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On the mechanism of the protection of mice by environmental hypoxia.*

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低酸素によるマウスの放射線防護のメカニズムについて

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低酸素の、致死効果に対する防護効果及び放射線感受性を、各種系統（RF, CF#1, LD50/30, 450—500 R; dd/YF, DBA/2, C57Bl/6 LD50/30 600—700 R）のマウスを用いて、これを比較検討した。

1) dd/YF, DBA/2, C57Bl/6 は、RF と CF#1 に比べて、低酸素でよく防護された。

2) 900R 照射3日後における腸管重量の減少に対する低酸素の防護効果は、RF と dd/YF で同様であった。

3) 照射中の環境が低酸素分圧状態にあると、

各系統のマウスの内因性脾コロニー（ESC）数は増加し、平均ESC数は、低酸素によるマウス防護程度とよく一致していた。

4) RF と CF#1 は、他の系統に比べて、低酸素に対する抵抗性が高かった。低酸素に対する抵抗性は、10%酸素による防護程度や平均ESC数と一定の関係をもっていた。

5) 空气中又は低酸素中で700R照射後の脾及び骨髄における⁵⁹Fe uptakeの時間的消長は、低酸素が骨髄細胞を防護し、脾の造血系組織は防護していないことを暗示している。

Introduction

The reduction of radiation sensitivity of mammals by hypoxia was demonstrated under a variety of experimental conditions^{1,2,3}. It has been shown that, for protecting mammals against lethal dose of radiation, the critical level of hypoxia (5% O₂ or less), is as effective as any other chemical protective agents⁴.

When 10% and 8% oxygen content in environment was used for protection, strain and species differences in survival of mammals exposed by X ray were observed³.

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In the present experiments a comparison of protection by hypoxia of gut and hematopoietic tissue was made. The results obtained so far are not extensive enough to verify every details of the protection of hematopoietic tissue, but seem to support the proposed role of protection of hematopoietic tissue in survival of mice irradiated in environmental hypoxia.

Main study was done in mice RF and dd/YF strains, and for comparison were used mice of other strains.

Material and Methods

Mice of strains RF, CF#1, dd/YF, DBA/2, and C57B1/6, of both sexes, were used, at 60-70 days of age.

Before irradiation mice were adapted in animal room at least for 3 weeks at normal physiological conditions, with free access to food and water.

There were used 20-25 mice in each group of experiments, except the measurement of the weight of intestine where 10 mice was used, and in determination of ^{59}Fe incorporation 8-10 mice in a group was used.

Mice were irradiated by X-ray machine at 200 kV, 25 mA, filtration 1.0 mmCu and 0.5 mm Al., dose rate 50R/min. Doses used are given in figures and table.

In experiment with irradiation of mice in nitrogen atmosphere a 6 MeV linear accelerator was used (LINAC—Toshiba), with dose rate of about 6000R/min.

During irradiation mice were kept in air or in various level of environmental hypoxia. Decrease of the oxygen content in environment was done by reduction of the barometric pressure of air, or by using the gas mixture of 10% O_2 + 90% N_2 , or 8% O_2 + 92% N_2 , or nitrogen gas was used. During breathing 10% or 8% oxygen mice were kept in normal conditions, while when nitrogen was used, mice were anesthetized by intraperitoneal injection of nembutal (2 mg/10g).

In experiments with the use of the low barometric pressure of air, 480 mB or 380 mB of air was used, which corresponds to 10% or 8% oxygen content in air respectively. Two control groups were used: the first breathed 10% or 8% of oxygen in gas mixture at normal barometric pressure, and the second was placed in chamber filled with oxygen at the decreased pressure of oxygen to 480 mB or 380 mB.

Measurements of oxygen tension in tissues was done by our modification of Clark microelectrode⁵⁾. During measurement of oxygen tension in tissue (spleen and muscle), mice were anesthetized by intraperitoneal injection of nembutal (2mg/10g).

The weight of the small intestine was used for the estimation of the effects of irradiation on the gastrointestinal tract. Animals were killed by cervical dislocation on the third day after irradiation of 900R, the intestine was, removed, dissected, cleaned from excretion, and dried for 7 hrs. at 130°C. At the same time the wet weights of the spleen and thymus were determined.

The effects of irradiation on the hematopoietic tissue were determined by i) counting endogenous spleen colonies, ii) by measurement of ^{59}Fe incorporation in the femur and spleen.

i) For counting endogenous spleen colonies (ESC), the spleen was harvested 10 days after irradiation, and the number of nodules in the spleen was counted after fixation in Bouin's solution⁶⁾.

ii) ^{59}Fe incorporation in the spleen and femur was determined 24 hrs after the intraperitoneal injection of ^{59}Fe , (ferrous citrate, spec. act. 12.8 mC/mg Fe) at a dose 0.5 μC /mice. The femur was cleaned from muscle tissue, and the spleen was washed in water, in order to eliminate the possible contamination of the organ surface by intraperitoneal injection of ^{59}Fe ⁷⁾. Measurement was done with a single channel

pulseheight analyser with a digital scaler and timer MP-GA. Results are presented as percentage of the ⁵⁹Fe uptake in experimental animals to that of control animals.

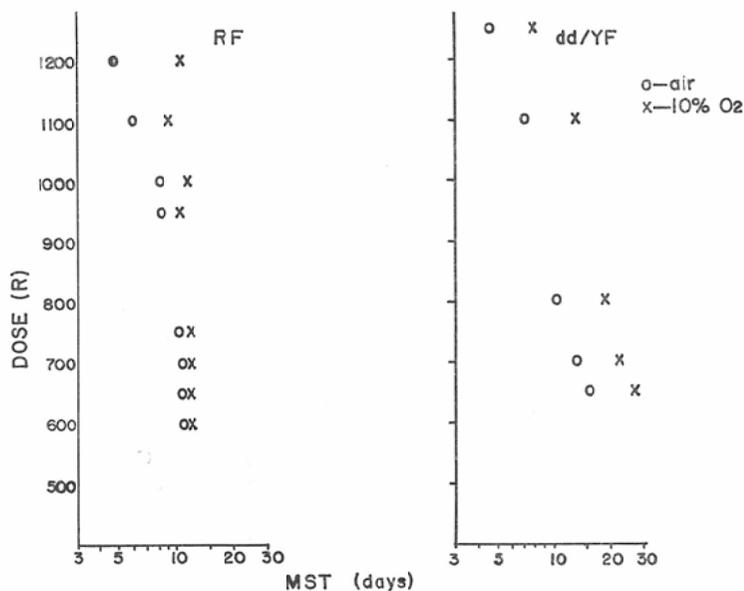
Tolerance of mice in hypoxia was estimated by percentage of survival of mice at the end of 15 min. stay in chamber at 6% of oxygen (280 mB of air).

Results

1. Survival of irradiated mice.

The median survival time (MST) after used doses of radiation given in air and 10% oxygen to RF and dd/YF strains are shown in Fig. 1. After high doses of radiation in air pattern of death of mice of both group indicate damage of gut. When mice were irradiated in 10% oxygen prolongation of MST in RF

Fig. 1 Median survival time of mice irradiated in air and in 10% O₂.



as well as in dd/YF was observed. In doses lower than 800R irradiation in 10% oxygen did not changed MST in RF strain, as compared with irradiation in air, but in dd/YF strain hypoxia prolonged MST after all doses used. So the MST of mice irradiated in hypoxia was dose-dependent in dd/YF but not in RF.

Survival curves after lethal doses of radiation given in air and 10% oxygen in different strains of mice are shown in Fig. 2a, 2b, 2c. Because of the differences in radiosensitivity 500R to RF mice, 550R to

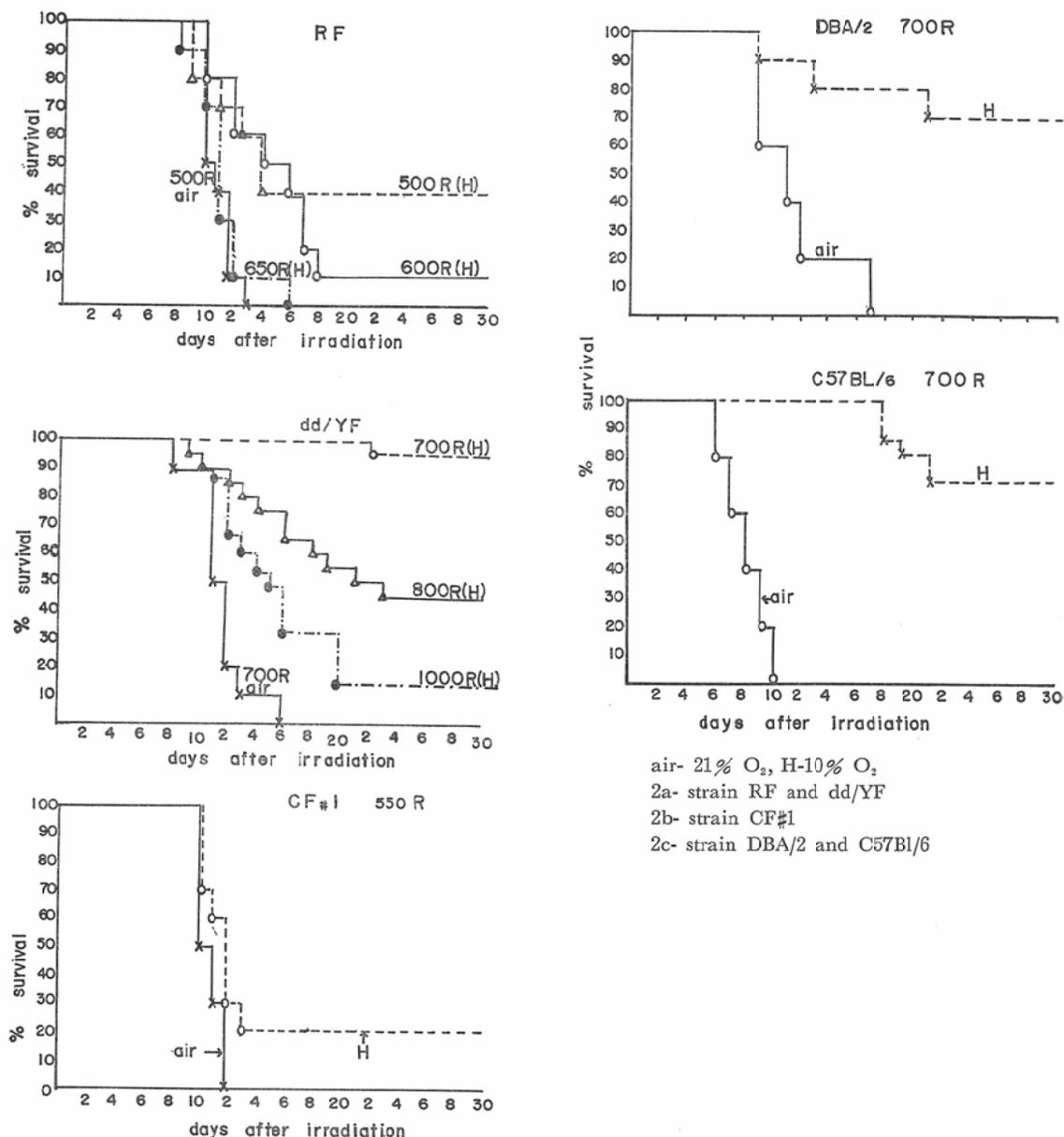
Table 1. Change of LD50/30 after irradiation in hypoxia.

	air	10%O ₂	DRF	8%O ₂	DRF	LINAC			
						air	RBE	N ₂	DRF
RF	460	510	1.11	610	1.32	—	—	—	—
dd/YF	620	800	1.3	1050	1.7	920	0.68	2350	2.56

LD50/30 was estimated by probit method
RBE —relative biological effectiveness

DRF — dose reduction factor

Fig. 2 30 day survival of different strains of mice after irradiation in hypoxia.

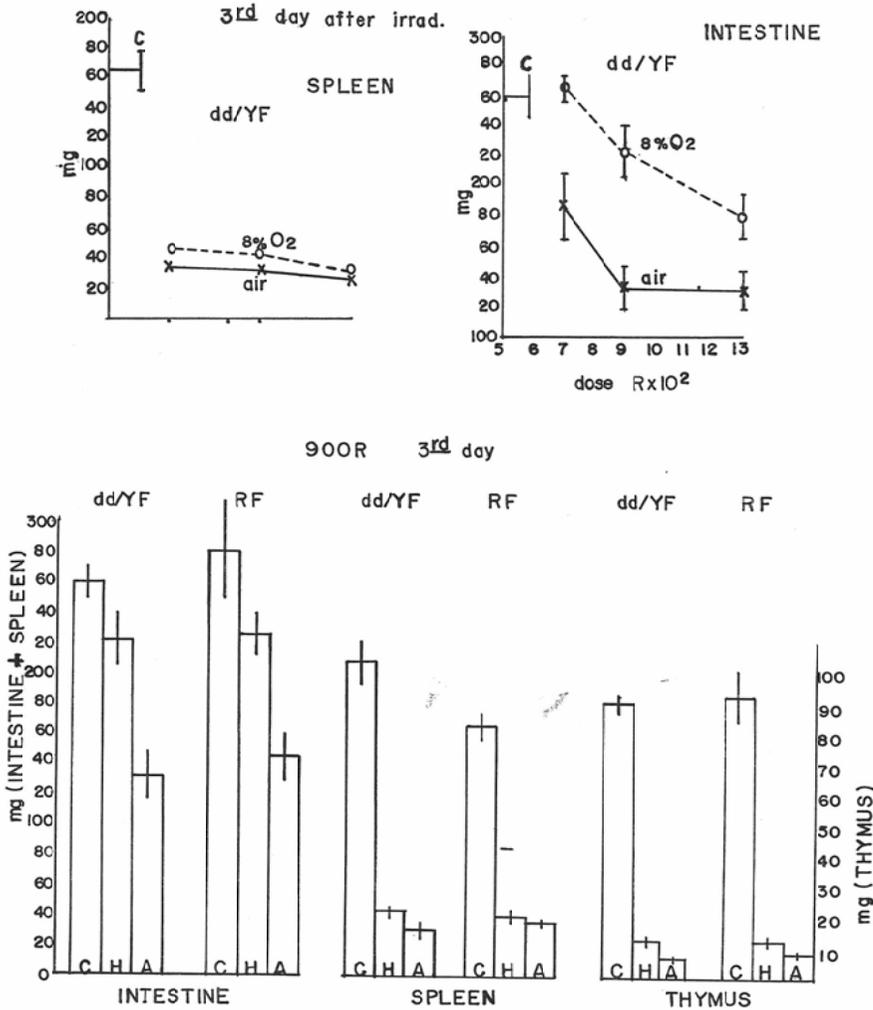


CF#1 mice, and 700R to dd/YF, DBA/2, and C57Bl/6 mice gave similar patterns of hematopoietic death when irradiated in air. But the same doses given in 10% oxygen resulted in different patterns, i.e. the percentage of 30 day survival are low in RF and CF#1 as compared with that in the other three strains. Thus it may be indicated that the protective effect of hypoxia on hematopoietic tissue is higher in DBA/2, C57Bl/6 and dd/YF than in CF#1 and RF strains. For more detailed comparison LD50/30 in various conditions in RF and dd/YF mice are shown in Table 1. Differences in DRF of hypoxia (10% oxygen) and 8% oxygen were observed, between the two strains, which also indicated higher protective effect of

Table 2. Oxygen tension in the spleen during environmental hypoxia (% of initial value)

strain	oxygen content	
	10%	8%
dd/YF	40	25
C57Bl/6	40	15
DBA/2	45	20
RF	60	35
CF#1	55	30

Fig. 3 Weight of tissues in the 3rd day after irradiation in air and hypoxia (8% O₂).



3a- Weight of spleen and intestine in dd/YF
 C- non irradiated control
 | - standard error of the mean

3b- Comparison of the weight of spleen, intestine and thymus in mice RF and dd/YF. C: control H: irradiated in hypoxia A: irradiated in air

hypoxia in dd/YF strain.

Results of Linac experiments showed that RBE of high energy and high dose rate X-ray was 0.68 in the present conditions of experiments, and DRF as high as 2.56 is attained by irradiation in short-term nitrogen breathing. It may be suggested that by breathing 8% oxygen, the oxygen tension in tissues may be higher than in case of breathing nitrogen gas.

2. Oxygen tension in the spleen of mice decreased during breathing 10% or 8% oxygen (Table 2). Decrease of tension was lower in the spleen in mice RF and CF#1, than in other strains of mice, but the differences are not statistically significant.

3. Decrease in the weight of gut on the third day after irradiation seemed to depend on the dose of irradiation up to 900 R (Fig. 3a). Further increase of the dose till 1300R did not result any more decrease of the weight of intestine. Hypoxia (8% O₂) during irradiation decreased the drop of the weight of intestine at all doses used.

No statistically significant differences in the weight of intestine in control groups was observed between RF and dd/YF strains of mice. Also the weight of intestine on the third day after irradiation 900R in air or 8% oxygen did not indicate significant differences among both of used strains. Hypoxia protected the mice from the drop of the weight of intestine at comparable level in both strains of mice (Fig. 3b).

Fig. 4 Dependence of the amount of endogenous spleen colonies in different strains of mice on the O₂ content during irradiation.

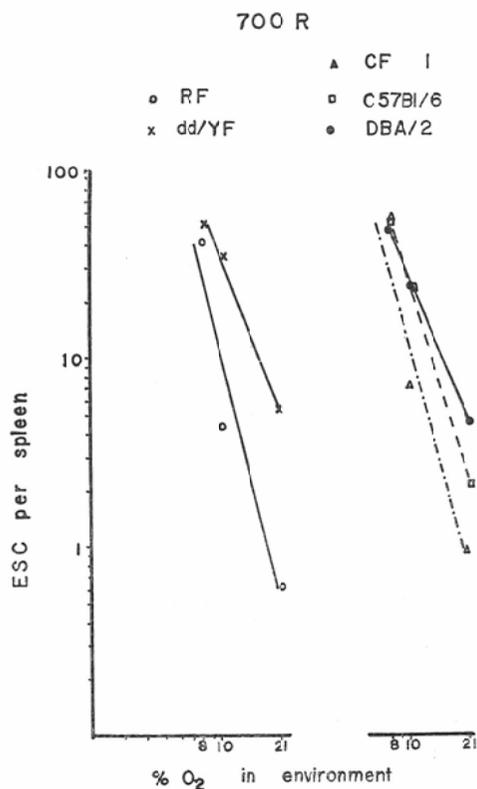
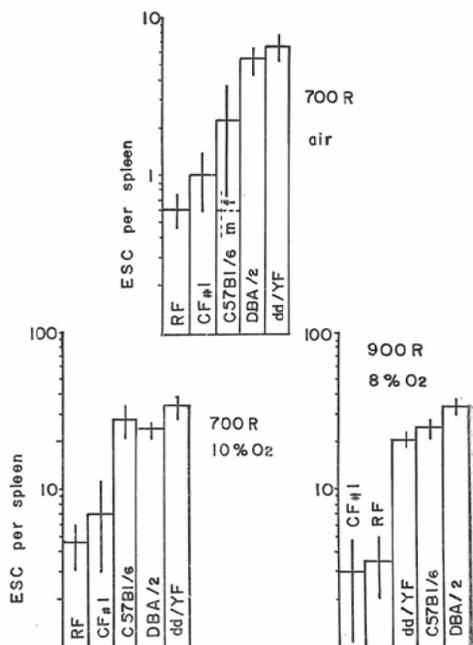


Fig. 5 Mouse strain differences in endogenous spleen colony count after irradiation in air and in hypoxia.



The weight of the spleen and thymus of both strains is slightly higher if irradiated in hypoxia.

4. In the spleen of mice survived 10 days after irradiation endogenous spleen colonies (ESC) were observed. Comparison of the mean ESC count after irradiation of 700 R in air, 10% and 8% oxygen is given in Fig. 4 and 5. Fig. 4 indicates approximately linear dependence of the increase in the ESC count per spleen in all mice on the decrease of the oxygen content in environment.

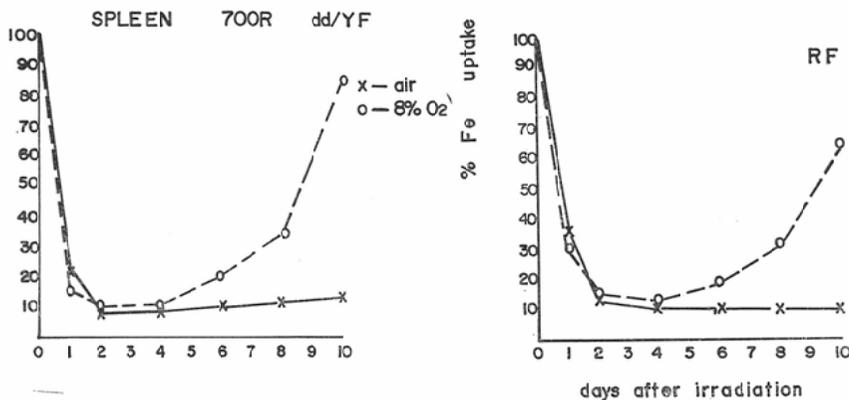
No differences in ESC count were observed when environmental hypoxia was attained by using the gas mixture at normal barometric pressure or when low barometric pressure of air was used.

In comparison of the mean ESC count between male and female sex difference in ESC count was observed only in C57B1/6 strain when irradiated in air (lower value in male 0.6 ± 0.21 than in female 2.2 ± 1.5) (Fig. 5), but not after irradiation in hypoxia- 10% O₂—(male 24.7 ± 4.3 ; female 28.8 ± 7.7). In other strains no sex differences was observed in ESC count and in figures pooled data of both sexes are given. After irradiation 700R in air or in 10% oxygen, the mean ESC count was lower in RF and CF#1 than in other strains, but when 8% oxygen was used the mean ESC count for 700R increased in all strains to more than 50; the spleen was full of colonies and it was difficult to count them accurately. When mice were irradiated 900R in 8% oxygen the mean ESC count was lower in RF and CF#1 strains as in case of irradiation of 700R in 10% oxygen.

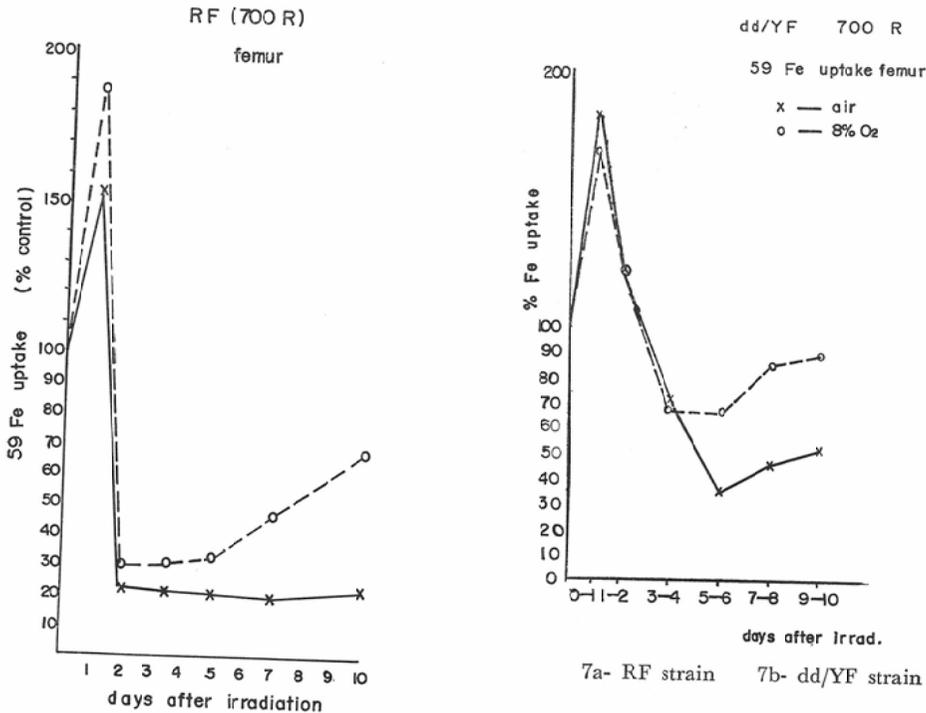
5. Determination of the 24 hour ⁵⁹Fe incorporation in the spleen and femur of mice dd/YF, DBA/2, RF, and CF#1 after 20 min stay in 8% oxygen did not show any increase in the radioiron uptake within 4 days after exposure to hypoxia.

In the spleen of mice irradiated 700R ⁵⁹Fe uptake decreased already within the first day after irradiation, and there were no differences in the initial decrease of radioiron uptake even if mice were irradiated in air or in hypoxia (8% O₂) (Fig. 6). In mice irradiated in air radioiron uptake remained in the low value till the end of observation (10 days), while in mice irradiated in hypoxia ⁵⁹Fe uptake started to increase from the 4th day after irradiation.

Fig. 6 Uptake of ⁵⁹Fe by spleens of mice irradiated 700R in air and in 8% O₂.



⁵⁹Fe uptake in the femur increased within the first day after irradiation 700R, but decreased from the second day (Fig. 7a, b). In all strains the decreased level of radioiron uptake in the femur was higher in mice irradiated in hypoxia, than irradiated in air (Table 3), but till 10 days after irradiation radioiron uptake did not restore the control level of non irradiated mice.

Fig. 7 Changes in uptake ^{59}Fe in femur after irradiation 700R in air and in 8% O_2 .Table 3. ^{59}Fe uptake* in femur and spleen on 5th day after irradiation 700R.

strain	femur		spleen	
	air	8% O_2	air	8% O_2
RF	20	33	10	17
CF#1	24	36	5	12
dd/YF	30	65	3°	5°
DBA/2	39	66	4	6

* 24 hour uptake in percentage of non irradiated control.

° used data from 3-4 day after irradiation.

6. Tolerance to hypoxia.

Survival for 15 min. stay in acute hypoxia were 16% in dd/YF, 20% in DBA/2, and 25% in C57B1/6 strains, while in strain RF survived 60% and in CF#1 40% of mice. In Fig. 8 and 9 the tolerance to hypoxia of various strains of mice is compared with their survival after irradiation 700R in 10% oxygen (Fig. 8), and with the mean ESC count (Fig. 9). Results indicate that mice with higher tolerance to hypoxia (RF and CF#1) were less protected by 10% oxygen, and have lower ESC count as compared with other strains used.

Fig. 8 Relation between survival in acute hypoxia (6% O₂) and intensity of protection by stay in 10% O₂ during irradiation.

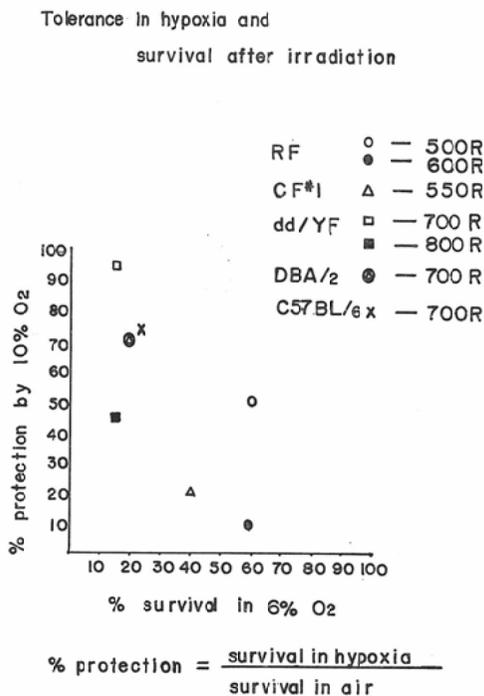
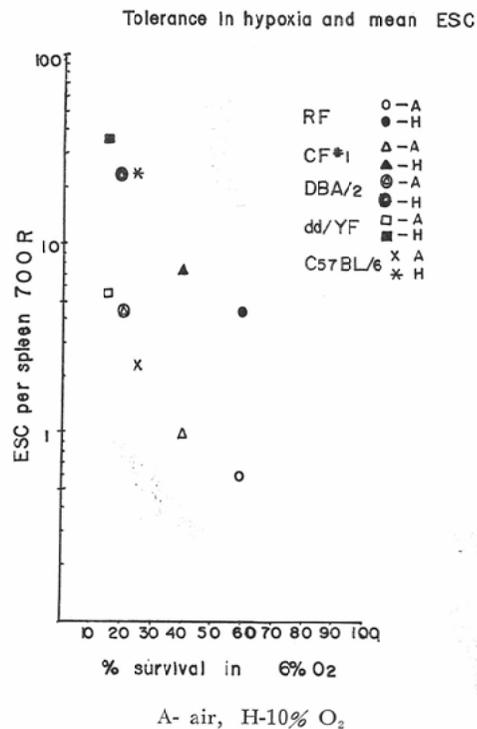


Fig. 9 Relation between survival in acute hypoxia and mean endogenous spleen colony count after irradiation in air and in 10% O₂.



Discussion

In the present study the difference in survival of mice irradiated in environmental hypoxia was demonstrated among strains of mice with different radiosensitivity (RF and CF#1 LD50/30 450—500R; dd/YF, DBA/2 and C57B1/6 LD50/30 600-700R). Mice of strain dd/YF, DBA/2 and C57B1/6 seem to be well protected by reduction of oxygen content in environment as compared with those of strain CF#1 and RF. These results may explain the controversial results that van den Meer et al.²⁾ observed protection of mice when irradiated in 10% oxygen, while Dowdy et al.¹⁾ used 10% oxygen for protection without effect, since the difference in strains used can be assumed.

Decrease of oxygen content in environment reduced oxygen tension in tissues of mice, and in agreement with above mentioned authors¹⁾²⁾ we observed in each strain the correlation between the decrease of oxygen tension in the spleen and the intensity of protection by environmental hypoxia. When a similar level of hypoxia is used for protection in different strains of mice, the decrease of oxygen tension in the spleen showed some differences among strains, though they were not statistically significant.

Among the same strains used, in strain RF and CF#1 were reported⁸⁾ previously to have higher oxygen consumption in tissues (spleen, bone marrow a.c.) than C57B1/6, as measured *in vitro*. Though it is very difficult to extrapolate from the results *in vitro* to conditions *in vivo*, it was assumed that, when a similar level of environmental hypoxia was used, in strains with higher oxygen consumption in tissues (RF and CF#1) oxygen tension in tissues would be reduce to lower level than in C57B1/6 strain in which

lower oxygen consumption in tissues was reported. But slightly higher level of oxygen tension in the spleen of mice RF and CF#1, when breathed 10% oxygen, and their higher survival in acute hypoxia may indicate higher compensation activity for maintaining the oxygen tension in tissue in these strains than in the other strains used. High tolerance to hypoxia may to some extent explain lower decrease of oxygen tension in the spleen of RF and CF#1 strains of mice and their lower protection by 10% oxygen in environment.

In previous experiments⁹⁾ differences in survival were not observed between RF and dd/YF strains, irradiated 700R after injection of para-aminopropiophenone (PAPP) and propylene glycol (PG). As PAPP and PG as environmental hypoxia decreased oxygen tension in tissues of mice. In case of PG the level of decreased oxygen tension was comparable with the decrease of oxygen tension in the spleen when 10% O₂ in environment was used. It is supposed that after the injection of PG oxygen tension in tissues decreased as a result of an increase of oxygen consumption in tissues and evoked primary tissue hypoxia. Reduction of oxygen content in environment evokes primary arterial hypoxia, and change in oxygen supply and oxygen tension in tissues depends on the ability of circulatory and respiratory systems to maintain oxygen tension in tissues at available level. We suppose that this different way of the decrease of oxygen tension in tissues can explain different results of both experiments.

In sheeps was found different hemoglobin types with considerably different oxygen affinities¹⁰⁾, which influence their tolerance in hypoxia. This possibility in used strains of mice can not be excluded.

As far as the protective effect of environmental hypoxia on the damage of the gut are concerned, differences in protection were not observed between strains RF and dd/YF. These results are supported by our data of MST and delta Death Rate⁹⁾.

In hematopoietic tissue the reduction of oxygen content in environment, during irradiation, can be correlated with the ESC count in each strain. A comparison of the mean ESC count with survival of mice irradiated in 10% oxygen indicates lower amount of ESC and lower protection by hypoxia in strain RF and CF#1 than in the other three strains. But when 8% oxygen was used for protection, mean ESC count increased in all strains to similar values. Comparison of the ESC count after irradiation in 10% with that in 8% oxygen indicate, in RF and CF#1 strains of 9-10 fold increase while, in other strains used, the ESC count increased in average 2 times. This high level of increase in ESC count after irradiation in 8% oxygen may indicate the overcompensation of the hemopoietic production in strain RF and CF#1. Despite of the high increase of the ESC count in RF strain, DRF of protection by 8% oxygen showed lower value in RF than that in strain dd/YF.

It was shown¹¹⁾ that the 4 day stay of mice in hypoxia increased ⁵⁶Fe uptake in the spleen but not in femur. Our results indicate, however, that a short-time hypoxia used in ours experiments did not increased hematopoietic activity of the spleen and bone marrow.

The decrease of ⁵⁶Fe uptake was observed, after irradiation, in the spleen as well as in femur. In the spleen the decrease reached at a similar level when mice were irradiated either in air or in hypoxia. But in bone marrow of femur the result may indicate higher damage of bone marrow cells in mice irradiated in air than in mice irradiated in hypoxia. After 700R the intensity of the decrease of radioiron uptake was higher in strain RF and CF#1, than in dd/YF and DBA/2; while the intensity of protection (i.e. difference between the level of radioiron uptake after irradiation in hypoxia and in air), was higher in strains dd/YF and DBA/2 than in strains RF and CF#1. The fact that differences in the decrease in

^{59}Fe uptake between mice irradiated in air and in hypoxia were observed in the bone marrow but not in the spleen may suggest that a dose 700R damaged all pools of stem cells in the spleen, and observed endogenous spleen colonies were resulted from the cells migrated from the bone marrow.

Mechanism of the increase of the ^{59}Fe uptake within the first 24 hrs after irradiation is under study and will be reported elsewhere.

Summary

The protective effect of environmental hypoxia against lethal effect of X-ray exposure was compared in mice of different strains and radiosensitivity (RF, CF#1 LD50/30 450-500R; dd/YF, DBA/2, and C57B1/6 LD50/30 600-700R).

1) Mice of strain dd/YF, DBA/2 and C57B1/6 were well protected by hypoxia as compared with those in strains RF and CF#1.

2) Hypoxia protected decrease of the weight of intestine, on the 3rd day after irradiation, in comparable level as in RF as in dd/YF strain.

3) Reduction of oxygen content in environment during irradiation correlates with mean endogenous spleen colony counts in each strain of mice used. The mean ESC count is in good agreement with the intensity of protection of mice by environmental hypoxia.

4) In mice RF and CF#1 was observed higher tolerance to hypoxia, than in other strains. Tolerance of mice to hypoxia correlates with protection by 10% oxygen and mean ESC count.

5) The course of ^{59}Fe uptake in spleen and femur after irradiation 700R in air or hypoxia suggests that hypoxia protected bone marrow cells while no protection of hematopoietic tissue of spleen was observed.

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