CHAPTER 12

CLASTOGENIC FACTORS, BYSTANDER EFFECTS AND GENOMIC INSTABILITY IN VIVO

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Abstract: For the last 15 years, we have investigated low dose radiation genetic effects on human populations affected by the Chernobyl accident. Cytogenetic longitudinal investigations showed that amount of radiation markers for clean-up workers remained at the elevated level and had trend to grow up with the time. A dynamic profile of the amount of aberrations confirms that this group has symptoms of the genomic instability. State of the genomic instability correlates with accumulation of clastogenic factors, responsible for increased genomic instability in clean-up workers peripheral blood. As a model for clastogenic activity testing, we used human keratinocyte cell line with blocked 1st check point of cell cycle. Our results confirm that cytogenetic and molecular effects of irradiation can be fixed even 20 years after the Chernobyl accident.

Keywords: Chernobyl accident; liquidators; affected populations; clastogenic factors; bystander effect; cytogenetic and molecular effects; low dose

Introduction

Today problem of genomic instability is crucial point of modern radiobiology. In a lot of publications they have shown that people suffered from additional irradiation manifest the stable long time elevation of chromosomal aberrations (Melnov, 2004). Mechanism of this event is not clear until now.

From the other side, in a last few years phenomenon of radiation-induced bystander effect has been fixed (Nagasawa and Little, 1992; Mothersill and Seymour, 1997).

C. Mothersill et al. (eds.), Multiple Stressors: A Challenge for the Future, 171–182.

In our understanding, bystander effect could be a key point of the genomic instability formation in vivo. In such situation, clastogenic bystander inducers may be spreaded out via blood serum in body. Possibility of spreading of such factors via liquid phase previously has been shown in vitro (Mothersill and Seymour, 1997).

Basing on this idea, we investigated the clastogenic effect of the serum samples from the patients affected by radiation from different sources and compared it with serum samples from patients suffered from acute and chronic virus diseases.

Materials and Methods

CELL CULTURES

HPV-G cells

The HPV-G cell line is a human keratinocyte line, which has been immortalised by transfection with the HPV virus, rendering the cells *p53* null. They grow in culture to form a monolayer, display contact inhibition and gap junction intracellular communication.

HPV-G cells were cultured in Dulbecco's MEM: F12 (1:1) medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine and 1 μg/mL hydrocortisone. The cells were maintained in an incubator at 37°C, with 95% humidity and 5% carbon dioxide and routinely subcultured every 8–10 days. When 80–100% confluent, the medium was poured from the flask and replaced with 1:1 solution of versene (1nM solution) to trypsin (0.25% in Hank's Balanced Salt Solution) (Gibco, Irvine, UK) after washing with sterile PBS. The flask was placed in the incubator at 37°C for about 11 min until the cells started to detach. The flask was then shaken to ensure that all cells had been removed from the base of the flask. The cell suspension was added to an equal volume of DMEM F12 medium to neutralize the trypsin. From this solution new flasks could be seeded at the required cell quantity.

Human peripheral blood lymphocytes

Blood samples were collected by standard venous puncture and stored with heparin (20 units/mL) no longer than 2–3 h before treatment. Human peripheral blood lymphocytes were cultured in RPMI-1640 medium (100 mL), including fetal bovine serum (20%), and gentamycin (0,2%). 4.5 mL of mixtures were aliquoted into 50 mL culture flasks and frozen at −20°C, before cultivation.

SAMPLE DONORS

The victims of the Chernobyl accident included three main categories: Chernobyl liquidators 1986–1987, workers from Polessky State Radiation and Environment Reserve (PSRER workers) and people living in territories of Gomel region contaminated by radionuclides. The control group were clinically healthy people corresponding to other groups in age and sex aspects.

BLOOD SERUM EXTRACTION

The blood samples were taken and placed in Vacutainers for serum extraction (Becton Dickinson), centrifuged at 2,000g for 10 min, and the serums were frozen and stored at −20°C before use. Before freezing the serum was filtered through Nalgene 0.22 μm filters in order to remove all residual cell components of the blood.

ROUTINE CYTOGENETIC TEST

At the day of analysis flasks with medium were defrosted, 0.5 mL of blood was added and 0.13 mL of PHAM (Sigma, 1 mg/1 mL). Flasks were cultivated in incubators for 48 h at 37°C. About 3 h before the end of cultivation 30 μL/flacks (standard stock solution) of colchicine was added. Lymphocytes were centrifuged for 5 min at 1,500 rpm, supernatant was removed and cells are washed with warm 0.55% KCl solution (at 37°C) for 20–25 min at incubator. Cells were fixed using Karnua solution (1 part of glacial acetic acid and 3 parts of methanol, 5 mL of solution per flask). Cold fixator was changed three times for 10, 20 and 10 min.

Suspensions were mounted on microscope glasses (3–4 drops of sample per glass) and dried at 40–42°C. Cells were stained using 10% Giemsa solution for 8 min and after drying analyzed under light microscope. Only cells with the chromosomes number between 44 and 47 were analyzed.

MICRONUCLEI TEST

After seeding human keratinocytes, cells were left at 37°C in the CO_2 incubator to attach for 12 h. The blood serum from affected populations was added to 24 cm^2 flasks (NUNC, USA) (6000 cells per flask) 1–2 days after seeding, and cells were cultivated in the incubator for 1–2 h. Then cytochalasine B was added (7 μg/mL concentration) and the cells were incubated for 24 h, additionally.

After this the cell-culture medium was removed, the cells were washed with PBS and fixed with chilled Karnua solution (1 part of glacial acetic acid and 3 parts of methanol, 10–15 mL three times for 10–20 min). Later flasks were dried and stained with 10% Giemsa solution.

The micronuclei count was carried out under inverted microscope (×400). Micronuclei were counted only in binucleated cells (1,000 binucleated cells per flask). All the data is calculated as the micronuclei number recorded per 1,000 binucleated cells (micronuclei were analyzed only in binucleated cells).

Results and Discussions

Results of the dynamic investigation of the main group (liquidators) has been analyzed (Marozik et al., 2007) and presented on Figs. 1 and 2.

Shortly summarizing this data, one can conclude that with time the sum of aberrations is growing up constantly, and maximal level has been fixed in 2004–2005 (Fig. 1). At the same time, we did not fix prominent dynamics for markers (Fig. 2), dicentric and ring chromosomes – main irradiation specific cytogenetic markers.

It means that the main increase of the total number of aberrations took place because of the elevated levels of unspecific aberrations (predominantly single and double fragments). In other words, investigated group manifested the presence of genomic instability symptoms in somatic cells.

This conclusion is absolutely reliable in comparison with the same data for control group (Figs. 3 and 4), were dynamics of the same parameters are absolutely different.

In control group statistically reliable dynamics for both total number of aberrations and for the level of marker aberrations were absent.

So, basing on this one can conclude that even 20 years after the Chernobyl accident liquidators had the cytogenetic effects in peripheral blood lymphocytes.

This situation was typical not only for liquidators, but also for children-prominent residents of contaminated areas.

Our results presented on Fig. 5 show the results of the investigation of the children from the special areas of Brest region where situation is characterized by quick migration of radionuclides along the food chains, resulting in their high accumulation in human body (children – "accumulators").

This group has been investigated three times with interval 2–3 years (whole period – 8 years).

Analyzing data from Fig. 5, we were able to confirm that dynamics of the investigated parameters of cytogenetic status in this group is very close to liquidator situation – practically all types of aberrations increased with the

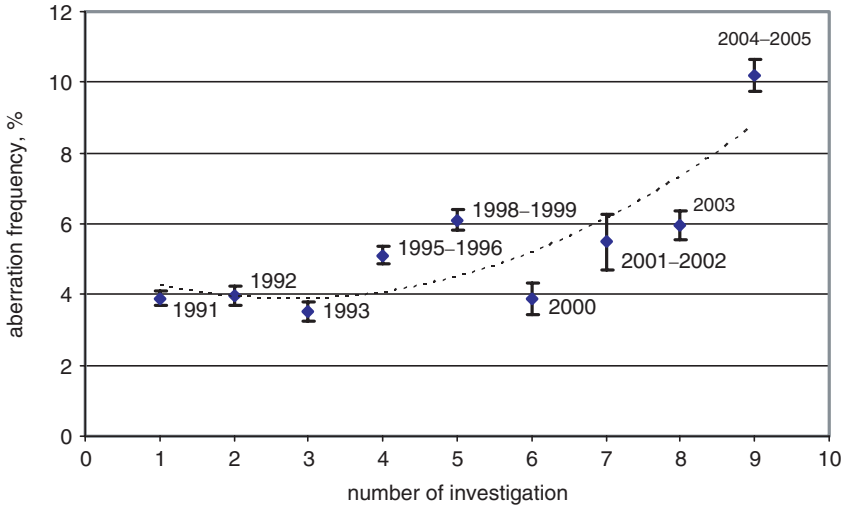

Fig. 1. Dynamics of the total number of aberrations in liquidators lymphocytes.

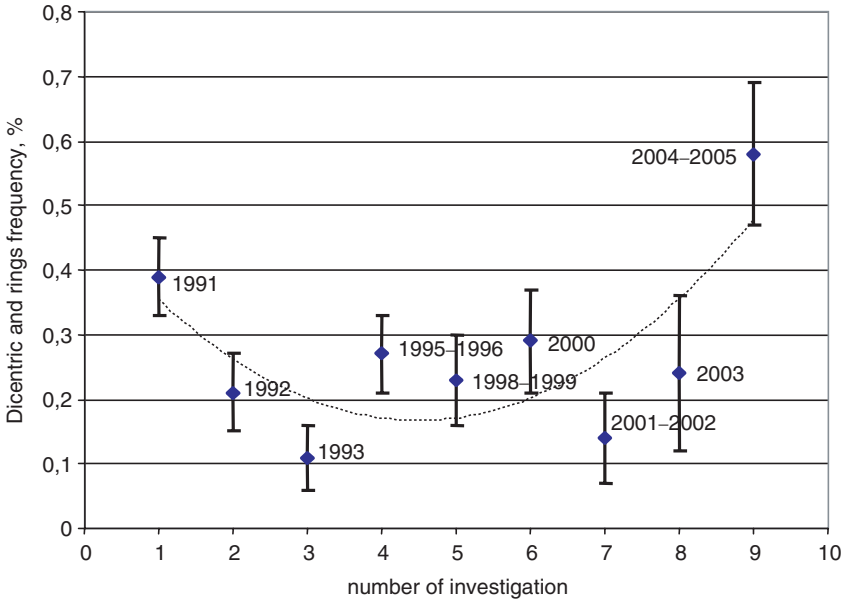

Fig. 2. Dynamics of the level of dicentric and ring chromosomes in liquidators lymphocytes.

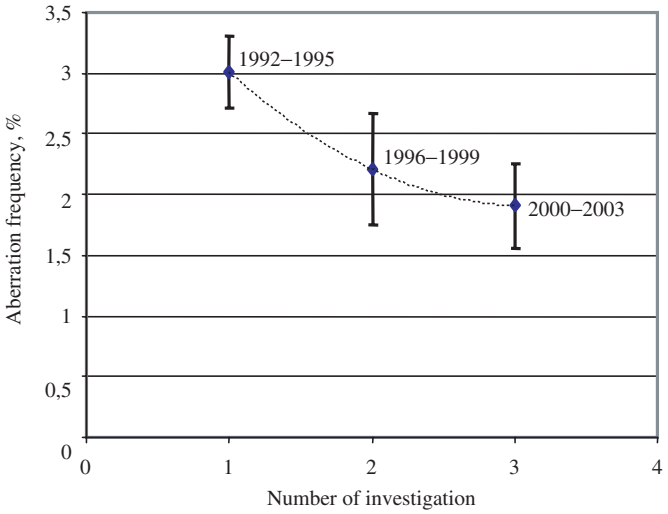

Fig. 3. Dynamics of aberrations frequency in control group.

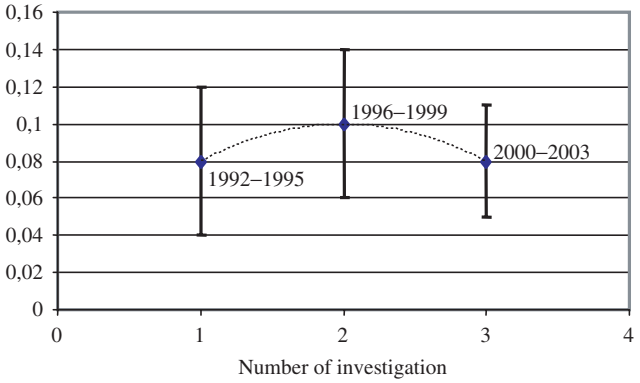

Fig. 4. Dynamics of dicentrics and rings frequency in control group.

time, but main elevation was connected with unspecific aberrations (single and double fragments) predominantly.

So we can conclude that the genomic instability is a typical phenomenon for human somatic cells in delayed period of time after irradiation as for short-time middle dose (liquidators) so as for low dose chronic irradiation (children from contaminated areas).

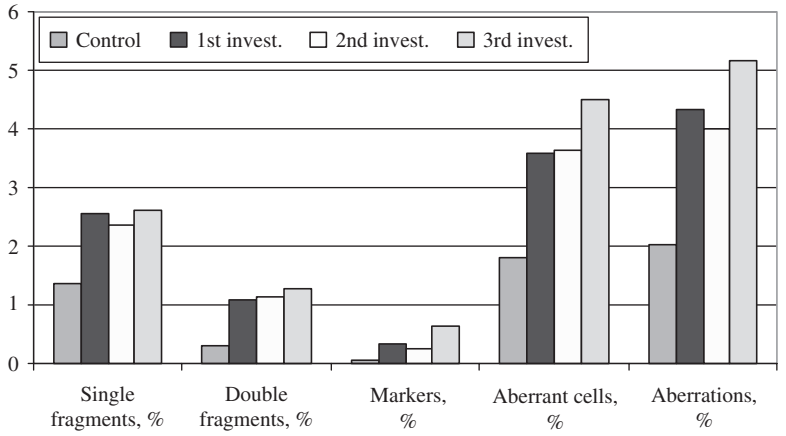

Fig. 5. Dynamics of cytogenetic status of children – "accumulators".

During the investigation we collected blood samples from four groups of patients: liquidators (short-time irradiation), workers of Polessky State Radiation and Environmental Reserve (PSRER, 30 km area around Chernobyl station – low dose chronic irradiation), people from contaminated territories of Gomel region suffered with hepatitis C and children from Gomel region with acute virus infection (flu).

Results of the incubation of keratinocytes in the presence of serum samples from liquidators are summarized in Fig. 6.

Basing on collected data, we can conclude that liquidators serum samples showed real clastogenic effect – the amount of induced micronuclei (in comparison with control group serum samples) increased approximately in three times (273.7 ± 22.4 and 91.8 ± 12.4, correspondingly; $p < 0.01$).

Specially important that these serums induced elevated levels of polymicronuclei cells (e.g., in control group cells with three micronuclei were absent absolutely).

Similar situation took place for workers of PSRER (Fig. 7).

Again the discrepancies between main and control groups were statistically reliable (the total micronuclei frequency 260.3 ± 18.3 vs. 91.8 ± 12.4; frequency of cells with micronuclei 232.2 ± 13.1 vs. 84.2 ± 11.5; $p < 0.05$). At the same time, the discrepancy between levels of micronuclei for liquidators and PSRER workers was statistically unreliable, but at the same time, for PSRER workers induced levels were constantly a little bit lower than for liquidators.

Similar situation has been fixed for two other investigated groups – for patients with chronic (hepatitis C) and for children with the acute (flu) virus infection (Figs. 8 and 9). Maximal induced level has been fixed for patient with acute virus infections (Fig. 9).

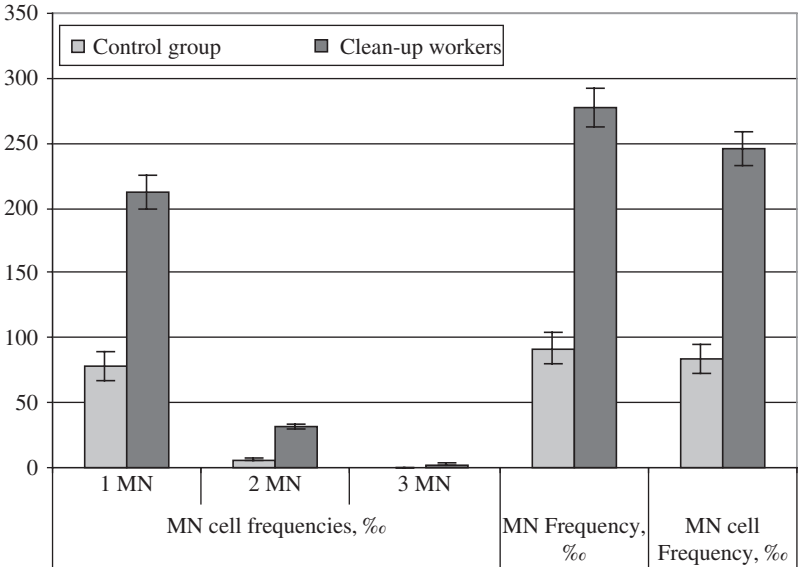

Fig. 6. Comparison of micronuclei induction by serums from people of control group and liquidators.

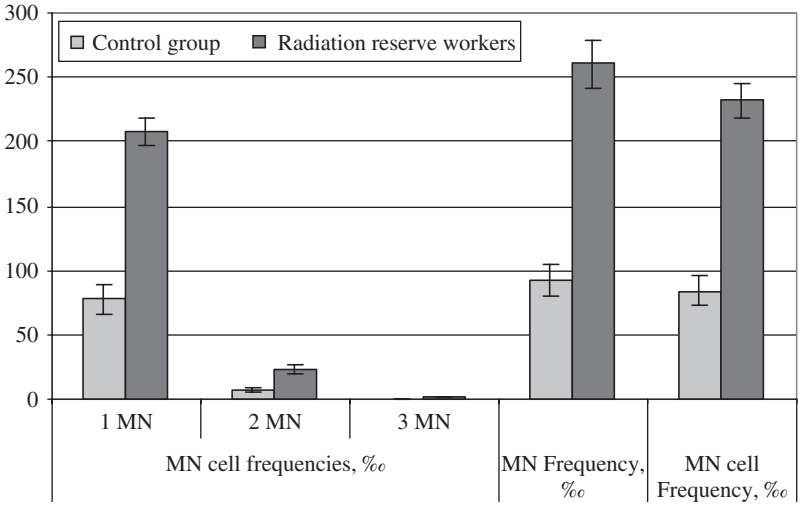

Fig. 7. Comparison of the levels of micronuclei induction by serums from patients of control group and PSRER workers.

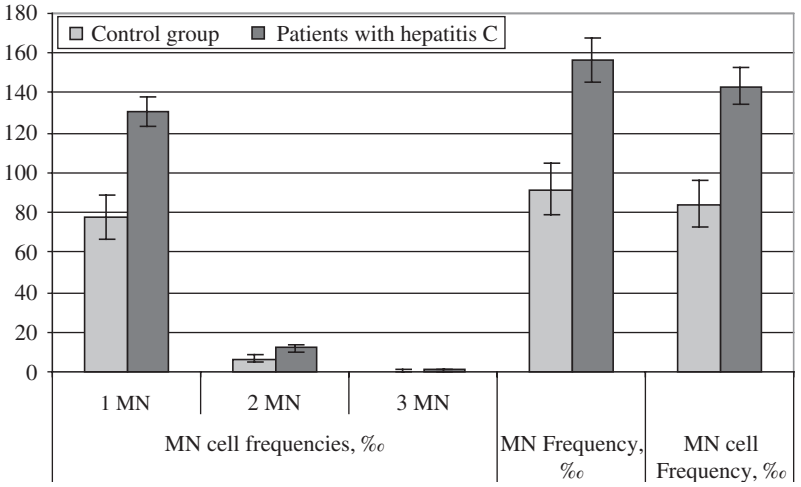

Fig. 8. Comparison of micronuclei induction by serum samples from patients of control group and with hepatitis C.

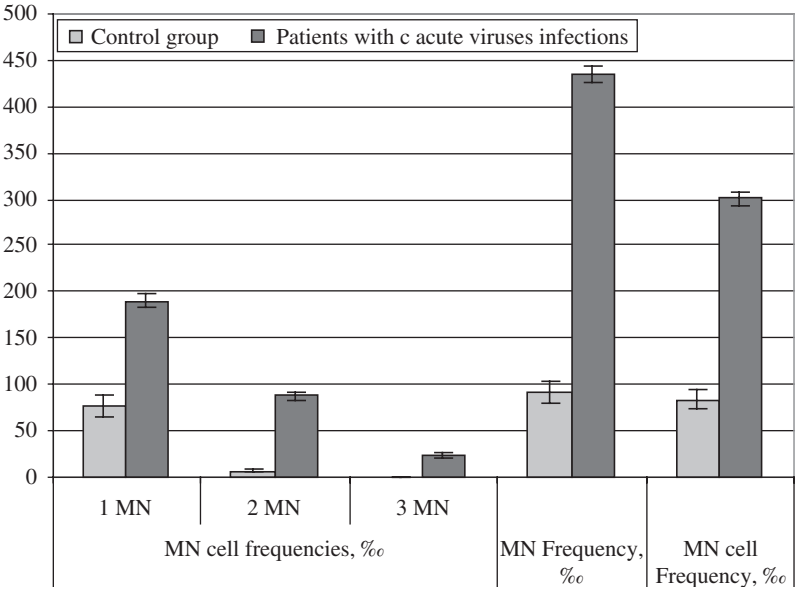

Fig. 9. Comparison of micronuclei induction by serum samples from patients of control group and with acute virus infection.

But we need to mention that 2 weeks later after virus infection the level of clastogenic factors for patients recovered from virus infection moved to norm, and serums did not induced additional micronuclei. In the serum samples from patients with chronic virus infection (hepatitis C) these factors continued to exist.

The similarity of clastogenic factor profiles for people affected by radiation and virus infection could be rooted in the similar role and higher induction of free radicals. Their effect for radiation effects is of common knowledge today. At the same time, it is well known that free radicals are generated in excess in a diverse array of microbial infections. Free-radical induced pathogenicity in virus infections is of great importance, because evidence suggests that NO and oxygen radicals such as superoxide are key molecules in the pathogenesis of various infectious diseases and have practically radio mimetic effect (Kash et al., 2004). Although oxygen radicals and NO have an antimicrobial effect on bacteria and protozoa, they have opposing effects in virus infections such as influenza virus pneumonia and several other neurotropic virus infections. The unique biological properties of free radicals are further illustrated by recent evidence showing accelerated viral mutation by NO-induced oxidative stress (Akaike, 2001).

Neutrophil host defense mechanisms are categorized as oxidative and non-oxidative. Oxidative mechanisms rely upon the production of superoxide, primarily by the multisubunit enzyme NADPH oxidase. ROS are also implicated in the activation of transcriptional factors (NF-κB and activator protein 1) leading to the transcription of genes that accentuate the inflammatory process (Swaun et al., 2004). Expression microarray analysis performed on lung tissues isolated from the infected animals showed activation of many genes involved in the inflammatory response, including cytokine, apoptosis and lymphocyte genes that were common to different infection groups.

Our data above gave us possibility to suggest that elevated level of chromosome aberrations will be stimulated by clastogenic factors and at the same time with activated their synthesis. If such idea is correct, there should be direct correlation between clastogenic effect of serum samples and the level of aberrations in the lymphocytes from the same blood samples.

In Table 1, shown earlier correlation between cytogenetic status parameters and the level micronuclei induction is presented.

Our results confirm that there is stable and deep correlation between the number of induced micronuclei and unspecific aberrations (single fragments $r = 0.50$; double fragments $r = 0.49$; $p < 0.005$) and total number of aberrations and aberrant cells ($r = 0.76$ and $r = 0.65$, correspondingly; $p < 0.005$ for both). In our opinion, it means that there is direct correlation between genomic destabilization and accumulation of clastogenic factors.

TABLE 1. Correlation matrix (by Spearman) for cytogenetic parameters compared to induced effect

Parameters of comparison, %	Number of cells with MN, ‰	Total MN number, ‰
Single fragments	0.50*	0.62*
Double fragments	0.49*	0.55*
Dicentric and ring	0.07**	0.08**
Atypic chromosomes	−0.24**	−0.24**
Polyploid cells	0.13**	0.10**
Number of aberrant cells	0.65*	0.71*
Total number of aberrations	0.76*	0.84*

*$p < 0.005$; **$p > 0.1$

Conclusions

Shortly summarizing our results, we can confirm the next:

- After irradiation destabilized genome initiate the cell production of bystander factors, able to stimulate the increase of the genomic destabilization of other somatic cells, transforming this situation in long time effect, possibly responsible for delayed health effects;
- Virus infections, which are able to increase the level of free radicals, can stimulate the same effect. But duration of its manifestation is depended upon the type of virus: if the decease is temporal, such situation will take place only during the acute phase; in the chronic situation (hepatitis C) it is a prominent effect.

Shortly speaking, our data confirm that radiation-induced bystander effect, induced in vivo, is a standard biological phenomena, responsible for delayed health effect of radiation.

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