Abstracts of Conference on Radiation Protection and Recovery

Author abstracts presented November 22 and 23, 1963 at the Holiday Inn, Oak Ridge, Tenn.

I. Chemical Protection and Related Topics

β-MERCAPTOETHYLAMINE EFFECT ON EARLY POSTIRRADIATION LEUKOCYTE COUNTS

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Treatments may produce regenerative changes in the hemopoietic system of irradiated animals without affecting initial depressions in cell count. Several investigators thought this was true for glutathione, β-mercaptoethylamine (MEA), and 2-mercaptoethylguanidine (MEG), but conclusions were based largely on total rather than differential counts, and dose-response characteristics were not always considered. In the first of the present experiments, MEA-treated (BALB/c x DBA/2)F1 mice from NIH and Bagg-Swiss mice from WRAIR were given the sublethal dose of 560 rads of Co60. [Only the (BALB/c x DBA/2)F1 mice were used in subsequent experiments.] Both strains had significantly higher granulocyte counts than controls 3 and 4 days after irradiation and higher lymphocyte counts 2, 3, and 4 days after irradiation. This was also true of the mice given 770 rads (LD30) even though the granulocyte counts were still falling between days 3 and 4 and the lymphocyte counts between days 2 and 3. With the supralethal dose of 1000 rads, granulocytopenia was reduced in the treated group 4 days after irradiation, also while the counts were still falling. Lymphocyte counts, on the other hand, were not higher in the treated group. The dose of 1000 rads is beyond the range of dose-dependence for lymphocytes even with MEA protection. These findings would ordinarily be interpreted to indicate a diminution of initial damage rather than an effect limited to recovery. Tracer technics, however, might reveal a recovery component in the early falling counts.

MERCAPTOPYRIDOXINE AS A RADIATION PROTECTIVE AGENT IN THE RAT AND MONKEY

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In 1958 Langendorff, Langendorff, and Koch (Strahlentherapie, 107:121, 1958) reported on the effectiveness of mercaptopyridoxine as a chemical protective agent against ionizing radiation in mice. We used mercaptopyridoxine (B6I) in rats and found that it was very effective against radiation damage with an acute gamma exposure of 1000 r. We then investigated the effectiveness of B6I in rats when they receive fractionated doses of 200 r per week over a period of several weeks to a total accumulated dose of several thousand roentgens (50 per cent mortality at 3800 r). With this fractionated exposure, B6I was not effective against the radiation damage. This finding confirms the hypothesis of several workers that, in general, chemical protective agents are not effective against fractionated or chronic exposures as compared with the protection provided against a single acute exposure. It is recognized that there are species differences in radiation sensitivity as well as species differences in the effectiveness of chemical protective compounds. We then irradiated a series of Macaca mulatta (rhesus) monkeys and found that B6I is effective against an acute irradiation exposure of 900 r.
SELECTIVE PROTECTION IN IRRADIATED TUMOR-BEARING MICE

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This work is a continuation of efforts to determine the possibilities of obtaining selective protection of different tissues in tumor-bearing mice. We previously found that mammary adenocarcinomas growing subcutaneously in C57BL mice are not protected against 3000 r (local) by pretreatment with 2-mercaptoethylguanidine (MEG). The absence of protection is associated with low tumor concentrations of the protein-bound and free disulfide forms of the protective agent; these also appear to be required for the protection of normal tissues. It is possible to delay radiation deaths in mammary tumor recipients following whole-body exposure if 200 or 280 mg/kg. of MEG are injected i.p. prior to whole-body exposure. Evidence of dose reduction in critical radiosensitive tissues such as bone marrow and small intestine explains this enhancement of survival. Unfortunately, the amount of whole-body exposure that can be given even under these conditions is not adequate to significantly retard the growth of the mammary tumor and death from this cause. A myeloid leukemia (C1498) has been similarly studied. This neoplasm is more radiosensitive and, as is true for the mammary tumor, is not protected by MEG against local X-radiation. Because C1498 rapidly disseminates throughout the body, whole-body exposure is necessary for its control. It has been found that the oral route of MEG administration leads to the longest extension of survival with leukemia in whole-body irradiated mice. This outcome is correlated with optimal differential concentrations of the protective forms in normal and neoplastic tissues.

CHEMICAL PROTECTION WITH MIXTURES OF PROTECTIVE COMPOUNDS

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Mixtures of protective compounds have been used previously to obtain increased protection against short term radiation mortality. In this study we selected glutathione (GSH) as a promising additional agent to use with 5,2- aminoethylisothiourea (AET) or 2-mercaptoethylamine (MEA) because of its low toxicity and implication in a host of biological reactions including disulfide-thiol interchange. GSH could conceivably increase the quantity of -SH compound at radiation-sensitive sites. It is protective when given i.v. (Patt et al., Proc. Soc. Exper. Biol. & Med. 73:18, 1950) and subcutaneously (Chapman and Cronkite, Proc. Soc. Exper. Biol. & Med. 75:318, 1950). We found that when given i.p. to BC3F1 mice (LD50/30 700 r), 1.6 Gm./Kg. 15 minutes prior to X-radiation the LD50/30 was 950 r while 3.2 Gm./Kg. yielded an LD50/30 of 1300 r. Chemical toxicity studies revealed that one-third more AET [as 2-mercaptoethylguanidine (MEG)] could be given i.p. when combined with 1-2 Gm./Kg. GSH (from 360 mg./Kg. to 480 mg./Kg. AET). Similarly, GSH in combination with MEA raised the MEA dose from 150 mg./Kg. to 200 mg./Kg. Both combinations consistently gave LD50/30 of 1700 r. GSH also reduced the toxicity of the disulfides of both compounds although increased radiation protection was not obtained. Mixed disulfides with GSH and MEG or MEA or with oxidized glutathione were also ineffective as protective agents. Paper chromatography of the effective mixtures showed them to be mixtures of the free thiols with only traces of disulfides present. The mixed disulfide of GSH and MEG or MEA is therefore not the effective agent in providing the increased radiation protection.
A REVIEW OF THE CARDIOVASCULAR TOXICITY OF BETA-MERCAPTOETHYLAMINE

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The cardiovascular toxicity of beta-mercaptoethylamine (MEA) in the dog is characterized by a severe, delayed and prolonged hypotension and hemoconcentration. The delayed fall in arterial blood pressure (12 minutes after the beginning of an injection) appears to be unique and has led to the studies reviewed in this report. Cardiac contractile force is increased during the development of hypotension, peripheral resistance in the innervated extremity and portal pressure is increased with no change in central venous pressure. This suggests hepatic-splanchnic pooling of blood which, in turn, causes poor return to the right heart, a decrease in cardiac output, and lowering of arterial pressure (MacLean and Weil, Circulation Res. 4:546, 1956; Mundy and Heifer, Radiation Res. 13:381, 1960). Histamine release is a mechanism which induces portal-splanchnic pooling. The model of histamine release as the causative agent of MEA-induced hypotension has been tested. First, most of the adverse hemodynamic effects of MEA administration can be prevented by antihistaminic compounds (Mundy et al., Am. J. Physiol. 204:997, 1963). Second, it is possible to detect increased histamine levels in the blood of dogs during the development of hypotension (Mundy et al., Fed. Proc. 22:424, 1963). Third, four daily doses (50–75 mg./Kg.) of MEA or of nicotinic acid (100 mg./Kg.) protects 50 per cent of the dogs (2 of 4) against hypotension following a dose of MEA (100 mg./Kg. base) which produced hypotension in all of 50 nontreated dogs. When an injection of MEA or cystamine is given to the dog, rat, or rabbit an equilibrium of sulphydryl and disulfide materials is established in the blood (Fischer and Goutier-Pirotte, Arch. Int. Physiol. 62:76, 1954; Mundy et al., Radiation Res. 14:421, 1961; Heifer et al., Radiation Res. 16:165, 1962). Work with in vitro histamine release from rat peritoneal cells shows that 50 per cent of MEA is converted to disulfide before significant histamine release occurs. The possibility will be discussed that conversion of MEA to cystamine is necessary for production of hypotension in the dog. The relationship of the time delay before hypotension to this conversion will also be evaluated.

EFFECTS OF GAMMA RADIATION ON BOVINE PANCREATIC RIBONUCLEASE

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Purified bovine pancreatic ribonuclease in aqueous solution aggregated upon irradiation with Co60 gamma rays. The aggregation process was inhibited by oxygen and by chemical protective agents. In the presence of oxygen, partially oxidized, partially active ribonuclease was formed during irradiation. In the absence of oxygen, aggregated enzymically inactive ribonuclease was produced. 3,3'-guanidinopropyl disulfide completely inhibited the aggregation process, but had little effect upon the formation of oxidized ribonuclease. Aggregation was observed from pH 1 to 8. At the neutral range, the irradiated solutions were turbid. Performic acid oxidized ribonuclease also formed aggregates upon irradiation. The biological significance of this aggregation process will be discussed.

CHEMICAL PROTECTION AGAINST HIGH ENERGY PHOTON RADIATION IN MICE

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Studies have been carried out to determine whether or not the lethal effects of whole-body exposure to 440 Mev proton radiation can be modified in CF1 male mice by the pre-irradiation administration of p-aminopropiophenone (30 mg./Kg. i.p. in
propylene glycol) or 2-mercaptopropionylalanine hydrochloride (225 mg./Kg. i.p. in water) and to compare the effects with those obtained in mice exposed 250 Kvp X-rays. In these studies, groups of 30 to 40 mice between 14 and 24 weeks of age were given single doses of between 235 and 1400 rads of proton radiation or doses of 222 to 1844 rads of X-radiation and the subsequent mortality in the control (vehicle only) and protected groups was observed daily for 30 days. The regression of the mortality probit vs. absorbed radiation dose calculated by the maximum likelihood method gave 30-day LD₅₀ values of 848 ± 27 rads for the mice exposed to 440 Mev protons and 561 ± 14 rads for the animals exposed to X-rays (RBE = 0.66); the regression on the logarithm of the absorbed dose gave values of 839 ± 36 rads and 538 ± 15 rads for protons and X-rays respectively (RBE = 0.64). The administration of PAPP at 10 minutes prior to the radiation exposures increased the 30-day LD₅₀ in the proton-irradiated mice to 1257 ± 71 rads and to 875 ± 22 rads in the X-rayed animals. The 30-day LD₅₀ values for the MEA-treated groups were 1364 ± 83 rads for proton radiation and 80 ± 13 rads for the X-ray exposure. It is evident that PAPP is somewhat more effective in preventing the acute-lethal effects of X-ray exposure (DRF = 1.56) than those of proton exposure (DRF = 1.48), whereas the reverse is true for MEA (DRF = 1.39 for X-rays and 1.61 for protons) at the dosage levels used for these studies. The histopathologic findings in the tissues of the proton-irradiated mice resembled those seen in the X-rayed animals except for an increased frequency of hemorrhages in the brain and liver of the proton-exposed animals.

THE ABILITY OF AET AND PAPP TO PROTECT MICE AGAINST 30 MEV ELECTRONS

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The ability to protect CF No. 1 female mice against high-energy (30 Mev) electrons from a linear accelerator by pre-irradiation injection of S,2-aminoethylisothiuronium (360 mg./Kg. i.p. in saline) and of p-aminopropiophenone (30 mg./Kg. i.p. in propylene glycol) has been compared with the effects in mice irradiated with 250 Kvp X-rays. Mice were confined in Lusteroid tubes suspended in a water phantom, and were irradiated either awake or under Nembutal anesthesia. Nembutal had no effect on survival of mice irradiated with electrons, but was slightly protective against X-rays.

Our experiments indicated that pre-irradiation treatments with AET or with PAPP are able to reduce 30-day lethality in mice exposed to high-energy electrons. AET afforded better protection than did PAPP against electrons as well as against X-rays. The 30-day LD₅₀ value for mice exposed to 30 Mev electrons was 954 ± 20 rads; pre-treatment with PAPP increased the LD₅₀ to 1323 ± 28 rads, and with AET to 1528 ± 85 rads. The LD₅₀ for anesthetized X-rayed mice was 826 ± 57 rads; pre-injection of PAPP increased this to 985 ± 25 rads, and of AET to 1027 ± 36 rads.

THE NATURE OF THE RADIATION-INDUCED PROTEIN-BINDING OF BIS(µ-GUANIDOETHYL) DISULFIDE (GED) IN AQUEOUS BUFFERED SOLUTIONS

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Buffered solutions of 10⁻³ M GED-S³⁵ or GED-C¹⁴ and 2 per cent bovine serum albumin, human serum albumin, urease, proteamine, gamma globulin, polyglycine, and polyaspartic acid were irradiated by a Co⁶⁰ gamma-ray source. Total protein binding of GED and the nature of the binding were determined by dialysis against water, hydroxylamine, and hydroxylamine plus cysteine. All proteins and polyamino acids showed radiation-induced binding of GED by an unknown type of linkage. In addition, gamma globulin and urease showed radiation-induced binding of GED by mixed disulfide
linkage. There was no radiation-induced binding of GED by thioester linkage. Chromatography of hydrolyzed bovine serum albumin containing GED-S\(^{35}\) or GED-C\(^{14}\), bound by the unknown type of linkage only, revealed a single radioactive peak suggesting binding to only one or a few amino acid moieties of the protein. The possibility that radiation-induced protein binding of GED may be one mechanism of the protective action of GED is discussed.

II. Bone Marrow Transplantation and Related Topics

THE USE OF CHEMICAL COMPOUNDS TO MODIFY BONE MARROW TRANSPLANTATION IMMUNITY IN LETHALLY IRRADIATED MICE

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Our group has been studying bone marrow treatment of mice lethally irradiated with Co\(^{60}\) gamma rays under high dose rate. With our irradiation facility, 900 r of gamma rays can be given to mice in less than half a minute. With this irradiation condition and the strain combination, in which Na2 strain mice are used as donors and dd/s strain mice as recipients, survival rate of 900 r irradiated dd/s mice given 10 million Na2 strain bone marrow cells is usually less than 50 per cent at 14 days, less than 30 per cent at 21 days, and less than 10 per cent at 60 days. Therefore in our experimental condition there are initial difficult take and delayed homologous disease. Pretreatment of homologous Na2 donor mice 6 days previously with 6 mg. of AET per mouse and 100 r of gamma radiation resulted in 45 per cent 21-day survival of the recipient 900-r irradiated dd/s mice. Sixty-day survival was 25 per cent better than the control. In the next experiment, AET was given to the recipient mice followed by 1500 r gamma irradiation, the pretreatment to the donor mice being the same as described. Although AET plus 1500 r was not an LD\(_{100}\) dose, six out of seven mice survived at 21 days and three out of seven mice survived at 90 days.

CURRENT STATUS OF LEUKOCYTE INFUSION STUDIES WITH LETHALLY IRRADIATED GUINEA PIGS

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Fresh pooled homologous leukocytes (1 \(\times\) 10\(^8\) nucleated cells) were infused via the intracardiac route into 26 lethally irradiated (550 r Co\(^{60}\), total body) Princeton guinea pigs. Eighteen animals exposed to the same irradiation source were used as
EFFECTS OF IRRADIATION ON ERYTHROCYTE CHIMERISM IN DZYGOTIC CATTLE TWINS
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Effect of irradiation on erythrocyte chimerism in Dzygotic cattle twins, which occurs in a high proportion of cattle twins, was stimulated by the observation that the proportion of the two cell types in such twins was not constant as had been assumed, but shifted markedly with age. Furthermore, in one twin, greater than 95 per cent of his erythrocytes were of a type containing a new combination of antigens. In other words, antigens which were previously on the two different cells in the mixture were now on the same cell. This observation suggested some kind of genetic exchange (recombination) between the hemopoietic tissues that produced the original types. This report describes our first year's progress in an experiment to determine if sublethal irradiation of cattle with erythrocyte chimerism can cause a shift in the proportion of cell types and the occurrence of recombinant types. We have given a single dose of 300 to 350 r to one member of each of 12 pairs of chimeric twins, the co-twins serving as controls. One member of the first group of four twins died at 4 weeks. Of the remaining three, one showed a shift in cell proportions at 8 weeks; similarly, another twin shifted at 20 weeks, and the last twin showed a shift at 28 weeks postirradiation, all of which are holding at 48 weeks. One of the second group of four twins showed a gradual shift beginning at 8 weeks postirradiation and is still changing at 36 weeks. The other three remained constant. No changes have been observed in the irradiated twins of the third group after 26 weeks of observation; however, one nonirradiated twin of this group has shown a 10 per cent shift. A fourth group of four twins has received from 650 to 1150 r in fractionated doses (50 r/week), but has not shown any marked changes after 17 weeks. None of the twins has shown recombinant cell types. Reciprocal skin grafts made between co-twins prior to irradiation have not been affected by the irradiation.

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controls. All controls died by the 16th day. Four treated animals died within 24 hours of the infusion; four other animals died during the control-animal survival period. Three animals lived longer than the control survival period, and 15 animals survived beyond 30 days. The survivors in this study have failed to demonstrate any evidence of homologous disease (well beyond the 5th month). One would expect to observe symptoms of a secondary disease within the first 90 days following irradiation and infusion. The failure to demonstrate such a syndrome suggests that these animals were not provided with a hemopoietic graft, but rather were provided with a transient therapeutic treatment which prevented a lethal bacterial infection. Studies are continuing in an attempt to evaluate the mechanism of recovery following the infusion of pooled homologous peripheral blood leukocytes.

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W. H. Stone, Audrey Fregin, Joan Caulton, R. G. Cragle and E. W. Swanson. From the Department of Genetics, University of Wisconsin, and University of Tennessee AEC Agricultural Laboratory, Oak Ridge, Tenn.

Renewed interest in erythrocyte chimerism, which occurs in a high proportion of cattle twins, was stimulated by the observation that the proportion of the two cell types in such twins was not constant as had been assumed, but shifted markedly with age. Furthermore, in one twin, greater than 95 per cent of his erythrocytes were of a type containing a new combination of antigens. In other words, antigens which were previously on the two different cells in the mixture were now on the same cell. This observation suggested some kind of genetic exchange (recombination) between the hemopoietic tissues that produced the original types. This report describes our first year's progress in an experiment to determine if sublethal irradiation of cattle with erythrocyte chimerism can cause a shift in the proportion of cell types and the occurrence of recombinant types. We have given a single dose of 300 to 350 r to one member of each of 12 pairs of chimeric twins, the co-twins serving as controls. One member of the first group of four twins died at 4 weeks. Of the remaining three, one showed a shift in cell proportions at 8 weeks; similarly, another twin shifted at 20 weeks, and the last twin showed a shift at 28 weeks postirradiation, all of which are holding at 48 weeks. One of the second group of four twins showed a gradual shift beginning at 8 weeks postirradiation and is still changing at 36 weeks. The other three remained constant. No changes have been observed in the irradiated twins of the third group after 26 weeks of observation; however, one nonirradiated twin of this group has shown a 10 per cent shift. A fourth group of four twins has received from 650 to 1150 r in fractionated doses (50 r/week), but has not shown any marked changes after 17 weeks. None of the twins has shown recombinant cell types. Reciprocal skin grafts made between co-twins prior to irradiation have not been affected by the irradiation.

HOMOLOGOUS BONE MARROW TRANSPANTATION AFTER 14.1 MEV FAST NEUTRON IRRADIATION IN MICE
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As the result of our previous experiments it was found that the extent of medullary hemopoietic disturbances in mice was greater with 14 Mev fast neutron than with X-ray exposure, even when the absorbed dose was the same and even when the dose was of equal relative biological effectiveness in respect to acute death. Also, in general, regeneration of medullary hemopoietic tissue in animals exposed to fast neutrons was delayed when compared with animals exposed to X-rays. Therefore the observations were made on the protective effect of homologous bone marrow transplantation against acute
death due to exposure to 700 rads of fast neutrons. The animals used in the experiments were random-bred 8-week-old ddN mice. Each group had 20 mice of both sexes. All control animals developed severe diarrhea with weight loss and died between the 3rd and 8th day after irradiation. In mice given bone marrow transplantation, although half died within 6 days after irradiation, the subsequent survival rate remained unchanged until after the 30th day following irradiation, and the survival rate on the 60th and 90th day was 40 per cent. The granulocyte count and lymphocyte count of the peripheral blood show a rapid decrease following irradiation, but the recovery of decrease of granulocytes was rapid. New formation of sporadic small hemopoietic foci was seen even in animals that had died 4 days after irradiation. As mentioned above, homologous bone marrow transplantation was effective to protect early acute death due to 14 Mev fast neutrons exposure in our experiment. This does not seem unreasonable in view of recent reports which consider generalized infection to be an important influence in the cause of early acute death due to fast neutron irradiation. It may be concluded that disturbance of hematoipoiesis in the bone marrow plays an important role not only in late acute death but also in early acute death. Moreover, it was noted that the occurrence of secondary disease following bone marrow transplantation in animals exposed to fast neutrons was few as compared with animals exposed to X-rays. It is considered that this fact shows some differences in the effect on the immune system between both radiations.

**LYSOZYME ACTIVITY IN RADIATION CHIMERAS**

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To obtain more information about the metabolic disorder in secondary disease, lysozyme activity was studied in different organs of X-irradiated mice treated with homologous bone marrow (HBM). Lysozyme activity was markedly elevated in the kidneys of radiation chimeras during the 2nd week, reaching a peak at 9 days after irradiation and transplantation, compared to the normal, isologous bone marrow (IBM) treated or controls which were irradiated (950 r total-body irradiation) only. Lysozyme activity was normal in kidney homogenates of old radiation chimeras at different intervals between 100 and 322 days after irradiation and bone marrow treatment. Levels of lysozyme activity on day 9 in the normal, IBM, HBM, and irradiated control groups were 180, 350, 450, and 29 µg./Gm. of bone marrow, respectively. In the same groups on day 9, levels of lysozyme activity in the kidney were 28, 42, 196, and 21 µg./Gm. of tissue, whereas in the spleen the values were 17.3, 39, 56, and 3.8 µg./Gm. of tissue, respectively. The lysozyme activity was also measured in the small intestine, lung, colon, lymph node, submaxillary gland, thymus, liver, and plasma. In normal mice, lysozyme activity was highest in bone marrow, small intestine, lung, kidney, spleen, and colon. In contrast, no activity was found in brain and skeletal muscle. In X-irradiated mice not given bone marrow, a decrease in activity was found in 7 of the 11 organs examined. Four organs showed no change or increase. The biological significance of lysozyme and the meaning of the changes found in radiation chimeras are not known. Since the highest increase in activity at 9 days was found in the bone marrow of the HBM mice, this presumably was the source of the elevated kidney lysozyme in these mice, according to the assumption that the kidney only filters out the lysozyme rather than produces it. Further work is needed to understand the lysozyme activity changes and its role in secondary disease.

**A COMPARISON OF THE SENSITIVITY TO STORAGE AT 37 C. IN TYRODE SOLUTION OF IMMUNOLOGICALLY COMPETENT CELLS AND OF MYELOID CELLS**

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In a hemopoietic graft, preservation for 2 hours at 37 C. in Tyrode solution does not notably influence the number of cells required to bring about myeloid restoration in
an irradiated recipient; to obtain the same biological result (50 per cent restoration) which is the result for which the determination of the required number of cells can be the most accurate, there must not be more preserved than fresh medullary cells. On the other hand, this preservation has a considerable influence on the immunologically competent cells which, when they are immunized against a semi-allogenic host, kill it through a graft-to-host reaction: 1,000,000 fresh lymph node cells kill 50 per cent of the animals, but of the cells that have been stored for 2 hours at 37°C in Tyrode solution 40,000,000 are not enough to bring about the same effect; accordingly it may be concluded that this preservation affects more than 97.5 per cent of those cells that are capable of evoking a graft-to-host reaction.

Regression of (C57BL × 101)F2 Marrows in Irradiated F1 Mice

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Ten million strain 101 bone marrow cells infused into a lethally irradiated (C57BL × 101)F1 mouse establish a permanent hemopoietic graft. Although a similar inoculum of strain C57BL marrow cells rescues a lethally irradiated F1 mouse from hemopoietic death, the grafted C57BL marrow usually begins to regress within a few months as residual hemopoietic cells of the host supplant the donor marrow graft. The purpose of this study was to determine whether the contrasting abilities of the two parental marrows to provide long-term grafts in F1 recipients were inherited as simple Mendelian traits. A total of 162 (C57BL × 101)F2 mice was classified for sex, color, hemotype, and H-2 serotype. Bone marrow from each F2 donor was injected intravenously into four F1 recipients. Each recipient was tested for the presence or disappearance of donor erythrocytes at 60, 120, and 180 days after marrow infusion. Permanent grafts were not obtained from 12.7 per cent of the F2 donors, which presumably inherited factors associated with, or that mimic, the failure of C57BL marrow grafts in F1 recipients. The nature of these factors is not known, but the factors are not sex-linked and they segregate independently from Hb, A, and H-2, which are loci of linkage groups I, V, and IX.

Comparison of the Acridine-Orange Bone Marrow Cell Viability Test with Dye Exclusion, Tissue Culture Transformation, and Transplantation

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A need exists for a simple in vitro test to determine the viability of hemic cells which have been preserved for human and animal transplantation. The ultraviolet-induced fluorescence of bone marrow cells stained with acridine orange (A-O) was studied to differentiate live from dead or injured cells. Supravital stained coverslip-slide preparations of fresh murine bone marrow cell suspensions were compared with similar suspensions of cells treated to produce injured and dead cells. Fresh cell suspensions were subjected to heat, change of pH, freezing (with and without protective agents), nutrient media deprivation, and cellular poison (NaCN) in order to produce dead and injured cells. The morphology and A-O staining characteristics of these cells were compared with those of fresh marrow cells. The fibroblastic transformation of each specimen of cells in tissue culture was compared with the staining results as an index of viability. Results indicated that “live” cells displayed a bright-green nucleus with varying amounts of small, red granules in a pale-green cytoplasm. Both the nucleus and cytoplasm of “dead” cells stained a diffuse red, or a dull-green without intracellular detail. Questionable or “injured” cells showed a diffuse red-orange hue. In general, the A-O test demonstrated the viability of cells preserved by freezing as effectively as the other in vitro tests. The A-O test may be more sensitive in determining viability of cells where metabolic processes have been injured by poisons, nutrient depletion, or pH change. Preliminary results of comparison of the in vitro A-O and dye exclusion tests and radiation protection effect of marrow preserved by freezing show good correlations.
Preliminary Efforts to Type for Histocompatibility in Dogs

From the Mary Imogene Bassett Hospital, Cooperstown, N. Y.

Sera for typing histocompatibilities were produced by immunizing one dog with the tissues of another. Several schedules of immunization were used. Repeated grafts of skin and repeated injections of marrow, lymph node, and splenic tissue were given over varying periods of time. In several experiments to simplify the patterns of reaction the tissues of one dog were used to immunize several dogs. The sera of immunized dogs were tested for reaction against the erythrocytes and leukocytes of the pertinent donors of tissue. Hemagglutinins were determined by three procedures: (1) against normal erythrocytes suspended in saline, (2) against trypsinized erythrocytes suspended in saline, and (3) against normal erythrocytes suspended in serum and dextran. The latter was selected as the most sensitive and its specificity was confirmed by the Coombs antiglobulin technic. A major problem with hemagglutination of canine erythrocytes has been their tendency to lyse. This difficulty was overcome by inactivating the dog sera at 56 C. for 25 hours and by using glassware cleaned in acid rather than in detergent. The leukocytotoxic, leukoagglutinating and thrombocoagglutinating titers of reactive sera were also measured. The cytotoxic test using dog serum complement was selected for standard procedure. The reactive antibodies, both hemagglutinins and leukocytotoxins, were found to be absorbable by erythrocytes, leukocytes, lymph nodes, and liver tissue. In this respect they differed from Swisher's canine blood group antibodies and resembled antibodies against strong tissue antigens such as the H-2 antigens in the mouse. A panel of antisera that showed good hemagglutinating and leukocytotoxic titers was used to type 40 dogs. The initial observations showed that the typing sera had selectively different patterns of reactivity. This was true even within groups of sera produced by immunizations with the tissues of a single donor. Attempts to simplify reactions by rendering the sera specific for single antigens or groups of antigens are in progress. For this purpose the sera are being absorbed with the leukocytes of selected dogs. The patterns of reaction with the 40 dogs will then be redetermined. Dogs whose reactions are similar with a number of sera will be selected for skin grafting to see whether the implications of histocompatibility are justified.

Preliminary Efforts to Type for Histocompatibility in Random-Bred Mice

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Matching of the H-2 antigens is critically important in securing survival of tissue transplanted between strains of inbred mice. The H-2 and H-3 antigens are of less and variable importance. The following experiments were undertaken to determine to what extent H-2 antigens could be matched in random-bred Swiss-Webster mice and to what extent the matching of donors and recipients for known H-2 antigens might increase survival rates following lethal exposure to radiation and the transplantation of marrow. The antisera for typing H-2 antigens were prepared in inbred mice of known H-2 composition in the laboratory of G. Hoecker at the University of Chile in Santiago. They were absorbed in vitro with tissues of known H-2 content to make them selectively reactive with known H-2 antigens. Sixty Swiss-Webster mice were obtained from a large commercial colony in New York where these mice have been random-bred for 25 years. Their H-2 antigens were determined with the known sera by the erythrocyte agglutination technic of Goris and Mikulski. The significant observations regarding H-2 types in this population were as follows: The animals lacked H-2 antigens D and S and, accordingly, the alleles H-2a, H-2d, and H-2'. Some known H-2 antigens occurred alone, a situation not found in inbred strains. Some mice had peculiar combinations of antigens that could be attributed to their being heterozygous. Others gave re-
Metabolism of Serum Glycoproteins in the Rat-Mouse Chimera

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Concentrations of protein and protein-bound carbohydrate and incorporation of glucosamine-1-C\(_{14}\) in serum fractions were measured in X-irradiated (950 r) mice implanted with rat bone marrow, irradiated mice treated with isologous marrow, and normal mice. Observations were made 23 days after irradiation when atrophy of the splenic white pulp and loss of body weight indicated the rat-mouse chimeras were undergoing the foreign-bone-marrow reaction. At this time the splenic white pulp of the isologous-marrow-treated mice was repopulated and body weight had returned to approximately the pre-irradiation level. Rates of glucosamine incorporation into serum fractions were highest in the rat-mouse chimeras. This group had the lowest concentration of \(\alpha_1\)-globulin-bound carbohydrate and the highest concentration of \(\beta\)-globulin-bound carbohydrate. The chimeras also had the lowest concentrations of albumin and \(\gamma_1\)-globulin. Differences among treatment groups in other serum fractions were not statistically significant (P > 0.05). These observations indicate abnormal serum glycoprotein metabolism in the rat-mouse chimera.

Oxygen Consumption in Lethally Irradiated Mice Given Bone Marrow Intravenously

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Whole-animal metabolism studies following foreign bone marrow transplantation are few in number. McRae (Ph.D. thesis, Univ. of Calif., Berkeley, 1960) has measured food intake, body weight changes, and reticuloendothelial activity. Preliminary studies by McArthur et al. (Abstract, Blood 22:502, 1963) have shown that mice given foreign marrow after lethal irradiation stay in positive nitrogen balance for 60 days, even in the presence of a small weight loss. A very mild clinical secondary disease was present in these animals. As a continuation of whole-animal metabolism studies in radiation
chimeras, the present report summarizes oxygen consumption in lethally irradiated mice given foreign homologous bone marrow. The measurements were made over a period of 90 days after irradiation and marrow transplantation. Measurements on normal and isologous bone marrow-treated animals extended through the 44th week. Three groups of animals were studied. One group was a normal control, and two groups received 900 r total-body X-irradiation followed within one-half hour by an intravenous injection of $40 \times 10^6$ bone marrow cells of isologous (IBM) or homologous $(101 \times C3H)F_1$ (HBM) origin. A total of 34 animals were used in this investigation.

There were no deaths among the normal Rats were irradiated with either 600 or $1000$ r whole-body irradiation and partially hepatectomized 24 or 36 hours after irradiation. The effect of irradiation on DNA synthesis of regenerating liver was investigated by measuring the incorporation of orotic acid $C_{14}$ into DNA and by determining thymidylic kinase and DNA polymerase activities in the liver supernatants. The administration of $1000^2$ blocks DNA synthesis in vivo (40 per cent) and reduces thymidylic kinase activity ($\sim 50$ per cent) but not that of DNA polymerase. The administration of $600^2$ inhibits DNA synthesis in vivo (70 per cent) but is without effect on either thymidylic kinase of DNA polymerase activities. The differences in the radiosensitivity of the two enzyme-forming systems responsible for the synthesis of thymidylic kinase and DNA polymerase will be discussed.

**SOME MOLECULAR ASPECTS OF RADIATION INJURY AND RECOVERY IN MAMMALIAN TISSUES**

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Rats were irradiated with either 600 or 1000 r whole-body irradiation and partially hepatectomized 24 or 36 hours after irradiation. The effect of irradiation on DNA synthesis of regenerating liver was investigated by measuring the incorporation of orotic acid $C_{14}$ into DNA and by determining thymidylic kinase and DNA polymerase activities in the liver supernatants. The administration of 10002 blocks DNA synthesis in vivo (40 per cent) and reduces thymidylic kinase activity ($\sim 50$ per cent) but not that of DNA polymerase. The administration of 6002 inhibits DNA synthesis in vivo (70 per cent) but is without effect on either thymidylic kinase of DNA polymerase activities. The differences in the radiosensitivity of the two enzyme-forming systems responsible for the synthesis of thymidylic kinase and DNA polymerase will be discussed.

**SEQUENCE OF EVENTS IN DEPRESSION AND RECOVERY OF GRANULOCYTES FOLLOWING TOTAL BODY IRRADIATION**

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The compartments or stages of normal development of the precursor granulocytic elements formed in bone marrow are described in terms of number of cells and time spent in each compartment. This self-perpetuating system, initiated at the stem cell level, suggested a pattern of granulocyte proliferation with orderly growth and development (steady state). Following total-body irradiation, the normal process is modified. Some stem cells are destroyed and some proliferate according to the normal cycle. A few develop by means of an altered proliferation pattern (non-steady state) and these are responsible for recovery. Comparison of the steady-state cycle with the non-steady-state proliferaton led to a quantitative analysis of post-irradiation granulocyte concentration in the peripheral circulation. Exposure ranged from 50 r to 250 r for patients receiving therapeutic total-body irradiation and calculations were based on granulocytes in peripheral blood during depression and recovery. In this dose range, the relation between stem cells destroyed and amount of radiation followed a sigmoid curve similar to the LD curve for humans. Surviving stem cells continuing normal proliferation were calculated; many of these disappear after four or five divisions. Following a single
exposure of 200 r, approximately 7 per cent of the stem cells continue development according to the modified proliferation pattern. This study finds application to bone marrow transplantation in the treatment of radiation injury. It provides (1) an indication of the time following radiation exposure when bone marrow transplant would be most beneficial and (2) a basis for estimating the amount of successfully transplanted marrow necessary for effective treatment.

CELL DIVISION IN A SPECIES OF ERWINIA: SOME EFFECTS OF PANTOYL LACTONE

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Cell division can be inhibited in a species of Erwinia by d-serine, penicillin, vancomycin, mitomycin C, or ultraviolet (UV) light. Individual cells can attain lengths of 100 μ or more (1–2 μ is considered normal). All these division inhibitions can be reversed by adding pantoyl lactone to the growth medium. Glucosamine (GA) and muramic acid (MA) content have been studied as parameters for cell wall composition in division-inhibited cells and cells made to divide "normally" in the presence of pantoyl lactone. Mitomycin C, vancomycin, penicillin, and d-serine increase the GA/MA ratio. Preliminary studies using UV light indicate minimal, if any, changes. Each agent lowers the amount of MA, and pantoyl lactone creates conditions for an increase in the level of MA. Thus one effect of pantoyl lactone is the restoration of the cell wall mucopolysaccharide to near-normal levels. Large amounts of two keto acids accumulate in the growth medium in the presence of all division-inhibiting agents. One of the keto acids is pyruvic acid; the other is not identified. Accumulation of both keto acids is greatly reduced when pantoyl lactone is present. All division-inhibiting agents allow for large amounts of 260/280 absorbing material to accumulate in the growth medium. Presence of pantoyl lactone causes a significant decrease of 260/280 absorbing material in all situations. The levels of 260/280 absorbing material and keto acids present in the medium of cells growing after UV irradiation are inversely related to the concentration of pantoyl lactone in the growth medium.

GENETIC CONTROL OF RADIATION SENSITIVITY AND CELL DIVISION

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The capacity for normal cell division is an important criterion of radiation damage in bacteria as well as other cells. In the bacterium Escherichia coli, cell division may be considered complete when a wall completely separating the daughter cells has been synthesized. The bacterium Escherichia coli K-12 AB1899 was first recognized as an organism sensitive to ultraviolet and ionizing radiations. Experiments have established that the basis of its radiation behavior is a genetic alteration that has resulted in a disturbance in the mechanism by which cells produce that section of cell wall separating the daughter cells. The genetic locus involved has been mapped by conventional techniques. After irradiation, cells of this organism continue to grow, synthesize DNA and RNA, and produce nuclei but fail to form cross-walls. These filaments do not regain the capacity for normal division except in the presence of certain biochemicals related to the synthesis of pantothenic acid. Pantoyl lactone and omega methyl pantoyl lactone are particularly effective in triggering the normal division of filaments. Once normal division has resumed, the presence of the lactones is not required for its maintenance.
GENIC CONTROL OF CHROMOSOME BEHAVIOR IN DROSOPHILA: CLARET-NONDISJUNCTIONAL

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It is generally assumed that the regulation of chromosome behavior in cell division is controlled by genes. Ordinarily the precision is great, so that chromosomes divide and pass to the poles regularly, failing only very rarely (less than 0.1 per cent). Thus when mutants arise which fail to maintain the regular precision of division, some normally rare types of chromosome misbehavior may be studied with relative ease. Drosophila melanogaster females homozygous for the mutant claret-nondisjunctional (ca"1) were tested to determine the nature and extent of chromosome nondisjunction and loss during egg maturation and embryonic development. In addition to that already known for the X chromosome, nondisjunction and loss of each of the autosomes were examined. Different chromosome pairs tended to be concomitantly involved in nondisjunction much more often than expected if nondisjunction were a random event. Likewise, chromosomes from different pairs tended to be concomitantly lost much more often than expected if loss were a random event. Nondisjunction and loss were found to be separate and distinct events sharing the property of nonrandomness and differing in times of occurrence. Tests of nondisjunction of dominantly marked fourth chromosomes, structurally heterozygous X chromosomes, and a compound X and a Y chromosome showed that nondisjunction occurred at the first and, within the limits of analysis, never at the second meiotic division. Nondisjunction occurred when nonexchange pairing occurred as well as when exchange was permitted. Since nondisjunction and loss shared the property of nonrandom involvement of different chromosome pairs and since nondisjunction and loss differed in the times at which they occurred, a mechanism was indicated having alternate results depending upon the mechanics of the divisions involved. Abnormal chromosome behavior in eggs was attributed to defective achromatic components of the spindle that would cause nondisjunction of bivalents at anaphase I and loss of separated daughter chromosomes at anaphase II and subsequent cleavage mitoses. It is believed, therefore, that the basic defect in ca"1 eggs is malfunctioning, and possibly malformed, spindle fibers which cause instability of both meiotic and mitotic divisions. This work will be published in detail elsewhere.

THE FATE OF PLASMA CELLS DURING AN IMMUNE RESPONSE

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Electron microscopic observations are reported that have been made on (101 × C3H)F1 mouse splenic tissue fixed at daily intervals following a single intravenous injection of either 2 × 10⁶ sheep erythrocytes or 100 × 10⁶ rat bone marrow cells. The development of plasma cells characterized ultrastructurally by a lamellar system of ergastoplasm filling the entire cytoplasm from large primitive lymphatic cells was followed. Many immature plasma cells were present in the spleen on day 4, whereas by day 10 few plasmacytes were found. As early as 3 days after antigen injection and with increasing frequency to day 7, phagocytized plasma cells with pyknotic nuclei, swollen mitochondria, and condensed cytoplasm were observed in thin sections of tissue. Histiocytes and reticular macrophages in both red and white pulp contained engulfed plasma cells. The phagocytosis of plasma cells parallels their disappearance from the spleen and is correlated with the log phase of antibody appearance in the serum. Support is given to the concept that plasma cells are terminal cells. The role of migration, de-differentiation, or transformation under conditions used in this experiment is undetermined.
The femoral artery of six beagle dogs was clamped. Immediately after arresting circulation, H³-thymidine was injected in a dose of 1 μC/Gm. body weight by sublingual vein. After 40 minutes the clamps were released. Bone marrow samples were aspirated from the clamped leg (experimental marrow) and unclamped leg (control marrow) 10, 20, 30, and 45 minutes; 1, 3, 6, 12 hours, and 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, and 16 days after the injection of H³-thymidine. Venous blood smears were made at the same time. Preparations were processed for autoradiography (exposure 50 days). No labeled cells were found in the experimental marrow of all dogs during clamping and up to 3 hours, indicating the effectiveness of clamping. Thereafter heavily labeled small lymphoid cells appeared in the experimental marrow, increasing in number up to day 6. The labeling indices of these cells, however, were significantly lower than those of the control marrow. From 7 hours through the last day of the experiment, erythroblasts of any stage of maturation had a very low labeling index and mean grain count (never higher than 5), whereas the latter were significantly higher in the control marrow up to day 3. On the other hand, the mean grain count of the erythroblasts of experimental marrow was significantly lower than that of the small lymphoid cells during the first 48 hours. The data suggest that the dog bone marrow may contain two populations of small lymphoid cells. One population apparently originates from initially labeled (unknown) precursors within the bone marrow. The other reaches the marrow from other areas via the blood stream. No evidence has been obtained so far that small lymphoid cells, which have migrated, can transform into erythroblasts or other hemopoietic cells within 2 days.
lymphocytes, normoblasts with pyknotic nuclei, metamyelocytes and granulocytes. The lymphocyte enrichment was inversely related to the total cell yield and directly related to the compactness of the columns. Known amounts of the original and of the filtered marrow were transplanted into isogenic recipient mice exposed a few hours earlier to 900 r of X-rays. The proliferative capacity of the transplanted marrow was estimated by measuring the marrow's ability to promote DNA synthesis in the spleens of the recipients, as judged by incorporation of labeled 5-iododeoxyuridine ($^{131}$I UdR), a specific DNA precursor, 5 days after transplantation. The hemopoietic function of the transplanted marrow was estimated by examining histologic sections of the recipients' hemopoietic sites 10 to 30 days later. Neither the filtration procedure nor the absence of blast cells was observed to reduce the proliferative and hemopoietic competence of the marrow, since a normal degree of $^{131}$I UdR incorporation, hemopoietic colony formation and 30-day survival were accomplished by the filtered marrow. The uptake of $^{131}$I UdR in the recipient spleens appeared, furthermore, to be correlated with the content of small and medium lymphocytes in both the unfiltered and in the filtered marrow, and not with any other cell type. The results indicate, therefore, that the marrow lymphocyte is indeed a multipotent hemopoietic stem cell in the mouse.

**Bone Marrow Lymphocytes of the Rat and Guinea Pig**

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Experiments using tritiated thymidine (TTH) and radioautography were designed to investigate the origin, life span, and possible developmental potential of bone marrow small lymphocytes in rats and guinea pigs. The bone marrow was found to have the most rapid turnover rate of small lymphocytes of the lymphocytopoietic tissues, including the thymus. By 3 days of continuous TTH infusion, 97 per cent of marrow small lymphocytes in the rat were labeled as compared with 5 per cent of the small cells in the thoracic duct lymph. Two types of experiments, using guinea pigs, showed that marrow small lymphocytes have an in situ origin. First, bone marrow was removed from animals with 2½ hours after giving TTH and cultured in millipore chambers. (At this time essentially none of the small lymphocytes are labeled.) Small lymphocyte development in the cultured marrow was comparable to that in marrow of intact controls as evidenced by the per cent of labeled cells and their rate of appearance. In the second experiment, guinea pigs having the circulation of one hind limb occluded were given a single intravenous injection of TTH. The occlusion bandage was removed after 20 minutes, by which time the TTH is largely cleared from the circulation. By 72 hours post-TTH, only 2 per cent or less of the small lymphocytes of the occluded limbs were labeled. In contrast, 40 per cent of the small lymphocytes were labeled in the marrows of nonoccluded limbs. In other experiments, using an inbred strain of rats (Lewis), labeled small lymphocytes from thoracic duct lymph or labeled thymocytes were transfused to irradiated and nonirradiated animals. No labeled thoracic duct cells were observed in the marrow of recipients, although many labeled thymocytes were present in the marrow of both irradiated and nonirradiated animals between 4 and 48 hours post-transfusion. Examination of a series of marrows from irradiated and nonirradiated rats which had received labeled thymocytes provided no evidence that these cells serve as stem cells for the myeloid elements. Experiments in which the thymus of guinea pigs was shielded during lethal irradiation provided no evidence that the thymus influenced the recovery of bone marrow hemopoiesis.