, though new information has been gained in recent years on this subject, the LNT model can be considered as a pragmatic guideline (it is worth noting it implies additivity of effects due to exposures occurred at different times during all one's life span), but it could be too simplistic.

As a matter of fact, the reaction of cells and tissues at low doses is quite complex, as demonstrated by the increasing importance given in recent years to the so-called "non targeted effects", such as the adaptive response, the genomic instability, the low dose hypersensitivity, and the bystander effect.

By the term "Adaptive response" (for a review, see [1] and [2]) is usually meant the induction in a biological system of resistance to moderate or high doses of a genotoxic agent by a previous exposure to a small dose of the same agents, called conditioning dose. The adaptive response to ionising radiation was first described by Olivieri *et al.* [3] in 1984 but has received new attention in view of identifying common mechanisms underlying a number of non-targeted effects and related to intra- and inter-cellular signalling [4]. Such a phenomenon implies that small doses of radiation may reduce the natural incidence of cancers and the question arises about the possible role of the "natural" radiation background. However, there is small information on the biological response after exposure to such protracted exposures. Collection of experimental data on this point is important not only to assess the risks related to chronic occupational exposure, but also to understand the role of natural radiation background in the evolution of life.

The tunnel at the Gran Sasso National Laboratory (LNGS) of the Istituto Nazionale di Fisica Nucleare (INFN), located under the Gran Sasso d'Italia mountain, which is an excellent shielding against cosmic rays and neutrons, offers a unique opportunity for investigating whether a significant reduction in the background exposure can change the susceptibility to acute exposures of genotoxic agents.

2. – Previous radiobiological experiments on adaptive response under the Gran Sasso tunnel

A short description of the preceding radiobiological experiments performed at the LNGS with a discussion of their results is here in order. In the first one [5], which can be considered as a feasibility study, the biological model was the yeast *Saccharomyces Cerevisiae*, strain D7. The experiments was proposed in 1993 to INFN by L. Satta ("La Sapienza" University in Rome and INFN-LNF), on behalf of a research group which included researchers of the INFN-Laboratori Nazionali di Frascati, of the Cell Biology Department at "La Sapienza" University in Rome, and of the "Centro di Genetica Evoluzionistica", CNR in Rome. This was a multidisciplinary group involving both

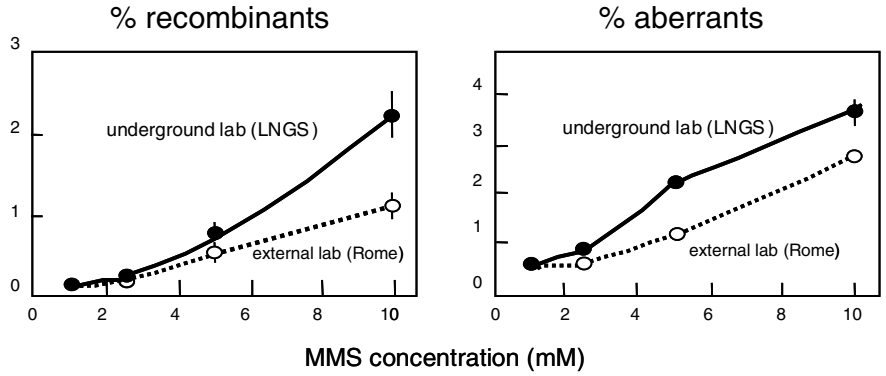

Fig. 1. – Percentage of recombinant and aberrant cells in the yeast *S. Cerevisiae* after treatment with various concentrations of MMS. Cultures were grown for more than 100 generations under the Gran Sasso tunnel (solid lines) or at the Rome University (dashed lines). Redrawn from [5].

physicists and biologists. The experiment, named PULEX (from the Latin word for flea, to mean the small space required under the Gran Sasso tunnel in contrast to the huge dimensions of other experiments performed there) was approved by the INFN and yeast cells were cultured in parallel under the tunnel and in the Molecular Genetics Institute of "La Sapienza" University in Rome. After more than 100 generations the cultures were treated with increasing concentration of methyl-methane-sulphonate (MMS), a genotoxic agent, and the percentage of wrong repair was measured for both cultures. Cells grown under the tunnel exhibited a reduced repair efficiency with respect to those cultured in the external laboratory as measured by the percentage of recombinant and aberrant cells (fig. 1).

This finding stimulated the interest of other researchers and suggested to extend the study to mammalian cells. A second collaborative study started which involved, in addition to L. Satta, various groups at the Istituto Superiore di Sanità (ISS) in Rome (M. Belli and co-workers), at INFN-LNGS (M. Balata and co-workers), and at L'Aquila University (M.P. Cerù, L. Conti Devirgiliis and co-workers). This second experiment was approved by the LNGS at the end of 1998. A cell culture laboratory was then set up in a single room building located in the gallery, equipped with a CO_2 incubator shielded by an iron box with 10 cm thick walls to further reduce the background radiation during cell growth and a continuous flush of air taken from outside the mountain was provided to avoid accumulation of radon gas. Under these conditions the gamma dose rate and the radon concentration inside the incubator in the tunnel were reduced by factors of about 70 and 25, respectively, with respect to the incubator at the ISS in Rome. For this study Chinese hamster cells of the V79 strain were chosen. The cultures were monitored for growth capacity, enzymatic activity and basal mutations at the *hprt* locus. Several biological end-points were determined, namely apoptosis induction by a protein synthesis inhibitor, *hprt* mutation induction after acute irradiation, expression of apoptosis-related genes (p53 and c-myc). The two cultures were monitored for 9 months and during this time a number of differences between them were detected [6].

The cells kept for 9 months in the tunnel showed: a) a significant increase of the cell density at confluence b) a significant differences in their capacity to scavenge reactive oxygen species (ROS) caused by the aging processes; c) a greater apoptotic sensitivity at the third month of culture that was however no longer detected after 9 months; d) an

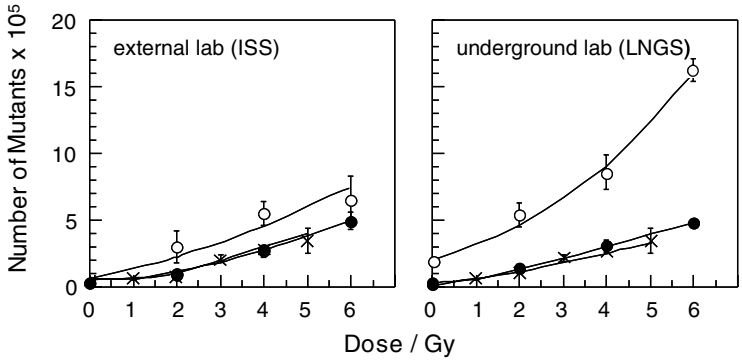

Fig. 2. – Number of mutants as a function of γ-ray dose. Cells were grown for 3 (closed circles) and 9 (open circles) months at the ISS and under the LNGS tunnel. Results obtained at zero time of culture (crosses) have been included in both panels. Redrawn from [6].

increase, after 9 months, in both the basal *hprt* mutation frequency and sensitivity to the mutagenic effect of γ-rays (fig. 2).

Overall, these data suggest a role of ionising radiation background in determining an adaptive response, which is lost after many generations spent in a low radiation environment. However, from this experiment the possibility cannot be excluded that, after many generations (9 months correspond to roughly 540 cell duplications), mutant clones having different characteristics have been selected in the two cultures, independently of the different radiation background.

Therefore we decided to plan a new experiment, aimed to disentangle these two possibilities. The experiment, PULEX-3, was submitted to INFN and approved in 2003. However, its start was postponed several times due to logistic reasons related to the extensive civil engineering works which were necessary inside the underground Gran Sasso Laboratory. In effect, the long observation time required for this kind of experiments is not compatible with repeated stop and go. For this reason, the PULEX biological laboratory went into operation at the end of March 2006. Moreover, the long culture time, increasing the possibility of external contamination, made it necessary to adopt some contingency plans which slowed down the experimental work.

3. – The PULEX-3 approach

In PULEX-3 the problem of possible random mutations was faced by setting up two independent cultures of V79 cells both inside the Gran Sasso tunnel and in the external laboratory. Furthermore, to reduce margins for errors, the external laboratory was also installed at the Gran Sasso Laboratory outside the tunnel, in the Chemical Department building. This assured a uniform treatment of the four cultures since the operators in charge for the four cell cultures could be the same, differently from the case of laboratories located at a long distance (*i.e.* ISS-Rome and LNGS-L'Aquila). The constant presence of the same operators for the manipulation of the biological samples is a crucial point for the quality of such a critical experiment. However, this arrangement reduced the ratio of external/internal radiation background, since the gamma contribution at the LNGS external laboratory was found to be less than at the ISS in Rome, and the radon concentration was found to be approximately the same in the external and in the underground laboratories.

TABLE I. – *Extent of apoptosis, as measured by the TUNEL assay, induced by CHX treatment in V79 cells after 3 months and 10 months of culture in "normal" (cultures A&B) and in low (cultures C&D) radiation background conditions. The average values for the culture pairs are listed with their semidispersions.*

	% apoptosis			
	3-month culture		10-month culture	
	Control	CHX treated	Control	CHX treated
Av. A&B ("normal" bk)	0.45 ± 0.15	7.2 ± 5.9	0.65 ± 0.25	32 ± 21
Av. C&D ("low" bk)	0.35 ± 0.25	11.7 ± 8.9	3.2 ± 2.4	53 ± 19

An effort was made to evaluate the relative contributions to the dose received by the cells in the two different laboratories, coming from exposure to external gamma radiation and to radon and its progeny.

Duplicate and independent cultures of Chinese hamster V79 cells, grown in both the external laboratory at LNGS (cultures A and B) and in the underground laboratory under the tunnel (cultures C and D) were monitored for 10 months. Apart from the radiation background, all other parameters, such as culture medium, serum, buffers, plastics, etc., were kept identical (*i.e.* prepared in the same way from the same batch) for all the cultures. The background γ-ray dose rate was 40.1 ± 4.2 nGy/h in the external laboratory and 4.3 ± 0.9 nGy/h in the underground laboratory. The radon concentration in both laboratories was about 5 Bq/m^3. If the background radiation acts as a priming dose eliciting an adaptive response subsequent to a challenging dose, the behaviour of the two cultures should differ as time goes by, at least for some biological end-points.

In the following we briefly comment on the experimental results obtained for the various biological end-points examined.

4. – Experimental results

4˙1. *Micronuclei induction.* – Chromosome damage, which can be caused by exposure to ionizing radiation, was determined with the cytokinesis-block micronucleus assay [7], in V79 cells either grown inside the underground laboratory or in the external laboratory. All the cell cultures were irradiated with 1 Gy of X-rays at the Ospedale San Salvatore at Coppito in the L'Aquila area, using a 6 MV medical linear accelerator at a dose rate of 2 Gy/min. Then, the number of induced micronuclei per 2000 binucleated cells were scored. After 10 months of culture there is a slight increase in the normalized number of induced micronuclei with respect to the zero time for both growth conditions and no significant difference is found between the V79 cells grown in the presence of low background radiation and that grown under "normal" background conditions (data not shown).

4˙2. *Apoptosis induction.* – Apoptosis induced by cycloheximide (CHX) treatment was assayed by flow cytometry, analyzing the labelled DNA strand breaks with the TUNEL (Terminal Deoxynucleotide Transferase dUTP Nick End Labeling) assay [8]. All the cultures showed an increasing sensitivity to the drug with the time, probably dependent on cellular aging. Table I lists the extent of apoptosis measured in the different cultures after 3 and 10 months under "normal" (A and B) and in low (C and D) radiation background

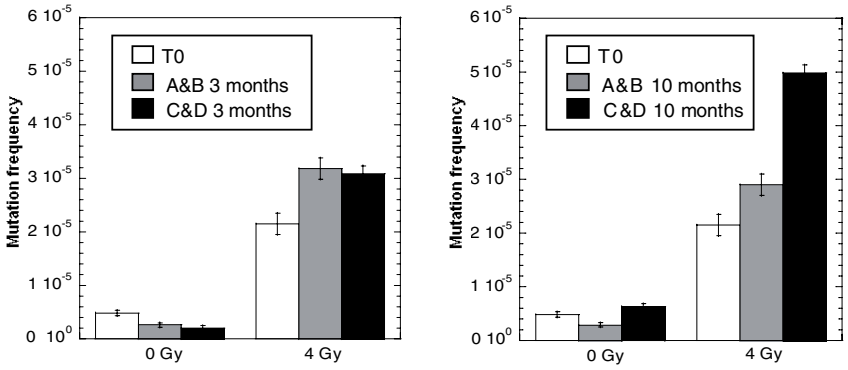

Fig. 3. – Number of mutants as a function of X-ray dose. A&B are underground cultures; C&D are external cultures; T0 refers to the cultures at zero time. Left panel: 3-month culture. Right panel: 10-month culture.

conditions. It can be seen that cells cultured in the underground laboratory are on average more sensitive to apoptosis induction than those grown in the external laboratory. This effect, hinted at 3 months, is still maintained after 10 months of culture. However, the variability within each pair of cultures resulted comparable to the differences between cultures in different conditions, so that it is hard from these results to draw conclusive indications, even if the average values suggest that apoptosis induction may be easier for cells grown under the tunnel.

4˙3. *Mutation induction.* – To study the mutagenic sensitivity to ionising radiation, all four cultures (*i.e.* the two pairs growing underground and in the external laboratory) were exposed to 4 Gy of X-rays after 3 months and 10 months and the dose-response curves for induction of mutations at the *hprt* genetic locus was measured. Also the culture at zero time was assayed, carrying out several experiments. Irradiations were performed at the Ospedale San Salvatore at Coppito (L'Aquila) with the same accelerator and dose rate used for micronuclei induction.

The results are presented in fig. 3. Since there was a consistent response of the cultures within each pair, the average value for each pair of cultures is here presented. Also the measurement at zero time is reported. After 3 months of culture (left panel) the responses, in terms of mutation frequencies induced by X-irradiation, of the underground and of the external cultures were rather similar. After 10 months of culture (right panel) the responses of the underground cultures are significantly higher than the corresponding values of the external cultures, indicating the onset of an increased radiosensitivity for mutation induction in comparison to the cells grown in the external laboratory.

It must be noted that an additional pair of cultures were grown in the laboratory at the Istituto Superiore di Sanità (ISS) in Roma, where the dose rate from background radiation was 287 ± 30 nGy/h and the average radon concentration about 120 Bq/m^3. When they were assayed for mutation induction, one of them showed an anomalous response, as its background mutation frequency became larger by an order of magnitude with respect to all other cultures, while the response of the second one was similar to that of the other external cultures (data not shown).

4˙4. *Anti-oxidant enzyme activity.* – Anti-oxidant enzymes are involved in the processing of the reactive oxygen species (ROS), known to be damaging species also induced by

radiation. In order to see if there were differences between the two background radiation conditions, the biochemical activities of superoxide dismutase (SOD), catalase (CAT) and selenium-dependent glutathione peroxidase (GPx) were measured in all the V79 cell cultures.

The ratios CAT/SOD and GPx/SOD are related to the scavenging efficiency of cells against ROS. An increase in one or both of these ratios means an increase in ROS scavenging efficiency, while the opposite holds for a reduction in these ratios.

We found that after 10 months the ratio GPx/SOD increases by a factor of about 2.5 in the cultures grown in the external laboratory, while we found no significant changes for those grown in the underground laboratory. This suggests that cells cultured in the presence of "normal" background radiation have a more efficient enzymatic pool for ROS removing in comparison to those cultured in a reduced background radiation environment.

5. – Conclusions

Two experiments aimed at studying the influence of the background radiation on living matter by exploiting the low radiation background environment in the underground Gran Sasso Laboratory of the INFN gave results consistent with the hypothesis that the "normal" background radiation determines an adaptive response, although they cannot be considered conclusive. The PULEX-3 experiment addressed a number of biochemical and biological aspects in the attempt to obtain a wide picture of the role played by the background radiation on a cell population. The main goal was to prove (or disprove) its role in the maintenance of a cell response of the adaptive type.

Among the assays performed, measurement of mutation induced by X-rays showed that V79 cells cultured for long time under reduced background radiation conditions became more sensitive, and antioxidant enzymes determination showed they are less protected against ROS produced by ionising radiation, compared to the parallel external cultures. These features, which are mutually consistent, were found after 10 months but not after 3 months.

Other assays did not give consistent indications, but we never found opposite results, *i.e.* it never happened that cells cultured under the Gran Sasso tunnel exhibited a greater resistance with respect to those grown in the external laboratory.

The value of the "positive" results obtained from PULEX-3 is higher than those from previous experiments, thanks to its design which involved pairs of cultures. This makes very unlikely that the observations are due to the random selection of mutants with such properties capable to mimic the loss of an adaptive response after 10 months inside the tunnel, since this should occur in both sister cultures. In effect, if one assumes that the mutation frequency in V79 cell cultures is of the order of 10^{-4}–10^{-5} for a given genetic locus, and that it is sufficient one mutated locus for giving the features observed (this is clearly an overestimation), the probability of observing the same mutant in two independent cultures is very small, of the order of 10^{-8}–10^{-10}.

Overall, the results obtained from PULEX-3 reinforce the hypothesis suggested by our previous studies that the background radiation induces an adaptive response in living matter. In effect, we observed that V79 cells cultured for long time under reduced background radiation conditions became more sensitive and less protected against ionizing radiation. This can be explained by the loss of the adaptive response gained in the presence of the "normal" background.

In order to give an even more firm support for this conclusion, we are presently exploring performing the following tests:

1) to extract the cells grown under the Gran Sasso tunnel and culture them in the external laboratory, together with those already there, to check if, in the presence of the "normal" background radiation, they will be able to recover the "lost" adaptive response;

2) as above, but giving an acute priming dose first, "equivalent" to that received by the external culture during the conditioning time and then a challenging dose to check if they show an adaptive response similar to that exhibited by the external culture.

* * *

This work was partly supported by INFN-CSN5 (PULEX and PULEX-3 experiments) and by INFN-LNGS (PULEX-2 and PULEX-3 experiments). The authors are grateful to Prof. V. Tombolini and to the technical staff at the Ospedale San Salvatore, Coppito, L'Aquila, for allowing irradiations of biological samples at the 6 MV linear accelerator.

REFERENCES

[1] Tapio S. and Jacob V., *Radiat. Environ. Biophys.*, **46** (2007) 1.
[2] Wolff S., *Environ. Health Perspect.*, **106** Suppl. 1 (1998) 277.
[3] Olivieri G., Bodycote J. and Wolff S., *Science*, **223** (1984) 594.
[4] Kadhim M. A., Moore S. R. and Goodwin E. H., *Mutat. Res.*, **568** (2004) 21.
[5] Satta L., Augusti-Tocco G., Ceccarelli R., Esposito A., Fiore M., Paggi P., Poggesi I., Ricordy R., Scarsella G. and Cundari E., *Mutat. Res.*, **347** (1995) 129.
[6] Satta L., Antonelli F., Belli M., Sapora O., Simone G., Sorrentino E., Tabocchini M. A., Amicarelli F., Ara C., Cerú M. P., Colafarina S., Conti Devirgiliis L., De Marco A., Balata M., Falgiani A. and Nisi S., *Radiat. Environ. Biophys.*, **41** (2002) 217.
[7] Fenech M., *Nature Protocols*, **2** (2007) 1084.
[8] Bortner C. D., Oldenburg N. B. and Cidlowski J. A., *Trends Cell Biol.*, **5** (1995) 21.