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The in vivo Effects of a Culture Medium. II. Influence of Culture Medium Administered Prior to Irradiation on Hemopoietic Recovery of Gamma-Irradiated Mice.

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Summary

The culture medium administered to C57B1/6 mice 18 h and 8 h before a single irradiation (9 Gy) had a radioprotective effect and clearly influenced postirradiation changes in haemopoiesis. Haemopoiesis recovery appeared to be faster in culture medium-pretreated animals than in those irradiated without such pretreatment. By 12–15 days after irradiation, the thymus cortex appeared to be repaired, on day 21 a multiple increase in extramedullar erythropoiesis, myelopoiesis and megakaryocytopoiesis in the red pulp was found and later, on day 28, the lymphopoiesis in the white pulp of spleen was restored. The rate of haemopoiesis proliferation of predominantly myeloid cells which reached a control level on day 28 following irradiation. Consequently, the regenerative processes in blood-forming organs were accompanied by considerable reticulocytosis and complete recovery of neutrophil and platelet counts in the peripheral blood as seen on day 21. Despite a slower rate complete recovery of the total leukocyte count was reached by day 180 after irradiation.

Key words
Radioprotection – Recovery – Haemopoietic organs – Peripheral blood

Introduction

Reparative processes of haemopoiesis and the manner in which they are influenced by radioprotective substances are of practical and theoretical importance in radiobiology. It is known that the application of various diets, milk, vitamins, mineral salts and other structural constituents apparently influence the haemato-immune system of irradiated or pathologically altered organisms (Jurášková 1971, Bell *et al. 1976*, Gallicchio and Murphy 1979, Pospíšil *et al.* 1980, Fernandes 1989, Majumdar and Boylan 1989). The culture medium (CM) that contains a large scale of different components such as amino acids, vitamins and inorganic salts was administered at various time intervals before and after irradiation damage (Fedoročko *et al.* 1991). When estimating the survival of mice and the occurrence of endocolonies, the results of the above study showed that CM administration 18 h

and 8 h before irradiation had a considerable radioprotective effect. On the basis of these results, our aim was to assess the influence of culture medium administration on the course of haemopoiesis recovery in haemopoietic organs and of blood renewal in the peripheral blood of irradiated animals.

Material and Methods

Mice. Female C57B1/6 mice (Velaz, Prague) weighing about 20 g were used throughout this study. They were held in the new environment for 14 days after delivery to allow for equilibration and to recover from any stress of transport. Mice 10–12 weeks old were housed in rodent cages five to seven animals per cage. They were given Larsen diet (Velaz, Prague) and tap water *ad libitum*.

Irradiation. Mice were placed in plexiglass containers and exposed to a 9 Gy of whole body gamma rays at a dose rate of 0.3 Gy/min. The Chisostat (Chirana, CSFR) ^{60}Co source was used for all irradiations.

Experimental design. Detailed composition of the culture medium used (minimal essential Eagle medium supplemented with amino acids, vitamins, etc.) is given by Fedoročko et al. (1991). Culture medium was lyophilized using Multi-Dry freeze-dryer (FTS Systems Inc. USA). The dry lyophilisate was immediately dissolved after lyophilization in a five-fold smaller volume of sterile deionized water as compared to the original volume. Approximately 18 h and 8 h before irradiation the mice received an i.p. injection of the culture medium in a volume of 1 ml. Control mice received i.p. saline in the same volume and at the same time intervals as the treated group. Animals (6–7 mice per group) were examined at various time intervals within one year after irradiation. After killing by decapitation, the blood was sampled for quantitative and qualitative analyses. The values of leukocytes, erythrocytes and haemoglobin were counted using automatic Coulter Counter Model ZF and that of platelets using a Bürker chamber. Reticulocytes were evaluated after staining the blood smears by brilliant cresyl blue. White blood cell differentials were performed by counting 100 white blood cells on May-Grünwald-Giemsa stained smears. Further, the spleen and thymus were extirpated, weighed and then processed by routine histological methods. Histological sections of 5–6 μm were stained with haematoxylin-eosin. Bone marrow smears were stained with May-Grünwald-Giemsa.

Statistical significance of differences in organ weights and peripheral blood parameters was evaluated by Student t-test.

Results

Haemopoietic organs

At the time of irradiation, i.e. 18 h and 8 h after culture medium administration, the weights of the thymus and spleen decreased approximately by 20–30 % as compared with initial values (Fig. 1, $P<0.01$). The debris, both scattered and phagocytosed in great macrophages, was observed namely in the thymus cortex and in some cases also in lymphatic follicles of the spleen. In the bone marrow, a slight hypoplasia was found largely as a result of the depletion of segmented granulocytes. Irradiation in CM-pretreated animals, as in the controls, caused marked injury to all haemopoietic organs with a maximum on day 7 after exposure. It was characterized by an atrophy of the thymus cortex and lymphatic follicles of the spleen as well as by a profound depression of haemopoietic activity in the bone marrow and red pulp of the spleen. While haemopoiesis inhibition tended to persist in animals irradiated only, the thymus cortex in CM-pretreated animals was seeded again with small lymphocytes, and partial recovery in thymus weight also took place by days 12–15 after irradiation. At this time, an increased occurrence of mononuclear cells of the lymphoid type in the bone marrow and of endogenous

erythroid colonies in the spleen was observed. Bone marrow haemopoiesis reparation progressed slowly and by day 28 only the myelopoiesis was fully recovered. In contrast to the bone marrow, the extramedullar haemopoiesis in the spleen sharply increased from day 15 accompanied by a gain in spleen weight and by hyperaemia. Not only extramedullar erythropoiesis, but also myelopoiesis and megakaryocytopoiesis reached a maximum on day 21 following irradiation when the spleen weight surpassed that of non-irradiated animals by 144 % (Fig. 1, $P<0.01$). Lymphatic follicles of the spleen also began to by seeded with small lymphocytes and on day 28 the active germinal centres were detected indicating the recovery of lymphopoiesis. At later intervals, extramedullar erythropoiesis and also lymphopoiesis in the white pulp remained slightly enhanced. On the contrary, the bone marrow was infiltrated with fat cells and contained a lower number of cells with a myeloid to erythroid cells ratio 3 : 1. The thymus weight diminished again on day 21 before remaining at a level significantly lower that of the control ($P<0.01$), despite the fact that thymus morphology was completely restored. The thymus cortex was found to be seeded densely with lymphocytes in various phases of mitotic division on day 28, 180 and 365 after irradiation.

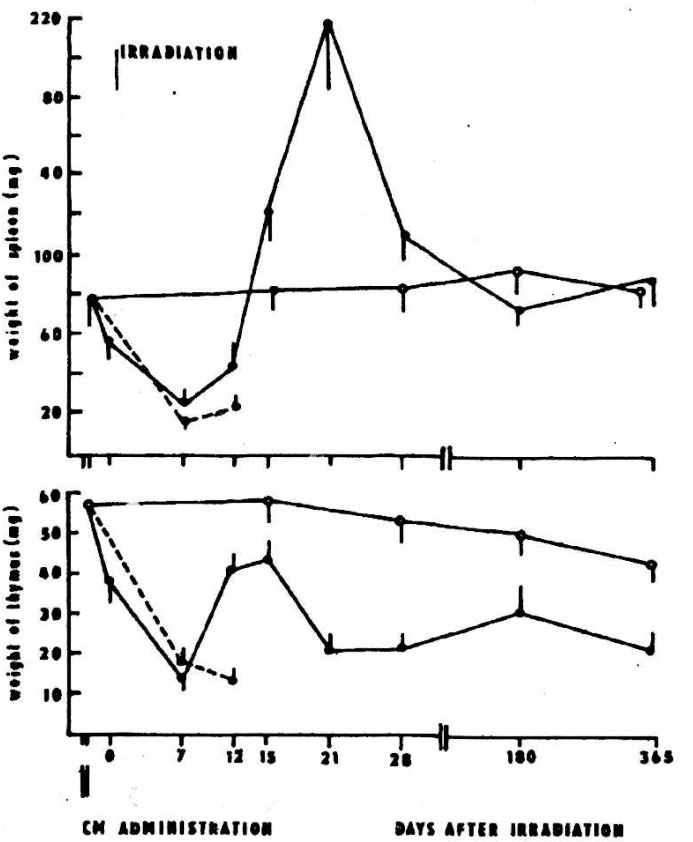

Fig. 1

Changes of spleen and thymus weight after whole-body gamma irradiation of unprotected (closed circles with broken line), CM-protected (closed circles with solid line) and control nonirradiated mice (open circles with solid line). Mice were irradiated on day 0. Very few saline-treated mice survived until day 12 after irradiation so that sufficient data could not be obtained after this time.

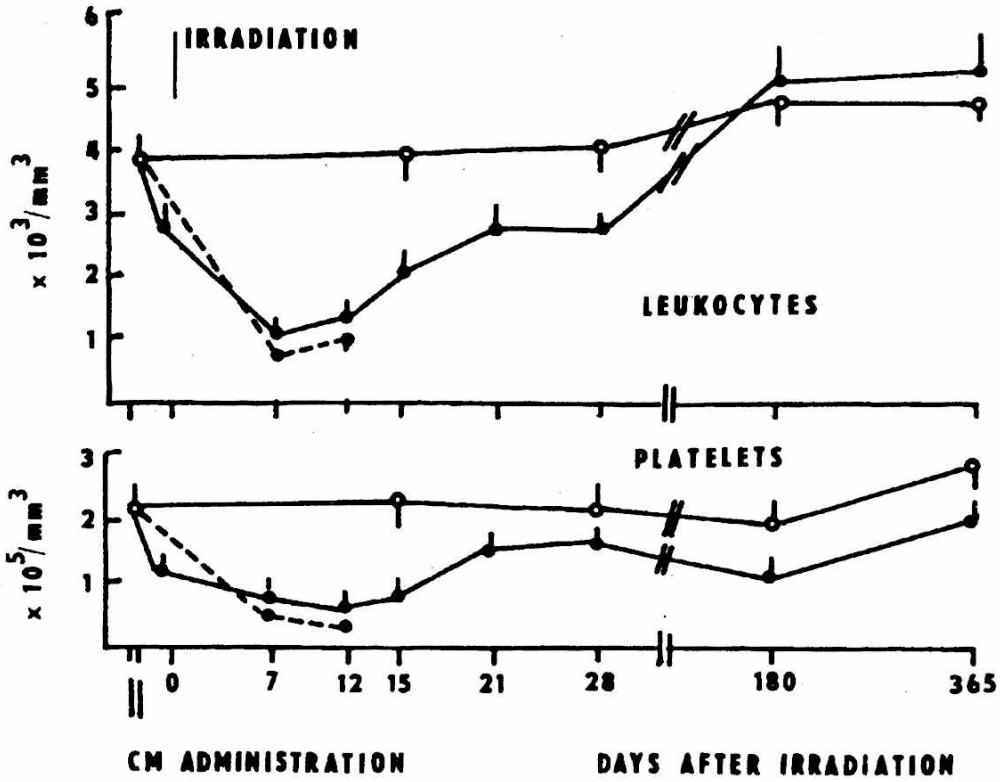

Fig. 2
Changes of leukocyte and platelets count after whole-body gamma irradiation of unprotected (closed circles with broken line), CM-protected (closed circles with solid line) and control nonirradiated mice (open circles with solid line). Mice were irradiated on day 0. Very few saline-treated mice survived until day 12 after irradiation so that sufficient data could not be obtained after this time.

Peripheral blood

In the blood 18 h and 8 h after CM administration, the leukocyte count was decreased by 29 % (reflecting mainly a lymphocyte decline) and the platelet count by 46 % were found (Fig. 2, $P<0.01$). The subsequent irradiation of these animals produced a considerable leukopenia, reticulocytopenia and thrombocytopenia, and on day 12 after irradiation also a 50 % decrease in erythrocytes and haemoglobin values (Figs. 2 and 3, $P<0.01$). A similar pattern of changes within the first days after irradiation described above was also seen in animals irradiated only, with the exception of a somewhat slower decrease in the erythrocyte count and haemoglobin values. However, the recovery of peripheral blood parameters was seen only in CM-pretreated animals. On day 21, a marked reticulocytosis paralleling a complete recovery of the erythrocyte count and haemoglobin values occurred. The number of platelets and also of neutrophil granulocytes reached the control levels. The rate of recovery of the total leukocyte count was slower since the rate of recovery of high radiosensitive lymphocytes proceeded slowly. However, 180 days after exposure, the total number of leukocytes as well as that of lymphocytes was fully recovered.

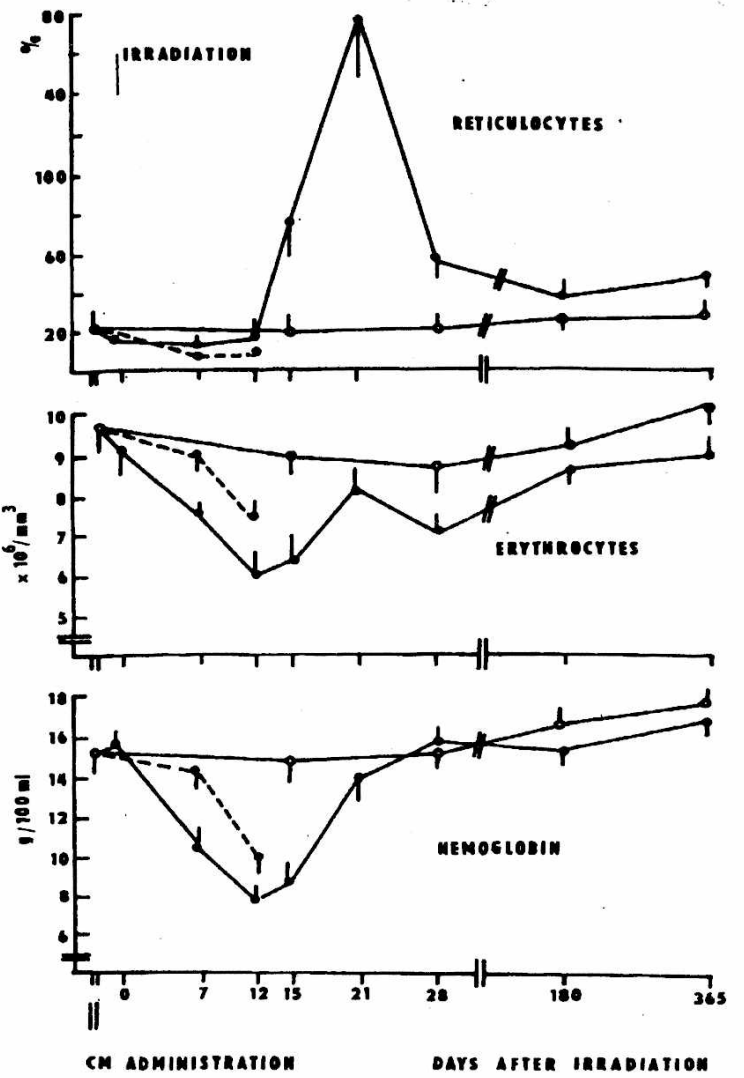

Fig. 3
Changes of reticulocyte and erythrocyte count and haemoglobin values after whole-body gamma irradiation of unprotected (closed circles with broken line), CM-protected (closed circles with solid line) and control nonirradiated mice (open circles with solid line). Mice were irradiated on day 0. Very few saline-treated mice survived until day 12 after irradiation so that sufficient data could not be obtained after this time.

Discussion

As follows from the results obtained, culture medium administration alone within 18 h after the first dose caused thymolymphocytolysis and a decrease in leukocytes (namely lymphocytes) in the peripheral blood. We assume that the changes mentioned above reflect a physiological response of the organism to the administered medium which may elicit a stress reaction and subsequently an increased secretion of adenocortical hormones to which lymphoid tissues respond very sensitively (Dougherty and White 1945, Lundin and Schelin 1968, Jensen 1969,

Cohen *et al.* 1970, Fauci and Dale 1975, Hedman and Lundin 1977, Rogers and Matison-Rogers 1982 and others). Radiation-induced changes in CM-treated mice were similar to those seen in only irradiated animals, but in contrast to these the former were reparable. This resulted in 95 % survival of the protected mice (Fedoročko *et al.* 1991). The relatively rapid mobilization of reparative processes led to a regeneration of the cortical area of the thymus within the second week after irradiation. One of the components of the culture medium are magnesium salts which could influence the reparative processes since they play a specific role in stimulating the thymolymphocyte mitotic activity (Whitfield *et al.* 1969). In the bone marrow, there was a transient increase in the occurrence of mononuclear cells of the lymphoid type as was also found after single irradiation with sublethal doses (Haot and Barakina 1969, Simar *et al.* 1975) or after protracted irradiation (Macková and Praslička 1981). In the spleen, erythroid endocolonies appeared and this haemopoietic organ gradually produced haemopoietic cells in all the developmental lines. Enhanced extramedullar erythropoiesis, myelopoiesis and megakaryocytopoiesis in the spleen with a maximum by the end of the third week after irradiation, were accompanied by considerable reticulocytosis and complete recovery of the number of neutrophil granulocytes and platelets in the peripheral blood. Though the bone marrow cellularity was still significantly decreased by 25 % the number of marrow colony-forming units (CFU-S) was completely repaired in this period (Fedoročko *et al.* 1991). Based on this disproportion between the course of CFU-S and cellularity recovery as well as on a simultaneous vigorous proliferation of haemopoietic cells in the spleen, we can assume that the haemopoietic stem cells recirculated from the marrow into the spleen. Probably the spleen provided a more appropriate haemoinductive microenvironment for seeding and proliferation of the stem cells.

One of the factors contributing to the improvement of haemoinductive microenvironment is hyperaemia. It is known that hyperaemia is an accompanying factor in erythropoietic stimulation (McCuskey *et al.* 1972) as has also been shown in our experiments. The hyperaemia of an organ is influenced by cyclic adenosine monophosphate (cAMP) that has a relaxation effect on smooth musculature (Anderson and Nilson 1972, Lugnier *et al.* 1972, Pöch and Kukovetz 1972, Somlyo *et al.* 1972, Shepherd *et al.* 1973) and increases vasodilatation in the bloodstream of the spleen red pulp (Reilly and McCuskey 1977). Gidari *et al.* (1971), Schooley and Mahlman (1971) and Dukes (1971) have found that cAMP initiates erythropoiesis, probably the differentiation of red blood cells as in the spleen (Winkert *et al.* 1971) and the bone marrow (Bottomley *et al.* 1971). The effect of erythropoietin, as a primary regulator of erythropoiesis (Krantz and Jacobson 1970), is mediated through cAMP. After CM administration, the level of cAMP might be influenced by nicotinamide, one of the CM components. Nicotinamide was found to increase cAMP concentration in the kidneys after a relatively short time after administration (Campbell *et al.* 1989). Pospíšil *et al.* (1988) have found that adenosine and adenosine monophosphate, if administered shortly before or after irradiation, have a radioprotective effect which may be intensified by further components of CM, magnesium and potassium salts. These elements, as such, in oral administration, accelerate the postirradiation regeneration of haemopoietic organs (Pospíšil *et al.* 1980, 1988). It also follows from the study of Gallicchio and Murphy (1979) that potassium may play a basic role in the mechanism by which erythropoietin acts on

the erythroid stem cells to induce the differentiation of these progenitor cells into morphologically recognizable erythroblasts. Magnesium levels influence B6 vitamin (Majumdar and Boylan 1989) that is involved in haemoglobin synthesis already in the initial activation of glycine. Furthermore, it plays a crucial role in the recovery of chemically-induced lymphopenia (Gobin *et al.* 1989). Another component of culture medium, namely pantothenic acid, that participates in porphyrin synthesis, may contribute to postirradiation recovery of haemopoiesis since porphyrins are necessary for synthesizing haeme.

It can be concluded that the culture medium used, as a source of many constituents can influence the postirradiation reparation of haemopoiesis in a complex way. The proper time of application or the mutually combined compensatory mechanisms might cause that, despite the high irradiation dose, the animals survived and their haemopoietic activity remained normal for a long time after irradiation.

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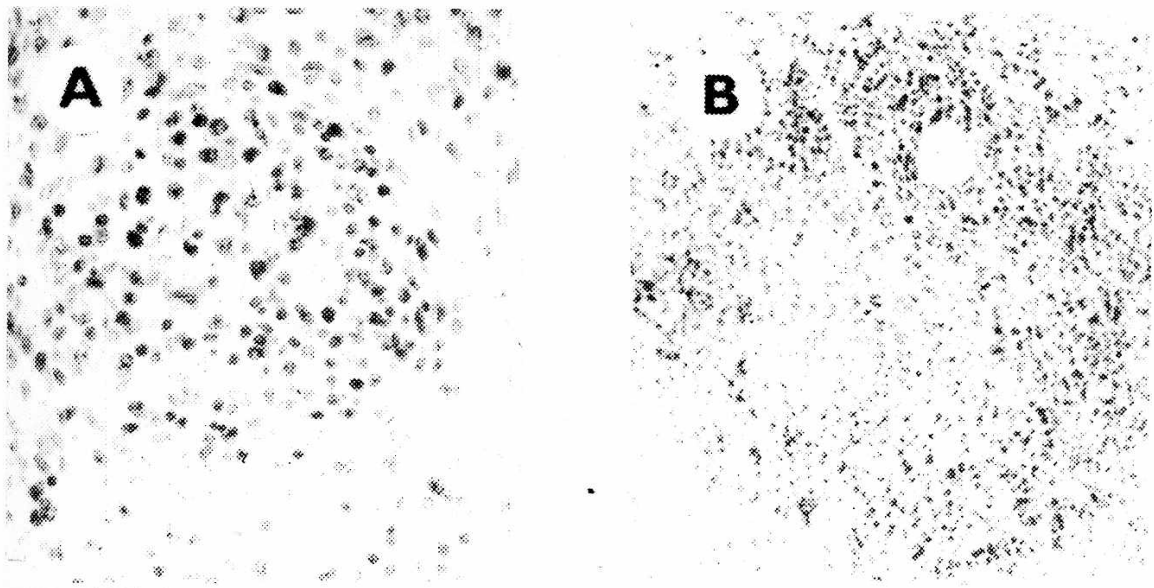
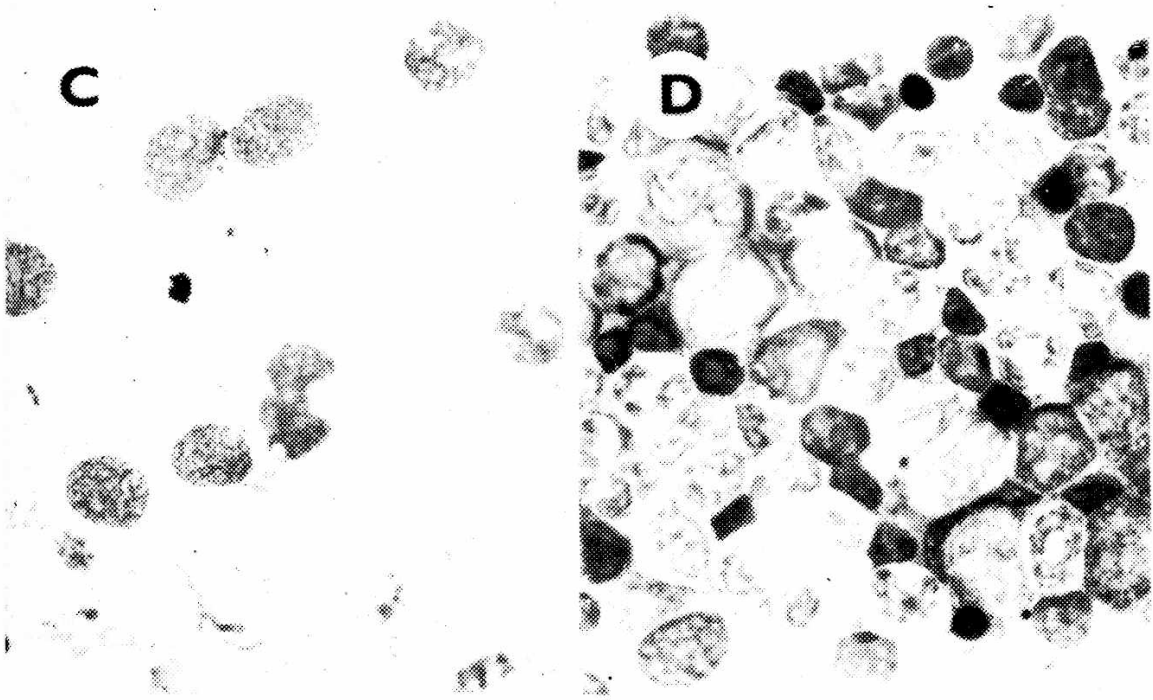
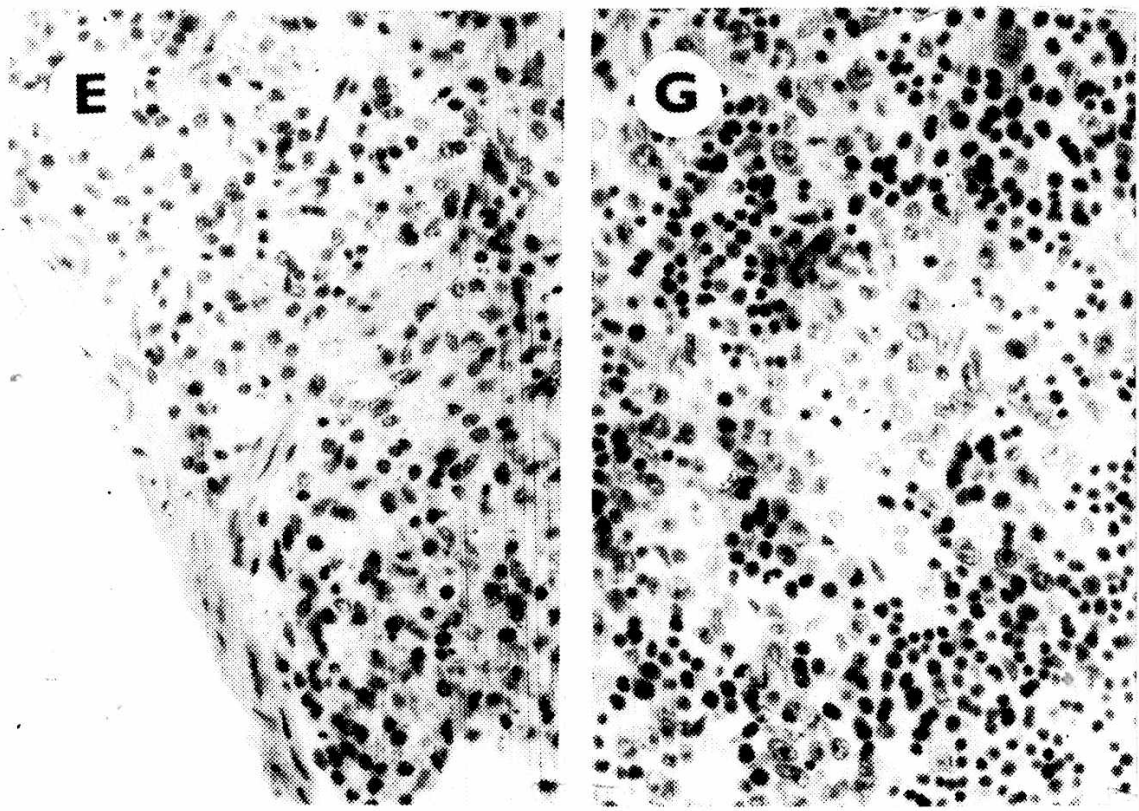


A. White pulp of the spleen 7 days after irradiation and administration of culture medium. lymphatic follicles were small with considerably low numbers of lymphocytes. Haematoxilin-eosin (x 450). B. White pulp of the spleen 28 days after irradiation and administration of culture medium. The lymphatic follicles contained active germinal centres with a large number of medium-sized and small lymphocytes. Haematoxilin-eosin (x 250).



C. Bone marrow 7 days after irradiation and administration of culture medium. Aplasia of the bone marrow. May-Grünwald-Giemsa (x 1000). **D.** Bone marrow 28 days after irradiation and administration of culture medium. The proliferation of haemopoietic cells, namely the myeloid series. May-Grünwald-Giemsa (x 1000).



E. Red pulp of the spleen 7 days after irradiation and administration of culture medium. The number of erythropoietic and granulopoietic cells is decreased. Haematoxilin-eosin (x 450). **G.** Red pulp of the spleen 21 days after irradiation and administration of culture medium. Note the significant proliferation of erythropoietic and granulopoietic cells. Haematoxilin-eosin (x 450).