ABSTRACTS

Bone Marrow Transplantation and Irradiation Protection

Abstracts of Presentations at a Discussion Group Meeting during the Sessions of the Federation of Societies of Experimental Biology, Atlantic City, N. J., April 15, 1962.

L. M. Tocantins, Chairman

Combined Treatment of Irradiated Mice with Bone Marrow and Thymus Cells

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Recent studies on the embryologic development of the immune system in the chicken, rabbit, and mouse suggest a unique origin in the bursa of Fabricius or the thymus. This report is a preliminary account of the experiments we carried out to use these ideas in circumventing secondary disease in lethally irradiated mice treated with homologous bone marrow. It was reasoned that fetal thymus cells might proliferate preferentially and establish a tolerant immune system in the host. All recipient animals were LAF1 mice exposed to 900 r x-rays. Each group, unless otherwise noted, contained 79 to 100 mice. Of the LAF1 mice given 10^6 A.CA bone marrow cells, 86 per cent died by day 90. Three similar groups were given in addition the following kinds of thymus cell (5-17 x 10^6) suspension intravenously on the day after the A.CA marrow injection: adult isologous LAF1 thymus cells, fetal isologous LAF1 thymus cells, fetal homologous A.CA thymus cells. All the mice (45) receiving adult isologous thymus were dead by day 17, presumably through marrow failure by rejection of the A.CA foreign cells. Of the group that received fetal isologous thymus cells, 83 per cent died by 90 days, and thus showed no effect of the thymus injection on secondary disease. This group did, however, demonstrate the lack of immunologic competence of the fetal thymus cells as compared to the adult thymus cells. In the third group which was given fetal homologous A.CA thymus cells (30 mice), 17 per cent died by 60 days compared to 67 per cent in the control (bone marrow only) group. This result looks favorable, but too much emphasis cannot be placed on it since the number of mice is small as yet, and a similar experiment with a different homologous marrow and thymus donor did not show any beneficial effect of the fetal thymus cells on secondary disease. Further experiments were initiated in which the fetal homologous thymus was transplanted subcutaneously in pieces instead of being given as an intravenous cell suspension. Results are not yet available. This method of transplantation is based on J.F.A.P. Miller's claim that whole thymus is necessary to restore immunologic competence of mice thymectomized at birth.

Transfer of Antitoxin Producing Cells in Homologous Disease


Using tetanus toxoid as the antigen and the direct challenge with a minimum lethal dose of tetanus toxin as a measure of antitoxin production, the capacity for bone marrow cells from hyperimmunized 101 X C3H donor mice to protect two different host strains was studied. Hosts were members of the LAF1, (C3H x A/He) and BDF1 (C3HBl/6 x DBA/2) strains, which were given whole body x-radiation (LD98) prior to the transfer of the bone marrow. Both strains of hosts developed a comparable, moderately severe, delayed irradiation sickness with a mortality of 33 per cent in the fourth week after irradiation at the time of toxin challenge. The percentage of 7-day survivors after toxin challenge was used as a semiquantitative measure of the amount
of antitoxin produced by the donor marrow. Controls consisted of irradiated isologous (101 x C3H) mice as hosts and a group of normal mice receiving only tetanus toxin. The results after toxin were as follows: 40 per cent of LAF1 hosts survive 7 days after toxin; 78 per cent of isologous hosts survive 7 days after toxin; 17 per cent of BDF1 hosts survive 7 days after toxin. These results indicate that 101 x C3H marrow remains viable and its antibody producing cells continue to function after transfer to the LAF1 host during the development of homologous disease, in contrast to a marked loss of capacity to produce antitoxin in a more hostile BDF1 environment.

The Correlation of Unsaturated Lipid Level and Successful Heterologous Bone Marrow Therapy in X-irradiated Mice

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Efforts to find a source of heterologous bone marrow with therapeutic value for x-irradiated mice, and accessibility superior to marrow from rats, have led to the discovery of such a system in cattle. Marrow suspensions from the shank-bone cavity of Hereford, Holstein, Guernsey, and mixed breed animals have been injected i.v. following exposure of 101 x C3H male mice to 950 r. Marrow from the Guernsey donors supported survival in a higher percentage of irradiated hosts. Complete survival was noted in one experiment where approximately 50 per cent of bone cavity contents in the donor had an oily consistency. Although variations in amount of unsaturated lipid were observed between marrow samples from isobred donors, the Guernsey marrow was as much as 1.8 times higher in this respect than marrow from other breeds in the study. Comparative decoloration of bromine water indicates that the standard 0.5 ml. injection of marrow suspension contains unsaturation equivalent to 4.2 \times 10^{-3} ml. of pure corn oil. Survival (60 days +) at the 30 per cent level in repetitive experiments with the cattle marrow has been generally noted; as high as 20 per cent survival has been obtained after holding the suspension at 4 C. for 1 week. This bone marrow system offers the advantage of a) producing no evidences of initial shock at the levels used; b) maintaining a noticeably higher activity level in the host for a longer period; c) reducing mortality rates; d) not demanding immediate utilization; e) being easily accessible for experimentation.

Modification of Homologous Disease in the Mouse by Methotrexate

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Uphoff has reported that she was able to modify homologous disease occurring in the mouse by the administration of the antifolic Methotrexate for six or seven injections beginning on the 14th day after x-ray and homologous marrow administration. We have extended these observations using male inbred mice of the strain C57Bl/6J as marrow donors for lethally irradiated (850 r) BDF1 mice of the same sex. In this donor-host combination a severe form of homologous disease occurs resulting in severe weight loss and death in 90 per cent of the mice between 15 and 60 days postirradiation. Methotrexate given in dosages of from 1.5 mg./Kg. to 3.0 mg./Kg. IP three times weekly, beginning on the 7th, 14th, or 21st day postirradiation, produced no modification of homologous disease. Methotrexate 0.25 mg./Kg. to 1.5 mg./Kg. given three times weekly beginning on the first day postirradiation and continuing to days 35–40, resulted in 50–90 per cent survival to 60 days. Methotrexate 1.5 mg./Kg. given on days 1, 3, 5, and 7, for only four injections, resulted in complete avoidance of the weight loss and mortality usually seen. Hemoglobin electrophoretic pattern in the homografted mice revealed the presence of donor type hemoglobin, indicating persistence of the marrow homograft after Methotrexate treatment. Administration of Methotrexate during the initial period of exposure of the foreign homografted cells to the host antigens appeared to be necessary for the prevention of the homologous disease.
ABSTRACTS

MODIFICATION OF SECONDARY DISEASE BY METHOTREXATE ADMINISTRATION

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Last year we reported observations on a group of dogs given 1200 to 1600 r of whole-body irradiation followed by infusions of marrow from unrelated dogs. Twenty-six given methotrexate lived beyond the first week, and 21 exhibited successful marrow engraftment. Sixteen dogs died in the first 3 months. Five dogs lived beyond 120 days and appeared to have survived the period of "secondary syndromes" and regained health. One of these long-term survivors was sacrificed in another experiment. We wish now to report that the remaining four long-term survivors are in excellent health, more than 1 year post-irradiation. In three dogs where markers existed to distinguish donor and host, the long-term survivors were shown to be radiation chimeras. We postulate that these chimeras represent a fortuitous choice of donor and recipient not widely differing in histocompatibility factors. It appears that Methotrexate, and probably other agents suppressing immune mechanisms, may suppress successfully a relatively mild graft-versus-host reaction allowing the development of a long-term compatibility between donor and recipient. Efforts are currently directed toward a better means of selecting donor and recipient that do not differ with regard to major histocompatibility factors.

REDUCTION OF SECONDARY DISEASE INCIDENCE IN RADIATION CHIMERAS BY INJECTION OF ANTI-DONOR ISOANTISERUM


Recent experimental results suggest that the cellular events associated with graft-versus-host reactions and leading to secondary disease in radiation chimeras occur relatively early after establishment of the chimera. Thus, in the case of parental strain-F1 hybrid lymphoid cell chimeras, it has been found that the donor cells (derived from nonirradiated adult A strain mice) become specifically unreactive—i.e., tolerant, to the LAF1 host during the first week after initial implantation. Similarly, in radiation-induced bone marrow chimeras, donor type immunologically competent cells can be shown to be tolerant of host type grafts during the second month after bone marrow transplantation. On this basis, attempts have been made to prevent or diminish the occurrence of secondary disease in radiation chimeras, by means of injections of anti-donor isoantiserum. LAF1 hybrid mice received 880 r of x-rays followed by a single intravenous injection of \( 6 \times 10^6 \) bone marrow cells from adult C3H strain donors. This was followed by two intravenous injections of 0.4 ml. isoantiserum (anti-C3H) at 16 and 18 days, or at 48 and 48 days after marrow injection. No secondary disease deaths have been observed by 7 months postirradiation. The incidence of secondary disease deaths (by 120 days) in a large series of control C3H-LAF1 chimeras was 51 per cent. That these animals are still chimeras is borne out by their acceptance of C3H and LAF1 tail skin grafts, and their rejection of BALB/c skin grafts. In experiments still in progress, radiation bone marrow chimeras have received injections of isoantiserum as early as 1 week after marrow injection. It is postulated that the rapidly dividing donor lymphoid cell elements (subjected to the proliferative stimulus of the host's isoantigens) in these chimeras are particularly sensitive to the cytotoxic and/or antimitotic effects of the injected isoantiserum, thus eliminating or inactivating the mature, immunogenic donor cells before they can mount and sustain a graft-versus-host reaction. These considerations serve to emphasize the need of early treatment of radiation chimeras in the attempt to prevent or forestall the occurrence of secondary disease.

PRESERVATION OF DOG BONE MARROW AT LOW TEMPERATURES IN DIMETHYL-SULPHOXIDE

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Glycerol has been the principle additive used in the preservation of bone marrow, but recently it has been shown that dimethyl-sulphoxide (DMS) has superior pro-
There is evidence, in work done in mice, that 10 per cent DMS is the concentration of choice, being far superior to 5 and 15 per cent DMS as well as corresponding percentages of glycerol. Dimethyl-sulphoxide seems equally effective in the dog. Marrow was aspirated from the long bones and centrifuged to remove supernatant fat. The concentrated DMS was diluted with the supernatant fluid and added to the marrow sediment to make a final DMS percentage of 5, 10, or 15 per cent. The marrow sample was frozen at 1 C. per minute to -20 C. and then placed at -80 C. for storage.

Dogs were irradiated with 1200 r just prior to thawing and intravenous administration of the marrow. No toxic symptoms were noted in relation to the DMS infusions. Dogs receiving marrow preserved in 10 per cent DMS showed prompt and complete hematologic recovery. Marrow preserved in 5 or 15 per cent DMS was less effective. We conclude that dimethyl-sulphoxide is the preservative of choice for freezing marrow because 1) the drug need not be removed prior to infusion, 2) slime formation is reduced to a minimum, and 3) marrow preserved in dimethyl-sulphoxide is at least equal to glycerol in its ability to protect the irradiated host.

Iron Utilization in Mice Protected with Frozen Bone Marrow


A major handicap in bone marrow preservation is the lack of an accurately reproducible method, which allows a quantitative determination of viable cells in a frozen sample. Past reports have indicated that the iron utilization in lethally irradiated mice injected with fresh bone marrow is directly proportional to the protective cell dose for a given period of time and can be considered as a measure of "take" of the transplanted cells. To apply this observation as a viability test for frozen bone marrow, the blood volume of irradiated and protected C₃H and A male mice was determined with iron-tagged red blood cells. Following irradiation, the blood volume in these animals is found insignificantly decreased, in sharp contrast to their body weight, which declines in an irregular manner. When, therefore, the blood volume is determined as a per cent fraction of the body weight, a different factor must be used for each irradiated animal. Bone marrow aliquots frozen in 15 per cent dimethyl-sulphoxide over liquid nitrogen vapor are used to protect lethally irradiated mice at standard doses of 1 × 10⁶ cells. During the sixth postirradiation day, the animals transplanted with marrow frozen at rates from 1.2 to 48 C. per minute, the 24-hour iron utilization is found to be of approximately the same magnitude. Normal control animals and animals protected with fresh bone marrow show much greater iron utilization; irradiation controls on the other hand show significantly lower levels of iron utilization. Recovery of peripheral WBC is also similar among animals protected with marrow frozen at different rates. The survival, however, is greater among groups protected with slowly frozen bone marrow. So far the method has failed to show significant differences in the survival of marrow frozen at rates of 1.2 to 48 C. per minute. The dose-recovery correlation in animals protected with frozen marrow is being determined.

Controlled Rate Freezing of Bone Marrow

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Many methods have been devised for the controlled rate cooling of biological specimens. One method involves placing the biological specimen in a cold environment and controlling the rate of specimen cooling using heated or insulated containers. Another category of controlled rate cooling involves placing the specimen in an environment at ambient temperature and then cooling the environment at a desired rate (e.g., 1 C./min.). We have studied a number of cooling regimes with bone marrow specimens and have found the phase transition on freezing to be a critical part of the cooling
process. Using a biological controlled rate freezer we can control both the rate of specimen cooling and length of time spent in the latent heat of fusion by the specimen. With bone marrow samples suspended in such intracellular additives as 15 per cent glycerol and 15 per cent dimethyl-sulfoxide, we have found that the shortest period in the heat of fusion is better than longer periods for optimum viability based on two metabolic assay methods, C14 glycine incorporation and cellular respiration. The effect of the rate of cooling prior to the phase transition does not appear to be significant. Once the latent heat of fusion has been removed, however, cooling of the specimen to —25 C. at a rate of 1 C./min. was better than faster rates. These studies show the importance of adequately controlling the cooling rate of the specimen and not just the environment, inasmuch as the length of time the specimen in the latent heat of fusion should be kept to a minimum for optimal viability of the cells.

FREEZING AND PRESERVATION OF MOUSE TUMOR SLICES

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A previous report outlined the advantages to be derived by the freezing and preservation of human tumor tissue. Successfully preserved, such tissue could later be used for biochemical, histochemical, and in vitro chemotherapeutic tests. Carcinoma 755 tissue from 14-day transplanted mouse tumors was used for developmental studies. Slices of tumor tissue approximately 20 x 10 x 3 mm. were manually cut under aseptic conditions. The slices were next equilibrated by shaking at 37 C. for 15 minutes in a freezing medium consisting of Basal Medium (Eagle) with Hanks' salts, serum, and 20 per cent glycerol. The freezing process consisted of a gradual decrease in temperature within the range of 0 C. to —25 C., followed by a rapid fall to about —60 C. Frozen slices were then stored in a dry ice refrigerator at —75 C. or in a liquid nitrogen refrigerator at —190 C. Tumor slices of Sarcoma 180, and Sarcoma 37, as well as Carcinoma 755, were rapidly thawed and tested for viability. For this purpose the slices were cut into 2-mm. fragments and the latter observed for outgrowth in explant culture. A semiquantitative evaluation demonstrated that viability was successfully preserved by the freezing method in the three tumors studied. Frozen-thawed tissues from these three tumors were also tumorigenic when inoculated in susceptible mice.

POTENTIALITES TO TRANSPLANT IN VITRO CULTURED HUMAN BONE MARROW

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The conventional method of storing bone marrow by the freezing process exerts a considerable physical trauma on the marrow. The visible signs of this trauma are diminished viability of the nucleated cells and hemolysis of the erythrocytes. To avoid this damage to the bone marrow, we studied the possibilities of using cultured bone marrow for transplantation. The standard methods of cultivating marrow results in formation of spindle cells and loss of their original characteristics. In our studies, we had cultured rat and also human bone marrow. In our bone marrow cultures we used heparinized autologous whole blood as the culture medium. The bone marrow cells and bone marrow particles are suspended in the heparinized autologous whole blood. In our "circulating-dialysing bag," the blood with the suspended bone marrow is kept in constant motion, and is supplied with oxygen and dialyzed against homologous plasma throughout the entire time of cultivation. With this method the bone marrow with the blood is kept for 2 weeks without any appreciable hemolysis. After two weeks of cultivation, blast cells, progranulocytes, normoblasts, and reticulum cells are well preserved. No spindle cell formation is seen. The mitotic activity indicates cell propagation. Normoblasts are increased and erythroblasts are reduced in numbers, suggesting erythroid maturation. The reappearance of neutrophils after 6 days of cultivation points to myeloid maturation. After 20 days of cultivation the culture is terminated and the bone marrow particles are fixed in formalin. The paraf-
fin section of this 20-day old culture shows histologically normal bone marrow architecture. Thus far, for transplantation, we have used cultured rat bone marrow. The 6-day old cultured rat bone marrow at 37°C is injected into sublethally radiated mice. Eight days after transplantation the recipient mice were sacrificed. Alkaline phosphatase positive leukocytes may be found in the spleen and bone marrow of the mice, indicating the maintenance of the cultured rat bone marrow. Studies are under way to use the above described human bone marrow cultures for transplantation.

**Autologous Bone Marrow Replantation**

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Storage of the patient's own bone marrow with subsequent replantation, in cases with marrow aplasia resulting from the disease itself or from drug toxicity, has been resorted to. This procedure was the outgrowth of an earlier program of attempted homologous bone marrow transplantation in acute leukemia formerly treated with irradiation with isotopes. Bone marrow was taken during the hematologic and clinical remission in patients with acute leukemia and prior to chemotherapy in other malignant diseases. Treatment after storage included antimetabolites, alkylating agents, radioactive phosphorous, antibiotics, Vinblastine and steroids. Marrow was returned when aplasia persisted for several days or when a profound bleeding tendency or severe infection occurred that did not respond to therapy such as antibiotics, blood transfusions or steroids. Of 156 bone marrows preserved, 36 were returned. In ten cases, a second marrow storage was done after complete recovery from an earlier procedure. Twenty patients showed bone marrow response in an average of 16.4 days. Of these, four were acute leukemia and 16 were other malignancies. No recovery was observed in 15 and in these death occurred from 1 to 14 days after replants. Viability was studied by means of a stathmocytotic index after 1-hour incubation with Colcemide. Seventy-seven per cent of the stored marrows by this method were considered viable after intervals of from 6 to 954 days of preservation. We were not able to prove recovery of bone marrow after storage and replantation because of the lack of distinguishing features or markers. Nor was a similar control group available. After bone marrow storage, larger doses of chemotherapy in malignant disease were used. The average recovery of bone marrow function in 16 days and the stathmocytotic studies are suggestive of cell survival and viability and seem to justify further studies.
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