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EARLY POST-RADIATION CHANGES OF RED BLOOD CELL SHAPE IN RATS

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Abstract

Scanning electron microscopy (SEM) of red blood cells in whole blood samples from rats was performed following acute γ-irradiation of animals with 0.25 to 1 Gy. Increased incidence of echinocytosis was observed and found to be dose- and time-dependent. At a higher radiation dose (1 Gy), echinocytosis was revealed within 5 minutes after treatment and persisted up to 3 weeks. The data demonstrate the applicability of SEM for detecting minimal radiation-induced lesions of red blood cells.

Key Words: Erythrocytes, rats, radiation effects, echinocytosis, scanning electron microscopy.

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Introduction

The diagnosis of acute radiation pathology in mammals is generally based on the studies of bone marrow, blood leukocytes and platelets. Among different markers of radiation injury, membrane changes are considered to be very sensitive, however, quite unstable (Koteles, 1982; Chukhlovin, 1995).

Red blood cells (RBCs) are considered, however, as relatively radioresistant cells, thus, poor indicators of early radiation damage. Only after *in vitro* treatment with high doses of X- or γ-rays (> 100 Gy), do high levels of lipid peroxidation occur along with disturbances in K/Na ion balance, membrane leakiness and other features of gross membrane pathology (reviewed by Lee and Ducoff, 1994). Donor's blood can be treated with up to 30 Gy of ionizing radiation and successfully transfused without excessive reduction in RBCs life-span (Button *et al.*, 1981). *In vivo* studies of RBCs after low-dose (< 1 Gy) irradiation, are uncommon. For example, Kubasova *et al.* (1981) found a transient, dose-dependent increase of ConA (Concanavalin A) binding to RBCs irradiated with 1-2 Gy of X-rays. The long-lasting decrease in RBC membrane charge, along with increased potassium loss from the cells was revealed after treatment of rats with 3 or 6 Gy of X-rays (Badginian *et al.*, 1995). Scanning electron microscopy (SEM) was occasionally used to assess radiation effects upon circulating RBCs (Linke *et al.*, 1985; Thompson and Johnstone, 1987). In this regard, this technique has proven to be an effective tool for studying fine membrane alterations following irradiation (Somosy *et al.*, 1989).

The aim of the present work was to conduct a primary *in vivo* evaluation of the effects of relatively low doses of whole body γ-irradiation on the shape of rat erythrocytes, employing SEM of whole blood samples. Dose- and time-dependent echinocytosis was the major finding in this study.

Materials and Methods

White Wistar rats (120-150 g) were subject to whole body irradiation in glass cylinders using a Cs-137 source

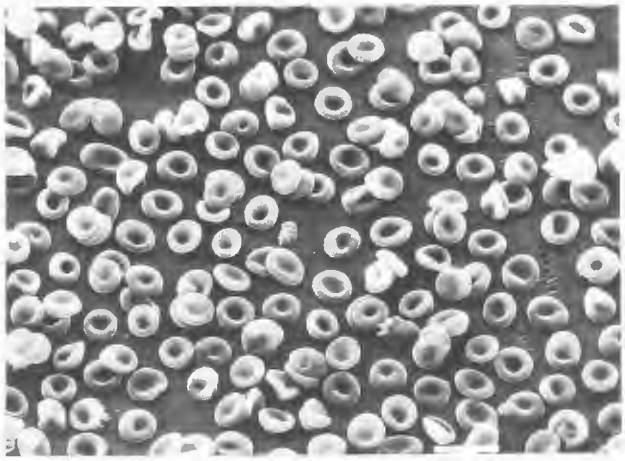
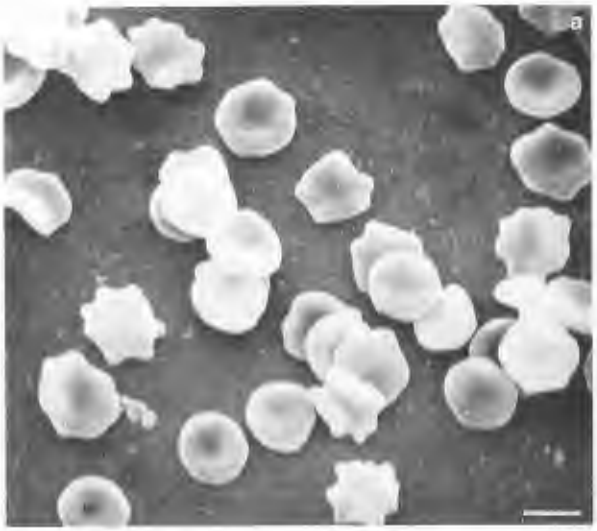
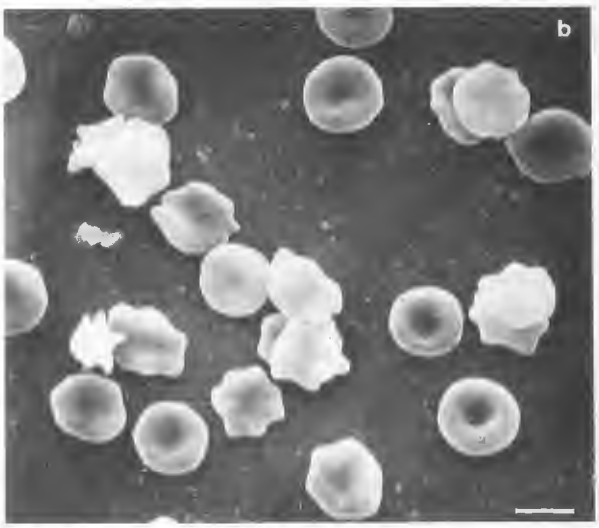


Figure 1. Scanning electron micrographs of whole blood from normal control rats. Bar = 10 μm.





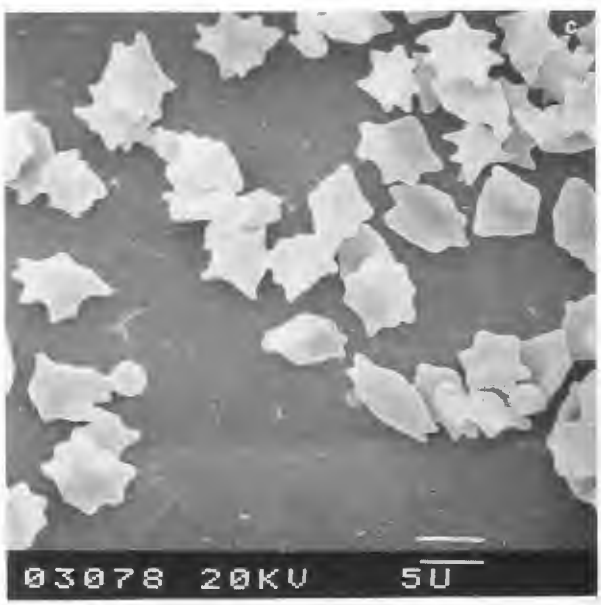


Figure 2 (at right). Scanning electron micrographs of peripheral blood red blood cells 3 days after whole-body irradiation of rats with 0.25 Gy (a); 0.5 Gy (b); and 1.0 Gy(c). Bars = 5 μm.

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at a dose-rate of 0.12 Gy/min. Uniformity of dose within the irradiated volume was ± 10%, as routinely assessed by an ionization chamber dosimeter. Basically, dosimetry was performed in a tissue-equivalent phantom. Total doses applied were 0.25 to 1.0 Gy.

Freshly taken 0.1 ml specimens of venous blood (37°C) were immediately fixed with 1.25% glutaraldehyde solution in 0.1 M phosphate buffer; pH 7.4. Fixation procedure was performed at 20°C, while the temperature of the fixative was 4°C. Cell suspensions were sedimented upon glass coverslips, dried and dehydrated in ethanol of ascending concentrations. Fixed and dried cell layers were evaporation-coated with a layer of gold. The samples were examined using either Hitachi 300 or JEOL S SEM operated at accelerating voltages of 15-20 kV. Cell images obtained have been studied for incidence of RBCs with altered shapes and/or sizes. The typical pathology of RBCs was determined qualitatively or, in some cases, as a percentage of affected cells (at least 200 RBCs per sample). The visual inspection of micrographs was done with coded samples. Identification and classification of RBC pathology was performed according to commonly used SEM standards for blood cells (Bessis, 1973; Castoldi and Beutler, 1988). The quantitative data were presented as mean values ± the standard error (SE).

Results

The SEM of whole blood samples taken from normal rats revealed normal discoid erythrocytes (Fig. 1).

Leukocytes and platelets were also observed in selected microscopic fields (not shown). Rounded extracellular objects of submicron size, either single or grouped, presented a peculiar feature of whole blood in the normal animals. The numbers of abnormal RBCs were uniformly low in both control and sham-irradiated animals (6.1 ± 0.6%, the mean of 9 rats).

Radiobiological experiments have been performed with rats acutely treated with 25 to 100 cGy of γ-rays. The early sign of radiation-induced RBC damage was reflected by the elevated frequency of echinocytosis compared with those in normal animals. For a minor RBC subpopulation, this pathology was slightly expressed as early as 5 minutes after exposure to 1.0 Gy.

The rise in frequency of echinocytic RBCs proved to be dose-dependent; the echinocytic RBC frequency was at its maximum after 1 Gy of γ-rays (Fig. 2). This pathologic response included an increased incidence of crenated erythrocytes, as well as the more prevalent echinocytic changes. The peak occurrence of these shape changes occurred 3 and 7 days after all the irradiation doses were tested (Fig. 3).

With longer post-irradiation times, the numbers of altered RBCs decreased (Fig. 3). At lower radiation doses (0.25 to 0.5 Gy), the increased echinocytic counts were observed until 7-14 days. However, after 1 Gy of γ-rays, the recovery of cell population proceeded until the end of the observation period (40 days). Hence, the incidence of RBC echinocytosis observed *in vivo* by SEM seems to be an informative index of acute radiation pathology, even at relatively low doses of γ-irradiation.

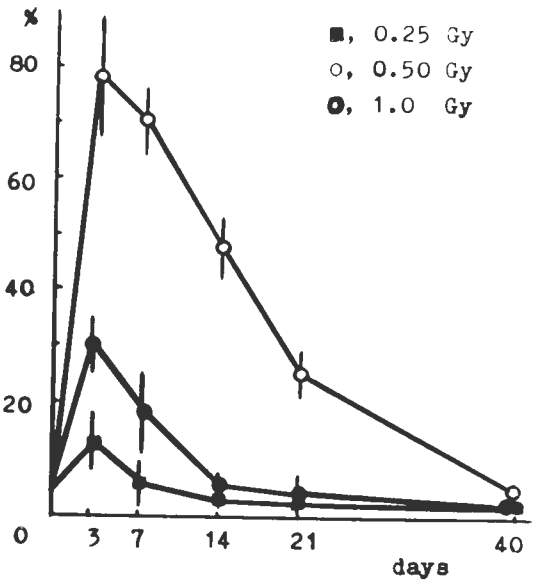

Figure 3. Incidence of rat echinocytosis after acute whole-body irradiation, % of total. Abscissa: time after treatment (in days); ordinate: echinocytic cells, percent of total cells.

Discussion

Acute radiation pathology of mammalian cells is induced to a large extent by reactive oxygen species (ROS), which cause initial peroxidation of membrane lipids, cross-linking and breaks within proteins and DNA (Riley, 1994). The mechanisms of oxidative damage to RBCs may be caused by the generation of endogenous ROS, e.g., by activating nitric oxide synthase (Delikonstantinos *et al.*, 1995). ROS-induced damage to RBCs may also cause deficiency of aminophospholipids at the inner membrane leaflet, due to their suppressed transport from outer membrane (Zachowski, 1993). The latter process coincides with intracellular calcium accumulation, depletion of endogenous adenosine triphosphate (ATP) and dephosphorylation of some lipids, which are thought to cause the failure of energy-dependent interactions between membranes and cytoskeletal networks (Reinhart and Chien, 1987; Lin *et al.*, 1994; Gedde *et al.*, 1995). The dissociation of membrane from the underlying cytoskeletal network is the most likely reason for red blood cell spiculation and crenation, described as echinocytosis (Liu *et al.*, 1989). The dynamic interactions between spectrin-actin-band 4.1 cytoskeleton complexes and membrane built-in proteins may be severely disturbed by oxidative treatment and by some genetic diseases (Mohandas, 1992; Bennett and Gilligan, 1993).

Small numbers of echinocytes are regularly found in peripheral blood of healthy humans and animals (Simpson, 1989). Increased number of echinocytes occur as a non-specific feature in various haematological conditions of known peroxidative mediated origins, e.g., in thalassemia and other hereditary anemias, along with more characteristic cell deformities (Bunyaratvej *et al.*, 1988; Castoldi and Beutler, 1988; Malorni *et al.*, 1993). Increased rates of RBC crenation are also revealed in severe metabolic disturbances, e.g., uremia and liver jaundice, thus being connected to hyperoxidized state of lipoproteins on the surface of RBCs (Agroyannis *et al.*, 1995). Significant increases in echinocytic RBCs were noted in rats after ozone inhalation, further supporting the role ROS plays in the *in vivo* induction of echinocytosis (Larkin *et al.*, 1978). Meanwhile, our study did yield echinocytic RBC changes following *in vivo* irradiation of white rats, thus suggesting similar oxidative mechanisms of membrane toxicity.

In the present study, increased rates of RBC echinocytosis were found at doses that do not induce gross haematological changes in laboratory rodents. Previously, RBC crenation was not considered an inherent trait of the acute radiation syndrome. However, in early SEM studies, similar distinctive RBC shape changes were re-

vealed, i.e., multiple echinocytic transformations of hamster RBCs were detected by SEM following sublethal (8 Gy) γ-ray irradiations (Thompson and Johnstone, 1987). Similarly, Linke *et al.* (1985), observed microblebing on the surface of rat RBCs several hours to days after *in vivo* γ-irradiation with 0.05 to 5 Gy.

The indirect detection of minimal RBC oxidative damage by SEM is based on its high resolution. A potential problem is, that echinocytosis may be an artifact, caused by the initial events associated with glutaraldehyde fixation, adhesion to the glass surface, or the alkaline pH of the suspending medium in the fixation step (Gedde *et al.*, 1995). However, the normal discoid appearance of RBCs from intact rats did not show any procedure-associated membrane damage. Therefore, it is quite possible that the pathology of the *in vivo* irradiated RBCs is of a latent nature and only fully expressed following full processing of the RBCs for SEM. Thus, even minimal radiation damage to cell membranes would be detected using the SEM. A similar approach to detection of the latent radiation-induced RBC changes was used by Mikhailov and Potemkin (1985). These authors have demonstrated an increased porosity of rat and canine erythrocytes after irradiation of animals with moderate doses of γ-rays. However, the differences between irradiated and control RBCs proved to be quite evident, but only following the permeabilizing of the RBC specimens with mild detergent.

Increased numbers of echinocytic RBCs after low-dose irradiation appear to be the result of a minimal, dose-dependent damage to plasma membranes. However, the indirect action of blood plasma- and leukocyte oxidants on circulating RBCs may indeed play a role in amplifying these echinocytic transformations (Reinhart and Chien, 1987). It is now recognized that oxidized lipoproteins from the plasma can be readily absorbed by the RBC surface and, in turn, recognized by circulating antibodies (Sambrano *et al.*, 1994). Furthermore, the noted shape transformations *in vivo* may be amplified by selected classes of autoantibodies that specifically recognize damaged cells; for example, significant levels of echinocytosis were found in human patients sensitized to certain drugs (Rahamim *et al.*, 1990).

In summary, *in vivo* echinocytic transformations of rat RBCs following relatively low dose exposures to whole body γ-irradiation is clearly observable by SEM. This noted cell-shape alteration probably stems from a direct oxidative effect of the irradiation and is probably mediated by exogenous factors that amplify the initial latent damage within limiting plasma membranes.

References

Agroyannis B, Dalamangas A, Zanatos H, Fortounas C, Kopelias I, Koutsikos D (1995) Relation between spiculated red cells and platelets in uraemic patients. Br J Haematol **89**: 686-687.

Badginian SA, Kazarian PA, Akopov SE, Saarian AV (1995) Changes in structure and functional state of the membrane of erythrocytes after ionizing irradiation. Radiobiol Radiat Ecol **35**: 364-369. (in Russian)

Bennett V, Gilligan DM (1993) The spectrin-based membrane skeleton and micron-scale organization of the plasma membrane. Ann Rev Cell Biol **9**: 27-66.

Bessis M (1973) Living Blood Cells and Their Ultrastructure. Springer-Verlag, Berlin, Germany. pp. 183-284.

Bunyaratvej A, Sahaphong S, Bamarapravati N, Nitiyanant P, Wasi P (1988) A scanning electron microscopic study of red cells of thalassemic patients. Birth Defects **23**: 169-175.

Button LN, DeWolf WC, Newburger PE, Jacobson MS (1981) The effects of irradiation on blood components. Transfusion **21**: 419-426.

Castoldi GL, Beutler E (1988) Erythrocytes. In: Atlas of Blood Cells. (Vol. 1). Zucker-Franklin D, Greaves M, Grossi C, Marmont A (eds.). Fisher, Stuttgart, Germany. pp. 49-156.

Chukhlovin AB (1996) Coupling of membrane pathology and DNA damage in irradiated lymphoid cell populations. In: NATO ASI Symposia Series, Subseries H "Cell Biology." Vol. 94. Springer Publishers, Berlin. pp. 399-404.

Delikonstantinos G, Villiotou V, Stavrides JC, Salemes N, Gogas J (1995) Nitric oxide and peroxynitrite production by human erythrocytes: A causative factor of toxic anemia in breast cancer patients. Anticancer Res **15**: 1435-1446.

Gedde M, Yang E, Huestis WH (1995) Shape response of human erythrocytes to altered cell pH. Blood **86**: 1595-1599.

Koteles GJ (1982) Radiation effects on cell membranes. Radiat Environ Biophys **21**: 1-18.

Kubasova T, Koteles GJ, Varga LP (1981) Surface alterations of mammalian cells upon ionizing radiation as detected by lectin-binding technique. Int J Radiat Biol **40**: 187-194.

Larkin EC, Kimzey SL, Siler K (1978) Response of rat erythrocyte to ozone exposure. J Appl Physiol **45**: 893-898.

Lee SW, Ducoff HS (1994) The effects of ionizing radiation on avian erythrocytes. Radiat Res **137**: 104-110.

Lin S, Yang E, Huestis WH (1994) Relationship of phospholipid distribution to shape change in Ca-crenated and recovered human erythrocytes. Biochemistry **33**: 7337-7344.

Linke A, Schmidt W, Muhlig P, Angsten K, Frick G, Nadrowitz R (1985) Phasenkontrastmikroskopische

und rasterelektron-mikroskopische untersuchungen zur variabilitat des plasmaproteinfilms von rattenerythrozyten nach ganzkorper-bestrahlungen unterschied licher dosierung (Phase contrast and scanning electron microscopy of plasma protein films upon rat erythrocytes following whole-body irradiation at different doses). Radiobiol Radiother **26**: 123-132.

Liu SC, Derick LH, Duguette MA, Palek J (1989) Separation of the lipid bilayer from the membrane skeleton during discocyte-echinocyte transformation of human erythrocyte ghosts. Eur J Cell Biol **49**: 358-365.

Malorni W, Iosi F, Donelli G, Caprari P, Salvati M, Cianciulli P (1993) A new, striking morphologic feature for the human erythrocyte in hereditary spherocytosis: The blebbing pattern. Blood **81**: 2821-2822.

Mikhailov VF, Potemkin LA (1985) Evaluation of radiation-induced membrane lesions in the rat erythrocytes, according to their altered sedimentation properties. Radiobiologia **25**: 784-786. (in Russian)

Mohandas N (1992) Molecular basis for red cell membrane viscoelastic properties. Biochem Soc Trans **20**: 776-782.

Rahamim E, Kahane A, Sharon R (1990) Electron microscopy of red blood cells affected by autoimmunity-inducing drugs. Vox Sang **58**: 292-299.

Reinhart WH, Chien S (1987) Echinocyte-stomatocyte transformation and shape control of human red blood cells: Morphological aspects. Am J Hematol **24**: 1-14.

Riley PA (1994) Free radicals in biology: Oxidative stress and the effects of ionizing radiation. Int J Radiat Biol **65**: 27-33.

Sambrano GR, Parthasarathy S, Steinberg D (1994) Recognition of oxidatively damaged erythrocytes by a macrophage receptor with specificity for oxidized low-density lipoprotein. Proc Nat Acad Sci **91**: 3265-3269.

Simpson LO (1989) Blood from healthy animals and humans contains nondiscocytic erythrocytes. Br J Haematol **73**: 561-564.

Somosy Z, Czuka O, Kubasova T, Kovacs J, Koteles GJ (1989) Plasma membrane heterogeneity of tumor cells and its changes upon ionizing radiation. Scanning Microsc **3**: 579-590.

Thompson JC, Johnstone AC (1987) The effects of γ-irradiation on haemopoiesis and red blood cell destruction in hamsters infected with Leptospira interrogans serovar ballum. Br J Exp Path **68**: 389-398.

Zachowski A (1993) Phospholipids in animal eukaryotic membranes: Transverse asymmetry and movement. Biochem J **294**: 1-14.

Discussion with Reviewers

T.M. Seed: If RBC shape changes do indeed provide an "informative index" of acute radiation pathology, the authors should state the nature of these correlates (e.g., correlated change in RBC numbers, hematocrit, etc.)?
W. Malorni: Do the alterations found correspond to other clinical findings?
Authors: The changes of RBC shape in irradiated rats did not correlate with results of routine blood analysis, including blood smears, since radiation was applied at lower doses, which may produce only marginal lymphopenia in blood without observable signs of acute radiation syndrome. Post-radiation echinocytosis found by SEM may, thus, reflect a minimal damage of irradiated RBC revealed after additional cell treatment (Mikhailov and Potemkin, 1985). Sufficient changes of RBC shape, using SEM technique, were observed for animals irradiated at moderate and high doses (Linke *et al.*, 1985; Thompson and Johnstone, 1987).

W. Malorni: Can the authors better discuss the results in terms of mechanisms?
Authors: As possible factors contributing to RBC crenation, the increased oxidation of membrane lipids, cross-linking and SH-blockage of membrane and cytoskeleton proteins, as well as membrane damage by the oxidized serum lipoproteins are suggested.

W. Malorni: In your micrographs, only a few RBCs are flattened on the coverslips and neither lymphocytes nor activated platelets are visible, why? Please provide micrographs showing changes in lymphocytes.
Authors: Leukocytes and platelets were sometimes observed, but we preferred to show the fields containing erythrocytes only. The effects of low-dose irradiation upon lymphocyte shape deserve a special study.

W. Malorni: A careful analysis of some clinical parameters, e.g., the effect on rheology, data on oxygen/CO clinical parameters, could strongly improve the paper. Do the authors think that the RBCs of treated rats and humans are good oxygen carriers?
Authors: The tendency to increased RBC agglutination in irradiated rats, though qualitatively evaluated, should be an indicative for possible rheological changes *in vivo*, correlating with higher rates of echinocytic transformation. We have no definite data about oxygen balance in blood of these animals. Oxygen-carrying properties of minimally damaged RBCs may be disturbed, depending on very probable metabolic depletion of ATP and 2,3-diphosphoglycerate accompanying echinocytic transformation of RBCs (reviewed by Reinhart and Chien, 1987).

Z. Somosy: As known, the cytoskeletal network in the shape changes of RBC plays an essential role!
Authors: The altered interactions between membrane

lipids and cytoskeletal proteins are critical to RBC shape changes, including echinocytosis (Bennett and Gilligan, 1993). The detailed mechanisms of red cell crenation are very well discussed, e.g., by Zachowski (1993).

Z. Somosy: As some *in vitro* experimental data presented, the intact erythrocytes and erythrocyte ghosts are very radioresistant even after irradiation with up to 30-50 Gy. How do you explain your data, i.e., you found morphological changes up to 0.25 Gy?
Authors: A severe membrane damage of RBCs after high doses of irradiation is commonly assessed, e.g., increased membrane porosity, cell flattening, blebing, or haemolysis (Lee and Dukoff, 1994). After RBC irradiation *in vivo*, a minimal oxidation of membrane lipids and proteins may occur, thus triggering the low dose-induced echinocytosis (see **Discussion**).

E. Rahamim: It is well established that treatment with OsO_4 is essential in the preparation of biological specimens for SEM. Why was this process absent?
Authors: Yes, we omitted treatment with OsO_4. It was empirically found that such modification does not induce any significant shape alterations of RBC specimens from healthy individuals (humans or animals).

E. Rahamim: When RBCs are sedimented upon the glass coverslips, a few precautions should be considered, since a transformation may occur from biconcave discs to echinocytes, due to the high pH induced by the glass. Did the authors examine this phenomena?
Authors: In fact, the specific example, "glass echinocytosis" is a very common artifact. In our experience, the rates of such transformation among freshly taken specimens of normal RBCs did not exceed the literature data (Simpson, 1989). However, the role of processing procedures for SEM may be significant in case of slightly damaged, e.g., irradiated, RBCs (see **Discussion**). We controlled this process steadily, and each experiment was paralleled by appropriate intact controls.

E. Rahamim: Much of the success of technique depends on proper drying of the samples at a temperature higher than the boiling point of the drying agent. Using ethanol alone, can cause some distortion and shrinkage of specimens. An improved air-drying technique has been described using a graded series of ethanol-freon-113 which provides better surface preservation.
Authors: We treated whole blood specimens uniformly, at low temperature, employing the technique of CO_2 critical point transition. Graded series of ethanol were used in the drying procedure. The regimen you recommended was not used, but we will try it in future work.

D. Tolle: Did you measure depth dose to target tissue, i.e., bone marrow, with Cs-137, since it will rapidly fall in air if measured by ionization chamber only?
Authors: Routine dosimetry was performed by ionization chamber dosimeter. However, in preliminary experiments, the tissue-equivalent phantom was used, and thermoluminescent dosimetry provided a more accurate assessment of dose distributions.

D. Tolle: Are there any findings in literature on RBC morphology in humans following total body irradiation (12-15 Gy) for bone marrow transplantation (BMT)?
Authors: Such data exist in every BMT unit but appropriate RBC changes have not yet been properly classified. Such patients cannot be regarded as "pure" clinical cases of acute radiation syndrome because of previous intensive chemotherapy and near-simultaneous administration of irradiation and potent cytostatic drugs, e.g., total irradiation plus cyclophosphamide or etoposide.

D. Tolle: Are there observations from hemograms of the 203 Chernobyl victims with acute radiation syndrome?
Authors: To our knowledge, the data about clinical course in these patients do not contain information about erythrocyte size, shape and functions, except of total RBC counts, whereas details were obtained about cytokinetics and recovery of bone marrow, lymphocytes, PMN's and platelets.

W. Malorni: Do the authors consider their results of some prognostic importance?
Authors: The prognostic importance of our results, both in animals, still cannot be assessed, because of short observation terms in this experimental series.

W. Malorni: It would be of interest to consider only rat RBCs in detail and provide more quantitative evidence regarding the alterations observed in rat; also, results obtained in humans could be of great relevance.
Authors: Indeed, in the article, we only consider rat RBCs. Human data representing chronic and mixed pathology will be submitted in the near future.

Z. Somosy: As suggested by data, the development of apoptotic cell death is not only a radiation-induced process, and the membrane changes during apoptosis are related to different levels of membrane-mediated transduction processes.
Authors: Indeed, the pathways of apoptosis may be different, depending upon the nature of toxic agent. Some common membrane changes in apoptotic cells and echinocytic RBCs are worth of a special review which we are planning to submit to **Scanning Microscopy**.