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Authors

Aceto, Henry
Springsteen, Robert
Gee, W.
et al.

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Henry Aceto, Jr., Robert Springsteen, W. Gee,[1] H. S. Winchell, and C. A. Tobias

Donner Laboratory and Lawrence Radiation Laboratory
University of California, Berkeley, California

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[1]Radiobiology Laboratory, School of Veterinary Medicine, University of California, Davis, California.

Aceto, Henry, Jr., Springsteen, Robert, Gee, W., Winchell, H. S., and Tobias, C. A. Erythropoietic Response in Dogs Given Sublethal Whole Body Proton Irradiation Followed by Hypoxic Hypoxia. Radiation Res. ____, pp. ___-___, ____.

ABSTRACT

Serial iron kinetics studies and radiophosphorus determinations of total red cell volume were performed in three groups of beagle dogs exposed to hypoxia (pO_2 = 72 mm Hg), sublethal whole body proton irradiation (200 rads MTD), and a combination of these two stresses, respectively. Hypoxia alone resulted in an increase, within one day, in plasma iron turnover rate (PITR), an increase in fractional incorporation of radioiron in red cells and a corresponding elevation in total red cell volume. Sublethal whole body proton irradiation alone resulted in diminution within one day in PITR, and a corresponding marked depression of fractional incorporation of radioiron in red cells. Dogs receiving a combination of whole body proton irradiation followed by hypoxic hypoxia demonstrated an initial increase in plasma iron turnover rate (PITR) for the first 7 days approximating the response seen with hypoxia alone. At 14 days the PITR and the fractional radioiron incorporation into red cells rapidly decreased, approaching, but not reaching, that seen in the dogs receiving radiation alone. Thereafter, the increase in the PITR in these dogs was greater than that seen following radiation alone and by 29 days again approximated that seen in dogs subjected to hypoxia alone. At all times total red cell volume in dogs subjected to combined whole body radiation and hypoxia was maintained at a level

intermediate between that seen in dogs subjected to whole body radiation or hypoxia alone.

We conclude that following sublethal whole body proton irradiation, cells responsible for synthesis of hemoglobin are capable of normal or increased rates of hemoglobin synthesis in response to hypoxic stimulation despite severe radiation damage. Red cells produced under such hypoxic stress following whole body irradiation are not extremely short lived and are functionally significant as evidenced by normally shaped red cell iron-59 incorporation curves and red cell volumes which are greater than that noted in dogs receiving radiation alone. Those cells present in the bone marrow at the time of irradiation which were "responsible" for hemoglobin synthesis during the ensuing 7 days are little affected by 200 rads (MTD) of whole body proton irradiation, with regard to their capability of synthesizing hemoglobin in response to hypoxia. Additionally, while those cells present in the bone marrow at the time of irradiation which are "responsible" for hemoglobin synthesis beyond 7 days following irradiation are more affected by 200 rads of whole body proton irradiation than those cells "responsible" for hemoglobin synthesis during the initial 7 days, even these cells are capable of being stimulated by hypoxic hypoxia.

Key words: hypoxia
erythropoiesis
proton irradiation

INTRODUCTION

Diminution in plasma iron turnover rate (PITR) and calculated hemoglobin synthesis rate is a well documented quantitative change in erythropoiesis noted following whole body irradiation (1). The finding of elevated plasma erythropoietin levels following whole body irradiation (2) suggests that irradiated erythropoietic cells are incapable of greater hemoglobin synthesis than that observed in the non-stimulated postirradiation state. Indeed, Newsom and Kimeldorf (3) have attributed their observed increased mortality of animals maintained at altitude following radiation exposure to an inadequate erythropoietic response during exposure to hypoxia. The present studies demonstrate that irradiated (200 rads) erythropoietic cells are capable of normal or increased rates of hemoglobin synthesis when subsequently stimulated by hypoxic hypoxia. The results of these findings are discussed both in regard to relative radiosensitivities of erythropoietic cells of different ages as well as in regard to erythropoiesis in astronauts subjected to combined radiation and hypoxic stress.

MATERIALS AND METHODS

Female beagle dogs, approximately 2 years of age, were obtained from the Radiobiology Laboratory, University of California, Davis. These animals are essentially worm free and are immunized for distemper and hepatitis. They were transferred to individual cages upon arrival and after a 3 week holding period, were subjected to a 4 week baseline study. In order to circumvent the complications of red cell measurements introduced by splenic activity in the dog, all of the

animals were splenectomized two weeks prior to commencing the baseline work. The 12 animals represented 3 separate experiments each consisting of a group of 4 animals. Upon completion of the baseline study, each group of four animals was divided as follows:

(1) Irradiated Control: These animals received a sublethal whole body exposure of 200 rads (MTD) delivered by the degraded 730 MeV proton beam whose residual mean energy is 265 MeV. The animals were anesthetized with sodium pentobarbitol, 30 mg/kg, given intravenously immediately prior to irradiation. They were placed in a specially constructed styrofoam holder, positioned with their long axis normal to the beam, and rotated to ensure omnilateral irradiation. Dosimetric measurements utilizing Teflon-encapsulated thermoluminescent lithium fluoride (LiF) dosimeters positioned in a frozen dog cadaver indicate a uniform dose distribution to within 10% over the entire body. The radiation was delivered at a rate of 26 rads/min. Upon completion of the irradiation, the animals were returned to the animal room.

(2) Irradiated-Hypoxic: The irradiating procedure for these animals was identical to that of the irradiated-control animals. The animals were immediately transferred to the high altitude chamber within 30 to 60 minutes following irradiation and maintained at a simulated altitude of 18,000 ft (pO_2 = 72 mm Hg) for a period of 57 days. The altitude exposure was interrupted each day for a period of less than an hour for animal maintenance.

At periodic intervals this daily period of recompression was extended to 3 or 4 hours in order that radioisotopic hematological

assays could be performed. The total time spent at altitude amounted to approximately 94% of the 57-day period. Decompression and recompression rates were 2,000 ft/min.

(3) Hypoxic-Control: These animals were anesthetized and sham-irradiated following the procedures for the irradiated animals. They were then immediately transferred to the chamber and maintained at a simulated altitude of 18,000 ft for a period of 57 days. The temperature and relative humidity in the chamber were maintained at 25°C and 50% RH, respectively. The gaseous environment of the chamber was continuously monitored for O_2 and CO_2 levels with a Beckman analyzer and its associated recorder. The periods of decompression and recompression were the same as those for the irradiated-hypoxic animals.

(4) Control: These animals were anesthetized and sham-irradiated following the procedures for the irradiated animals and then returned to the animal room.

The following assays were performed on each animal:

1. Red Blood Cell Volume (RBCV). The RBCV was determined by the radiophosphorus tagged cell method of Berlin et al. (4).

2. Serum Iron. The concentration of iron in plasma was determined by the modification of Peters and co-workers of the Ramsey method (5).

3. Plasma Volume. The plasma volume was determined by the ^{59}Fe dilution technique of Apt and co-workers (6).

4. Red Cell Radioiron Uptake. With some modification, the methods described by Huff et al. were employed for these experiments (7).

5. Plasma Iron Clearance Rate (PCR). The measurement of clearance rate of radioiron from the plasma has been described by Huff et al. (8).

Plasma iron turnover rate (PITR) was calculated as the product of plasma volume, net plasma iron concentration, and plasma iron clearance rate.

Effective hemoglobin synthesis was calculated as the product of plasma iron turnover rate, maximal radioiron incorporation in circulating red cells, and the ratio of molecular weight of hemoglobin to four times the molecular weight of iron.

Datum points for these parameters represent the average of values obtained from three separate experiments each of which is within about 10 or 15% of the mean value.

RESULTS

Figure 1 graphically presents serial changes in plasma iron turnover rates (PITR) expressed as a fraction of baseline values obtained in each individual animal at various time intervals following whole body exposure to proton radiation (200 rads MTD) and/or hypoxia (pO_2 = 72 mm Hg). Within one day following exposure to hypoxia alone, PITR increased significantly and remained elevated for the duration of the hypoxic exposure (57 days). Thereafter it immediately fell to below baseline levels.

Within one day following exposure to 200 rads whole body proton radiation the PITR diminished and continued to fall until the 14th day. There is a definite increase in the PITR by the 21st day and it approaches baseline levels by the 28th day. Thereafter, there is an "overshoot" to above normal levels and it again approaches baseline levels by the 80th day.

Within one day following combined exposure to whole body radiation plus hypoxia there was an increase in PITR which continued through the 7th day at a level roughly equivalent to that seen with hypoxia alone.

At 14 days the PITR rapidly diminished to a level somewhat greater than that seen with whole body radiation alone. Thereafter the PITR again increased at a greater rate than that seen following radiation alone and by 29 days approximated that seen in dogs subjected to hypoxia. Subsequent to discontinuance of hypoxia at 57 days the diminution in PITR in animals receiving combined radiation followed by hypoxia is comparable to that seen with hypoxia alone.

The pattern of radioiron incorporation into circulating red cells was obtained in animals subsequent to radioiron administration at 1, 7, and 14 days following radiation and/or hypoxia. In each case the radioiron incorporation in the circulating red cells increased rapidly, achieving maximum levels 2 to 3 days following intravenous administration of ^{59}Fe and remained at the maximum levels for the ensuing 3 to 4 days of measurement. In no case was an early decrease in the radioiron content of circulating red cells observed, thus precluding the presence of an early (within 3 to 4 days) destruction of newly formed red cells.

Figure 2 presents red cell ^{59}Fe incorporation curves obtained on animals administered ^{59}Fe 7 days subsequent to exposure to whole body proton irradiation and/or hypoxia. It can be seen that 7 days subsequent to whole body proton irradiation and/or hypoxia the pattern of radioiron incorporation into circulating red cells is essentially identical for the control animals and those subjected to hypoxia alone or total body irradiation followed by hypoxia. There is a marked diminution in the ^{59}Fe radioiron incorporation into circulating red cells in those animals given whole body proton irradiation alone.

Figure 3 is a plot of the incorporation of the radioiron in the circulating red cells as measured on the 6th day following administration of radioiron as a function of days after irradiation and/or hypoxia on which the radioiron was administered. This time (6th day) is taken as "maximum incorporation of ^{59}Fe in red cells" and is expressed as percent of radioactivity injected. The "maximum radioiron incorporation" into red cells in animals exposed to hypoxia alone or whole body proton irradiation followed by hypoxia was close to normal values 1 and 7 days following exposure to irradiation and/or hypoxia. However, on the 14th day, dogs exposed to hypoxia alone achieved a greater than normal "maximum incorporation of radioiron" in circulating red cells (99%) while those treated with whole body proton irradiation followed by hypoxia had a marked diminution in "maximum radiation" incorporation into red cells approaching that seen in animals given whole body radiation alone. Dogs given whole body radiation alone had an immediate diminution in "maximum radioiron incorporation" into circulating red cells (10%), which increased linearly to approximately 35% by day 14.

Figure 4 presents results of the calculated effective hemoglobin synthesis rates by using the method of Huff et al. (9). An estimate of effective hemoglobin synthesis in this case was obtained by multiplying the "maximal fractional incorporation of radioiron" into circulating red cells by the plasma iron turnover rate and the known relationship between grams hemoglobin synthesized and milligrams iron incorporated into hemoglobin. The data are expressed as a ratio of the calculated effective hemoglobin synthesis rate at various times following irradiation and/or hypoxia to that obtained during the pretreatment observation

period. It can be noted that the calculated effective hemoglobin synthesis rate in animals subjected to hypoxia alone increases in essentially a linear fashion from day 1 to day 14. The calculated effective hemoglobin synthesis rate in animals given radiation alone was markedly depressed at 1 day subsequent to such radiation and over the ensuing 2 weeks there was only modest increase in the effective hemoglobin synthesis rate. In animals exposed to sublethal whole body proton radiation followed by hypoxia, the effective hemoglobin synthesis rate was not diminished on the first day following radiation and by the 7th day there was actually significantly increased effective hemoglobin synthesis, approaching that seen in animals subjected to hypoxia alone. At the 14th day there was a rapid diminution in effective hemoglobin synthesis rate to a level approaching that seen in animals treated by radiation alone.

Figure 5 presents the results of serial red cell volume determinations on 3 groups of dogs at various time intervals following whole body proton irradiation and/or hypoxia expressed in milliliters/kilogram body weight and normalized to baseline values. In animals exposed to hypoxic hypoxia alone there was a progressive increase in red cell volume, reaching maximal levels at about the 52nd day. On the 57th day hypoxia was discontinued and there was a brisk drop in the circulating red cell volume. Although the rate at which the red cell volume decreased following the discontinuance of the hypoxia suggests that these cells were being removed from the circulation at a rate faster than that which would be anticipated from normal red cell senescence, the data are too meager to reach any conclusions on this particular point. Animals exposed to whole body proton irradiation alone had a

progressive fall in their total red cell volume until approximately the 24th day following irradiation. Since the rate of this fall also appeared to be somewhat steeper than that anticipated from normal red cell senescence, the possibility of blood loss must be entertained in these irradiated animals. Subsequent to 24 days there was a slow increase in the red cell volume in irradiated dogs approaching the pre-irradiation levels by the 72nd day. Animals exposed to whole body proton irradiation followed immediately by hypoxic hypoxia maintained red cell volumes intermediate between that seen in animals treated with hypoxia or whole body irradiation alone.

DISCUSSION

In the present experiment, within 24 hours after exposure to hypoxic hypoxia, a significant increase in plasma iron turnover rate and calculated effective hemoglobin synthesis was noted in dogs. Similar results were previously noted in a human subject (10). Since hemoglobin synthesis occurs largely in the later stages of normoblast development, this early and significant increase in hemoglobin synthesis following hypoxia is most probably due to stimulation of cells well along in their commitment to the formation of red cells rather than to stimulation of an uncommitted or stem cell pool to enter the erythroid cell line.

Previous estimates of the turnover rate of erythroid precursors within the bone marrow are generally less than 5 to 7 days (11). The finding that hypoxic hypoxia following sublethal total body radiation results in an increase in hemoglobin synthesis during the first 7-day period following the application of these stresses suggests that most

of the recognizable erythroid precursors in the bone marrow at the time of radiation participated in this erythropoietic response. If this indeed is the case, one must conclude that the majority of cells committed to, or recognizable as, erythroid precursors at the time of radiation were insufficiently damaged by 200 rads to preclude their responding to hypoxic hypoxia with increased synthesis of hemoglobin. Indeed, the response to this hypoxic hypoxia was very close to the response noted in unirradiated animals.

Although it is generally accepted that irradiated erythropoietic cells are incapable of normal or increased proliferation, the contrary results of the present paper actually might be anticipated from theoretical considerations. The viability and functional integrity of the red cells merely requires a prior ability to synthesize a single heme protein, hemoglobin, the structural proteins of the red cell membrane, and possibly stroma, and sufficient enzymes involved in anaerobic glycolysis to produce a source of reducing materials (e.g., TPNH) and a source of energy (e.g., ATP). The production of these constituents accounts for only a small fraction of the total information contained within the DNA of any progenitor cell. Thus a cell, once committed to the red cell line, should be able to endure a great deal of damage to DNA coding for nonessential processes within the cell. As long as a red cell precursor is able to maintain its own integrity, undergo division, synthesize hemoglobin and structural and enzymatic protein, it should be able to give rise to functional red cells. If this indeed is the case, it would explain the present observations.

The finding in the present paper that hemoglobin synthesis diminishes markedly 7 days following irradiation in animals exposed to hypoxia subsequent to whole body radiation is consistent with the suggestion that uncommitted stem cells are significantly more radiosensitive than those committed to the erythroid series. According to this view the initially increased hemoglobin synthesis during the first 7 days post irradiation is occurring in relatively radioresistant cells already committed to the erythroid line. Subsequent to the "exhaustion" of a pool of these cells at the end of a 7-day period, there is a net diminution in hemoglobin synthesis due to radiation damage to the stem cell pool, thus preventing replenishment of the red cell precursor cell series. The finding that hemoglobin synthesis in the animals given hypoxia shortly after whole body radiation is greater than that seen in animals given radiation alone even after the 14th postirradiation day further suggests that even "stem cells" are able to be stimulated to proliferate following radiation by hypoxic hypoxia and thus that they are not being maximally stimulated following radiation in the absence of hypoxic hypoxia.

These results have important implications with respect to space travel since combined stress studies relating the interactive processes induced by the multiple physical and environmental factors of space flight represent perhaps one of the more exigent aspects of space-related research. Bender reported a synergistic effect during the Gemini-3 manned space flight between radiation and "some spaceflight parameter" for the production of human chromosome aberrations in whole blood samples (12). Biomedical data obtained during the Gemini

series indicate an "adaptive" red cell volume loss of at least 20% during missions of several days duration (13). The full significance of these observations should be evaluated in terms of those space factors which might serve to further compromise the functional integrity of the erythropoietic system. The radiation-hypoxia problem is an important aspect of such an evaluation. The present results indicate that the combined effect of radiation and hypoxia is not synergistic. Indeed, hypoxia appears to favorably modify the damage induced by radiation on the erythropoietic system and to accelerate recovery.

The observations in this paper also have implications with regard to possible mechanisms by which recovery rates can be improved following whole body radiation. If indeed following whole body radiation the cells throughout the body are capable of greater proliferation and repair than that spontaneously seen after irradiation, a search is indicated for possible methods of stimulating such proliferative processes. Unfortunately hypoxic hypoxia, while stimulating erythropoiesis in the irradiated animal, represents an additional undesirable stress. If, however, other means of stimulating cell proliferation and repair could be developed it might be possible to improve postirradiation survival by the use of these agents. Such agents would be particularly useful for astronauts who might unwittingly be exposed to lethal levels of radiation in locations far removed from adequate medical care.

In an additional set of experiments not discussed in the present communication, similar results were obtained in non-splenectomized dogs similarly exposed to whole body proton irradiation and/or hypoxic hypoxia. Thus the results demonstrated in the present communication

cannot be ascribed to any effects induced by the absence of the spleen in the animals in the present study.

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FIGURE LEGENDS

Fig. 1. Serial changes in plasma iron turnover rates (PITR) at various time intervals following whole body exposure to proton radiation (200 rads MTD) and/or hypoxia (pO_2 = 72 Hg).

Fig. 2. Red cell ^{59}Fe incorporation curves obtained on animals 7 days subsequent to exposure to whole body proton irradiation and/or hypoxia.

Fig. 3. Plot of the incorporation of radioiron in the circulating red cells as measured on the 6th day following administration of radioiron as a function of days after irradiation and/or hypoxia on which the radioiron was administered. This time (6th day) is taken as "maximum incorporation of ^{59}Fe in red cells" and is expressed as percent of radioactivity injected.

Fig. 4. Results of the calculated effective hemoglobin synthesis rates, using the method of Huff et al. (9). The data are expressed as a ratio of the calculated effective hemoglobin synthesis rate at various times following irradiation and/or hypoxia to that obtained during the pretreatment observation period.

Fig. 5. Results of serial red cell volume determinations on 3 groups of dogs exposed to whole body proton irradiation and/or hypoxia expressed in milliliters/kilogram body weight and normalized to baseline values as a function of days after initial exposure to whole body proton irradiation and/or hypoxia.

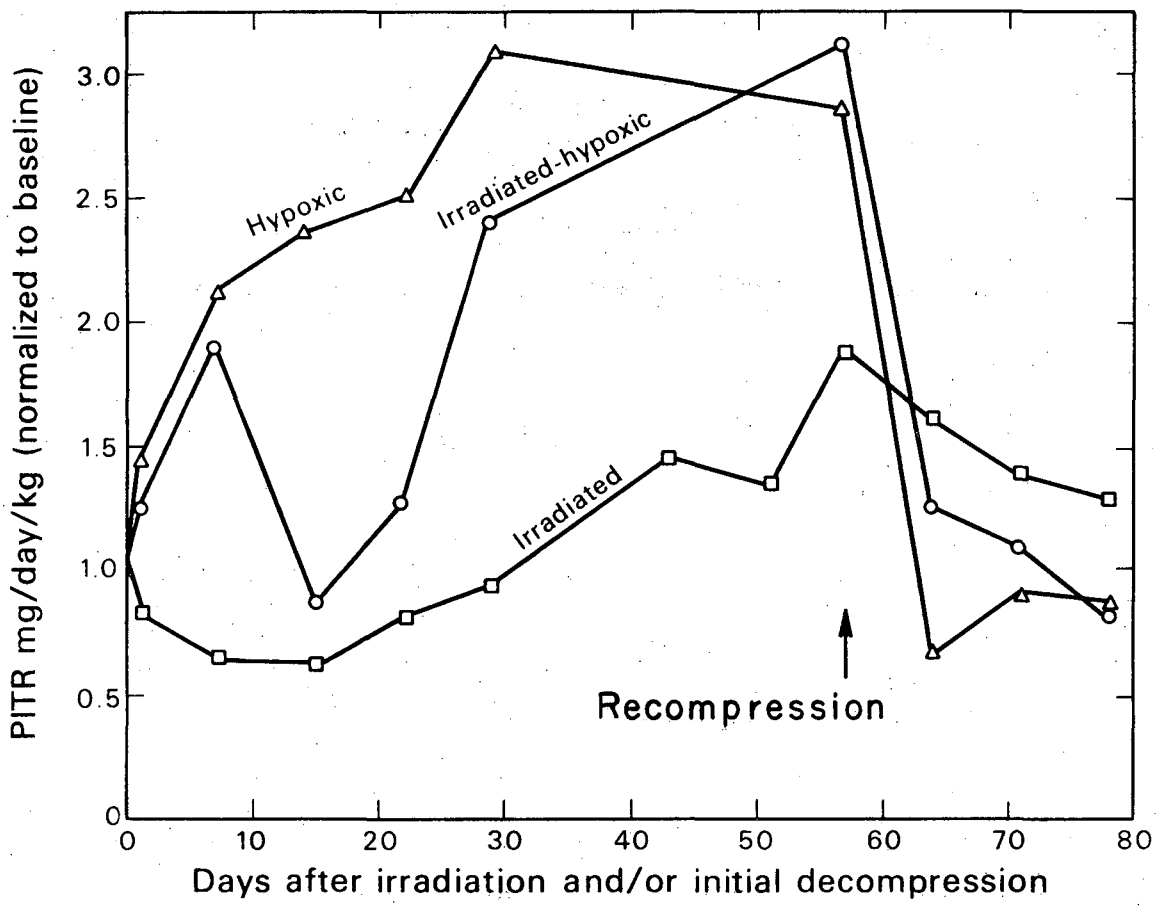

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Fig. 1

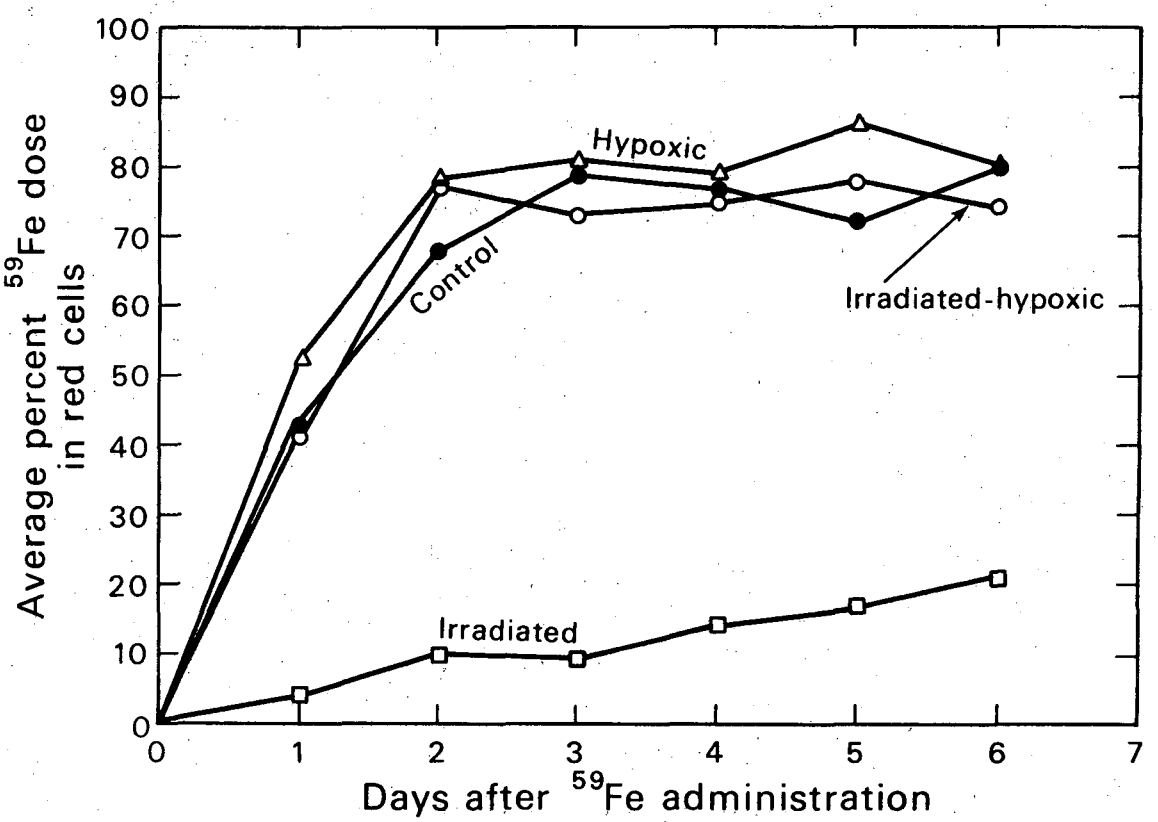

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Fig. 2

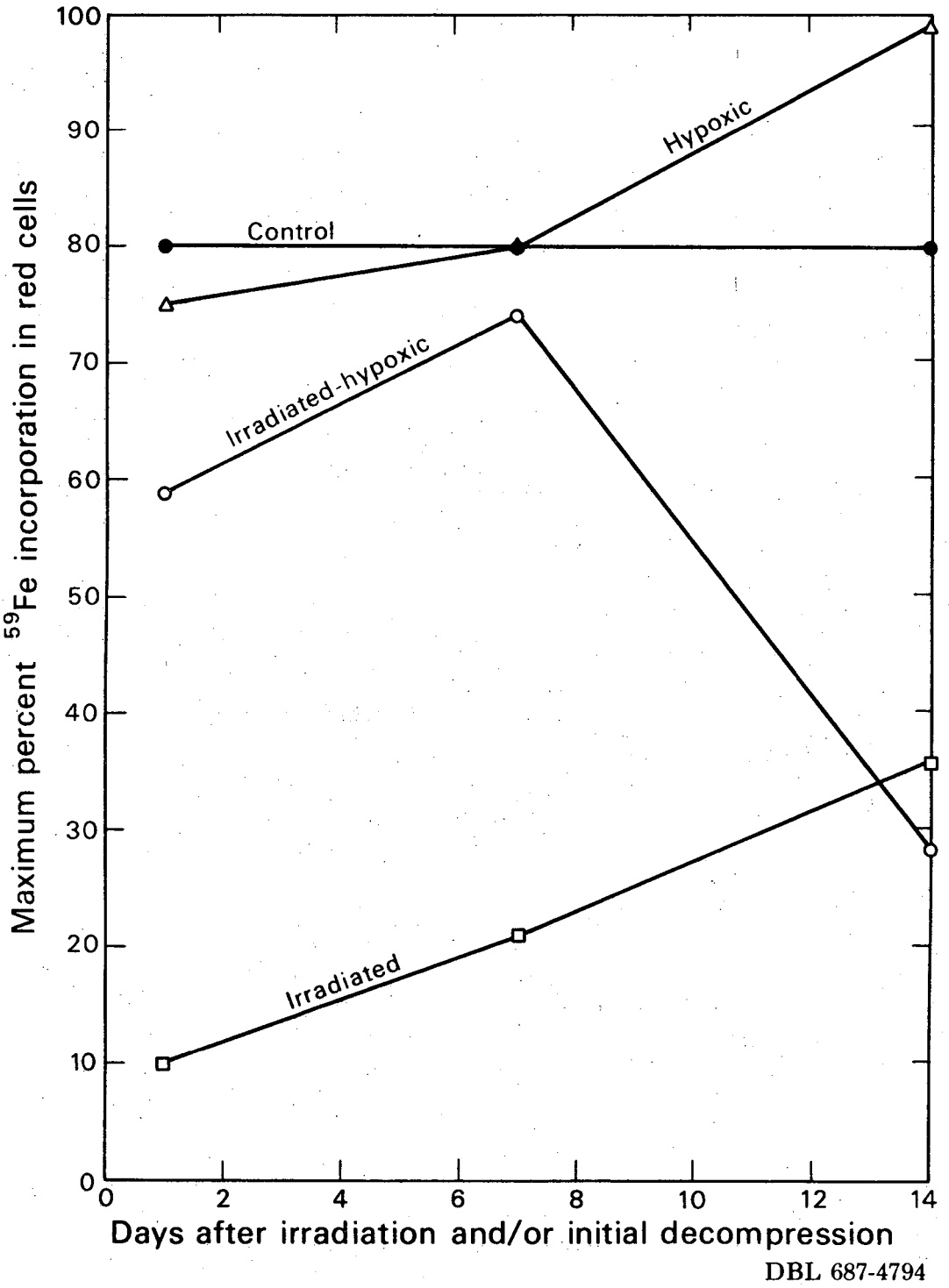
Maximum percent ^{59}Fe incorporation in red cells
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
Days after irradiation and/or initial decompression
Hypoxic
Control
Irradiated-hypoxic
Irradiated
DBL 687-4794

Fig. 3

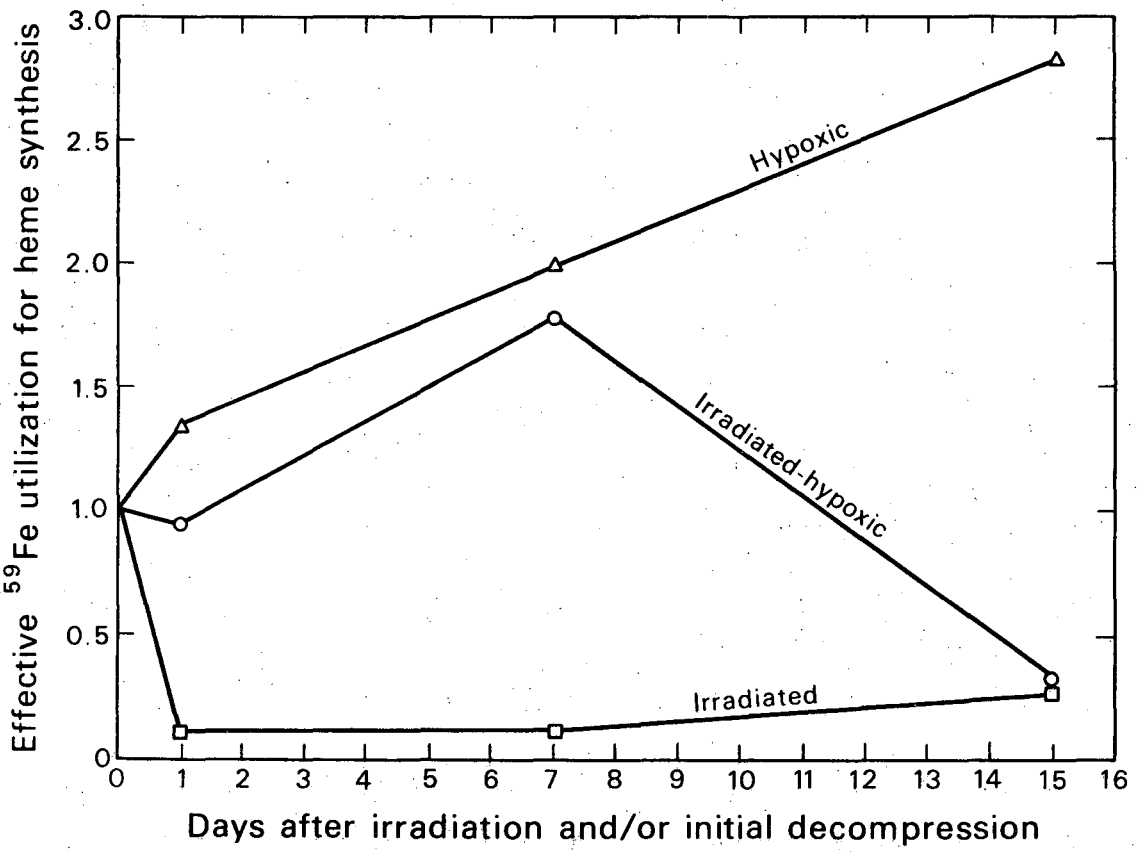

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Fig. 4

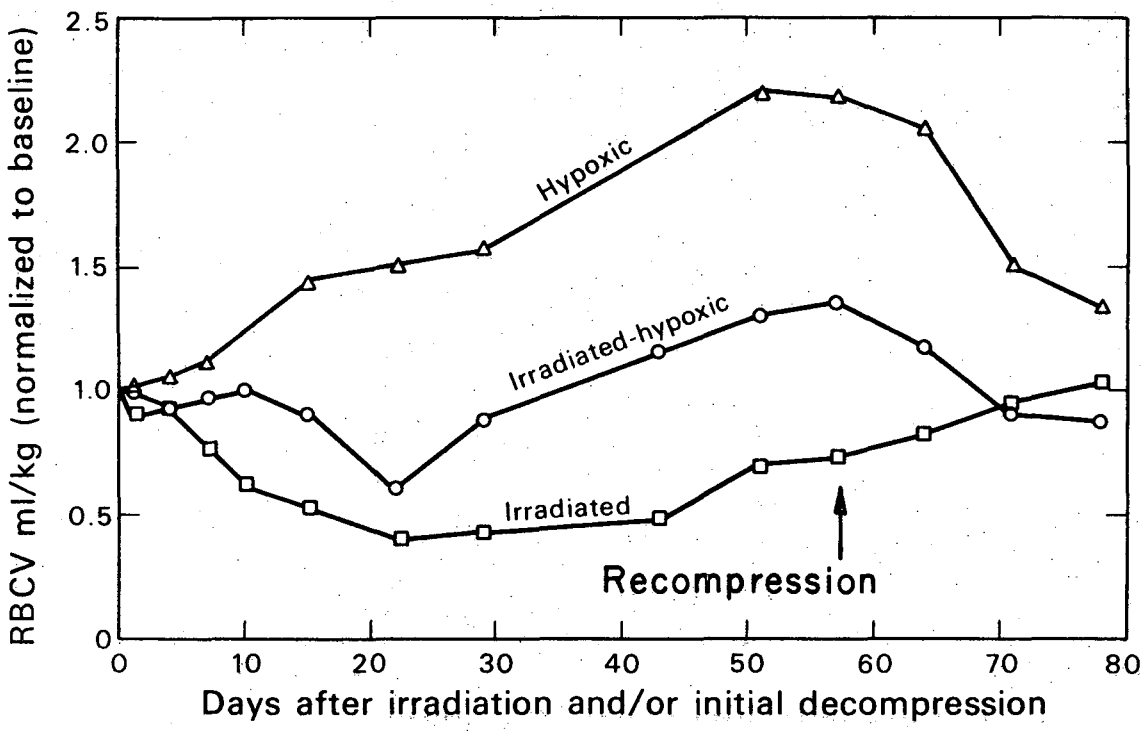

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Fig. 5