SYMPOSIUM

Recent Developments in Studies of Blood Platelets


INTRODUCTION

This conference was held at the Biology Division of the Oak Ridge National Laboratory, June 22 and 23, 1962. It was organized by Drs. T. T. Odell, Jr. and Mario Baldini, and supported by the Oak Ridge National Laboratory. Drs. G. A. Andrews, L. M. Tocantins, and W. Dameshek served as Chairmen of the sessions. Approximately 85 persons attended. The program included papers on platelet components and their function, immunology, preservation, survival, and platelet and megakaryocyte dynamics and regulation. The enthusiasm shown at the meeting was evidenced by suggestions that additional conferences of the same general nature be held in the future.—Alexander Hollaender

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SYMPOSIUM: STUDIES OF BLOOD PLATELETS

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Platelet Components and Their Function

G. A. Andrews, Chairman

Platelet Phospholipids and Blood Clotting

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Pure platelet phosphatidyserine (PS) and phosphatidylethanolamine (PE) are compared with brain cephalin (Ceph.) in 1) two-stage (thrombin-generating) tests, using as "thromboplastic enzymes" a) styphven, b) trypsin, and c) autoprothrombin C; 2) PTT (plasma-Ca-lipid clotting time) tests; and 3) thromboplastin generation tests (TGT), including the Pool-Robinson modification. Except for the unmodified TGT, which does not work with PE, all tests show clot-accelerating (prothromboplastic) activities with each of these phosphatides. A validated bioassay has been worked out with a standardized styphven eluate two-stage system. Log-log plots of end-point clotting times against phospholipid concentrations are rectilinear. Results may be expressed, percentagewise, as equivalents of a standard lipid preparation, usually cephalin. End-points may also be related to equivalents of prothrombin activated (EPA), or of thrombin yield (ETY). These are valuable in computing expected activities in mixtures of assayable phosphatides. In mixtures of weak assayable phospholipids, simple quantitative summation of activities is proved. Compared with a less refined brain PS preparation (with antithromboplastic properties, as well as activity), pure platelet PS is devoid of a demonstrable inhibitor. Alterations in the reactivity of the lipids are demonstrated. These may be 1) decreases during storage, 2) increases in the presence of desoxycholate (both of which are assayable), or 3) an unsuspected (oxidative?) increase (in the thromboplastic enzyme tests), which usually invalidates bioassays. The modified TGT does not show the above 3) hyper-reactivity phenomena, suggesting that the Russell's viper venom can utilize certain acquired reactive groups in altered lipids, whereas the intrinsic system of the TGT is unable to do so. The modified TGT, however, can assay activator reactivities. Both tests agree that the present platelet phospholipids are less active than brain cephalin, but this needs further explanation. Besides indicating that the platelet phospholipids may serve a prothromboplastic role in blood clotting, the new methods, particularly the styphven and autoprothrombin C tests, offer a means for further exploration of the biochemical basis of reactivities in identifiable lipids, which are activators (or inhibitors) of blood clotting. The ability of autoprothrombin C to work with pure platelet lipids supports a "thromboplastic enzyme" theory.
Phospholipid preparations obtained from bovine brain, liver, and lung tissue, from porcine liver, from butter and from soybeans were found to be thromboplastic in in vitro coagulation systems. These phospholipids have an activity comparable to platelet suspensions but are less activating than tissue thromboplastin. Phospholipids from fresh beef liver were fractionated according to the procedure of Folch. The fraction V material was found to have thromboplastic activity and the fraction III phospholipid was found to have antithromboplastic activity in the thromboplastin generation test and in a recalcified plasma assay. In the latter assay, 80 µg. fraction III/0.1 ml. of plasma caused a 100 per cent increase in clotting time, 40 µg. a 50 per cent increase, and 15 µg. a 25 per cent increase. No antithromboplastic activity and no thromboplastic activity were obtained at concentrations less than 3 µg./0.1 ml. plasma. The antithromboplastic phospholipid inhibits the thromboplastic activity of platelets and of the thromboplastic phospholipids described above. Intravenous administration of 12 mg./Kg. to rabbits causes a significant increase in whole blood clotting time, and intravenous administration of 40 mg./Kg. causes a 100 per cent increase in whole blood clotting time. Intravenous administration of 25 mg./Kg. to dogs causes a significant increase in whole blood clotting time.

Plasmalogens are phosphoglycerides that generate higher fatty aldehydes on hydrolysis. Early studies from this laboratory on total lipid extracts of human blood platelets revealed that 16 per cent of the dry weight was in the plasmalogen form. In addition, it was noted that these phosphoglycerides were associated with platelet fractions showing thromboplastic activity. More recently, platelet phospholipids have been completely separated and the plasmalogens further studied by a combination of chemical and gas-liquid chromatographic methods. It was found that platelet phosphatidylethanolamine was 66 per cent in the plasmalogen form. Phosphatidylserine and the "choline" phosphoglycerides contained 3 and 5 per cent plasmalogen respectively. It is of interest that human erythrocytes and myelin also contain large amounts of plasmalogen and it is possible that these lipids share common functions in cell (plasma) membrane metabolism. From present evidence it is not likely that plasmalogens play a role in blood coagulation. Phosphatidylserine promoted blood thromboplastin formation in all clotting systems studied. Phosphatidylethanolamine was inert in the thromboplastin generation test, but active in other coagulation systems.

Factors Influencing Platelet Stability


Alteration of stability does not mean that platelets are destroyed. Platelets, for instance, can clump and go into viscous metamorphosis or a number of platelets can diminish after exposure of blood to glass contact, without any observable clump. Our study deals only with some of the factors that can influence the stability of platelets: surface, temperature, movement, pH, ionic strength, thrombin, heparin, calcium, ADP, ATP, EDTA, sodium oxalate, sodium citrate. It deals also with the influence of red cells on the stability of platelets, with or without calcium, and also with the importance of sex, if any. Low temperatures make easier aggregation of platelets by any agent. Clumping of platelets with aspect of viscous metamorphosis is obtained with ADP, ATP, either on ice or at 37°C, no matter if platelets are platelet-rich native plasma or plate-
Activation of Platelet Factor 3 by Bacterial Endotoxin and by Immune Reactions


Previous studies from this laboratory have indicated that incubation of platelet-rich rabbit plasma in vitro induces platelet aggregation with activation of platelet factor 3. Changes in platelet factor 3 activity have been measured by a test we have designated as the product I substrate time, employing a stable product I concentrate prepared by the method of Spaet which results from the interaction of clotting factors VIII, IX, X, XI, and XII. The speed of coagulation on addition of product I and calcium to a plasma has been found to be inversely proportional to the concentration of available platelet factor 3. In the present studies it has been found that immune reactions will substitute completely for bacterial endotoxin in increasing platelet factor 3 activity of platelet-rich plasma. Incubation of human γ-globulin and Coombs’ serum with rabbit platelet-rich plasma markedly shortened the product I substrate time. Two immune reactions involving human platelets were studied. Addition of rabbit anti-human platelet serum to human platelet-rich plasma partially increased the platelet factor 3 activity during 2 hours of incubation at 37 C. Serum containing the antibody of quinidine-induced thrombocytopenic purpura increased the platelet factor 3 activity of human platelet-rich plasma when quinidine was added to the incubation mixture, but not in the absence of the drug. The latter finding proved to be a highly sensitive and reproducible method for demonstrating the presence of drug-induced purpura. It appears likely that certain immune reactions involving platelets injure the platelets with release of platelet-specific factors, such as platelet factor 3. Endotoxin-induced platelet injury, which appears to account for the accelerated coagulation noted on addition of bacterial endotoxins to clotting blood or platelet-rich plasma, may be mediated by such an immune reaction.

A Streptokinase Cofactor in Human Platelets

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Well washed human platelets, platelet extracts, and certain fractions of these extracts plus streptokinase are not fibrinolytic, but when a bovine serum fraction is added a potent fibrinolysin appears. The bovine serum fraction, a euglobulin, plus streptokinase alone produces no fibrinolytic activity. Well washed human platelets are essentially free from antifibrinolytic and tissue fibrinolytic activities. The streptokinase cofactor activity of human platelets was studied on bovine fibrin plates and in a new assay in which fibrin I\(^{131}\) serves as the substrate. The cofactor was found in the γ-β globulin fractions of platelet extract. The cofactor was heat labile (56 C.) and disappeared slowly on storage at −20 C.

The Effect of Anti-Platelet Antibodies on the In Vitro Uptake of 5-Hydroxytryptamine by Blood Platelets

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The effect of anti-platelet antibodies on the in vitro uptake of 5-hydroxytryptamine (5-HT) by human blood platelets was studied. Various antibody-containing sera
The inhibition of in vitro platelet functions to detect antiplatelet antibodies is of proven usefulness. Inhibition of the clot retraction ability of platelets has been used to measure as in two patients whose sera gave a negative result, this was normal. The ability of the isimmune sera to inhibit 5-HT uptake occurred in the absence of platelet agglutination and lysis. This inhibiting factor could be removed by absorption with donor platelets. Comparative studies by the use of the Dausset, Colombani, and Colin agglutination test and Schulman’s complement fixation test suggested that the serotonin method is at least as sensitive as these standard procedures. Plasma or serum from patients with ITP, supposedly containing an “autoantibody,” showed no effect. The platelet uptake of radioactive 5-HT in vitro may be usefully adopted in studies on immunity to blood platelets.

Platelet Immunology

L. M. Tocantins, Chairman

ISOIMMUNITY TO BLOOD PLATELETS

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The mode of occurrence of isoimmunity to blood platelets, methods for its detection, and attempts at its correction were the object of investigations. The following is a review of the results obtained thus far: 1) Sensitization to homologous blood platelets, as determined by reduction in survival of Cr51-labeled donor’s platelets, occurred in each instance in which fresh platelet concentrates were infused two to eight times in normal volunteers. Survival of homologous platelets was also studied in multitransfused patients. It was found to be shortened in the majority of cases and normal in only occasional patients. 2) Experiments in humans demonstrated that platelet antigens are not identical to red cell antigens and not completely identical to leukocyte antigens. Demonstration of platelet groups as well demarcated as the ABO, Rh groups for the red cells was not obtained. Experiments in rabbits and dogs demonstrated that blood platelets contain tissue antigens and histocompatibility antigens. 3) Depression of the in vitro uptake of radioactive serotonin by donors’ platelets in the presence of serum from sensitized patients was found to be a useful indicator of antiplatelet isosensitization. 4) Preliminary experiments on modification of the rapid destruction of homologous platelets in isoimmune stages gave encouraging results. The short (4 hours) survival time of homologous platelets in a dog previously sensitized by seven platelet infusions returned to normal values (6 days) following splenectomy and corticosteroid treatment. Splenectomy gave a similar result in a multitransfused patient with hypoplastic anemia who was under corticosteroid treatment and who was shown to be sensitized to homologous blood platelets.

DISCUSSION

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The inhibition of in vitro platelet functions to detect antiplatelet antibodies is of proven usefulness. Inhibition of the clot retraction ability of platelets has been used to measure
antibody in the past. However, this is a complex event with many intermediate steps, some of which may compensate for others, raising the question of inadequate sensitivity. The inhibition of serotonin uptake involves a more specific platelet function, and it seems reasonable to suppose that this will enhance the sensitivity. Platelet isoantibodies have been implicated in cases of neonatal thrombocytopenia, and in thrombocytopenia resulting from transfusion of incompatible platelets. Their major importance, however, is in the patient receiving multiple transfusions. Platelets must be capable of circulating to be hemostatically effective in the thrombocytopenic recipient. This capability depends on many factors, and may be altered by the presence of immune mechanisms which prevent the circulation of otherwise viable platelets. Further study of the effect of splenectomy and steroids on this will be of practical and theoretical importance. A deterrent to the understanding of the over-all importance of platelet antibodies is the difficulty in satisfactorily demonstrating their presence by current technics. In idiopathic thrombocytopenic purpura there is circumstantial evidence that autoantibodies might be important, but the laboratory demonstration of the etiologic significance of such antibodies is not conclusive. The hypothesis that this disease is due to an autoimmune process remains unproven.

**Platelet Preservation**

**L. M. Tocantins, Chairman**

**IN VIVO CIRCULATION OF RAT PLATELETS STORED IN THE FROZEN STATE**


Studies by Gardner (Conference on Platelets—National Research Council, 1959) and subsequently by this group (Federation Proceedings, 1959) on freezing of platelets in glycerol-containing media have indicated that preservation of platelets at low temperatures may be possible. Other methods of freezing platelets in a viable state have therefore been investigated. Supercooling of rat platelets resulted in rapid loss of viability, exceeding that of control preparations stored at 4°C. Dehydration with hypertonic concentrations of sucrose prior to freezing at -79 or -195°C permitted actual elevation of platelet counts in thrombocytopenic animals by infusion of stored frozen platelets. The increase was slight, although significant (p < 0.01), and lasted very briefly. A number of other sugars, gelatine, PVP, albumin, and dimethylsulfoxide were less or not effective. A combination of 5 per cent dextrose and 5 per cent dimethylsulfoxide in plasma, however, resulted in preservation of the integrity and ability to circulate of as much as 70 to 89 per cent of platelets stored at -195°C for periods varying from 1 to 4 weeks. The net increase of the platelet counts varied from 300,000 to 800,000 platelets per mm³. Marked increases were usually still present 5 to 6 hours following the infusion. The platelet counts 24 hours later were higher than the pretreatment levels. The abnormally prolonged bleeding time of the animals was reduced to normal values following the transfusion of preserved platelet preparations.

**PRESERVATION OF PLATELETS WITH DIMETHYLSULFOXIDE IN THE FROZEN STATE**


Attempts to freeze and store platelets for prolonged periods of time have met with only moderate success. Good results obtained on some occasions could not be reproduced. In these experiments, glycerol in concentrations varying from 7 to 10 per cent, with or without the addition of plasma, served as the preservative of choice. In the hope of improving results, investigations were carried out using di-
methylsulfoxide (DMS) as additive. In preliminary experiments, the preservation of the clot retracting activity of platelets was studied. Human platelets were isolated by differential centrifugation, and were resuspended in 50 per cent saline and original plasma, following three saline washings. Control clot retraction, performed on a system of 1.3 ml. total volume containing $2 \times 10^5$ fresh platelets, was recorded at 88 per cent. Addition of 15 per cent DMS resulted in insignificant reduction of clot retraction. Freezing over liquid N$_2$ vapor

Patients with thrombocytopenic complicating disease, bleeding times over 12 minutes, no clot retraction of whole blood clot, and prothrombin consumption times below 15 seconds, were transfused with normal, human, intact platelets in concentrates from 16 donors. The platelet concentrates were prepared according to the standard blood bank procedures, with blood collected in ACD solution in a closed plastic system. The function and ultrastructure of intact platelets of the patient, of the concentrate and the transfused, surviving platelets in the patient were studied. Detection of platelet antibodies before and after the infusions were carried out using our modification of the technic of Tullis. Differential platelet counts under the electron microscope (Rebuck) reveal that most normal platelets are in the dendritic form (82–93 per cent) with very few intermediate or spread forms and at rates of 10 C. per minute showed that DMS will protect the clotting activity of platelets only in the presence of plasma. Further experiments showed that clot retraction activity was best maintained when the platelets were suspended in 50 per cent plasma and 15 per cent DMS. Storage in liquid N$_2$ for 11 days resulted in an insignificant loss of clot retraction activity, but approximately 30 per cent loss was observed after storage for 15 days. In vivo studies of platelet survival are under way.

Platelet Survival and Function

L. M. Tocantins, Chairman

Further Experience with C$^{14}$ 5HTA Platelet Survival Data

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Initial experience (J. Clin. Invest. 40: 2134, 1961) suggested that C$^{14}$ serotonin (5-hydroxytryptamine, 5HTA) labeling of platelets might be a suitable and simple method for studying in vivo platelet survival. Serotonin, however, is concentrated
by circulating platelets from sites of serotonin production in vivo, and active exchange of the label between platelets and serotonin depots may occur. Further, in the first 24 hours, 40–50 per cent of the $^{34}$C$^{14}$ 5HTA infused bound to platelets cannot be accounted for by totaling blood and urine radioactivity. In four infusions of platelet-rich plasma (PRP) and platelet-poor plasma (PPP), urine, stool, and blood samples for periods of 8–30 days were collected and radioactivity measured. Five to 10 per cent for periods of 8–30 days were collected and, ma (PPP) (rich plasma accounted for by totaling blood and urine, PRP) and platelet-poor plasma (PPP), urine, stool, and blood samples for periods of 8–30 days were collected and radioactivity measured. Five to 10 per cent for periods of 8–30 days were collected and, ma (PPP) (rich plasma accounted for by totaling blood and urine.

The unaccounted-for activity may be different fate than when infused free in plasma. The unaccounted-for activity may be bound to platelets which are not actively circulating but which disappear at the same rate as circulating platelets, or it may be present in another metabolic pool of 5HTA which is exchanging with platelet 5HTA.

The data do not allow us to distinguish between these possibilities. Infusion of ABO type incompatible platelets, a situation in which the $^{14}$C$^{14}$ 5HTA-tagged infused platelets were expected to be rapidly destroyed, seemed to offer an opportunity to study disappearance of tag after PRP infusions in recipients with normal circulating platelet levels. In five cross-transfusions of donor-recipient AB incompatible platelets, the results were generally similar to those seen with AB compatible platelet infusions.

There was a slightly reduced yield of AB incompatible platelets, but disappearance rates were similar. The results indicate that: 1) infusion of $^{14}$C$^{14}$ 5HTA-labeled platelets is a good indicator of viability of infused platelets; 2) survival curves may reflect metabolic turnover of $^{14}$C$^{14}$ 5HTA as well as death of platelets; 3) AB donor-recipient incompatibility exerts little effect on survival of platelets.

RAPID EXCHANGE OF PLATELET $^{14}$C-LABELED SEROTONIN IN PATIENTS WITH FUNCTIONING CARCINOID TUMORS

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Heyssel found that the disappearance curve of platelets labeled with $^{14}$C$^{14}$ serotonin is a straight line when radioactivity is plotted logarithmically, and that the half-time is 5–6 days (J. Clin. Invest. 40:2134, 1961). Six studies were done on five subjects either by injecting labeled platelets (in vivo label) or infusing serotonin directly (in vivo). Platelet radioactivity disappeared along a logarithmic curve in all. Half-times in a hematologically normal subject with multiple sclerosis were 3.2 days (in vivo labeling) and 1.8 days (in vivo labeling). In a patient with rectal carcinoid without carcinoid syndrome and with normal 5-hydroxyindoleacetic acid (HIAA) excretion, half-time was 4.5 days (in vivo labeling). In three patients with carcinoid syndrome, the label disappeared more rapidly, with half-times of 0.2 (in vitro), 0.4 (in vivo), and 1.3 days (in vivo). Urinary 5-HIAA levels were respectively 450, 300, and 46 mg/24 hours. Platelet serotonin was elevated in all three patients, but platelets in vitro took up serotonin readily. Rabbits with platelets labeled in vitro show a logarithmic disappearance with a half-time of about 1.2 days. Platelet radioactivity dropped precipitously after intraperitoneal administration of 50 mg. of 5-hydroxytryptophane, a serotonin precursor. It thus appears that platelet serotonin exchanges with body serotonin depots, as it does with serotonin in vitro (Born and Gillson; J. Physiol. 146:472, 1959). Consequently, disappearance curves of platelet serotonin radioactivity do not necessarily reflect platelet survival because they depend upon the pool size and turnover of serotonin in the body.
A USEFUL RABBIT MODEL FOR STUDIES OF PLATELET SURVIVAL

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A rabbit model for measurement of platelet viability in vivo was studied, and its usefulness as a research tool was determined. Survival studies of fresh homologous platelets were evaluated by five methods involving transfusion of 1) nonradioactive platelets to thrombocytopenic rabbits, 2) platelet concentrates labeled in vitro with Cr\(^{51}\), 3) concentrates of platelets labeled in vivo with P\(^{32}\), 4) whole blood labeled in vivo with P\(^{32}\), and 5) concentrates of platelets labeled in vivo with S\(^{35}\). Comparison of results obtained with these methods led to the following conclusions: 1) Three values were important to determine platelet viability for each method: a) per cent recovery of transfused platelets in the recipient's circulation, b) survival time of transfused platelets, and c) shape of the survival curve. 2) Platelet viability did not appear to be reduced by labeling and preparation into concentrates. 3) With Cr\(^{51}\) or P\(^{32}\)-labeled platelets, recoveries were apparently less than 100 per cent because of a) presence of small numbers of isotopically labeled red cells in the sample from which injected radioactivity was measured, and b) elution of part of the isotope from Cr\(^{51}\) labeled platelets immediately after infusion. 4) It appeared that neither P\(^{32}\) nor Cr\(^{51}\) was eluted from platelets during the remainder of their life span. 5) Methods 4) and 5) gave less reproducible results than did method 1), 2), or 3). 6) In studies of platelet storage, the rabbit model, utilizing Cr\(^{51}\)-labeled platelets, was shown to be useful for predicting the behavior of human platelets.

THE PATTERN OF PLATELET DESTRUCTION

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There are many conceivable mathematical models to describe the pattern of platelet destruction. Two main models have been proposed: 1) Platelets are randomly destroyed and survival is, therefore, mainly determined by their external environment; 2) platelets have a natural life span which follows some such distribution as the gaussian and that this is mainly determined by their internal environment. Because of experimental error, it is not possible from simple examination of the platelet survival curve to determine the pattern of destruction. We adduce the following evidence that random destruction is the more important mechanism. 1) Simultaneous cohort (S\(^{35}\)) and population (DFP\(^{32}\)) tagging of platelets in the pig and the dog yield survival curves which are straight and parallel on semilogarithmic paper. This indicates that the rates of destruction of these two populations of different ages are the same. 2) Alteration of the external environment of the platelet by drugs, diet, etc., changes platelet survival computed under either model. 3) Results are more reproducible and give better discrimination when the exponential model is used. 4) From biological considerations, it seems more plausible that platelets should be consumed as they are needed regardless of their age. The known metabolic activity of the platelet, however, makes it probable that intrinsic factors set a limit on the duration of platelet survival, but that the importance of this factor is ordinarily masked by the demands of its external environment.

FACTORS INFLUENCING THE FATE OF THE PLATELET

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Three factors have to be examined in considering the fate of the platelet: 1) senescence; 2) deposition on the endothelium; 3) consumption in blood coagula-
The last two factors have provoked the most controversy and in consequence more attention has been paid to them. We have been interested in the relationship of the platelet to the endothelium because of its possible role in atherogenesis. Examination of endothelium membranes prepared from the aorta and coronary arteries of swine of all ages has shown that platelet thrombi occur on endothelium which is at least morphologically intact. Such thrombi are distributed at vessel orifices and bifurcations; sites at which incipient atherosclerosis often begins. Cross-sections through these sites have shown that some platelet thrombi have been covered with endothelium and incorporated into the intima. Platelet survival has been manipulated through changes in its external environment. 1) Diets rich in eggs and dairy fat shorten platelet survival. 2) Dicumarol in low doses tends to shorten platelet survival, while large doses prolong it. 3) Heparin in doses of 8,000 units t.i.d. prolongs platelet survival. All these mechanisms are associated with corresponding changes in blood coagulation. 4) Smoking shortens platelet survival. This is not associated with change in blood lipid or persistent alteration in blood coagulation. These findings indicate that the endothelium and blood clotting are important in platelet survival.

**Effects of Total Body Irradiation on Platelet Values in Human Beings**


Single doses to human beings, normal hematopoietically, in the 250-350 r range produce a fall to very low levels by the 21st day. After a period of depression lasting about 10 days, recovery of platelet values occurs over a 10-day period (6 weeks after exposure). This recovery phase begins earlier in patients with leukemia who develop remissions after total body radiation. Comparisons of these various curves with platelet values after nitrogen mustard show that the nitrogen mustard produces more rapid depression and earlier recovery. As compared with the higher doses of radiation, single doses of 100 r produce a more moderate depression with a less striking recovery phase. After single 50-r doses, patients with leukemia or lymphoma show a mild depression in platelet values during the fourth and fifth week, falling from a mean pretreatment value of 177,000 to 98,000 per mm.³ by the 28th day, then returning to a mean level of 131,000 by the 42nd day.

**Premammalian Thrombocytes**

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The platelet, a cellular fragment with potential hemostatic function, is unique to the mammalian species. Blood samples from premammalian vertebrates contain thrombocytes—complete nucleated cells—which appear to have certain hemostatic functions similar to those of the mammalian platelet. Whole blood coagulation times were varied among the different species. Fish and bird bloods clotted rapidly; turtle, skate, and shark very slowly. Clot retraction, usually less marked than in human blood, occurred in alligator, fish, and skate blood, but was absent from most of the turtle blood samples studied. Recalcification times of cell-poor plasma were markedly shortened by adding buffy coat (turtle). Erythrocytes of lower species are oval and nucleated and may be very large. Thrombocytes are smaller, oval, nucleated, and contain one or more specific granules. They do not differ greatly in appearance or sedimentation characteristics from lymphocytes. The thrombocytes of all premammalian species showed strong reactions on PAS stain for glycogen as do human platelets. However, lymphocytes of all species showed negative or faint reaction. On observing the native or recalcified whole blood under phase microscope, the thrombocytes agglutinated together making clumps similar to the viscous morphosis of human platelets. The cytoplasm of thrombocytes spread out thinly, while the nuclei moved to locate at the center of the thrombocyte clump. These morphologic changes did not occur in lym-
Platelet and Megakaryocyte Dynamics and Regulation

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Morphology of Megakaryocytes

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In order to perform basic research relating to platelet formation, it is necessary to identify megakaryocytes at different stages of maturation. The most reliable criteria are size, number of nuclei, granularity of cytoplasm, and platelet differentiation. The most primitive cell, megakaryoblast, has a single round or bilobed nucleus. The chromatin is finely linear or has a granular appearance. Nucleoli are usually visible. The cytoplasm is nongranular and stains blue with Romanovski stains. Blunt pseudopods and ectoplasmic protrusions which are darker blue than the endoplasm, and which have a sponge-like appearance, may be present. The second cell in the maturation sequence is the promegakaryocyte which usually has two to four attached nuclei. Fine granules are present next to the nucleus. This cell, like the megakaryoblast, has multiple blunt pseudopods which have a "bubbly" appearance. The third cell in the maturation sequence is the megakaryocyte without platelet differentiation. This cell is larger than adjacent granulocytes. There are four or more nuclei. Cytoplasm is diffusely granular. In some megakaryocytes there is a peripheral symmetrical circle of large chromophobic spaces (vacuoles) which separate the granular cytoplasm from the marginal nongranular ectoplasm. The fourth stage in the maturation of megakaryocytes is characterized by the differentiation of thrombocytes. Relatively light and nongranular spaces appear between and around areas of granular substance. The electron microscope reveals these structures as "demarcation membranes." At the periphery of the megakaryocytes, well-defined platelets with central granular substance and peripheral hyaline material appear. The term "metamegakaryocyte" is recommended for the megakaryocyte with multiple nuclei and platelet differentiation.

Kinetics of Megakaryocyte Proliferation in Rat Bone Marrow

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The kinetics of the megakaryocytic cell line of the rat bone marrow are being studied using tritiated thymidine as a cell label. Normal adult rats, given a single intravenous injection of tritiated thymidine, were killed at intervals of time following tracer injection. The changes in the percentage of labeled cells as a function of time after injection of the tracer were registered separately for arbitrarily chosen successive recognizable stages of megakaryocytic differentiation. Emphasis was on the development of initially labeled cells into a stage of maturation corresponding to initially non-labeling cell forms. The following results were obtained: 1) The transit time from the most immature recognizable stage of megakaryocytic development to megakaryocytic disintegration was approximately 40 hours. 2) Evidence was obtained that the recognizable megakaryocytic elements originate from unrecognized precursors which continuously synthesize DNA for a period of at least 1 to 3 days prior to maturation into recognizable megakaryocytic precursors. 3) The process of nuclear lobulation was not accomplished by the end of DNA synthesis, thus being comparable to nuclear segmentation in neutrophilic granulocytes. This latest phase of matura-
MEGAKARYOCYTE LABELING WITH TRITIATED THYMIDINE

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Rats were injected with 50 µc. of tritiated thymidine intraperitoneally at 30-minute intervals for 10 hours in order to label a large percentage of the megakaryocyte population. The rats were killed at 1, 3, 4, 5, 7, and 9 days after injection and autoradiographs were made of smears and sections of their bone marrow. Approximately 30 per cent of the marrow megakaryocytes was labeled (over 3 gr./cell) 24 hours after beginning injections. There was a rise to nearly 100 per cent on days 3, 4, 5, and 7, but a decline by day 9 to about 80 per cent. Although we have not classified megakaryocytes according to stage of maturation in this study, it seems likely that the cells that were not labeled on day 1 (70 per cent of the population) were megakaryocytes that had matured beyond the period of DNA replication and nuclear division. By day 3 this segment of the population had presumably produced platelets and was replaced by maturing labeled cells. Both the mean and maximum grain counts declined with time. Successive reductions in maximum grain counts with time after labeling may indicate maturation and loss of megakaryocytes that were in successively earlier stages of maturity at the time of labeling.

DISCUSSION

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Our study of megakaryocyte life span with tritiated thymidine is in preliminary stages. Three rabbits have been given single intravenous injections of 0.8, 1.0, and 1.5 µc./Gm., body weight. Bone marrow samples were aspirated at 1 and 6 hours and daily for 7 days. In addition, two other marrow samples were incubated for 2 hours in vitro with 20 µc. of tritiated thymidine. Autoradiographs were processed in the routine manner and stained with Giemsa. Megakaryocytes were classified by morphologic criteria into three compartments: megakaryoblasts, intermediate cells, and mature megakaryocytes. At 1 or 2 hours almost no megakaryocytes are labeled in vivo or in vitro. In one in vitro experiment, four of 50 blasts were tagged and in another, one of several hundred mature cells had a heavy label. By 4 to 6 hours, labeling of the blasts reaches a maximum of 45 per cent and the relative number of tagged cells declines over the next few days. Up to 50 per cent of the intermediate cells are labeled by day 1 and 70 per cent of the mature megakaryocytes by day 3. Then there is a decrease until day 7 when only scattered cells with significant grain counts are observed. Since tritiated thymidine is available for incorporation into DNA for only a few minutes, one explanation of our data is that primitive unrecognized cells are labeled; these early forms then divide and the daughter cells differentiate in a few hours to megakaryoblasts which mature in 3 days to adult cells. The mature megakaryocytes have a life span of 2 to 3 days. Apparently, the megakaryocyte does not incorporate thymidine during its maturation.

STUDIES ON THE HUMORAL CONTROL OF THROMBOPOIESIS

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The existence of a humoral erythropoietic regulatory mechanism is now established. Recent observations on the thrombocytosis-promoting effects of certain plasmas support the thesis that thrombopoiesis may be similarly influenced. Ten daily doses of the
EFFECTS OF VARIOUS AGENTS ON BLOOD PLATELET PRODUCTION
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thermostable fraction of phenylhydrazine-induced anemic rabbit plasma administered by gastric tube evoked 70 per cent increases in the thrombocyte counts of normal recipient rats. Similar responses have been observed in rats given extracts of plasmas from 34 patients with polycythemia vera and from one patient with essential thrombocythemia. Normal plasmas (human or rabbit) in daily doses equivalent to 2 per cent of the recipients’ weights induced approximately 30 per cent increases in the rats’ thrombocyte counts. When the dose of normal plasma was halved, thrombocytosis did not ensue. The agent (or agents) responsible for the thrombopoietic activity of these plasmas withstands prolonged boiling, is ether-soluble, and is active both orally and parenterally. Although the presence of multiple humoral factors, each affecting a single cell type, cannot be excluded, the thrombopoietic stimulus has not yet been dissociated from the thermostable plasma erythropoietic or leukopoietic factors which have been studied. Many challenging problems remain unanswered. On the basis of data now at hand, however, it is proposed that a humoral regulator does exert physiologic and pathophysiologic control over thrombopoiesis; available evidence also suggests that a single humoral substance may affect the proliferation of all blood cell precursors. Future developments in this field of hematologic research promise to be most exciting.

Studies on Thrombopoiesis: Effect of Non-dialyzable Spleen Extract on the Blood Platelet Level
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A non-dialyzable, protein-rich extract has been obtained from bovine spleen. This material elevates the blood platelet level of mice and rabbits. This elevation is significant in magnitude (p < .01), delayed 4 days after injection and sustained for the duration of the platelet life span (3 to 4 days in the rabbit). Post-depression thrombocytosis has been eliminated as a cause of the elevation. It is hypothesized that the increase in platelet level results from new platelet production. The elevator material causes no change in the hematocrit or megakaryocyte numbers or differential. Two hours after injection an acute leukopenia develops; however, the white count is normal by 24 hours and remains unchanged for the next several days. The test animals appear well and do not lose weight even with repeated injections. An assay method utilizing rabbits has been devised. The assay appears to be uninfluenced by a number of nonspecific stimuli, i.e., bovine serum and albumin, human serum, celite, citrovorum factor, and hydrocortisone. A number of the bovine organs besides the spleen have been assayed; none possesses elevator activity.

Effects of Various Agents on Blood Platelet Production
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We and others have reported that serum of platelet-depleted donor animals (active serum) injected subcutaneously into normal assay animals of the same species produced an increase in the number of circulating blood platelets. The peak platelet activity was attained 5 days after initiating injections. Normal serum was ineffective. In our experiments, soluble egg albumin and powdered glass produced a response similar to that observed after injection of active serum. Serum obtained from donor animals so treated also stimulated an increase in platelet numbers, implying that these materials can initiate production or release into the circulatory system of a platelet-stimulating agent. Injection of cortisone acetate did not increase platelet levels. We have assayed filtrates of acidified, boiled serum, prepared according to the procedure used in preparing erythropoietin. Such filtrates derived from active serum were less potent than the untreated active serum. It thus appears that the agent that stimulates production is at least partially heat sensitive.
Our working hypothesis accepts the presence of hematopoietic regulators which are circulated in the blood stream, and the platelet regulator is a part of this mechanism. The two paramount problems associated with isolation and identification of these regulators are inadequate chemical procedures and lack of reliable criteria. These studies are concerned with chemical separation of thrombopoietic regulators from human serum proteins. One fraction, associated with a small component of serum albumin, a molecular weight of 60,000, and thermostability to 60 °C. for 30 minutes, was found to increase platelets in normal animals, normal people and some leukemic patients with thrombocytopenia. The platelet count revealed a definite pattern of increase in 2 days with two peaks and the increase lasting for 2 or more weeks. No other serum protein fractions showed a similar response. Inadequate dosages altered the platelet pattern response. Platelet increases were found in so-called nonspecific experiments, but actually represented responses where platelets were required for hemostasis. Another fraction, associated with a component of serum albumin showing an ultraviolet absorption spectrum differing in optical density of other albumin components, produced an increase in megakaryocytes in normal animals and leukemic patients with a blast marrow devoid of megakaryocytes. In the light of existing inadequate methods of protein fractionation and inadequate criteria, these studies represent probing investigations, which offer suggestive evidence of the presence of circulating hematopoietic regulators concerned with production of platelets and megakaryocytes.

Chemical separation and properties of platelet regulators

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Chemical separation of fractions containing platelet regulators was accomplished by several procedures. Ammonium sulfate, continuous flow electrophoresis, zinc acetate, sodium tetrametaphosphate, and ion-exchange column chromatography were used for the separation of the regulators in normal human and rabbit sera. Experimental conditions, such as pH, temperature, ionic strength, and composition of reagents, should be rigidly controlled. Active fractions representing a concentration of about two hundred times from original sera have been partially characterized. The megakaryocyte regulator is found to be associated with a part of the albumin component and indicative of a lipoprotein. Its ultraviolet absorption spectrum shows a relatively high optical density value at 260 μm compared to serum albumin. The platelet regulator appears to be associated with an electrophoretically fast moving albumin component and is one of four components as seen in starch-gel electrophoretic patterns. Ultracentrifugal analyses show that it is a single component having a molecular size typical for that of albumin. Adequate methods for the fractionation of serum proteins are still needed in order that sufficient quantities of fractions can be procured for extensive biological testing.

Discussion: Stimulation of thrombopoiesis in rats by injections of human plasma extracts

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Filtrates of acidified, boiled (30 min.) fresh normal human plasmas regularly induced thrombopoiesis in Sprague-Dawley rats after repeated intraperitoneal injection. Injections of the extract in a dose of 1.0 ml./100 Gm. rat body weight resulted in a rise in platelet...
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Increasing the dose to 2.0 ml./100 Gm. rat weight for 3 days raised the platelet counts by 39.0 ± 25.5 per cent. With five daily injections of 1.0 ml./100 Gm. rat weight, an increase in platelet count of 45.5 ± 28.0 per cent was induced. Five daily injections of 2.0 ml./100 Gm. rat weight induced a rise of 72.3 ± 32.8 per cent. Injections of saline as a control (2.0 ml./100 Gm. rat weight for 5 days) induced a rise of 3.9 ± 17.1 per cent. Filtrates of aged normal human plasma in the same dose were also found to be devoid of thrombopoietic activity (rise of 6.3 ± 15.8 per cent) and served as a second type of control. Using the above technic the filtrate of fresh plasma from the previously described patient (Blood 16:943, 1960) thought to be deficient in platelet stimulating factor was found to have no thrombopoietic activity (rise of 2.1 ± 9.5 per cent). On the other hand, plasmas from four children with acute ITP were found to have normal thrombopoietic activity. More recently the effects of a single intravenous injection of the extract (2.0 ml./100 Gm. rat weight) have been studied. Seventy-two hours after injection, saline induced a rise of 7.8 ± 19.2 per cent, filtrates from fresh normal plasmas 64.5 ± 14.2 per cent, filtrates from aged normal plasmas 8.1 ± 32.3 per cent, and filtrate from the patient described above 30.8 ± 23.8 per cent. Further evaluation of this technic is in progress.

DISCUSSION: STUDIES ON PLASMA THROMBOPOIETIC FACTOR

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Spector (Proc. Soc. Exper. Biol. & Med. 108:146, 1961) found that the platelet count of normal recipient rabbits rose to an average of 166 per cent of control value 4 days after intravenous injection of 25 ml. fresh heparinized plasma from thrombocytopenic rabbits. Moderate thrombocytopenia was induced in the donors by injection of dog antirabbit platelet serum 2 to 20 hours earlier, or by administration of Myleran. Control plasma from normal donors caused no rise. Subsequent studies did not reveal as great an effect of active plasma (121 per cent of control level; p < 0.02). Plasma boiled 30 minutes at pH 5.5 was effective. Variability between recipient rabbits given the same batch of active plasma made correlation of dose and response virtually impossible. A further difficulty encountered was elevation of platelet count when a rabbit became ill, or 4 days after surgical stress or ether anesthesia. Megakaryocyte counts were performed on marrow sections before injection of active plasma and 2 or 3 days later in two animals respectively. Increased numbers of megakaryocytes (72 per cent rise) were noted in only one rabbit; this animal had by far the highest rise in platelet count on day 4 (224 per cent of control value). These results indicate the existence of a circulating substance which stimulates platelet production but also demonstrate difficulties in quantification and specificity of the assay in the rabbit.
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