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Blood-Bone Marrow Tissue Culture and Cell Separation

Abstracts of presentations at a conference held on October 20 and 21, 1961 at the Clinical Center Auditorium, National Institutes of Health, Bethesda, Maryland

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AND C. C. CONGDON, Co-chairmen

INTRODUCTION

The purpose of the conference was to bring together different groups of investigators in experimental hematology, including those working with white blood cell tissue culture, bone marrow tissue culture, cell separation, and bone marrow transplantation. In white blood cell tissue culture the major effort has been devoted to cytodiagnostic uses of the technic. Besides this aspect, work was presented at the present conference on improvements in the culture method, use of white blood cell cultures to investigate cell growth in vitro by autoradiographic and other methods, effects of hormonal treatment on growth of white blood cells in culture and application of the culture technic to white blood cells of various animal species. Perhaps the most interesting points in the discussion of these papers concerned the origin and nature of the cell that grows out in white blood cell tissue cultures, and the mechanism of action of phytohemagglutinin in stimulating cell proliferation. One suggestion was that the proliferating cells came from immunologically competent elements derived from lymphatic tissue and that phytohemagglutinin acted as a special antigenic material to stimulate cell division. Rather intriguing new developments have occurred in bone marrow tissue culture suggesting that changing the temperature or growing cells in special dialysis bags might lead to actual tissue culture of differentiating bone marrow cells. Ecological problems in bone marrow tissue culture were also discussed. New developments in separation of white blood cells, platelets, bone marrow cells, and peritoneal exudate cells give promise of being of great importance in experimental hematology. This was especially clear from evidence presented on the presence of stem cells among the separated white blood cells of mice.

CHROMOSOME STUDIES IN CHRONIC MYELOID LEUKEMIA

Patricia A. Jacobs and Ishbel M. Tough, Edinburgh, Scotland

Chromosome studies on peripheral blood cultures from 14 untreated cases of chronic myeloid leukemia are reported. The Phi^1 chromosome was always present, the frequency of cells carrying it varying from 38 per cent to 100 per cent. In four of the patients a further chromosome abnormality was seen. Two had a developmental chromosome abnormality shown to be present in skin as well as blood—one was an XY/XXY mosaic (Tough et al.: Lancet 1:411, 1961), while the other is presumed to have a reciprocal translocation between one chromosome number 16 and one in the medium size range. The remaining two patients had a chromosome abnormality which is possibly restricted to the leukemic cells, as there is at present no evidence for the abnormality occurring in cells not carrying the Phi^1 chromosome. In five of the patients it was possible to culture blood serially throughout a course of x-irradiation to the spleen, and details of chromosome studies during treatment are given. In all five cases the frequency of the Phi^1 chromosome fell during treatment, and could not be detected at all in the peripheral blood at the end of therapy.

INDUCED AND SPONTANEOUS CHROMOSOME ABERRATIONS IN HUMAN LEUKOCYTES

IRRADIATED IN VIVO AND IN VITRO

P. Carolyn Gooch, Oak Ridge, Tenn.

The peripheral leukocyte culture method has made it possible to study spontaneous and induced chromosomal aberrations in vivo and in vitro. Measurement of aberra-
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IMPROVEMENT IN WHITE CELL CULTURE INCLUDING DIFFERENTIAL LEUKOCYTE SEPARATION
Herbert L. Cooper and Kurt Hirschhorn, New York, N. Y.

There are currently several variations of peripheral blood leukocyte culture techics in use. All share the same basic elements, and some of the same problems. The features common to all methods are: 1) Obtaining an anticoagulated venous blood specimen; 2) separation of erythrocytes from leukocytes; 3) addition of suitable culture medium; 4) addition of a vegetable extract found empirically to possess the properties of panhemagglutination and mitotic stimulation; 5) after adequate period of incubation, a mitotic inhibitor is added; 6) cells are harvested, treated with hypotonic salt solution, fixed and prepared for examination by staining and squashing, or by air-drying and staining. Experience of most workers with these techics has been that approximately 25 per cent of cultures fail to grow. In many, this failure may be related to the appearance shortly after the beginning of incubation of a dense coagulum which appears to enmesh the growing cells and either prevents growth or harvesting of growing cells. Such coagula consist of dense agglutinates of polymorphonuclear leukocytes. By utilizing a technic involving phagocytosis of iron particles and subsequent exposure to a magnetic field, we have been able markedly to reduce the proportion of polymorphonuclear leukocytes in white cell suspension. The resulting suspensions contain mainly lymphocytes, and culture of such suspensions never produces a coagulum. We have by this method increased our percentage of successfully grown cultures to over 95 per cent.

HORMONE STUDIES WITH LEUKOCYTE CULTURES

Inhibition by prednisolone of mitotic activity of normal human mononuclear leukocytes in short-term culture has been demonstrated, with a roughly linear relation between the log dose of steroid and the percentage decrease in mitoses. Over a concentration range of 0.002 μg./ml. to 10 μg./ml., the mitotic index decreased from 100 per cent to 25 per cent of control values. Further studies indicate that most of this inhibitory effect of the steroid operates during the first 24 hours in tissue culture, although the effect is not demonstrable until the second and third day when mitosis begins. The findings indicate that prednisolone does not significantly inhibit mitosis directly in these cultures, but rather inhibits the conversion of partially differentiated circulating mononuclear leukocytes to a state wherein they are capable of mitosis. This action occurs at the same time as that of phytohemagglutinin, which initiates mitosis in these cultures by stimulating the conversion process. Epinephrine (0.1 μg./ml. to 10 μg./ml.) has shown an inhibitory effect on the initiation of mitosis in these cultures similar to that of prednisolone. Growth hormone (monkey) and insulin have failed to produce any demonstrable effects on mitosis in this system.
PHYSICOCHEMICAL CONDITIONS AFFECTING MITOSES OF PERIPHERAL LEUKOCYTES

T. Sato, Y. Ohnuki, A. Awa and C. M. Pomerat, Pasadena, Calif.

The quest for more efficient phytohemagglutinins led us to examine the growth-promoting capacity of a series of crystalline proteins isolated from legumes by Dr. Paul Melnychyn of the Lima Bean Growers Association of California and the U. S. Department of Agriculture. Populations of standard human amnion (Fernandes) and the HeLa cell lines seeded in T30 flasks were studied with the aid of the Coulter counter. Treatment for five days with proteins from lima, kidney, navy and pinto beans was not found to enhance growth. Neither was stimulation obtained with phytohemagglutinin M or P at 0.01, 0.1, and 1.0 per cent per ml., although the latter dose of the P compound proved relatively toxic. Human amnion maintained on a diet of human serum, and growing luxuriantly, showed marked injury when horse serum was substituted as the protein factor in a parallel line, even in the second passage. Lima bean protein at 0.1 mg. per ml. appeared to relieve this injury, as measured by increase in population size. In contrast with phytohemagglutinin “Exp” and M, the P fraction yielded the poorest growth. The amount of plasma obtained from centrifuging 10 ml. of blood under comparable conditions gave 0.8, 3.8, and 1.0 ml. for control, phytohemagglutinin M, and lima bean protein, respectively. The corresponding number of white cells in these sera were $5.0 \times 10^5$, $17.0 \times 10^5$, and $9.7 \times 10^5$.

From these results, phytohemagglutinin M (Lot No. 451224) proved most favorable for obtaining high mitotic counts. Employing the most suitable procedure for studying the human leukocytes, we were unsuccessful in obtaining useful cultures of the monkey blood (Macaca mullata). Among systematic variations in technics were: phytohemagglutinins M and P at various concentrations, centrifugation rates and time, noninactivated calf, horse, and monkey sera, and the ratio of cells to medium.

CELLULAR KINETICS OF A PERIPHERAL BLOOD CELL SYSTEM TREATED WITH PHYTOHEMAGGLUTININ


The technics of high resolution autoradiography, ultraviolet (UV) absorption of cellular materials in the nucleic acid range, and isotope analysis (planchette counting) were used in an attempt to explain the mechanism and kinetics of cellular changes in a human peripheral blood cell system treated with phytohemagglutinin. Autoradiographic findings using tritium-labeled thymidine indicated a relatively low uptake of DNA precursor by cells of this system until about 48 hours, when both the proportion of labeled cells and the average grain count per labeled cell increased substantially. These parameters further increased at 72 hours. Cellular materials absorbing in the UV region of 2800 A, however, showed an average increase of 100 per cent after 48 hours’ culture. Uptake of C14-adenine by these cells followed the aforementioned changes in UV absorption, while the uptake of C14-thymidine confirmed the pattern of tritium-labeled thymidine uptake noted after the autoradiographic studies. Present data appeared to favor transformation of pre-existing cellular elements rather than the outgrowing of an originally small, but potentially fast-growing, population of cells.

A BLOOD CELL CULTURE TECHNIC FOR THE STUDY OF THE SATELITES AND SECONDARY CONSTRICIONS OF 10 PAIRS OF HUMAN CHROMOSOMES

O. J. Miller and W. R. Berg, Southbury, Conn.

Correct interpretation of the observed variation in human somatic metaphase chromosome morphology is essential. The purpose of the present study has been to evaluate different methods of fixation and of flattening cells in terms of the clarity of such details of chromosome morphology as the satellites and secondary constrictions. Two fixatives were compared: (1) 45 per cent acetic acid, and (2) a 3:1 mixture of
DNA, RNA, AND PROTEIN SYNTHESIS, AND THE ONSET OF MITOSIS IN HUMAN LEUKOCYTES IN VITRO

M. A. Bender and D. M. Prescott, Oak Ridge, Tenn.

Experimental studies on human leukocyte chromosomes require information concerning the timing of DNA, RNA, and protein synthesis, the time of the onset of mitosis, duration of the mitotic cycle, and the number of mitoses undergone by individual leukocytes in vitro. We have used tritium labeling and autoradiography to study some of these parameters. The number of cells synthesizing DNA in peripheral blood is very low. When leukocytes are placed in a culture medium containing phytohemagglutinin, they show an increased DNA synthesis beginning at about 24 hours. Mitosis starts between 42 and 48 hours. These events occur in two types of cells: a large mononuclear cell, and a smaller cell similar to a lymphocyte. Our experiments establish limits of a minimum of 24 hours, a minimum of 12 hours, and a maximum of 6 hours, respectively for the lengths of the pre-DNA-synthesis period, the synthesis period, and the post-DNA-synthesis period. Also, they show conclusively that the same leukocytes undergo at least four consecutive divisions. RNA synthesis becomes progressively more and more intense in leukocyte cultures, leveling off at about 24 hours. The active cells are probably the same as those that synthesize DNA. Incomplete studies suggest that this is also true of protein synthesis.

STUDIES ON PHYTOHEMAGGLUTININ-STIMULATED LEUKOCYTE CULTURES


A number of variables in the previously described technic (Exp. Cell Res. 20:613, 1960) of cultivating peripheral blood leukocytes have been examined. These studies were undertaken to determine what modifications would result in the maximum yield of successful chromosome preparations from human material, and permit shipment of cells from outlying communities to a central laboratory for culture and karyotype analysis. Leukocytes suspended in plasma were obtained by gravity sedimentation of heparinized blood at room temperature. The cells in these suspensions remained viable at 5 C. for up to 96 hours, but did not survive for 24 hours at room temperature. Phytohemagglutinin P (0.04 ml.) was added directly to each 8-ml. culture. Higher concentrations induced agglutination of the leukocytes, interfering with the metabolism and subsequent preparation of chromosome spreads. Storage of cells in the refrigerator for up to 96 hours did not eliminate the need for phytohemagglutinin as a mitotic squash preparations and permanent slides made from them showed that the temporary preparations were superior. Further use of the 2a procedure permits the following conclusions to be reached. The number and size of the satellites or the acrocentric chromosomes in man are relatively constant from cell to cell in each individual, but vary markedly from individual to individual. Negatively heteropycnotic regions, or secondary constrictions, have been observed on at least five of the nonacrocentric chromosomes i.e., numbers 1, 3, 4, or 5, at least one chromosome in group 6-12, and at least one chromosome in group 16-18. Individual variation is a striking feature in both satellites and secondary constrictions, as studied by this technic.

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absolute ethanol-glacial acetic acid. Two methods of flattening cells were compared: (1) squashing a suspension of cells in 45 per cent acetic acid, and (2) air-drying. All preparations were stained with acetic orcein. The squash preparations were examined and photographed as temporary wet mounts with no seal around the cover slips. Combinations of the above treatment schemes can be designated 1a, 1b, 2a, and 2b. In other respects, the blood cell culture technic that was used was no different from other technics in common use, except that the period of treatment with 1 per cent sodium citrate was never longer than 6 minutes. In terms of the clarity of chromosomal detail, 2a was the best combination, followed by 2b, 1b, and 1a. A further comparison between the temporary wet squash preparations and permanent slides made from them showed that the temporary preparations were superior. Further use of the 2a procedure permits the following conclusions to be reached. The number and size of the satellites or the acrocentric chromosomes in man are relatively constant from cell to cell in each individual, but vary markedly from individual to individual. Negatively heteropycnotic regions, or secondary constrictions, have been observed on at least five of the nonacrocentric chromosomes i.e., numbers 1, 3, 4, or 5, at least one chromosome in group 6-12, and at least one chromosome in group 16-18. Individual variation is a striking feature in both satellites and secondary constrictions, as studied by this technic.
LONG TERM CULTURE OF HUMAN BONE MARROW AT 27 C. TO STUDY HEMOGLOBIN BIOSYNTHESIS
Lorraine M. Kraus, Memphis, Tenn.

Aspirated human bone marrow or bone fragments containing red marrow were incubated at 27 C. with Tyrode's solution containing 10 per cent patient's plasma in sealed culture dishes. The level of the culture media was adjusted to provide an air space of 4-6 mm beneath the cover. Throughout the incubation period the cultures were allowed to stand without being shaken or inverted. Tyrode's media gradually becomes acid as the cells grow in sheets or clumps on the bottom of the dish. There is a visible increase in the density of the cellular layer, while the supernatant media remains clear. In addition, bone fragments during growth produce a lipid material which accumulates on the surface of the media. Mitotic figures were found in direct smears of the cultured cells after 11 and 16 days of incubation. Viability of all types of cells was demonstrated throughout the culture period by staining with brilliant cresyl blue. The erythrocytic series of cells incorporate iron-59 into hemoglobin after 10 days of culture. The hemoglobin synthesized in tissue culture for 10 or more days is under investigation.

CULTIVATION OF HUMAN BONE MARROW AND CANCER CELLS IN AUTOLOGOUS-HEPARINIZED WHOLE BLOOD
Lester Jankay and Nathaniel B. Kurnick, Los Angeles, Calif.

The purpose of these studies was to maintain bone marrow, peripheral blood cells and cancer cells for a prolonged period of time in vitro with retention of normal morphology. Whole blood deteriorates in one day if incubated at 37 C. To maintain blood at 37 C., we have constructed a disposable "circulating-dialyzing-chamber," which keeps the heparinized whole blood continuously agitated, oxygenated and dialyzed. A mixture of 95 per cent air and 5 per cent CO₂ is used as gas phase. The dialyzing fluid consists of normal human plasma heated at 56 C. for 20 min. The dialyzing fluid is changed every 3-4 days. In this system the blood is free of hemolysis during the first 2 weeks at 37 C. Bone marrow cells maintain normal morphology over 20 days. Erythroid and myeloid maturation takes place. Mitotic figures are observed in reticulum cells and myeloid cells during the cultivation period. Regional blood containing cancer cells is obtained from cancer patients during surgery. The cultures of this regional blood and also fresh fragments of cancer tissue maintain good cellular and tissue morphology over 10 days. We conclude that (1) heparinized whole blood can be used effectively as culture medium, (2) blood can be kept at 37 C. without any appreciable hemolysis over 2 weeks, and (3) bone marrow cells maintain normal morphology without spindle cell formation.

PROBLEMS RELATED TO THE USE OF TISSUE CULTURED HEMIC CELLS IN MAMMALIAN TRANSPLANTATION EXPERIMENTS
Daniel Billen, Houston, Texas

Several of the problems associated with the use of the tissue culture technic in the hemic tissue transplantation experiment will be discussed. One of the conditions to be satisfied for successful hemic-induced recovery in mice is infusion of a sufficient number of functional cells. Following ex-planting of bone marrow, there is recorded
a sharp drop in surviving cells during the first week of in vitro existence (25 per cent eosin-negative survivors by the seventh day). Qualitative changes in the population also occur. A maturation of the immature forms occurs early. This is followed by degeneration of the terminal cells without replacement. By the end of the third week, large epithelial-like cells, monocytes, and macrophages are in the majority. Time-lapse cinematographic records of these changes have been made. By the end of the third week of in vitro culture, infusion of tissue cells into lethally irradiated mice in sufficient numbers, as determined with fresh bone marrow cells, brings about little, if any, sparing of death. Using appropriate conditions of culture, population growth will occur between one and two months. Epithelial-like cells usually predominate in this phase and eventually form a population which can be subcultured indefinitely. We have found that special nutritional environments may favor the development of cells morphologically resembling monocytes. All cell lines so far tested have been found incapable of reversing otherwise fatal radiation-induced injury in recipient animals. In addition to the problem of selective growth of "non-effective" hemic cells, there is the phenomenon of chromosomal change, both quantitative and qualitative in nature, occurring at about the same time as the establishment of marked cell proliferation. Such chromosomal alterations may preclude normal function of initially effective cell types. The use of the tissue culture method for propagation of functional hemic cells requires further extensive investigation into the factors controlling cell morphogenesis.

**Experience with Tissue Culture and Transplantation of Fetal Liver Cells**

**J. H. Githens, Louisville, Ky.**

Studies have been carried out to determine the application of various tissue culture methods for the preservation of hematopoietic cells from fetal liver of mice. Several methods have been evaluated for growing colonies of cells. The best growth has been obtained by plating small tissue particles in a media of T 199, chick embryo extract and fetal calf serum (method of Cooper). Study of cell growth with this method has revealed that the gestational age of the fetal liver determines in large part the cell type that ultimately predominates in the culture. Livers from fetuses of 12-16 days' gestation develop into almost a pure culture of small mononuclear cells derived from hematopoietic tissue, even though there is an early proliferation of liver cells. Cell growth from livers of fetuses of 18-21 days gestation, on the other hand, is characterized by the proliferation of fibroblast-like cells derived from liver cells. At the same time there is a gradual disappearance of hematopoietic cells. Study of the radiation protection effect of tissue cultured fetal liver cells has been, in general, unrewarding. Good protection has been obtained with cells cultured by the method of Cooper for a few days, but difficulty in harvesting cells has limited protection studies with long-term cultures. Several organ culture methods have been applied to fetal liver tissue. Approximately 25-40 per cent protection has been obtained with isologous tissue cultured up to four days on tantalum screen in T 199 and fetal serum (method of Reisner), and on agar clots (method of Las Fargues). Cultures of six days or more have not shown protection and the tissue from older cultures has been lethally toxic.

**Ecology of Human Hemic Cells**

**E. E. Osgood, Portland, Ore.**

The importance of thinking in terms of an ecology of cells in planning and interpreting all experiments either in culture or in vivo is emphasized. Ecology is defined as the interaction of living things with each other and their total environment. Measurable criteria of effects are outlined and the importance of the alpha, 2 alpha; alpha, n and n, 2 n types of cell division in the interpretations of such effects are stressed. The major ecologic factors are outlined and illustrated. These ecologic factors and their subdivisions are: (1) *physical factors*—space, time, surface, fluid suspension, viscosity of media, flow rates, shearing forces, charge distribution and radiation; (2) *micro-
CHROMOSOME PREPARATIONS BY THE BLOOD TISSUE CULTURE TECHNIC IN VARIOUS LABORATORY ANIMALS

Warren W. Nichols and Albert Levan, Camden, N. Y. and Lund, Sweden

Chromosome preparations from the blood of laboratory animals have been possible, utilizing the technic of Moorhead et al. with some modifications. These modifications consisted of using either 6 per cent dextran in PSS (MW75,000) or 6 per cent filtered fibrinogen as a separating agent and supplementing the growth media with calf serum in all cases, rather than isologous serum. After separation with either dextran or fibrinogen the white cell suspension must be spun down and the separating agent decanted and then the white cells resuspended in growth media. This prevents clotting and/or membrane formation in the final culture.

Some of the variables important in the separation of granulocytes from normal human blood by their selective attachment to glass bead columns have been described previously (Garvin: J. Exper. Med. 114:51, 1961). Further study of these variables has been carried out in the somewhat simpler system obtained by suspending the cells from rat peritoneal exudates, chiefly polymorphonuclear neutrophils (PMN) in balanced salt solution plus serum. Using unsiliconized glass beads as the adsorbant, an unexpected effect of serum was observed. It was found that ethylenediaminetetra acetic acid (EDTA) at 10 mM blocked the adhesiveness of the PMN only if serum was present. Under identical conditions, but in the absence of serum, the PMN were strongly adherent. This serum factor was found to be nondialyzable and fully active down to a concentration of 5 per cent serum. Human serum was found to be as active for rat PMN as rat serum. Human serum fractions II, III, IV, V, and VI showed very low activities, either singly or together. The locus of the serum activity does not appear to be on the PMN directly; since hard-packed buttons of PMN obtained by centrifugation suspended easily without clumping when 10 mM EDTA was added in the absence of serum, whereas, under identical conditions, the PMN were strongly adherent to the unsiliconized glass beads. On the other hand, when, instead of unsiliconized glass beads, an adsorbant surface coated with whole serum protein was used, the PMN were nonadhesive in the presence of EDTA even when no serum was added. These data are consistent with the hypothesis that there is a serum fraction which coats naked density; infection; and (5) pharmacologic—innumerable agents with dose rate = conc./time as an equally important variable. Among the illustrations are the uncoiling and dissociation of chromosomes dependent on the monovalent-divalent cation balance, changes in colony pattern, labeling and cell proportions dependent on the gradient factor, and the evolutionary changes in life span and doubling time with time since isolation.

BIOCHEMICAL MECHANISMS IN THE SEPARATION OF LEUKOCYTES BY SELECTIVE ADSORPTION

James E. Garvin, Chicago, Ill.

Some of the variables important in the separation of granulocytes from normal human blood by their selective attachment to glass bead columns have been described previously (Garvin: J. Exper. Med. 114:51, 1961). Further study of these variables has been carried out in the somewhat simpler system obtained by suspending the cells from rat peritoneal exudates, chiefly polymorphonuclear neutrophils (PMN) in balanced salt solution plus serum. Using unsiliconized glass beads as the adsorbant, an unexpected effect of serum was observed. It was found that ethylenediaminetetra acetic acid (EDTA) at 10 mM blocked the adhesiveness of the PMN only if serum was present. Under identical conditions, but in the absence of serum, the PMN were strongly adherent. This serum factor was found to be nondialyzable and fully active down to a concentration of 5 per cent serum. Human serum was found to be as active for rat PMN as rat serum. Human serum fractions II, III, IV, V, and VI showed very low activities, either singly or together. The locus of the serum activity does not appear to be on the PMN directly; since hard-packed buttons of PMN obtained by centrifugation suspended easily without clumping when 10 mM EDTA was added in the absence of serum, whereas, under identical conditions, the PMN were strongly adherent to the unsiliconized glass beads. On the other hand, when, instead of unsiliconized glass beads, an adsorbant surface coated with whole serum protein was used, the PMN were nonadhesive in the presence of EDTA even when no serum was added. These data are consistent with the hypothesis that there is a serum fraction which coats naked density; infection; and (5) pharmacologic—innumerable agents with dose rate = conc./time as an equally important variable. Among the illustrations are the uncoiling and dissociation of chromosomes dependent on the monovalent-divalent cation balance, changes in colony pattern, labeling and cell proportions dependent on the gradient factor, and the evolutionary changes in life span and doubling time with time since isolation.
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glass, and that the attachment of the PMN to this coating can be blocked by EDTA, whereas if this coating is not present, the PMN firmly adhere to the glass in spite of the EDTA. Work aimed at the identification of the serum component is in progress.

CENTRIFUGAL SUBFRACTIONATION OF BONE MARROW

Sadek K. Hilal, Tauno E. Keta, Bertram F. Woolfrey and Donn G. Mosser,
Minneapolis, Minn.

The separation of various species of cells of the dog’s bone marrow was approached using the density gradient centrifugation technic. A cell suspension solution that keeps the cells dispersed without clumping was developed. The bone marrow cells so suspended were layered above a combined viscosity and density gradient in specially developed plastic tubes. The density and viscosity gradients were prepared using various concentrations of various molecular weights of dextran. The change in the concentration provided the density gradient and the change in the molecular weight provided the viscosity gradient. All the solutions used are nontoxic and isotonic. They were buffered to a slightly alkaline pH; their surface tension was lowered by a combination of surface active agents; and they could withstand sterilization by autoclave. At the end of the procedure, 90 per cent of the original viable cells were still living, as determined by the trypan blue technic and the tritiated thymidine incorporation. The lighter layers contained the immature forms of both the myelocytic and normocytic series. The more mature forms were in the heavier layers. Autoradiographs indicated that the lighter layer contained a population of cells that was more heavily labeled by tritiated thymidine.

EVIDENCE FOR STEM CELLS IN THE PERIPHERAL BLOOD OF MICE

Joan Wright Goodman and George S. Hodgson, Oak Ridge, Tenn. and Santiago, Chile

Interest in the question of stem cells in peripheral blood has been stimulated recently by data from leukocyte culture experiments and by the demonstration, in several species, of transplatable immunologically competent cells in blood. We have shown that leukocytes from peripheral blood of normal mice can be transplanted to an irradiated host and can give rise to lymphopoiesis, granulopoiesis, and erythropoiesis. Lethally irradiated mice were injected with a suspension of leukocytes prepared by centrifugation in saline from heart blood pooled from many donor mice. Recipients in nonisologous experiments were inbred strains, either parents of or closely related to, the F₁ hybrid blood donors. This donor-host combination was selected to avoid graft-versus-host reactions and, at the same time, to permit identification of grafted cells by virtue of antigenic differences between donor and recipient. Isoimmune sera were prepared and used in classical agglutination, hemolysis, and cytotoxicity tests for identification of cell types. Long-term survival (more than 3 months after x-rays and treatment) has been observed in one F₁ hybrid isologous and two nonisologous experiments. Donor-type erythrocytes and leukocytes were observed in the nonisologous chimeras 1½ to 2 months after transplantation. Donor-type cells were also identified in suspensions of chimeric lymph nodes, spleens, and bone marrow within the first two weeks after transplantation. Histologic examination of hematopoietic tissues of these mice revealed proliferation of all blood elements. Lymph nodes and spleen white pulp were cellular and contained many transitional and plasma cells. Tissues of radiation control mice showed no evidence of cellular proliferation. Fe²⁹ was taken up by spleen and erythrocytes of chimeras but not by tissues of radiation control mice. When these newly formed, Fe²⁹-containing chimeric erythrocytes were reacted with donor-specific isoimmune serum, the Fe²⁹ was released from the cells. No comparable release was seen from these erythrocytes in nonimmune serum.
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Separation of Eosinophils and Macrophages for In Vitro and In Vivo Production of Antibody
R. S. Speirs, New York, N. Y.

We are attempting to determine how the formation of antibody is initiated in cells by the presence of antigen. By injecting antigen into the peritoneal cavity of immunized and nonimmunized mice it is possible to determine quantitatively the cellular response by washing the inflammatory peritoneal exudate. These suspended inflammatory cells are being extensively studied both in vivo and in vitro to determine physiologic and morphologic changes during inflammation, and to measure their capacity to produce antibody. Procedures have been developed for markedly altering the cellular composition of the exudate by treatment with cortisone, x-irradiation, etc. In spite of the fact that the exudative cells were suspended in a fluid medium and could be removed from the animal with a minimum of trauma and temperature change, it was evident that the majority of cells failed to survive more than a few hours when cultured in glass or plastic chambers. However, when a substrate of formalin treated gelatin or cross-linked thiolgel (thiolated gelatin) was used to coat the glass or plastic, the cells did not become necrotic. Instead they showed evidences of amoeboid motion within 20 minutes. As shown in photographs, these cells were highly active and exhibited chemotaxis and phagocytosis for bacteria, crystalloid material and injured cells. Eosinophils exhibited marked chemotaxis to certain injured macrophages. Later these eosinophils were actively phagocytized by viable macrophages.

Separation of Platelets and Granulocytes for Transfusion Studies

The technic of plasmapheresis is useful for obtaining platelets and granulocytes for transfusion studies in humans. Utilizing plastic “double packs” 500-cc. units, and an International PR-2 centrifuge at speeds of 2300-2500 rpm for 3 minutes results in separation of platelet-rich plasma (PRP) from red cells. Two units (500 cc.) of PRP can be obtained in 90 minutes from a single venipuncture. The resultant plasma contains over 80 per cent of the platelets and less than 10 per cent of the granulocytes of the donor blood. With the same equipment, plasmapheresis of donor with chronic granulocytic leukemia using speeds of 400 rpm for 20 minutes is used to produce granulocyte-rich plasma (GRP). Over 50 per cent of the granulocytes of the donor blood are recovered. Plasmapheresis of donors at rates of two liters or less of plasma per week does not result in any depletion of serum proteins or formed elements of the blood. At rates of five liters per week, depletion of serum proteins, particularly gamma globulins, and platelets have been observed; however, rapid recovery follows. These technics have been used for studies of survival, and function of formed elements in vivo. They are particularly valuable for control of donor variables in such studies.

Mechanical Removal of Leukocytes from Whole Blood
J. Souto and D. L. Anderson, Oak Ridge, Tenn.

A simple procedure for obtaining blood with a low leukocyte content is described. Centrifugation and physiologic saline was used in this process because of the low sedimentation rate of sheep blood and the necessity of avoiding the introduction of nonphysiologic large molecular substances. Polyvinyl chloride bags of blood containing an anticoagulant (sodium ethylenediaminetetra acetate) were centrifuged at 1500 rpm for 15 minutes. The platelet-enriched plasma was expressed and stored at 5 C. The remaining red blood cells and buffy coat were transferred to glass bottles, washed with isotonic saline, centrifuged at 2000 rpm for 25 minutes and the saline and buffy coat were removed with a pipette. The cells were again washed, centrifuged and pipetted. The areas of the buffy coat in the bottles were swabbed and the residual erythrocytes,
plasma, and platelets were reconstituted. Resulting reconstituted blood had an average leukocyte count of less than 1,000 per mm.³, or about 10 per cent of that of original blood. Lymphocytes were reduced to about 3 per cent of original numbers, granulocytes to 22 per cent, and monocytes to 20 per cent. The remaining leukocytes appeared morphologically normal when examined in stained and phase contrast preparations. To obtain six liters of reconstituted blood required about six hours.

**Separation of Lymphocytes from Blood**

R. I. Walker and J. G. Palmer, Chapel Hill, N. C.

In 1951, Fichtelius reported that lymphocytes could be eluted with saline from heparinized rabbit blood previously incubated on a cotton column. This technic was evaluated particularly regarding facility of handling large volumes of blood to yield sufficient lymphocytes for chemical fractionation. Heparinized whole blood was poured into a prewarmed 2 × 60 cm. glass tube loosely packed with ordinary cotton. These were stoppered to prevent drying, incubated one hour at 37 C., and eluted with warm isotonic saline containing heparin. Twice the volume of blood used was collected. Suction was used to aid loading and elution.

**Observations on Cells Found in the Rejecting Renal Homograft**


Cytologic characteristics of cells infiltrating canine renal homografts were studied on Wright's stained cover slip imprints of kidney tissue. It is possible with this technic to obtain information regarding the morphologic characteristics of the individual cells that participate in the homograft rejection. The most prominent "invading" cell type is a large mononuclear cell resembling the monocyte. Normal, as well as bizarre lymphocytes are commonly seen. Typical plasma cells are not common during the first week in the canine renal homograft, but some of the cells seen might be called plasma cell precursors. Polymorphonuclear leukocytes are customarily found in moderate numbers. Large, primitive reticulum cells similar to those described by Scothorne (Ann. N. Y. Acad. Sc. 64:1029, 1957) and Andre and Schwartz (personal communication) in regional lymph nodes draining skin homografts are occasionally seen. Using the immune-adherence phenomenon (Nelson: Science 118:733, 1953) as a test system, more recent studies on cell suspensions from rejecting kidneys seem to indicate the presence of an antigen-antibody reaction involving renal tubular cells as well as vascular endothelial cells in the advanced rejection, but vascular endothelium only in the early (three days) stages of rejection. Prior immunization of the recipient dog with washed platelets from the donor in small doses (10⁸ platelets given intravenously) produces a typical second-set rejection of kidney within 24-48 hours after grafting. This is attended by a prominent cellular infiltration of the kidney as well as an immune-adherence phenomenon involving both renal tubular cells and vascular endothelial cells when these are incubated with complement and washed human erythrocytes.

**Effect of Humoral and Hormonal Factors on Hemopoiesis in Vitro**

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Using the well technic developed in our laboratory, normal marrow was cultured in the presence of prednisolone, testosterone, estrone, and human pituitary growth hor-
Phytohemagglutinin Preparations
Leland H. Pence, Detroit, Mich.

Phytohemagglutinin was prepared by the method of Rigas and Osgood and assayed for its red blood cell agglutinating ability. Its ability to agglutinate erythrocytes provided a ready means of obtaining white blood cells. Nowell reported that bacto-phytohemagglutinin stimulated division of white blood cells in tissue culture. Red blood cell agglutination was reproducible, but the variability of mitotic stimulation of blood cultures from one lot to the next made it appear that there were two different factors. We had not been examining the product for this hitherto unknown mitotic-stimulating property. We are now assaying phytohemagglutinin for its mitotic-stimulating activity according to the procedure of Moorhead et al. (Exp. Cell Res. 20:613, 1960). Because of the well known variability of blood tissue cultures, a preparation of bactophytohemagglutinin must exhibit a mitotic index of at least 30 cells in mitoses per 1000 cells on four different samples of blood before it is now released. Usually the mitotic indices are considerably higher than this minimal figure. In cultures of leukocytes for the preparation of chromosomes it has not been necessary to have a sterile phytohemagglutinin since penicillin and streptomycin kept the bacterial contamination of the cultures under control. The original phytohemagglutinin was not sterilized. Now, however, investigators wish to extend the use of phytohemagglutinin to sterile cultures in which they do not want antibiotics present. The P-form as a sterile product should be available in the near future.
Symposia: Blood-Bone Marrow Tissue Culture and Cell Separation

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