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RADIATION DOSE DEPENDENCE OF THE SENSITIZATION BY OXYGEN AND OXYGEN MIMIC SENSITIZERS

L. RÉVÉSZ and B. PALCIC

Abstract

New and old evidence is discussed which suggests that the oxygen enhancement ratio (OER) is decreased at lower radiation doses as compared with that of higher radiation doses. In addition, there is evidence that cells irradiated in severe hypoxia have an impaired ability to recover from sublethal damage. However, there is also contrary evidence to these observations. Thus the suggestions that oxygen is not strictly a dose modifying agent have been controversial from the very beginning. It is hoped that new experimental undertakings currently performed in many different laboratories will resolve these issues as well as explain the controversy which has lasted more than 25 years in this field of radiobiologic research.

The development of the clonogenic technique (36) which facilitates quantitative measurements of cell survival as a function of radiation dose of mammalian cells grown in tissue culture, signifies a milestone in modern radiation biology. In general terms, dose/response curves (logarithm of cell survival S as a function of dose D of ionizing radiation) of aerobic cells have been characterized by, a) an initial shoulder at lower doses and b) nearly exponential cell kill at higher doses, which results in an approximate straight line (Fig. 1). The slope of the straight line is defined by the quantity *Do*, which represents the dose reducing cell survival by approximately 37 per cent (1/e). Extrapolation of the exponential part of the curve to zero dose gives the 'extrapolation number' *n* which is a measure of the shoulder. Since a given increment of radiation dose kills fewer cells in the shoulder region than in the exponential region of the survival curve, the shoulder implies a certain protection of the cells at lower radiation doses. The pioneering work of ELKIND & SUTTON (9), using split-dose experiments, indicated that this protection can be explained by the recovery (repair) of sublethally damaged cells.

The fact that hypoxic cells were found in the deficiently vascularized neoplastic tissues stimulat-

From the Department of Tumor Biology II, Karolinska Institutet, S-104 01 Stockholm, Sweden, and Medical Biophysics Unit, British Columbia Cancer Research Centre, Vancouver, B.C., Canada V5Z 1L3. Accepted for publication 21 March 1985.

ed widespread interest of radiation response of hypoxic cells. Despite this interest, a relatively long period of time elapsed between the introduction of the clonogenic techniques is 1956 and the publication of the first report (6) in which this technique was used in studies of survival of hypoxically irradiated mammalian cells. At that time, it was not known that oxygen, which is dissolved in plastic material, would prevent attached cells from becoming hypoxic even in a carefully de-aerated medium (5). Therefore, the cells in the reported experiments were probably not as hypoxic as originally assumed. The Department of Radiation Therapy at Stanford University was among the first institutions in which a systematic investigation of the survival characteristics of cultured mammalian cells was initiated whereby the cells were irradiated under severely hypoxic conditions (45). It was by chance that glass dishes were used instead of plastic dishes to maintain the cells in these studies. Furthermore, although the radiobiologic importance of oxygen contamination in commercially available nitrogen was not recognized until much later, the nitrogen used for the de-aeration of the cellular medium was carefully purified by leading it through heated copper chips in an air-tight system. The results obtained with different mammalian cell lines indicated that the survival curves of cells in the case of hypoxic irradiations have a 2.5 to 3 times larger *Do* (i.e. decreased cellular sensitivity) in comparison to aerobically irradiated cells. Furthermore, the hypoxic survival curves also showed a greatly decreased shoulder portion: the entire survival curve became nearly exponential with an extrapolation number close to unity (Fig. 2, curve b). These results were reported by ROBINSON and RÉVÉSZ at two meetings held in 1961 (37, 45). Neither of the authors could foresee that the results of the radiation response of hypoxic cells would become a controversial issue lasting many years; some questions remain unresolved even after more than two decades.

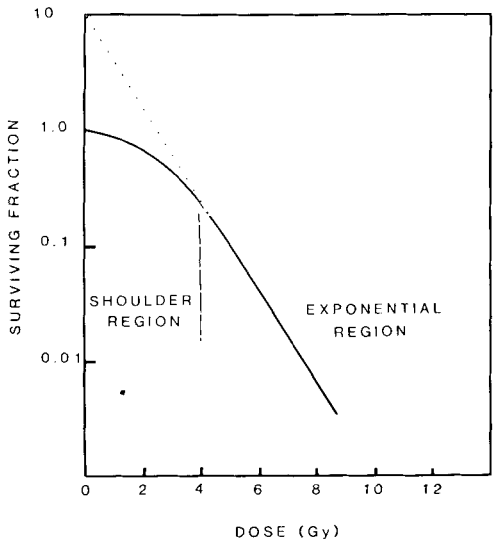


Fig. 1. Typical survival curve of mammalian cells grown in vitro irradiated with graded doses of roentgen rays in the presence of oxygen. The dose/response curve is characterized by the shoulder region and the exponential region. This type of survival curve can be adequately fitted by many mathematical equations. In this work the discussion is limited to the 'single-hit multi-target equation' $S=1-(1-\exp(D/Do))^n$ which has been most often used in the past to fit survival data. Extrapolation number n is obtained by extending the straight line to zero dose. The value of Do can be calculated from the slope of the straight line. For this case $Do=0.9$ Gy and $n=10$.

Survival curve of hypoxically irradiated cells

It was originally proposed (1) that the effect of oxygen on cell survival is simply a dose-modifying effect, thus oxygen enhancement ratio (OER) will be constant throughout the dose range. The results of ROBINSON and RÉVÉSZ, however, suggest that the shape of the shoulder will change in the low dose region; this implies that OER will at least be decreased and possibly becomes equal to one, or even negative (protective effect). These suggestions initiated extensive investigations to experimentally prove various possibilities. At the Department of Tumour Biology in Stockholm, an experimental setup was constructed to permit a precise control of different grades of oxygenation of cells during radiation exposure. In the early sixties, argon was commercially available at a higher purity than nitrogen and thus argon was used in most cases to create hypoxic conditions. Great attention was given to the statistical analysis of the experimental data, particularly, as extrapolation of data from high doses had to be employed in order to establish n. By that time, it was well known that radiation sensitivity of cells can vary from experiment to experiment, thus the data were collected from a great number of replicate tests, many of which were performed as 'paired experiments' keeping all but one of the variables constant. For example, cells from the same culture were treated in an identical way and the only difference was the exposure of cells to radiation under aerobic or hypoxic conditions. Generally, the data were fitted to the 'single-hit multi-target' survival

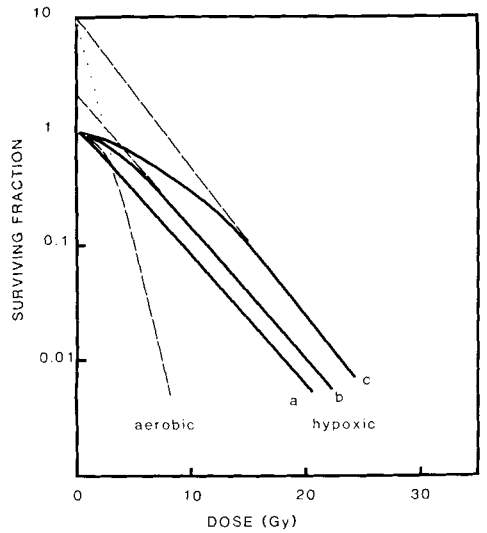


Fig. 2. Schematic presentation of possible survival of cells irradiated at various degrees of hypoxia. In this case, *Do* remains approximately constant (2.7 Gy) but the change is expressed in the extrapolation number *n* which assumes values of 1, 2 and 10 for the curves a, b and c, respectively.

model expressed by the relationship $S=1-(1-\exp(-D/Do))^n$. In a great majority of the experiments, the clonogenic capacity expressing reproductive integrity was selected as a general criterion for cellular survival. In some experiments other end-points were also chosen, such as colony size, cell population growth, or the frequency of micronuclei formation. The experimental procedures, the equipment used and the methods of statistical analysis have been described in detail in a series of articles by the Stockholm group and are referenced in this review.

The evidence which has accumulated over the past years from a great number of experiments performed in different laboratories is concordant with the hypothesis that the survival curve of cells irradiated in nearly complete absence of oxygen, has the *Do* value which is approximately three times larger than the *Do* obtained using aerobically irradiated cells. Less clear is the picture of the extrapolation number *n*. In many instances reports in the literature of carefully conducted experiments show no difference in the extrapolation number *n* (10, 14, 33). However, there is accumulating evidence which suggests that the extrapolation number *n* is lower for hypoxic survival curves than aerobic curves. The mean value of *n* was close to one when argon was used (17, 21, 39), and slightly above one, when nitrogen was used (44) for obtaining hypoxic conditions. The lower *n* of hypoxic survival curves was found to be independent of culture age: this was noted not only with cells in the plateau phase of growth, but also with cells from young exponential phase cultures (40) which have a particularly large *n* after aerobic irradiations. When traces of oxygen (e.g. 200–400 ppm) were present in the gas phase during irradiation, *n* was increased (22). The presence of oxygen in a concentration as low as 0.6 μmol/l (0.6 μmol/l corresponds to approximately 600 ppm of O_2 in solution of 37°C) yielded the value of *n* close to that found from the survival curves of aerobically irradiated cells (see Fig. 2, curve c, for a schematic illustration). This concentration of oxygen is not sufficient to modify *Do* to any appreciable extent and is more than twenty times lower than that needed to induce the maximum change in *Do*. The lack of a significant change in *Do* at oxygen concentrations that are sufficient to significantly affect the extrapolation number *n*, and the fact that standard commercial nitrogen is often contaminated by various amounts of oxygen, may partly explain the controversies which concern the effect of oxygen in the dose range corresponding to the shoulder portion of the survival curve.

Several hypotheses were drawn from these observations, and many of them were experimentally tested. One important hypothesis concerns the OER, which is defined as the ratio of dose of hypoxic and aerobic irradiations which yield the same biologic effect, e.g. the same level of cell survival. If the aerobic and hypoxic survival curves have the same extrapolation number, OER would be constant throughout the dose range and equal to the ratio of slopes of the survival curves. Thus, oxygen would have the same sensitizing (i.e. 'dose modifying') effect at any given dose. However, if the survival curve of hypoxically irradiated cells extrapolates to a lower *n* compared with the curve of aerobically irradiated cells, OER will be decreased in the low dose region. At higher doses it will gradually increase and reach the maximum value (about 3) at very large doses (Fig. 3). This dose dependence of the OER was tested in experiments in which the OER was determined separately in the regions of high and low doses. The observations (44) of an increased OER in the large dose regions (more than about 10 Gy), and the absence of or considerably decreased OER in the shoulder region (0–4 Gy) was in agreement with the theoretical predictions.

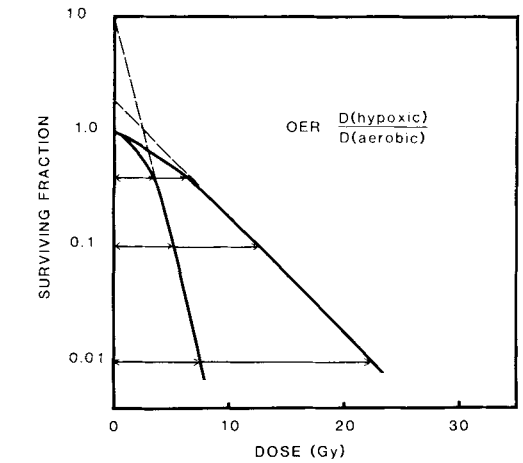
A decreased OER at lower doses was measured in detail by PALCIC and colleagues (27, 31) who

showed that OER decreases at doses below 1 Gy to approximately half of the value of that measured at higher doses (3–30 Gy). In these experiments, no extrapolation of data was used. Instead, in order to obtain sufficient precision of data at low doses, both surviving fraction and killed fraction of cells were assayed, thus eliminating any counting, pipetting and dilution errors. Purified nitrogen (less than 5 ppm O_2 present) was used to deplete oxygen. At the start of hypoxic irradiations the oxygen level was always less than 0.1 μmol/l as measured by a sensitive Clark electrode.

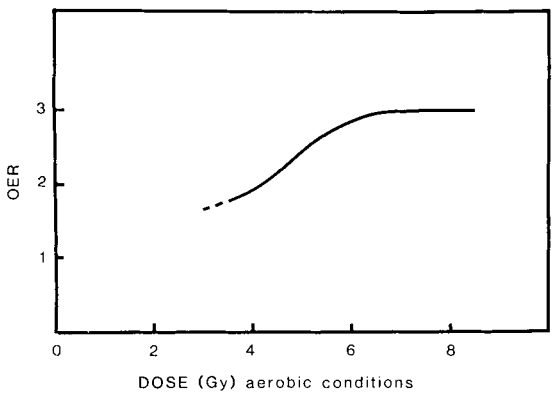
Recovery after hypoxic exposure

Another main hypothesis is concerned with the ability of the cells to recover from sublethal radiation injury. In view of the decreased *n* value of the survival curve of hypoxically irradiated cells, these cells can be considered to lack sublethal damage repair, or they have a decreased capacity to do so. This possibility was tested in a series of split-dose experiments in which radiation exposures were made aerobically or in different degrees of hypoxia; the cells were routinely incubated under aerobic conditions between exposures. Clonogenic survival (20), cell population growth (17), or cell colony size (46) were used as the end-points. In all cases the results were consistent with the hypothesis that cells irradiated in the complete absence of oxygen fail to recover to any significant extent during the split-dose interval. When traces of oxygen were present in the gas phase during irradiation (22, 43), some recovery occurred but it was less than that found in cells irradiated under aerobic conditions.

The inability of severely hypoxic cells to repair sublethal radiation damage and the ability of moderately hypoxic cells to repair a considerable amount of sublethal damage could lead to a greater resistance to radiation of the latter cells under certain conditions. Such conditions, for example, can be expected to occur after treatment with low radiation doses: recovery due to presence of some oxygen can compensate for radiosensitization of that amount of oxygen, and the net effect may be an overall increase in cell survival. If cells are exposed to larger doses, at some point, sensitization cannot any longer be compensated for by the repair, and the moderately hypoxic cells become more sensitive to radiation than severely hypoxic cells. These considerations are illustrated schematically in Fig. 4.



a



b

Fig. 3. Frequently, when purified nitrogen is used to obtain hypoxic conditions, survival curves of hypoxic cells show a decrease in extrapolation number *n*. In this schematic representation, *n* is decreased from 10 (in aerobic cells) to 2 (in hypoxic cells). In such a case, OER will be a function of dose as approximated in (b).

In order to test the suggestion that oxygen might actually protect at low doses of radiation, specific experiments were designed in which cells were exposed, with or without a low oxygen concentration (120 ppm), to repeated small radiation doses in order to magnify the possible difference due to the presence or absence of repair of sublethal injury (22). Statistical analysis of the data from these fractionated survival curves yielded a significant difference between the survival of cells in severe hypoxia and the survival of cells in the presence of 120 ppm oxygen. This suggested that traces of oxygen may indeed have a protecting effect. Another independent confirmation of this suggestion was reported

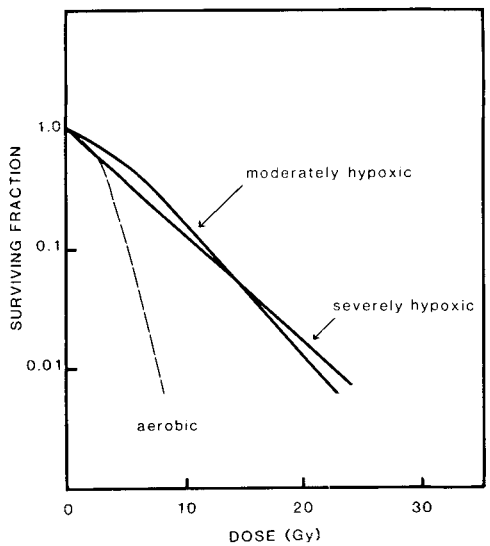


Fig. 4. A schematic presentation of protection of traces of oxygen in moderately hypoxic cells. At low doses, moderate hypoxia allowed some repair of hypoxic damage and thus moderately hypoxic cells survived better than severely hypoxic cells. At higher doses, the sensitizing effect of oxygen cannot be compensated any longer with repair and the moderately hypoxic cells become more sensitive than severely hypoxic cells.

more recently using a different experimental system (16).

Oxygen mimic sensitizers

Several substances with oxygen mimic properties have been synthesized for the purpose of substituting oxygen to radiosensitize hypoxic cells. In view of the dual effect of oxygen in both enhancing radiation lethality and recovery as discussed above, it was of particular interest to investigate the action of hypoxic cell sensitizers which are assumed to mimic action of oxygen. The first experiments of this type were conducted using a nitroxyl-free radical TAN (triacetoneamine-N-oxyl) under conditions of severe hypoxia (41). The clonogenic radiation survival curve of TAN-treated cells showed a decreased *Do* and an increased *n* in comparison to the untreated controls. Furthermore, in split-dose experiments, TAN-treated cells exhibited some recovery which was absent, or greatly decreased in the hypoxic controls. The results obtained in similar experiments with cells treated with an electron affinic sensitizer, misonidazole (42), were in general agreement with TAN results. The main conclusion that can be drawn from these experiments is that the sensitizing efficiency of these hypoxic sensitizers is dose-dependent, thus similar to that of oxygen. This conclusion received experimental support in subsequent tests in which the sensitizing efficiency of misonidazole was investigated separately in low and large-dose regions measuring micronucleus formation as the end-point (24). More recently, using the low dose survival assay (28), we have also found a decreased effect of misonidazole at low doses of ionizing radiation.

Possible mechanisms

Already from the very beginning of this research it was considered (20) that the presence of oxygen during irradiation not only affects the yield of the lesions (quantitative differences), but also induces different types of lesions (qualitative differences). It is conceivable that the different radiolysis products which arise under the two conditions lead to some different vital biochemical injuries. This concept received support in observations (47, 48, 50) which indicate that radiation induced DNA damage is qualitatively different in hypoxically and aerobically irradiated cells. Furthermore, recent observations show that there may be different mechanism(s) involved in the repair of these lesions (47).

The concept of two types of damage, oxic and hypoxic, was also supported by the result of experiments (21) in which the combined effect of oxically and hypoxically irradiated cells was tested in split-dose experiments using the clonogenic survival assay. The results showed that the total repair capacity of the cells is not shared after oxic and hypoxic irradiations, but is limited to exposure of cells under oxic conditions.

A possible mechanism for the decreased oxygen enhancement ratio and the decreased effectiveness of other electron affinic radiosensitizers (e.g. misonidazole) at low doses was recently presented by PALCIC (26).

Agreements and controversies

Until recently a great deal of controversy surrounded the question whether sensitization by oxygen and oxygen mimic hypoxic cell sensitizers is dose-dependent, or whether the same dose modifying factor applies over a wide range of doses. Similarly, it was questioned whether hypoxically irradiated cells have a similar capacity to repair sublethal injury after irradiation with split-doses as have aero-

bically irradiated cells. In this context, it is important to emphasize the distinction between split-dose experiments in which cells are returned to aerobic conditions between fractions, and the experiments in which cells are maintained under hypoxic conditions throughout the experimental procedure. There appears to be a consensus (11, 15) that, in the latter case, repair of sublethal injury does not occur. However, there is still no agreement of the effect of hypoxia (at the time of irradiation) on the repair of sublethal damage.

The majority of published literature described observations of OER and split-dose ratios which are independent of the radiation dose (e.g. 10, 14, 33). There have been, however, several important reports to the contrary. PHILLIPS & HANKS (34) irradiated bone marrow cells in vitro in the presence of oxygen or nitrogen, and subsequently determined their survival curve in vivo with a spleen colony technique. The survival curve of the anoxically irradiated cells extrapolated to $n=1$ while the aerobic survival curves had a greater extrapolation number. In the experiments of HALL & CAVANAGH (12) Vicia faba seedlings showed a considerably decreased recovery factor when irradiated with split-doses in nitrogen than in air, although the seedlings were stored in both cases aerobically for varying intervals between the irradiations. The work of PETTERSEN et coll. (32) using ultra-pure nitrogen and a stringent control of experimental conditions has confirmed that, in almost complete absence of oxygen, an extrapolation number of one is obtained. After addition of an organic nitroxyl-free radical (TMPN), the extrapolation number n was increased and Do decreased as it has been found earlier with TAN (41). As a consequence, a net protection for doses below 3 Gy and a net sensitization for higher doses was noted. NIAS et coll. (25) also reported that the logarithm of survival of cells, irradiated hypoxically, was a linear function of dose at all doses while the survival curve of aerobically irradiated cells had a shoulder. In the experiments of TOLKENDORF & EICHHORN (51) the survival curve of cells exposed in nitrogen showed a decreased n in comparison to that of cells irradiated under aerobic conditions, but the decrease in n in this case was from 6 to 3 and not to 1. CHAPMAN et coll. (4) who fitted survival data using the CHADWICK & LEENHOUTS (3) survival model (linear-quadratic equation), noted a decreased oxygen enhancement ratio at lower doses. Similarly, UNDERBRINK & WOCH (52) presented evidence that the OER at pink somatic mutations in Tradescantia stamen hairs is dose-dependent, and approaches unity at low roentgen doses. In recent studies, MARSHALL & BIANCHI (23) investigated the micronucleus formation in Vicia faba roots as a function of dose, dose rate, fractionation and oxygen level. In the low dose region (in contrast to the large dose region) dose rate, fractionation and oxygen did not affect the formation of micronuclei.

Some of these experiments may not be entirely conclusive from the point of view of: a) statistical problems involved in estimating a small fraction of lethally damaged cells in a large population of surviving cells after exposure to small doses; b) the assumptions required to extrapolate data obtained from the region of large doses into the range of small doses; c) many technical problems involved in controlling hypoxia, such as the permeability of rubber tubing to oxygen, the high solubility of oxygen in plastics, the back-flow of air at the exit of the oxygen-free gas, the risk of contaminating a pure, oxygen-free gas cylinder when attaching a decompression head which contains air in the 'dead space' etc; d) error in estimation of the level of oxygen during irradiation which could arise due to the fact that oxygen is depleted by radiation (2). The latter, for example, can result in a biphasic survival curve consisting of an initial steep portion (some oxygen present) and a second, less steep portion (oxygen consumed) of the survival curve. If n is derived by extrapolation of the second portion of the biphasic curve, an artificially low value could be obtained.

These types of criticism may be relevant to many of the observations which are considered in support of, or contrary to, the concept of a dose-dependence of OER and the effect of some hypoxic cell sensitizers. Some novel technical developments appear to be helpful in resolving much of the existing controversies. For example, Dr Cameron Koch of the Cross Cancer Institute in Edmonton, Alberta, Canada has recently developed very sensitive Clark electrodes for measurements of very low levels of oxygen in suspensions. At the British Columbia Cancer Research Centre in Vancouver, a semi-automatic computer aided system (ALDAS) was developed (28) that permitted a direct examination and classification of survivors and non-survivors in an irradiated cell population. With this instrument, in combination with a Clark electrode, data have been obtained that can be regarded as evidence for a decreased oxygen enhancement ratio at low radi-

ation doses (27, 31). Furthermore, in testing the effectiveness of misonidazole, the sensitizer enhancement ratio was shown also to be decreased at low doses (29). Despite the semi-automatic approach, it took nearly two years to obtain several complete survival curves with sufficient statistical accuracy. For that reason, a completely automated device, Dynamic Microscope Image Processing Scanner (DMIPS), has been designed and developed (30). Using DMIPS, it is now possible to measure the fate of more than 30000 individual cells in a single experiment. It is expected that further studies which will take advantage of DMIPS will clarify some remaining controversies such as the question of recovery of hypoxically irradiated cells, radioprotection of oxygen at low oxygen concentrations and others.

Practical aspects

In addition to theoretical interest, the effectiveness of radiosensitizers, including oxygen, is of particular practical importance. If the enhancement of radiosensitivity by oxygen and oxygen mimic drugs is greater in the larger than in the lower dose region, a greater efficacy of these sensitizers in radiation therapy of tumours can be expected when they are used in treatment protocols where larger doses per fraction are employed. This consideration is supported by many clinical observations (7, 13, 35) which indicate that radiation therapy in hyperbaric oxygen is more efficient when larger doses are used instead of those of conventional therapy. However, the enhancement of radiosensitivity by oxygen and/or other oxygen mimic radiosensitiers is only one aspect which determines the final therapeutic response. Thus, the use of larger doses per fraction must be considered together with all other factors which govern the final outcome of radiation therapy. In general, many other considerations argue for use of smaller doses per fraction instead of vice versa (e.g. 8). A decreased OER at lower doses renders both oxygenated and hypoxic cells more equally radiosensitive. This is at least one of the advantages which was considered in the rationale for current multi-fractionation regimes with smaller doses (e.g. 8, 19).

Most recent literature (e.g. abstracts presented in the Book of Abstracts of the 32nd Annual Meeting of the Radiation Research Society in 1984), demonstrates a renewed interest in many laboratories to study the question of the dose-dependence of radiobiologic phenomena. It can be expected that this interest will further clarify the problems dealt with in this article, among them also the question, why the issue has been controversial for such a long time.

ACKNOWLEDGEMENTS

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