Arh. hig. rada, *19* (supl. 1) (1968) 117.

THE EFFECT OF CERTAIN AGENTS ON RADIOSENSITIVITY OF AMOEBA AND ON RECOVERY FROM RADIATION DAMAGE

YVETTE ŠKREB

In order to evaluate damages caused by ultra-violet and gamma radiation, we studied the survival of whole amoebae and their nucleate and anucleate fragments, and the changes observed in the synthesis and quantity of RNA and proteins. The research led us to the following conclusions:

– To minimise the radiotion damage, and to obtain the maximal effect from the two agents – actinomycin D and cooling – the presence of the nucleus and entire cytoplasm is imperative.

– Actinomycin D, if used prior to the irradiation, will increase the radiation sensitivity and inhibit the spontaneous recovery, while cooling immediately after radiation enhances the recovery.

– The cytoplasmic DNA takes no part in the recovery phenomena.

Amoebae (Amoeba proteus) have for years served as a very convenient experimental material in cellular biology, because of their specific properties:

They are easy to cultivate, their division cycle is quite slow, their radiation resistance high, and in addition, they can be dissected in two parts, one of which contains the nucleus and the cytoplasm, the other one only the cytoplasm. Among the many researchers who had worked with the amoebae in the field of cellular biology, photobiology and radiation biology, we shall mention only those whose results represented the starting point of our work:

Brachet (1) has through a series of biochemical analysis, performed upon amoeba fragments, shown that fragments without the nucleus can not only survive two weeks but that they even possess a form of a metabolic activity. He thus refuted a number of hypotheses giving a central role to the nucleus, and minimizing the role and significance of the cytoplasm.

Mazia and *Hirshfield* (2) have exposed to the ultraviolet rays, in increasing dosages, whole amoebae and their fragments. Observing their survival rate, they saw that irradiated whole amoebae lived longer than their fragments, and that anucleate fragments were far more sensitive.

By application of X-rays, *Ord* and *Danielli* (3) have established a high radiation resistance of amoebae. By a method of transplanting irradiated nuclei into a non-irradiated cytoplasm, and the other way around, they pointed at the sensitivity of the cellular nucleus, and at a specific role of its polyploidy.

On the basis of these key experiments, we studied radiation damage in amoeba from the following angle:

NUCLEO-CYTOPLASMIC RELATIONSHIP AND RADIOSENSITIVITY OF AMOEBA

In duplicating the experimental conditions of *Mazia* and *Hirshfield* (2), after having observed the survival rate, we have concluded (4) that ultraviolet rays are more harmful to anucleate fragments than to the whole amoebae or their nucleate fragments. Length of the starvation period preceeding irradiation, increases the sensitivity to irradiation. Cytochemical study (staining of cells by the Unna-Brachet method) has shown that radiation causes a decrease of basophilia in the cytoplasm and nucleus, which is also proportional to the dosage of ultraviolet radiation. Large dosages of irradiation very quickly disrupt the vital cellular functions, hence we aimed our attention at the effect of lower dosages which do not cause evident morphological changes but only modify biochemical processes in a cell.

Accordingly, our interest was pointed at the metabolic changes of the ribonucleic acid (RNA) and of proteins following radiation dosages ranging from 1,200 to 4,800 erg/mm square. We have used biochemical micromethods to establish quantities of those components in whole amoebae, in nucleate and anucleate fragments, at various times after irradiation. Whether we used UV radiation (5) or gamma rays (6), there occured a decrease of the RNA quantity which in time increased again. This decrease is, as a rule, lesser in whole amoebae, and after 2–3 days there appears a recovery.

Under identical experimental conditions (7) it was noticed that the cellular oxygen intake drops off only in the anucleate fragment, while in the nucleate fragment or in the whole amoeba, it remains at the same level as that in the control, non-irradiated amoebae.

A specific protein such as the acid phosphatase, behaves somewhat differently (8). Enzyme activity after irradiation shows different degrees of inhibition of the same order of magnitude both in nucleate and anucleate fragments.

Incorpotation of specific precursors such as ^{14}C adenine, and ^{14}C phenylalanine, as observed by the autoradiographic method following the UV (9) or gamma (10) radiation, confirmed the quantitative data (5, 6) obtained earlier.

From these results we were able to conclude that the presence of a nucleus, and the integrity of the cytoplasm are essential if a minimal damage from a certain radiation dosage is to be achieved. These data alone can serve to show the important role of the nucleus which – although irradiated – takes an active part in repairing the cell after irradiation.

THE INCREASE OF RADIOSENSITIVITY OF AMOEBA UNDER THE INFLUENCE OF ACTINOMYCIN D

After the importance of the metabolism of the ribonucleic acid and proteins within nucleocytoplasmic relationships in irradiated amoebae has been evaluated, a new problem emerged: Can irradiation resistance of a cell be increased by interrupting the DNA-RNA-proteins reaction chain, by using specific agents such as actinomycin D which binds in a cell with the deoxyribonucleic acid (DNA) and inhibits the synthesis of the messenger RNA? Thus, whole amoebae, their nucleate or anucleate fragments, were exposed to the action of actinomycin D (50 ug/ml) for two hours, or to gamma radiation (100 Kr), or to both factors applied consecutively, though actinomycin D was washed out before gamma rays were applied.

In whole amoebae which were in actinomycin D for two hours, division was inhibited for 5 days, and afterwads the amoebae divided normally. When both agents were applied in the same experiment, cellular division was irreversibly inhibited (11). The surviving rate of fragments followed the same pattern as in the preceding case: anucleate fragments, treated with both agents, were most heavily damaged and died quickly.

To be able to observe more closely the effect of those agents on RNA synthesis, *Horvat* (12) used ^{3}H-cytidine as a percursor. Inhibition of RNA synthesis in the cells treated with actinomycin D is clearly demonstrated in a decreased number of beta traces in the autoradiogram: the cytoplasm retains 65–70% of radioactivity as compared with the control group, while the nucleus retains a bit less than that. In irradiated amoeba the cytoplasm retains 50–60% radioactivity while the nucleus retains a little more.

Cells treated with both agents retain only 15–30% of ^{3}H-Cytidine in the cytoplasm of whole amoebae and nucleate fragments, the nucleus itself retaining virtually none. Anucleate fragments show no traces of tritiated precursor. It would appear that actinomycin D causes heavier damage to the nucleus, while radiation produces a more harmful effect on the cytoplasm.

As a tracer in the protein synthesis we used ^{14}C-phenylalanine. The results of counting beta traces in the autoradiogram are more scattered, but under closer observation they show that the effects are of the same

kind and of the same order of magnitude, and that the synthesis inhibition is even more pronounced. In described reactions, amoebae react more in the manner of microorganisms than of mammalian cells (13).

It appears that actinomycin D has a twofold effect on the radiation sensitivity of amoeba. First, it increases radio-sensitivity – this is why some damage on the biochemical level is heavier (RNA and proteins), and second, it prevents the spontaneous restoration i.e. recovery which is a normal occurrence after gamma radiation. These conclusions coincide with data by *Elkind* (14), obtained in mammalian cells.

REPARATION OF RADIATION DAMAGE IN AMOEBA BY COOLING

Having learned one way how to increase radiation damage, we wanted the reverse – to try speeding up the mentioned spontaneous recovery. In our earlier work (15) we found out that, in the case of amoebae, there exists a possibility of photorestoration by visible light, which is accompanied by a significant increase of the RNA and protein quantity in whole amoebae and their fragments.

From the results obtained by other authors who have studied effects of various external agents upon the recovery from radiation damage in other types of cells, we have expected that low temperatures, too, might present a suitable agent for the restoration of radiolesions in amoeba. It is known that cooling can temporarily slow down the cellular metabolism, and in some cases prevent the development of radiation damage (16, 17, 18). We presumed that a spontaneous recovery, we had earlier observed in amoebae, could be speeded up and enhanced under the conditions of low temperature applied immediately after irradiation. This was confirmed by our preliminary experiments (19).

In continuing the research, *Eger* (20) worked with whole amoebae, their nucleate and anucleate fragments concurrently in four groups: control group kept at room temperature; cell group which was for 2 hours cooled at 6°C and returned to room temperature; UV-irradiated cells at room temperature, and cells which immediately after irradiation were cooled at 6°C. for 2 hours, and then returned to room temperature.

The cooled cells divided slowly at first, but in 3 days the division rhythm came back to the normal. Irradiated amoebae did not divide for the first 18 days. Compared to these, the amoebae that were both irradiated and cooled resumed their division after only 3 days.

Survival curves for nucleate fragments in all four experimental groups, fall off together. The fastest decline was observed in irradiated fragments, a much slower one in irradiated and cooled fragments. The

survival rate of double-treated fragments does not differ from the survival curve of the control group i.e. cooled fragments. Anucleate fragments presented an identical picture, though the dying off is much faster.

Quantitative measurement of RNA in all irradiated cells has shown that the RNA decreases as soon as two hours after irradiation, and that after 24 hours there remained only 60% of the acid. Subsequently, the RNA quantity in whole cells gradually increases, while in fragments it continues to decrease. In whole amoebae and nucleate fragments which were cooled after irradiation, the RNA quantity remains virtually unchanged. In irradiated anucleate fragments, after irradiation, the RNA quantity decreases continually, even more so than in untreated fragments.

Protein quantity changes are very much like those for the RNA. Whole cells and nucleate fragments react in the same way. Irradiated cells show a very definite drop, and after 2 hours there remains only 70% of protein, after 48 hours only 50%. Doubly-treated cells retain 80–90% of protein as compared with normal cells. Anucleate fragments show a significant decrease of proteins, while cooling remarkably increases this drop in irradiated fragments.

The results can be compared with data obtained by other authors. *Kovaleva* (16) has established a similar temperature effect in irradiated paramecia. The same conclusion was arrived at by *Giese* (17) with the ciliate Blepharisma. *Schrek* and *Elrod* (18) have established that low temperature affects irradiated lymphocytes in the rat by inhibiting the progress of radiation damage.

The obtained data show that low temperature partially and temporarily inhibits the whole metabolism of a cell, and thereby also the processes which accompany the progress of radiation damage. Under those conditions, a spontaneous recovery can evolve faster, but to achieve this, it is imperative that a cell have its nucleus and entire cytoplasm.

THE ROLE OF NUCLEAR AND CYTOPLASMIC DEOXYRIBONUCLEIC ACID IN THE PROGRESS OF RADIATION DAMAGE

Though we were able to offer certain explanations about the metabolism of RNA and proteins after irradiation and after application of other agents, we had no data about the DNA in the Amoeba proteus. We have therefore undertaken a quantitative measurement of DNA in amoebae by comparing the amounts in nucleate and anucleate fragments. *Benzinger* (21) has found out that the nucleus contains very little DNA, while the cytoplasm is relatively rich with it.

We have tried to correlate the quantitative data for the nuclear and cytoplasmic DNA in the amoeba with the changes of RNA and proteins in the processes following irradiation and in recovery processes. We have reached a conclusion that in the described phenomena the cytoplasmic DNA takes no part, while all those processes depend solely on the DNA of the nucleus (2).

This research was performed by cooperation of the Cellular Biology Laboratory staff. We had always enjoyed the useful advice and support of Professor J. Brachet of the Laboratory for Animal Morphology, and Professor M. Errera, Biophysics and Radiobiology, Université libre de Bruxelles, Rhode-S. T.-Genèse, Belgium.

References

1. *Brachet, J.:* Recherches sur les interactions biochimiques entre le noyau et le cytoplasme chez les organismes unicellulaires. I. Amoeba proteus, Biochim. Biophys. Acta, *18* (1955) 247.
2. *Mazia, D., Hirshfield, H. I.:* Nucleus-cytoplasm Relationships in the Action of Ultraviolet Radiation on Amoeba Proteus, Exptl. Cell Research, *2* (1951) 58.
3. *Ord, M. J., Danielli, J. F.:* The Site of Damage in Amoebae Exposed to X-rays, Quart. J. Micr. Sci., *97* (1956) 29.
4. *Škreb, Y., Errera, M.:* Action des rayons U. V. sur des fragments nucléés et anucléés d'amibes, Exptl. Cell Research, *12* (1957) 649.
5. *Škreb, Y., Bevilacqua, Lj.:* Acide ribonucléique et protéins sur les fragments d'amibes irradiés, Biochem. Biophys. Acta, *55* (1962) 250.
6. *Škreb, Y.,Eger, M., Horvat, Đ.:* Effets des rayons gamma sur la teneur en acide ribonucléique et en protéines des amibes, Biochim. Biophys. Acta, *103* (1965) 180.
7. *Škreb, Y., Škreb, N.:* Effets des rayons U. V. sur la respiration des fragments d'amibes, Biochim. Biophys. Acta, *39* (1960) 540.
8. *Škreb, Y., Lončar, B.:* Activité de la phosphatase acide chez les fragments d'amibes irradiés, Biochim. Biophys. Acta, *80* (1964) 523.
9. *Errera, M., Ficq, A., Logan, R., Škreb Y., Vanderhaege, F.:* Nucleocytoplasmic Relationships in Irradiated Cells, Exptl. Cell Research, Suppl., *6* (1958) 268.
10. *Škreb, Y., Horvat, Đ.:* Influence of Gamma-Radiation on the Incorporation of ^{14}C–adenine and ^{14}C–Phenylalanine Into Whole Amoebae and Nucleate and Anucleate Fragments, Exptl. Cell Research, *43* (1966) 639.
11. *Škreb, Y., Horvat, Đ.:* Interactions de l'actinomycine D et de l'irradiation gamma chez les Amibes, C. R. Acad. Sc. Paris, *264* (1967) 340.
12. *Horvat, Đ.:* Eksperimentalne modifikacije sinteze nukleinskih kiselina i proteina kod Amoeba proteus, Magistarski rad, Sveučilište u Zagrebu, 1967.
13. *Denis, H.:* Effets de l'actinomycine sur le développment embryonnaire. III. Etude biochimique: influence de l'actinomycine sur la synthèse des protéines. Develop. Biol., *9* (1964) 473.
14. *Elkind, M. M., Whitmore, G. F., Alescio, I.:* Actinomycin D: Suppression of Recovery in X-Irradiated Mammalian Cells, Science, *143* (1964) 1454.
15. *Škreb, Y., Bevilacqua, Lj.:* Photorestauration des fragments d'amibes irradiés, Exptl. Cell Research, *28* (1962) 430.
16. *Kovaleva, N. E.:* Vlijanie temperaturi na soderžanie ribonukleinovoi kisloti u Paramecium caudatum posle rentgenovskogo oblučenija, Citologija, *6* (1964) 709.

17. *Giese, A. C., McCaw, B., Cornell, R.:* Retardation of Division of Three Ciliates by Intermittent and Continuous Ultraviolet Radiations at Different Temperatures, J. Gen. Physiol., *46* (1963) 1095.

18. *Schrek, R., Elrod, L. M.:* Radioresistance of Lymphocytes Incubated at Low Temperatures, Rad. Res., *24* (1965) 657.

19. *Škreb, Y., Eger, M.:* Effet restaurateur d'une basse température sur la survie, le taux du RNA et des protéines des amibes irradiées, C. R. Acad. Sc. Paris, *264* (1967) 477.

20. *Eger, M.:* Modifikacije efekta UV zračenja niskom temperaturom u Amoeba proteus, Magistarski rad, Sveučilište u Zagrebu, 1967.

21. *Benzinger, Lj.:* Kvantitativno određivanje deoksiribonukleinske kiseline kod amebe (Amoeba proteus), Magistarski rad, Sveučilište u Zagrebu, 1966.

22. *Škreb, Y., Eger, M., Horvat, Đ.:* Study of Deoxyribonucleic Acid in the Cytoplasm of Amoeba proteus, Biochem. J., *105* (1967) 53.