Conference on Bone Marrow Transplantation and Irradiation Protection

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Chairman: C. C CONGDON

INTRODUCTION

The protection and recovery conferences held during the past few years were organized to bring laboratory workers in these fields into close contact with those clinical researchers interested in the treatment of whole-body radiation injury or similar chemical injury situations involving severe damage to bone marrow, lymphatic tissues and the gastrointestinal tract.

One of our major efforts each year in maintaining frequent communication between the two groups of investigators has been to arrange an evening discussion at the annual meeting of the Federation of American Societies for Experimental Biology. The late L. M. Tocantins organized and served as chairman of these Federation sessions from their inception.

The present series of abstracts is a result of the continuation of Tocantins's plan to offer the investigator the opportunity to present briefly his most recent and cogent research results to two specially interested groups.

I. Work on Small Animals

PROTECTION OF RHESUS MONKEYS AGAINST LETHAL DOSES OF X-RADIATION

R. C. Wolf, J. R. Allen and J. L. Van Lancker. From the Primate Center, University of Wisconsin, Madison, Wis.

Lethal doses of x-radiation (800 r) were given to *Macaca mulatta* monkeys, injected with AET and cysteine prior to exposure. The 30-day survival was 81 per cent for the animals injected with sulfhydryl compounds, and 21 per cent for unprotected animals. Hematologic, histologic, and biochemical studies of bone marrow and spleen demonstrated marked cellular regeneration within 30 days after irradiation in the protected monkeys. The hemoglobin of *Macaca mulatta* has the electrophoretic mobility characteristic of human hemoglobin A; in contrast, the mobility of *Macaca nemestrina* hemoglobin is distinctly greater. No variation between either group can be detected. This difference in the electrophoretic pattern of *Macaca mulatta* and *Macaca nemestrina* hemoglobin is being used to study the development of bone marrow transplants in monkeys receiving 800 r total-body doses of x-irradiation.

SOME OBSERVATIONS ON THE HISTOLOGY OF LETHALLY IRRADIATED GUINEA PIGS PROTECTED WITH NUCLEATED ELEMENTS OF HOMOLOGOUS BLOOD

T. I. Malinin, V. P. Perry and M. F. Dolan. From the National Naval Medical Center, Bethesda, Md.

This investigation presents a study of histological alteration in lethally irradiated (550 r) guinea pigs protected with nucleated elements of pooled homologous blood (1×10^8 cells). All control guinea pigs which did not receive cells died within 16 days. These demonstrated typical changes associated with ionizing radiation. The bone marrow from sternum, rib, vertebra and femur was always hypocellular with scat-

tered degenerated hemopoietic cells found at random. The lymphatic tissue throughout the body was always absent and most animals succumbed to infection. Animals which survived lethal irradiation received pooled homologous nucleated elements from peripheral blood (1 x 10^8 cells). Animals were killed periodically following 30-day survival for histologic study. Blood counts were made at time of irradiation, at time of hematopoietic depression, and immediately prior to killing. Histologically, the bone marrow of protected animals was hypercellular and contained many immature hematopoietic cells. Megakaryocytes were always abundant. The spleen and lymph nodes showed actively proliferating germinal centers surrounded by lymphocytes. The lungs of many animals contained foci of large immature, reticuloendothelial-like cells. An attempt will be made to correlate histologic changes observed in the bone marrow and throughout the reticuloendothelial system with changes seen in peripheral blood.

Colony-forming Ability of Hematopoietic Cells

H. Yamamoto. From the Veterans Administration Hospital, East Orange, N. J., and Seton Hall College of Medicine, Jersey City, N. J.

The colonal-growth potential of freshly isolated bone marrow cells with no previous in vitro history was studied with single-cell platings. Plating efficiencies were determined with and without feeder layers of fibroblast-like cells (FLC) derived from bone marrow. Present data indicate plating efficiencies with feeder layers to be 5 to 10 orders of magnitude greater than those without feeder layers. The results indicate that the use of bone marrow FLC feeder layers not only enhances plating efficiencies but also aids in overcoming the initial crises of cell degeneration and provides a system for the establishment and maintenance of bone marrow cells.

Complete Change of Circulating Erythrocytes in WW^v Genetically Anemic Mice Receiving $W+W+$ Normal Bloodforming Tissue

C. C. Lushbaugh and Elizabeth S. Russell. From the Los Alamos Scientific Laboratory, Los Alamos, N. M., and Roscoe B. Jackson Memorial Laboratory, Bar Harbor, M.

The macrocytic anemia characterizing WW^v mice may be completely cured by i.p. injections of isologous or parent-strain cells from the hematopoietic liver of 15-day normal $w+w+$ fetuses (Bernstein and Russell: Proc. Soc. Exper. Biol. & Med. 101:769, 1959; Russell; Fed. Proc. 19:573, 1960). After C57BL/6-$w+w+$ fetal hematopoietic cells, capable of making electrophoretically "single" hemoglobin, were given to (WB/Re x C57BL/6)F_1-WW^v mice, with electrophoretically "diffuse" hemoglobin, the hemoglobin pattern of 17 hosts changed to the single type as their number of erythrocytes increased and MCV decreased to normal values. Three injected WW^v mice remained anemic with diffuse hemoglobin. Thus the anemia "cure" resulted from functioning of implanted donor hematopoietic cells rather than from transformation of host hematopoietic cells. Do small numbers of macrocytic WW^v erythrocytes continue to be formed along with very large numbers of normocytic $w+w+$ erythrocytes in chimeric implanted $w+w+/WW^v$ individuals? The size distribution of macrocytic erythrocytes from WW^v anemic mice and of normocytic erythrocytes from their $w+w+$ normal litter mates were determined at Los Alamos, in a specially modified Coulter counter with added 100-channel analyzer. Blood from both anemic and normal mice showed a bimodal curve of erythrocyte volumes with a small peak of relatively large cells, and a much higher peak of relatively small cells. Both of these peaks were displaced sharply to the right (macrocytic) in blood from WW^v anemic mice. These curves of erythrocyte size distribution were compared to similar curves obtained from four WW^v mice 90 days after injection of $w+w+$ hematopoietic cells. The WW^v stem cells and erythroid precursors should not have been destroyed by any experimental procedure used. The curve for one injected WW^v mouse which

was still anemic could not be distinguished from those obtained with untreated WW^v mice. However, the curves for three "cured" anemics could be superimposed exactly upon the curves for untreated $w+w+$ mice. These findings imply that practically all of the circulating erythrocytes in successful chimeric $w+w+/WW^v$ mice come from the implanted $w+w+$ hematopoietic cells.

II. Separation of Marrow Elements

Attempts to Separate Cellular Elements of Mouse Bone Marrow by Differential Centrifugation

I. L. Stoloff and A. J. Weiss. From Jefferson Medical College, Philadelphia, Pa.

Attempts have been made to fractionate the cellular elements of mouse bone marrow and to define their physiologic role by observing the ability to protect lethally irradiated isologous hosts. Method of fractionation was by density-gradient centrifugation in a sucrose medium using 1.5 ml. volumes of sucrose varying in concentration from 17 to 50 per cent. Centrifuging was performed in siliconized centrifuge tubes, 15 X 100 mm., at speeds of 650 to 750 rpm for 12 minutes. Each sucrose layer was separated, sufficient material removed for cell counts and morphologic staining, and equal numbers of cells from each layer injected into small groups of irradiated isologous hosts. Layering of marrow cells was achieved by this technic. Morphologic examination of cells in each of the layers suggested that larger mononuclear cells tended to settle in the denser sucrose layers, but pure lines of cells were not obtained. Viability of separated marrow cells was demonstrated by the protection for 30 days of irradiated hosts injected with equal numbers of cells from all except the densest sucrose layers. The osmotic effect of high sucrose concentrations was not destructive to whole bone marrow cells, for such material retained its capacity to protect irradiated hosts. Marrow cells from certain of the lighter sucrose layers more effectively protected irradiated hosts in equal numbers than those from other layers. Whole bone marrow was not superior to cells from these more effective layers. Cells from other layers protected irradiated hosts for 30 days after irradiation, but in the 4th or 5th week, many of the animals lost weight and died. The clinical appearance superficially resembled that which has been described in mice with "homologous disease."

Cellular Fractions from Stable-Flow Free Boundary Sedimentation of Rat Bone Marrow

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In preparation for evaluating the proposition that transplantability of bone marrow cells varies according to cell type, fractionation studies of whole rat marrow have been undertaken. The method chosen was stable-flow free boundary (STAFLO) sedimentation as suggested by previously reported studies using STAFLO electrophoresis (Mel: J. Chem. Phys. 31:559, 1959; Science 132: 1255, 1960). In this method, marrow from one rat is mechanically brought into a suspension of single cells in isotonic saline and introduced into the top of the horizontally flowing system. Sedimentation (under 1 g only) occurs vertically at right angles to the flow. During the 32-minute steady-state throughput time, cells follow separate paths according to their sedimentation rates and exit through corresponding outlets, primarily into 7 of the 12 collection tubes. The necessary flow stability depends upon hydrodynamic principles in free solution, including self-balancing feedback, rather than on use of any supporting medium. Particularly noteworthy is that the non-nucleated cells sediment less rapidly than the nucleated cells. Peaks appear in descending order from top to bottom as follows: erythrocytes; erythroblasts (often a second, lower peak also appears); immatures; myelocytes and

granulocytes. Major changes in composition relative to the starting marrow are effected. For example, fraction No. 5 contains as much as 46 per cent immature cells compared with 15 per cent in the starting sample. In the experiments reported, sample flowed at the rate of 2 x 10^6 cells/minute.

III. Antibodies-Immune Responses

Comparative Action of Certain Cytotoxic Agents on the Primary Antibody Response in Man and Rodents

G. W. Santos and A. H. Owens, Jr. From the Department of Medicine, Johns Hopkins Hospital, and Oncology Service, Baltimore City Hospitals, Baltimore, Md.

Recently, studies of the chemical suppression of the immune response have been actively pursued in many laboratories and even more recently also in the clinical setting. It would seem, therefore, that a comparison of the action of several agents on the primary immune response in man and rodents would be of current interest. BDF_1 female mice, aged 10 to 12 weeks, and female Sprague-Dawley rats, aged 10 to 12 weeks, comprised the rodent species studied. The animals were immunized with one injection of 1 per cent sheep red cells by the intraperitoneal route 4 hours after administration of a cytotoxic agent. The individual animals were bled and titers performed in a manner quite analogous to that described previously (Fed. Proc. 21:25, 1962). Patients with untreated metastatic cancer were immunized with Vi antigen or tularemia vaccine 4 hours after initial drug dosage. Details of this clinical study were described previously (Ann. New York Acad. Sc., in press). Single doses of mechlorethamine, 6 mg./Kg. i.p. for mice, 0.5 mg./Kg. i.p. for rats, and 0.8 mg./Kg. i.v. for man, had little effect on antibody production as compared to controls On the other hand, single doses of cytoxan, 300 mg./Kg. i.p. for the mouse, 100 mg./Kg. i.p. for the rat; had a profound effect on delaying induction time and depressing peak titer in the rodents. The four patients treated with Cytoxan, 7.5 mg./Kg. i.v. for seven consecutive doses, demonstrated no measurable antibody production during the 28 days of observation. It is interesting to note that in the rat and the mouse, 500 r whole-body x-ray had less effect than Cytoxan on antibody production, even though the toxicity was comparable as judged by survival studies and white count depression. A tentative summary of other studies from this laboratory would suggest the following comparisons of various agents on the primary antibody response in the mouse, rat and man.

	Mouse	Rat	Man
Cyclophosphamide	+++	+++	+++
Mechlorethamine	−	−	−
Methotrexate	++	+++	+++
5-Fluorouracil	−	−	++
6-Mercaptopurine	+	+	+++
X-ray	++	++	?

−, no effect; +++, maximum effect.

IV. Biochemical Observations

Study of Nitrogen Balance in Irradiated Mice Given Foreign Bone Marrow

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Lethally irradiated mice treated with foreign bone marrow frequently show a lethal secondary disease process as soon as they recover from the acute radiation syndrome. The unusual body weight loss and the failure of hair to grow in the foreign marrow

chimera suggest the mice are suffering from a metabolic starvation. In the present experiments, nitrogen intake and excretion are measured in normal, x-ray control, isologous marrow, and foreign marrow-treated groups. Nitrogen intake and excretion are about the same in both marrow-injected groups. The isologous and homologous animals are able to maintain a positive nitrogen balance for the first 30 to 60 days after x-ray exposure. Normal animals maintain a positive nitrogen balance throughout the experiments. The x-irradiated animals not given bone marrow show progressively negative nitrogen balance until death. The homologous marrow-treated animals, while maintaining positive nitrogen balance, lose some weight. It is possible that the nitrogen within the body of the homologous animal is retained in abnormal compartments which are not present in the isologous animal. Antigen-antibody complexes, bacterial foci, and parasitic organisms might constitute such abnormal compartments. Our data show no difference between nitrogen excreted in isologous and homologous animals. These data agree with conclusions reached in earlier experiments.

RNA and DNA in Liver of Irradiation Chimeras

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RNA-phosphorous concentration was increased in liver of mice 9 and 14 days after 950 r total-body x-irradiation and 40 x 10^6 homologous bone marrow cells. The concentration in similarly irradiated animals given isologous cells was the same as unirradiated controls. When the RNA-phosphorus content was calculated (mg. per liver) from the concentration, the total RNA of liver of homologous chimeras was found to be increased on day 9 and 14; 1.15 and 1.08 mg. respectively, compared to 0.87 mg. at 7 days after treatment and 0.87 mg. in unirradiated controls. All x-ray controls died before the 14th day but on day 9 there was increased RNA-phosphorus concentration in liver. When content was calculated from the concentration, however, there was no increase: 0.84 mg. at 9 days and 0.85 at 7 days.

Further Studies of the Effects of AET and Cysteamine on Murine Bone Marrow Nucleic Acid Metabolism in Vitro

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A previous report from this laboratory had shown that AET and cysteamine administered 15 or 30 minutes before addition of labeled thymidine caused a marked inhibition of incorporation of the isotope into the DNA of mouse bone marrow cells in vitro. Recent experiments on uridine incorporation into RNA have shown that AET and cysteamine administered under similar conditions have only a slight effect on RNA synthesis. Thus, at concentrations sufficient for in vitro radioprotection of bone marrow cells, administration of these chemicals results in a selective inhibition of DNA synthesis as compared to RNA synthesis.

Screening for Radiation Modifiers

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The Cancer Chemotherapy National Service Center is undertaking a program to search for drugs which will modify responses to ionizing radiation. Materials will be sought which may either enhance or protect from radiation effects. Emphasis will be placed on types of compounds other than those already known to produce such effects. Routine screening will be started in the near future, utilizing bacterial and cell culture systems, as well as mice.

V. Tissue Preservation

Hematologic Recovery of Irradiated Mice Protected with Frozen Marrow

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As a first step in evaluating the possible damage induced by the accepted technics of freeze-thawing on bone marrow cells and with the hope on gaining some insight into the kinetics of the surviving cells within the animal body, the hemopoietic recovery of lethally x-radiated mice was studied following infusion with preserved bone marrow. C3H mice given 900 r of x-radiation served as recipients. A/Jax animals were used as homologous donors. Protection dose was 10 x 10^6 cells. Freezing of marrow cells was carried out at a slow rate in a buffered EDTA medium with 10 per cent dimethyl sulfoxide (DMSO). Thawing was rapid. The erythropoietic recovery was expressed as the per cent value of the 24-hour Fe^{59} utilization and was plotted against the post-protection time. The recovery curves obtained with the fresh and frozen marrow appeared parallel but frozen marrow showed a lag period of 24 hours. Beyond day 10 the 24-hour iron utilization in both groups returned and continud to be within normal range. In homologous combinations the results were similar until day 25 when the first symptoms of secondary disease appeared. Leukopoietic recovery was measured by WBC counts in the peripheral blood. Recovery started at day 5 and normal values were reached 10 days later. The lag period, however, shown by the frozen cells was in the proximity of 72 hours. In homologous combinations the results were the same but the study of the peripheral blood smears revealed the forthcoming secondary disease by the absence of lymphatic cells. The results leave little doubt that actual implantation of the frozen marrow has taken place. The faster recovery of the erythroid elements is probably a reflection of the faster maturation cycle of the red cell forming units. Interesting is the observed lag period in the recovery with frozen bone marrow cells. It might represent a selective elimination of the more mature bone marrow elements during the preservation procedures, placing the frozen marrow one generation behind the fresh. If this is so, recovery of the surviving elements of the frozen marrow appears to be prompt and, in fact, not different from that of the fresh. Another explanation for the delay is a possible deprivation by freezing of functional intracellular units, e.g., mitochondria, which need to be replaced before any mitotic cycle can be initiated in the preserved bone marrow samples.

Studies on Freeze Drying Animals of the Lower Orders

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The search for the achievement of a state of latent life in cells and animals has continued since the time of Leeuwenhoek, Spallanzani and Needham, who watched the return to the actively moving living state of dried preparations of rotifers, tardigrades and nematodes. During the 1920's and 1930's it was demonstrated that such metazoans in their anabiotic (latent life) phase were undergoing minimal metabolic activities. Only when they were stored at refrigerator temperatures or much lower would this process be halted and complete suspended animation obtained (Becquerel: Compt. rend. (Paris) 231:261, 1950). In the anabiotic phase the animal, often in the larval stage, looks like a collapsed version of itself and returns to life directly from this form which is highly resistant to heat, additional drying, cold, noxious gases and sterilizing solutions. Obviously such cells have particular biochemical and physiologic properties which enable them to withstand drying and subsequent storage. A study of these cells may give valuable clues as to the technics which would aid us in the preservation of mammalian tissues in a stable, relatively temperature-*independent*

form. To find out how resistant to drying and freezing the animals actually are, an experimental freeze dryer was built after the pattern of Professor R. I. N. Greaves' machine [Preservation of living cells by freezing and drying. *In* Progrès Récentes en Lyophilisation, edited by L. Rey. Paris, Hermann, 1962]. This device enabled ultrarapid or very slow freeze-drying, with observation and recording of the temperature and vacuum at any instant. Problems were that the themocouple necessarily measured the temperature of the organism mixture, not of the individual organisms; and the heat input to the process is uncertain as evaporation proceeds in a chamber at a variable room temperature. The nematode *Panagrellus redivivus* is not known to be resistant to freezing or drying in nature. On experimentation, the worm suspended in distilled water did not stand an ultrarapid freezing, freeze-drying, but did stand the process where they were gradually desiccated to the freezing point, from which normal freeze-drying proceeded. Optimal results are obtained by drying from −4 C., the pre-freezing cooled state. The youngest larvae are the principal survivors, many being found alive within the dried and dead female. This may explain how the worm persists in adversity. Whereas the *Euglena gracilis* work (Greaves, *op. cit.*) indicated the protozoan was dead at the end of a drying curve, the worms were not; they survived drying to 20 C. and to 2 μ Hg vacuum. It can be noted that the worm has a fairly impenetrable cuticle; also the larvae are composed of approximately the same number of cells as the adult, but on histochemical examination the cells are smaller and the whole larvae very dense. Resistance of free living cells may be a function of unusually small size and protoplasmic density. A movie of a rotifer rehydrating from the anabiotic phase showed its marked swelling as water was absorbed and before motion began. Current studies with a worm naturally resistant to desiccation, *Ditylenchus myceliophagus,* illustrates the same thing. Therefore it would seem that a mechanism of resistance in nature, besides cell size, colloidal density, and cuticles is one of dehydration—but before freezing, not after.

In the 1950's tumor cells were successfully dried in 20 per cent glucose [Passey and Dmochowski; *In* Freezing and Drying Symposium, 1951 Institute of Biology, edited by R. J. C. Hams. New York, Hafner Publ. Co., 1952, p 63]. Total dryness was probably not achieved; further experiments showed that the cells did not survive total drying after freezing. Since then, utter dryness of products has been the aim, since this confers maximum stability. For fundamental science this should be. But cysts and anabiotic forms may retain considerable moisture. The stability of products with a fair degree of water content has not been investigated by modern means.

The Recovery of Lethally Irradiated Dogs Given Infusions of Autologous Leukocytes Preserved at −80 C. in 10 Per Cent Dimethyl Sulfoxide

J. A. Cavins, S. C. Scheer, E. D. Thomas and J. W. Ferrebee. From The Mary Imogene Bassett Hospital, Cooperstown, N. Y.

Nine mongrel dogs were exposed to 1200 r air-dose total-body iradiation continuously at 4 r per minute from paired Co^{60} sources. They were then given infusions of 2.12 to 20 billion autologous leukocytes that had been previously obtained from their individual peripheral bloods and stored at −80 C. in 10 per cent dimethyl sulfoxide. The return of peripheral white cell and platelet counts was slower by 2 to 3 weeks than that observed in dogs given similar amounts of bone marrow. Three dogs given 2.12 to 5 billion cells died on post-irradiation days 14 to 16. One dog given 6.04 billion cells died on day 28 but showed evidence of marrow regeneration. Three of five given 9 to 20 billion cells are alive and well 50 to 150 days after their irradiation exposures. Their return of white counts and platelets was proportionately more rapid than that seen in the dogs given smaller numbers of cells. The two that died after infusions of 20 billion received their cells in split dosage over a period of 36 hours.