Conference on Bone Marrow Transplantation and Chemical Protection in Large Animals and Man

Abstracts of papers presented at the Veterans Administration Hospital, Long Beach, California, on June 1 and 2, 1962
C. C. Congdon and N. B. Kurnick, Co-chairmen

The objective of the meeting was to review the present work on chemical protection and marrow transplantation in large animals and man and to provide an opportunity to discuss the new field of white blood cell transfusion and transplantation in man and experimental animals. Special attention was devoted to preservation of autologous marrow and of white blood cells. Under chemical protection, toxicity of AET in man and its use in protecting monkeys against radiation were discussed. The toxicity of AET has greatly limited clinical testing of the drug.

Recovery of Lethally Irradiated Dogs Following Infusion of Autologous Marrow Stored at Low Temperature in Dimethyl Sulfoxide
J. A. Cacins, S. Kasakura, E. D. Thomas and J. W. Ferreebey, Cooperstown, N. Y.

Sixteen dogs were exposed to 1200 r of Co$^{60}$ radiation continuously at 4 to 6 r/minute. They were then given infusions of 1 to 5 billion autologous marrow cells that had been removed prior to their radiation exposure and preserved at $-80\,\text{C.}$ in dimethyl sulfoxide (DMSO). Nine dogs received marrow preserved in 10 per cent dimethyl sulfoxide. Eight survived and did well. One made a normal recovery but died of chronic exocrine pancreatic insufficiency 5 months post-radiation. Restoration of hematopoiesis was prompt and complete in each. Three dogs were given marrow preserved in 5 per cent DMSO. One died. Three were given marrow preserved in 15 per cent. Two died. Deaths were associated with slow restoration of hematopoiesis.

The Use of Frozen Autologous Bone Marrow for the Protection of Lethally Irradiated Dogs

Thirty mongrel dogs received frozen autologous bone marrow 24 hours after lethal total-body radiation. The bone marrow was obtained by multiple aspirations from the long bones and ribs. The freezing technique used was essentially that described by Ferreebey and Thomas. Both glycerol and dimethyl sulfoxide (DMSO) were used as protective agents for the freezing of marrow in a final concentration of 10 per cent. The marrow specimens were stored for 10 days at $-80\,\text{C.}$ All dogs received 600 r total-body radiation in a Co$^{60}$ irradiator with a single exposure of 30 minutes which was fatal for eight control dogs. Eleven dogs survived 30 days or longer. There were no survivors in 13 dogs receiving less than 2.5 billion cells. Sixty-four per cent of the dogs survived that received more than this number of cells. All dogs survived which had a pre-freezing count of over 5 billion cells. DMSO as a protective agent for the freezing of marrow compared favorably with glycerol in terms of animal survival. Pre-freezing cell counts showed a satisfactory correlation with survival.

Evaluation of Liquid Nitrogen Preserved Bone Marrow Cells by Metabolic Techniques
A. W. Ronce and J. Felliq, Tonawanda, N. Y.

The viability of bone marrow cells preserved at liquid nitrogen temperatures ($-196\,\text{C.}$) was evaluated by measuring some of their metabolic reactions. Bone
marrow cells from the femurs of young rabbits were frozen in the vapor of liquid nitrogen in the presence of various intracellular and extracellular additives. Bone marrow cells were assayed by several technics including vital staining (2-C14), glycine incorporation, and cellular respiration. Vital staining was carried out with both eosin and trypan blue. A more stringent criterion of viability was that of incorporation of (2-C14) glycine into the TCA insoluble fraction of bone marrow cells. Protective additives had to be removed prior to the incorporation assay because they inhibited uptake of the metabolites. Good correlation of survival between vital staining assay and glycine incorporation was found when cells were frozen in an intracellular additive such as dimethyl sulfoxide. However, cells frozen in the presence of polymeric extracellular additives such as polyvinylpyrrolidone, dextran, bovine serum albumin, and homologous serum appeared viable by vital staining, but were essentially inactive metabolically. A low molecular weight extracellular additive, mannitol, was also found to preserve cell viability by both assays. Another metabolic criterion of viability, cellular respiration, was measured by standard Warburg manometric technics. Viability of bone marrow cells frozen in the additives described above and assayed by respiration were found to correlate well with viabilities as assayed by glycine incorporation. Based on irradiation studies reported in the literature, the metabolic methods of evaluation, glycine incorporation and cell respiration showed a greater correlation as criteria of viability than did vital staining.

**The Viability of Human Marrow Preserved in Dimethyl Sulfoxide**

H. M. Pyle and H. Boyer, Jamaica Plain, and Boston, Mass.

Viability of human stored marrow is difficult to quantitate on the basis of clinical response. In this presentation, a procedure for preserving autologous human bone marrow and a method of determining the post-thawed viability of the marrow are presented. The bone marrow is aspirated from the posterior ileum and diluted with equal volumes of the following preservation media: dimethyl sulfoxide (DMSO), 20 per cent; glucose, 1 per cent; Eagle’s medium, adjusted to pH 7.8; heparin, 60 units ml.

Approximately 250 ml of marrow tissue is aspirated in aliquots of 10-15 ml, each from multiple sites. The marrow is slowly frozen in refrigerated boxes to −84 C. over a 4½-hour period and maintained at this temperature for the duration of storage. When the marrow is needed for reinfusion, it is thawed at room temperature, approximately 20 minutes, centrifuged at 2000 rpm for 15 minutes, and the supernatant fat and preservation media removed. The sedimented cells are resuspended in an isotonic 5 per cent albumin solution with 400 mg. per cent added. An aliquot is removed for viability studies, and the remaining is administered through an intravenous transfusion set with nylon filter. To determine the viability of the stored marrow, the cells are incubated with 0.5 µc. H3-thymidine/ml. in a medium of 5 per cent albumin, 10 per cent serum, and 4 per cent fresh recipient red cells. The incubation period is 3 hours at a temperature of 37 C. The number of cells incorporating H3-thymidine into DNA and the degree of granulation per cell is compared to the nonfrozen counts and this is taken as a semiquantitative viability index.

Results show nearly the same viability of frozen marrow as fresh aliquots. Viability is maintained after storage for as long as 9 months, the oldest marrow specimens currently available. Information on the degree of viability of the older marrows is not available yet. The value of the recipient red cells to the incubation mixture seems to be as a source of ATP. DMSO inhibits or destroys the ATP of nucleated cells; physiologic amounts of ATP can be substituted for the fresh recipient red cells.

**Clinical Effects of Autologous Marrow Preserved in DMSO**

H. M. Pyle and Hilda Boyer, Boston, Mass.

Bone marrow for storage purposes was aspirated from 25 patients selected on the basis of potential tumor sensitivity to chemotherapy. Five patients received their bone marrow back after periods of storage ranging from 5 days to 6 weeks, and two addi-

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tional patients received supernatant dimethyl sulfoxide (DMSO) solutions. Despite the significant toxic effects of the chemotherapy, each patient had normal peripheral counts within 2½ weeks of bone marrow reinfusions. The patients had several systematic effects from chemotherapy, but no immediate or long-term irreversible toxic effects of DMSO. Each patient received a DMSO bone marrow solution with a concentration of DMSO less than 1.5 per cent (largest total Gm. DMSO administered was 7). Fifty per cent of the DMSO is cleared through the kidneys; the remaining is exhaled through the lungs over a 12-hour period. No change in renal or liver function studies was noted in follow-ups up to 1 year.

INFUSION OF MARROW IN THE MOUSE AND DOG AFTER THIO-TEPA

H. L. Locht, Jr., S. Kasakura, E. D. Thomas and J. W. Ferrebee, Cooperstown, N. Y.

Studies in animals to date indicate that isogeneic marrow is of benefit in reducing lethality caused by some chemotherapeutic agents but not by others. This report describes the use of fresh isologous marrow in the BDF₁ mouse and the use of autologous marrow preserved in dimethyl sulfoxide in the dog in attempts to modify mortality resulting from the administration of thio-TEPA. A single intravenous dose of 0.7 mg. thio-TEPA in the mouse resulted in survival of 9 to 42 control animals; isologous marrow allowed 17 of 45 to survive. Isologous marrow after higher single doses and after the chronic administration of thio-TEPA in a variety of dose schedules did not improve survival. Stored autologous marrow in the dog improved survival at single intravenous doses of 5.0 mg./Kg. and 6.0 mg./Kg. but not at 8.0 mg./Kg. At 4.0 mg./Kg. thio-TEPA, all animals survived without marrow treatment. It is concluded that isogeneic marrow is of value against a narrow dose range of a chemotherapeutic agent that causes mortality primarily by marrow aplasia. At lower doses of such agents, autogenous marrow recovery occurs. At higher doses, lethality results from causes other than marrow depression, and cannot be prevented by marrow infusions. A careful evaluation of dose, dose schedule, marrow toxicity, and tumor toxicity will be required for each agent employed.

HOMOLOGOUS MARROW TRANSPLANTATION IN X-IRRADIATED DOGS TREATED WITH 6-MERCAPTOPURINE AND URETHANE


Experience has now been accumulated with homologous marrow transplantation in 15 mongrel dogs (Group 1) treated with 6-mercaptopurine (6-MP) or urethane prior to x-irradiation (900 r, delivered at a dose rate of 15 4/minute). The marrow dose was 7-11 x 10⁶ cells, given on the day following irradiation; urethane (175 or 350 mg./Kg.) and 6-MP (12.5 or 25 mg./Kg.) were administered at three or four daily intervals during the week prior to irradiation. Mean survival time (MST) in Group 1 was 23 days, with a maximum of 63 days. MST in a group of dogs (Group 2) given homologous marrow after 900 r, but not treated with the chemicals, was 10 days. Group 1 animals characteristically showed good recovery of peripheral blood granulocyte count by 8-10 days, together with objective evidence of marrow "take"; recovery of mononuclear cell count was not observed, except in the single case which survived for 63 days. None of the Group 2 animals showed any rise in the peripheral blood count after initial depression, and all died with marrow aplasia. Secondary disease in Group 1 dogs was characterized by anorexia, weight loss, infection, and lymphoid tissue aplasia in all the animals; in some of the animals skin atrophy, liver lesions, jaundice and anemia were seen. The marrow showed active hematopoiesis and from moderate to good cellularity in most of the group 1 animals, although megakaryocyte activity was deficient in some. Pneumonia and pulmonary edema were found in many of the dogs at autopsy. It is evident that the use of these antimetabolites permits the successful transplantation of homologous marrow in dogs at a dose of x-radiation.
(900 r) which, by itself, is insufficient. Therefore, these compounds are additive to x-radiation with respect to suppressing the homograft reaction in dogs, as well as in mice (Cole and Davis; Science 135:792, 1962).

**Experiences with Infusion of Autologous and Isologous Bone Marrow in Man**

**N. B. Kurnick,** Long Beach, and Los Angeles, Calif.

Our experience with 101 bone marrow collections and storage by freezing in 15 per cent glycerol solution, according to the Lovelock curve, and infusion of 23 autologous and 2 isologous bone marrows has led us to the conclusion that repopulation of radiotherapeutically depressed bone marrow function in man can be achieved by autologous or isologous bone marrow infusion. The marrow was thawed slowly and diluted with one-half volume 35 per cent glucose, infused intravenously without other manipulation or filtration. Asymptomatic hemoglobinuria occurs due to lysis of the red cells contained in the bone marrow aspirates. Evidence for repopulation consists of the much more rapid return to normal of the formed elements of the peripheral blood in infused patients compared to noninfused controls, early reticulocytosis, hyperplasia of the irradiated bone marrow sites in 3 to 5 weeks following infusion compared to long-term hypoplasia in the controls, and the repopulation of specific bone marrow sites which have suffered long-standing hypoplasia from past local radiotherapy following generalized bone marrow depletion and infusion of autologous marrow. In the case of the isologous marrow infusions, bone marrow hyperplasia followed in a patient who had been suffering from severe pancytopenia for over a year due to intensive radio- and chemotherapy. We anticipate that improved results will be obtainable by more intensive and extensive radiotherapy followed by bone marrow infusion for radiosensitive malignancies such as seminoma and lymphoma. We suggest that cells capable of repopulating the bone marrow circulate in man in the absence of leukemia only when bone marrow is infused intravenously, unlike the situation in the mouse. In man, intensive radiotherapy of local sites is usually followed by long-term hypoplasia. In contrast, mice exposed to 1000 r daily for 10 days (total 10,000 r) showed rapid fall in the cell number in marrow of the irradiated leg to 6 per cent of normal 1 week after the completion of radiation, with prompt return to 45 per cent 1 week later, and 72 per cent the following week. The opposite limb, which presumably provided some of the bone marrow for the repopulation, fell to 45 per cent of normal cell count 1 week after the completion of radiation, and returned to 85 per cent of normal 2 weeks after that. A dose of 10,000 r, administered in the course of 2 weeks to one extremity in man, would have been expected to produce permanent aplasia of the irradiated bone marrow.

**Antibody Formation by Transplanted Bone Marrow, Spleen, Thymus and Lymph Node Cells in Isologous, Homologous and Heterologous Recipients**

**R. D. Stoner and V. P. Bond,** Upton, N. Y.

It has been postulated that wasting disease and delayed deaths in irradiated animals which are bone-marrow protected may result from an immunologic potential of marrow cells following their transplantation to irradiated animals. Isologous, homologous, and heterologous bone marrow cells were obtained from mouse and rat donors previously immunized to tetanus toxoid. Immunologic competence was determined by the ability to elicit antitoxin responses from the transplanted cells following an antigenic stimulus of fluid tetanus toxoid. The appearance of antitoxin in the sera of the recipients indicated that antibody was produced by the transplanted cells since the irradiated recipient mice were unable to produce antibody in response to the soluble antigen. Cell preparations of peripheral leukocytes, spleen, thymus and lymph node tissues were also obtained from the same donor animals to compare antibody responses by bone mar-
row to other lymphoid tissues. Antitoxin formation was elicited from transplanted bone marrow cells in isologous and homologous mouse-to-mouse donor-host combinations. We were unable to demonstrate antitoxin formation by bone marrow cells in heterologous rat-to-mouse combinations. These findings show immunologic competence of bone marrow when donor and host are of the same species, indicating that elements of the marrow have the capacity to respond to antigenic stimulus, with an inferred potential to respond to antigens present in the host resulting in wasting disease and delayed deaths. Failure to elicit antibody from bone marrow cells in rat-to-mouse combinations may result from poor survival of donor cells or massive antigenic challenge of the donor cells by host antigens. Antibody formation was also demonstrated by cell preparations of spleen, thymus, lymph nodes and peripheral leukocytes in mouse-to-mouse combinations. Minimal antibody responses were obtained with spleen and thymus in rat-to-mouse combinations. In a series of experiments in which the same number of cells were transplanted, the order of cell preparations may be arranged as follows in order of high to low titer of antitoxin produced: spleen, lymph node, thymus, and bone marrow. Consistent findings have not been obtained in the ability to elicit antibody formation from transplanted peripheral leukocytes. Differing viability of the various cell preparations may account for this.

ROLE OF AUTOLOGOUS MARROW INFUSION IN THE MANAGEMENT OF HUMAN CANCER

B. H. Feder, Long Beach, Calif.

With the assurance that stored autologous marrow has given us, we have been able to double both the volume irradiated and the rate of irradiation, and to increase somewhat the dose levels attained in the treatment of metastatic seminoma of the testis. Of 55 testicular tumors, 21 have had marrow stored, but we have felt that infusion was necessary in only five of these. It is still too early to evaluate salvage, but increase of volume, rate, and dose, without increased morbidity, may be expected to improve the cure rate in metastatic seminomas. In general, autologous marrow infusion may be indicated for those patients with whom extensive marrow-suppressive radiotherapy and/or chemotherapy is justified. Palliation may thus be enhanced in widely disseminated malignancies of many varieties. The ideal subject, of course, has a radiosensitive and potentially radiocurable neoplasm with a predictable route of spread; and he has a reasonable life expectancy. Few neoplasms meet these conditions as well as testicular seminoma does. Availability of autologous marrow facilitates the management of these neoplasms.

AUTOLOGOUS BONE MARROW TRANSFUSION AFTER CHEMOTHERAPY

Leo M. Meyer, Theodore F. Fliedner and Eugene P. Cronkite, Oceanside, and Upton, N. Y.

The present study was designed with the purpose of tracing reinfused autologous bone marrow cells in subjects with moderately advanced cancer receiving chemotherapy, using H\(^3\) thymidine-labeled cells. In two instances marrow was labeled in vivo, after which it was withdrawn and preserved in 15 per cent glycerol at -80 C. prior to intravenous administration. Four other patients received autologous marrow which were labeled in vitro. After reinfusion of marrow cells, radioautographs of daily aspirates from bone marrow and concentrates of peripheral leukocytes were prepared and examined. A control group of patients received identical doses of the same drug but no marrow was infused. Time of maximal depression of leukocytes and platelets and recovery to normal levels of both groups were compared. Although occasional labeled cells were found in bone marrow or blood of patients reinfused with their own cells, recovery was attributed to spontaneous regeneration of marrow. There was no significant difference in recovery from leukopenic and thrombocytopenic states.
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COMPARATIVE METHODS FOR STUDYING RADIOPROTECTIVE AGENTS IN IN VIVO AND IN VITRO SYSTEMS


Reports in the literature indicate both positive and negative radioprotection of mice and rats given lethal doses of x-ray or gamma radiation following administration of nucleic acids. Current in vivo studies in our laboratory indicate the degree of mouse survival (following 800 r gamma radiation 30 minutes after an intraperitoneal injection of ribonucleic acid [RNA]) varied, depending upon the source of RNA. Yeast RNA appeared to enhance radiosensitivity, while increased survival was obtained with rat spleen, liver or thymus RNA from moderate to substantial amounts. In vitro studies revealed that a 24-hour preirradiation treatment of an amnion cell line with yeast RNA increased cell survival in proportion to the RNA content added to the medium. Conversely, ribonuclease increased radio-sensitivity in this system. When the preirradiation treatment period was varied from 4 to 24 hours, the protective response was not observed before 8–10 hours with yeast RNA. Some increase in cell survival was recorded at 4–6 hours if an alkaline hydrolysate of yeast RNA was used. Microspectrophotometric measurements of cytoplasmic basophilia suggested that yeast RNA was ingested by the cells in substantial amounts within 4 hours. The delay in a protective response during this period may have been due to digestion and resynthesis into an active radioprotective agent. A strong correlation between RNA synthesis (as seen during depolymerized RNA ingestion) and the radioprotective response seemed to confirm this hypothesis. It was concluded from in vivo and in vitro observations that the effectiveness of RNA as a radioprotective agent may depend upon its specific molecular configuration.

LYMPHATIC TISSUE RECOVERY IN IRRADIATED ANIMALS PROTECTED WITH AET

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How well AET protects the immune mechanism from irradiation is a problem that has been discussed in recent protection and recovery conferences. The idea that AET does not protect the immune system the same as it does the blood-forming tissues is supported by a limited number of experimental transplantation studies using irradiated and AET-protected irradiated tissues. Morphologic studies on the recovery of the lymphatic tissues after AET protection during radiation indicate that recovery is prompt and quite comparable to that in bone marrow and spleen red pulp of the mouse. In an experiment, mice were given 16 mg. of AET orally before 900 r. The thymus, spleen white pulp, and lymph nodes were microscopically examined daily for 30 days and compared to similar tissues in a control group that got 900 r only. The control group could be followed for only 12 days. Recovery of the thymus was most striking in the chemically protected group. The cortex was normal histologically at 6 days. It was extremely atrophic in the control group at the same interval. The spleen white pulp and lymph nodes were extremely atrophic at the 7-day interval, both in AET-protected and control animals. Regeneration of bone marrow was well-developed at 7 days. By day 13 the spleen white pulp showed some recovery, and by day 30 all lymphatic tissues appeared normal. In view of the evidence that the thymus regenerates first in AET-protected mice, it may be that this structure is the source of cells for lymphatic tissue recovery in the spleen and lymph nodes. This would mean that even in the adult mouse the thymus may be the source of a regenerated immune mechanism after radiation damage. Recent evidence suggests that the immune system develops embryologically in the thymus. It might also be suggested in some bone marrow transplantation experiments that the lymphatic tissues are eventually repopulated by the way of the thymus which receives its donor cells from the injected marrow.
ABSTRACTS

BONE MARROW CULTURE

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A preliminary experiment has been carried out with rats to grow isolated hematopoietic tissue in the abdominal cavity. For this purpose, a double-deck diffusion chamber was designed. The bottom is made of a piece of coverslip glass, and the top and middle decks are sheets of millipore membrane. The middle deck separates the chamber into upper and lower compartments. A tiny piece of marrow tissue excised from the femur of a rat was placed in the lower compartment, and minced pieces of epithelial tissue of the urinary bladder were placed in the upper compartment. The chamber was then inserted under the mesentery of a large intestinal loop of another rat, with the top deck facing the ventral side of the animal. For the control experiment, a single-deck chamber with the lower marrow compartment only was used. Three, 7, and 10 days after the operation, microscopic preparations were made from the bottom coverslip and the fluid in the marrow compartment. For staining, the Giemsa method was generally employed. Fresh materials were examined by phase-contrast microscopy, and later, after staining with acridine orange, by fluorescence microscopy. These methods were useful for differentiation of cell types. In the controls, the cellular elements were degenerative 3 days after implantation while specimens from the double-deck experiment appeared different. Many apparently normal myeloid cells of various cell types were observed, some of them in the mitotic phase. Seven days after implantation, cellular growth became more evident in the double-deck experiment. Tissue growing in a thin layer on the coverslip surface showed the organized myeloid structure. The sinusoidal network, the granulocytic series of cells at various stages of modulation, and various sizes of megakaryocytes were observed. The normoblasts at different stages of maturation were relatively scarce in the tissue on the coverslip but abundant in the chamber fluid. Ten days after implantation, marrow tissue growing on the coverslip was well-developed. A technic has been developed which permits growth of organized hematopoietic tissue from a piece of excised bone marrow in a diffusion chamber implanted in the abdominal cavity. Minced urinary epithelium placed near the piece of marrow apparently was responsible for proliferation and differentiation of the hematopoietic cells to form the organized marrow tissue.

COMPARATIVE STUDY OF PRESERVING HUMAN BONE MARROW BY CULTIVATION VERSUS CONVENTIONAL FREEZING PROCESS

L. Jankay, Long Beach, Calif.

To preserve bone marrow by the freezing method, an equal amount of 30 per cent glycerol in Osgood solution is added to the bone marrow aspirate. The freezing is done at the rate of 1 °C per minute. The degree of hemolysis of the bone marrow aspirate depends, to a certain extent, on the length of time of contact of the bone marrow aspirate with the glycerol solution before the freezing process is started. Thus, if the freezing is started immediately after adding the glycerol solution, the hemolysis is much greater than if the glycerol is in contact with the bone marrow aspirate 2 hours before the freezing is started. On the other hand, the glycerol contact time has a reverse effect on the viability of the nucleated cells. However, the end result, regardless of the contact time with the glycerol solution, is massive hemolysis and pyknotic appearance of the nucleated cells after thawing. For cultivation of bone marrow we used our "circulation and dialyzing method." This consists of the following: 10 ml of heparinized (0.02 mg heparin per 1 ml of blood) bone marrow aspirate is placed into a U-shaped dialyzing cellophane tube. Both ends of the cellophane tube are connected alternately (with the aid of a timing device) to the pressure of a 95 per cent air and 5 per cent CO2 gas phase. The alternating pressure keeps the
blood (bone marrow aspirate) in constant motion and supplies it with oxygen. The dialyzing tube is immersed in an outer bag which holds the dialyzing fluid (homologous plasma is used as dialyzing fluid). Samples are taken from the bone marrow culture daily. The test of these samples shows a minimal hemolysis in comparison with the freezing method. The optical density of the supernatant with the freezing method is 0.900 and by the culture method is 0.014. The cultured bone marrow cells show normal morphology and mitotic activity up to 20 days. No pyknosis or spindle-shaped cell formation appears. Cultured rat bone marrow has been injected into irradiated mice. After killing these mice, alkaline phosphatase positive white cells are found. This indicates maintenance of the cultured rat bone marrow cells in the irradiated mice. The "circulating and dialyzing" method offers a new approach to preserve bone marrow cells, whereby hemolysis is minimal and normal morphology of the bone marrow cells is maintained without spindle-shape cell formation.

EFFECTS OF PORT TELETHERAPY ON BONE MARROW


Eight patients receiving "cancerocidal" doses of Co-60 teletherapy were studied for changes in peripheral blood values and bone marrow. Bone marrow in unirradiated control sites before, during, and after irradiation show no morphologic change that can be regarded as an "indirect effect" of the heavy port irradiation. At the site of teletherapy there is the expected panhypoplasia with relative increase in plasma cells, lymphocytes, and "mononuclear" cells. This is usually accompanied by increased hemosiderin at the irradiated site but not generally elsewhere. Although the irradiated marrow tends to be hypocellular for some time, if not permanently, after therapeutic doses, it is noteworthy that proliferative activity does occur. Megakaryocytes, granulocytic precursors, and erythrocytic precursors are identified after 1 and 2 months following doses as high as 6900 r. Marrow cells from these irradiated sites also show some in vitro incorporation of H3-thymidine. Autoradiograms of aspirated marrow cells from irradiated sites are of less uniformly good quality than normal cellular marrows; the labeling is as high as 5–10 per cent of the nucleated cells present, but invariably is less than from non-irradiated sites. Labeled cells in the irradiated sites include spindle-shaped nuclei judged to be fixed tissue elements of the marrow, either fibroblasts or reticuloendothelial cells, monocytic forms, and red cell precursors.

SOME OBSERVATIONS ON THE COLLECTION AND PRESERVATION OF BUFFY COAT

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The combination of helical flow centrifugation and density differential sedimentation has proved effective in the selective separation of nucleated cellular elements of both guinea pig and human peripheral whole blood. The technic employs the use of the International Centrifuge Helical Flow Unit Model 2303 and the addition of a dextrose solution to peripheral whole blood. During centrifugation, a solution under the effect of gravity is passed through a coiled tubing. The density differentials of the dextrose fluid medium, the red blood cells, and nucleated cells force the heavier red cells to the wall of the tubing, permitting the free flow of the nucleated cell-enriched solution. The use of helical flow centrifugation simplifies the problem of nucleated cell separation from whole peripheral blood and permits the quantitative yield of apparently uninjured cells. Following the addition of dimethyl sulfoxide, and subsequent freezing and storage at liquid nitrogen temperatures (−196 C.), cellular populations have been recovered which have demonstrated eosin exclusion rates of 65 per cent for the guinea pig leukocytes and 80 per cent for human leukocytes. Bioassay in the irradiated guinea pig is incomplete at this time.
IMMUNOLOGICAL OBSERVATIONS WITH LEUKOCYTES PRESERVED IN LIQUID NITROGEN

E. Cohen, Arthur W. Rowe and J. Gorczyca, Buffalo, and Tonawanda, N. Y.

Human chronic lymphocytic leukocytes were preserved in liquid nitrogen. Antigenic properties were retained as evidenced by leukoagglutination by human sera containing leukoagglutinins. Sera of normal subjects did not agglutinate preserved or fresh leukocytes. The lymphocytic leukemic leukocytes were harvested from heparinized blood by dextran sedimentation and washed with buffered saline. The leukocyte concentration was adjusted to 20,000 cells/mm.³ and suspended in 15 per cent dimethyl sulfoxide (DMSO) solution in saline. The leukocyte suspensions were frozen at rates varying from approximately 0.03 C./second to 30 C./second and stored for various periods of time at liquid nitrogen temperature (−196 C.). Rapid thawing was accomplished by gentle agitation in a 37 C. water bath. After thawing it was found advantageous to reduce the DMSO concentration by gradual dilution with saline. The leukoagglutination test was done by mixing equal volumes of test sera with the leukocyte suspension containing 20,000 cells/mm.³ and incubating for 1 hour at 37.5 C. Mixtures were examined microscopically for leukoagglutination and scored from 1+ to 4+. Titers and scoring indicated that sera containing leukoagglutinins consistently agglutinated preserved or fresh cells. Non-specific (false) agglutination was not observed with leukocytes tested with undiluted normal sera. Only the slower cooling rates resulted in antigenically active cells. These results indicate the feasibility of using leukocytes preserved in liquid nitrogen for routine clinical testing of patients' sera suspected of containing leukocyte antibodies.

PRESERVATION OF FUNCTIONAL WHITE BLOOD CELLS

Joan W. Goodman, Oak Ridge, Tenn.

Recent work indicating that peripheral leukocytes have a great capacity for proliferation and differentiation suggests that white cell preservation may become important clinically. In a feasibility experiment we have frozen mouse leukocytes and a month later thawed and used them in biological tests of their functional capacity. The leukocytes were concentrated from pooled, heparinized heart blood from many mice, and the suspensions contained many contaminating red cells and platelets. Glycerol (3 per cent in saline) was added to the suspensions to be final concentration of 15 per cent, and the mixture was sealed in 5 ml. vials and frozen slowly (−1 C./minute) to −25 C. They were then placed in a liquid nitrogen refrigerator for 4-6 weeks. Cells were thawed rapidly at 37 C. and recovered by centrifugation at 1000 g for 30 minutes, resuspended in 10 per cent glycerol, followed by centrifugation at 60 g for 60 minutes, resuspended in 5 per cent glycerol, followed by centrifugation at 1000 g for 30 minutes. All discarded supernatants were dark red. The final pellet of cells representing 20-30 per cent of the number originally frozen, was resuspended in 10 per cent mouse serum in saline for injection. Preservation of immunologic competence was demonstrated by an experiment in which frozen white cells were injected either intravenously or intraperitoneally into lethally irradiated isologous (BC3F₁) mice that had been injected with 10 million homologous (BALB/c) bone marrow cells. Fresh leukocytes were injected into a similar group of marrow-injected mice as a control. Mice given homologous marrow only survived 30 days, but those given in addition either fresh (1 million) or frozen (3-5 million) leukocytes died from rejection of the homologous marrow graft. When the frozen leukocytes were irradiated (2000 r) before injection, this immune capability was lost, and the marrow-transplanted mice lived. Erythropoietic function of frozen leukocytes was preserved and demonstrated by injection of thawed cells into isologous, irradiated recipient mice (B6D2F₁): Several mice died immediately after intravenous injection of the cell suspension.
ABSTRACTS

Three survived the injection of 12 million intraperitoneally. Only one of these recipients lived 9 days after transplantation and was injected with Fe59. When erythrocytes and spleen were counted 24 hours later, this mouse was found to have iron uptake slightly greater than that measured in the control that had received 10 million fresh white cells. These data, although limited in number, do indicate that functional leukocytes can be preserved by freezing. Further work is needed on technics of leukocyte separation, freezing, and cell recovery after freezing to reduce the loss in cell number and to eliminate extensive red cell hemolysis.

TRANSFUSION OF GRANULOCYTES FROM DONORS WITH CHRONIC MYELOCYTIC LEUKEMIA TO LEUKOPENIC RECIPIENTS

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The technic of plasmapheresis has increased the availability of fresh platelets and granulocytes for transfusion studies. While sufficient quantities of platelets can be obtained from normal donors for control of hemorrhage, the relatively small numbers of circulating granulocytes preclude use of normal donors for granulocyte transfusion. However, patients with chronic myelocytic leukemia (CML) and white blood cell counts of 100-300,000/mm³ can donate adequate numbers of granulocytes in relatively small plasma volumes. Five patients with CML donated 500 ml. of granulocyte-rich plasma as often as 5 days a week for 3 weeks. The median dose was 5 x 10¹⁰ granulocytes per transfusion (range 2 x 10¹⁰ to 1 x 10¹¹). Fifty such transfusions were given to 10 leukopenic recipients, 17 of whom had acute leukemia. This resulted in a median 1 hour post-transfusion increment in circulating granulocytes of 1000/mm³ (range 0-11,000). The median recovery in the circulation was 5 per cent (range 0-20) of the dose given. Circulating granulocytes returned to pre-transfusion levels over the next 1 to 4 days (median 2). With repeated daily treatment, granulocyte levels could be maintained above 1,000/mm³. Transfusions have been repeated as often as 14 times for a single recipient. Thirty-two granulocyte transfusions were given during febrile episodes. In 14 of these defervescence occurred within 12 hours. Ten patients with fever had positive blood cultures for pseudomonas organisms prior to transfusion. Nine had received colimycin B or polymixin for 2 days or more prior to transfusion. In six of these patients, temperature returned to normal within 12 hours of transfusion, and they remained afebrile for more than 2 weeks.

EFFECTS OF BLOOD REPLACEMENT IN SHEEP USING IRRADIATED DONORS

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Blood replacement in the sheep was used to ascertain the effects of exsanguinotransfusion (E-T) using lethally irradiated (Co⁶⁰-γ ray source) sheep as donors. Comparison was made with E-T using normal donors or normal blood from which leukocytes had been mechanically removed in order to get leukocyte figures approximating those of lethally irradiated donors. In each case the recipient blood was replaced twice. Blood with low leukocyte content (mechanical removal) evokes a mobilization of leukocytes from the stores to peripheral blood. Neutrophils, lymphocytes, and monocytes are involved, with a maximal mobilization of mature neutrophils following the E-T. When about the same number of leucocytes are infused from irradiated sheep, there is also a mobilization of leukocytes to the peripheral blood. However, the absolute number of neutrophils in peripheral blood is significantly lower than in the case of E-T using blood mechanically deprived of leucocytes. At the same time, when blood of irradiated sheep is used, there is an increase of pre-band myeloids in peripheral blood. In comparison with the E-T from Normal to Normal, in the case of sheep in which blood is replaced by that of irradiated donors, there is a clear decrease in the number of immature myeloids and an increase in the immature red cells of the bone marrow immediately after the E-T.
ABSTRACTS

GROWTH AND DIFFERENTIATION OF LEUKOCYTES IN DIFFUSION CHAMBERS

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Studies on mononuclear leukocytes of blood of man and animals indicate the capacity of certain of these cells to synthesize DNA, undergo mitotic division in tissue cultures, to be immunologically competent, and, under certain environmental conditions, to possess a mesenchymal capacity for differentiation to fibroblasts and hematopoietic tissues. Recently, investigations were made on the ability of mononuclear leukocytes of human blood to survive prolonged storage under blood-bank conditions (N. L. Petrakis and G. Politis; New England J. Med. In press). Tissue cultures of leukocytes were made from units of ACD-preserved bank blood over a 3-week period of time. These studies demonstrate that mitotically competent leukocytes survive in blood during this period of storage. Calculations suggest that a unit of bank contains approximately $6 \times 10^8$ mitotically competent mononuclear leukocytes at the start of storage. These findings may have important clinical implications for immunologically suppressed patients who receive large leukocyte homografts by way of multiple blood transfusions. Under the immunologically suppressing influence of severe surgical stress, chronic illness, corticosteroids, chemo- and radiation therapy, it is conceivable that large numbers of transfused mononuclear leukocytes could colonize and proliferate for varying periods of time, resulting in beneficial hematopoietic differentiation, or more likely, harmful effects, such as graft vs. host reactions. Further clinical and epidemiologic studies are indicated.

EFFECTS OF ENDOXIN ON BONE MARROW

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A preparation of endotoxin was obtained from Serratia marcescens and purified. Its mouse LD$_{50}$ was $190 \mu g$ by intraperitoneal injection. Bone marrow from mice which died 1 day after injection was intensively hyperemic and partially hemorrhagic. The granulocytes and mature myelocytes were intensively degenerative. Bone marrow from mice surviving 100 $\mu g$ of endotoxin and sacrificed 3 days after the injection was injured to a less degree. Mitoses were frequent in the granulocytic series of cells. Toxicity of the endotoxin was considerably reduced by splitting some part of the ester groups in its structure by treating with potassium methylate in boiling methanol. The mouse LD$_{50}$ became over 4000 $\mu g$, whereas other biological activities remained unchanged. Long survival of mice to a large dose permitted investigation of pronounced biological response. Bone marrow 1 day after intraperitoneal injection with 1000 $\mu g$ of detoxified endotoxin was not hyperemic but still degenerative. Although some granulocytes were degenerative, many of the myelocytes looked normal. Erythropiesis showed no change. Three days after the injection, cell-rich foci were eminent. There were many myelocytes and granulocytes. Seven days after the injection, bone marrow was well restored in architecture. The granulocytic series of cells at various stages were especially abundant. By that time, the spleen had more than doubled in size. Hyperplasia of reticulum cells and granulocytic hematopoiesis was conspicuous. The blood pictures well reflected the changing patterns of bone marrow and spleen. Similarly treated mice survived 600 r whole-body irradiation until sacrificed at the end of 4 weeks, increasing weight and showing a quick recovery of leukopoiesis, while the control animals lost weight and 33 per cent died within the same period.

AUTOLOGOUS MARROW TRANSPLANTATION AS PROTECTION FOR MASSIVE CHEMOTHERAPY IN MAN

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Efficiency of cytotoxic antitumoral agents is limited by their bone marrow toxicity. The possibility of overcoming this barrier by autologous marrow transplantation is
the rationale for the present paper. In 12 cases of widespread malignant disease, bone marrow was obtained by needle aspiration by different technics (4.7 to 25.2 x 10^9 nucleated cells were obtained). Immediately afterward, nitrogen mustard (0.8 to 1.7 mg. Kg. bw) was injected intravenously. Thirty minutes later the patients' own marrow was rein infused. A constant hematologic picture followed: (1) Marked lymphopoiesis within 24 hours, which tended to recover slowly by the end of the 3rd week. (2) Marked neutropenia and thrombocytopenia, starting by day 6-8 and persisting to day 14-17, when recovery started (closely preceded by circulation of few immature cells). GI-tract symptoms and other side effects did not constitute a problem in the present series. Three patients died, one apparently without a clear-cut cause of death but for minor tubular damage due to hemolysis in the transfused aspirated marrow; a second one as a result of marked necrosis of lung tumor masses which produced suffocation; the third by sepsis from an "endogenous" source (residual empyema, underestimated). Time sequence, circulation of immature cells shortly before recovery, and the finding of extramedullary erythropoiesis in one case, led the authors to believe in a true "seeding" effect. Effect on the malignant disease was constant; however, it is considered poor when compared to the magnitude of the procedure. It is emphasized that only terminal widespread cases were studied. Earlier cases, new dose schedules, and new cytotoxic agents deserve further trial, since a protective effect of the autologous marrow transplant appears evident.

**STUDIES IN MACACA MULATTA PRIMATES USING BONE MARROW AND/OR CHEMICAL PROTECTION**

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Protection against damage from x-rays has been accomplished in *Macaca mulatta* primates using aminoethylisothiurea (AET). Studies in our primates indicated that a mixture of AET and cysteine HCl provides enhanced protection when compared to either individual drug. This treatment, given intravenously to animals receiving 800 r of x-rays, was 50 per cent effective and also allowed a one-third reduction of the dose of the more toxic material—AET. At 900 r of x-rays a post-irradiation symptomatic therapeutic regimen was combined with the chemical radioprotectants; which treatment, in itself, was a combination of mechanisms of action, routes of administration, and sites and rates of absorption of the drugs. The combined treatment is 50 per cent effective and affords protection to one animal which lived nearly 2 years after irradiation. Autologous bone marrow infusions were accomplished and were 50 per cent effective at 30 days after 900 r; all these animals died by day 180. In a final experiment, marrow infusion, chemical protection, and therapy were all utilized to save four of eight primates at 900 r and further, three of these animals have survived 13 months post-irradiation and are in superb clinical condition.
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