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RADIATION DAMAGE TO THE ERYTHROCYTE MEMBRANE: THE EFFECTS OF MEDIUM AND CELL CONCENTRATIONS

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Human erythrocytes suspended in plasma, or in phosphate buffered saline (PBS), were exposed to ionizing radiation. Potassium leakage from irradiated erythrocytes is significantly higher in PBS than in plasma. The potassium leakage decreases when PBS is gradually replaced by plasma. These findings suggest that some of the plasma constituents have radioprotective properties. The potassium leakage per cell is independent of the hematocrit, Hct. The potassium leakage is attributed to the formation of radiation defects in the membrane. Analysis of the effect of radiation dose, plasma and cell concentrations on the product of the number and surface area of the radiation defects indicates that the radiation damage is mainly due to the direct formation of free radicals in the cell membrane. The radioprotective effect of plasma is attributed to surface reactions of these free radicals with plasma constituents adsorbed on the membrane.

KEY WORDS: erythrocyte membrane, irradiation, hematocrit, external medium, potassium leakage.

INTRODUCTION

Radiation treatment of blood units is currently used to prevent graft-versus-host-disease, GVHD, as a complication of transfusion.[1] This procedure is based on the large difference between the radiation sensitivities of T-lymphocytes and red blood cells.[1,2] Thus, one has to apply a dose high enough to destroy almost all the T-lymphocytes while causing as little as possible damage to the red cells. There is a debate about the minimal dose required to fulfill the first requirement.[1,3,4] Recent studies indicate that doses larger than 30 Gy, which are usually applied, are required.[3] Doses of this order of magnitude cause only minor changes in the

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concentration of the essential constituents of the red cells, e.g. ATP, DPG[4-6] catalase, glutathione peroxidase and SOD.[7] However, they cause potassium leakage from the red blood cells attributed to damage to the cell membranes.[6,8] Thus, there is a need to achieve an optimal compromise regarding the dose applied.

Most of the studies reported up to date deal with the irradiation of packed cells with high hematocrit, (Hct $\geq$ 60).[5,6,9] The effects of the nature of the medium in which the cells are suspended and their concentration were not studied systematically.

In the framework of a program directed towards finding conditions which maximize the difference in radiation sensitivity between T-lymphocytes and red blood cells it is important to get more information on the source of the damage caused to the membranes of red blood cells. It was therefore decided to study systematically the effects of radiation dose, the nature of the medium in which the cells are suspended and the cells' concentration on the rate of potassium leakage. The data indicate that the major source of damage to the red blood cells are the free radicals formed directly by the radiation in the membrane.

MATERIALS AND METHODS

Blood units, within seven days from donation, were supplied by the blood bank of the Soroka Hospital. The units were obtained after small samples had been used for transfusion to newborns.

The samples were prepared from packed cells collected in Citrate-Phosphate-Dextrose-Adenine (CPDA-1) bags. Thus, the plasma medium is enriched with some citrate, glucose and adenine. To obtain cells in artificial external solutions, plasma was removed from the units by aspiration after centrifugation for 6 minutes at 2000 g, the red blood cells were washed by isotonic phosphate buffer (PB), PBS or 0.9% NaCl solution three times and then resuspended in the appropriate medium.

Irradiations were performed in a ^{60}Co γ source, supplied by Noratom. The dose rate was 0.25 $Gy \cdot s^{-1}$. Blood samples were irradiated in sterile syringes. The irradiations were carried out at room temperature. After irradiation the samples were stored at 4°C. In order to obtain a homogeneous distribution of RBCs in suspension the samples were shaken carefully immediately before irradiation.

24 hours after irradiation, or at other specified intervals, the erythrocyte suspensions were centrifuged in order to obtain supernatant samples for the measurement of concentrations of extracellular ions and hemoglobin (Hb), or to replace the external medium. Extracellular Na^+ and K^+ were measured by Flame Photometer 343 (Instrumentation Laboratory Inc., USA). Calibration curves were carried out with industrially produced standards. The accuracy of these determinations was $\pm 5\%$. Extracellular hemoglobin concentrations were assayed with Drabkin's reagent.[10] The optical density was measured using a Baush & Lomb Spectronic 2000 (NY, USA) spectrophotometer. Blood analyzer Technicon H*1 (Miles Inc., NY, USA) was employed for the determination of mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) of the erythrocytes. The hematocrit values were determined using a microcentrifuge KH-1200, (Kubota, Japan).

RESULTS

The effect of the total radiation dose on potassium leakage, as measured 24 hrs. after irradiation, from red blood cells suspended in plasma or in PBS was studied. The change of external potassium concentration $\Delta[K^+_{ext}] = [K^+_{ext}]^{ir} - [K^+_{ext}]^{cont}$ was calculated, (where ir and cont refer to irradiated and control samples respectively). The results are depicted in Figure 1a. Curves i and i′ represent data obtained by irradiation of samples from the same unit suspended in PBS and plasma respectively. Samples from three different blood units taken from three different donors were used, the numbers on the curve indicating the unit number. The results clearly point out that an increase of the absorbed dose increases the potassium leakage both in PBS and plasma suspensions. The cellular loss of K^+ was balanced by a gain of Na^+ (data not shown) in accord with reported data.[6] However, the rate of external K^+ accumulation is considerably higher in PBS than in plasma. The results also clearly indicate that the sensitivity of red blood cells to radiation is an individual property of the donor.

The observed damage to the red blood cells could plausibly be attributed to hypochlorite, or other Cl^- radiolysis products formed in the external medium. The role of the latter radiolysis products in radiobiological processes was recently demonstrated.[11,12] In order to check this possibility the suspensions of RBCs in plasma, PB, PBS and saline were irradiated (D = 225 Gy). The measured $\Delta[K^+_{ext}]$ values in PB, PBS and saline suspensions are identical within experimental accuracy (data not shown) and are larger than those observed in suspensions of RBCs in plasma. In addition, 24 hrs storage of nonirradiated erythrocytes in irradiated PB, PBS and saline did not induce any additional potassium leakage. (D = 225 Gy). The time interval between irradiation and preparation of RBCs suspensions was 20–30 min. Thus, the results indicate that the radiolytic damage to RBCs is not due to chloride radiolysis products in the external medium.

The effect of cell concentration on the potassium leakage was measured, the results are plotted in Figure 2a. (Samples from three different units were used, these samples were taken from different units than those used in the previous experiment.) The results clearly indicate that an increase in hematocrit leads to an increase of $\Delta[K^+_{ext}]$ in both PBS and plasma (Figure 2a). The change of external potassium is greater in PBS suspensions than in plasma ones, in accord with the previous experiment. As in Figure 1a, the absolute value of $\Delta[K^+_{ext}]$ depends not only on the external medium composition (plasma or PBS) but also on the properties of the unit.

In view of the fact that the potassium leakage is lower in samples suspended in plasma than in PBS, the effect of plasma concentration on this leakage was measured. For these experiments blood units from two additional donors were used. The erythrocytes were washed with PBS and then resuspended in the appropriate mixture, the range of plasma concentrations was 0–93%. The data are summed up in Table 1. As there is a higher potassium concentration in plasma than in PBS, it should be noted that also in the PBS control samples some potassium leaks out within 24 hrs, the measured potassium concentrations are higher both in the control and in the irradiated samples in the plasma suspensions. The results clearly point out that the radiation induced potassium leakage, $\Delta[K^+_{ext}]$, gradually decreases with the increase in plasma concentration.

No radiation induced hemoglobin release into the external medium was observed under all the experimental conditions. Consequently, the diameter of the radiation

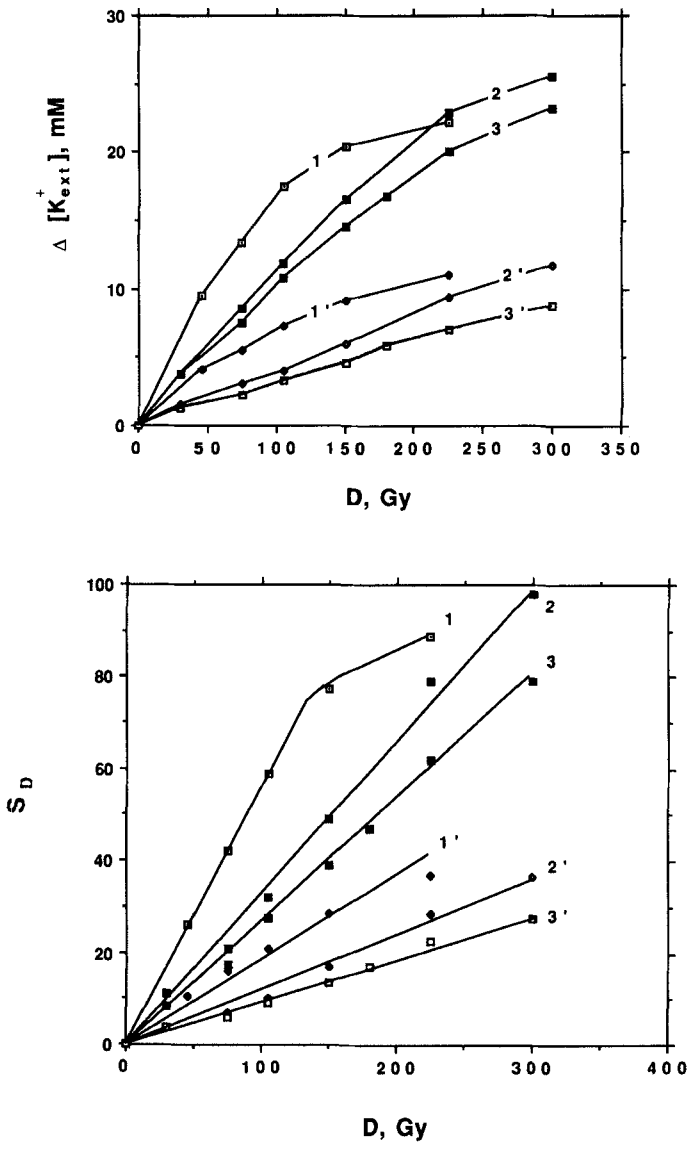

FIGURE 1 a. Effect of radiation dose on potassium leakage, within 24 hrs., in PBS (1, 2 and 3) and in plasma (1′, 2′ and 3′) for erythrocytes taken from three donors. The Hct values were 27%, 29% and 30% in samples 1, 2 and 3 respectively. b. Effect of radiation dose on the product of the number, surface area of the radiation defects and time, S_D, calculated from the data in Figure 1a applying equation IV.

defects must be less than 5 nm, the diameter of the Hb molecule. In addition, no measurable change of the mean corpuscular hemoglobin concentration and the cell volume due to the radiation was observed.

The larger radiation induced leakage of potassium from cells suspended in PBS than from those suspended in plasma could be attributed to several causes:

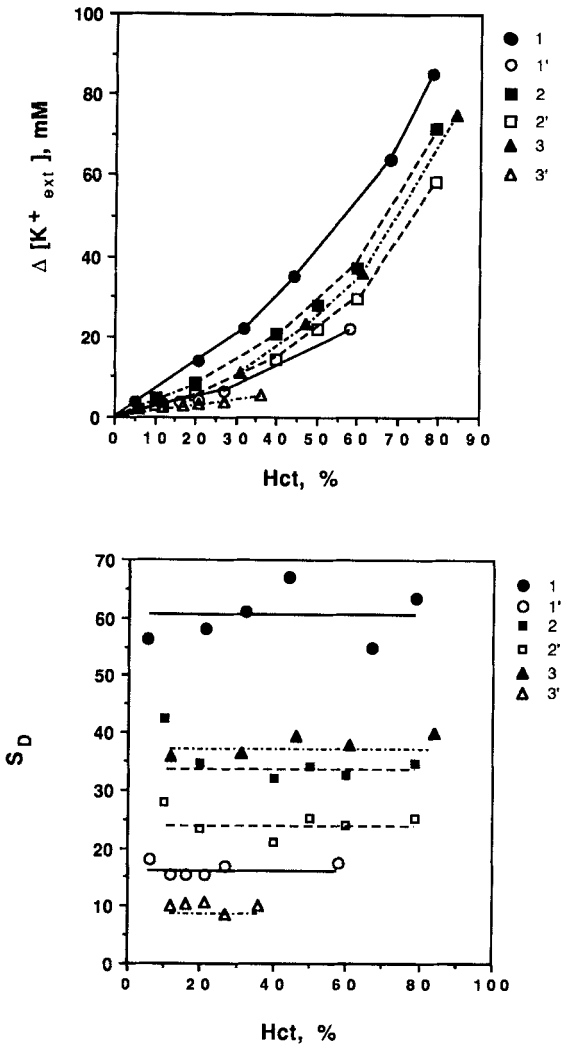

FIGURE 2 Effect of cell concentration on radiation induced potassium leakage (a), and surface area of membrane defects (b) within 24 hrs, in PBS (1, 2 and 3) and in plasma (1′, 2′ and 3′). Total dose 225 Gy.

1. Ion flux through the membrane might depend on Donnan's potential, E_m, and the observed effect could be due to differences in Donnan's potential in cells suspended in PBS and plasma.
2. The potassium leakage is expected to be affected by the trans-membrane concentration gradient. Thus, the observed effect might be due to the higher concentration of potassium in plasma.
3. The presence in the plasma of primary free radical scavengers which act as radioprotectors.
4. One of the plasma constituents might act as a post-irradiation protective agent.

TABLE I
The influence of plasma concentration on radiation induced potassium leakage. (D = 225 Gy).

Blood # 1					Blood # 2				
[plasma]	Hct.	$[K^+]_{ext}$, mM		$\Delta[K^+_{ext}]$	[plasma]	Hct.	$[K^+]_{ext}$, mM		$\Delta[K^+]_{ext}$
%	%	control	irradiated	mM	%	%	control	irradiated	mM
0	30	2.7	24.5	21.8	0	24	2.2	20.8	18.6
2.3	32	3.4	26.5	23.1	1.2	23	2.5	21.1	18.6
5.6	29	3.8	23.4	19.6	2.3	24	2.6	20.9	18.3
11.3	29	4.9	23.6	18.7	11.6	23	4.3	21.6	17.3
22.6	30	7.7	26.3	18.6	23.2	24	6.3	23.0	16.7
33.9	30	10.3	28.2	17.9	46.5	26	11.3	26.5	15.2
45.2	31	13.4	30.3	16.9	93.0	29	20.9	34.5	13.6
67.8	31	19.0	34.9	15.9					

To analyze which of these plausible causes is the source of the observed effect the following experiments were carried out:

1. To clarify whether the difference in the Donnan's potential causes the observed effect, the following experiment was performed. Two samples of erythrocyte suspensions, taken from the same blood unit were irradiated in PBS; 15 minutes later the PBS from one sample was removed by centrifugation and the RBCs were suspended in saline to attain the same Hct value, (30%). After this protocol the composition of the external medium was 24% PBS and 76% saline. Donnan's potentials, E_m, in these media differ considerably. However, the concentration of the external potassium determined after storage for 24 hrs. remained unchanged. Thus E_m as the source for the medium effect on radiation induced potassium leakage is ruled out.
2. In order to check whether primary free radical scavengers present in the plasma are responsible for the observed effect of the medium, samples of erythrocytes suspensions in mixtures of PBS and CPDA (the CPDA concentration was varied within 0 to 87%) were irradiated. The value of $[K^+_{ext}]$ measured after 24 hrs. of storage was found to be independent on CPDA concentration (data not shown). The data point out that free radical scavengers such as citrate and glucose have no radioprotective effect on the radiation induced potassium leakage from the erythrocytes.
3. In order to probe for the possibility that the effect of the medium stems from a post-irradiation effect of plasma constituents, the following experiment was carried out: Samples irradiated in PBS or plasma were divided into two portions. 10–15 min. after the irradiation the medium of one portion, from each type of medium, was replaced by the second type of medium. After this treatment the composition of the external solutions of the cell suspensions irradiated in PBS and plasma was 86% plasma + 14% PBS and 86% PBS + 14% plasma, respectively. The potassium concentration in the external medium was measured 24 hrs. later. The results are summed up in Figure 3a. These data were obtained by investigation of six blood units. The substitution of the extracellular medium clearly has some effect on $\Delta[K^+_{ext}]$. These results are discussed below.

The rate of radiation induced potassium leakage was measured over a period of 19 hrs. The results are plotted in Figure 4a. The results clearly indicate that the increase in $[K^+]$ in the external medium is a gradual and rather slow process, in accord with earlier reports.[5,6,9]

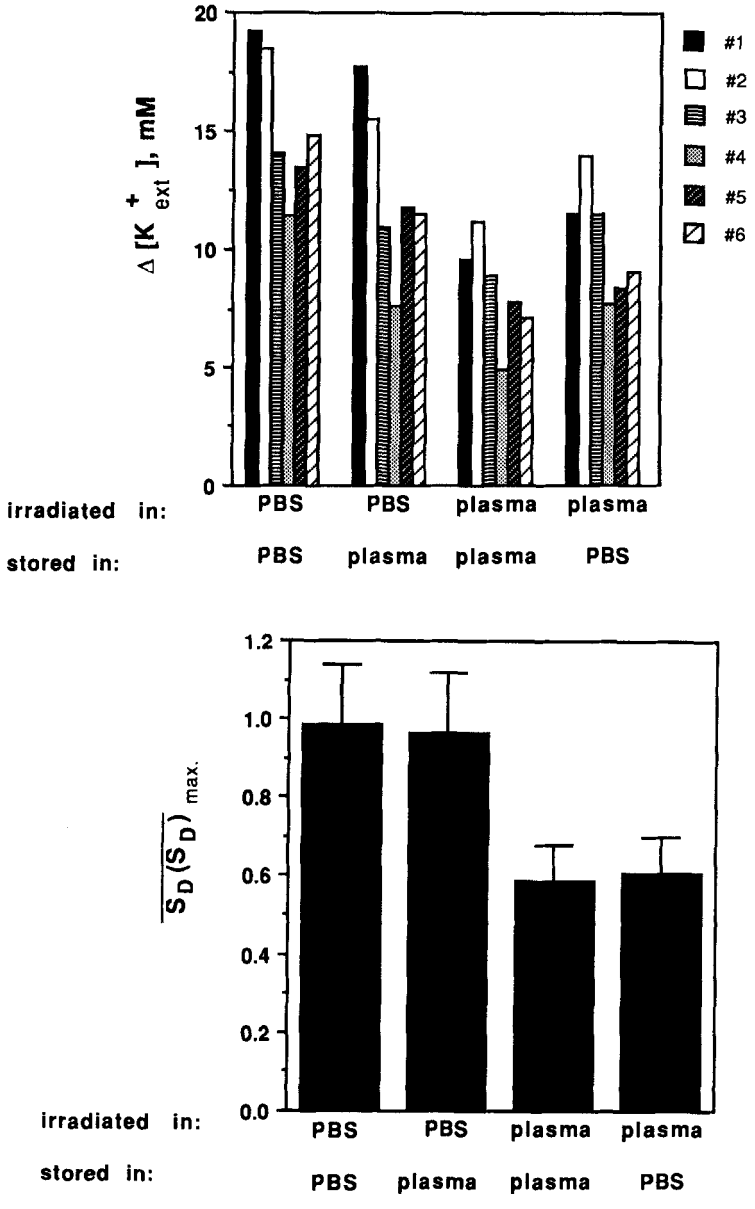

FIGURE 3 a. Influence of external medium during irradiation and post-irradiation storage, on potassium leakage, within 24 hrs. Total dose 225 Gy, Hct 30%. Data for RBCs taken from six donors are shown; the number of blood unit is indicated on the figure. b. Influence of external medium during irradiation and post-irradiation storage on $\overline{S_D}/(\overline{S_D})_{max}$, calculated from the data in Figure 3a applying equation IV. The results represent the mean values ±SD.

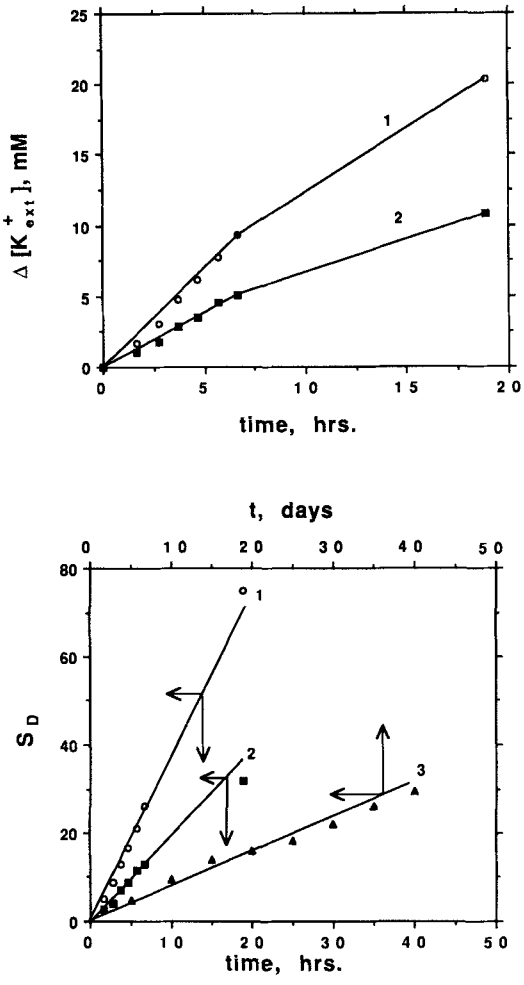

FIGURE 4 a. Kinetics of radiation induced K^+ leakage as a function of storage time in PBS (curve 1) and plasma (curve 2). Total dose 225 Gy, Hct 30%. b. S_D as a function of time after radiation. Curves 1 & 2 calculated from the data in Figure 4a applying equation IV. Storage time in hrs. D = 225 Gy, Hct = 30%. Curve 3 calculated from the data reported by Brugnara and Churchill[6] in AS-1. Storage time in days, D = 10 Gy, Hct = 68%.

DISCUSSION

The fact that radiation up to 200 Gy causes no significant changes in the concentration of any of the essential constituents of the red blood cells[5-7] but increases considerably the rate of potassium leakage indicates that the major site of radiation damage is in the cell membrane. The results, Figure 4a, clearly indicate that the potassium ions leakage is a relatively slow process. The observation that the radiation causes potassium leakage but does not cause a significant release of hemoglobin into the medium may be explained by one of the following arguments:

1. The radiation damages the Na^+-K^+ pump. However, other reports[5,6] point out that radiation up to 200 Gy does not affect the activity of the Na^+-K^+ pump and the ATP level.
2. The radiation forms holes with a diameter of < 5 nm in the membrane thus enabling passive cation passage through the membrane.

As the first possibility is ruled out by data in the literature, the results indicate that the second possibility is the correct one. The rate of passive potassium leakage is expected to obey equation (I):

$$\frac{dK^+_{ext}}{dt} = D\pi r_{ir}^2 N_{ir} \frac{[K^+]^{ir}_{int} - [K^+]^{ir}_{ext}}{1} \tag{I}$$

Where D is the diffusion coefficient of K^+, r_{ir} and N_{ir} are the average radii of the radiation defects and their number, respectively, and 1 is the membrane thickness. In solving equation (I) one has to remember that the material balance is kept, i.e.:

$$K^+_{tot} = K^+_{ext} + K^+_{int} = [K^+]_{int} \frac{Hct}{100} + [K^+]_{ext}\left(1 - \frac{Hct}{100}\right) \tag{II}$$

where K^+_{ext} and K^+_{int} are the total amounts of potassium ions in the medium and in the cells, respectively. The total amount of radiation defects in a volume unit is given by equation (III):

$$N_{ir} = N_D * Hct/(MCV * 100) \tag{III}$$

where N_D is the mean value of radiation defects on a single erythrocyte. After substitution of $[K^+]_{int}$ from equation (II) into equation (I) and taking into account equation (III) one can integrate equation (I) using the boundary conditions $t \to 0$, $[K^+]^{ir}_{ext} \to [K^+]^{cont}_{ext}$. (The results presented in Figure 4a clearly confirm the correctness of this boundary condition.) Thus one obtains:

$$(100 - Hct) \ln \frac{K^+_{tot} - [K^+]^{cont}_{ext}}{K^+_{tot} - [K^+]^{ir}_{ext}} = \frac{10^2 D * N_D * \pi * r_{ir}^2 * t}{MCV * 1} \tag{IV}$$

Measurements of MCV, within one day after irradiation, indicate that there is no significant difference between irradiated and control cells, i.e. equation (IV) predicts that the rate of K^+ leakage depends only on the total surface area of the radiation defects. Therefore, it is preferable to use as an ordinate:

$$(100 - Hct) \ln \frac{K^+_{tot} - [K^+]^{cont}_{ext}}{K^+_{tot} - [K^+]^{ir}_{ext}} = S_D$$

instead of $\Delta[K^+_{ext}]$ when the experimental results are plotted. Such plots normalize the experimental data for differences in Hct or $[K^+]$ in the individual samples. The experimental results were therefore replotted in Figures 1b, 2b, 3b, 4b, and 5. These plots are indeed a measure of the effect of the experimental conditions on the product $N_D * r_{ir}^2$ in PBS and plasma.

Figure 1b clearly points out that the product $N_D * r_{ir}^2$ is linearly correlated to the total dose absorbed by the suspension. The results for sample 1, the most sensitive to radiation, indicate a saturation effect at high doses.

The results in Figure 2b point out that the product $N_D * r_{ir}^2$ is independent of the hematocrit, up to Hct = 80. This result indicates that the damage to the membrane

is not caused by free radicals formed in the medium as, at least in PBS, the number of free radicals formed in the medium and expected to interact with each cell should decrease with increasing Hct.

As the potassium leakage after irradiation varies for different donors (Figure 3a) the arithmetic mean value $\overline{SD/(SD)_{max}}$ for these units is presented in Figure 3b. The results depicted in this figure demonstrate that the effect of the external medium on the total surface area of the radiation defects occurs during irradiation. The replacement of an external solution after irradiation does not influence the total surface area of the formed defects.

The results in Figure 4b clearly indicate that the product $N_D{*}r_{ir}^2$ remains constant over an extended period of storage both in plasma and in PBS suspensions. We have introduced into the figure the line calculated from the data obtained by Brugnara and Churchill.[6] The latter data are for a dose of 10 Gy, i.e. a dose lower by a factor of 22.5, and a different medium (containing mannitol, glucose and saline) AS-1.[13] It is of interest to note that after correcting the slopes for the relative doses, the slopes of lines 1 & 3 differ by less than a factor of 3. Furthermore, the results presented in Figure 3b demonstrate that the effect of the nature of the medium is not affected by the exchange of the medium after the irradiation. These results clearly point out that the nature of the radiation defects does not change with time, though some changes in the first minutes after the radiation is absorbed cannot be ruled out.

It is of interest to analyze the reason for the large difference in the value of the product $N_D{*}r_{ir}^2$ in plasma and in PBS. As stated above the different results in the two media cannot be attributed to:

1. A post irradiation effect.
2. Free radical reactions in the medium.
3. Chloride radiolysis products in the external medium.
4. Differences in Donnan's potentials.

The results, as stated above, indicate that the radiation defects are formed by the direct absorption of the radiation by the cells. Due to the large concentration of free radical scavengers in the cytosol it is reasonable to assume that the cause of the radiation defects are free radicals formed in the membrane via the direct absorption of the radiation by it. If so, what is the mechanism of the radioprotective effect of the plasma? A plot of the results presented in Figure 5, clearly points out that the product $N_D{*}r_{ir}^2$ is inversely proportional to the plasma concentration in the medium. It is reasonable to suggest that the source of this effect is as follows: the radiation defects are formed via a chain reaction at each site where radiation is absorbed in the membrane. A chain reaction is certainly required in order to form a hole large enough for the leakage of potassium ions. This chain process can be terminated by the reaction of a free radical with a constituent of the plasma adsorbed to the surface of the membrane.

CONCLUDING REMARKS

The results clearly indicate that radiation induced defects are formed in the membranes of the red blood cells. The number of these defects depends linearly on the radiation dose. The number of the defects decreases with the increase in plasma concentration in the external medium. The defects are large enough to enable

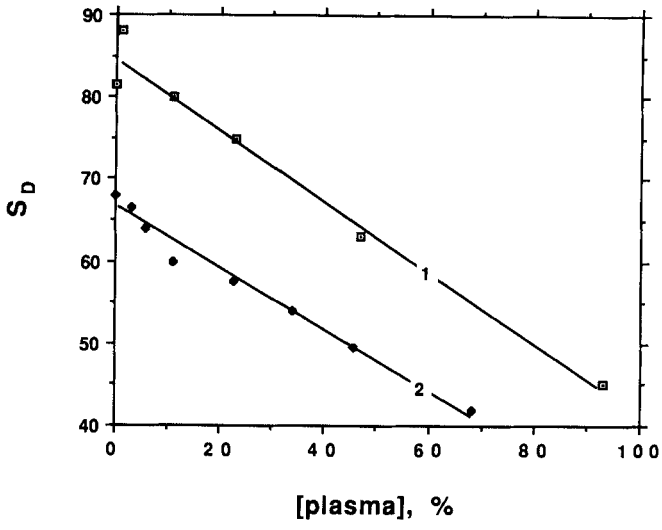

FIGURE 5 Dependence of S_D on plasma concentration for two blood units, D = 225 Gy, calculated from the data in table 1.

passive leakage of potassium from the cells. The leakage is a slow process but within few days an equal concentration of potassium inside the affected cells and in the medium will be achieved. Thus, even low radiation doses have a deleterious effect on blood units.

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